Possible Interrelationship between Vitamins E and B₁₂ in the Disturbance in Methylmalonate Metabolism in Vitamin E Deficiency

By ANURADHA S. PAPPU, PREMA FATTERPAKER
and ARUNACHALA SREENIVASAN*

Biochemistry and Food Technology Division, Bhabha Atomic Research Centre,
Bombay 400 085, India

(Received 31 August 1977)

1. The disturbance in 2-methylmalonate metabolism resulting in its increased urinary excretion observed in vitamin E deficiency is not caused by increased formation of methylmalonate from propionate as is evident from the activity of the enzyme propionyl-CoA carboxylase (EC 6.4.1.3), but can be traced to an impairment in the conversion of methylmalonate into succinate by the vitamin B₁₂-requiring enzyme, methylmalonyl-CoA mutase (EC 5.4.99.2) in rat liver. 2. It is shown that the decrease in the activity of methylmalonyl-CoA mutase in vitamin E deficiency is not a consequence of a secondary vitamin B₁₂ deficiency. Peroxidative destruction of the coenzyme in vitamin E deficiency was also ruled out. The results suggest a defect in the conversion of cyanocobalamin into its coenzyme form.

It is well known that increased urinary excretion of 2-methylmalonate is associated with deficiencies of both vitamin E and vitamin B₁₂. Premature infants, suffering from vitamin E deficiency, excreted large amounts of methylmalonate in their urine. This symptom is reported to be reversed by vitamin E therapy (Osaki *et al.*, 1966). Similar observations on increased amounts of this metabolite in the urine of vitamin E-deficient rats also have been reported by Barness (1967). Urinary excretion of methylmalonate is well recognized as a diagnostic tool for the detection of pernicious anaemia, a state of vitamin B₁₂ deficiency (Williams *et al.*, 1969; Gurnani *et al.*, 1960; Reed & Tarver, 1970; Barker, 1972a; Vivacqua *et al.*, 1966). The disturbance in methylmalonate metabolism in vitamin B₁₂ deficiency has been traced to a defective conversion of methylmalonyl-CoA into succinyl-CoA by an isomerase requiring adenosylcobalamin (Gurnani *et al.*, 1960). It was therefore felt desirable to ascertain if the increased amounts of methylmalonate in the urine of vitamin E-deficient rats could result from alterations in vitamin B₁₂ metabolism in this deficiency condition. An interdependence of vitamin E and vitamin B₁₂ was indeed postulated by Hove & Hardin (1951). However, the molecular mechanisms responsible for these interrelationships were not fully elucidated.

In the present investigation the possible causes of increased methylmalonate excretion in vitamin E deficiency have been investigated by studying the enzymes responsible for its production and utilization. An impairment in the activity of vitamin B₁₂-requir-

ing methylmalonyl-CoA mutase, responsible for the conversion of methylmalonate into succinate, has been observed. This has been traced to an imperfect conversion of cyanocobalamin into its coenzyme form (adenosylcobalamin).

Materials and Methods

Chemicals

ATP, CoA, glutathione and adenosylcobalamin were from Sigma Chemical Co., St. Louis, MO, U.S.A. α -Tocopherol acetate and cyanocobalamin were from E. Merck, Darmstadt, Germany. Butylated hydroxytoluene was from Koch-Light Laboratories, Colnbrook, Bucks. SL3 0B2, U.K. Casein was from Amul Products, Anand, India. [1-¹⁴C]Propionate (sp. radioactivity 20 mCi/mmol) and cyano[⁵⁷Co]-cobalamin (sp. radioactivity 13.1 mCi/mmol) were from the Isotope Division of this Research Centre.

Vitamin E-deficient animals

Weanling male albino rats of the Wistar strain weighing between 30 and 40 g were raised either on a vitamin E-deficient or a vitamin E-supplemented diet (Adhikari *et al.*, 1968). The vitamin E-deficient diet contained vitamin-free casein (20%), sucrose (8%), salt mixture USP XIV (1950) (4%), ground-nut oil (8%), vitamin mixture in sucrose (2%) and starch (58%). The vitamin mixture contained (per 100 g), thiamin (10 mg), riboflavin (20 mg), pyridoxine (20 mg), choline (5 g), inositol (5 g), *p*-aminobenzoic acid (1.5 g), nicotinamide (0.5 g),

* Present address: 72 Pali Hill, Bombay 400050, India.

calcium pantothenate (50mg), folic acid (12.5mg), vitamin B₁₂ (0.25mg), biotin (0.5mg), menadione (2.5mg) and cholecalciferol (5.0mg). Retinyl palmitate was included in the diet, at 25mg/kg of diet. The tocopherol-supplemented diet was identical with the deficient diet except for the addition of 150mg of DL- α -tocopherol/kg of diet. The animals were fed *ad libitum* for about 5–6 months. The onset of vitamin E deficiency in rats was monitored by determining the susceptibility of erythrocytes to peroxidative haemolysis (Rose & Gyorgy, 1952) and from the decrease in weight of the testes of the animals.

Isolation of mitochondria

Rat liver mitochondria were obtained from a 10% (w/v) liver homogenate in 0.25M-sucrose containing 0.01M-Tris/HCl, pH7.4, and 1mM-EDTA (Satav *et al.*, 1976).

Acetone-dried powders of homogenate and the twice-washed mitochondria were prepared by the method of Halenz & Lane (1960).

Measurement of methylmalonate in urine

Methylmalonate was measured quantitatively in urine by the method of Reed & Tarver (1970), with some modifications. Urine of rats, maintained on 10g of diet/day, was collected for a 24h period. The volume of the urine was measured and it was filtered through Whatman no. 1 paper, after adjusting the pH to 1 with 5M-HCl. A portion (50ml) of the urine was extracted with diethyl ether continuously for 24h. The ether extract was evaporated under N₂ and the residue was dissolved in a minimum quantity of water. The insoluble residue was removed by centrifugation at 3000rev./min for 10min, and the supernatant was used for the measurement of methylmalonate by the procedure of Giorgio & Plaut (1966).

Microbiological assay of cyanocobalamin

Cyanocobalamin in serum and liver homogenate was determined by using the vitamin B₁₂-requiring algal flagellate *Euglena gracilis*, as test organism. The assay was carried out by the method of Hunter *et al.* (1956). The values were expressed as ng/ml of serum and ng/g of liver.

Conversion of [1-¹⁴C]propionate into succinate via methyl[1-¹⁴C]malonate

The assay procedure followed for studying the conversion of [1-¹⁴C]propionate into [1-¹⁴C]succinate was essentially that of Flavin & Ochoa (1957) with some modifications. The supernatants obtained by extracting the acetone-dried powders of liver homogenate and mitochondria with 4mM-Tris/HCl,

pH7.2, for 30min and centrifuging at 12800g for 10min at 4°C, were used as sources of enzymes in the assay systems. The experiments were carried out in two sets.

Set (i). The incubation mixture for studying the conversion of propionate into succinate contained (final volume 1ml): 100mM-Tris/HCl buffer, pH8.5, 4mM-MgCl₂, 4mM-ATP, 5mM-glutathione, 15mM-KHCO₃, 1mM-CoA, 10mM-propionate, 0.5 μ Ci (9.5 $\times$ 10⁵c.p.m.) of sodium [1-¹⁴C]propionate and supernatant from acetone-dried powders of either liver homogenate (5mg of protein) or mitochondria (2.5mg of protein).

Set (ii). A parallel set of experiments was run simultaneously in which further conversion of methylmalonate into succinate was inhibited by including additionally 20mM-sodium malonate (Flavin *et al.*, 1955).

Incubation of both sets was carried out at 37°C for 30min in a shaking water bath. The reaction was stopped by addition of 0.2ml of 1M-NaOH which would hydrolyse thioesters of dicarboxylic acids. The incubation mixtures were then neutralized with 10% (w/v) trichloroacetic acid and filtered. The filtrates from the tubes of set (i) were heated with three drops of 3% KMnO₄, in a water bath for 10min. This step was introduced to destroy volatile acids like methylmalonic acid, which may otherwise interfere with determination of succinic acid.

Samples of filtrates were extracted continuously for 24h with diethyl ether (peroxide-free) to recover dicarboxylic acids. The ether extracts were evaporated to dryness under N₂ and the residues were dissolved in a minimum amount of water.

The dicarboxylic acids from the reaction mixtures were separated by descending paper chromatography on Whatman no. 1 paper, with butanol/acetic acid/water (4:1:5, by vol.) as solvent system (Macek, 1963). Authentic samples containing known amounts of succinic acid and methylmalonic acid were spotted alongside for reference. The chromatograms were run for about 10h and then dried. The acidic spots were detected after a light spray of Bromocresol Green (0.1% in ethanol, made alkaline with NaOH and diluted 1:4 with acetone before use). The spots corresponding to standard succinic acid and methylmalonic acid were cut out and placed in counting vials containing 0.3% PPO (2,5-diphenyloxazole) and 0.01% dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] in 10ml of toluene as scintillator. Radioactivity was counted in a Beckman LS-100 liquid-scintillation spectrometer with 95% instrument efficiency for ¹⁴C. In the presence of filter-paper strips the counting efficiency was, however, 38%. All values reported are actual c.p.m. measured without quench corrections being applied. The first set of experiments provide data for the conversion of propionate into succinate, via inter-

mediate methylmalonate, and the second set of experiments give the amount of methylmalonate formed from $[1-^{14}\text{C}]$ propionate.

Effect of addition in vitro of adenosylcobalamin on $[1-^{14}\text{C}]$ propionate conversion into $[1-^{14}\text{C}]$ succinate

About 20 μg of adenosylcobalamin was introduced into the above reaction mixture [set (i), without malonate] and the experiments were repeated to determine its effect on $[1-^{14}\text{C}]$ succinate production in liver homogenate and mitochondria of vitamin E-deficient animals.

Conversion of intraperitoneally administered cyano- $[^{57}\text{Co}]$ cobalamin into its coenzyme analogues in liver

Cyano- $[^{57}\text{Co}]$ cobalamin (3 μCi) in 0.9% NaCl was injected intraperitoneally into rats. Rats were killed after 24 h and cyanocobalamin and its coenzyme analogues were extracted from the liver by a combination of the methods used by Cardinale *et al.* (1969) and Toohey & Barker (1961).

Extraction of cyanocobalamin and its coenzyme analogues from rat liver. Liver (2.0–5.0 g) was homogenized in 5 ml of glass-distilled water for 30 s in the presence of either α -tocopherol (200 $\mu\text{g/g}$ of liver) or butylated hydroxytoluene (200 $\mu\text{g/g}$ of liver) or in the absence of any anti-oxidant. The homogenate was then poured into 50 ml of 95% ethanol in a 250 ml flask and boiled for 5 min. The solution was cooled and filtered through fluted filter paper. The residue was extracted once again with 25 ml of boiling ethanol (3 min). The combined filtrates were almost dried under vacuum on a rotary evaporator, and the residue was taken up in sufficient water to make a final volume of 5.0 ml. All operations were carried out in the dark. A small torch was used for illumination where necessary.

The aqueous solution was extracted three times with equal volumes of ether to remove lipids and was evaporated to dryness. The residue was dissolved in a known volume of water.

Separation of adenosylcobalamin from other analogues. Adenosylcobalamin was separated from other analogues by paper electrophoresis as described by Ford *et al.* (1955). The electrophoresis was performed at 20 V/strip for 16 h at 20–25°C with acetic acid (0.5 M) as solvent. Samples were applied to Whatman no. 3 paper strips with reference solutions containing cyanocobalamin and adenosylcobalamin. Immediately after electrophoresis, the strips were rapidly dried by a stream of warm air and were hung in a fume-hood to remove residual acetic acid. The spots corresponding to standard cyanocobalamin and adenosylcobalamin detected by u.v. light were cut out and counted for radioactivity. The counting was carried out in a well-shaped gamma counter with efficiency of 15%.

Proteins were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Assessment of vitamin E deficiency

The onset of deficiency was assessed from the extent of peroxidative haemolysis of erythrocytes which was 95% of control values at the time of killing the rats. Also, the testes showed a decrease in weight from 1.8 g in the normal to 1 g in the deficient rats. α -Tocopherol content of the livers of the deficient rats was less than 2 $\mu\text{g/g}$ as compared with 200 $\mu\text{g/g}$ in the control rats (A. S. Pappu, P. Fatterpaker & A. Sreenivasan, unpublished work).

Urinary excretion of methylmalonate

The excretion of methylmalonate in vitamin E-deficient rats was $41.2 \pm 5.5 \mu\text{mol}/24\text{h}$ in contrast with $10.6 \pm 1.02 \mu\text{mol}/24\text{h}$ in control rats. The observations of Barness (1967) that excretion of methylmalonate over the control values up to 5 mg (42.5 μmol) can be considered as due to nutritional disorders, especially vitamin E deficiency, are relevant in this connexion. In separate experiments, $[1-^{14}\text{C}]$ propionate was injected intraperitoneally into rats and the concentrations of methylmalonate in liver and urine of rats were measured. The radioactivity recovered in methylmalonate was much higher in both liver and urine of the deficient rats, the increase being 3- and 2-fold respectively, as compared with control rats.

Cyanocobalamin concentrations in serum and liver

To find out whether the increased excretion of methylmalonate in vitamin E deficiency could be a result of secondary vitamin B₁₂ deficiency, the cyanocobalamin concentrations in serum and liver in the vitamin E-deficiency condition were measured. The cyanocobalamin concentration in serum (ng/ml) was 0.13 ± 0.01 (mean $\pm$ S.E.M.) and 0.12 ± 0.01 for the vitamin E-deficient and control rats respectively. The corresponding values in the livers were $150 \pm 16 \text{ ng}$ and $140 \pm 15 \text{ ng}$ per g of tissue. Thus there is no significant difference between cyanocobalamin concentrations either in serum or liver of the deficient and control rats. This would mean that vitamin E deficiency apparently does not interfere with the absorption, transport or storage of cyanocobalamin in the rat. However, these results do not provide any information about the interference, if any, of a lack of α -tocopherol in the utilization of cyanocobalamin in cellular processes. Involvement of α -tocopherol in cyanocobalamin metabolism can be best studied

by following some of the metabolic pathways requiring cyanocobalamin coenzyme.

Conversion of [1-¹⁴C]propionate into [1-¹⁴C]succinate via methyl[1-¹⁴C]malonate

Increased excretion of methylmalonate in vitamin B₁₂ deficiency has been correlated with a decrease in the activity of methylmalonyl-CoA mutase, an isomerase requiring adenosylcobalamin. An increased conversion of propionate into methylmalonate by propionyl-CoA carboxylase in liver can also give rise to increases in methylmalonate concentrations in liver, which would lead to its enhanced excretion in urine. These possibilities were therefore examined by studying the conversion of [1-¹⁴C]propionate into methyl[1-¹⁴C]malonate and subsequently into [1-¹⁴C]succinate by the liver enzymes, propionyl-CoA carboxylase and methylmalonyl-CoA mutase respectively. Results detailed in Table 1 indicate that the extent of conversion of [1-¹⁴C]propionate into methylmalonate by the propionyl-CoA carboxylase of the homogenate and mitochondria is unaffected in vitamin E deficiency. However, further isomerization of methylmalonic acid into succinic acid, regulated by the vitamin B₁₂-requiring methylmalonyl-CoA mutase, seems to be impaired by 45% in homogenate and 31% in mitochondria. The decreased ability to metabolize methylmalonic acid by vitamin E-deficient rats may thus be responsible for its increased hepatic accumulation and subsequent excretion in the urine.

Effect of adenosylcobalamin addition, in vitro, on the conversion of [1-¹⁴C]propionate into [1-¹⁴C]succinate

Increased production of methylmalonate in liver can be due to: (i) a decrease in methylmalonyl-CoA mutase activity; (ii) a decrease in methylmalonyl racemase activity; or (iii) an increase in methylmalonyl-CoA hydrolase activity (Vivacqua *et al.*, 1966).

To decide between the above possibilities, the formation of [1-¹⁴C]succinate from [1-¹⁴C]propionate by vitamin E-deficient rat liver homogenate and mitochondria in the presence of exogenously added adenosylcobalamin was studied. In these experiments, conversion of propionate into methylmalonate was not examined, since this step was not influenced in the deficiency condition. Results of these experiments show that the conversion of [1-¹⁴C]propionate into [1-¹⁴C]succinate by enzymes of liver homogenate and mitochondria from the deficient rats is considerably enhanced when the coenzyme is added to the incubation medium, the values in the deficient group being almost restored to normal concentrations (Table 2). Addition of the coenzyme also results in increased formation of [1-¹⁴C]succinate by the homogenate enzyme from control rats. However, this is explicable on the basis of the reported observations (Cardinale *et al.*, 1969) that the apoenzyme under normal physiological conditions is not completely saturated and hence exhibits higher activity on addition of the coenzyme.

The results of these experiments thus point out that the impairment in the conversion of methylmalonate into succinate in the vitamin E-deficiency condition is a result of the impairment in methylmalonyl-CoA mutase activity owing to cyanocobalamin-coenzyme being limiting. Any possible oxidative degradation of the apoenzyme, either *in situ* or during processing of the liver homogenate, thus seems to be unlikely.

Conversion of cyanocobalamin into adenosylcobalamin

Decreased methylmalonyl-CoA mutase activity with normal liver concentrations of cyanocobalamin may be due to defective production of the adenosylcobalamin, since the coenzyme is known to be an absolute requirement for the enzyme activity, or to peroxidative destruction of the coenzyme in the deficiency condition. These possibilities were therefore investigated. Cyanocobalamin is converted into

Table 1. *Effect of vitamin E deficiency on conversion of [1-¹⁴C]propionate via methyl[1-¹⁴C]malonate into [1-¹⁴C]succinate in vitro by rat liver enzymes*

The incubation mixture contained 100mM-Tris/HCl buffer, pH 8.5, 4mM-MgCl₂, 4mM-ATP, 4mM-reduced glutathione, 15mM-KHCO₃, 1mM-CoA, 10mM-sodium propionate, 0.5μCi (9.5×10⁵c.p.m.) of sodium [1-¹⁴C]-propionate and enzyme suspension from homogenate and/or mitochondria equivalent to 5mg or 2.5mg of protein respectively. Sodium malonate (20mM) was added to inhibit conversion into succinic acid wherever necessary. Results are means±S.E.M. for four independent experiments. Values are significantly lower than controls at: *P<0.005 and **P<0.025.

Enzyme source	10 ⁻³ × Radioactivity (c.p.m./mg of protein of acetone-dried powder)				Efficiency of conversion into succinic acid (%)		Decrease (%)
	Methylmalonic acid		Succinic acid		Control	Deficient	
	Control	Deficient	Control	Deficient			
Homogenate	3.6 ± 0.5	3.9 ± 0.4	3.15 ± 0.36	1.5* ± 0.2	96.0	53.0	44.6
Mitochondria	31.0 ± 4.0	34.0 ± 3.0	32.0 ± 3.0	21.0** ± 2.0	94.4	67.8	30.8

Table 2. *Effect of adenosylcobalamin in vitro on conversion of [1-¹⁴C]propionate into [1-¹⁴C]succinate by liver enzymes in vitamin E deficiency*

Conversion of [1-¹⁴C]propionate into [1-¹⁴C]succinate was studied in the presence of 20 µg of a denosylcobalamin. The results are means ± S.E.M. of four independent experiments. Values are significantly lower than controls at: **P* < 0.005 and ***P* < 0.025.

Addition	10 ⁻³ × Radioactivity in succinic acid (c.p.m./mg of protein of acetone-dried powder)			
	Homogenate		Mitochondria	
	Control	Deficient	Control	Deficient
None	3.6 ± 0.4	1.7* ± 0.2	32 ± 3	21** ± 3
Adenosylcobalamin	5.6 ± 0.4	4.7 ± 0.4	36 ± 2	33 ± 2

Table 3. *Conversion in vivo of cyano[⁵⁷Co]cobalamin into adenosylcobalamin coenzyme in vitamin E deficiency*
 Cyano[⁵⁷Co]cobalamin (3 µCi; 13.1 mCi/mmol) was injected intraperitoneally to vitamin E-deficient and control rats. The rats were killed after 24 h and vitamin B₁₂ analogues were extracted from liver homogenates either in the presence of vitamin E (200 µg of α-tocopherol/g of liver) or butylated hydroxytoluene (200 µg/g liver) or in the absence of any added anti-oxidant. Results are means of four independent experiments ± S.E.M. Values are significantly different from controls at: **P* < 0.001 and ***P* < 0.025.

Group	10 ⁻⁵ × Uptake of cyano[⁵⁷ Co]cobalamin (d.p.m./g of liver)	Radioactivity recovered in liver (% of total)		
		Adenosylcobalamin	Cyanocobalamin	Other cobalamin analogues
Control	2.7 ± 0.2	71.1 ± 8.2	15.5 ± 1.8	13.4 ± 1.5
Vitamin E-deficient	2.4 ± 0.3	8.91 ± 0.92*	26.8 ± 3.1**	64.3 ± 8.3*
Vitamin E-deficient	—	7.45 ± 0.62*†	31.5 ± 3.2**†	61.0 ± 4.5*†
Vitamin E-deficient	—	7.12 ± 0.78*‡	28.8 ± 1.8**‡	64.1 ± 7.8*‡

† Extraction was done in the presence of α-tocopherol.

‡ Extraction was done in the presence of butylated hydroxytoluene.

its coenzyme forms in liver. The amount of label appearing in the adenosylcobalamin fraction, 24 h after an intraperitoneal injection of cyano-⁵⁷Co]cobalamin is taken as a measure of coenzyme-synthesizing capacity of liver. Table 3 shows that the uptake of the vitamin, as determined by the total radioactivity (d.p.m./g of liver), appears to be identical in both control and deficient animals. About 70% of the radioactivity in liver appears to be in the coenzyme fraction in control animals, which is consistent with reported observations (Quadros *et al.*, 1976). However, the radioactivity recoverable in adenosylcobalamin in the deficient liver is only one-ninth of that in the control. Addition of the anti-oxidants, α-tocopherol and butylated hydroxytoluene, to liver homogenates before extraction of the cobalamin and its analogues has no effect on the recoverable radioactivity, suggesting that the observed decrease in the coenzyme concentration is not due to oxidative breakdown *in vitro* of the coenzyme during the isolation procedure. Cyanocobalamin concentrations as reflected in the percentage of the total radioactivity in this fraction are increased nearly 2-fold, which probably represents decreased conversion of the vitamin into its coenzyme. The increase in other

cobalamin analogues is 5-fold, which may be due to decreased conversion of the vitamin into the adenosylcobalamin form or due to increased breakdown of the coenzyme into these analogues.

Discussion

The observed increase in excretion of methylmalonate in urine cannot be due to dietary vitamin B₁₂ deficiency since the diet of rats contained sufficient amount of vitamin B₁₂ (0.05 mg/100 g of diet). Besides, induction of dietary vitamin B₁₂ deficiency is difficult, for it has been shown that rats maintain residual vitamin B₁₂ tenaciously and can procure it from intestinal flora or by coprophagy (Thomas & Stalder, 1958). In fact, it has been suggested that vitamin B₁₂ need be included in the diet only when intestinal synthesis is inhibited (Jelinek, 1967). A secondary deficiency of vitamin B₁₂, though plausible in view of reported selective decreases in the amino acids and fatty acids transport across the intestinal epithelium during vitamin E deficiency (Imami *et al.*, 1970), was also ruled out from the observations on normal cyanocobalamin concentrations (total cyanocobalamins inclusive of coenzyme forms) in the serum and livers of deficient animals and those on the uptake

of cyano[⁵⁷Co]cobalamin in livers of normal and deficient animals.

The results of the present investigation demonstrate that the increased production of methylmalonate in the livers of vitamin E-deficient animals is due to an impairment in methylmalonyl-CoA mutase activity arising out of a limited availability of the adenosylcobalamin. The possibility that the enzyme activity is lowered either by oxidation of the apoenzyme or of the coenzyme or by a defective binding of the apoenzyme to coenzyme through thiol groups (Barker, 1972b) is ruled out in view of the observations detailed in Tables 2 and 3. There is a full recovery of the enzyme activity after addition of adenosylcobalamin *in vitro* to the enzyme system, suggesting that the impairment in methylmalonyl-CoA mutase activity is caused by a deficiency of the coenzyme (Table 2). Further, the lowered concentrations of the coenzyme in vitamin E-deficient rat liver after intraperitoneal injection of cyano[⁵⁷Co]cobalamin and a failure of its reversal by addition *in vitro* of vitamin E or butylated hydroxytoluene to liver homogenates strongly suggest that there is a defective conversion of cyanocobalamin into its coenzyme form and that the lower values for the coenzyme in the deficiency condition is not due to procedural artifacts of oxidative destruction (Table 3). It is possible that the coenzyme may get oxidized *in situ* in vitamin E deficiency and thus become non-functional. Our findings on increased concentrations of cyanocobalamin and other vitamin B₁₂ analogues (Table 3) do not, however, favour this possibility.

The enzymic steps involved in the conversion of cyanocobalamin into its coenzyme form have not been worked out fully in animal systems (Brady & Barker, 1962), although in micro-organisms it has been shown that it involves enzymic reductions of cyanocobalamin (Walker *et al.*, 1969). If we assume that the conversion of cyanocobalamin into its coenzyme in the animal tissues also involves intermediate reducing steps as for micro-organisms, then it is likely that α -tocopherol may have a role at these reducing steps. Lack of α -tocopherol would then result in lowered coenzyme(s) like adenosylcobalamin and methylcobalamin leading to interference respectively in methylmalonate and sulphur-containing amino acid metabolism. Disturbance in the latter can in turn affect C₁ metabolism (Levander & Morris, 1970). These possibilities need further investigation.

Methylmalonic acid accumulation in liver in vitamin E deficiency can interfere with succinate oxidation (Schwartz, 1972). It is reported that succinyl-CoA formed from methylmalonyl-CoA is preferentially utilized for haem synthesis (Cavender, 1971). The decreased formation of succinate could, perhaps, partly explain the impairment in haem synthesis in vitamin E deficiency (Nair, 1972).

Studies by Cardinale *et al.* (1970) suggested that

methylmalonyl-CoA could be utilized by rat liver preparations to produce branched-chain fatty acids. Incorporation of abnormal fatty acids like branched-chain fatty acids into membranes can cause alterations in membrane function (van Deenen, 1969). The observed aberrations in the membrane structure and function in vitamin E deficiency (Molenar *et al.*, 1972; Lucy, 1972) may be partly due to incorporation of branched-chain fatty acids derived from methylmalonyl-CoA precursors. Increased accumulation of methylmalonate and propionate in brain and cerebrospinal cord were observed in vitamin B₁₂-deficient patients with neurological syndrome (Gammon & Agranoff, 1963). Branched-chain and odd-numbered fatty acids accumulation was also found in brains of patients with deranged vitamin B₁₂ metabolism (Kishimoto *et al.*, 1973). How far these abnormal metabolites are responsible for the myopathies observed in vitamin E deficiency is yet to be ascertained.

The megaloblastic anaemia in vitamin E deficiency is similar in some respects to that occurring as a result of vitamin B₁₂ deficiency (Dinning, 1962; Beck, 1968). A feature shared by both is increased incorporation of [³H]thymidine into DNA of bone marrow. How the decrease in cyanocobalamin coenzyme is responsible for the observed anaemia is yet to be investigated.

Although several of the known interrelationships of vitamin E and vitamin B₁₂ would seem to be explicable on the basis of the observations reported here, more detailed studies of the molecular mechanisms involved are necessary.

References

- Adhikari, H. R., Vakil, U. K. & Sreenivasan, A. (1968) *Indian J. Biochem.* **5**, 132-136
- Barker, H. A. (1972a) *Annu. Rev. Biochem.* **41**, 55-90
- Barker, H. A. (1972b) *Enzymes 3rd Ed.* **6**, 509-537
- Barness, L. A. (1967) *Am. J. Clin. Nutr.* **20**, 573-577
- Beck, W. S. (1968) *Vitam. Horm. (N.Y.)* **26**, 413-442
- Brady, R. O. & Barker, H. A. (1962) *Biochem. Biophys. Res. Commun.* **4**, 464-468
- Cardinale, G. J., Drefus, P. M., Auld, P. & Abeles, R. H. (1969) *Arch. Biochem. Biophys.* **131**, 92-99
- Cardinale, G. J., Carty, T. J. & Abeles, R. H. (1970) *J. Biol. Chem.* **245**, 3771-3775
- Cavender, F. L. (1971) *Biochem. Med.* **5**, 515-520
- Dinning, J. S. (1962) *Vitam. Horm. (N.Y.)* **20**, 511-519
- Flavin, M. & Ochoa, S. (1957) *J. Biol. Chem.* **229**, 965-979
- Flavin, M., Ortiz, P. J. & Ochoa, S. (1955) *Nature (London)* **176**, 823-826
- Ford, J. E., Holdsworth, E. S. & Kon, S. K. (1955) *Biochem. J.* **59**, 86-93
- Gammon, G. D. & Agranoff, B. W. (1963) *Proc. Soc. Exp. Biol. Med.* **114**, 600-602
- Giorgio, A. J. & Plaut, G. W. E. (1966) *J. Lab. Clin. Med.* **66**, 667-676

- Gurnani, S., Mistry, S. P. & Johnson, B. C. (1960) *Biochim. Biophys. Acta* **38**, 187-188
- Halenz, D. R. & Lane, M. D. (1960) *J. Biol. Chem.* **235**, 878-884
- Hove, E. L. & Hardin, J. C. (1951) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **10**, 385
- Hunter, S. G., Bach, M. K. & Ross, G. I. H. (1956) *J. Protozool.* **3**, 101-106
- Imami, R. H., Reiser, S. & Christeinson, P. A. (1970) *J. Nutr.* **100**, 101-109
- Jelinek, V. (1967) in *Husbandry of Laboratory Animals* (Conalty, M. L., ed.), pp. 97-114, Academic Press, New York
- Kishimoto, Y., Williams, M., Moser, H. W., Hignite, C. & Biemann, K. (1973) *J. Lipid Res.* **14**, 69-77
- Levander, O. A. & Morris, V. C. (1970) *J. Nutr.* **100**, 1111-1117
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Lucy, J. A. (1972) *Ann. N.Y. Acad. Sci.* **203**, 4-11
- Macek, K. (1963) in *Paper Chromatography* (Hais, I. M. & Macek, K., eds.), pp. 344-353, Academic Press, New York and London
- Molenaar, I., Vos, J. & Hommes, F. A. (1972) *Vitam. Horm. (N.Y.)* **30**, 45-82
- Nair, P. P. (1972) *Ann. N.Y. Acad. Sci.* **203**, 53-61
- Oski, F. A., Myerson, R., Barness, L. A. & Barness, W. J. (1966) *Am. J. Clin. Nutr.* **18**, 307
- Quadros, E. V., Matthews, D. M., Wise, I. J. & Linnell, J. C. (1976) *Biochim. Biophys. Acta* **421**, 141-152
- Reed, E. B. & Tarver, H. (1970) *J. Nutr.* **100**, 935-947
- Rose, C. S. & Gyorgy, P. (1952) *Am. J. Physiol.* **168**, 414-420
- Satav, J. G., Katyre, S. S., Fatterpaker, P. & Sreenivasan, A. (1976) *Biochem. J.* **156**, 215-223
- Schwartz, K. (1972) *Ann. N.Y. Acad. Sci.* **203**, 45-52
- Thomas, K. & Stalder, K. (1958) *Hoppe-Seyler's Z. Physiol. Chem.* **312**, 22-24
- Toohy, J. I. & Barker, H. A. (1961) *J. Biol. Chem.* **236**, 560-563
- van Deenen, L. L. M. (1969) in *The Molecular Basis of Membrane Function* (Tosteson, D. C., ed.), pp. 47-78, Prentice-Hall, Englewood Cliffs
- Vivacqua, R. J., Myerson, R. M., Prescott, D. J. & Rabinowitz, J. L. (1966) *Am. J. Med. Sci.* **251**, 507-515
- Walker, G. A., Murphy, S. & Huennkens, F. M. (1969) *Arch. Biochem. Biophys.* **134**, 95-102
- Williams, D. L., Spray, G. H., Newman, G. W. & O'Brien, J. R. P. (1969) *Br. J. Nutr.* **23**, 343-351