An abnormality of the bone marrow associated with vitamin E deficiency in sheep

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(*Received* 19 *March* 1974 – *Accepted* 16 *May* 1974)

1. Sheep fed on a maintenance ration of wheaten-hay chaff or of wheaten-hay chaff–lucerne-hay chaff (1:1, w/w) became deficient or incipiently deficient in vitamin E.

2. Degenerative changes were observed in bone marrow and muscle, and liver function was impaired in some animals. These abnormalities were not influenced by the vitamin B_{12} status of the animals or by a shortage of cobalt in the rumen.

3. Plasma ascorbic acid levels may not have been optimum, and folic acid may not have been fully utilized by some sheep.

4. Liver function responded fairly rapidly to α-tocopheryl acetate, but skeletal muscle had not returned to normal after 28 weeks of treatment. A variable trend towards normal cellularity was found in bone marrow following supplementation with α-tocopheryl acetate.

5. A secondary deficiency or, alternatively, inefficient excretion or metabolism of a toxic material, may occur in vitamin E deficiency as a result of degenerative changes in the absorptive or excretory areas of the intestinal tract and be responsible for the bone marrow abnormality.

Hypoplasia of the bone marrow of sheep given a ration of wheaten-hay chaff deficient in cobalt was reported by Ibbotson, Allen & Gurney (1970). The abnormality was not reversed by treatment of the animals with vitamin B_{12} intramuscularly or with Co intravenously, but had been found only in animals in which the stores of vitamin B_{12} had at some time been depleted.

The present study was designed to determine whether a hypoplasia of the bone marrow similar to that reported by Ibbotson *et al.* (1970) is reversible, and whether the abnormality is influenced by depletion of the animal's reserves of vitamin B_{12}, or by the presence of adequate concentrations of Co in the rumen. The intention was to test the reversibility of the condition by substituting 50% of a maintenance wheaten-hay-chaff diet with lucerne-hay chaff; this diet, with the addition of small quantities of retinol and cholecalciferol, is usually thought to supply all the nutrients required to keep an adult sheep in good health. However, during the course of the experiment it became apparent that some of the sheep were suffering from the effects of vitamin E deficiency. Treatment with this vitamin, therefore, became the test of reversibility of the bone marrow abnormality.

* On secondment from the Commonwealth Scientific and Industrial Research Organization for the duration of the experiment.

Table 1. *Details of diets and treatments for groups of sheep*

(No. of sheep in each group in parentheses)

Group	Daily ration
1 Control (6)	500 g wheaten-hay chaff* + 500 g lucerne-hay chaff
2 Cobalt-deficient (12) (+vitamin B_{12} after deficiency established)	1 kg wheaten-hay chaff* + 50 g wheat gluten + 11 g salt mixture†
3 Co-deficient + vitamin B_{12}‡ (2)	As for group 2
4 Co-deficient + Co (2)	As for group 2 + 1 mg Co (orally as chloride in 16 ml water)

In addition to the daily ration, retinol and cholecalciferol were supplied orally as 'Vetemul' (Nicholas Pty Ltd, Melbourne, Victoria); 2 ml twice/week to the individuals of group 1, and 4 ml twice/week to those of groups 2–4; 1 g 'Vetemul' contained 1·5 mg retinol and 25 μg cholecalciferol.

* The wheaten-hay chaff was particularly selected, legume-free chaff low in Co (0·024–0·04 μg/g).

† 11 g salt mixture supplied (g): NaCl 3, Na_2SO_4 1, $CaCO_3$ 2, $CaHPO_4.2H_2O$ 2·5, $MgSO_4.7H_2O$ 2, KCl 0·5. In addition, supplements of iron and zinc, equivalent to 30 mg of each/d, and of copper, equivalent to 5 mg/d, were administered as an oral drench three times/week.

‡ Vitamin B_{12}, 250 μg as cyanocobalamin or hydroxocobalamin (Glaxo Australia Pty Ltd, Boronia, Victoria) in 1 ml physiological saline solution, was administered once/week by intramuscular injection to the individuals of group 3, and later, after their stores of the vitamin had been depleted, to those of group 2.

EXPERIMENTAL

Animals and treatments

Twenty-six healthy, 18-month-old Merino ewes were taken off pasture. Four animals were killed and sections of bone marrow prepared for reference. The remaining animals were dipped, drenched for worms and infected with *Eperythrozoön ovis* (Sheriff, Clapp & Reid, 1966) to ensure their subsequent immunity to this blood parasite. They were then placed in individual pens and offered daily 500 g wheaten-hay chaff + 500 g lucerne-hay chaff. Seven weeks later the sheep, having proved not to be susceptible to infection with *E. ovis*, were divided at random into groups which received the treatments shown in Table 1.

The concentration (mean and SE) of α-tocopherol in five samples of wheaten-hay chaff was 2·3 ± 0·16 mg/kg. A sample of lucerne-hay chaff contained 5 mg α-tocopherol/kg. Supplementary α-tocopheryl acetate was provided for some sheep at the beginning of week 89. The vitamin was administered as an oral drench of an aqueous suspension of 'Dry Vitamin E 25%' (Roche Products Pty Ltd, Dee Why, New South Wales); 1 g 'Dry Vitamin E 25%' contains 250 mg DL-α-tocopheryl acetate. In calculating the dose, allowance was made for 10–20% destruction in the rumen (Alderson, Mitchell, Little, Warner & Tucker, 1971). Between weeks 90 and 111, inclusive, the weekly dose of α-tocopheryl acetate was approximately 250 mg, administered as 0·7 g 'Dry Vitamin E 25%' twice/week; twice this amount was administered during week 89. Between weeks 112 and 116, inclusive, the weekly dose of α-tocopheryl acetate for the animals of groups 2–4 was increased to approximately 1 g (1·6 g 'Dry Vitamin E 25%' administered three times/week).

Throughout the experiment the animals were weighed weekly and bled every 2

weeks for haematological examination and every month for the estimation of serum folic acid and vitamin B_{12}.

All sheep of group 1 and those of groups 2–4 that were not treated with α-tocopheryl acetate were slaughtered between weeks 110 and 112; the treated sheep of groups 2–4 were killed at the end of week 117.

Methods

Haematological indices. The methods were those used routinely in the Division of Haematology of this Institute.

Cellular enzymes. Creatine kinase (ATP:creatine phosphotransferase; *EC* 2.7.3.2) (CPK) activity was estimated by the technique described by Rosalki (1967), using a Calbiochem CPK Stat-Pack (Calbiochem, San Diego, California); glutamic–oxaloacetic transaminase (L-aspartate:2-oxoglutarate aminotransferase; *EC* 2.6.1.1) (GOT) activity was estimated by a colorimetric method using azoene fast red salt, and based on that of Babson, Shapiro, Williams & Phillips (1962); lactate dehydrogenase (L-lactate:NAD oxidoreductase; *EC* 1.1.1.27) (LDH) activity was estimated by the method described by Hochella & Weinhouse (1965). One unit of enzyme activity was defined as that amount which converts 1 μmol substrate/min at 37°.

Selenium. Whole blood Se was estimated by the method of Watkinson (1966).

Amino acids. The standard procedure recommended for use with the TSM Amino Acid Analyzer (Technicon Instruments Corp., Tarrytown, New York) was used for estimating amino acid concentrations in serum.

Vitamins. Vitamin B_{12} levels were estimated by a radioisotopic method based on the principle of saturation analysis (Tibbling, 1969), and folic acid content was estimated by a procedure using *Lactobacillus casei*, adapted from that of Baker, Herbert, Frank, Pasher, Hutner, Wasserman & Sobotka (1959).

A method based on that of Hansen & Warwick (1966), with the following modifications recommended by G. B. Storer (personal communication), was used for the estimation of α-tocopherol concentration of the plasma. Plasma (1 vol.) was added to 3 vol. ascorbic acid solution (10 g/l; adjusted to pH 4·5 with NaOH). Precipitation of protein by the addition of ethanol was done whilst the contents of the tube were agitated, using a vortex mixer, and the tube was then, and after each subsequent operation, flushed with nitrogen gas. Estimations were done in a partially darkened room and all the apparatus used was of glass and before use was rinsed twice with ethanol. Plasma ascorbic acid content was estimated by the method of Owen & Iggo (1956).

Ubiquinone-10 (*Q*-10). A 10 g sample was taken from the ventricular muscle of the heart of each sheep, after it was killed, and stored at −20° in the ethanol subsequently used for homogenization (cf. Mervyn & Morton, 1959; Diplock & Haslewood, 1967). *Q*-10 content was estimated by the method described by Crane & Barr (1971) except that six extractions with *n*-heptane were done, and instead of drying the heptane extract with Na_2SO_4, the residue, after removal of the heptane by evaporation under reduced pressure, was taken to dryness twice, each time after the addition of 5 ml ethanol. Two drops of *n*-octanol were added to the mixture before saponification.

Liver function test. A solution of sulphobromophthalein (BSP) (50 g/l) was injected intravenously (15 mg/kg body-weight) and four blood samples were removed at successive 4 min intervals after the injection. Concentrations of BSP in plasma samples were estimated by the method described by Fleck & Morrison (1970) except that sodium *p*-toluene sulphonate was not added to the pH 11·2 buffer and, where necessary, plasma samples were diluted with bovine serum albumin solution (10 g/l). The relative clearance rates were compared using coefficients obtained from the regression analysis for log plasma BSP concentration *v.* time.

Bone marrow biopsy. Biopsies of the sternums were done at various times on animals under anaesthesia with pentothal (Abbott Laboratories Pty Ltd, Kurnell, New South Wales), using a trephine designed by Dr J. A. Bonnin of this Institute. A core 6 mm in diameter was obtained.

Histology. Sections for histological examination were fixed in formalin (100 ml/l; pH 7·0), stained with haematoxylin and counterstained with eosin.

RESULTS

General performance of animals

Loss of appetite, the first symptom of vitamin B_{12} deficiency to appear in sheep (Marston, 1970), became apparent in one of the animals of group 2 after 15 weeks on the experimental diet and, thereafter, during the next 4 months each animal in the group lost its appetite. To ensure virtually complete depletion of the stores of vitamin B_{12}, each sheep of group 2 was allowed to lose about 5·5 kg body-weight before being treated with the vitamin. During the period of depletion the concentration of vitamin B_{12} in the serum of each animal fell to 0·1 μg/l or less. Values of $< 0{\cdot}2$ μg vitamin B_{12}/l are indicative of a state of deficiency (Dawbarn, Hine & Smith, 1957). By the 39th week all sheep of group 2 (Co-deficient) were being treated with vitamin B_{12}, and 5 weeks later the mean concentration was 2·1 μg/l serum and thereafter it remained between 2 and 3 μg/l.

The mean concentration of serum vitamin B_{12} in the animals of group 1 (controls) was 1·2 μg/l (range 0·25–2·2 μg/l). The mean serum concentration of the vitamin for individuals of group 4 (Co-deficient + Co) tended to be higher than that for those of group 3 (Co-deficient + vitamin B_{12}); the mean values with their SE for six observations between weeks 85 and 105 were $5{\cdot}5 \pm 0{\cdot}23$ and $2{\cdot}6 \pm 0{\cdot}22$ μg/l, respectively.

The maintenance ration provided to all sheep kept them at a steady body-weight. At the 12th and 103rd weeks the mean post-shearing body-weights of the sheep from all groups did not differ by more than 1 kg; at 48 weeks the mean post-shearing body-weight of group 2 sheep was affected by vitamin B_{12} deficiency.

Vitamin E deficiency was suspected when, about 1 year after the start of the experiment, high concentrations of the cellular enzymes CPK, GOT and LDH were found in the serums of the sheep (see Table 3). Half the animals from each group were then allocated for treatment with α-tocopheryl acetate. Treatment was started after week 88 when it had been established by bone marrow biopsy that the bone marrows of each of the sheep of groups 2–4 (except nos. 4 and 24) that were to be treated were abnormal

Table 2. *Haemoglobin content and white cell count in blood from sheep given a control diet* (1) *or cobalt-deficient diet* (2) *and, for some sheep, supplementary α-tocopheryl acetate* (+*E*) *from week* 89

(Mean values with their standard errors for three determinations)

Group*	No. of sheep	Experimental period (weeks)	Haemoglobin (g/l)		White cell count (× 10^9/l)	
			Mean	SE	Mean	SE
1	3	5–9	110	2·7	5·8	0·190
1+E	3	5–9	107	2·1	4·9	0·233
1	3	83–87	102	2·6	4·4	0·190
1+E	3	83–87	100	2·6	4·3	0·260
1	3	103–108	101	3·8	5·5	0·330
1+E	3	103–108	99	3·7	5·0	0·410
2	6	6–10	98	1·8	5·8	0·340
2+E	4	6–10	95	2·0	5·2	0·310
2	6	84–88	100	1·2	5·4	0·230
2+E	4	84–88	102	1·3	4·6	0·360
2	6	104–108	97	1·4	5·4	0·290
2+E	4	104–108	105	2·0	4·6	0·320
2+E	4	114–116	104	1·2	4·3	0·190

* For details of groups and dietary treatments, see Table 1; for α-tocopheryl acetate treatment, see p. 358.

Sections from specimens taken by trephine from the sternums of two sheep at week 55 and at week 67 from another two sheep of group 2 showed incipient to unequivocal hypoplasia. By week 86 sections of trephine specimens from all sheep of groups 2–4 (except no. 24) that had been allocated for treatment with α-tocopheryl acetate were showing loss of cellularity. The bone marrow of sheep no. 24 (group 2) showed no gross abnormality after 96 weeks on the diet and the animal was not treated with α-tocopheryl acetate.

Two sheep of group 2 became very sick during the course of the experiment and were killed; no. 4 at week 87, and no. 17 at week 95, although the latter had been treated with α-tocopheryl acetate for 6 weeks. Of the remaining animals none showed any obvious signs of disability except three, nos. 4 and 13 of group 2 and no. 9 of group 3, that suffered from laminitis, which responded to corticosteroid (Betsolan; Glaxo Australia Pty Ltd, Boronia, Victoria) therapy. Towards the end of the experiment, however, those animals of groups 2–4 that were not supplemented with α-tocopheryl acetate seemed to be more lethargic and spent more time lying down than the treated animals in their respective groups.

Haemoglobin content and white cell count

No significant differences were observed between treatments for either haemoglobin or white cell count (Table 2). Values for other red cell indices showed a pattern similar to that of haemoglobin and therefore are not reported. Variations between results for any one animal, and between animals, were in most instances greater than those between treatments, for both haemoglobin content and white cell count, and

Table 3. *Activities (U†/l) of creatine kinase (ATP:creatine phosphotransferase; EC 2.7.3.2) (CPK), glutamic–oxaloacetic transaminase (L-aspartate:2-oxoglutarate aminotransferase; EC 2.6.1.1) (GOT) and lactate dehydrogenase (L-lactate:NAD oxidoreductase; EC 1.1.1.27) (LDH) in serums from sheep given a control diet (1) or cobalt-deficient diet (2), with supplementary vitamin B_{12} (3) or Co (4) and, for some sheep, α-tocopheryl acetate (+E) from week 89*

(Mean values with their standard errors)

Group‡	Stage of experiment (weeks)	No. of sheep	CPK Mean	CPK SE	GOT Mean	GOT SE	LDH Mean	LDH SE
1	46	6	55	13	190	47	370	38
1	83	6			140	28	390	17
1	109	3			150	14	400	30
1+E	109	3			70**	6·7	350	24
2	46	12	375**	100	800*	140	510	61
2	83	12			230	30	460	33
2	109	6			250	83	400	29
2+E	109	4			80	6·9	360	38
3	46	2	785	55	3030	1300	990	150
3	83	2			240	70	480	75
3	109	1			110	—	350	—
3+E	109	1			75	—	340	—
4	46	2	505	365	690	37	520	7·5
4	83	2			310	0	700	20
4	109	1			770	—	520	—
4+E	109	1			75	—	460	—

At week 46 the mean serum activity of CPK for the sheep of group 2 was significantly higher (** $P < 0.01$, SE of difference 102) than that for group 1; the corresponding comparison for GOT was significant: * $P < 0.05$, SE of difference 204. At week 109 the mean serum activity of GOT for the sheep of group 1+E was significantly reduced compared with that for group 1 (** $P < 0.01$, SE of difference 15).

† For definition of units, see p. 359.

‡ For details of groups and dietary treatments, see Table 1; for α-tocopheryl acetate treatment, see p. 358.

for this reason results for the two sheep that were killed during the experiment have not been included in the results shown in Table 2.

Cellular enzymes in serum

Analysis of variance of the results obtained at week 46 for the serum activities of CPK, GOT and LDH in the four groups of sheep (Table 3) indicated that the mean activities of CPK and GOT for the animals of group 2 (Co-deficient) were significantly higher than those for group 1 (controls); the corresponding comparison for LDH indicated no significant difference between the enzyme activities for the two groups.

The mean activity of GOT in the serums from the sheep of group 1 was significantly reduced following treatment with α-tocopheryl acetate (Table 3); however, although the mean activity for the treated sheep of group 2 was very much lower than that for untreated animals, the difference was not statistically significant.

CPK activities after treatment with α-tocopheryl acetate were not measured.

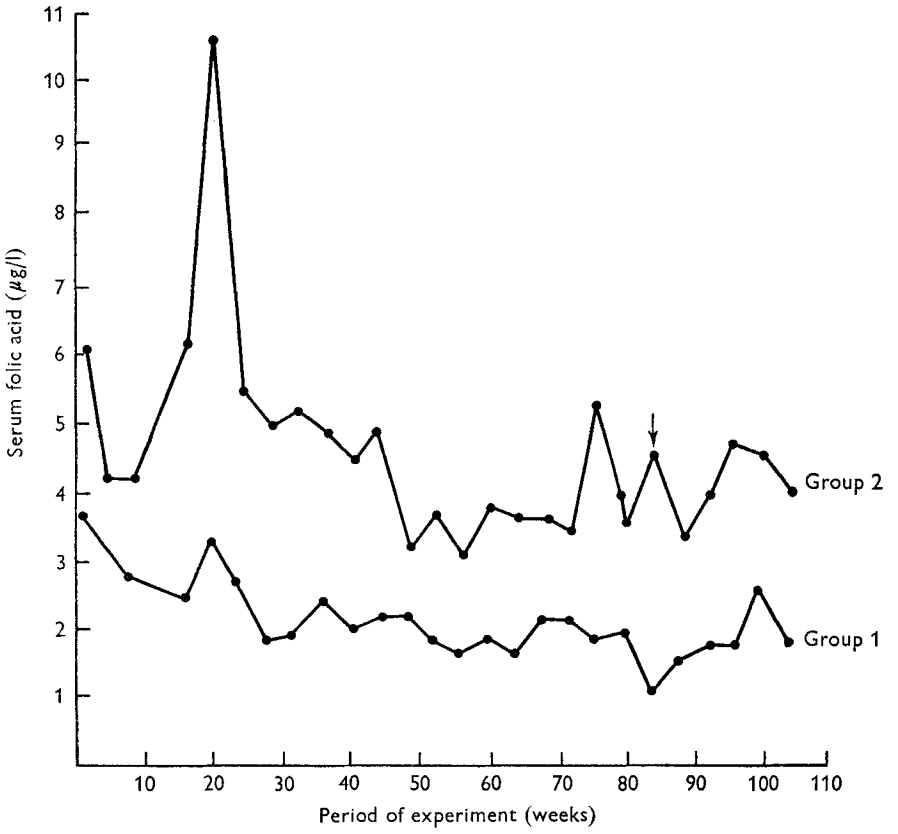

Fig. 1. Mean concentrations of folic acid in the serum of sheep given a control diet (500 g wheaten-hay chaff + 500 g lucerne-hay chaff/d; group 1) or cobalt-deficient diet (1 kg wheaten-hay chaff + 50 g wheat gluten + 11 g salt mixture/d; group 2), with, for some animals, supplementary α-tocopheryl acetate from week 89(↓). For details of diets, see Table 1; for α-tocopheryl acetate treatment, see p. 358.

Se in blood

Approximately 1 year after the start of the experiment the mean values with their SE for the concentrations of Se in the bloods from four sheep from each of groups 1 and 2 were (μg Se/l) 230 ± 27·0 and 310 ± 8·50 respectively. The normal range is 60–340 μg Se/l (Underwood, 1971).

Amino acids in serum

At week 56 the serum concentrations of amino acids for two sheep from each of groups 1 and 2 were estimated. Differences between the two groups were small with most of the values falling near the upper ranges reported by Hogan, Weston & Lindsay (1968). In particular, the concentration of sulphur-containing amino acids seemed to be adequate.

Folic acid in serum

Throughout the experiment the mean serum concentrations of folic acid for sheep of group 2 (Co-deficient) were significantly higher than those for sheep of group 1

Table 4. *Concentrations of α-tocopherol in plasma from sheep given a control diet* (1) *or cobalt-deficient diet* (2) *and, for some sheep, supplementary α-tocopheryl acetate* (+*E*) *from week* 89

(Mean values with their standard errors; no. of samples taken during experimental period in parentheses)

Group†	No. of sheep	Experimental period (weeks)	α-Tocopherol (mg/l) Mean	SE
1	6	56–84	1·0***	0·06 (3)
2	12	56–84	0·6	0·02 (3)
1	3	93–108	0·9	0·01 (4)
1+E	3	93–108	1·5***	0·1 (4)
2	6	93–108	0·6	0·02 (4)
2+E	4	93–108	1·3***	0·1 (4)
2+E	4	115–116	1·8	0·2 (2)

For weeks 56–84 the mean concentration of α-tocopherol for the sheep of group 1 was significantly higher (*** $P < 0{\cdot}001$, SE of difference 0·06) than that for group 2. For weeks 93–108, for the sheep of groups 1 and 2, treatment with α-tocopheryl acetate resulted in significantly higher (SE of difference 0·1) plasma concentrations of α-tocopherol compared with those for non-treated animals.

† For details of groups and dietary treatment, see Table 1; for α-tocopheryl acetate treatment, see p. 358.

(Fig. 1). The significance of the difference was as follows: at weeks 53, 57 and 73, $P < 0{\cdot}01$; at weeks 61 and 65, $P < 0{\cdot}001$; at week 69, $P < 0{\cdot}05$. Treatment with α-tocopheryl acetate appeared to have no affect on folic acid concentrations.

Ascorbic acid in plasma

Analysis of variance of five values for the plasma concentration of ascorbic acid for the sheep of groups 1 and 2 between weeks 48 and 84 indicated that the concentration was significantly higher ($P < 0{\cdot}01$) for group 1; the mean values with the SE of difference were 6·4, 4·2 and ±0·56 mg/l, respectively. Analysis of variance of the change in concentration of ascorbic acid in the plasmas of sheep following treatment for 20 weeks with about 250 mg α-tocopheryl acetate/week indicated that treatment at this level had no apparent effect on the concentration of ascorbic acid in the animals of either group 1 or 2.

α-Tocopherol in plasma

Plasma concentrations of α-tocopherol for groups 1 and 2 are shown in Table 4. Analysis of variance of values for α-tocopherol concentration for three samples taken between weeks 56 and 84, before treatment with α-tocopheryl acetate and for four samples taken between weeks 93 and 108, after treatment of some sheep with α-tocopheryl acetate indicated that the concentration was significantly higher ($P < 0{\cdot}001$) for group 1 between weeks 56 and 84, and for the treated animals of both groups between weeks 93 and 108.

Table 5. *Regression coefficients for the clearance of sulphobromophthalein (liver function test)† for sheep given a control diet (1) or cobalt-deficient diet (2) and, for some sheep, supplementary α-tocopheryl acetate (+E) from week 89*

(Weighted mean values with their standard errors; no. of sheep in parentheses)

Group‡	Experimental period (weeks)	Regression coefficient	
		Mean	SE
1	50–53	−0·0771	0·000556 (6)***
2	50–53	−0·0486	0·000717 (12)
2	50–53	−0·0439	0·00224 (4)
2+E	99–101	−0·0614	0·00244 (4)*

The regression coefficient for group 2+E was significantly different (* $P < 0·05$, SE of difference 0·0062) from that for the same four sheep before treatment and the regression coefficient for group 1 was significantly different (*** $P < 0·001$, SE of difference 0·00292) from that for the twelve sheep of group 2.

† For details, see p. 360.

‡ For details of groups and dietary treatments, see Table 1; for α-tocopheryl acetate treatment, see p. 358.

Liver function test

The rate of clearance from the blood stream of intravenously administered BSP was determined between weeks 50 and 53, and again between weeks 99 and 101. After an experimental period of about 1 year the mean rate of clearance for the sheep of group 1 (controls) was significantly higher ($P < 0·001$) than that for group 2 (Co-deficient). Following treatment with α-tocopheryl acetate for about 10 weeks the mean rate for the four treated sheep of group 2 was just significantly higher ($P < 0·05$) than that for the same sheep at the previous test (Table 5). Between tests the rates did not change significantly for the untreated sheep of group 2, nor was there any significant difference between the treated and untreated sheep of group 1.

Post-mortem findings

General. The skeletal muscles from all sheep not treated with α-tocopheryl acetate were pale compared with those from treated animals, particularly when compared with those from animals that had been treated with the vitamin for 28 weeks; the latter sheep were in a better condition than the former.

The following comments refer to sheep of group 2 (Co-deficient). Evidence of white muscle disease was apparent in the biceps muscle from one leg of sheep no. 24; the fat from sheep no. 23 was oily; sheep no. 14 was emaciated, and sheep no. 4 was in a state of extreme weakness and lethargy when killed at week 87. This animal had pin-point haemorrhages of the duodenum, very thin-walled abomasum and small intestine and no subcutaneous fat. Sheep no. 17, which was killed after 6 weeks of treatment with α-tocopheryl acetate because it was very reluctant to stand and would eat only if attended, was in a relatively good condition. No gross lesions were apparent in skeletal muscle apart from slight atrophy of the brachio cephalic muscle from the neck and the latissimus dorsi muscle from the back. The heart muscle appeared very fatty and the liver was pale and fatty. One sheep (no. 13) had no spleen.

Table 6. *Concentrations of ubiquinone-10 (Q-10) in heart muscle of sheep given a control diet (1) or cobalt-deficient diet (2) and, for some sheep, supplementary α-tocopheryl acetate (+E) from week 89*

(Mean values with their standard errors; no. of samples in parentheses)

Group†	Q-10 (μg/g fresh tissue) Mean	SE
1	26	3·2 (3)
1+E	85	— (1)
1+E+sodium phenobarbital	22	7·8 (2)
2	20*	3·9 (6)
2+E	87	13 (4)

The value for group 2 was significantly lower (* $P < 0{\cdot}02$, SE of difference 13) than that for group 2+E.

† For details of groups and dietary treatments, see Table 1; for α-tocopheryl acetate treatment, see p. 358.

Bone marrow histology. Sections of bone marrow from the rib, sternum and vertebra were examined. Assessment of these sections showed that none of the sheep in the experiment had completely normal marrow; typical sections showed patchy loss of cellularity, i.e. normal areas alternating with hypoplastic, and, in some instances, hyperplastic areas; sections of the ribs from some of the sheep of group 1 were normal. A greater degree of abnormality was observed in the bone marrows from groups 2–4 than in those from group 1; the bone marrows from groups 3 and 4 were just as badly affected as those from group 2. A variable but definite return towards normality was found when α-tocopheryl acetate supplements were added to the diets. Representative photomicrographs of sections of normal bone marrow, aplastic bone marrow from a sheep of group 2 (Co-deficient) and patchy regeneration after treatment with α-tocopheryl acetate, all taken *post mortem*, are shown in Plate 1 *a*, *b* and *c*, respectively.

Skeletal muscle histology. A section of the muscle from only one of the sheep, an animal that had been treated with α-tocopheryl acetate for 28 weeks, was considered normal; sections from all other sheep indicated varying degrees of degeneration, associated with scattered swollen, hyalinized fibres; loss of internal structure of the fibre; an increase in cellularity and, occasionally, proliferation of sarcolemmal nuclei and indications of an inflammatory reaction. A picture of classical muscular dystrophy was provided by a section from sheep no. 4 (group 2, Co-deficient) (cf. Plate 1 *d*). There was no clear-cut evidence of a response to treatment with α-tocopheryl acetate.

The muscles from all the sheep were infested with *Sarcocystis tenella*.

Liver histology. No abnormalities were found in sections of liver.

Q-10. The mean concentration of Q-10 for the heart muscle from the sheep of group 2 that had been treated with α-tocopheryl acetate was significantly higher ($P < 0{\cdot}02$) than that for untreated sheep of group 2 (Table 6). Some sheep of group 1 and some of the vitamin E-deficient sheep of group 2 were dosed with sodium phenobarbital before slaughter (see Discussion). Dosing with phenobarbital had no apparent

effect on the Q-10 concentrations of the heart muscle from the vitamin E-deficient sheep of group 2, nor on the untreated sheep of group 1. However, the concentrations for the two vitamin E-treated sheep of group 1, which were also dosed with phenobarbital, fell within the range for the untreated sheep of group 2 (Table 6).

DISCUSSION

High serum concentrations of the cellular enzymes CPK and GOT, normal serum concentrations of amino acids and normal blood concentrations of Se for the sheep of groups 2–4, about 1 year after the start of the experiment, led us to suspect that the animals were suffering from vitamin E deficiency. This suspicion was subsequently confirmed by analysis of their plasmas for α-tocopherol content.

Although the sheep of group 1, receiving 500 g wheaten-hay chaff + 500 g lucerne-hay chaff/d, had about 1 mg α-tocopherol/l plasma this was not sufficient to protect them completely from the effects of vitamin E deficiency; sections of both muscle and bone marrow showed signs of degeneration.

Comparison of sections of bone marrow taken from sheep killed at the beginning of the experiment (comparable with sections from normal 3–4-year-old sheep) with those taken after they had been given the experimental diets for just over 2 years showed that none of the latter was completely normal. All were characterized by patchy loss of cellularity alternating with normocellular areas.

Assessment of sections of bone marrow from the three sheep of group 1 that were treated with about 250 mg α-tocopheryl acetate/week for 23 weeks suggested that, if the deficiency is not too severe, treatment will bring about recovery in time. In those animals (groups 2–4) for which the deficiency was more severe, and which were treated for a total of 28 weeks, first with about 250 mg and later with about 1 g α-tocopheryl acetate/week, the results were equivocal, and long-term studies are needed to determine whether the hypoplasia is reversible. Higher doses of the vitamin might have accelerated the rate of bone marrow regeneration. The sheep's daily requirements for vitamin E are not known (Alderson *et al.* 1971), and the dose of 250 mg α-tocopheryl acetate/week was chosen to supply about ten times the intake for a sheep of group 1.

The vitamin B_{12} status of the animal and the lack of Co in the rumen were each without effect on the abnormality of the bone marrow.

Although there was a high degree of bone marrow hypoplasia in the animals of group 2, neither the concentration of haemoglobin nor the white cell count was affected. The decrease in haemoglobin concentration over a period of 6 months reported by Ibbotson *et al.* (1970) for sheep given a ration similar to that of group 2 probably resulted from spuriously high values at the beginning of the experiment, before the animals had become accustomed to being handled (Turner & Hodgetts, 1959; Dooley, Hecker & Webster, 1972).

Muscular dystrophy is the most commonly observed disability associated with vitamin E deficiency and has been reported in many species (Roels, 1967; Pennington, 1971; Wasserman & Taylor, 1972). In the sheep, muscular damage resulting from a

low intake of vitamin E is probably more widespread than has previously been supposed. Sections taken at autopsy from apparently healthy sheep were found to have marked degenerative changes.

Liver function, as measured by the BSP test, was impaired at a fairly early stage of the deficiency; it did not worsen and responded relatively rapidly to treatment with α-tocopheryl acetate. Degenerative changes were not apparent in sections of the livers from any of the sheep used in the experiment.

The higher mean plasma ascorbic acid concentration for the sheep of group 1 (control) compared with that for group 2 (Co-deficient) may have resulted from differences in intake or may have been influenced by a lack of vitamin E. The concentrations for both groups were considerably lower than that (12–14 mg/l plasma) quoted as normal by Pope, Phillips & Bohstedt (1947).

The role of vitamin E in ascorbic acid synthesis is controversial (Mapson, 1967; Brown, Sharp, Young & Van Dreumel, 1971). In the experiment reported here treatment with α-tocopheryl acetate had no apparent effect on ascorbic acid concentration of the plasma. The synergistic properties of ascorbic acid and vitamin E have been discussed by Tappel (1968).

Throughout the experiment the mean serum folate concentrations for the sheep of group 2 (Co-deficient) were significantly higher than those for the controls, a difference that may have reflected the difference in diets, or may have been an indication that in those animals with lower concentrations of ascorbic acid and α-tocopherol less of the folic acid was available in a physiologically active form (Hoffman, 1970; Sim, 1972).

In eight of the twelve sheep that were depleted of vitamin B_{12} there was a marked decrease in the concentration of folic acid after treatment with vitamin B_{12} (cf. Smith, 1965).

The pathogenesis of laminitis has been discussed by Coffman & Garner (1972). Vitamin E (Wasserman & Taylor, 1972) and ascorbic acid (Mapson, 1967; Edgar, 1969; Peterkofsky, 1972) are both thought to be involved in the synthesis of collagen. It is tentatively suggested that a shortage of one or of both of these vitamins may have been the predisposing factor that led to three of the sheep given the wheaten-hay-chaff ration developing laminitis.

Reports about the ability of vitamin E-deficient animals to synthesize Q-10 are conflicting (Hemming & Pennock, 1965; Jáuregui-Adell, 1966; Poukka, 1968; Folkers, 1969). Concentrations of Q-10 in the heart muscles from our experimental sheep were undoubtedly higher in those from animals which had been treated with α-tocopheryl acetate.

The role of vitamin E in porphyrin synthesis is not clear (cf. Porter & Fitch, 1966; Nair, Mezey, Murty, Quartner & Mendeloff, 1971). Red blood cells from sheep given a diet similar to that of the animals of group 2, but not supplemented with vitamin B_{12}, were reported (Allen, 1956) to have high concentrations of free protoporphyrin, which gradually returned to normal levels after treatment of the sheep with vitamin B_{12}. If the vitamin B_{12}-deficient sheep were also deficient in vitamin E, which now seems probable, it is likely that the decrease in free protoporphyrin

concentration of the red blood cells resulted not from a direct effect of treatment with vitamin B_{12} but from an increase in the vitamin E status of the animals following restoration of appetite.

Carpenter (1967) found that there was a relationship between the activity of the hepatic microsomal mixed-function oxidase system in rats and their vitamin E status; a similar finding was reported by Cawthorne, Bunyan, Sennitt, Green & Grasso (1970) and Jeffery & Diplock (1972); and electron microscopy studies have indicated that there are changes in mitochondrial and microsomal structures in vitamin E-deficient animals (Schwarz & Baumgartner, 1969).

The hepatic microsomal enzymes isolated from sheep used in this experiment, some isolated after the animals had been dosed with phenobarbital for several days prior to killing, were studied by Gourlay, Savage & Stock (1975). No significant differences were found between preparations from the sheep of group 1 (controls) and those from group 2 (Co-deficient). However, the rates of reaction for several of the enzymes were higher for microsomes from the sheep of group 1, and significant differences were found in preparations from sheep treated with phenobarbital compared with untreated animals.

The optimum concentration for plasma ascorbic acid does not seem to have been established for sheep. No conclusion can therefore be drawn about the influence, if any, of the ascorbic acid status of the experimental animals on the various abnormalities reported. The occurrence of muscle dystrophy was almost certainly attributable to a lack of vitamin E. Loss of cellularity in the bone marrow, on the other hand, may have been a direct result of a deficiency of vitamin E, possibly an effect on the microenvironment, or may have resulted from a secondary deficiency arising from malabsorption of another nutrient. Absorption of nutrients has been shown to be influenced by degenerative changes in cell membranes brought about by vitamin E deficiency (Imami, Reiser & Christiansen, 1970). Malexcretion of a toxin or abnormal metabolite is a third possible cause of loss of cellularity in the marrow.

The authors thank Glaxo Australia Pty Ltd, Boronia, Victoria, for the gift of vitamin B_{12} and Roche Products Pty Ltd, Dee Why, New South Wales for 'Dry Vitamin E 25%'. Analyses of the α-tocopherol content of the diet were kindly made by Roche Products Pty Ltd, and those of the amino acid content of serum by Dr A. C. Pollard of The Adelaide Children's Hospital (Inc.), Adelaide, South Australia. We are indebted to the late Dr E. A. Cornish, Chief of the Division of Mathematical Statistics, Commonwealth Scientific and Industrial Research Organization, and other members of that Division for the statistical analyses, and to many members of the Institute of Medical and Veterinary Science's Divisions of Animal Science, Clinical Chemistry, Haematology and Histopathology for help in a variety of ways, and, in particular, to Dr J. W. Macaulay for the post-mortem examinations and to Dr D. A. Purcell for a report on the histology of muscle. S.H.A. wishes to express her gratitude to Dr J. A. Bonnin, Director, IMVS, for providing the facilities to carry out the experiment reported, and to Dr J. R. Price, Chairman, CSIRO, for approving her secondment.

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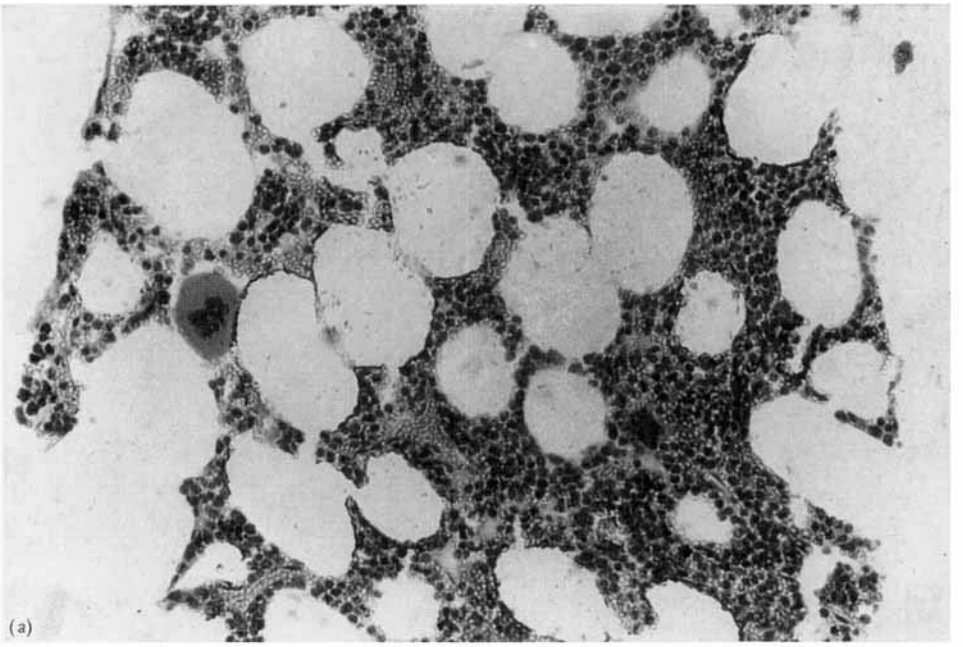

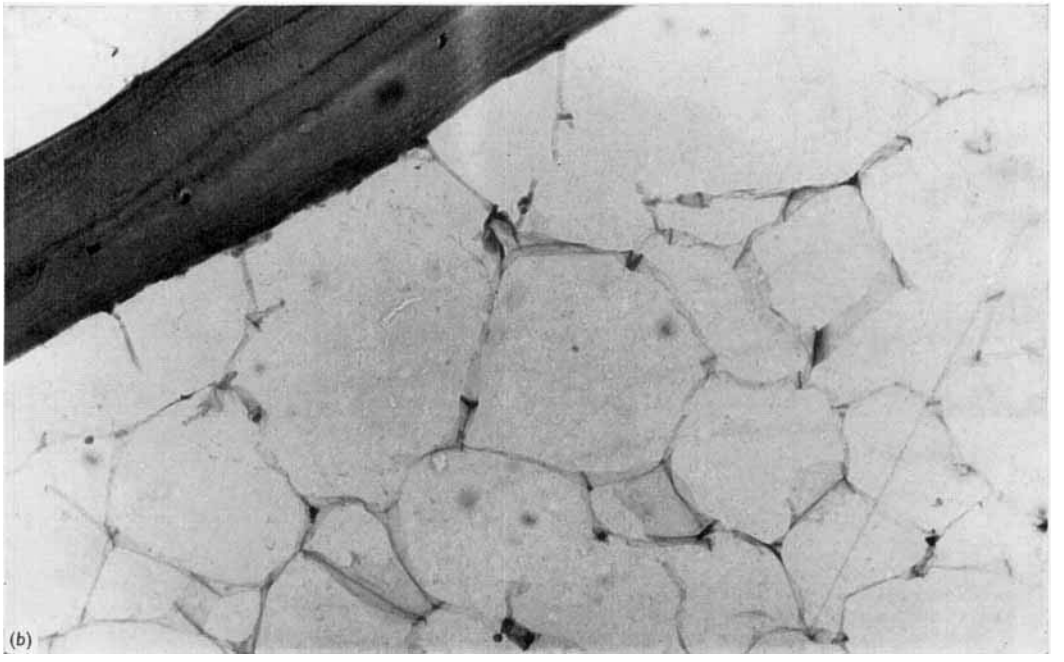

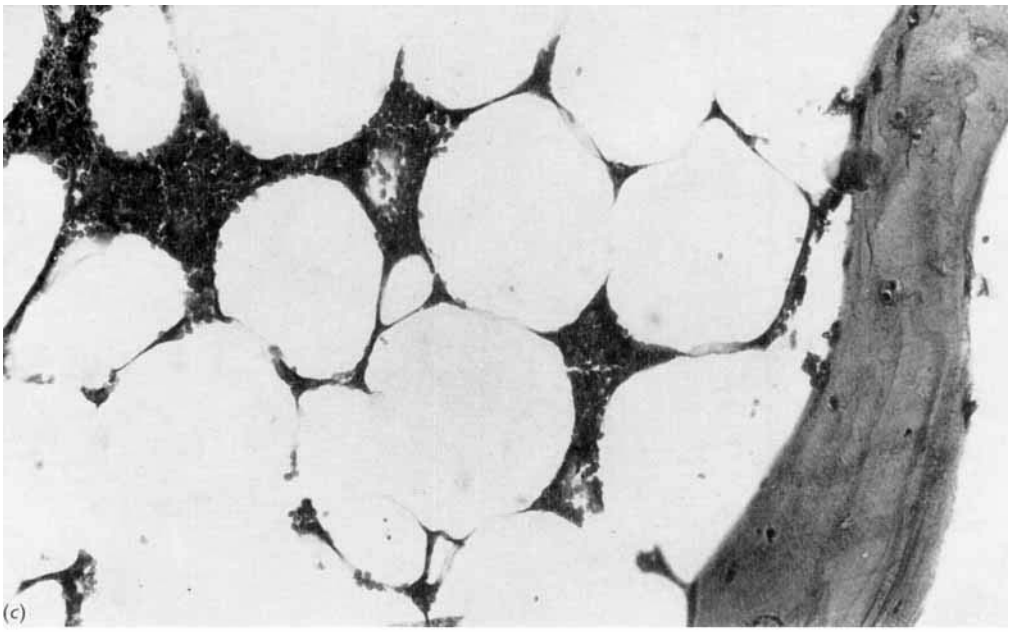

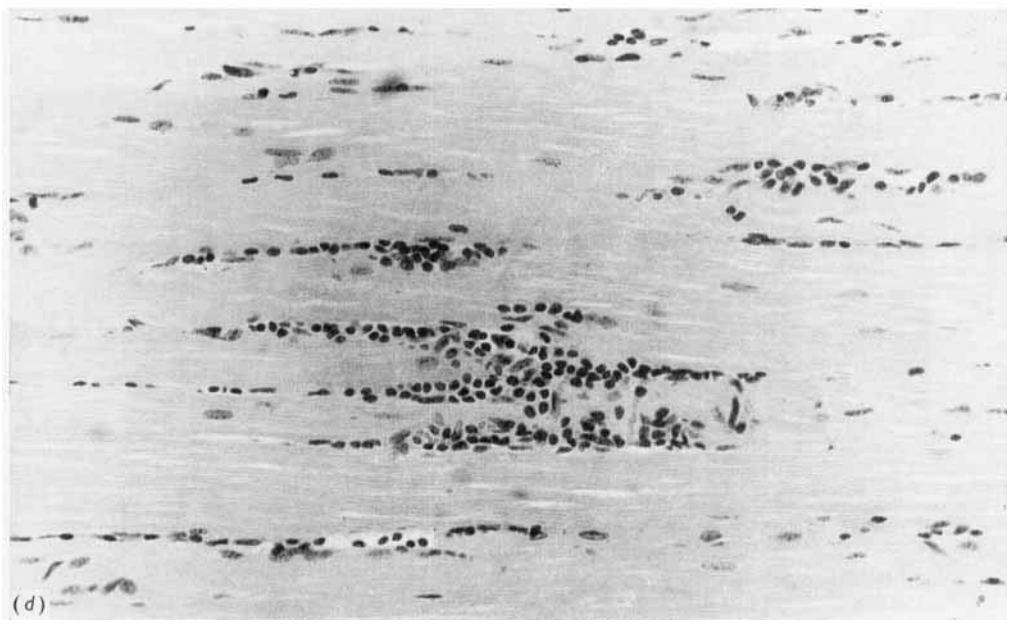

EXPLANATION OF PLATE

(*a*) Appearance of bone marrow section from a healthy sheep taken off pasture; (*b*) marrow aplasia in a bone marrow section from a sheep given a cobalt-deficient diet with supplementary vitamin B_{12} (intramuscularly); (*c*) patchy regeneration of bone marrow in a section from a sheep given a Co-deficient diet with supplementary vitamin B_{12} (intramuscularly) and after α-tocopheryl acetate supplements (orally); (*d*) muscle degeneration and cellular infiltration in a section of skeletal muscle taken at autopsy at week 87 from sheep no. 4 (group 2) given Co-deficient diet with supplementary vitamin B_{12} (intramuscularly). For details of diets, see Table 1; for α-tocopheryl acetate treatment, see p. 458.