

## Some factors affecting the level of vitamin D in the blood of sheep

By J. QUARTERMAN, A. C. DALGARNO AND AGNES ADAM

*Rowett Research Institute, Bucksburn, Aberdeen*

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Disorders such as rickets and hypocalcaemic staggers in sheep or other animals can be cured or prevented by the timely administration of vitamin D. It is not known if a deficiency or an excess of vitamin D is a significant factor in the origin of other disorders or how severely sheep in practice are deficient in the vitamin. In natural conditions sheep must find their vitamin D from the plants eaten or from sunlight activating the provitamins in their body. There is almost no information about the relative importance of these sources to the sheep and only indirect evidence to show how great are the variations in the supply during the year and throughout the country. Neither is there much information on vitamin D requirements of the sheep or how the requirement may be altered, as is known for the pig, by other nutritional factors such as the amounts and relative proportions of calcium and phosphorus in the diet (Dunlop, 1935), or by possible rachitogenic factors (Grant & O'Hara, 1957). Andrews & Cunningham (1945-6) gave a figure of 180 i.u./100 lb live weight as the vitamin D requirement of sheep but the diet they used had an unusually high Ca:P ratio.

Benzie, Boyne, Dalgarno, Duckworth & Hill (1959) have examined the interactions of Ca, P, energy and vitamin D intakes on the skeletal composition of growing sheep. Their sheep were given either no vitamin D supplement or 2 ml cod-liver oil (containing about 300 i.u. vitamin D) daily per head. When Ca, P and energy supplements were included, this amount of vitamin D in the diet gave rise to normal levels of Ca in the serum and to well-formed bones. Without these supplements there was a drop in the serum Ca level accompanied by skeletal resorption. It is not possible to say from their work whether 2 ml cod-liver oil supplied the optimum amount of vitamin D since no other levels were tested. Blood levels of vitamin D were not determined during this study, but in a later experiment Dalgarno, Hill & McDonald (1962) estimated the level in sheep receiving the same basal diet with or without cod-liver oil supplement. In general, the sheep receiving the cod-liver oil supplement had blood levels of vitamin D greater than about 9 i.u./100 g and those receiving no supplement had lower blood levels. From these two experiments it appears possible that, when the blood levels of vitamin D fall below about 9 i.u./100 g, the supply is insufficient to maintain normal skeletal development. In natural conditions, however, the blood level is probably below this value for several months during the winter (Dalgarno *et al.* 1962). There is an interval of several months between the introduction of the low-vitamin D diet and the onset of any signs of vitamin D deficiency, and even when severe resorp-

tion of skeletal mineral has taken place, as may occur in the lactating ewe, the animal has a great capacity for repairing its skeleton when the diet improves or when the period of physiological stress is over (Benzie *et al.* 1959).

This paper describes experiments designed to give further information on variations in the concentration of vitamin D in the blood of sheep in relation to seasonal and climatic changes. Blood was chosen as the tissue to be sampled, first, because of the convenience of taking successive samples, and secondly, because there is evidence, presented in the preceding paper (Quarterman, Dalgarno, Adam, Fell & Boyne, 1964), that in the pig and probably also in the sheep the blood is the largest pool of vitamin D in the body. Since the use of a massive dose of vitamin D is becoming important as a prophylactic against hypocalcaemic tetany in cattle (Hibbs & Pouden, 1955) and has been suggested as a means of improving the thriftiness of sheep (Green, 1953; Franklin, 1953; Curran & Crowley, 1961), the influence of such treatment on the blood vitamin D level was also examined.

#### EXPERIMENTAL

In all the experiments groups of seven or eight sheep were used for each treatment and blood samples were pooled from three or four sheep, thus making two subgroups in each experimental group.

*Expt 1.* The experiment was in two parts. Fourteen Blackface ewes that had wintered together in 1959–60 were divided into two groups. Seven were shorn in May 1960 and the other seven were left unshorn. A blood sample was taken for vitamin D assay from each sheep just before shearing in May and again in June, August and November 1960 and February 1961. During the experiment all the animals were on pasture.

In 1961 the experiment was repeated with fourteen wethers (castrates). They were bled at shearing in June and again in August and November.

*Expt 2.* A group of ten ewes and six wether hogs (castrates under 1 year old) that had been together all winter was divided into two groups balanced according to sex and weight. Every animal was shorn in April 1961 and thereafter one group was kept outside on a concrete run and the other indoors with no direct sunlight. Each group was given grass *ad lib.* from the same field cut freshly each day. Blood samples were taken at the beginning of the experiment in April and then in July and September 1961. Two of the sheep that had been outside were slaughtered in October and blood and liver samples taken for vitamin D assay.

*Expt 3.* Twenty-four wethers that had overwintered at this Institute were divided into three groups of eight. One group was retained on normal grazing at the Institute farm, a second group was sent to graze on the University of Reading farm, Sonning, Berks, and the third group on a hill farm in Glen Isla, Perthshire, at an altitude of 1200 ft. Blood samples were taken from these groups of sheep before separation in April 1961 and again in June, September and October 1961 and in March 1962.

*Expt 4.* Sixteen mated ewes wintered at this Institute were put into two groups, similar in weight distribution. The ewes in one group were each given 1 million i.u.

cholecalciferol in ethyl oleate (2 ml Y-Vit; R. Young and Co. Ltd), by injection into the longissimus dorsi muscle about 1 in. deep in the region of the first lumbar vertebra and about 2 in. to one side of dorsal mid-line. Blood samples were taken from all the ewes just before injection on 10 January 1961. Samples were taken subsequently on 18 January and on 14 February, 8 May and 16 October.

*Blood samples.* Blood samples (125–150 ml) were taken from the jugular vein of each animal. The procedures used for blood and liver saponification and extraction are described in the preceding paper (Quarterman *et al.* 1964). The curative, radiographic assay with rats used to measure vitamin D and the method for the statistical assessment of the measurements have been described by Dalgarno *et al.* (1962). Serum Ca was measured by the method of Clark & Collip (1925) and inorganic P in whole blood by that of Fiske & Subbarow (1925).

*Sunshine.* Information about sunshine (presented in Table 4 and Fig. 1) was derived from daily readings at Dyce, Aberdeenshire, at Reading University and at Blairgowrie, Perthshire, the nearest meteorological stations to each of the three farms in Expt 3.

#### RESULTS

*Expt 1.* The results of this experiment have been published in part by Quarterman, Dalgarno & McDonald (1961); the complete results are given in Table 1 for the first part conducted in 1960–1 and in Table 2 for the second part in 1961.

Table 1. *Expt 1. Blood levels of vitamin D (i.u./100 g) in shorn and unshorn sheep during 1960–1 estimated by rat assays*

No. of sheep in subgroups	19. v. 60	24. vi. 60	23. viii. 60	1. ix. 60	15. ii. 61
Shorn sheep					
3	28	69	86	28, 23*	11
4	15	60	61	18, 16*	6
Unshorn sheep					
4	13	18, 20*	30	15, 13*	8
3	10	15, 18*	30	19, 29*	9

The mean 95 % fiducial limits of these assays were 55–180 % of the estimates.

\* Duplicate assays on same sample.

In 1960 the blood vitamin D levels of both groups of sheep rose during the period from May to August (Table 1), the greatest increase being in shorn animals. By November values had fallen to the preclipping levels of May and by February 1961 they were still lower. The difference between the estimates for the shorn and unshorn sheep was significant in June and August. The two subgroups of shorn sheep were consistently different throughout the whole year though the difference between the estimates was significant only in May. The greatest rise in the blood levels of vitamin D in the shorn sheep coincided with a period of strong sunshine in the second half of May and June, as shown in Fig. 1.

When this experiment was repeated in 1961 with a different flock of wethers the same clear picture was not obtained (Table 2). The 1961 experiment began in June and at first there was a period of dull weather. By the beginning of August the vitamin D blood level in both groups had increased considerably, but the level of one group of shorn sheep was as low as that of one group of the unshorn sheep. The reasons for this result are unknown. The levels of vitamin D in the unshorn sheep in June 1961 were lower than those in June 1960, but the August and November levels were similar in the 2 years.

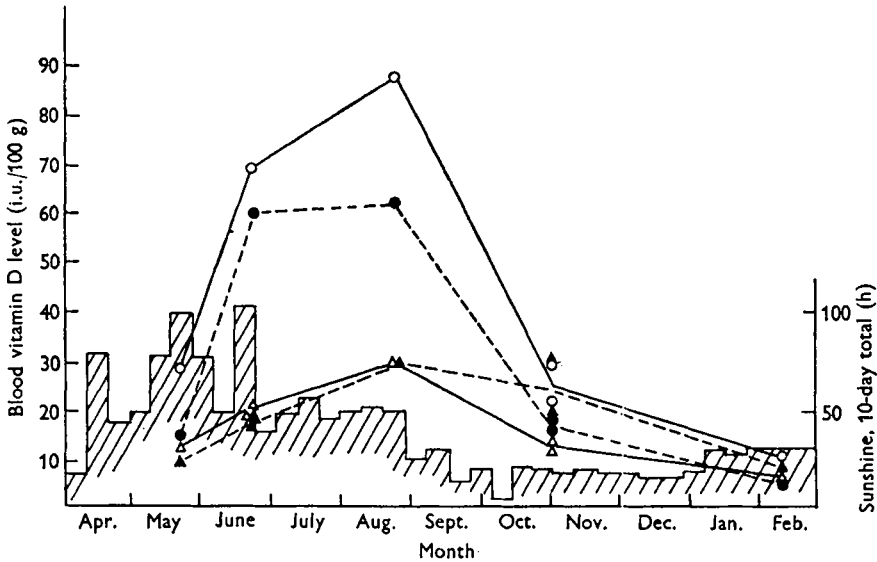


Fig. 1. Changes in blood level of vitamin D in two groups of shorn (○, ●) and two groups of unshorn (△, ▲) sheep during 1960, and the hours of sunshine at a nearby meteorological station.

Table 2. *Expt 1. Blood levels of vitamin D (i.u./100 g) in shorn and unshorn sheep during 1961 estimated by rat assays*

No. of sheep in subgroups	14. vi. 61	1. viii. 61	5. xi. 61
Shorn sheep			
3	6	60, 72*	29
4	7	42, 32*	19, 71*
Unshorn sheep			
4	11	23, 31*	14, 29*
3	12	44	23

The mean 95% fiducial limits of these assays were 49–229% of the estimates.

\* Duplicate assays on same sample.

*Expt 2.* In April before the sheep were separated they all had less than 5 i.u. vitamin D activity/100 g blood (Table 3). By July vitamin D blood levels of the sheep indoors had fallen whereas those of the sheep outside receiving the same fresh grass

had risen to about 10 and 31 i.u./100 g. However, during the next 2½ months, when there were fewer hours of sunshine than during the first 3 months, the blood levels of the sheep outside rose to about 37 and 62 i.u./100 g and of those inside to 29 and 68 i.u./100 g.

Table 3. *Expt 2. Blood levels of vitamin D (i.u./100 g) in sheep, given fresh-cut grass and kept outside on concrete or indoors out of sunlight, estimated by rat assays*

No. of sheep in subgroups	25. iv. 61	11. vii. 61	12. ix. 61
Indoors			
4	< 5	< 2	68
4	< 5	4	29
Outside			
4	< 5	9	37
4	< 5	31	62

In five of these assays the dosage levels were such that it was possible only to give an upper limit. The mean 95 % fiducial limits of the other assays were 41–227 % of the estimates.

Table 4. *Expt 3. Blood levels of vitamin D (i.u./100 g) in sheep at Aberdeen, Reading and a hill farm in Glen Isla, Perthshire (1961–2) estimated by rat assays, and the total hours of sunshine between each sampling date recorded at Dyce (Aberdeenshire), Reading University and Blairgowrie, Perthshire*

	No. of sheep in subgroups	25. iv. 61	15. vi. 61	13. ix. 61	27. x. 61	10. iii. 62
Aberdeen						
Blood level	4	3	26	26, 27*	15	8
Sunshine (h)	4	5	20	25	14	—
		250	373		185	345
Reading						
Blood level	4	5	29	63, 37*	32	—
Sunshine (h)	4	3	27	47, 33*	21	5
		314	575		182	294
Glen Isla						
Blood level	4	3	13	30	10	8
Sunshine (h)	4	5	32	19	7	—
		304	448		140	300

In three of these assays the dosage levels were such that it was possible only to give an upper limit. The mean 95 % fiducial limits for the other assays were 42–200 % of the estimates.

\* Duplicate assays on same sample.

In the two sheep slaughtered in October the blood contained 21 and 22 i.u./100 g and the liver 12 and 8 i.u./100 g. The weights of the sheep were 39 and 35 kg and their livers weighed 658 and 614 g, respectively. On the assumption that the blood amounts to 8.0 % of the body-weight (Kolb, 1962), it can be calculated that there was, respectively, 8.3 and 12.6 times as much vitamin D in the blood as in the livers.

*Expt 3.* Before the sheep in this experiment left this Institute for their various destinations in April their blood levels of vitamin D were about 3–5 i.u./100 g (Table 4).

Sheep at all centres showed the characteristic rise in blood vitamin D level during the summer; the highest value was in September at Reading, when the levels were significantly greater than in the other two places. A rapid drop in levels occurred up to late October and continued until values of from 5 to 8 i.u. were recorded in March.

Table 5. *Expt 4. Blood levels of vitamin D (i.u./100 g) in sheep that had received an intramuscular injection of 1 million i.u. vitamin D<sub>3</sub> in oil, and in a group not given the injection, estimated by rat assays*

No. of sheep in subgroups	10. i. 61	18. i. 61	14. ii. 61	8. v. 61	16. x. 61
Group given injection on 10 January					
4	15, 29*	236, 175, 199*	203, 91*	221, 121, 73*	46
4	15	133, 109, 116*	227, 194, 150*	247, 51, 60, 90*	45
Group not given injection					
4	10, 11*	11, 11*	6	9	32
4	8	15	5	9	36

The mean 95 % fiducial limits of these assays were 53–188 % of the estimates.

\* Replicated assays on same sample.

*Expt 4.* The ewes in this experiment that had not received an injection of vitamin D showed the normal seasonal changes in blood vitamin D concentration (Table 5), but in the sheep that had received an injection of 1 million i.u. in January the level had risen to between 109 and 236 i.u./100 g in a week and was still as high a month later. Four months after the injection in May the blood levels were still very high. Reproducibility in assays of stored blood extracts from injected sheep was poor, owing we believe to a loss of potency during storage. This decline in potency has not been observed when the concentration of the vitamin was less than about 50 i.u./100 g blood.

After 9 months from injection the blood level of the group that had had vitamin D injections was not significantly different from that of the control group.

The mean values for blood inorganic P at the times of the first three bleedings were 5.25, 5.18 and 4.42 mg/100 ml for the uninjected animals and 5.62, 6.69 and 5.28 for the injected animals. The corresponding serum Ca values were 10.11, 10.72, 10.01 and 10.62, 10.95, 10.17 mg/100 ml, respectively.

#### DISCUSSION

Throughout all this work the blood level of vitamin D activity has been assumed to be a measure of the total amount of the vitamin in the whole body. Support for this assumption is given in the preceding paper (Quarterman *et al.* 1964) in which it is shown that, per unit weight, over a wide range of vitamin D level, the vitamin D concentration of the blood of the pig is always as great as or greater than that of the liver, and since the blood has a much greater mass than the liver, it must always contain a much larger amount of the vitamin. Other organs do contain vitamin D (Kodicek, 1958), and the vitamin has demonstrable metabolic functions in the intestine

(Schachter & Rosen, 1959) and probably in the kidney (Kodicek, Darmady & Stranack, 1961), but in the sheep no tissue has as high a concentration of vitamin D as the blood (New Zealand Department of Agriculture, 1949-50).

In two sheep in Expt 2 the blood was shown to contain about ten times as much vitamin D as the liver. The use of blood samples in this work has thus given information about what is probably the largest pool of vitamin D in the sheep.

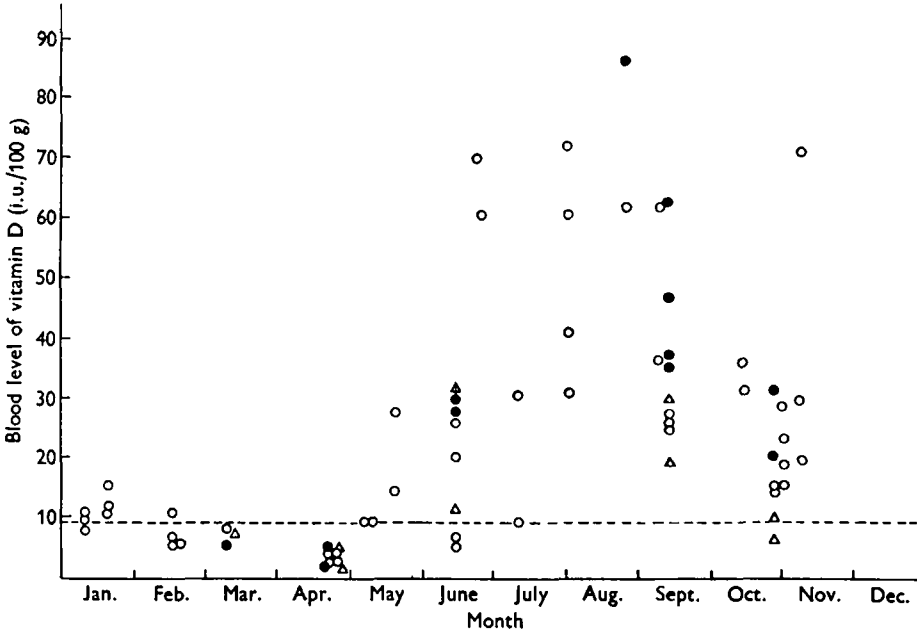


Fig. 2. Normal seasonal changes in the blood level of vitamin D in sheep kept out of doors, clipped at the normal time and given no injections of vitamin D, at Aberdeen (○), Reading (●) and Glen Isla (△). The broken line is at 9 i.u./100 g blood (see p. 79).

From the work reported here and from that of Dalgarno *et al.* (1962) the annual cycle of changes (Fig. 2) in the vitamin D blood level of sheep at pasture has been found to consist of a period of a low level, usually below 9 i.u./100 g from February to the beginning of May, a rise after this time to a maximum in August or September, which can vary from 25 to 80 i.u./100 g, and then a rapid decline during October and November. This is so also at the three farms of the Hill Farming Research Organization in Scotland, Glensaugh, Sourhope and Lephinmore (B. W. Simpson, 1956, private communication). Therefore for 5 or 6 months of the year sheep have a level of vitamin D in the blood at or below the level which is considered adequate by the criteria discussed in the introduction. The rapid decline in blood vitamin D level from the high values found in August and September to values at or below 10 i.u./100 g in January suggests that the sheep is unable to store naturally acquired vitamin D until the time of parturition and lactation. However, it is noteworthy that once the blood levels have dropped to about 10 i.u./100 g further reduction occurs very slowly indeed; no values lower than 2 i.u./100 g have been found.

From our work there is a strong suggestion that large individual differences in blood level of vitamin D occur, at least during the summer. For instance, in 1960 of the two subgroups of sheep that were clipped, the pooled blood of one had a higher level of vitamin D at every sampling (Table 1) although the difference was significant only at the first sampling in May.

Grazing animals can obtain their vitamin D either from solar irradiation of the skin and sebaceous secretions converting any provitamins D present into vitamins D or from the vitamin D in pasture. It is possible that ingested provitamin D<sub>2</sub> (ergosterol) can be transported to the skin and then irradiated. The value of sunlight to an animal with such a thick protection to its skin as the fleece may be questioned. Bekemeier & Pfennigsdorf (1959) and Bekemeier (1959) found that, as the thickness of an animal's hair increases, the vitamin D activity in the skin produced by u.v. irradiation decreases. After a given dose of u.v. irradiation pigs had 30–110 i.u./cm<sup>2</sup> of vitamin D activity in the skin, rats had 5–15, rabbits 2–5 and guinea-pigs 0–3 i.u./cm<sup>2</sup>. Even if the skin is completely shielded, it is possible that there are provitamins in the wool wax that can be activated and reabsorbed. Truter (1956) quotes three workers who confirm the presence of such provitamins or vitamins in wool wax. Vitamin D can be absorbed through the skin as readily as through the intestinal wall (Schaefer, Sassaman, Slocum & Greene, 1956). The factors that affect the amount of the u.v. light having antirachitic activity, i.e.  $\lambda$  about 295–315 nm, that reaches the ground are discussed by Abrams (1952). The sun begins to have significant antirachitic activity when it is more than 35° above the horizon, which occurs at latitude 60° N (Aberdeen is 58° 25' N) from early April to early September (Benford, 1947). This period is extended by about a month at either end at latitude 50° N (Reading is 51° 26' N). The intensity of the u.v. radiation will be affected by those factors that affect visible and thermal solar radiation, but the filtering effect is generally greater for the shorter wave-lengths than for the longer. Since it was not possible to obtain data for the very short solar u.v. radiation, Fig. 1 and Table 4 give values for normal 10-day total sunshine published by the Meteorological Office.

The vitamin D activity of fresh grass is small and very variable and does not bear any clear relationship to the sunshine it has received (Keener, 1954; Wallis, Kennedy & Fishman, 1958; Henry, Kon, Thompson, McCallum & Stewart, 1958) except that there is an increase after it has been cut and sun-dried. Scottish pastures were found to contain 0.05–1.09 i.u. vitamin D/g dry matter (Henry *et al.* 1958). If, therefore, a sheep ate 1.5 kg of dry matter a day its intake of vitamin D could range from 75 to 1635 i.u. a day.

May and June 1960 were very sunny months, and in blood level of vitamin D there was a clear difference between the shorn and the unshorn sheep (Table 1, Fig. 1). The contrast between the steep increase in vitamin D in the blood of the shorn sheep and the small increase in that of the unshorn sheep suggests that the animals obtained most of their vitamin D through u.v. irradiation and very little from their food. The sheep, however, that were not shorn probably ate less (K. L. Blaxter, personal communication) because of heat stress during the summer and, in fact, after both groups of sheep had been shorn in May 1961 those that had not been shorn in 1960



had a mean weight of 60 kg compared with 67 kg for the other, shorn, group. However, it is unlikely that differences in food intake could fully account for the large magnitude of the differences in blood vitamin D content.

Expt 2 in 1961 was specifically designed to estimate how much of the vitamin D supply came from the food and how much from irradiation, and the results for the April and July bleeding gave support to the conclusion drawn from Expt 1, but those obtained in the autumn did not. The explanation is not clear. The blood level of the sheep receiving no sunlight decreased whereas that of those outside increased, showing that during April, May and July the grass did not contain enough vitamin D to maintain the blood level even at the low value of 5 i.u./100 g. Access to sunlight caused the level to rise to as much as six times the original value in one subgroup although the rise in the other was trivial. However, in September the blood levels of both groups of sheep in this experiment had risen to quite high values and it seems as if the supply of vitamin D from grass was considerable. The grass used in this experiment was cut from an ungrazed field and was by this time high and seeding. The autumn of 1961, in contrast to that of the previous year, had weather conditions very suitable for the growth of fungi. Infection of grasses with ergot (*Claviceps* sp.) was reported from various places in north-east Scotland (Elizabeth Gray, personal communication) and was accompanied by many reports of abortion and other disorders in cattle diagnosed as due to this infection. If fungal infection had been present the amount of provitamin D (ergosterol), activated or not, consumed by the sheep might have been increased considerably.

Lenkeit, Brune & Günther (1958) found evidence of an annual cycle in the level of vitamin D in milk, independent of sunlight and changes in the food. Cows kept indoors and on the same food for the whole year had an increased concentration of vitamin D in the milk in the summer, and this vitamin D was found to be wholly cholecalciferol.

The results of Expt 3 show that a difference in altitude of 1000 ft had no influence on vitamin D levels in summer or winter. A southwards move of about 7° latitude approximately doubled the summer maximum value but had no influence on the low values found in winter. This absence of difference in the winter minimum between our three centres suggests that differences in latitude or altitude within the British Isles may have little or no influence on the blood vitamin D level of ewes during pregnancy and lactation and, consequently, may not account for locally occurring disorders of Ca and P metabolism found in ewes or young lambs.

The effects of a large intramuscular dose of vitamin D were for the first 4 months much greater than those arising from differences in pasture, climate or geographical situation. Such an injection given in December or January would probably have maintained the blood vitamin D level of a ewe above 50 i.u./100 g during lactation when the vitamin D requirement is likely to be greatest. The highest blood levels recorded were between 200 and 250 i.u./100 g, which is well below those observed in conditions of vitamin D intoxication in other animals. Warkany (1942) reports that one (human) patient tolerated a concentration of over 11000 i.u. ergocalciferol/100 ml serum for 2½ months without ill effects, and Thomas, Morgan, Connor, Haddock,

Bills & Howard (1959) produced a level of 5000 i.u./100 ml serum by giving a human subject 100000 i.u. ergocalciferol daily for 2 years. Warkany and also Thomas *et al.* noted very large variations in blood vitamin D level and in the manifestation of toxicity symptoms between individuals after oral administration of the vitamin.

Bots (1957) found that 10% of a similarly massive dose of cholecalciferol in oil injected into a chick muscle remained after 6 months. Fitch (1944) gave hoggets a subcutaneous dose of 1600000 i.u. ergocalciferol and found about 700 i.u./100 ml in the serum 3½ months later. Four months after a subcutaneous dose of 1000000 i.u. he found only small amounts of the vitamin present in thirty-five organs. In contrast Simpson (1956; quoted by Dalgarno *et al.* 1962), found that an intramuscular dose of 10000000 i.u. ergocalciferol in a sheep had no effect on the liver level of the vitamin 17 weeks afterwards.

In Expt 4 there were no significant differences in weight of ewe, breeding performance and fleece weight or in weight of lamb at birth and weaning between the two groups attributable to the massive dose of vitamin D. The absence of any effect on weight gain or lamb production has been the experience in many field trials with this type of intramuscular injection (National Agricultural Advisory Service, 1961; North of Scotland College of Agriculture: Agricultural Section, 1960, 1961).

There was no difference between the two groups in serum Ca values but the level of blood inorganic P was higher in the injected animals. These results are in agreement with those of Staskiewicz, Juskiewicz & Romanowska (1956) who found that a large dose of ergocalciferol raised both Ca and P blood levels in sheep on a 'winter diet' but only the P level of sheep on pasture.

#### SUMMARY

1. The vitamin D activity of the blood of sheep has been estimated by a radiographic, curative rat assay.
2. The blood level of vitamin D in the shorn, grazing sheep was at a maximum of 40-90 i.u./100 g whole blood in August or September. The level dropped to 10-30 in November and was at a minimum of about 5 i.u./100 g in April.
3. If the sheep were not clipped in the spring the summer rise was much reduced, indicating that sunlight was the major source of vitamin D for the sheep.
4. Vitamin D in the blood was reduced to a very low level in sheep that were housed out of sunlight during the summer, but a marked increase was found in early autumn. Possible reasons for this effect are discussed.
5. Comparison of sheep at Aberdeen and at 1200 ft above sea level in Scotland, both near latitude 58° N, with sheep at Reading near latitude 51° N indicated that the latter had a higher blood level of vitamin D in summer but the values for the three groups were not significantly different in late winter.
6. An intramuscular injection of 1 million i.u. vitamin D raised the blood level to over 100 i.u./100 g within a week. High levels were maintained for over 4 months.

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## REFERENCES

- Abrams, J. T. (1952). *Vet. Rec.* **64**, 151.  
 Andrews, E. D. & Cunningham, I. J. (1945-6). *N.Z. J. Sci. Tech.* (A), **27**, 223.  
 Bekemeier, H. (1959). *Hoppe-Seyl. Z.* **314**, 125.  
 Bekemeier, H. & Pfennigsdorf, G. (1959). *Hoppe-Seyl. Z.* **314**, 120.  
 Benford, F. (1947). *Illum. Engng*, **42**, 527.  
 Benzie, D., Boyne, A. W., Dalgarno, A. C., Duckworth, J. & Hill, R. (1959). *J. agric. Sci.* **52**, 1.  
 Benzie, D., Boyne, A. W., Dalgarno, A. C., Duckworth, J., Hill, R. & Walker, D. M. (1960). *J. agric. Sci.* **54**, 202.  
 Bots, J. P. L. (1957). *Rec. Trav. chim. Pays-Bas*, **76**, 209.  
 Clark, E. P. & Collip, J. B. (1925). *J. biol. Chem.* **63**, 461.  
 Curran, S. & Crowley, J. P. (1961). *Irish J. agric. Res.* **1**, 43.  
 Dalgarno, A. C., Hill, R. & McDonald, I. (1962). *Brit. J. Nutr.* **16**, 91.  
 Dunlop, G. (1935). *J. agric. Sci.* **25**, 22.  
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.  
 Fitch, L. W. N. (1944). *Aust. vet. J.* **20**, 303.  
 Franklin, M. C. (1953). *Aust. vet. J.* **29**, 302.  
 Grant, A. B. & O'Hara, P. B. (1957). *N.Z. J. Sci. Tech.* (A), **38**, 548.  
 Green, R. J. (1953). *Aust. vet. J.* **29**, 316.  
 Henry, K. M., Kon, S. K., Thompson, S. Y., McCallum, J. W. & Stewart, J. (1958). *Brit. J. Nutr.* **12**, 462.  
 Hibbs, J. W. & Pouden, W. D. (1955). *J. Dairy Sci.* **38**, 65.  
 Keener, H. A. (1954). *J. Dairy Sci.* **37**, 1337.  
 Kodicek, E. (1958). *Proc. int. Congr. Biochem.* iv. Vienna. Vol. 11, p. 198.  
 Kodicek, E., Darmady, E. M. & Stranack, F. (1961). *Clin. Sci.* **20**, 185.  
 Kolb, E. (1962). *Lehrbuch der Physiologie der Haustiere*, p. 367. Jena: Gustav Fischer.  
 Lenkeit, W., Brune, H. & Günther, K. (1958). *Z. Tierphysiol. Tierernähr.* **13**, 246.  
 National Agricultural Advisory Service (1961). *Great House Rev.*  
 New Zealand Department of Agriculture (1949-50). *Rep. Anim. Res. Div.* p. 12.  
 North of Scotland College of Agriculture: Agricultural Section (1960). *Field Trials Results*, p. 21.  
 North of Scotland College of Agriculture: Agricultural Section (1961). *Field Trials Results*, p. 15.  
 Quarterman, J., Dalgarno, A. C., Adam, A., Fell, B. F. & Boyne, R. (1964). *Brit. J. Nutr.* **18**, 65.  
 Quarterman, J., Dalgarno, A. C. & McDonald, I. (1961). *Proc. Nutr. Soc.* **20**, xxviii.  
 Schachter, D. & Rosen, S. M. (1959). *Amer. J. Physiol.* **196**, 357.  
 Schaefer, A. E., Sassaman, N. L., Slocum, A. & Greene, R. D. (1956). *J. Nutr.* **59**, 171.  
 Staśkiewicz, G., Juszkiewicz, T. & Romanowska, M. (1956). *Méd. vét. Varsovie*, **12**, 217.  
 Thomas, W. C. Jr., Morgan, H. G., Connor, T. B., Haddock, L., Bills, C. E. & Howard, J. E. (1959). *J. clin. Invest.* **38**, 1078.  
 Truter, E. V. (1956). *Wool Wax*. London: Cleaver Hume Press.  
 Wallis, G. C., Kennedy, G. H. & Fishman, R. H. (1958). *J. Anim. Sci.* **17**, 410.  
 Warkany, J. (1942). *J. Lab. clin. Med.* **27**, 557.