Vitamin D in the blood of sheep

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The vitamin D content of the tissues of mammals and birds is believed normally to be too small to be determined by any chemical method at present available. Information on the matter can be obtained only from biological assay; as this is laborious and time-consuming, it is perhaps understandable that few results have been recorded. The only published values for sheep are those of Warkany (1937), 46–67 i.u./100 ml plasma.

It is not clear in which tissues, if any, there is long-term storage of vitamin D. Heymann (1937) gave rabbits a large dose of vitamin D, 200000 i.u., and assayed the vitamin D in various tissues of animals killed at intervals up to 12 weeks after dosing. Vitamin D was lost most rapidly from the brain, next from red cells, small intestine, large intestine, skin, lungs, kidneys, liver and finally plasma, in that order. In a similar study with dogs, Morgan & Shimotori (1943) found vitamin D fairly uniformly distributed among the tissues assayed. In growing calves fed on hay and concentrates, almost equal concentrations of vitamin D were found in the liver and the blood (Guerrant, Morck, Bechdel & Hilston, 1938); although in the newborn calf a dose of 50000 i.u. caused a much larger increase in liver than in plasma vitamin D, the concentration in the liver 3 days after dosing was only some 50% higher than in plasma from the same animal (Eaton, Spielman, Loosli, Thomas, Norton & Turk, 1947*a*, *b*). Cruickshank, Kodicek & Armitage (1954) found 1440 i.u. in the liver as against a total of 620 i.u. in the muscle, skin and kidneys of rats killed 1 day after they had been given a large dose of vitamin D, but for those killed 2 days after dosing the amounts were 510 and 810 i.u., respectively. Later reports have confirmed that there is deposition of vitamin D in the liver of rats, in quantities of the order of 8–12% of the dose, 1 day after dosing by stomach tube or by intramuscular injection (Kodicek, 1956*a*; Cruickshank & Kodicek, 1956; Kodicek & Ashby, 1960*a*, *b*). Similar results were reported by Blumberg, Aebi, Hurni & Schönholzer (1960) for both rats and monkeys. The drop in vitamin D content of the rat's liver between 1 day and 2–4 days after dosing has also been confirmed, and it is suggested that destruction of vitamin D takes place in the liver (Kodicek, 1956*b*). Assay of the livers of sheep killed 1, 17 and 34 weeks after intramuscular doses of 0, 10^4, 10^5, 10^6 or 10^7 i.u. vitamin D suggested that liver storage was increased 1 week after the largest dose but that the effect had disappeared by 17 weeks (B. M. Simpson, 1956, personal communication).

In the absence of evidence for substantial long-term storage of vitamin D in body tissues it was considered that the concentration in the blood might be a useful guide

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to the vitamin D status of sheep. Measurement of blood concentration is experimentally convenient, because sufficiently large quantities for assay can be taken from groups of three or four sheep, and the progress of individual groups of sheep can be followed by taking serial blood samples.

In the experiment reported here, measurements were made on samples taken over a year from sheep kept indoors over winter, under several dietary régimes, and from others remaining outside throughout the year.

EXPERIMENTAL

Sheep

Thirty Blackface ewe lambs, aged 6 months and in moderate condition, were divided at random into three groups (A, B and C) of eight animals each and one group (D) of six. Groups A, B and C were housed in individual pens screened from sunlight from early November to early July. From early in November to early in May their diets were:

A, basal diet of low phosphorus content with no vitamin D supplement;

B, basal diet of low P content with a daily supplement of 500 i.u. vitamin D;

C, basal diet with added P but with no vitamin D supplement (P added as sodium dihydrogen phosphate).

The basal diet consisted of 50 g oat straw and 500 g of a concentrate mixture containing 75% dried mollassed sugar-beet pulp, 12% blood meal, 5% sugar, 6·5% starch, 0·5% limestone and 1% common salt. Several minor modifications to the diet were made: when straw consumption fell in the middle of the feeding period the quantity offered was temporarily reduced to 25 g daily; for short periods at the beginning and end of indoor feeding the quantities of concentrates were, respectively, 400 and 600 g daily.

The quantities of P offered daily to each sheep in groups A, B and C were 0·40, 0·40 and 1·46 g, respectively.

During the final 2 months of indoor feeding (early May to early July) the treatments were similar except that disodium hydrogen phosphate was added to the diets of groups A and B to provide levels of P similar to that of group C.

Over the whole period, group D remained out of doors and received hay and silage with a small quantity of dried grass during severe weather, but no supplements of calcium, phosphorus or vitamin D were given. The animals in groups A, B and C were put outside early in July and grazed together with group D from then until the last blood samples were taken in October. Half of the animals of each group were shorn in July and the others left unshorn.

Blood samples

Blood was taken for vitamin D assay at the beginning of the experiment in early November, and in the following February, May and October. A sample of 125–150 ml blood was withdrawn from the jugular vein of each animal. The samples from groups of three or four sheep were pooled for preparation and assay. Each pooled sample of

blood was saponified with alcoholic potash (50 g KOH and 250 ml ethanol to 500 g blood), the unsaponifiable fraction being extracted with diethyl ether, dried, ground with 5 ml arachis oil and stored at −20° under nitrogen until required for assay.

Biological assay of vitamin D

The method of assay was based on that of Bourdillon, Bruce, Fischmann & Webster (1931), in which the curative effect of doses of vitamin D is judged from radiographs of the tibial epiphyses of young rats reared on a rachitogenic diet. Eight litters of eight rats were used in each of nine separate assays. After weaning at 21 days of age, the rats were given a modified U.S.P. no. 2 vitamin D-deficient diet (Numerof, Sassaman, Rodgers & Schaefer, 1955) for 24 days. Their left tibias were radiographed to establish satisfactory development of rickets. Four rats from each litter were then given doses of vitamin D (international standard), at levels of 1, 2, 4 and 8 i.u. in assays nos. 1–5. There was some non-linearity of response at the level of the lowest dose, and the later assays, nos. 6–9, were therefore done with levels of 1·8, 2·9, 4·7 and 7·6 i.u. In assays nos. 2–5, the other four rats in each litter were given doses prepared from two blood samples, each at two levels; in assays nos. 1 and 6–9 each unknown was administered to only one rat from each litter, so that four blood samples were tested in each assay. In assays nos. 1–5 each dose, whether standard or unknown, was given in ten parts, on 10 consecutive days, but it was later concluded that there was no advantage in this procedure and in assays nos. 6–9 the whole dose was given on one day. After the rats had been dosed for 10 days, they were killed, and the left tibias were again radiographed.

The degree of recovery shown in the final radiographs was assessed on a 9-point scale of healing values. Each radiograph was scored separately and independently by two observers, without reference to the dose to which it referred. There was good agreement, in general, between the two scores, and these were averaged for statistical analysis. The potencies of the blood samples, and fiducial limits, were estimated by the methods described by Finney (1952) for parallel-line assays.

RESULTS

The vitamin D concentrations are given in Table 1, together with the number of the assay by which each was obtained. It was not possible to obtain a reliable value for some of the samples because the mean levels of healing they produced were outside the range of values for the standard doses. Either an upper or a lower 95% fiducial limit was calculated instead, and is given in Table 1 with a 'less than' (<) sign or a 'greater than' (>) sign, as appropriate.

Results were expressed in i.u./100 g, as it was more convenient to weigh the original samples of blood than to make accurate measurements of their volumes. Measurements on samples of blood from other sheep of the same breed gave a sp. gr. of 1·05 (I. F. Duthie, 1960, personal communication). This constant may be used to convert from i.u./100 g to i.u./100 ml without adding appreciably to the error of the determinations, even although there are slight differences in specific gravity of blood from sheep to sheep.

Table 1. *Concentrations of vitamin D in sheep's blood, obtained in assays nos. 1–9*

		Vitamin D concentration (i.u./100 g) in blood collected in				
Group*	Sub-group†	November 1957	February 1958	May 1958	Sub-group†	October 1958
A	1	7·7 (1)	—	7·7 (2)	s	>9·0 (9)
	2	16 (5)	<6·0 (6)	4·5 (7)	u	>9·0 (9)
B	1	7·0 (1)	7·0 (6)	18 (2)	s	19 (8)
	2	7·9 (3)	16 (7)	15 (3)	u	13 (8)
C	1	8·5 (1)	3·4 (7)	<9·0 (5)	s	>9·0 (9)
	2	8·5 (4)	<3·5 (6)	<5·0 (4)	u	>9·0 (9)
D		8·0 (1)	<4·8 (6)	16 (7)	s	30 (8)
(out of doors)					u	29 (8)

The assay nos. are given in parentheses.

Values prefixed < are upper 95 % fiducial limits and values prefixed > are lower 95 % fiducial limits for blood samples to which satisfactory values cannot be assigned because the dosage levels were poorly chosen.

* Groups A–C were given a low-phosphorus diet; A, without supplement; B, with vitamin D supplement; C, with P supplement (see p. 92).

† For the purpose of the first three bleedings the sheep in groups A, B and C were each split into two replicate subgroups, 1 and 2. After the third bleeding all four groups were split into two new subgroups, of which one was shorn (s) and the other left unshorn (u).

The precision of the results can be judged from Table 2, and is primarily dependent on the ratio, λ, which ranged in these nine assays from 0·15 to 0·32, satisfactory small values for vitamin D assays. Bliss & Cattell (1943) quote values of 0·07, 0·18, 0·25, 0·27 and 0·34 for five different techniques, and Jones (1945) gives mean values of 0·42, 0·45 and 0·59 for large numbers of radiographic assays over different periods of time. The breadth of the fiducial limits is determined by λ and also by the design of the assay and by the extent to which the assay result can be foreseen so that the most advantageous dosage levels can be chosen for the unknowns.

When vitamin D concentrations measured in different assays are compared in the light of the fiducial limits recorded in Table 2, comparatively few of the differences are

Table 2. *Precision of the assays and approximate* 95 % *fiducial limits for vitamin D contents*

Assay	λ*	Approximate 95 % fiducial limits (as percentage of estimate)
1	0·24	60–160
2	0·30	50–170
3	0·24	65–150
4	0·15	80–125
5	0·15	80–125
6	0·17	65–140
7	0·31	45–210
8	0·24	60–210
9	0·32	—

* $\lambda = \dfrac{\text{Residual standard deviation of healing scores}}{\text{Estimated increase in healing score with unit increase in } \log_{10} \text{(dose)}}$.

found to be significant. However, any two estimates obtained in the same assay may be compared more precisely, provided that the dosage levels were the same, since such comparison depends only on the radiographic scores and is not affected by the error in estimating the dosage–response relationship.

The first blood samples were taken in November, just before the animals were put on experiment; with the exception of group A2, they contained about 8 i.u. of vitamin D/100 g blood (see Table 1). The higher value exhibited by group A2 may reflect some lack of homogeneity in the sheep; no explanation has been found. By May, the vitamin D level in the blood had fallen for groups A and C, kept indoors without vitamin D supplementation, but had risen for group B, given such a supplement, and for group D, kept out of doors. These changes were significant, when tested on a within-assay basis, for groups B2 ($P < 0.05$) and C2 ($P < 0.01$). Further confirmation of the changes is provided by the significant differences in the May values between groups A1 and B2 ($P < 0.01$) and between groups A2 and D ($P < 0.01$). It may be noted that group A2 was the one giving the high initial value, but that by May it showed no difference from the other groups on treatments A and C. The results for the blood samples taken in February are more fragmentary and cannot be compared with either the November or the May values on a within-assay basis. They suggest that the falls noted in the May values for groups A and C had already taken place by February, but that the rise for group D had not. The October results, obtained after all the sheep had been grazing for 3 months, showed some recovery for groups A and C from the relatively low vitamin D concentrations found in May, but the doses given in assay no. 9 proved too high for calculating precisely the levels in those two groups. The October values for group D, which had been outside throughout the experiment, were the highest of all and significantly higher than those for group B ($P < 0.01$). There were no significant differences between the shorn and unshorn subgroups, either in group B or group D.

DISCUSSION

The vitamin D concentrations showed a considerable range, from about 3 to 30 i.u./100 g, in sheep's blood. Although part of the variation undoubtedly arose from the imprecision of the assay technique, most of the larger differences were consistent between replicates and were in directions that could reasonably be expected in the light of differences in treatment and in time of year. It may be concluded that the concentration in the blood can be taken as a guide to the vitamin D status of sheep. In particular, the May concentrations were higher for group D kept out of doors, and for group B given a vitamin D supplement, than for the other two indoor groups. The difference was small in relation to the amount of the vitamin D supplement, which was 500 i.u. daily for each sheep during the 6 months from November to May. On the assumption that about 8% of a sheep's body-weight is blood (Dukes, 1947), the supplement was at the rate of approximately 20 i.u./100 g blood daily, yet the total rise in concentration over 6 months was only about 8 i.u./100 g blood for group B, against a fall of possibly 4 i.u./100 g blood for the indoor sheep not receiving the supplement. In May the levels were about 16 i.u./100 g blood for groups B and D; by October the

value for group D had risen to about 30 i.u./100 g whereas that for group B was unchanged. It would appear either that group D had benefited greatly from the outdoor conditions in June or else, more likely, that the response of group B to summer grazing from July onwards was influenced by recovery from the disturbance to mineral metabolism associated with the low-P diet of the winter months. The mineral status of the sheep was assessed radiographically in November 1957, May 1958 and July 1958. Results of this study have been given in detail elsewhere (McRoberts, 1961), but they may be summarized as showing that from November to May there was considerable loss of mineral from the skeletons of groups A and B and a smaller loss from those of group C, whereas group D was relatively unaffected. By July there had been considerable recovery but it was by no means complete. It is likely that group B had a higher vitamin D requirement than group D while this repair was taking place and was for this reason unable to match the rise in blood level of the latter group.

The October measurements showed no difference between the vitamin D levels in the blood of shorn and unshorn sheep. There may, however, have been a period between May and October when the shorn subgroups had higher levels. A later experiment (Quarterman, Dalgarno & McDonald, 1961) showed that shearing had an effect on vitamin D levels in June and August but that the effect had disappeared by November. In an experiment with rats, Cruickshank & Kodicek (1955) showed that there was increased formation of vitamin D, under irradiation, when fur was removed.

The results for group D show a rise in vitamin D level from about 8 to 30 i.u./100 g blood over the year, during which the sheep were kept out of doors in the same region of the country as that in which they had been reared from birth to 6 months of age. Such a rise in vitamin D level may be normal between the ages of 6 and 18 months, and possibly associated with the decrease in the rate of skeletal growth, but at present this can be no more than surmise.

All the values obtained by us were lower than those found for sheep in the U.S.A. by Warkany (1937). The difference may be due to differences in exposure to sunshine, since that author's measurements were made in the months of May and June in a more southerly latitude than that of Aberdeen, or else to differences in type of sheep or diet.

SUMMARY

1. Thirty ewe lambs were divided into four treatment groups in November 1957. One group remained outside on pasture, but the other three were kept indoors on diets differing in their contents of phosphorus and vitamin D until early in July 1958, after which all four groups grazed together outside.

2. Blood samples were taken from the sheep in November 1957 and in February, May and October 1958, and the vitamin D contents of these samples were determined in a series of nine rat assays by a radiographic technique.

3. The vitamin D concentrations ranged from about 3 to 30 i.u./100 g blood (sp. gr. 1·05). Several significant differences in concentrations were found. These are discussed in relation to the corresponding differences in treatment or in time of sampling,

and it is concluded that the concentration in the blood can be taken as a guide to the vitamin D status of sheep.

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REFERENCES

Bliss, C. I. & Cattell, McK. (1943). *Annu. Rev. Physiol.* **5**, 479.
Blumberg, A., Aebi, H., Hurni, H. & Schönholzer, G. (1960). *Helv. physiol. acta*, **18**, 56.
Bourdillon, R. B., Bruce, H. M., Fischmann, C. & Webster, T. A. (1931). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 158.
Cruickshank, E. M. & Kodicek, E. (1955). *Proc. Nutr. Soc.* **14**, viii.
Cruickshank, E. M. & Kodicek, E. (1956). *Biochem. J.* **64**, 24*P*.
Cruickshank, E. M., Kodicek, E. & Armitage, P. (1954). *Biochem. J.* **58**, 172.
Dukes, H. H. (1947). *The Physiology of Domestic Animals*, 6th ed. New York: Comstock Publishing Corp.
Eaton, H. D., Spielman, A. A., Loosli, J. K., Thomas, J. W., Norton, C. L. & Turk, K. L. (1947*a*). *J. Dairy Sci.* **30**, 787.
Eaton, H. D., Spielman, A. A., Loosli, J. K., Thomas, J. W., Norton, C. L. & Turk, K. L. (1947*b*). *J. Dairy Sci.* **30**, 795.
Finney, D. J. (1952). *Statistical Method in Biological Assay*, 1st ed. London: Charles Griffin and Co. Ltd.
Guerrant, N. B., Morck, R. A., Bechdel, S. I. & Hilston, N. W. (1938). *Proc. Soc. exp. Biol., N.Y.*, **38**, 827.
Heymann, W. (1937). *J. biol. Chem.* **118**, 371.
Jones, J. I. M. (1945). *Quart. J. Pharm.* **18**, 92.
Kodicek, E. (1956*a*). In *Bone Structure and Metabolism*, p. 161. *Ciba Found. Symp.* London: J. and A. Churchill.
Kodicek, E. (1956*b*). *Biochem. J.* **64**, 25*P*.
Kodicek, E. & Ashby, D. R. (1960*a*). *Biochem. J.* **75**, 17*P*.
Kodicek, E. & Ashby, D. R. (1960*b*). *Biochem. J.* **76**, 14*P*.
McRoberts, M. R. (1961). The effects of calcium, phosphorus and vitamin D upon bone and tooth development in sheep. Ph.D. Thesis, University of Aberdeen.
Morgan, A. F. & Shimotori, N. (1943). *J. biol. Chem.* **147**, 189.
Numerof, P., Sassaman, H. L., Rodgers, A. & Schaefer, A. E. (1955). *J. Nutr.* **55**, 13.
Quarterman, J., Dalgarno, A. C. & McDonald, I. (1961). *Proc. Nutr. Soc.* **20**, xxviii.
Warkany, J. (1937). *Biochem. Z.* **293**, 415.

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