Methylmalonic Acid and Coenzyme A Concentrations in the Livers of Pair-Fed Vitamin B₁₂-Deficient and Vitamin B₁₂-Treated Sheep

By R. M. SMITH, W. S. OSBORNE-WHITE AND G. R. RUSSELL

*Commonwealth Scientific and Industrial Research Organization, Division of Nutritional Biochemistry,
Adelaide, S. Austral. 5000, Australia*

(Received 6 December 1968)

The concentrations of CoA in the livers of severely vitamin B₁₂-deficient ewes were about 2.6 times those in pair-fed animals treated with vitamin B₁₂. When the feeding rates of the pair-fed animals were closely similar, the concentrations of methylmalonic acid in deficient livers were about twice those in vitamin B₁₂-sufficient livers. The molar concentrations of CoA present were more than three times those of methylmalonic acid in both deficient and treated animals, and it is concluded that the elevated concentrations of CoA in the deficient livers were not primarily due to accumulation of methylmalonyl-CoA.

Increased concentrations of pantothenic acid or CoA have been reported in the livers of vitamin B₁₂-deficient chicks (Evans, Groschke & Butts, 1951), rats (Boxer, Shonk, Gilfillan, Emerson & Oginsky, 1955) and sheep (Dawbarn, Forsyth & Kilpatrick, 1963), but the cause of the increase is unknown. Accumulation of methylmalonyl-CoA occurs during propionate metabolism in liver homogenates of vitamin B₁₂-deficient rats (Smith & Monty, 1959) and sheep (Marston & Smith, 1961), and has been attributed to depletion in the liver of 5'-deoxyadenosyl-cobalamin, the specific coenzyme for methylmalonyl-CoA mutase (EC 5.4.99.2).

The unusual stability of the thiol ester bond of methylmalonyl-CoA (Stadtman, Overath, Eggerer & Lynen, 1960) could permit its accumulation in the liver, and the present work was undertaken to discover whether the elevated concentrations of CoA in the livers of vitamin B₁₂-deficient sheep could be attributed to this cause.

MATERIALS AND METHODS

Animals. Six Australian Merino ewes, aged 17 months (mean body wt. 32 kg.), were fed on a cobalt-deficient diet in open-air pens. The daily ration consisted of 1 kg. of wheaten-hay chaff (cobalt content 0.03 µg./g. dry wt.), 50 g. of washed commercial gluten (cobalt content <0.02 µg./g. dry wt.) and 10 g. of NaCl, supplemented weekly with 70 mg. of copper (as CuSO₄ in water) and 25 ml. of cod liver oil. After 14–16 weeks of this treatment, the mean body weight was 33 kg. and the appetite of the sheep began to fail, and from that time food intake and body weight progressively declined. From 23 weeks, when food intakes were 400–500 g./day and mean body weight was 25 kg., two of the animals (nos. 385 and 318) were treated with vitamin B₁₂ (250 µg./week) injected intramuscularly in water, and pair-fed with animals

nos. 305 and 348 respectively (Expts. 2 and 3 in Table 1). From 32 weeks, when food intake of the deficient animals was 300–400 g./day and mean body weight was 23 kg., animal no. 392 was treated similarly with vitamin B₁₂ and pair-fed with animal no. 357 (Expt. 1). Five weeks later, the latter pair of animals was killed, and after a further 5 weeks the former two pairs were killed.

Animals were pair-fed on a daily basis. As a routine food was given *ad libitum* to deficient animals at 10.00 hours and removed and weighed on the following morning at 09.00 hours, the treated animal being scheduled 1 day later than its deficient counterpart. This procedure was followed in Expt. 1, the deficient animal (no. 357) being killed at 14.00 hours after starvation for 5 hr. and the treated animal at the same time on the following day. The treated animal, however, consumed all its ration within 4 hr. on the day before being killed and so was without food for 24 hr. In Expts. 2 and 3 the feeding procedure was varied to ensure comparable food intakes with time of the members of each pair. In these experiments the animals were fed for only 8 hr. on the days on which they were killed and on the 2 days immediately beforehand. Food intakes of the deficient animals were measured hourly and the appropriate quantities were given hourly to the respective treated animals 23 hr. later. On the day of slaughter food was first given to deficient animals at 06.00 hours and intakes were checked hourly until they were killed at 14.00 hours. A similar schedule was followed on the next day for the respective treated animals.

Determination of CoA. CoA in liver was determined in duplicate at each of three dilutions by the method of Kaplan & Lipmann (1948) as modified by Novelli (1955). A sample of CoA (70–75% pure) was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) and found to contain 340 Lipmann units (Kaplan & Lipmann, 1948)/mg. Based on the equivalence of 1 Lipmann unit at 0.6 µg. of pantothenic acid (Novelli, 1955) the sample was about 71% pure, and CoA in liver was determined by comparison with this standard. The values obtained for CoA in livers of vitamin B₁₂-sufficient sheep (Table 2) were similar to those for total

CoA measured by Dawbarn *et al.* (1963), obtained by *Lactobacillus plantarum* assay of bound pantothenic acid [121 ± 4 (S.E.M.) μg . of CoA/g. liver].

Determination of methylmalonic acid. Methylmalonic acid in liver was determined by isotope dilution of a small amount of methyl[1- ^{14}C]malonic acid of high specific radioactivity added to a homogenate in 0.7M-NaOH (which would hydrolyse methylmalonyl-CoA); methyl[^{14}C]malonic acid was then recovered and purified and its specific radioactivity determined.

After the animal had been killed by cutting its throat, the liver was rapidly removed and samples were taken for determination of CoA and vitamin B₁₂. The remainder (176–432g.) was weighed, quickly cut into pieces with scissors and homogenized in a Waring Blender for 3 min. at top speed in 750 ml. of 0.7M-NaOH. The homogenate was washed into a 2l. flask and 5.6 μmoles (8.3 μC) of methyl[1- ^{14}C]malonic acid were added. The suspension was heated to 80° and mechanically stirred for 30 min. at this temperature, the pH being maintained above 12. The flask contents were then adjusted to about pH 4 with 5M-H₂SO₄ and the flask was heated in a boiling-water bath for 5 min. after reaching 90°. After being chilled, the suspension was centrifuged, the sediment was resuspended in water and again centrifuged, and the supernatants were combined and filtered. The filtrate (about 1l.) was adjusted to pH 9 with 5M-NaOH and extracted with three 250 ml. portions of butan-1-ol to remove unsaponified lipid. The solution was adjusted to pH 2 with 5M-H₂SO₄ and extracted with five 250 ml. portions of butan-1-ol. About 90% of the ^{14}C was recovered in the butan-1-ol, the remainder being discarded. The methyl[1- ^{14}C]malonic acid was extracted from the butan-1-ol into an excess of aqueous NaOH with a mechanical emulsifier (Vibro-Mischer; A.-G. für Chemie-Apparatebau, Zurich, Switzerland) and the butan-1-ol was washed similarly with two 5 ml. portions of water. The aqueous phases (recovered by centrifuging) were combined (25–30 ml.) and adjusted to pH 9 with 0.25M-H₂SO₄ before being evaporated to dryness *in vacuo* (complete removal of butan-1-ol was essential before column chromatography). The dried salts were dissolved in 1 ml. of 1.5M-H₂SO₄ and chromatographed on a column (40 cm. $\times$ 1.2 cm. diam.) of Celite with chloroform and chloroform–butan-1-ol mixtures. Fractions (10 ml.) were collected and ^{14}C was measured in 0.1 ml. samples. Chromatographic and counting procedures were as described by Smith, Osborne-White & Russell (1967). A single radioactive peak was eluted in butan-1-ol–chloroform (1:9, v/v) corresponding to the elution position of methylmalonic acid and lactic acid. The tubes containing most of the ^{14}C were combined, the solvent was evaporated and methylmalonic acid and lactic acid were separated by paper chromatography. The free acids were applied as a streak in 1 ml. of water to Whatman 3MM paper and chromatographed (descending) for 21 hr. with ethanol–aq.NH₃ (sp.gr. 0.88)–water (8:1:1, by vol.) (Jones, Dowling & Skraba, 1953). After being dried in air, the paper was cut into longitudinal strips, the single radioactive peak in each strip was located with a scanning device and the appropriate areas were cut out and eluted with water. After the combined eluates had been evaporated to dryness, the residue was dissolved in 1 ml. of 1.5M-H₂SO₄ and chromatographed on Celite as described above. The eluate was collected in fractions (10 ml.), ^{14}C determined in 0.1 ml. samples and the remainder titrated under N₂ with 0.1M-NaOH (low

carbonate content) by using a micro-burette. The purity of the recovered labelled material was indicated by the constancy of the specific radioactivity in successive fractions. The original methyl[1- ^{14}C]malonic acid was standardized in the same way, the dilution due to unlabelled methylmalonic acid in the liver being calculated from the fall in specific radioactivity.

Methylmalonic acid in blood (700 ml.) was determined similarly, except that the initial alkaline hydrolysis was omitted and half the above quantity of labelled methylmalonic acid was used.

Preparation of methyl[1- ^{14}C]malonic acid. This was prepared from diethyl[1- ^{14}C]malonate and unlabelled methyl iodide by malonic ester synthesis in the presence of stoichiometric quantities of sodium ethoxide (Weiner, 1943). Reagents were added to the reaction tube in an atmosphere of dry N₂, with desiccated equipment at 0°. To a partly drawn-out Pyrex test tube (11 mm. internal diam.) was added 10.7 mg. (67 μmoles) of diethyl[1- ^{14}C]malonate, which was washed in by 215 μl . of anhydrous ethanol. This was followed by about 71 μmoles of sodium ethoxide (37 μl . of a solution of 4.6 g. of metallic sodium in 100 ml. of anhydrous ethanol), and 5 μl . (about 80 μmoles) of methyl iodide (freshly distilled from silver wool). The tube was stoppered, rapidly sealed in a flame and immersed in a water bath at 80° for 2 hr. After being cooled, the tube was opened and the esters were saponified with 5 ml. of 0.1M-NaOH at 100° for 30 min. The solution was washed through a column of Dowex 50 (H⁺ form) to remove Na⁺, and evaporated to dryness. The residue was taken up in 1 ml. of 1.5M-H₂SO₄ and chromatographed on Celite as described above. A radioactive peak was obtained in butan-1-ol–chloroform (1:9, v/v) corresponding to the elution position of methylmalonic acid and well separated from that of malonic acid. Recovery of methyl[1- ^{14}C]malonic acid was 68%, based on consumption of [1- ^{14}C]malonic ester, and the specific radioactivity (1.5 $\mu\text{C}/\mu\text{mole}$) was identical with that of the [1- ^{14}C]malonic ester.

Measurement of ^{14}C . Scintillation counting of ^{14}C in effluent fractions from Celite columns was as described by Smith *et al.* (1967). Samples (0.1 ml.) of fractions (10 ml.) were counted in 5 ml. of Bray's (1960) liquid-scintillator solution. Counting efficiency with effluent containing butan-1-ol–chloroform (1:9, v/v) was 46.2%.

Assay of vitamin B₁₂. Vitamin B₁₂ activity in liver and blood serum was determined by microbiological assay with *Lactobacillus leichmanii* (Dawbarn & Hine, 1954; Dawbarn, Hine & Smith, 1957).

Materials. Diethyl[1- ^{14}C]malonate was obtained from The Radiochemical Centre, Amersham, Bucks. CoA and an acetone-dried powder of pigeon liver were obtained from the Sigma Chemical Co. Other materials were as described by Smith *et al.* (1967) or were reagent-grade chemicals.

RESULTS

The vitamin B₁₂ status of the animals at the time of slaughter is shown in Table 1. Deficiency symptoms appear in sheep fed on this diet when the liver vitamin B₁₂ concentration has declined to less than 0.15 $\mu\text{g/g}$. (Marston, 1961). The three deficient animals used in the present experiments showed

Table 1. *Liver and serum vitamin B₁₂ concentrations in pair-fed vitamin B₁₂-deficient and vitamin B₁₂-treated ewes*

In each case the treated animal was fed on the cobalt-deficient diet for the same period as the corresponding deficient animal, and was treated with vitamin B₁₂ and pair-fed after both animals had become deficient. Food intakes of all animals began to fall after 14–16 weeks on the experimental diet, from an initial value of 1050 g./day. Initial body weights were about 32 kg.

Expt. no.	Animal no.	Treatment	Age when killed (months)	Food intake for week before slaughter (g./day)	Body wt. (kg.)	Liver wt. (g. fresh wt.)	Vitamin B ₁₂ Content	
							Liver (μg./g. fresh wt.)	Serum (ng./ml.)
1	357	Cobalt-deficient diet for 37 weeks	26	250	21.3	256	0.035	—
	392	Cobalt-deficient diet for 37 weeks; treated with 250 μg. of vitamin B ₁₂ /week and pair-fed with animal no. 357 for final 5 weeks	26	250	20.3	208	0.65	—
2	305	Cobalt-deficient diet for 42 weeks	27	215	16.9	269	0.027	0.35
	385	Cobalt-deficient diet for 42 weeks, treated with 250 μg. of vitamin B ₁₂ /week and pair-fed with animal no. 305 for final 19 weeks	27	215	17.6	199	0.83	4.0
3	348	Cobalt-deficient diet for 42 weeks	27	339	19.4	466	0.015	0.25
	318	Cobalt-deficient diet for 42 weeks, treated with 250 μg. of vitamin B ₁₂ /week and pair-fed with animal no. 348 for final 19 weeks	27	339	24.0	259	0.78	7.2

liver vitamin B₁₂ concentrations well below this value, and marked loss of appetite and body weight had occurred. Vitamin B₁₂ concentrations in serum also reflected the severe depletion that had occurred. The pair-fed animals treated with vitamin B₁₂ showed liver vitamin B₁₂ concentrations well above 0.15 μg./g., but losses in body weight, due to the restricted food intakes, were similar.

Table 2 shows the concentrations of methylmalonic acid and CoA found in the livers. Methylmalonic acid concentrations were elevated in the livers of vitamin B₁₂-deficient animals in comparison with those of pair-fed controls, and in the single pair studied a similar difference was found in the concentrations in whole blood (Expt. 1). The large difference in concentrations of methylmalonic acid in the liver of these two animals was probably due in part to the longer period of starvation of the vitamin B₁₂-treated animal, brought about by the much more rapid consumption of its ration. When food intake was adjusted on an hourly basis on the day of slaughter and for 2 days beforehand, the difference between deficient and treated animals was smaller (Expts. 2 and 3). In the latter experiments

methylmalonic acid concentrations in livers of deficient animals were about twice those in pair-fed animals treated with vitamin B₁₂.

It is not known to what extent the various acyl-CoA compounds in the livers were hydrolysed to free CoASH during preparation of the samples for CoASH determination by the method of Kaplan & Lipmann (1948). If it is assumed that hydrolysis was complete and that all the methylmalonate found was present in the liver as methylmalonyl-CoA, then in Expts. 2 and 3 the latter substance contained no more than 20% of the total CoA in either deficient or treated animals. In Expt. 1, where the concentration of methylmalonic acid in the liver of the deficient animal was higher, the corresponding maximum concentration of methylmalonyl-CoA was 31% of the CoASH found. It is probable, however, that hydrolysis of acyl-CoA compounds was not complete, and from the appearance of the free acid in blood it is also probable that part of the methylmalonic acid found in the liver was present in the free state. In either case the fraction of total CoA that could be accounted for as methylmalonyl-CoA would be less.

Table 2. *Methylmalonic acid and CoA in the livers of pair-fed vitamin B₁₂-deficient and vitamin B₁₂-treated sheep*

In Expts. 2 and 3 the intakes of the pair-fed animals were adjusted on an hourly basis on the day of slaughter and for 2 days beforehand. The vitamin B₁₂ status of the animals is shown in Table 1.

Expt. no.	Animal no.	Treatment	Food intake immediately before slaughter	Methylmalonic acid in whole blood (nmoles/ml.)	Methylmalonic acid in liver (nmoles/g. fresh wt.)	CoA concentration found (nmoles/g. fresh wt.)
1	357	Vitamin B ₁₂ deficient	Consumed 278 g. over 23 hr., then starved 5 hr. before slaughter	118	126	415
	392	Vitamin B ₁₂ -treated and pair-fed with animal no. 357	Consumed 278 g. over 4 hr., then starved 24 hr. before slaughter	5	13	160
2	305	Vitamin B ₁₂ -deficient	Consumed 283 g. over 8 hr. immediately before slaughter; intakes checked hourly	—	73	420
	385	Vitamin B ₁₂ -treated and pair-fed with animal no. 305	Consumed 283 g. over 8 hr. immediately before slaughter; intakes equivalent to animal 305 on hourly basis	—	34	166
3	348	Vitamin B ₁₂ -deficient	Consumed 266 g. over 8 hr. immediately before slaughter; intakes checked hourly	—	64	442
	318	Vitamin B ₁₂ -treated and pair-fed with animal no. 348	Consumed 266 g. over 8 hr. immediately before slaughter; intake equivalent to animal 348 on an hourly basis	—	26	157

DISCUSSION

The concentrations of methylmalonic acid in the livers at slaughter were presumably affected by the quantities of food consumed over the few hours immediately preceding death. Ruminal production of short-chain volatile fatty acids, including propionic acid, rapidly follows the consumption of food (Gray, Weller, Pilgrim & Jones, 1967) and is followed by their absorption and metabolism. However, apart from the low concentration of methylmalonic acid in the liver of the vitamin B₁₂-treated animal that was starved (no. 392), there was no clear indication of this in the results. Thus the highest concentration was found in the deficient animal no. 357 after 5 hr. starvation, and from body weight and liver vitamin B₁₂ concentration this was the least severely affected of the deficient animals. When food intake was adjusted on an hourly basis and animals were fed for only 8 hr. each day, this was accompanied by a decreased daily food intake by one deficient animal (Expt. 3) and an increased daily food intake by the other (Expt. 2). The concentrations of methylmalonic acid found,

however, were similar in the livers of both these animals after 8 hr. of feeding and both values were lower than that of animal no. 357.

On the other hand the concentrations of CoA found were very similar in livers of all three deficient animals, and in no case did the maximum possible concentration of methylmalonyl-CoA exceed one-third of the total CoA. It is concluded that the extra CoA in the livers of vitamin B₁₂-deficient sheep was not in the form of methylmalonyl-CoA.

We record our thanks to the late Dr H. R. Marston for suggesting this work and for providing the pair-fed animals. Thanks are due to Dr M. C. Dawbarn, formerly of this Division, for the vitamin B₁₂ analyses.

REFERENCES

- Boxer, G. E., Shonk, C. E., Gilfillan, E. W., Emerson, G. A. & Oginsky, E. L. (1955). *Arch. Biochem. Biophys.* **59**, 24.
- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Dawbarn, M. C., Forsyth, H. & Kilpatrick, D. (1963). *Aust. J. exp. Biol.* **41**, 1.
- Dawbarn, M. C. & Hine, D. C. (1954). *Aust. J. exp. Biol.* **32**, 1.

- Dawbarn, M. C., Hine, D. C. & Smith, J. (1957). *Aust. J. exp. Biol.* **35**, 321.
- Evans, R. J., Groschke, A. C. & Butts, H. A. (1951). *Arch. Biochem. Biophys.* **31**, 454.
- Gray, F. V., Weller, R. A., Pilgrim, A. F. & Jones, G. B. (1967). *Aust. J. agric. Res.* **18**, 625.
- Jones, A. R., Dowling, E. J. & Skraba, W. J. (1953). *Analyt. Chem.* **25**, 394.
- Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
- Marston, H. R. (1961). *Nature, Lond.*, **190**, 1085.
- Marston, H. R. & Smith, R. M. (1961). *Nature, Lond.*, **190**, 1088.
- Novelli, G. D. (1955). *Meth. biochem. Anal.* **2**, 189.
- Smith, R. M. & Monty, K. J. (1959). *Biochem. biophys. Res. Commun.* **1**, 105.
- Smith, R. M., Osborne-White, W. S. & Russell, G. R. (1967). *Biochem. J.* **104**, 441.
- Stadtman, E. R., Overath, P., Eggerer, H. & Lynen, F. (1960). *Biochem. biophys. Res. Commun.* **2**, 1.
- Weiner, N. (1943). In *Organic Syntheses*, Coll. vol. 2, p. 279. Ed. by Blatt, A. H. New York: John Wiley and Sons Inc.