The effect of dose rate of 1-α-hydroxycholecalciferol on calcium and phosphorus metabolism in sheep

BY G. D. BRAITHWAITE

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT

(*Received* 28 *January* 1980 – *Accepted* 21 *February* 1980)

1. A combination of a mineral balance and a radioisotope technique has been used to study the relationship between dose rate of 1-α-hydroxycholecalciferol (1α-OH-D_3) and the magnitude and duration of its effect on the various processes of calcium and phosphorus metabolism in adult wether sheep.

2. The rate of absorption of Ca was markedly increased by treatment and maximum response occurred at the lowest dose rate.

3. Although sheep were already Ca-replete, the extra Ca absorbed was all retained and increased retention was brought about by a combination of an increase in bone accretion and a decrease in bone resorption. This finding conflicts with the generally-held belief that bone resorption is increased by cholecalciferol treatment.

4. The rates of absorption and retention of P were increased by 1α-OH-D_3 treatment and maximum response occurred at the lowest dose rate.

5. That P absorption could be increased by treatment suggests that not all the available dietary P was absorbed in the control period.

6. Although the loss of endogenous P in the faeces was unaltered by treatment, the secretion of P into the gut was increased, and the increase was directly related to increased serum inorganic P concentration.

7. Nearly all the extra P absorbed was retained and increased retention was achieved by a combination of an increased incorporation into and a decreased loss from the non-exchangeable pools of bone and soft tissues.

8. The time interval taken for absorption rates of Ca and P to return to normal after the end of each treatment was related to the dose rate of 1α-OH-D_3. Although higher dose rates had little effect on the magnitude of response, they did prolong slightly the duration of response.

1-α-Hydroxycholecalciferol (1α-OH-D_3) a synthetic analogue of 1-α-25-dihydroxycholecalciferol (1α, 25$(OH)_2D_3$) the biologically-active metabolite of vitamin D, has been used with some success to prevent post-parturient hypocalcaemia and reduce the incidence of milk fever in dairy cows (Sansom *et al.* 1976; Barlet, 1977; Gast *et al.* 1977; Sachs *et al.* 1977). Recently its mechanism of action has been clarified in studies with lactating ewes (Braithwaite, 1978). Treatment resulted in a marked increase in the absorption of calcium and in the apparent absorption of phosphorus and as a consequence, the negative mineral retention normally associated with peak lactation (Braithwaite *et al.* 1969), was prevented.

Studies of serum Ca and P concentration in cows treated with different doses of 1α-OH-D_3 suggest that larger doses increase the duration, rather than the magnitude of response (Sansom, 1977). The relationship between dose rate of 1α-OH-D_3 and the magnitude and duration of its effect on the various processes of Ca and P metabolism has now been studied in the sheep, here used as a model ruminant.

EXPERIMENTAL

Animals, housing and diet. Eight 2-year-old Suffolk × Scottish blackface wethers weighing 60–70 kg were housed in metabolism cages designed for the separate collection of urine and faeces. They were maintained on a diet of hay and concentrates (Table 1) and had free access to distilled water.

Experimental procedure. Animals were allowed 1 month to adapt to the experimental diet. They were then given in random order at monthly intervals each of the following four

0007-1145/80/3474-2102 $01.00

Table 1. *Composition of the basal diet given daily to wether sheep*

Ingredient*	Amount (g/kg body-wt)	Total Ca (mg/kg body-wt)	Total P (mg/kg body-wt)
Hay	10	45·0	18·7
Barley	5	2·8	18·3
Bran	1	0·6	13·0
Soya-bean meal	1·5	5·4	10·5
Dicalcium phosphate†	0·33	66·1	51·4
Total		119·9	111·9

* The diet also contained βeta vitamin No. 3a (Cooper Nutrition Products Ltd, Witham, Essex) to supply (/kg body-weight) 37·5 μg retinol equivalent and 0·775 μg cholecalciferol.

† For the experiment with a high calcium intake, this diet was supplemented with double the amount of dicalcium phosphate.

treatments: 0, 0·02, 0·05 or 0·1 μg 1α-OH-D_3 in propylene glycol/kg body-weight per d injected intramuscularly for 12 d.

Ca and P kinetic studies were carried out during the last 7 d of each treatment. A known amount of ^{45}Ca as $CaCl_2$ and ^{32}P as orthophosphate (2·5 and 6 μCi/kg body-weight respectively) in aqueous solution was injected into a jugular vein and samples of blood, urine and faeces were collected as previously described (Braithwaite *et al.* 1969). At the same time Ca and P balance measurements were made. Balance measurements were then continued for a further 21 d after the end of each treatment period.

Determination of Ca and P. The methods used for measurement of the Ca content of blood, food, urine and faeces have been described previously (Braithwaite *et al.* 1969). Total P content of urine and ashed samples of food and faeces were determined by the procedure of Fiske & Subbarow (1925) modified (Technicon Instruments Corporation, 1967) for use with an autoanalyser. Serum inorganic phosphorus (P_i) was measured by the same procedure after first precipitating the protein with trichloroacetic acid (200 g/l) (Manston, 1966).

Measurement of radioactivity. Radioactivity was measured in a Packard liquid-scintillation spectrometer (Model 2450B) by a dual-label technique with external standardization. Samples of serum (1 ml of the TCA-supernatant fraction), urine (1 ml acidified with three drops 2 M-hydrochloric acid) and ashed faeces in HCl (1 ml) were counted in 10 ml Insta-gel scintillator solution (Packard Instruments Co. Inc.).

Kinetic analysis. Kinetic analysis of the Ca results was done by the method of Aubert & Milhaud (1960) modified for use with sheep (Braithwaite *et al.* 1969; Braithwaite & Riazuddin, 1971; Braithwaite & Glascock, 1976).

A similar method of analysis was also used for the P results. The specific radioactivity of serum P_i was plotted *v.* time on semi-logarithmic co-ordinates and the curve was resolved by standard methods of curve analysis into five exponentials. The integral of the curve, which in effect gives the mean specific radioactivity of the exchangeable pool of P_i over the 7 d period of the experiment, together with the total radioactivity recovered in urine and faeces allows calculation of the endogenous loss of P in urine and faeces (faecal endogenous P). The rate of P absorption (V_a) was then obtained from the equation

$$V_a = V_i + V_f - F,$$

where V_i is the rate of ingestion of P, V_f is the rate of endogenous loss in faeces and F is the rate of total loss in faeces. This method, with minor modifications, has previously been used by other workers to calculate rates of P absorption and faecal endogenous loss of P in both sheep and cattle (Schroder & Hansard, 1958; Gueguen, 1963; Preston & Pfander, 1964; Young, Lofgreen *et al.* 1966; Symonds, 1969).

Only 80% of the total body P is present in the skeleton, compared with 99% of Ca. The remaining 20% of P is present in soft tissues, largely in an organic form and probably non-exchangeable. The rate of accretion of P into bone cannot therefore be calculated in the same way as for Ca and the equation of Aubert & Milhaud (1960) which describes the total loss of P from the exchangeable pool (V_T) has to be modified to include the additional loss of P into soft tissues. Thus:

$$V_T = V_u + V_f + V_o^+ + V_{ST},$$

where V_u is the rate of excretion in the urine, V_o^+ is the rate of accretion of P into bone and V_{ST} is the rate of incorporation of P into soft tissues. Although the total loss (V_T) can be calculated in the usual way by the method of Parsons (1968) it is not possible to distinguish between V_o^+ and V_{ST} and only a combined value for these two processes can be calculated. It is recognized that this combined value may be subject to error. One problem is that calculations are based on the assumption that no radioactivity returns from the non-exchangeable pools during the period of the experiment. Whilst this is probably true of bone P it may not hold for soft-tissue P. Nevertheless this value may be a useful indicator of changes in bone P metabolism particularly in comparative studies. This belief is strengthened by the present investigations, in which kinetic studies suggest similar changes in Ca and P metabolism in bone as a result of 1α-OH-D_3 treatment.

RESULTS AND DISCUSSION

The 1α-OH-D_3 treatment resulted in a marked increase in the rates of absorption and retention of Ca (Table 2). There was no significant difference, however, due to the level of dose given, maximum response occurring at the lowest dose rate. Since the efficiency of absorption of Ca during treatment approached 50% which is probably the maximum obtainable from hay and concentrate diets (Braithwaite, 1976), absorption may have been limited by the availability of dietary Ca. To eliminate this possibility, these same sheep, treated with the highest dose rate of 1α-OH-D_3 were given a Ca intake of 200 mg/d per kg body-weight, i.e. nearly double the previous intake. The resulting rates of absorption and retention of Ca were virtually identical with those obtained at the lower intake. The limiting factor in absorption therefore could not have been availability of dietary Ca but must rather have been the capacity of the intestine to absorb Ca, which presumably became saturated even at the lowest dose rate of 1α-OH-D_3 used.

Nearly all the extra Ca absorbed during treatment was retained. Faecal endogenous loss of Ca was unchanged and loss in urine increased only slightly at the higher dose rates. Since these sheep were already Ca-replete and in the control period absorbed only enough to meet their maintenance requirements (i.e. unavoidable losses in urine and into intestine), it is surprising that the extra Ca absorbed during treatment was retained and not immediately excreted. These findings, however, do support a previous suggestion (Braithwaite, 1979) that ruminants may lack a mechanism for eliminating surplus Ca.

The increased total body retention of Ca, which reflected an increase in skeletal retention, was brought about by a combination of a decrease in the rate of bone resorption and an increase in the rate of bone accretion. This finding disagrees with the generally-held belief that the major effect of 1α,25$(OH)_2D_3$ on bone is to increase bone resorption (Fraser & Kodicek, 1973; DeLuca, 1975, 1977). However, evidence is now accumulating which throws doubt on this belief and which instead suggests that the predominant effect of 1α,25$(OH)_2D_3$ is to increase bone accretion. Thus, increased bone accretion rates have been reported in man and in lactating ewes treated with 1α-OH-D_3 (Pierides *et al.* 1976; Braithwaite, 1978) and in mature rats treated with 1α, 25,$(OH)_2D_3$ (Larsson *et al.* 1977). Furthermore, it must be pointed out that the 1α-OH-D_3 and 1α,25$(OH)_2D_3$ mediated

Table 2. *Effect of dose-rate of* 1-α-*hydroxycholecalciferol* (1α-*OH-D*$_3$) *on calcium and phosphorus metabolism of wether sheep*

(Results in mg/d per kg body-weight are means of eight animals/group)

	1α-OH-D$_3$ (μg/d per kg body-wt)					standard error (residual) mean square)	Difference between mean values required for statistical significance: $P < 0.05$
	0	0·02	0·05	0·1	0·1		
Rate of ingestion of Ca	109·6	107·3	110·6	114·5	198·2	2·3	7·4
Rate of loss of Ca in faeces	111·1	72·8	72·0	80·0	160·8	3·5	11·0
Rate of excretion of Ca in urine	3·0	4·4	7·5	8·2	1·1	1·3	3·3
Rate of Ca retention	−4·5	+30·1	+31·1	+26·3	+36·1	2·8	8·9
Rate of endogenous loss of Ca in faeces	11·0	11·3	11·4	11·8	14·7	0·4	1·2
Rate of absorption of dietary Ca	9·6	45·8	50·0	46·3	52·0	2·6	8·1
Ca absorbed (% Ca ingested)	8·7	42·9	45·4	40·5	26·2	2·3	7·3
Rate of accretion of Ca into bone	38·5	54·1	50·4	47·0	54·8	4·5	14·3
Rate of resorption of Ca from bone	43·1	24·0	19·3	19·4	18·7	3·7	11·8
Rapidly-exchangeable pool of Ca (mg/kg body-wt)	55·2	58·5	52·7	51·2	62·5	3·3	10·3
Slowly-exchangeable pool of Ca in bone (mg/kg body-wt)	103·0	139·3	103·8	105·9	102·7	13·7	43·2
Rate of ingestion of P	99·7	102·5	100·7	99·0	145·4	1·8	5·7
Rate of loss of P in faeces	99·9	79·7	70·1	73·7	114·2	2·8	8·8
Rate of excretion of P in urine	1·0	1·2	2·6	1·6	9·4	1·3	4·2
Rate of P retention	−1·2	+21·6	+28·0	+23·7	+21·8	2·3	7·4
Rate of endogenous loss of P in faeces	39·5	38·3	33·9	34·6	36·7	1·7	5·4
Rate of endogenous secretion of P into intestine*	67·6	98·1	103·3	82·7	73·6	9·7	30·7
Rate of absorption of dietary P	39·1	61·2	64·4	60·0	67·8	3·5	11·0
P absorbed (% P ingested)	38·9	59·7	63·8	60·5	46·7	2·7	8·5
P incorporation into non-exchangeable pools of bone and soft tissues	33·7	50·9	47·0	44·5	50·3	3·2	10·0
P loss from non-exchangeable pools of bone and soft tissues	34·9	29·3	19·0	20·8	28·5	3·0	9·5
Serum Ca (mmol/l)	2·64	2·62	2·87	3·01	3·23	0·08	0·23
Serum inorganic P (mmol/l)	2·24	2·79	3·28	3·58	4·01	0·13	0·35

* Rate of endogenous secretion of P into intestine $= \frac{\text{rate of loss of endogenous P in faeces}}{1-(\%\text{ dietary P absorbed})/100}$ (from Young, Lofgreen *et al.* 1966).

increase in bone resorption was demonstrated in vivo only in animals maintained on Ca-deficient diets (Tanaka & DeLuca, 1971; Wong *et al.* 1972; Holick *et al.* 1976). The present results suggest that such an increase is unlikely to occur in mature animals maintained on adequate Ca intakes. In any case, it is difficult to understand how both Ca absorption and bone resorption could increase at the same time without either a concomitant increase in the rate of loss or a continuously-expanding pool of exchangeable Ca. In the present experiments, the loss of Ca increased only slightly and neither the rapidly-exchangeable Ca pool of soft tissues nor the slowly-exchangeable Ca pool of bone were altered by treatment. Serum Ca concentration, however, did increase slightly at the higher dose rates.

Results of the P kinetic studies are also shown in Table 2. The rates of absorption and retention of P, like those of Ca, were increased by the 1α-OH-D_3 treatment but again there was no direct relationship between magnitude of response and dose rate, maximum response occurring at the lowest dose.

In experiments with lactating ewes, it was found that 1α-OH-D_3 treatment increased the apparent absorption of P (Braithwaite, 1978) but it was not possible to decide whether this increase was due to an increase in true absorption or to a decrease in endogenous secretion. These results now show that the major effect of 1α-OH-D_3 treatment was to stimulate P absorption, though faecal endogenous excretion did decrease slightly at the higher dose rates.

The increase in P absorption during treatment suggests that not all the available dietary P was absorbed in the control period. A failure to absorb all the available dietary P was also suggested in animals given the higher Ca intake. Although P intake was also increased, no further increase in P absorption occurred and as a consequence the efficiency of absorption was decreased. These findings are of particular interest in view of previous studies (Lueker & Lofgreen, 1961; Preston & Pfander, 1964; Young, Richards *et al.* 1966), which show that P is absorbed in direct relation to intake, and suggest that absorption is normally limited by the availability of dietary P. Of course, the possibility exists that in the presence of large amounts of dietary Ca, much of the dietary P is precipitated in the intestine as insolublecalcium phosphate and is unavailable for absorption. During 1α-OH-D_3 treatment, the increased Ca absorption may then result in less precipitation of P and hence more being available for absorption.

A large number of studies in various species have now shown that P absorption is increased by vitamin D and its metabolites (Harrison & Harrison, 1961; Wasserman & Taylor, 1973; Chen *et al.* 1974; Fox & Care, 1976). There is controversy, however, over the actual mechanism by which this increase is achieved (Wasserman, 1975; Norman, 1978). For example, it is not yet firmly established whether it occurs as a result of a direct stimulation by 1α,25$(OH)_2D_3$, or whether P is absorbed as a co-ion with Ca. Certainly in the present experiments the ratio, Ca:P absorbed as a result of 1α-OH-D_3 treatment, i.e.

$$\frac{Ca_a \text{ (treated)} - Ca_a \text{ (control)}}{P_a \text{ (treated)} - P_a \text{ (control)}},$$

remained remarkably constant at approximately 1·6:1, irrespective of dose rate, which might be regarded as evidence of a coupled Ca:P absorption. However, further investigations of P metabolism in 1α-OH-D_3-treated sheep given high or low Ca intakes now suggest that the increase in P absorption is independent of Ca absorption (Braithwaite, unpublished results).

The slight decrease in endogenous loss of P in the faeces during treatment is surprising in view of the increased serum P_i concentration. At first sight this seems to suggest that endogenous secretion is not related to serum P_i concentration. However, if the total rate of

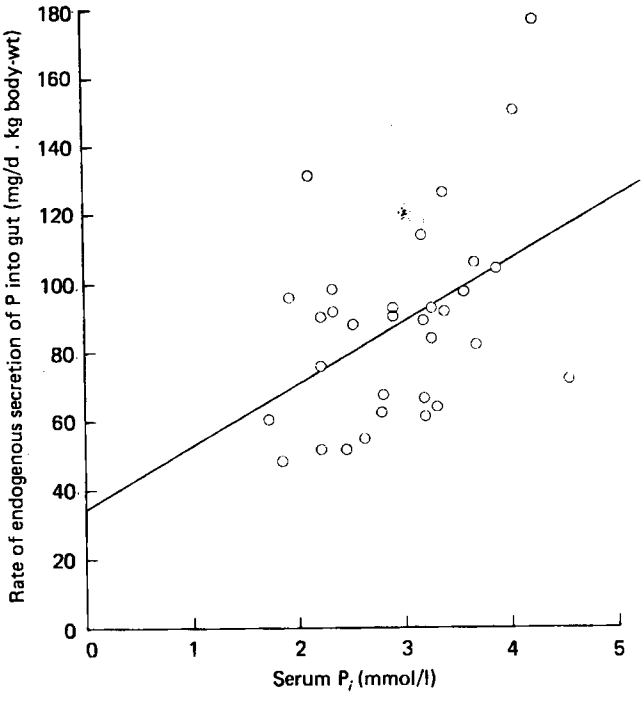

Fig. 1. Relationship between serum inorganic phosphorus (P_i) (mmol/l) and the rate of endogenous secretion of P (mg/d per kg body-weight) into the gut (P_s) of wether sheep. $P_s = 34{\cdot}5 + 18\ P_i$; r 0·45.

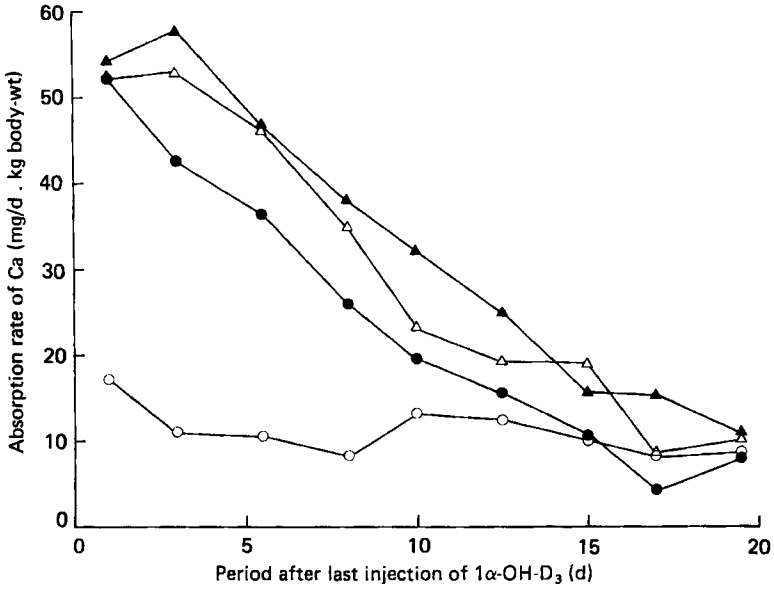

Fig. 2. Changes in the rate of absorption of Ca (mg/d per kg body-weight) in wether sheep during the 21 d immediately after the end of the 1α-hydroxycholecalciferol (1α-OH-D_3) treatment periods. ○, Control; ●, 0·02; △, 0·05; ▲, 0·1 μg 1α-OH-D_3/d per kg body-weight.

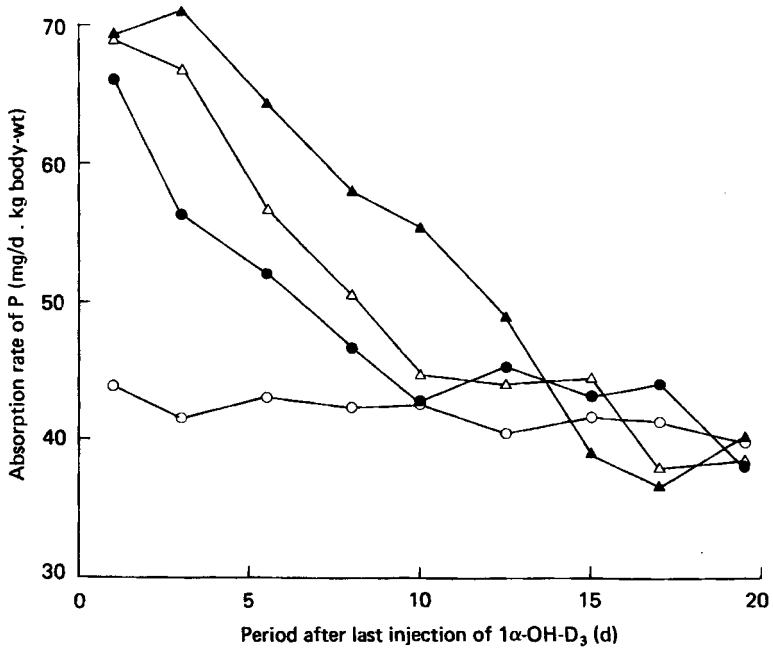

Fig. 3. Changes in the rate of absorption of P (mg/d per kg body-weight) in wether sheep during the 21 d immediately after the end of the 1α-hydroxycholecalciferol (1α-OH-D_3) treatment periods. ○, Control; ●, 0·02; △, 0·05; ▲, 0·1 μg 1α-OH-D_3/d per kg body-weight.

Table 3. *Total absorption of calcium and phosphorus (mg/kg body-weight) during the 21 d immediately after the end of 1-α-hydroxycholecalciferol treatment*

(Results are the mean of eight animals/group)

	1α-OH-D_3 (μg/d per kg body-wt)				Standard error of difference
	0	0·02	0·05	0·1	(21 df)
Total Ca absorption	238	490	608	669	31·1
Total P absorption	880	1007	1046	1120	65·7

secretion of P into the upper small intestine is calculated by the formula of Young, Lofgreen *et al.* (1966):

$$\text{rate of endogenous secretion} = \frac{\text{rate of endogenous loss in faeces}}{1 - \text{fraction of dietary P absorbed}}$$

which is based on the assumption that endogenous P is reabsorbed with the same efficiency as dietary P, then it is clear that total endogenous secretion was increased by 1α-OH-D_3 and was related ($P > 0{\cdot}01$) to serum P_i concentration (Fig. 1).

Nearly all the additional P absorbed during treatment was retained and the increased retention was achieved by a combination of an increased incorporation into the non-exchangeable pools of bone and soft tissues and a decreased loss from these pools. Although it was not possible to distinguish between the bone and the soft tissue P, changes in the rates of incorporation of P into and loss from the combined pool as a result of treatment did follow the same trend as changes in the rates of Ca accretion into and resorption from bone.

Recent work has suggested that it is not the magnitude of response that is altered by increased dose rates of 1α-OH-D_3 but rather the duration of response (Sansom, 1977).

Figs. 2 and 3 show the mean absorption rates of Ca and P respectively for the four treatments, calculated from balance measurements carried out during the 21 d period immediately after the end of the 1α-OH-D_3 administration. In these calculations, it was assumed that the faceal endogenous loss of Ca and P remained constant and equal to the mean values obtained in the control period.

The absorption rate of Ca was initially maintained at a high level, but then fell steadily, the time taken to return to normal being related to the dose rate of 1α-OH-D_3. Total absorption of Ca over the whole 21 d period was markedly increased by treatment ($P < 0{\cdot}001$, Table 3), the greatest increase occurring at the highest dose rate.

The pattern of changes in absorption rates of P was similar to that for Ca, except that the rates returned to normal levels earlier. Consequently, the increase with increasing dose levels, in total absorption over the 21 d period, while still significant ($P < 0{\cdot}05$) was less than for Ca.

These results confirm that the main effect of 1α-OH-D_3 is to increase Ca and P absorption. They also show that increased dose rates above 0·02 μg/d per kg body-weight have little effect on the magnitude of response but do prolong slightly the duration of response. There may therefore be little advantage in using doses greater than 0·02 μg/d for preventing deficiency disorders and possibly even lower doses may be just as beneficial.

I thank Miss F. J. McEwan and Mr R. J. Ranson for skilled technical assistance, Mr A. Mowlem for supervising the care of the experimental animals and Mr J. M. Pott of Leo Laboratories for the supply of 1α-OH-D_3.

REFERENCES

Aubert, J.-P. & Milhaud, G. (1960). *Biochim. biophys. Acta* **39**, 122.
Barlet, J.-P. (1977). *Annls Biol. Anim. Biochim. Biophys.* **17**, 363.
Braithwaite, G. D. (1976). *J. Dairy Res.* **43**, 501.
Braithwaite, G. D. (1978). *Br. J. Nutr.* **40**, 387.
Braithwaite, G. D. (1979). *J. agric. Sci., Camb.* **92**, 337.
Braithwaite, G. D. & Glascock, R. F. (1976). *Bienn. Rev. natn. Inst. Res. Dairy.*
Braithwaite, G. D., Glascock, R. F. & Riazuddin, Sh. (1969). *Br. J. Nutr.* **23**, 827.
Braithwaite, G. D. & Riazuddin, Sh. (1971). *Br. J. Nutr.* **26**, 215.
Chen, T. C., Castillo, L., Korycka-Dahl, M. & DeLuca, H. F. (1974). *J. Nutr.* **98**, 1056.
DeLuca, H. F. (1975). *Acta orthop. scand.* **46**, 286.
DeLuca, H. F. (1977). *Adv. clin. Chem.* **19**, 125.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fox, J. & Care, A. D. (1976). In *Calcified Tissues* '1975', p. 147 [S. Pors Nielson & Hjorting-Hanson, editors]. Copenhagen: FADL Publishing Co.
Fraser, D. R. & Kodicek, E. (1973). *Nature, New Biol.* **241**, 163.
Gast, D. R., Marquardt, J. P., Jorgensen, N. A. & DeLuca, H. F. (1977). *J. Dairy Sci.* **60**, 1910.
Gueguen, L. (1963). *Annls Biol. Anim. Biochim. Biophys.* **3**, 243.
Harrison, H. E., & Harrison, H. C. (1961). *Am. J. Physiol.* **201**, 1007.
Holick, M. F., Taleva, T. E., Holick, S. A., Schnoes, H. K., DeLuca, H. F. & Gallagher, B. M. (1976). *J. biol. Chem.* **251**, 1020.
Larsson, S.-E., Lorentzon, R. & Boquist, L. (1977). *Clin. Orthop. Relat. Res.* **127**, 228.
Lueker, C. E. & Lofgreen, G. P. (1961). *J. Nutr.* **74**, 233.
Manston, R. (1966). *Br. vet. J.* **122**, 443.
Norman, A. W. (1978). In *Vitamin D*, ch. 3, [D. E. M. Lawson, editor]. New York and London: Academic Press.
Parsons, D. H. (1968). *Mathl. Biosci.* **2**, 191.
Pierides, A. M., Simpson, W., Ward, M. K., Ellis, H. A., Dewar, J. H. & Kerr, D. N. S. (1976). *Lancet* i, 1092.
Preston, R. L. & Pfander, W. H. (1964). *J. Nutr.* **83**, 369.
Sachs, M., Bar, A., Cohen, R., Mazur, Y., Mayer, E. & Hurwitz, S. (1977). *Am. J. vet. Res.* **38**, 2039.
Sansom, B. F. (1977). *Vet. Sci, Commun.* **1**, 323.

Sansom, B. F., Allen, W. M., Davies, D. C., Hoare, M. N., Stenton, J. R. & Vagg, M. J. (1976). *Vet. Rec.* **99**, 310.
Shroder, J. D. & Hansard, S. L. (1958). *J. Anim. Sci.* **17**, 343.
Symonds, H. W. (1969). *Res. vet. Sci.* **10**, 584.
Tanaka, Y. & DeLuca, H. F. (1971). *Archs Biochem. Biophys.* **146**, 574.
Technicon Instruments Corporation (1967). *Technicon Method Sheet* N-4B. Tarry Town, New York: Technicon Instruments Corporation.
Wasserman, R. H. (1975). *Cornell Vet.* **65**, 3.
Wasserman, R. H. & Taylor, A. N. (1973). *J. Nutr.* **103**, 586.
Wong, R. G., Myrtle, J. F., Tsai, H. C. & Norman, A. W. (1972). *J. biol. Chem.* **247**, 5728.
Young, V. R., Lofgreen, G. P. & Luick, J. R. (1966). *Br. J. Nutr.* **20**, 795.
Young, V. R., Richards, W. P. C., Lofgreen, G. P. & Luick, J. R. (1966). *Br. J. Nutr.* **20**, 783.

Printed in Great Britain