The response of serum 25-hydroxyvitamin D concentrations to vitamin D intake and insolation in sheep

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1. Vitamin D-depleted, housed sheep were given diets providing fixed intakes of cholecalciferol ranging from 0·0 to 0·8 μg/kg body-wt per d for 220 d. Thereafter they were shorn, deprived of dietary cholecalciferol and turned out from 30 June to 30 November. The concentration of 25-hydroxyvitamin D (25-OHD) in serum was determined at frequent intervals.

2. The serum concentrations of 25-OHD took approximately 10 weeks to stabilize after which they reflected dietary intake over the range 0·1–0·4 μg/kg body-wt per d. For intakes of 0·4 and 0·8 μg/kg body-wt per d the mean maximum concentrations were similar, but the rates of increase differed. The latter was proportional to the logarithm of intake over the range studied.

3. Changes in serum 25-OHD due to insolation were similar in all sheep regardless of their starting values, and consequently in some animals reached levels considerably greater than from the dietary source.

4. Although the response of serum 25-OHD to the higher dietary intakes appeared to be limited there was no evidence of any such control over the response to endogenously-synthesized vitamin.

Although previous studies (Quarterman *et al.* 1961, 1964; Dalgarno *et al.* 1962) have shown that insolation has a pronounced effect on circulating concentrations of vitamin D in sheep, the results available do not permit 'a valid assessment of the amount of vitamin D likely to be synthesized by domestic livestock exposed to sunlight' (Agricultural Research Council, 1965), nor is it possible to assess the relative proportions provided by diet and endogenous synthesis. There is similarly very little information on which the requirement of sheep for the vitamin can be based. The only study designed specifically to determine this was that of Andrews & Cunningham (1945) using six-month-old lambs, the criterion of adequacy being the prevention of rickets. No measurements of circulating concentrations of vitamin D were made. There has been no quantitative work on the vitamin D status of sheep in the last decade since the advent of the more specific and precise methods of estimation which have resulted in the explosion of interest in the importance of vitamin D in human nutrition. The present study was designed to add to the meagre relevant information currently available.

The best available measure of vitamin D status is the circulating concentration of 25-hydroxyvitamin D (Stanbury & Mawer, 1978), but although this parameter is widely used in human medicine its relationship with vitamin D intake has been the subject of some controversy. Several investigators have shown a diminishing response in plasma 25-OHD to increasing oral doses of vitamin D and explain this in terms of a control of the hydroxylation in the liver which, however, may be over-ridden at high dose levels (Bhattacharyya & De Luca, 1973; Stanbury *et al.* 1973; Fraser, 1975; Mawer & Reeve, 1977). Others (Haddad & Stamp, 1974) have shown a linear relationship between plasma 25-OHD concentrations and vitamin D intake over a wide range of the latter which included massive therapeutic doses. Doubts about the existence of an effective control of 25-hydroxylation have been expressed by Tucker *et al.* (1973), and Clark & Potts (1977).

Before serum 25-OHD concentrations can be used as an indicator of adequate vitamin D status it is essential to establish whether a measurable response in the former results from

changes within the normal range of intakes for healthy animals. This study was designed to examine this relationship in sheep using dietary vitamin supplements ranging from 0 to 40 μg/d, i.e. from zero to approximately six times the recommended daily intake (Agricultural Research Council, 1965). The animals were housed to eliminate any contribution from insolation. When plasma 25-OHD concentrations had stabilized, the dietary source of vitamin D was removed and the sheep placed out of doors to compare the relative effect of exposure to sunlight.

EXPERIMENTAL

Twenty-five adult Blackface non-pregnant ewes (average weight 51·8 kg) were divided at random into five groups of five animals. During the first part of the experiment when diets of known cholecalciferol supplementation were fed (220 d) the sheep were kept indoors in individual pens and were given their respective diets (approximately 1 kg/d per sheep) to maintain constant body-weight. At 221 d the sheep were shorn, placed out-of-doors and the basal diet with no added cholecalciferol was given to all animals which were group-fed to maintain constant body-weight thereafter. Throughout the experiment blood samples were taken at intervals, and serum stored at $-20°$.

The diets were pelleted and of the following basic composition: bruised oats, 60 kg; ground oat hulls, 22 kg; sugar, 5 kg; dried skimmed milk, 5 kg; urea, 1 kg; ammonium chloride, 1 kg; sodium bicarbonate, 2 kg; potassium chloride, 1 kg; calcium carbonate, 0·75 kg; anhydrous sodium sulphate, 1 kg; water, 6 l; Pristerine (Price Chemical Company, Bebington, Merseyside), 0·8 kg; $FeSO_4.7H_2O$, 19·0 g; $MnSO_4.H_2O$, 6·0 g; $ZnSO_4.7H_2O$, 2·0 g; $CoCl_2.6H_2O$, 0·25 g; potassium iodide, 1·0 g; $CuSO_4.5H_2O$, 1·19 g; α-tocopheryl acetate, 20 g; and Rovimix (Roche Products Ltd., Dunstable, Bedfordshire) equivalent to 1·41 mg retinol per kg. Supplementary cholecalciferol (Rovimix) was added to the basal diet (D0) to give intakes of 50, 100, 200, 400 and 800 ng/kg body-weight per d when fed D2, D4, D8, D16 and D32 respectively at the previously-stated rate. All animals were initially given diet D0 to reduce circulating 25-OHD levels. After 4 weeks five animals were allocated at random to each of diets D0, D2, D4, D8 and D16. After a further 12 weeks the sheep on diet D2 were changed to diet D32. The calcium and phosphorus concentrations in the diets were 4·7 and 2·6 g/kg respectively.

Concentrations of Ca in serum were determined by atomic absorption spectroscopy and inorganic phosphate by a modified Fiske & Subbarow (1925) procedure.

The concentration of 25-OHD in serum was determined by the method of Belsey et al. (1974). The method was modified as follows: serum from normal rats at a dilution of approximately 1:6000 was used as a source of binding protein. 25-hydroxy[26 (27)methyl^{3}H]-cholecalciferol (specific activity 7–12 Ci/mmol; The Radiochemical Centre, Amersham, Bucks.) was diluted to 0·0625 μCi/ml for use in the assay. Protein in 0·2 ml samples was precipitated with 0·8 ml ethanol. A 0·2 ml portion of the supernatant fraction and 0·9 ml of the binding-protein solution were used. Recovery of tritiated 25-OH cholecalciferol from serum was shown to be quantitative, so routine monitoring of recovery in this stage of the assay was unnecessary. Samples (1·5 ml) were mixed with 12 ml of toluene based scintillator and counted using external standardization. The scintillator contained 1 l toluene, 500 ml Triton-X100, 5 g 2,5-diphenyloxazole and 0·3 g 1,4-di-2(5-phenyloxazolyl) benzene. The validity of the quench-correction procedure for this particular application was confirmed by demonstrating the identity of quench curves obtained with [^{3}H]hexadecane and ^3H_2O using the same proportions of toluene, Triton X-100 and water (Fox, 1974).

Results were routinely calculated on an IBM 175 computer using the Animal Breeding Research Organization radioimmunoassay computer package which employs a logit-log transformation and iterative weighted least squares regression analysis (Rodbard & Lewald,
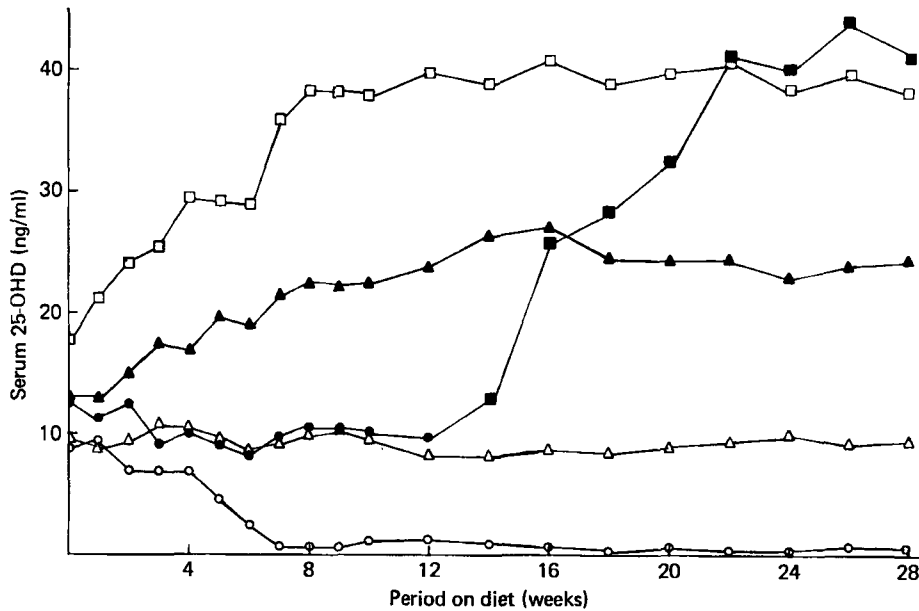
Fig. 1. Mean concentrations of 25-hydroxyvitamin D (25-OHD) in serum of sheep given varying amounts of cholecalciferol (ng/kg body-wt per d): (○), basal diet; (●), 50; (△), 100; (▲), 200; (□), 400; (■), 800; for details of diets, see p. 534.

1970). The program includes checks for linearity of the dose response curve, parallelism between standards and diluted control samples and provides for quality control and assay optimization. The computed minimum detectable concentration was 1·5 ng/ml. The program provides an estimate of the concentration in the sample together with 95% confidence limits based on the analyses of replicates and the characteristics of the standard curve for each individual assay. Using the previously-mentioned procedures it was possible to carry out approximately eighty determinations in duplicate daily. The mean coefficient of variation for repeated analyses of a pooled plasma sample containing 17·0 ng 25-OHD/ml was 3·5% for nine replicates on one occasion and 5·6% for single determinations on twenty-six occasions.

Relative monthly insolation intensities were calculated from sunshine records provided by the Royal Observatory, Edinburgh, corrected for azimuthal variation using the results of Beadle (1977).

RESULTS

The mean concentrations of 25-OHD in plasma during the first 220 d are shown in Fig. 1. Since none of the dietary treatments appeared capable of producing concentrations as high as those encountered under conditions of normal farming practice in summer (B. S. W. Smith and H. Wright, unpublished results), the sheep originally given diet D2 were transferred after 84 d to diet D32. Animals given the basal diet without supplementary cholecalciferol (diet D0) showed a decrease ($P < 0.001$) in circulating 25-OHD levels during the first 6 weeks on the diet, the values thereafter remaining constant at 2 ng/ml. Both diet D2, which was only fed for the first 12 weeks, and diet D4 which provided 80% of the currently-recommended supplement for sheep (Agricultural Research Council, 1965), produced no significant change from the low starting values. The other diets all caused
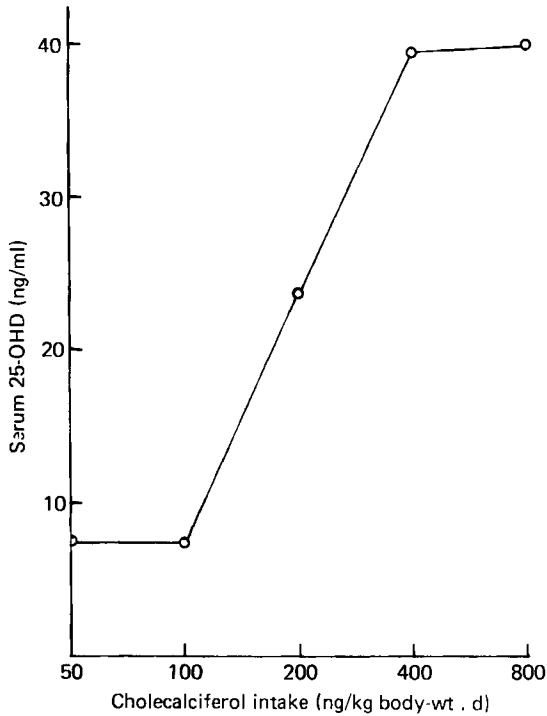
Fig. 2. Relationship between dietary cholecalciferol (ng/kg body-weight per d) and serum 25-hydroxyvitamin D (25-OHD) concentration (ng/ml) in sheep.

significant ($P < 0.001$) increases in concentration. Diet D8 gave values which reached a plateau after 12 weeks at a mean concentration of 23 ng/ml. Surprisingly, the maximal concentrations obtained on diets D16 and D32 were essentially the same (approximately 40 ng/ml) although the rate of increase on diet D32 was greater. From Fig. 2 it can be seen that the serum 25-OHD only provides a measurable response for intakes ranging from 100 to 400 ng/kg body-weight per d. The rate of change of serum 25-OHD concentrations during the first 10 weeks of the experiment did reflect the dietary intake and it can be seen from Fig. 3 that the rates were proportional to the logarithms of the intakes.

In Fig. 4 the mean serum concentrations are shown for the period from 30 June to 30 November 1977 when the sheep were kept out of doors on diet D0. The 25-OHD concentrations increased for the first 5 weeks and the extent of the increase was similar in all groups regardless of the widely-different initial concentrations. Values subsequently declined slowly throughout the remainder of the period of observation. Again, the extent and rate of decline were similar in all groups.

There were no significant changes with time in the concentrations of either Ca or inorganic P for any group and all treatments were therefore without effect on either of these parameters. The over-all mean ($\pm$ SE) concentrations were 2.49 ± 0.033 and 1.82 ± 0.058 mol/l for Ca and inorganic P respectively.
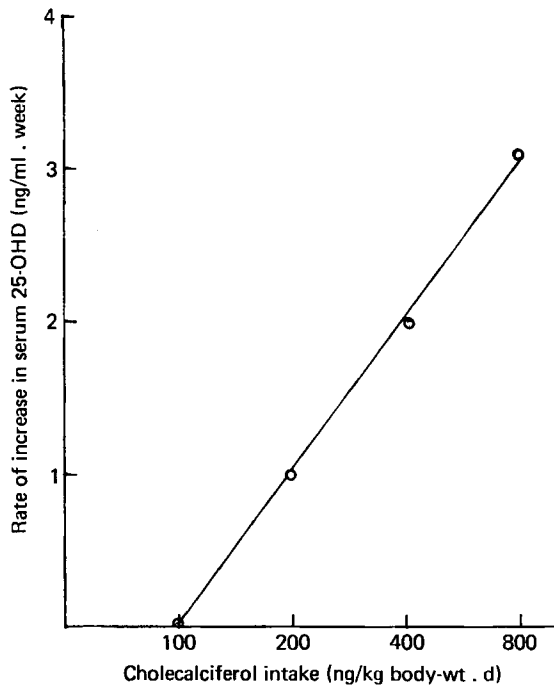
Fig. 3. Relationship between dietary cholecalciferol (ng/kg body-weight per d) and rate of increase of serum 25-hydroxyvitamin D (25-OHD) concentration (ng/ml per week) in sheep.
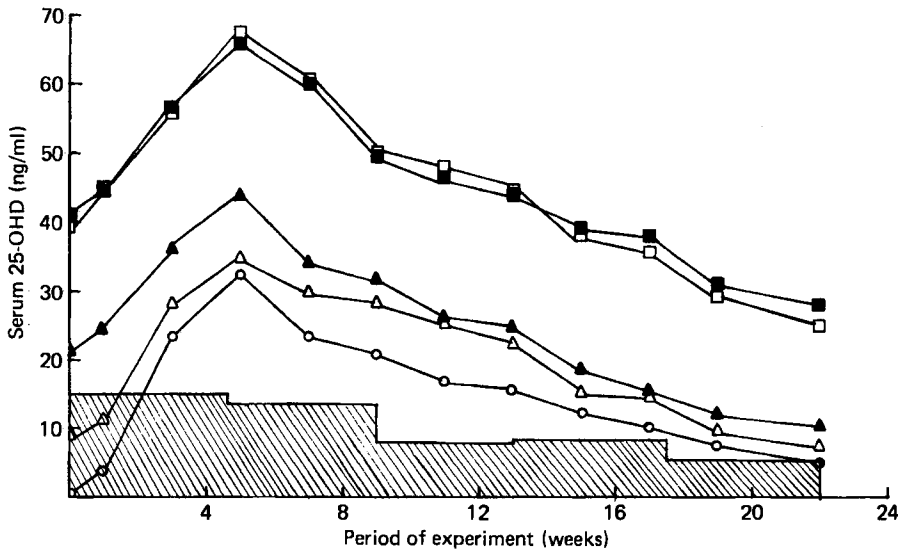
Fig. 4. The effect of exposure to sunlight, in the absence of dietary vitamin D, on mean serum 25-hydroxyvitamin D (25-OHD) concentration (ng/ml) in sheep: (○), basal diet; (△), 100; (▲), 200; (□), 400; (■), 800; for details of diets, see p. 534. ▨ Relative monthly sunlight intensity (arbitrary units).

DISCUSSION

The simplified assay procedure which omits the chromatographic separation has a significant advantage in allowing more samples to be processed in a given time. It does, however, estimate 24, 25-dihydroxyvitamin D (24, 25-diOHD) with an efficiency equal to that for 25-OHD, and vitamin D with a relative efficiency of 1 %. It has been shown that 24, 25-diOHD occurs at a concentration of rather less than 10 % of 25-OHD under normal physiological conditions (Taylor *et al.* 1976, 1977) and so the values we obtained for 25-OHD would be expected to be high to this extent. Offerman & Dittman (1974) have demonstrated a close correlation between this simplified assay and one using a chromatographic separation. Consequently this lack of specificity does not detract from the validity of the use of the simplified assay as an index of vitamin D status.

Our results indicate that over the range of intakes from 0·1 to 0·4 μg/kg body-weight per d the estimation of serum 25-OHD provides a useful measure of vitamin intake in sheep. Above and below these limits the response curve becomes less sensitive, and if current estimates of requirements are correct (0·125 μg/kg live-weight per d; Agricultural Research Council, 1965) serum 25-OHD concentrations are of limited value for indicating deficiency for this reason. However, information on which estimated requirements can be based is very limited and depends on only three studies with small numbers of animals. The recommended intake was chosen to be just sufficient to prevent rickets on the basis of these studies, but it is quite conceivable that under some conditions of farming practice requirements could exceed recommended intakes. It is questionable whether requirements should be based on prevention of overt disease rather than providing for maximum production, and it might therefore be considered prudent to provide a vitamin D intake in excess of the recommended requirement by for example 50–100 % in order to allow a safety margin which would be adequate for rapid growth, pregnancy or for sub-optimal Ca and P intakes. Such a provision would be unlikely to produce problems of toxicity for the following reasons. The lowest dose of vitamin D which has been reported to produce toxic effects is 12·5 mg (Clegg, 1976) which represents 5·5 years intake for 50 kg animals at the recommended level. Secondly, as shown in this study, intakes of six times requirements do not produce circulating concentrations as high as those encountered in the field in summer (B. S. W. Smith and H. Wright, unpublished results). Additionally, there is now some evidence that 25-OHD is the compound responsible for toxic effects (Counts *et al.* 1975; Hughes *et al.* 1976; Morrisey *et al.* 1977). If this is so, it is clear that a modest increase in intakes above recommended requirements would still allow a large safety margin. Under such circumstances the assay of circulating 25-OHD would provide a valuable indication of adequacy and the need for supplementation.

There was no difference between the mean serum 25-OHD concentration in the groups receiving 0·4 and 0·8 μg/kg body-weight per d. If this is the result of a control on 25-hydroxylation rather than increased catabolism it would be expected that at intakes greater than 0·4 μg/kg body-weight per d the excess vitamin would be stored, presumably as cholecalciferol. Storage of vitamin D has been reported after both high and low vitamin intakes in humans (Mawer *et al.* 1972) but there is very little information on the availability of this stored material. If storage did occur in these sheep on high intakes it did not delay the decrease of serum 25-OHD after the sheep had been exposed to sunshine. It may be that storage did not take place or that plasma concentrations during depletion did not reach sufficiently low levels for the stores to become available.

The results from our dietary trials appear to be in keeping with the widely-accepted view that 25-hydroxylation is controlled in vivo (Holick & De Luca, 1978). However, the relative responses of the different groups of sheep to insolation do not appear to be subject to the

same degree of control as in the dietary trial, in that the response was of the same order regardless of initial plasma values, some of which were already apparently limited by the control process. This implies that the control mechanism was either by-passed or swamped. The generally accepted site for control of 25-OHD formation is the liver and it is difficult to see how endogenously-synthesized vitamin could escape this control. However, endogenous production does by-pass the gut and control of absorption could explain our results although we are not aware of any direct evidence for such a process. Before considering whether swamping of the control process is a reasonable explanation it is necessary to get some estimate of the dietary equivalent of the endogenous vitamin D production. From Fig. 3 it can be seen that the rate of increase in plasma 25-OHD concentrations (y) is proportional to the logarithm of the intake ($\log x$), the equation for the regression being $y = 3.43 \log x - 6.89$. By extrapolation, the rate of increase obtained by insolation is equivalent to a daily intake of the order of 250–300 μg. This estimate is similar to that given by Stamp *et al.* (1977) for the dietary equivalent of a maximal response to u.v. in humans. Such an intake is within the range for which control of 25-OHD concentration appears to operate in humans (Stamp *et al.* 1977) and so it is possible that in our experiment the control mechanism was not swamped. This aspect of our results cannot therefore be adequately explained at the moment, and further experimentation will be necessary.

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