Methylmalonic acid metabolism and nervous-system fatty acids in cobalamin-deficient fruit bats receiving supplements of methionine, valine and isoleucine

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Cobalamin neuropathy was produced in Cape fruit bats (*Rousettus aegyptiacus*) by a cobalamin-free diet combined with intermittent exposure to nitrous oxide, which inactivates cobalamin. Cobalamin-deficient bats had low hepatic methylmalonyl-CoA mutase holoenzyme activity, with elevated plasma and urinary methylmalonic acid levels. No significant changes could be demonstrated in the concentration of odd- or branched-chain fatty acids in the nervous system. Supplementation of the cobalamin-free diet with methionine, valine or isoleucine delayed the onset of neuropathy, despite persistence of methylmalonic acid accumulation. Supplementation with any of the three amino acids was associated with elevation of hepatic methionine concentration. The action of valine and isoleucine in delaying the onset of neuropathy can be explained by their methionine-sparing effect. These results emphasize the central role of methionine in the cobalamin neuropathy and do not support the hypothesis that the neuropathy is related to an effect of cobalamin deficiency on the propionic acid pathway.

INTRODUCTION

The mechanism whereby cobalamin (Cbl) deficiency leads to neurological impairment remains uncertain. A requirement for the vitamin has been established in two biochemical reactions in man: as 5-deoxyadenosyl-Cbl in the isomerization of L-methylmalonyl-CoA to succinyl-CoA, the final step in the metabolism of propionate to succinyl-CoA, and as methyl-Cbl in the methylation of homocysteine to methionine.

Impairment of the adenosyl-Cbl-dependent reaction and its relationship to fatty acid metabolism has been suggested as the biochemical basis of the neuropathy in Cbl deficiency (Peifer & Lewis, 1979). Inadequate amounts of adenosyl-Cbl result in decreased activity of methylmalonyl-CoA mutase and a resultant accumulation of the intermediates propionyl-CoA and methylmalonyl-CoA. Methylmalonyl-CoA can substitute for malonyl-CoA, and propionyl-CoA for acetyl-CoA, in fatty acid synthesis, resulting in the formation of branched-chain and odd-chain fatty acids respectively. These fatty acids then accumulate in membrane lipids of neural tissue and so produce the neurological syndrome (Frenkel, 1973).

The demonstration of the protective effect of methionine on the development of neurological changes in experimentally induced Cbl deficiency in the monkey (Scott *et al.*, 1981) focused attention on the possible role of methyl group deficiency resulting from the impaired methyl-Cbl-dependent homocysteine-methionine methylation as the possible mechanism underlying the neuropathology of Cbl deficiency. The protective effect of methionine was confirmed in the experimental Cbl-deficient neuropathy of the fruit bat (van der Westhuyzen *et al.*, 1982a) and the pig (Weir *et al.*, 1988). However, defective methylation could not be demonstrated in the nervous system of the Cbl-deficient bat, in that neither the levels of S-adenosylmethionine (AdoMet) (van der Westhuyzen & Metz, 1983), nor the S-adenosylmethionine:S-adenosylhomocysteine methylation ratio was reduced (Vieira-Makings *et al.*, 1990a).

Valine and isoleucine also protect against both the clinical neurological changes (Vieira-Makings *et al.*, 1990b) and the electron-microscopically discernible changes in the bat model (Duffield *et al.*, 1990). If the neuropathy of Cbl deficiency is linked to accumulation of propionic acid intermediates, such as methylmalonic acid (MMA), and resultant changes in fatty acids in nervous tissue, it would be expected that the protective effect of methionine, valine and isoleucine would be accompanied by normalization of MMA production, and maintenance of a normal fatty acid profile in the nervous system. The present study reports the effect of these amino acids on MMA metabolism and nervous system fatty acids in the fruit bat with Cbl deficiency induced by a combination of dietary restriction and exposure to nitrous oxide (N₂O), which oxidizes active reduced cob (I) alamin to the inactive cob (III) alamin (Banks *et al.*, 1968). In this model, severe neurological changes occur and are associated with electron-microscopically discernible changes in the spinal-cord myelin, including distention, separation and vacuolation of myelin lamellae (Duffield *et al.*, 1990).

MATERIALS AND METHODS

Experimental animals

Colonies of the Cape fruit bat (*Rousettus aegyptiacus*) captured in the wild were maintained on a Cbl-free diet of fresh peeled fruit as previously described (van der Westhuyzen *et al.*, 1983). The diet consisted largely of bananas, supplemented with oranges, apples and papayas when available. The daily intake of methionine, valine and isoleucine was calculated as about 10, 80 and 70 mg respectively, according to tables of food values (Church & Church, 1966). The animals were fed *ad libitum* and were supplied with clean tap water daily, and their cage floors were kept clean. A multi-vitamin preparation (Abidec, Parke-Davis, Cape Town, South Africa) free of vitamin B₁₂ (cobalamin) was given orally to all the bats to ensure adequate

Abbreviations used: Cbl, cobalamin; MMA, methylmalonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingomyelin; AdoMet, S-adenosylmethionine.

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intake of vitamins A, B₆, thiamine, riboflavin and niacin (van der Westhuyzen *et al.*, 1982a).

Experimental groups

The bats were randomly divided into five groups: one control group and four experimental groups, each comprising five animals. The experimental groups were depleted of Cbl by exposure to N₂O and received the standard vitamin B₁₂-free fruit diet either (1) unsupplemented, or (2) supplemented with L-methionine (99%; E. Merck, Darmstadt, Germany) in the proportion of 0.6 g/kg of fruit, or (3) supplemented with L-valine (99%; Riedel-de Haen, Hannover, Germany) in the proportion of 0.46 g/kg of fruit, or (4) supplemented with L-isoleucine (99%; E. Merck) in the proportion of 0.55 g/kg of fruit, throughout the study. The methionine, valine and isoleucine supplements were equimolar. Control animals were fed the same diet, but were given intramuscular vitamin B₁₂ (5 µg of cyanocobalamin/kg body wt.) every 2 weeks to maintain normal Cbl nutrition.

Experimental procedure

The protocol for this study was approved by the Animal Ethics Committee of the University of the Witwatersrand, Johannesburg, South Africa.

Before commencement of the experiment all the animals were examined clinically and weighed. Animals were bled by cardiac puncture for measurement of Cbl and MMA. The bats were bled again at 6 weeks, 12 weeks and at the end of the experiment (18 weeks). The experimental groups were depleted of Cbl by exposure for the first 6 weeks to an N₂O/O₂ (1:1) atmosphere for 90 min/day in a specially constructed environmental chamber in which levels of water vapour and CO₂ were controlled (van der Westhuyzen *et al.*, 1982a). After the N₂O exposure period the bats were maintained on the Cbl-free diet for a further 12 weeks. Animals were examined regularly at weekly intervals for the development of neurological impairment, and flight characteristics were recorded. At the end of the experimental period the bats were killed by exsanguination and weighed. The brains and spinal cord were rapidly removed, weighed and homogenized in chloroform/methanol (1:2, v/v) for lipid analyses. The livers were removed under subdued lighting and placed immediately on to aluminium foil and then on to solid CO₂ for quick freezing. The livers were then tightly wrapped with double foil and placed in an air-tight container to avoid desiccation and stored at -20 °C until assayed.

Analytical methods

Cobalamin. Cbl was measured by radioisotopic dilution assay using a Simultrac-SNB kit (Beckton-Dickinson Co., New York, NY, U.S.A.).

MMA. Plasma MMA was extracted and assayed by capillary gas chromatography by the method of McMurray *et al.* (1986), using ethylmalonic acid as internal standard.

Methylmalonyl-CoA mutase. Enzyme activity was measured in the liver by a modification of the method of Cannata *et al.* (1965) as modified by Kolhouse & Allen (1977). After thawing, an aliquot of tissue was minced in 0.01 M-Hepes buffer containing GSH. Tissue was then homogenized, and after sonication, the homogenate was centrifuged at 106000 g and the supernatant removed.

Analysis of central-nervous-system fatty acids

Lipid extraction and phospholipid separation. Total lipids were extracted according to the method of Bligh & Dyer (1959) and

evaporated to dryness in a sand bath at 37 °C under a stream of N₂. The dried material was re-dissolved in 100 µl of chloroform/methanol (2:1, v/v) and spotted on to t.l.c. plates (Merck; silica-gel 60). The plates were developed by a modification (Thomas & Holub, 1987) of the method of Mitchell *et al.* (1986), sprayed with a saturated solution of 2,7-dichlorofluorescein in methanol/water (1:1, v/v) and exposed to NH₃ vapour. The phospholipid bands were visualized under u.v. light, and identified by comparing the retention times with those of known standards.

Preparation of fatty acid methyl esters. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SPH) were scraped from the plates, and fatty acid methyl esters were prepared by a modification of the method of Holub & Skeaff (1987). The phospholipids were scraped into screw-top glass tubes containing 3 ml of 6% H₂SO₄ in methanol by volume and 100 µg of monpentadecanoate (containing C_{15:0}) as an internal standard. The tubes were tightly sealed with Teflon-lined caps, vortex-mixed, and incubated at 80 °C for 2 h. After cooling to room temperature, 1.5 ml of hexane and 1 ml of water was added. After vortex-mixing and centrifugation, the upper hexane phase, containing the eluted fatty acid methyl esters, was removed and transferred to a Mini-Vial.

Analysis of fatty acid methyl esters. The methyl esters were analysed using a 60 m × 0.2 mm (internal diameter) SP2330 capillary column (Supelco) in a Hewlett-Packard 5890A gas chromatograph fitted with a flame-ionization detector. Fatty acids were identified by comparing retention times with those of known standards, and the areas of the peaks were computed using a Spectrophysics SP4270 integrator. By using the peak area and the known weight of the monpentadecanoate internal standard, the weight and amount in mol of the individual fatty acids were calculated. In all the fatty acid analyses, pentadecanoate (C_{15:0}) was used as an internal standard for quantification of methyl esters and was therefore not determined in tissue lipids.

Methionine

Methionine was assayed by the method of Kaldor *et al.* (1986) in which methionine bleaches platinate and the decrease in colour is proportional to the concentration of methionine. The liver homogenates were deproteinized by the addition of uranyl acetate, followed by centrifugation at 10000 g for 30 min.

Statistics

Significance of difference in group means was determined by Student's paired *t* test (two-tailed). The level of significance was chosen as *P* < 0.05. Fatty acid results were subjected to analysis of variance.

RESULTS

Neurological impairment

After 6 weeks all bats in the unsupplemented group began to show signs of neuromuscular impairment manifested by reluctance to use the hind limbs. By 9 weeks the bats were no longer climbing, the legs were held passively and the clutching reflex was lost. After 11 weeks, the flight was reduced to short hops. By 17 weeks they had ceased to fly, and spasticity was evident by head retraction and rigidity of the back.

Bats receiving supplemental methionine remained clinically normal up to 17 weeks, when they started showing signs of

Table 1. Plasma Cbl levels in the various experimental groups before and after the induction of Cbl deficiency

Results are expressed as means $\pm$ S.E.M. Abbreviations: Cbl-def., Cbl-deficient; Cbl-def., Met, Cbl-deficient supplemented with methionine; Cbl-def., Val, Cbl-deficient supplemented with valine; Cbl-def., Ile, Cbl-deficient supplemented with isoleucine.

Group	Time (weeks)...	[Cbl] (pmol/l)			
		0 (Initial)	8	12	18
Control ($n = 4$)		1890 $\pm$ 169	1900 $\pm$ 174	1907 $\pm$ 173	1909 $\pm$ 171
Cbl-def. ($n = 5$)		1997 $\pm$ 173	41 $\pm$ 9	12 $\pm$ 1	8 $\pm$ 4
Cbl-def., Met ($n = 5$)		1690 $\pm$ 190	36 $\pm$ 6	22 $\pm$ 1	18 $\pm$ 2
Cbl-def., Val ($n = 5$)		1750 $\pm$ 180	34 $\pm$ 6	18 $\pm$ 4	15 $\pm$ 2
Cbl-def., Ile ($n = 4$)		1759 $\pm$ 122	36 $\pm$ 5	18 $\pm$ 3	14 $\pm$ 3

impaired hind-limb use. Bats receiving either valine, isoleucine or vitamin B₁₂ supplementation remained clinically well throughout the experimental period.

Body weight

In the vitamin B₁₂-unsupplemented group, the mean body weight fell from 136.1 $\pm$ 7.6 g at the start of the experiment to 130.0 $\pm$ 11.6 g after 18 weeks. In all other groups, body weight was either maintained or increased during the experimental period.

Plasma cobalamin

After 6 weeks of daily exposure to N₂O, the levels were very low in all the experimental groups and remained low throughout the remaining 12 weeks on the Cbl-free diet (Table 1).

Methylmalonic acid

At the termination of the experiment, the mean level in the Cbl-deficient unsupplemented group was markedly elevated (Table 2). Supplementation of the diet with methionine, valine or isoleucine reduced the mean levels.

Methylmalonyl-CoA mutase activity

The mean value for liver methylmalonyl-CoA mutase holoenzyme activity was significantly lower in the Cbl-deficient animals (Table 3). Supplementation of the diet with methionine, valine or isoleucine resulted in slightly higher mean values.

Fatty acids

In the spinal cord, the major fatty acids in PE, PC and SPH were palmitic acid (C_{16:0}), stearic acid (C_{18:0}) and oleic acid (C_{18:1}). These three fatty acids comprised 64, 76 and 80 % of the total fatty acids in PE, PC and SPH respectively. The fatty acid composition of PE, PC and SPH was similar in the control, Cbl-deficient and methionine + valine + isoleucine-supplemented groups. No differences of statistical significance could be demonstrated.

The pattern was similar for fatty acids in the brain, where palmitic acid, stearic acid and oleic acid together comprised 62, 87 and 78 % of the total fatty acids in PE, PC and SPH respectively. There were no statistically significant differences in spinal-cord lipid fatty acid composition between the control and various test groups.

The values for branched-chain and odd-chain fatty acids in spinal cord and brain are shown in Tables 4 and 5. Branched- and odd-chain fatty acids (C_{17:0}, C_{17:1} and C_{23:0}, but not C_{19:0}) were detected in only trace amounts in both spinal cord and

Table 2. Plasma MMA levels in the various experimental groups before and after the induction of cobalamin deficiency

Results are expressed as means $\pm$ S.E.M. Significantly different from the control group: * $P < 0.001$, ** $P < 0.01$. Abbreviations are as defined in Table 1.

Group	Time...	Plasma [MMA] (μ mol/ml)	
		Initial	18 weeks
Control ($n = 5$)		6.5 $\pm$ 4.0	5.1 $\pm$ 0.9
Cbl-def. ($n = 5$)		6.1 $\pm$ 2.6	101.2 $\pm$ 42.2*
Cbl-def., Met ($n = 5$)		6.5 $\pm$ 3.3	48.4 $\pm$ 5.4**
Cbl-def., Val ($n = 5$)		5.1 $\pm$ 3.0	28.1 $\pm$ 9.7**
Cbl-def., Ile ($n = 5$)		5.9 $\pm$ 1.0	25.8 $\pm$ 14.1**

Table 3. Liver methylmalonic-CoA mutase activity of the various experimental groups at 18 weeks after the induction of Cbl deficiency

Results (in μ mol/g) are expressed as means $\pm$ S.E.M. *Significantly different ($P < 0.001$) from the control group. Abbreviations are as defined in Table 1.

Group	Holoenzyme	Apoenzyme	Total
Control ($n = 4$)	6.32 $\pm$ 0.74	270.38 $\pm$ 3.78	276.70 $\pm$ 1.78
Cbl-def. ($n = 4$)	2.53 $\pm$ 2.01*	218.76 $\pm$ 7.90	221.29 $\pm$ 10.83
Cbl-def., Met ($n = 4$)	3.32 $\pm$ 1.68*	244.50 $\pm$ 4.57	247.82 $\pm$ 2.90
Cbl-def., Val ($n = 4$)	3.12 $\pm$ 2.06*	296.84 $\pm$ 9.84	299.96 $\pm$ 7.82
Cbl-def., Ile ($n = 4$)	3.64 $\pm$ 1.68*	331.64 $\pm$ 2.90	335.28 $\pm$ 0.78

brain. No significant increase in branched- or odd-chain fatty acids in PE, PC or SPH could be demonstrated in the Cbl-deficient animals.

Liver methionine concentration

The mean value for liver methionine concentration in the Cbl-deficient bats was about half that of the control animals (Table 6). In the group receiving supplementary methionine, the mean value was markedly elevated. Supplementation with either valine or isoleucine showed a similar effect.

Table 4. Odd- and branched-chain fatty acid composition of PE, PC and SPH extracted from the spinal cords of the various experimental groups 18 weeks after the induction of Cbl deficiencyResults are expressed as the means $\pm$ S.E.M. Abbreviations are as defined in Table 1.

Lipid	Fatty acid	Composition (mol/100 mol)				
		Control group	Cbl-def.	Cbl-def., Met	Cbl-def., Val	Cbl-def., Ile
PE	C _{17:0β}	2.00 $\pm$ 0.67	1.85 $\pm$ 0.40	1.60 $\pm$ 0.25	0.60 $\pm$ 0.04	0.43 $\pm$ 0.06
	C _{17:0}	1.16 $\pm$ 0.25	0.71 $\pm$ 0.14	0.60 $\pm$ 0.15	0.32 $\pm$ 0.04	0.43 $\pm$ 0.07
	C _{23:0}	1.06 $\pm$ 0.02 <i>n</i> = 3	0.95 $\pm$ 0.06 <i>n</i> = 5	0.88 $\pm$ 0.07 <i>n</i> = 4	0.88 $\pm$ 0.07 <i>n</i> = 5	1.00 $\pm$ 0.09 <i>n</i> = 4
PC	C _{17:0β}	0.36 $\pm$ 0.09	0.43 $\pm$ 0.02	0.18 $\pm$ 0.03	0.63 $\pm$ 0.11	0.33 $\pm$ 0.06
	C _{17:0}	0.23 $\pm$ 0.05	0.30 $\pm$ 0.02	0.30 $\pm$ 0.02	0.25 $\pm$ 0.03	0.28 $\pm$ 0.04
	C _{23:0}	0.06 $\pm$ 0.02 <i>n</i> = 3	0.05 $\pm$ 0.01 <i>n</i> = 5	0.15 $\pm$ 0.04 <i>n</i> = 4	0.08 $\pm$ 0.02 <i>n</i> = 4	0.05 $\pm$ 0.00 <i>n</i> = 4
SPH	C _{17:0β}	0.38 $\pm$ 0.04	0.40 $\pm$ 0.06	0.50 $\pm$ 0.07	0.48 $\pm$ 0.10	0.30 $\pm$ 0.13
	C _{17:0}	0.20 $\pm$ 0.05	0.10 $\pm$ 0.03	0.26 $\pm$ 0.04	0.60 $\pm$ 0.09	0.53 $\pm$ 0.13
	C _{23:0}	0 <i>n</i> = 4	0.10 $\pm$ 0.03 <i>n</i> = 3	0.43 $\pm$ 0.13 <i>n</i> = 3	0.05 $\pm$ 0.01 <i>n</i> = 4	0 <i>n</i> = 4

Table 5. Odd- and branched-chain fatty acid composition of PE, PC and SPH extracted from the brains of the various experimental groups 18 weeks after the induction of cobalamin deficiencyResults are expressed as means $\pm$ S.E.M.. Abbreviations are as defined in Table 1.

Lipid	Fatty acid	Composition (mol/100 mol)				
		Control group	Cbl-def.	Cbl-def., Met	Cbl-def., Val	Cbl-def., Ile
PE	C _{17:0β}	1.00 $\pm$ 0.35	0.16 $\pm$ 0.03	0.38 $\pm$ 0.06	0.60 $\pm$ 0.14	0.65 $\pm$ 0.23
	C _{17:0}	0.23 $\pm$ 0.07	0.36 $\pm$ 0.02	0.43 $\pm$ 0.04	0.48 $\pm$ 0.02	0.20 $\pm$ 0.05
	C _{23:0}	0.80 $\pm$ 0.09 <i>n</i> = 3	0.95 $\pm$ 0.07 <i>n</i> = 5	0.90 $\pm$ 0.04 <i>n</i> = 4	1.07 $\pm$ 0.07 <i>n</i> = 4	0.50 $\pm$ 0.07 <i>n</i> = 4
PC	C _{17:0β} *	0.40	0.18 $\pm$ 0.02	0.18 $\pm$ 0.02	0.20 $\pm$ 0.04	0.27 $\pm$ 0.03
	C _{17:0} *	0.30	0.16 $\pm$ 0.03	0.20 $\pm$ 0.03	0.13 $\pm$ 0.03	0.27 $\pm$ 0.01
	C _{23:0} *	0.10 <i>n</i> = 1	0.08 $\pm$ 0.01 <i>n</i> = 5	0.13 $\pm$ 0.01 <i>n</i> = 4	0.13 $\pm$ 0.03 <i>n</i> = 4	0.20 $\pm$ 0.05 <i>n</i> = 4
SPH	C _{17:0β}	0.47 $\pm$ 0.08	0.10 $\pm$ 0.02	0.12 $\pm$ 0.01	0.30 $\pm$ 0.06	0
	C _{17:0}	0.37 $\pm$ 0.04	0.40 $\pm$ 0.07	0.16 $\pm$ 0.01	0.08 $\pm$ 0.02	0
	C _{23:0}	0.13 $\pm$ 0.04 <i>n</i> = 3	0.32 $\pm$ 0.06 <i>n</i> = 5	0.14 $\pm$ 0.03 <i>n</i> = 5	0 <i>n</i> = 5	0 <i>n</i> = 3

* Owing to contamination with water, three samples were unable to be analysed.

Table 6. Liver methionine concentrations in the various experimental group 18 weeks after the induction of cobalamin deficiencyResults are expressed as means $\pm$ S.E.M. Significantly different from the control group: **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Abbreviations are as defined in Table 1.

Group	[Methionine] (μ mol/g)
Control	25.2 $\pm$ 0.49
Cbl-def. (<i>n</i> = 4)	13.8 $\pm$ 1.25*
Cbl-def., Met (<i>n</i> = 4)	120.6 $\pm$ 1.29***
Cbl-def., Val (<i>n</i> = 4)	60.9 $\pm$ 3.49**
Cbl-def., Ile (<i>n</i> = 4)	54.4 $\pm$ 2.03**

DISCUSSION

In the present study, the combination of Cbl-free diet and exposure to N₂O induced severe Cbl deficiency with neurological changes in the test animals, as reported in previous studies (van der Westhuyzen *et al.*, 1982a). The Cbl deficiency was accompanied by markedly decreased activity of the Cbl-dependent enzyme methylmalonyl-CoA mutase in the liver. A decrease in holoenzyme activity in all the organs studied in Cbl-deficient bats, with an absence of activity in the spinal cord, has been reported previously (Green *et al.*, 1983). In the present study, decreased enzyme activity was accompanied by accumulation of MMA in the plasma. Despite the accumulation of MMA, we were unable to demonstrate excessive odd-chain or branched-chain fatty acids in either brain or spinal-cord lipids.

The results of the assay of liver methionine stores suggest a possible explanation for the methionine-like effect produced by valine and isoleucine. As expected, methionine levels in the liver were markedly elevated in bats receiving methionine supplementation, but were also substantially increased in the animals whose diet was supplemented by valine or isoleucine, compared with

Abbreviations: CH₂, methylene; CH₃, methyl; H₄, tetrahydro; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.

The results of the present study reaffirm the central role of methionine in the pathogenesis of the Cbl neuropathy. Although it is unlikely that this action is mediated via the propionic acid pathway, the results in the fruit bat do not offer evidence for an alternative mechanism for the methionine effect. In the fruit bat, in contrast with the pig (Weir *et al.*, 1988), we have not been able to show a defect in methylation reactions attendant upon Cbl deficiency. This would suggest that the methionine effect could be mediated via its role in formate metabolism or by some as yet unidentified pathway.

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