# The requirement of sheep for cobalt or for vitamin B$_{12}$

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1. Sheep confined to pens and given a cobalt-deficient ration which supplied about 30 $\mu$g Co/d required for maintenance of normal growth rate a Co supplement approaching 40 $\mu$g administered *per os* daily; for maintenance of what appeared, under the conditions of the experiments described, to be the maximum vitamin B$_{12}$ status of a sheep, namely 3 ng vitamin B$_{12}$/ml serum and 1·4 $\mu$g vitamin B$_{12}$/g liver tissue, a supplement of between 0·5 and 1·0 mg Co/d *per os* was necessary.

2. For maintenance of normal growth rate the minimum daily requirement for vitamin B$_{12}$ of sheep given the Co-deficient diet was about 11 $\mu$g: about 5 $\mu$g absorbed from the gut and about 6 $\mu$g injected parenterally.

3. Extrapolation of the linear regression obtained by plotting the amount of vitamin B$_{12}$ injected/d against concentration of vitamin B$_{12}$ in the liver tissue of a sheep indicated that to attain a concentration of 1·4 $\mu$g vitamin B$_{12}$/g liver (wet weight) injection of 34 $\mu$g vitamin B$_{12}$/d was necessary.

4. Comparison of the relative effects on body-weight of the administration of 100 $\mu$g vitamin B$_{12}$/d *per os* and 3·12 $\mu$g vitamin B$_{12}$/d by injection to sheep given the Co-deficient diet indicated that the efficiency of absorption of the vitamin from the gut was < 3 %.

5. Loss of appetite, the first symptom of vitamin B$_{12}$ deficiency to appear in the sheep, occurred when the concentration of vitamin B$_{12}$ in the liver was reduced to about 0·1 $\mu$g/g wet weight.

6. In sheep given a supplement of 1 mg Co/d *per os* neither production in the rumen nor absorption from the gut but rather capacity for storage limited the concentration of vitamin B$_{12}$ in the liver.

7. Following treatment to restore the vitamin B$_{12}$ status of a sheep whose stores of the vitamin had been depleted, there was a linear negative correlation between the increase in the concentration of vitamin B$_{12}$ in the liver and the concentration prior to treatment.

8. Following withdrawal of treatment from a sheep whose stores of vitamin B$_{12}$ were adequate, there was a linear positive correlation between the rate of depletion of vitamin B$_{12}$ from the liver and the concentration prior to cessation of treatment.

9. The concentrations of vitamin B$_{12}$ in the serums of sheep given the Co-deficient diet, and given a supplement of 1 mg Co/d *per os* 0·5 h after feeding, remained relatively stable over the period 0–7 h after feeding; maximum concentration occurred 7–8 h after feeding.

10. Under the conditions of the experiments described a significant linear regression existed between concentration of vitamin B$_{12}$ in the liver of a sheep and the logarithm of the concentration in the serum.

The importance of the role which cobalt plays in the nutrition of ruminants has been discussed by Marston (1952). That this role is primarily one of providing a milieu in which those micro-organisms within the rumen which produce vitamin B$_{12}$ may flourish is supported by the observation that to be effective Co must be ingested (Marston, 1949; Lee & Marston, 1969), and by brief reports of the effectiveness of the parenteral administration of vitamin B$_{12}$ in relieving the deficiency syndrome suffered by sheep confined to rations deficient in Co (Marston & Lee, 1952; Marston & Smith, 1952; Marston, Allen & Smith, 1961). The subject has been reviewed more recently by Underwood (1962).

The experiments described below were designed to determine the minimum requirements of sheep for Co, the relative efficiencies of the administration of Co *per os* and of vitamin $B_{12}$ *per os* and parenterally in preventing the malady suffered by sheep confined to rations deficient in Co, the minimum requirement of sheep for vitamin $B_{12}$, and the relationship between the intake of Co or of vitamin $B_{12}$ and the concentrations of vitamin $B_{12}$ in the liver and in the blood serum.

## EXPERIMENTAL

### Expt 1. The requirement for Co of sheep fed in pens

The forty-eight Merino wethers used in this experiment were healthy animals about 3 years old and of an average weight of 39 kg when they were taken off normal pasture and placed in individual pens and given daily the standard Co-deficient ration. This ration consists of 1 kg of Co-deficient wheaten hay chaff (0·03–0·04 $\mu$g Co/g dry weight) plus 50 g of washed wheat gluten ( <0·02 $\mu$g Co/g dry weight) plus 5 g of sodium chloride. When supplemented with Co and copper these rations maintained sheep in good health for a period of at least 5 years (Dewey, Lee & Marston, 1969). After 5 weeks the animals were divided into eight groups, each of six animals, evenly matched on the basis of body-weight and concentrations of vitamin $B_{12}$ in the liver and blood serum.

Treatments were as follows:

| Group | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Supplement (mg Co/d *per os*) | None | 0·01 | 0·02 | 0·04 | 0·08 | 0·10 | 1·0 | 10·0 |

Co was administered as cobalt chloride in 10 ml solution just before feeding, six times a week. On Saturdays double the normal daily dose was given.

Cod-liver oil, 5 ml twice a week, and Cu, 25 mg as copper sulphate in 10 ml solution once a week, were administered to each animal *per os* after feeding.

Food intakes were measured daily, the animals were weighed weekly, blood samples were taken at intervals for the estimation of the concentration of vitamin $B_{12}$ in the serum and the concentrations of vitamin $B_{12}$ were estimated in samples of liver (about 100 mg of tissue) taken by aspiration biopsy at weeks 36 and 71.

Supplements were withdrawn from all groups at the end of week 71, and 11 weeks later liver samples for the estimation of the concentrations of vitamin $B_{12}$ were again taken by aspiration biopsy from all of the animals that remained in the experiment. On the basis of these results, twelve animals were chosen for a further experiment (Expt 2).

### Expt 2. Vitamin $B_{12}$ in liver: storage and depletion

The six pairs of sheep used in this experiment were chosen from the animals of Expt 1 on the basis of the amounts of vitamin $B_{12}$ stored in their livers, 11 weeks after treatments had been withdrawn. In three of the pairs the concentrations of vitamin $B_{12}$ in the livers were low ( <0·25 $\mu$g/g) and in three about half normal (about 0·65 $\mu$g/g).

One member of each pair was treated with 0·1 mg Co/d *per os* (A-series) and the

other member with 1·0 mg Co/d *per os* (B-series). Co (as cobalt chloride in 10 ml solution) was administered and the animals were fed between 09.30 and 10.00 hours each day; blood samples for the estimation of vitamin B$_{12}$ concentrations in the serums were withdrawn between 14.00 and 14.30 hours at frequent intervals over a period of 10 weeks.

At the start of the experiment all animals, except no. 626/1 A (700 g/d), were consuming daily 1050 g of the standard Co-deficient diet, and within 3 weeks of the administration of Co no. 626/1 A was also eating the whole of its ration, as did all of the animals throughout the duration of the experiment.

Treatments were continued for 10 weeks, when liver samples were taken by aspiration biopsy for estimation of the concentration of vitamin B$_{12}$. Samples of liver were also obtained 6 and 15 weeks subsequent to withdrawal of treatments.

### Expt 3. The requirement for vitamin B$_{12}$ of sheep fed in pens

The animals, twenty ewes and twenty wethers, used in this experiment were about 6 months old and of an average weight of 25 kg when they were placed in individual pens and given daily a Co-deficient ration consisting of 50 g of washed wheat gluten plus 5 g sodium chloride together with wheaten hay chaff offered *ad lib.* from automatic feeders. The gluten contained < 0·02 $\mu$g and the hay chaff < 0·04 $\mu$g Co/g dry weight.

After a period of 13 weeks on this regimen the animals were divided into groups evenly matched on the basis of body-weight and haemoglobin concentration. Each group comprised four animals: two ewes and two wethers.

Treatments were as follows:

| Group | Daily supplement |
|---|---|
| 1 | 1 mg Co *per os* |
| 2 | None |
| 3 | 25 $\mu$g vitamin B$_{12}$ by intramuscular injection |
| 4 | 12·5 $\mu$g vitamin B$_{12}$ by intramuscular injection |
| 5 | 6·25 $\mu$g vitamin B$_{12}$ by intramuscular injection |
| 6 | 3·12 $\mu$g vitamin B$_{12}$ by intramuscular injection |
| 7 | 100 $\mu$g vitamin B$_{12}$ *per os* |
| 8 | 50 $\mu$g vitamin B$_{12}$ *per os* |
| 9 | 25 $\mu$g vitamin B$_{12}$ *per os* |
| 10 | 12·5 $\mu$g vitamin B$_{12}$ *per os* |

Co was administered as cobalt chloride in 10 ml solution and vitamin B$_{12}$ (cyanocobalamin) in 0·5 ml solution (intramuscular injection) or in 10 ml solution (*per os*).

Co and vitamin B$_{12}$ supplements were administered daily except on Sundays; on Saturdays each animal was given double the normal daily dose. Supplements administered orally were given after feeding.

Cod-liver oil and Cu were administered to each animal as for Expt 1.

Food intakes were measured daily, the animals were weighed weekly and bled for the estimation of haemoglobin concentrations at approximately monthly intervals. At week 63, liver samples were taken by aspiration biopsy for estimation of the concentrations of vitamin B$_{12}$.

Treatment with Co was withdrawn from group 1, and treatment with vitamin B$_{12}$ from groups 3–6 inclusive from week 62, i.e. after 49 weeks of treatment.

Co was estimated by the method of Marston & Dewey (1940); vitamin $B_{12}$ by the *Lactobacillus leichmannii* assay procedure (Dawbarn & Hine, 1954; Hine & Dawbarn, 1954; Dawbarn, Hine & Smith, 1957); and haemoglobin by the alkaline haematin method (Marston & Lee, 1948).

<div align="center">RESULTS AND DISCUSSION</div>

<div align="center">*Expt 1. The requirement for Co sheep fed in pens*</div>

A supplement of Co adequate for the maintenance of body-weight and health of sheep fed on this Co-deficient diet was not sufficient to allow maximum storage of vitamin $B_{12}$ in the livers of these animals or to allow for a concentration of vitamin $B_{12}$ in the blood serum, which, from the observations of this experiment, would seem to be the maximum concentration for sheep under these conditions.

*Co requirement for maintenance of body-weight.* Eighteen weeks after treatment of the other groups had started, one animal in the negative control group which was un-treated began to lose weight, and subsequently over the next 13 weeks all of the animals in this group began to do likewise (Fig. 1) until at week 39 the group was withdrawn from the experiment and the individuals were treated with the appropriate amounts of Co or vitamin $B_{12}$ in order to restore them to normal health (see below).

Administration of 0·01 or 0·02 mg Co/d *per os* was not sufficient to maintain normal health and body-weight of sheep fed on the Co-deficient diet used in this experiment. During the period 31–53 weeks the means of the body-weights of the group treated with 0·02 mg Co/d became significantly less ($P < 0·05$) than those of the groups treated with greater amounts of Co, and the means of the body-weights of the group treated with 0·01 mg Co/d became significantly less ($P < 0·05$) than those of the group treated with twice this amount of Co (Fig. 1). Subsequent to week 53, three of the animals of the group which was treated with 0·01 mg Co/d were withdrawn from the experiment in an advanced stage of the vitamin $B_{12}$ deficiency syndrome and the Co supplements were increased in order to prevent their deaths from inanition.

That a supplement of 0·02 mg was close to the minimum requirement of sheep on this regimen was indicated, however, by the fact that, when at week 56 a new batch of hay chaff was drawn upon, the appetites of four of the animals of this group were gradually restored completely or partly to normal, with consequent increase in body-weight (Fig. 1).

From these observations it may be inferred that the minimum requirement of sheep for Co for the maintenance of body-weight is close to 0·07 mg Co/d *per os*—0·03 mg from the hay chaff and 0·04 mg administered as a supplement—provided that the supplement is administered each day.

Throughout the 71 weeks during which supplements were administered there was no statistically significant difference between the means of the body-weights of the groups which were treated with 0·04, 0·08, 0·10, 1·0 and 10 mg Co/d *per os*, and the animals remained in good health.

*Co requirement for maximum concentration of vitamin $B_{12}$ in blood serum.* The means of the concentrations of vitamin $B_{12}$ in the serums of groups treated with 0, 0·01,

0·02 and 0·04 mg Co/d *per os* never differed significantly from each other; nor did those of the groups treated with 0·08 and 0·1 mg Co/d *per os*, nor those treated with 1·0 and 10 mg Co/d *per os* (Fig. 1).
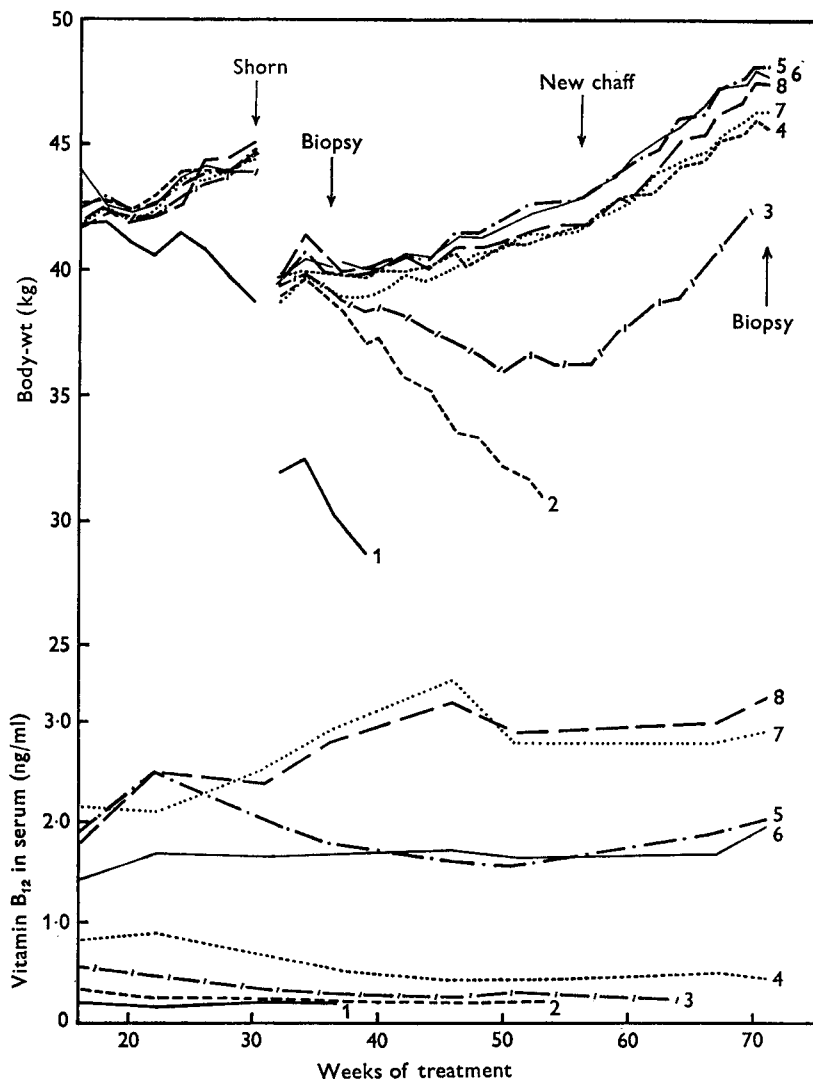


Fig. 1. Mean body-weight and mean concentration of vitamin B$_{12}$ in blood serum of sheep consuming a diet deficient in cobalt and given *per os* various Co supplements (mg Co/d): (1) none; (2) 0·01; (3) 0·02; (4) 0·04; (5) 0·08; (6) 0·10; (7) 1·0 and (8) 10·0.

At week 16 there was an acceptable degree of significance between the means of the concentrations of vitamin B$_{12}$ in the serums of groups treated with 0–0·04 mg Co/d *per os* and of those treated with 0·08 and 0·10 mg Co/d *per os* ($P < 0·01$), and at week 36 between the means of these latter groups and of those treated with 1·0 and 10 mg Co/d *per os* ($P < 0·01$).

During the 71 weeks of the experiment the concentrations of vitamin $B_{12}$ in the serums of those animals treated with 1·0 and 10 mg Co/d *per os* reached a relatively steady level of about 3 ng/ml, from which it may be inferred that this concentration is the maximum for sheep under the conditions of this experiment.

From the values plotted in Fig. 2a one may deduce that the micro-organisms within the rumen of a sheep subsisting on the standard Co-deficient diet require a minimum daily supplement of between 0·5 and 1·0 mg Co in order to produce sufficient vitamin $B_{12}$ for its concentration in the serum to reach and maintain the maximum level of about 3 ng/ml.
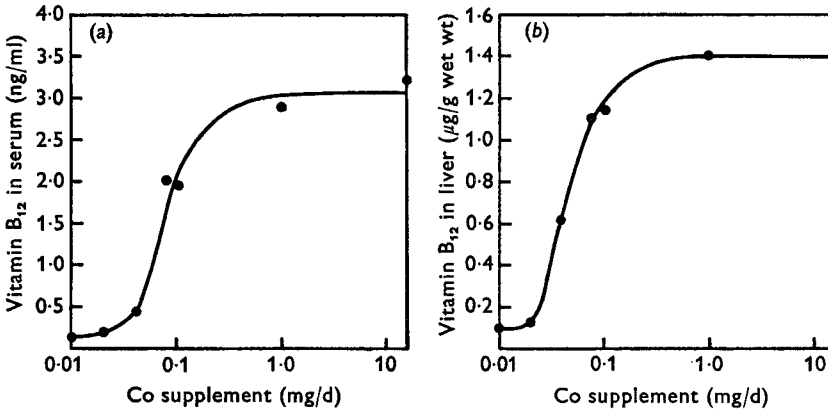


Fig. 2. Relationships between daily supplements of cobalt administered *per os* to sheep and the mean concentrations of vitamin $B_{12}$ at week 71 in (a) the blood serums and (b) the livers of the appropriate groups.

*Co requirement for maximum storage of vitamin $B_{12}$ in liver.* At week 36 there was no significant difference between the means of the concentrations of vitamin $B_{12}$ in the livers of the animals of the groups treated with 0·08–10 mg Co/d *per os*; they were, however, significantly higher ($P < 0·001$) than those of the group treated with 0·04 mg Co/d *per os* and those of the remaining two groups were correspondingly lower.

At week 71 the means of the concentrations of vitamin $B_{12}$ in the livers of the animals of the groups treated with 1 and 10 mg Co/d *per os* were significantly higher ($P < 0·01$) than those of the groups treated with 0·08 and 0·10 mg Co/d *per os* (Fig. 2b).

Under the conditions of this experiment storage of vitamin $B_{12}$ in the livers of animals given a supplement of 1·0 mg Co/d *per os* reached an apparent maximum concentration of 1·4 $\mu$g vitamin $B_{12}$/g tissue, a concentration which was not influenced by an increase of the Co supplement to 10 mg/d (Fig. 2b).

The question arises whether this 'maximum' concentration of vitamin $B_{12}$ in the liver is imposed by the capacity of the liver to store vitamin $B_{12}$, or by production of the vitamin by micro-organisms within the rumen, or by the ability of the animal to absorb that which is produced. That the limiting factor was the capacity of the liver to store vitamin $B_{12}$ was indicated by the results of Expt 2.

### Expt 2. *Vitamin B$_{12}$ in liver: storage and depletion*

*Storage.* Production of vitamin B$_{12}$ by micro-organisms within the rumen of a sheep fed on the standard Co-deficient diet is limited by the Co concentration when the animal is given a supplement of 0·1 mg Co/d *per os*. Comparison of vitamin B$_{12}$ concentrations in the serums of pairs of animals, chosen because of similarity in concentrations of vitamin B$_{12}$ in liver and serum prior to treatment with 0·1 and 1·0 mg Co/d *per os*, shows considerably higher concentrations in the animals supplemented with 1 mg Co/d *per os* (Fig. 3).
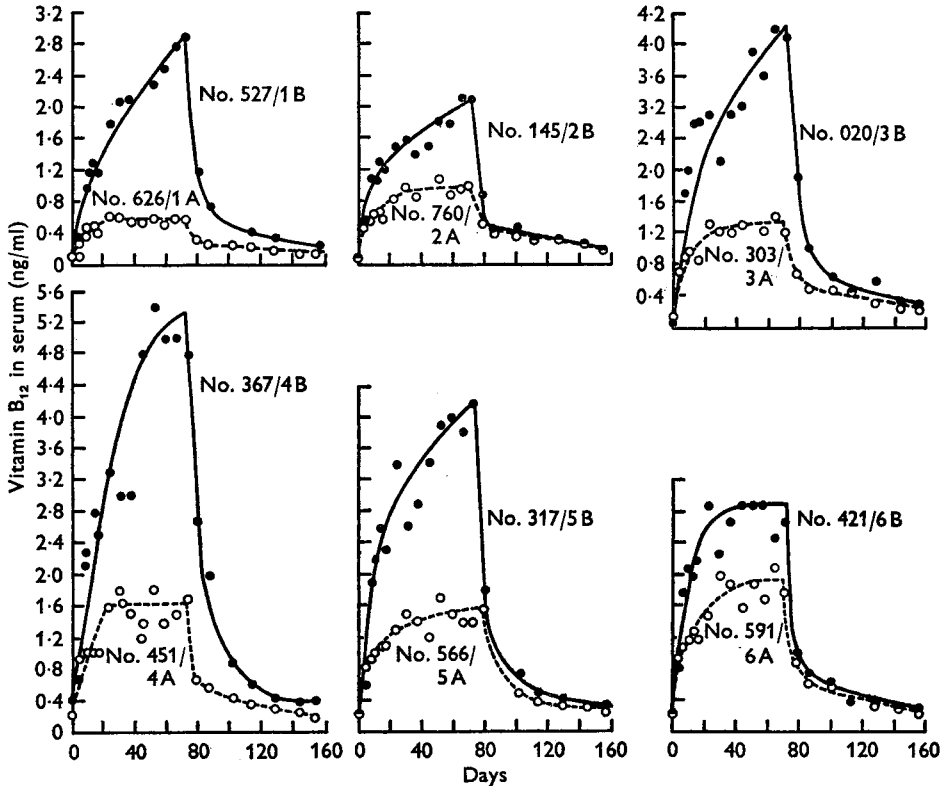


Fig. 3. Influence of cobalt intake and initial concentration of vitamin B$_{12}$ in the liver on the concentration of vitamin B$_{12}$ in the blood serum of sheep fed on a Co-deficient diet. Before commencement of treatments the concentrations of vitamin B$_{12}$ in the livers of nos. 626/1 A, 527/1 B, 760/2 A, 145/2 B, 303/3 A and 020/3 B were <0·25 µg/g tissue and of nos. 451/4 A, 367/4 B, 566/5 A, 317/5 B, 591/6 A and 421/6 B about 0·65 µg/g tissue. The A-series was treated with 0·1 mg, and the B-series with 1·0 mg Co/d *per os*, commencing on day 0; treatments were withdrawn on day 72.

Following administration of 0·1 mg Co/d *per os* there was an immediate increase in the concentration of vitamin B$_{12}$ in the serum, which within 3–4 weeks reached a relatively steady state apparently determined by the vitamin B$_{12}$ status of the animal when treatment started, and which presumably was the resultant of the factors production, absorption from the gut, absorption by the tissues and utilization. A marked and

relatively rapid rise in the concentration of vitamin $B_{12}$ occurred in the serums of animals treated with 1 mg Co/d *per os* following a period of limited Co intake during which the stores of vitamin $B_{12}$ had been depleted to varying degrees (Fig. 3).

The postulate that it is the capacity for storage which limits the concentration of vitamin $B_{12}$ in the liver is supported by the values plotted in Fig. 4. When adequate (1·0 mg Co/d), or nearly adequate (0·1 mg Co/d), amounts of Co were fed to sheep whose reserves of vitamin $B_{12}$ had been depleted, there was a linear negative correlation ($P < 0.05$) between the increase in the concentration of vitamin $B_{12}$ following 10 weeks of treatment and the concentration prior to treatment.
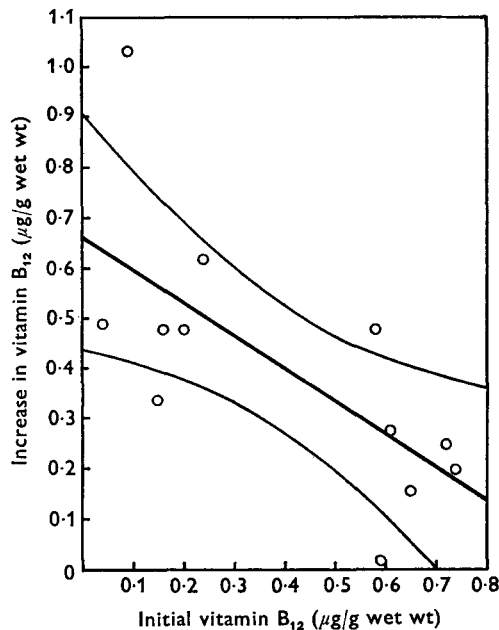


Fig. 4. Relation between initial concentration of vitamin $B_{12}$ in liver and the increase in concentration that occurs following administration of cobalt *per os* to sheep fed a diet deficient in Co. The period of treatment was 10 weeks. The curved lines on either side of the linear regression indicate fiducial limits at $P = 0.05$.

The rapid increase in the concentration of vitamin $B_{12}$ in the serums of vitamin $B_{12}$-deficient animals following treatment with adequate Co *per os*, noted above, further supports the hypothesis and indicates that neither production nor absorption from the gut limits the amount of vitamin $B_{12}$ available for storage in the liver.

*Depletion.* Within 7 d of withdrawal of treatments the concentrations of vitamin $B_{12}$ in the blood serums of all but one animal (no. 367/4 B) had fallen to about one-half of those prior to withdrawal, in reflection of a marked reduction in production of vitamin $B_{12}$ within the rumen; thereafter they fell more slowly as the animals' reserves of vitamin $B_{12}$ became depleted.

Mathematical analysis of the decreases that occurred in the concentrations of vitamin $B_{12}$ in the livers of these twelve sheep during periods in which no Co supplements were administered showed that there was a significant positive correlation

($P < 0.001$) between the rate of depletion of vitamin B$_{12}$ from the liver and the concentration prior to cessation of treatment (Fig. 5). Two periods of depletion are referred to in Fig. 5: the 11 weeks prior to choosing the animals for Expt 2 and the 15 weeks subsequent to withdrawal of the treatments used in this experiment.
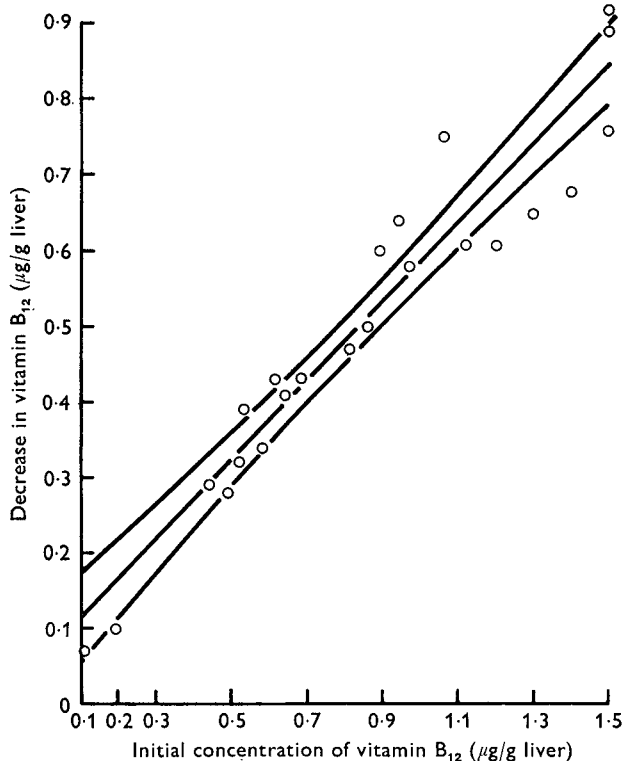


Fig. 5. Relation between initial concentration of vitamin B$_{12}$ in liver and the decrease in concentration that occurred following withdrawal of an oral supplement of Co to sheep fed a Co-deficient diet. The observations were made 11 and 15 weeks after cessation of treatments. The curved lines on either side of the linear regression indicate fiducial limits at $P = 0.001$.

### Vitamin B$_{12}$ in blood serum

Under conditions in which the stores of vitamin B$_{12}$ are depleted, little of the radioactivity associated with the $^{60}$Co of $^{60}$Co-labelled 5′-deoxyadenosylcobalamin injected intravenously, or of that associated with the $^{58}$Co of $^{58}$Co-labelled cyanocobalamin injected subcutaneously, is excreted in the urine (Smith & Marston, 1970a); the concentration of vitamin B$_{12}$ found in the serum when equilibrium was attained was thus the resultant of the balance set up between the amount required for circulation and that channelled into storage sites (Fig. 1). When, however, the concentration of vitamin B$_{12}$ in the liver was near maximum this condition was reflected in relatively steady and maximal levels of vitamin B$_{12}$ in the serum (Figs. 1 and 2).

The rate of disappearance of vitamin B$_{12}$ from the blood stream, during the first few hours following its injection intravenously into an animal whose stores were depleted, was not indicative of the degree of depletion, for the rate of disappearance

seemed to be limited by the rate at which the vitamin was absorbed by the tissues. The following experiment illustrates this phenomenon.

Two wethers, no. 609 (0·066 $\mu$g vitamin $B_{12}$/g liver tissue) and no. 590 (0·63 $\mu$g vitamin $B_{12}$/g liver tissue) fed on the standard Co-deficient diet, were fasted for 24 h and blood samples were collected immediately before, and at various intervals after, the injection of 100 $\mu$g vitamin $B_{12}$ into a jugular vein of each.

The concentrations of vitamin $B_{12}$ in the serums of both animals fell rapidly from the maximum attained immediately after the injection, and within 4 h had reached a relatively steady level which was essentially the same for both, irrespective of the concentrations of the vitamin in their respective livers (Fig. 6).
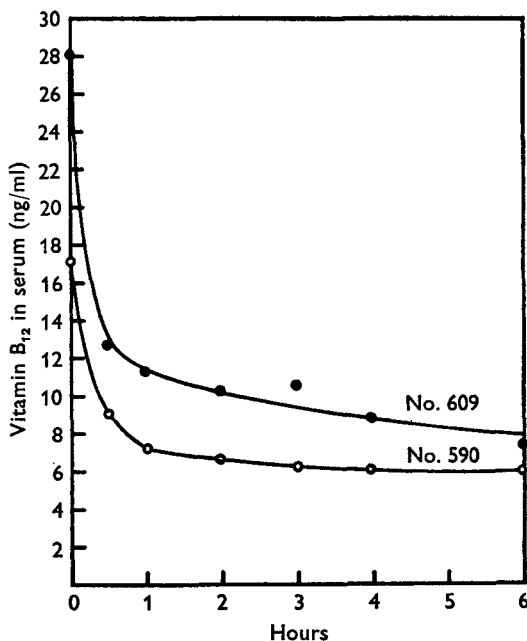


Fig. 6. Rate of disappearance of vitamin $B_{12}$ from the blood stream of sheep following injection of 100 $\mu$g vitamin $B_{12}$ into a jugular vein of each animal at time o. The concentrations of vitamin $B_{12}$ in the livers of these sheep were: no. 609, 0·066 and no. 590, 0·63 $\mu$g/g liver tissue.

The fact that the concentration of vitamin $B_{12}$ in the serum of each animal reached a relatively steady level at a concentration about twice that which occurs in animals in which there is maximum storage in the liver indicated that the rate of absorption by the tissues was the controlling factor in the distribution of vitamin $B_{12}$ following introduction of a plentiful supply of the vitamin into the blood stream, either by absorption from the gut or by injection.

*Diurnal fluctuation of the concentration of vitamin $B_{12}$ in blood serum.* Two wethers, no. 515 (0·063 $\mu$g vitamin $B_{12}$/g liver tissue) and no. 751 (0·67 $\mu$g vitamin $B_{12}$/g liver tissue) had been fed on the standard Co-deficient diet for many months, and Co, 1 mg/d *per os*, was administered daily to each, 0·5 h after food was offered, for 10 d before

withdrawal of blood samples for estimation of the concentrations of vitamin B$_{12}$ in the serums prior to and over a period of 8 h subsequent to drenching.

Examination of Fig. 7 shows that, under the conditions of this experiment, the concentration of vitamin B$_{12}$ in the serum decreased slightly over the period 2·5–4 h after feeding; it was, however, relatively stable over the period 0–7 h after feeding and maximum concentration occurred 7–8 h after feeding.
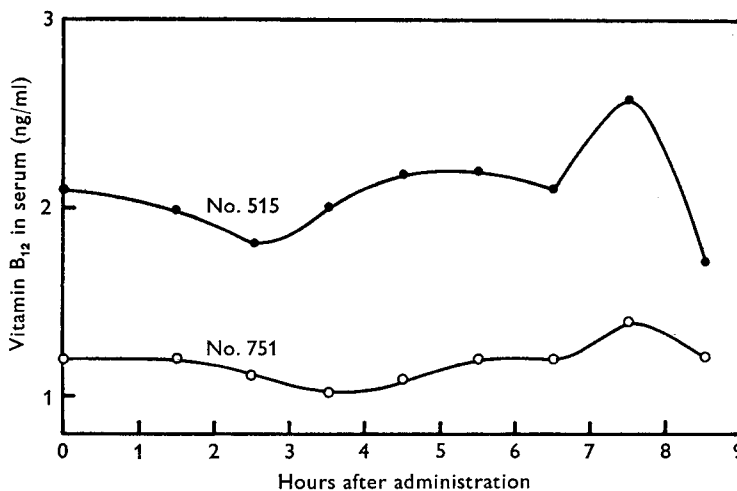


Fig. 7. Diurnal fluctuation in the concentration of vitamin B$_{12}$ in the serums of sheep fed on a Co-deficient ration and given supplements of 1 mg Co/d *per os*. The concentrations of vitamin B$_{12}$ in the livers of these sheep were: no. 515, 0·063 $\mu$g and no. 751, 0·67 $\mu$g vitamin B$_{12}$/g liver tissue.

### *Relationship between concentration of vitamin B$_{12}$ in serum and that in liver*

Equilibrium between the concentration of vitamin B$_{12}$ in the serum and that in the liver seemed to be established relatively quickly following change in treatment of animals, and examination of the results obtained from Expts 1 and 2 shows that the vitamin B$_{12}$ status of a sheep may be predicted with a considerable degree of accuracy from the concentration of vitamin B$_{12}$ in the serum.

Mathematical analysis of the results obtained at week 36 for the concentrations of vitamin B$_{12}$ in the serums and livers of individuals of the eight groups of Expt 1, and at weeks 71 and 82 for the individuals of groups 3–8, and those from the twelve animals of Expt 2, following 10 weeks of treatment and 6 and 15 weeks subsequent to withdrawal of treatments, showed that a significant linear regression ($P < 0·001$) existed between the concentration of vitamin B$_{12}$ in the liver, in $\mu$g/g, ($y$) and the logarithm of the concentration of vitamin B$_{12}$ in the serum, in ng/ml, ($x$), the equation for which is

$$y = 0·779 + 0·835x,$$

with 95 % limits of $\pm$ 0·36 for a prediction of a concentration of vitamin B$_{12}$ in liver tissue.

*Expt 3. The requirement for vitamin B$_{12}$ of sheep fed in pens*

*Groups treated with vitamin B$_{12}$ per os and the negative, untreated, control.* Vitamin B$_{12}$, 100 $\mu$g/d, administered *per os* was insufficient to meet the full nutritional requirements of the experimental sheep fed on these Co-deficient rations (Fig. 8). The four groups of animals dosed *per os* with 12·5, 25, 50 and 100 $\mu$g vitamin B$_{12}$/d began to
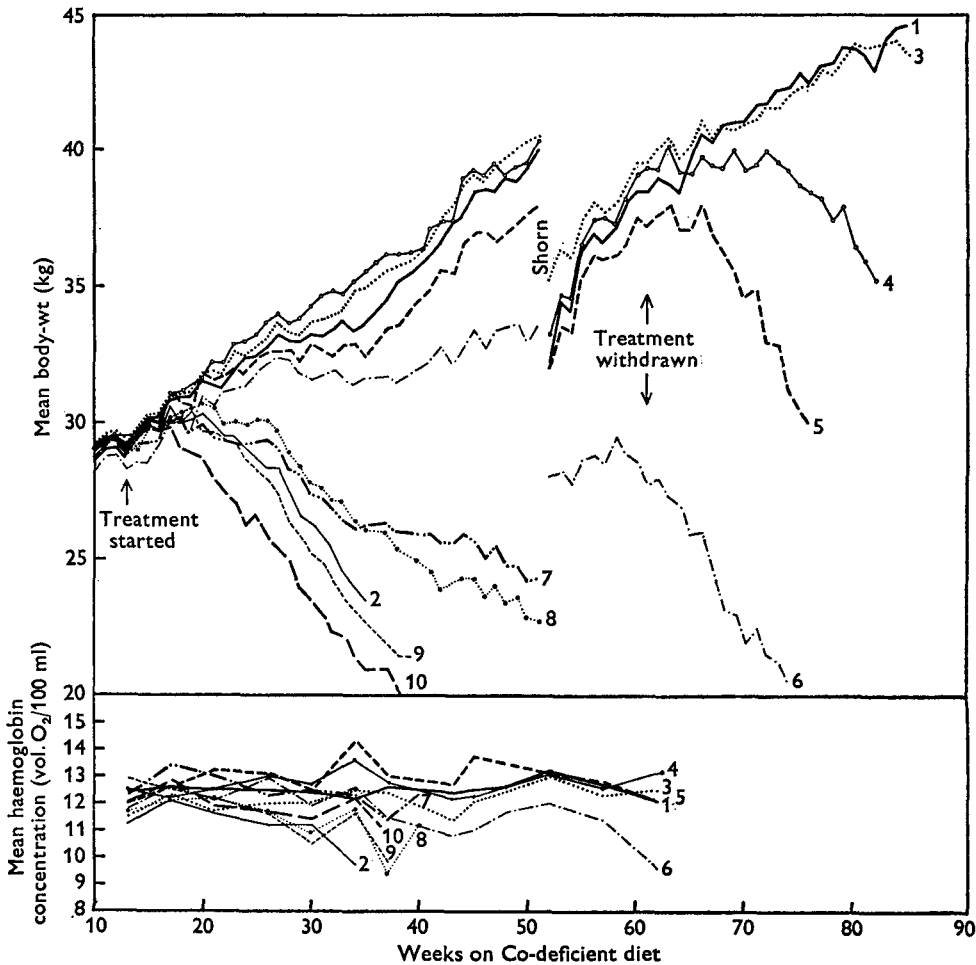


Fig. 8. The relative effectiveness (as determined by body-weight and haemoglobin concentration) of vitamin B$_{12}$ administered *per os* and parenterally and of Co administered *per os* to sheep fed on a ration deficient in Co. Group 1 received 1 mg Co/d *per os*; group 2 received no supplement; groups 3, 4, 5 and 6 were injected intramuscularly with 25, 12·5, 6·25 and 3·12 $\mu$g vitamin B$_{12}$/d respectively; groups 7, 8, 9 and 10 received *per os* 100, 50, 25 and 12·5 $\mu$g vitamin B$_{12}$/d respectively.

lose weight during the period 16–19 weeks after the beginning of the experiment, about the same time as the untreated control group, and subsequently the rate of depletion, as measured by the decline of body-weight, was not influenced significantly by any treatment until in the course of the 35th week the first death in the control

group occurred—analysis of variance of the body-weights of all five groups of this series over the period 31–35 weeks indicated the standard deviation of the difference at any time between two group means to be 2·23 kg. After 35 weeks it became clear that dosing with either 50 or 100 $\mu$g vitamin $B_{12}$/d retarded the progressive loss of weight,
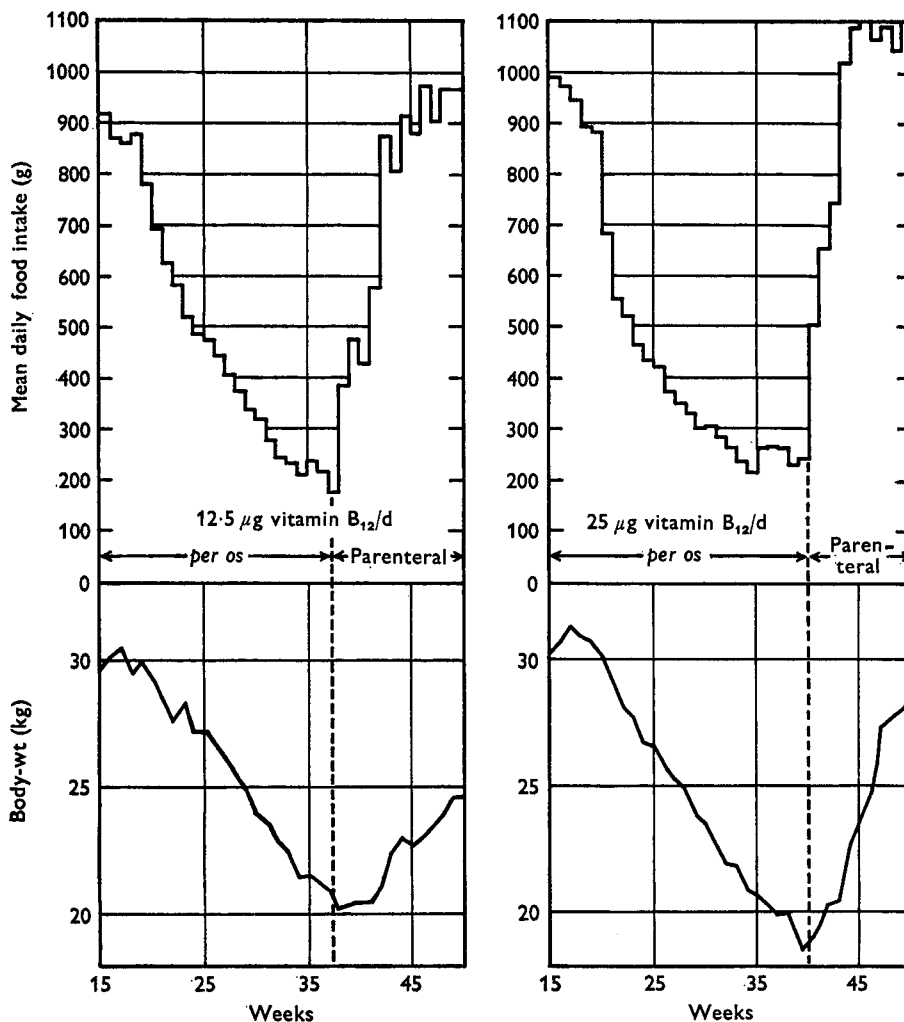


Fig. 9. The relative responses of vitamin $B_{12}$-deficient sheep to the administration of vitamin $B_{12}$ *per os* and parenterally: daily food intake and body-weight.

and, although the amounts absorbed were apparently not sufficient to make good the vitamin $B_{12}$ lost from the tissues in the normal course of metabolism, they prolonged life considerably. Variance within these two groups was high, however, and the significance of observed differences between them never reached $P = 0·01$. All individuals in these latter groups survived in a state of ill health until, at 50 weeks, when they were *in extremis*, they were removed from the experiment and treated by intramuscular injection with appropriate amounts of vitamin $B_{12}$. The dramatic

response which invariably supervened on resumption of a normal vitamin $B_{12}$ status within the tissues provided conclusive proof that the syndrome was primarily the result of vitamin $B_{12}$ deficiency. The performances of the two animals illustrated in Fig. 9 are typical of the response to parenterally administered vitamin $B_{12}$ shown by sheep whose stores of the vitamin have been depleted on the identical dose of vitamin $B_{12}$ given *per os*.

The means of the concentrations of haemoglobin of these groups did not differ significantly up to week 34; after that time, however, there was considerable variability between animals within groups and a relatively rapid decrease in the haemoglobin concentrations of some individuals of the groups that were treated with 12·5 and 25 $\mu$g vitamin $B_{12}$/d *per os* and in that which was not treated (Fig. 8).

*Groups treated with vitamin $B_{12}$ by parenteral injection and with Co* per os. Intramuscular injection of 3·12 $\mu$g vitamin $B_{12}$/d markedly retarded the advent of the deficiency syndrome; this amount, however, was not enough to fulfil the requirements of sheep on these rations. After about 30 weeks, some of the animals in the group treated in this way had already begun to lose their appetites, and 7 weeks later all were refusing to consume the whole of their rations. The mean body-weight of this group (group 6) then became significantly lower than those of the other four groups in which the animals had been injected each day with twice, four times or eight times this amount of vitamin $B_{12}$ or dosed with 1 mg Co/d *per os*. From analysis of variance of the body-weights of all five groups over the period 28–42 weeks, the mean weight of the individuals of group 6 contrasted at any time with the means of the other four groups of this series (groups 1, 3, 4 and 5) provided a SD = 0·92 kg. At 33 weeks the difference between these means was 2·2 kg and $P < 0·05$; at 37 weeks the difference was 3·7 kg and $P < 0·01$; subsequently the difference increased progressively. All animals in this group developed the obvious clinical symptoms of vitamin $B_{12}$ deficiency (see below), and would have died had they not been withdrawn and appropriately treated with a larger amount of the vitamin.

So long as treatment continued there was never any acceptably significant difference between the mean weights of the individuals of the other four groups (SD = 1·59 kg); all of these animals remained healthy and vigorous (Fig. 8).

Throughout the experiment the means of the concentrations of haemoglobin of the group treated with Co *per os* and those of the groups treated parenterally with 6·25, 12·5 and 25 $\mu$g vitamin $B_{12}$/d did not differ significantly; the means of the haemoglobin concentrations of the group treated parenterally with 3·12 $\mu$g vitamin $B_{12}$/d, however, were significantly lower ($P < 0·05$) than those of the groups which received larger doses of vitamin $B_{12}$ via the same route (Fig. 8).

*Relationship between the amount of vitamin $B_{12}$ injected and the amount stored in the liver.* The concentrations of vitamin $B_{12}$ in the livers of the animals at week 63 of the experiment, i.e. 50 weeks after treatments were started, are reported in Fig. 10 as a function of the amount of vitamin $B_{12}$ injected. Over the range studied, the relationship between the amount of the vitamin retained within the liver and the amount that had been injected each day was virtually linear.

Although more variable, the amount retained by individuals of the group treated

with Co *per os* was certainly higher than that retained by animals injected with 25 $\mu$g of vitamin $B_{12}$/d. If one omits from consideration possible losses into the urine contingent upon intramuscular injection, moderate extrapolation of the linear regression, $\mu$g vitamin $B_{12}$/g liver against $\mu$g vitamin $B_{12}$/d injected, implies that the Co-treated animals effectively absorbed between 28 and 42 $\mu$g vitamin $B_{12}$/d from their gastro-intestinal tracts—the observed mean was 33·7 $\pm$ 1·4 $\mu$g.
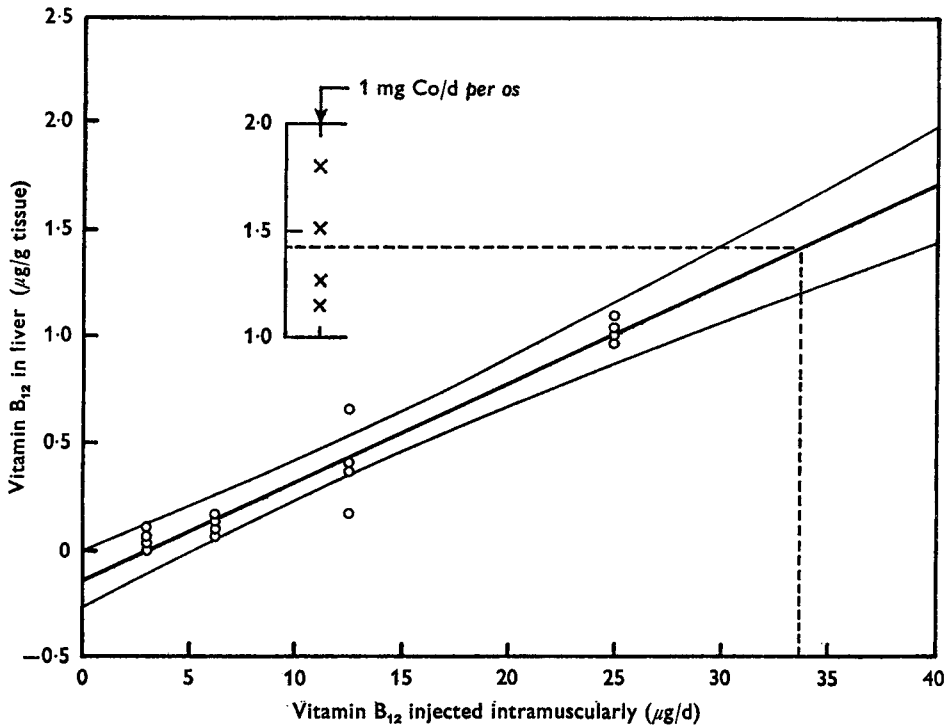


Fig. 10. Relationship in sheep between amount of vitamin $B_{12}$ administered parenterally and concentration of the vitamin in the liver after 50 weeks of treatment. Inset: concentrations of vitamin $B_{12}$ in the livers of the four sheep treated with 1 mg Co/d *per os*. The broken lines indicate the average concentration and the amount of vitamin $B_{12}$ estimated to be absorbed each day from the gut. The curved lines on either side of the linear regression indicate fiducial limits at $P = 0·01$.

The regression indicates that intramuscular injection of 6·25 $\mu$g vitamin $B_{12}$/d provided close to the minimum supplement necessary to maintain the physiological requirements of sheep on these Co-deficient rations, for, after meeting the immediate requirements, little if any remained for storage. The difference between the average amounts retained (0·123 and 0·052 $\mu$g vitamin $B_{12}$/g liver respectively) by individuals of the group receiving 6·25 $\mu$g and by those of the group treated with half this amount of vitamin $B_{12}$/d is relatively small and so is somewhat obscured in the figure. Actual differences in the total amounts in these livers may have been greater than the concentrations imply for, at the time when the biopsies were performed, the appetites of the latter animals had long since begun to fail, and, as a consequence of the

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reduction of the energy available from the food consumed, the animals had lost condition; in these circumstances, reduction of the mass of the liver could result in an increase in the concentration of the vitamin. The animals in the former group were in a state of incipient deficiency, for though they remained healthy so long as the injections continued their stores of vitamin $B_{12}$ were perilously low (see below). The animals in the latter group, i.e. those treated with $3 \cdot 12$ $\mu$g vitamin $B_{12}$/d, certainly had not sufficient vitamin $B_{12}$ in their tissues to ensure normal function. Thus it may be concluded that, for mature sheep on a diet similar to the one employed in these experiments, the least concentration of vitamin $B_{12}$ in the liver that is compatible with the full needs of intermediary metabolism lies between $0 \cdot 05$ and $0 \cdot 125$ $\mu$g/g, i.e. close to $0 \cdot 1$ $\mu$g vitamin $B_{12}$/g liver tissue.

*Vitamin $B_{12}$ depletion: effect on appetite.* Progressive failure of appetite is invariably associated with vitamin $B_{12}$ deficiency. It seems probable that during depletion of the stores of vitamin $B_{12}$ a stage is reached at which the capacity of one at least of the channels of intermediary metabolism in which vitamin $B_{12}$ is a functional unit becomes so restricted as to no longer allow the metabolites arising from a full feed to be cleared at a normal rate. The concentration of vitamin $B_{12}$ in the liver is then close to $0 \cdot 1$ $\mu$g vitamin $B_{12}$/g (wet weight). At this juncture appetite, apparently delicately poised by the concentration of these metabolites, or of intermediary products arising therefrom, in the tissue fluids, begins to fail. Subsequently, food consumption reflects the further depletion of vitamin $B_{12}$; it falls progressively as the status worsens, and the animal loses weight steadily (Fig. 8) until it dies, virtually of inanition. This aspect of vitamin $B_{12}$ deficiency is discussed in more detail by Marston, Allen & Smith (1971).

During the decline through failure of appetite, the vitamin $B_{12}$-deficient animal exhibits no obviously specific clinical symptoms other than, frequently but not invariably, an anaemia. A sheep that progressively loses its appetite through vitamin $B_{12}$ deficiency, and a normal sheep of similar initial weight whose vitamin $B_{12}$ status is maintained by repeated injections of vitamin $B_{12}$, apparently behave similarly if the food intake of the latter is perforce restricted to that of the former; both lose weight and both may die of inanition about the same time if appropriate treatment of the deficient animal is not instituted (Smith & Marston, 1970*b*). There are no gross specific pathological lesions—the condition of the viscera of a vitamin $B_{12}$-deficient animal *in extremis* is that of hunger oedema; occasionally, a fatty liver may be found at autopsy, but this is exceptional.

*Relationship between the concentration of vitamin $B_{12}$ in the liver and the onset of deficiency symptoms in sheep given these Co-deficient rations.* Progressive failure of appetite associated with vitamin $B_{12}$ deficiency may, under properly controlled experimental conditions, be employed as a sensitive indicator of the incidence of the deficiency syndrome.

As the food intake of each experimental animal in the series under discussion here was determined daily, the beginning of any progressive failure of appetite could be estimated with confidence with an error of $\pm 1$ week (Fig. 11) which was a somewhat better criterion of the deficiency than that indicated by the entailed progressive loss of body-weight. With this sensitive measure of the first consequence of the deficiency an

assessment of the mean rate of depletion of vitamin $B_{12}$ became possible without repeating the biopsy operations.

The appetite of one animal (o·o9 $\mu$g vitamin $B_{12}$/g liver) in the group that was treated with 6·25 $\mu$g vitamin $B_{12}$/d failed almost immediately following withdrawal of treatment and, within 6 weeks of cessation of the injections, the appetites of the five animals in whose livers the concentration of vitamin $B_{12}$ ranged between o·o9 and
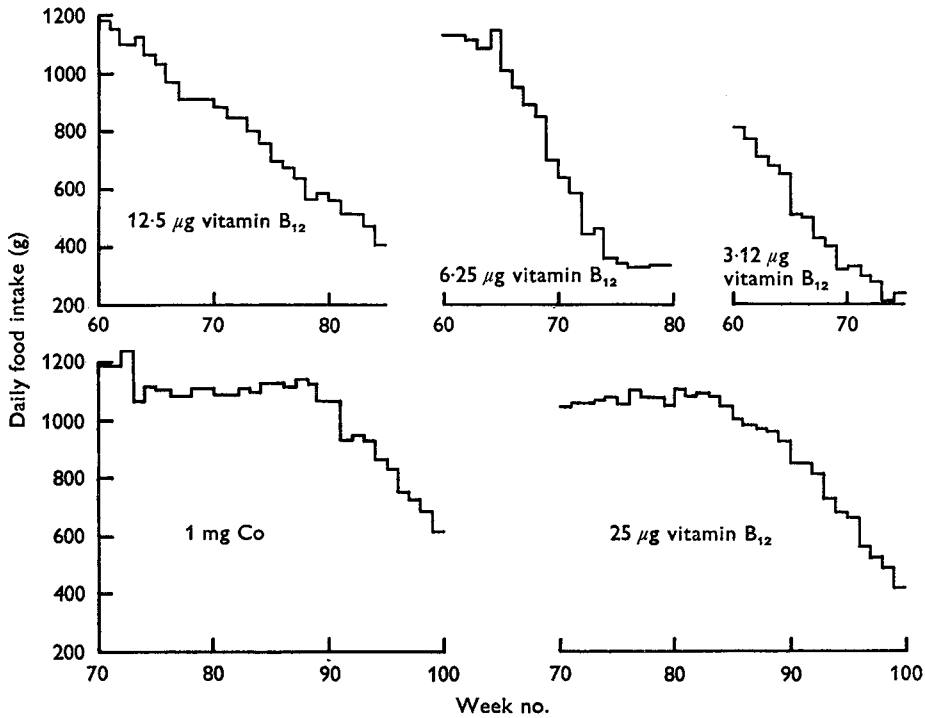


Fig. 11. Average daily food intake of groups of sheep fed on a ration deficient in Co. They had been given daily supplements as indicated (Co was given *per os*, vitamin $B_{12}$ was injected) until withdrawn at week 62.

o·17 $\mu$g/g had also begun to fail, and subsequently during the course of the ensuing 30 weeks, this first indication of a deficiency state within the tissues appeared and progressed in all of the others. The duration of the period through which an individual maintained normal health under these conditions was found to be related direct to the concentration of the vitamin in its liver when dosing was discontinued (Fig. 12). A sheep confined to the standard Co-deficient diet absorbs about 5 $\mu$g vitamin $B_{12}$/d from its intestinal tract (Smith & Marston, 1970*a*). For those animals whose daily complement of vitamin $B_{12}$ was about 11 $\mu$g—6 $\mu$g by injection and about 5 $\mu$g absorbed from that produced by ruminal fermentation—the amount utilized in the normal course of metabolism and lost to the animal each day must have been remarkably constant and close to 11 $\mu$g, for although normal function was maintained in these animals little of the vitamin was available for storage.

*Efficiency of absorption of vitamin $B_{12}$.* The vitamin $B_{12}$ activity, as estimated by assay with *Ochromonas malhamensis*, of the rumen contents of sheep fed on the standard

Co-deficient diet supplemented with Co represents at any time only a fraction of the total 'vitamin $B_{12}$ activity' estimated by the *Escherichia coli* (plate) assay (Hine & Dawbarn, 1954; Smith & Marston, 1970a), a procedure particularly sensitive to most of the known analogues of the vitamin (Smith, 1965).
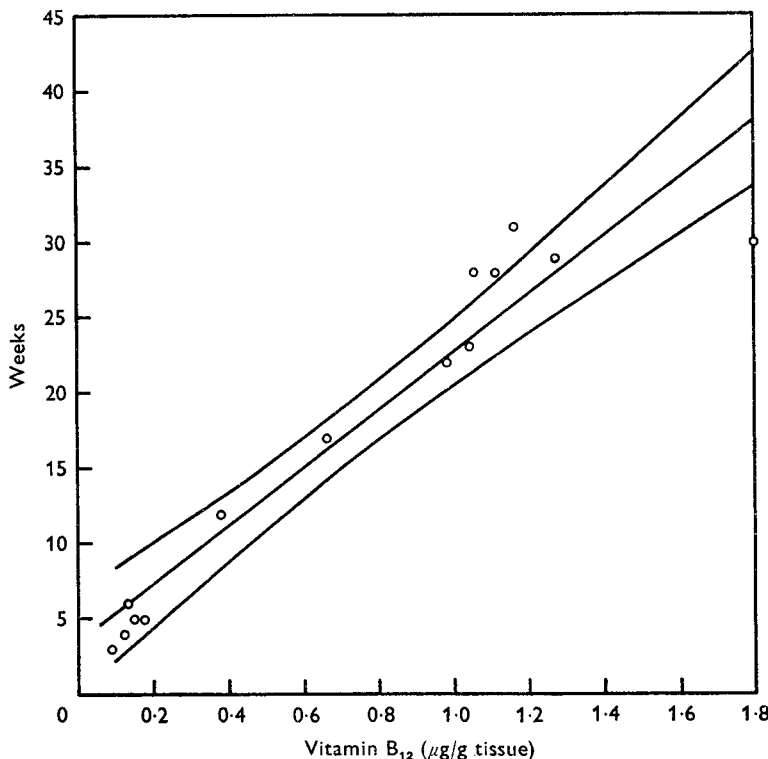


Fig. 12. Relationship in sheep between concentration of vitamin $B_{12}$ in liver at time of withdrawal of supplements and duration of period before deficiency symptoms (loss of appetite) first appeared. The curved lines on either side of the linear regression indicate fiducial limits at $P = 0.05$.

Despite production of relatively large amounts of the analogues of vitamin $B_{12}$ compared with that of vitamin $B_{12}$ itself within the contents of the rumen, absorption of the analogues is negligible or, if it does occur, destruction must be very rapid, for the vitamin $B_{12}$ activity of liver and serum is due almost entirely to vitamin $B_{12}$ itself (Smith, 1965) and only traces of the analogues are found in urine (Dawbarn & Hine, 1955).

Micro-organisms within the rumen of a mature sheep that is consuming a full maintenance ration of the standard Co-deficient fodder produce about 90 $\mu$g vitamin $B_{12}$/d when the concentration of Co is limited to that of the fodder and about 700 $\mu$g when adequate Co is supplied as an oral supplement (Smith & Marston, 1970a). The vitamin passes to the abomasum and thence to lower levels of the intestinal tract. Under the more favourable conditions, storage of the vitamin within the liver (Fig. 10) implied an effective absorption of about 35 $\mu$g vitamin $B_{12}$ each day, or 5% of the

estimated daily production. An efficiency of absorption from the intestinal tract of less than 3 % is implied by the observations illustrated in Fig. 8, which show unequivocally that 100 $\mu$g vitamin B$_{12}$/d, administered *per os*, failed to provide for the physiological requirements of sheep as effectively as 3 $\mu$g vitamin B$_{12}$/d injected intramuscularly, though no account was taken of possible losses contingent upon rapid diffusion from the injection site. Interpretation of the results is rendered difficult, however, by the fact that a portion of the vitamin B$_{12}$ introduced into the rumen may be modified before reaching the absorptive site (Smith & Marston, 1970*a*).

There is some evidence that analogues closely related to the vitamin combine with Castle's intrinsic factor (Smith, 1965); if this is so, they could compete with the vitamin itself in this essential prerequisite for absorption from the intestinal tract. Dilution of the vitamin with analogues that are useless to the animal organism might thus impose serious limitations in the absence of a considerable excess of intrinsic factor. The sheep's surprisingly poor capacity to utilize the vitamin B$_{12}$ produced by symbiotic flora within its rumen may be due in part to interference of this sort, but it is probably in greater part the consequence of the relative inefficiency with which the vitamin is liberated from the micro-organisms within the fermenting mass when this passes on from the rumen to be subjected to proteolysis by pepsin in the acid medium of the abomasum. Whatever the underlying cause, the efficiency of absorption of vitamin B$_{12}$ from the intestinal tract of the sheep is extraordinarily low.

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