Thus, while these results would support the possibility that any phospholipase C derived from complement may differ from the exogenous enzyme as regards accessibility to the sphingomyelin substrate, in our opinion, the same observations may be used to argue that phospholipase C activity is not produced upon activation of the terminal complement components.

Acknowledgments

We are indebted to Drs. T. Yamakawa, S. Handa, and A. Makita for the gifts of Forssman hapten and globoside I which made this investigation possible. Numerous discussions with Dr. J. Dittmer (Department of Biochemistry, St. Louis University Medical School, St. Louis, Mo.) and Drs. P. N. Needleman and R. M. Burton (this department) proved invaluable. Mrs. C. B. Kinsky provided her usual competent assistance in the execution of these experiments.

References

Alving, C. R., Kinsky, S. C., Haxby, J. A., and Kinsky, C. B. (1969), *Biochemistry 8*, 1582.

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37*, 911.

Bruno, G. A., and Christian, J. E. (1961), *Anal. Chem. 33*, 1216.

Dittmer, J. C., and Lester, R. L. (1964), *J. Lipid Res. 5*, 126.

Goldman, J. N., Ruddy, S., Austen, K. F., and Feingold, D. S. (1969), *J. Immunol. 102*, 1379.

Haxby, J. A., Götze, O., Müller-Eberhard, H. J., and Kinsky, S. C. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 290.

Haxby, J. A., Kinsky, C. B., and Kinsky, S. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 300.

Humphrey, J. H. and Dourmashkin, R. R. (1969), *Advan. Immunol. 11*, 75.

Kaller, H. (1961), *Biochem. Z. 334*, 451.

Kanfer, J. N., and Brady, R. O. (1969), *Methods Enzymol. 14*, 131.

Kinsky, S. C. (1970), *Annu. Rev. Pharmacol. 10*, 119.

Kinsky, S. C., Haxby, J. A., Zopf, D. A., Alving, C. R., and Kinsky, C. B. (1969), *Biochemistry 8*, 4149.

Kinsky, S. C., Haxby, J. A., Zopf, D. A., Alving, C. R., and Kinsky, C. B. (1970), *Biochemistry 9*, 1048.

Sessa, G., Freer, J. H., Colacicco, G., and Weissmann, G. (1969), *J. Biol. Chem. 244*, 3575.

Smith, J. K., and Becker, E. L. (1968), *J. Immunol. 100*, 459.

25,26-Dihydroxycholecalciferol, a Metabolite of Vitamin D_3 with Intestinal Calcium Transport Activity*

T. Suda, H. F. DeLuca,† H. K. Schnoes, Y. Tanaka, and M. F. Holick

ABSTRACT: A metabolite of vitamin D_3 (40 μg) has been isolated in pure form from the plasma of eight pigs given 250,000 IU of vitamin D_3/day for 28 days. It has been unequivocally identified as 25,26-dihydroxycholecalciferol by means of mass spectrometry and ultraviolet absorption spectra. This metabolite has some activity in intestinal calcium transport, but is virtually inactive in the cure of rickets and in the mobilization of bone mineral in rats.

Since the successful identification of 25-HCC[1] (Blunt *et al.*, 1968a) and 25-HEC (Suda *et al.*, 1969), it has been strongly suggested that all vitamin D compounds must be hydroxylated in the 25 position before they are active. Both 25-HCC and 25-HEC were 1.4–1.5 times more active than vitamin D_3 or D_2 in curing rickets in rats (Blunt *et al.*, 1968b; Suda *et al.*, 1969). Not only were they more active than their parent vitamins in the stimulation of intestinal calcium transport and in the mobilization of bone mineral, but they acted more rapidly (Blunt *et al.*, 1968b; Suda *et al.*, 1970a). In addition, 25-HCC was effective in isolated target organs while vitamin D_3 itself was without effect (Olson and DeLuca, 1969; Trummel *et al.*, 1969). Therefore, 25-hydroxy D vitamins seemed to be at least the circulating or hormonal active forms of the vitamins (DeLuca, 1969).

Recent studies on the metabolism of [^3H]vitamin D_3 have demonstrated that other metabolites, more polar chromatographically than either vitamin D_3 or 25-HCC, are present in certain tissues and blood following administration of physiological doses of the radioactive vitamin D or 25-HCC (Ponchon and DeLuca, 1969; Haussler *et al.*, 1968; Lawson

* From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received June 22, 1970.* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported by the H. Steenbock Research Fund of the Wisconsin Alumni Research Foundation, National Institutes of Health Grant No. AMO-5800-08, National Institutes of Health Training Grant No. GM00236 BCH from the National Institute of General Medical Sciences, the N. V. Philips Duphar Co. of the Netherlands, and Merck and Co., Rahway, N. J.

† To whom inquiries should be addressed.

[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 25-HEC, 25-hydroxyergocalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol; 25,26-DHCC, 25,26-dihydroxycholecalciferol.

et al., 1969; Cousins *et al.*, 1970a,b). Although the biological activity of these polar metabolites is still unclear, their identification appeared important to a thorough understanding of the mechanism of vitamin D action.

More recently one of these polar metabolites has been isolated in pure form from hog plasma and identified as 21,25-DHCC (Suda *et al.*, 1970b). It was found to have a marked action on mobilization of bone mineral while having a small effect on intestinal calcium transport and in the cure of rickets in rats (Suda *et al.*, 1970b).

Another polar metabolite has now been isolated in pure form from hog plasma and unequivocally identified as 25,26-DHCC. This metabolite was shown to have activity in intestinal calcium transport while having no effect on bone mineral mobilization and in the cure of rickets in rats. It is the purpose of this communicaion to report these results.

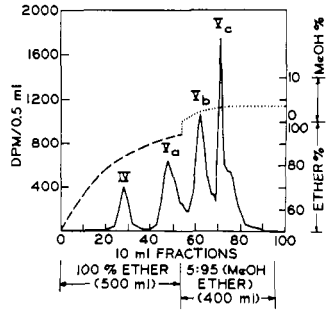

FIGURE 1: Silicic acid column chromatographic profile of plasma peak V. The column was eluted with an ether–Skelly B–methanol gradient as described in the text. (——) Radioactivity; (-----) gradient; 10-ml fractions were collected and tubes 70–90 were used in further purification.

Methods and Results

General Procedures. Ultraviolet spectra were recorded with a Beckman DB-G spectrophotometer. Samples were dissolved in ethanol. A molar extinction coefficient of 18,000 at 265 mμ was used for vitamin D_3 and 25-HCC (Blunt *et al.*, 1968a). A molar extinction of 18,000 at 265 mμ was assumed for 25,26-DHCC as well. Mass spectra were determined with an MS-902 mass spectrometer (electron energy, 70 eV; ionizing current, 450 μA; source temperature, 135–150° above ambient) using a direct introduction probe.

All radioactive determinations were carried out with a Packard Tri-Carb liquid scintillation spectrometer, Model 3003, equipped with an external standardization system. Samples for tritium determination were evaporated to dryness with a stream of air, dissolved in toluene-counting solution (2 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per l. of toluene), and counted.

Acetylation Reactions. About 1 μg of the metabolite was allowed to stand at room temperature for approximately 5 hr in a solution of 5 μl of acetic anhydride and 5 μl of pyridine. The mass spectrum of the derivative was obtained by transferring the entire mixture to the probe tip and allowing the solvent to evaporate.

Silyl Ether Formation. Complete silylation was accomplished by treating the metabolite with about 10 μl of silylating reagent (pyridine–hexamethyldisilazane–trimethylchlorosilane, 7:2:1, v/v) for approximately 2 hr.

Periodate Cleavage. To about 1.5 μg of the metabolite in 20 μl of methanol a drop of 5% aqueous $NaIO_4$ was added. After 20 min the solution was diluted with water and extracted with chloroform. The chloroform solution was concentrated to a few microliters which were transferred to the probe tip for mass spectral analysis.

Isolation of Metabolites. Plasma (14.1 l.) was obtained from eight hogs which were maintained on normal stock rations supplemented with 250,000 IU of vitamin D_3 daily for 28 days. The plasma was made 70% saturated with $(NH_4)_2SO_4$, and the precipitate was extracted with methanol–chloroform and partitioned to remove less polar metabolites of vitamin D_3 as previously described (Suda *et al.*, 1970b).

Exactly 100 IU (2.5 μg) of [1,2-^3H]vitamin D_3 which was prepared in this laboratory, was dosed intravenously to each of 51 chickens, which were maintained on a vitamin D deficient diet (Imrie *et al.*, 1967) for 27 days. Twenty hours after administration blood was collected by decapitation giving 133 ml of plasma after centrifugation. This was extracted with methanol–chloroform, and ^3H-labeled polar metabolites were partitioned and applied to a silicic acid column (Ponchon and DeLuca, 1969) to obtain the ^3H-labeled peak V as previously described (Suda *et al.*, 1970b).

The radioactive peak V taken from chicken plasma was then mixed with the extract from the hog plasma. The combined extract was then applied in 20 ml of chloroform to a large 150-g, 60-cm, multibore silicic acid column measuring stepwise in diameter 1.0, 2.0, 3.0, and 4.0 cm. The column was eluted with the Skelly B (*n*-hexane, bp 67–68°)–ether–methanol gradient as previously described (Suda *et al.*, 1970b). The peak V was eluted as a single peak in the 5% methanol region. The peak V was then rechromatographed on a regular straight silicic acid (20-g) column as described by Ponchon and DeLuca (1969) except that the constant-volume mixing chamber contained 250 ml of 50% diethyl ether in Skelly B and the holding chamber contained 500 ml of 100% diethyl ether. As soon as the holding chamber became empty, it was filled with 400 ml of 5% methanol in diethyl ether. Fractions of 10 ml were collected. The elution profile is shown in Figure 1. Clearly the original peak V has been resolved into at least three components, which were designated Va, Vb, and Vc. The peak Va has been identified as 21,25-DHCC (Suda *et al.*, 1970b). The peak Vc collected in tubes 70–90 was then rechromatographed on a Celite partition column, especially designed for peak V metabolites as previously described (Suda *et al.*, 1970b). The column was developed with the mobile phase (20% chloroform–80% Skelly B equilibrated with 90% methanol–10% water) with 5.5-ml fractions collected. The profile of radioactivity from that column is shown in Figure 2. The peak Vc has now been resolved into four peaks. The major peak Vc,3 (tubes 77–90) was then applied in 0.1 ml of methanol to a 60 × 1 cm Sephadex LH-20 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which was developed in methanol. Fractions of 1 ml were collected. Figure 3 shows the profile of radioactivity and 265-mμ-absorbing material which was eluted from that column. The absorbancy at 265 mμ exactly coincided with the radioactivity plot. The ultraviolet spectrum of the material is shown in Figure 4, giving maximum absorption at 265 mμ and minimum absorption at 228 mμ. A total of 40 μg of the metabolite was isolated.

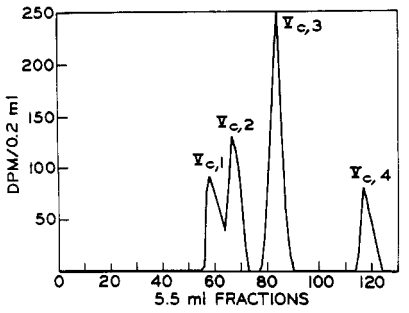

FIGURE 2: Partition chromatography of the peak Vc metabolite from Figure 1. Celite was used as the support. The mobile phase consisted of 20% chloroform–80% Skelly B equilibrated with the stationary phase (90% methanol–10% water). Fractions (5.5 ml) were collected and tubes 77–90 were used in further purification.

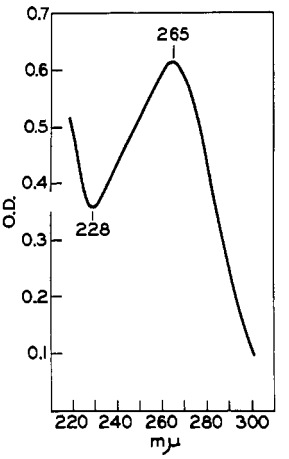

FIGURE 4: Ultraviolet absorption spectrum of the plasma peak Vc metabolite isolated from Sephadex LH20 columns.

Identification of the Metabolite. The ultraviolet spectrum of the metabolite which exhibited absorption at λ_{max}^{EtOH} 265 mμ, indicated an unchanged *cis*-triene chromophore (vitamin D_3 shows λ_{max}^{EtOH} 265 mμ). Peaks at *m/e* 136 and 118 (136-H_2O) in the mass spectrum of the metabolite (Figure 5) confirmed this assignment, since these ions are characteristic for the vitamin D triene system (see, for example, Blunt *et al.*, 1968a, and Suda *et al.*, 1969). The molecular weight of 416 suggested the incorporation of two additional oxygen functions into the vitamin D_3 ring system; both of these had to be located in the sidechain, since the peaks at *m/e* 271 and 253 (271-H_2O) which correspond to loss of the entire sidechain (cleavage of C-17–C-20 bond) in the mass spectrum of vitamin D_3 itself, are also present in the spectrum of the metabolite (Figure 5). The metabolite formed a diacetate (mol wt 500) on treatment with acetic anhydride in pyridine, but a trimethylsilyl ether (mol wt 632, Figure 5) resulted upon silylation of the compound. These transformations establish that both additional oxygen functionalities are present as hydroxy groupings and that one of these should be located on a tertiary carbon center. The exact structure of the compound followed from an interpretation of the mass spectra of the metabolite and its derivatives. The metabolite spectrum (Figure 5) in addition to peaks already discussed shows a peak at M − 18 − 31 (367) suggesting the presence of a CH_2OH group. Since the acetylation results suggested one tertiary hydroxyl function, the loss of 31 mass units pointed toward a 20,21- or 25,26-dihydroxylated side chain. The former possibility appeared unlikely since cleavage of the C-20–C-22 bond should be expected with elimination of the side chain fragment comprising carbons 22–27. (For example, 20-hydroxycholesterol shows a very intense peak at *m/e* 317 due to this cleavage.) The mass spectrum of the trisilylated metabolite confirmed a 25,26-substitution pattern. The relatively intense peak at *m/e* 529 corresponds to loss of 103 mass units [M − $CH_2OSi(CH_3)_3$] and the peak at *m/e* 219 can result by cleavage of the C-24–C-25 bond as shown in Figure 6. The peak at *m/e* 208 corresponds to *m/e* 136 in the spectrum of the metabolite shifted by one silyl grouping. The mass spectrometric evidence is summarized in Figure 6. Further proof for the presence of a vicinal glycol grouping was provided by periodate cleavage of the metabolite which gave a compound of mol wt 384 as expected for the transformation of a 25,26-dihydroxy compound to the corresponding 25-keto derivative. These data, therefore, establish the structure of this new metabolite as 25,26-DHCC (Figure 7).

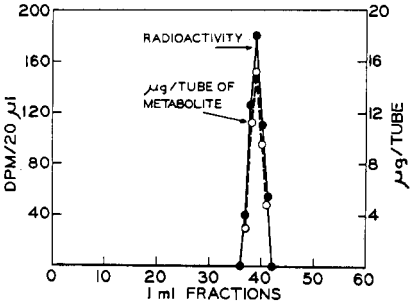

FIGURE 3: Chromatographic profile of the major peak Vc, 3 (tubes 77–90 of Figure 2) on a Sephadex LH20 column. The column was eluted with methanol. Fractions (1 ml) were collected. The micrograms per tube were calculated on the basis of a molar extinction coefficient of 18,000 at 265 mμ assumed for this metabolite.

Biological Activity of 25,26-Dihydroxycholecalciferol. The line test assay of 25,26-DHCC was carried out in rats as previously described (U. S. Pharmacopoeia, 1955) except that it was given intrajugularly in 20 μl of ethanol. 25,26-DHCC failed to show significant antirachitic activity, thus its antirachitic activity if any must be less than 4 IU/μg.

Bone mineral mobilization response to 25,26-DHCC intravenously was tested as previously described (Blunt *et al.*, 1968b) except that only 0.25 μg of 25,26-DHCC was administered. In addition, intestines were removed from these rats and intestinal calcium transport was measured by the everted sac technique as previously described (Blunt *et al.*, 1968b). Precise comparison of biological activity of 25,26-DHCC to that of 21,25-DHCC was not possible, because of the limited amount of the 25,26-DHCC isolated. As shown in Table I, 0.25 μg of 25,26-DHCC elicited no significant elevation of serum calcium concentration at 12 hr after administration. Under the same conditions, 0.25 μg of 25-HCC elevated serum calcium concentration from 4.7 to 6.6 mg %. On the other hand, 0.25 μg of 25,26-DHCC significantly

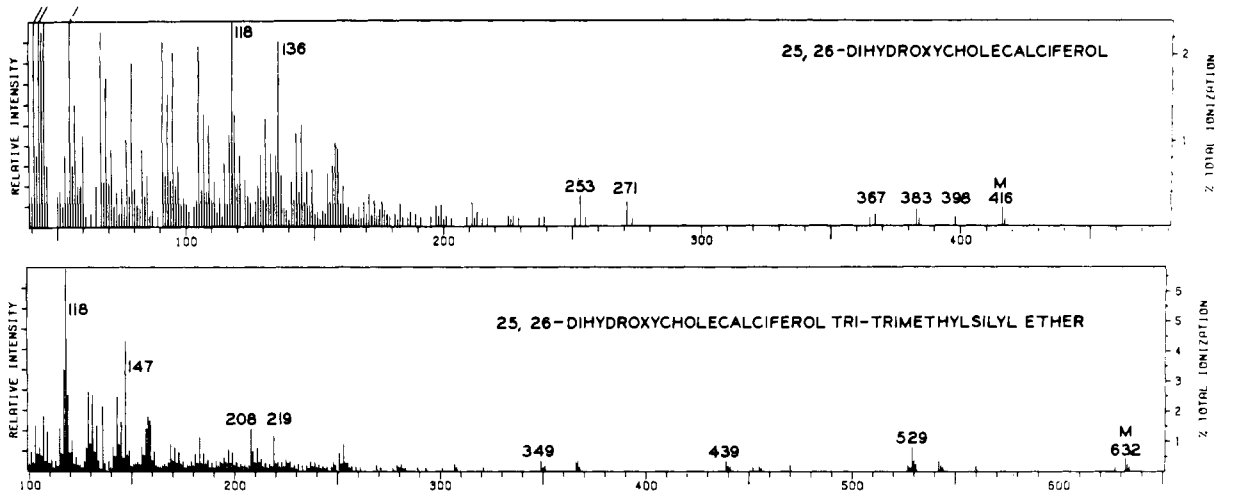

FIGURE 5: Mass spectra of the plasma peak Vc and its tritrimethylsilyl ether derivative.

TABLE I: Effect of Intrajugular Administration of 0.25 or 2.5 μg of 25,26-DHCC, 21,25-DHCC, or 25-HCC on Calcium Transport and Serum Calcium of Rats on a Low Calcium Diet.

	12 hr after Administration			
	Calcium Transport ^{45}Ca Serosal/^{45}Ca Mucosal		Bone Mobilization Serum Ca mg%	
Group	0.25 μg	2.5 μg	0.25 μg	2.5 μg
Control	1.6 ± 0.2[a] (4)	1.5 ± 0.3[a] (5)	4.7 ± 0.1[a] (4)	4.4 ± 0.4[a] (5)
25,26-DHCC	2.8 ± 0.6 (5)		5.1 ± 0.2 (5)	
21,25-DHCC		2.6 ± 0.8 (5)		6.5 ± 0.2 (5)
25-HCC	4.0 ± 0.5 (3)	5.9 ± 1.6 (5)	6.6 ± 0.6 (3)	7.3 ± 0.5 (5)

[a] Standard errors of the mean. Numbers in parentheses show the number of animals in each group.

increased intestinal calcium transport. 25,26-DHCC was about one-half as active as 25-HCC in the stimulation of intestinal calcium transport.

Discussion

In a series of experiments on the metabolism of vitamin D in this laboratory, it has been strongly suggested that all vitamin D compounds must be hydroxylated in the 25 position before they are effective (DeLuca, 1969). Further biological evidence established 25-HCC and 25-HEC at least as the circulating or hormonal form of vitamin D_3 and D_2, respectively (Blunt *et al.*, 1968b; Suda *et al.*, 1970a; DeLuca, 1969; Trummel *et al.*, 1969; Olson and DeLuca, 1969). In continuing investigation, Cousins *et al.* (1970a,b) demonstrated that 25-HCC is rapidly metabolized to a more polar metabolite (peak V) in intestine, bone, and kidney. This conversion in the case of intestine involves an even more polar metabolite (peak VI) as intermediate. Haussler *et al.* (1968) and Lawson *et al.* (1969) have also reported metabolites of vitamin D_3

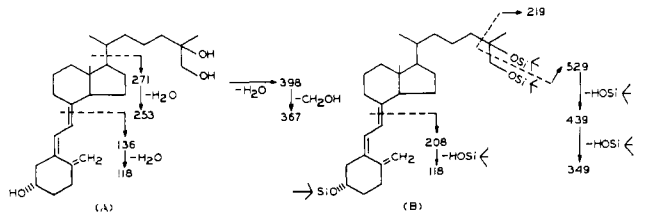

FIGURE 6: Schematic representation of bond ruptures leading to prominent ions in the mass spectra of 25,26-DHCC and its tritrimethylsilyl ether derivative.

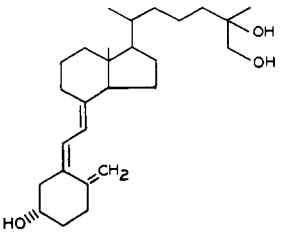

FIGURE 7: Structure of 25,26-DHCC.

more polar than 25-HCC. Myrtle *et al.* (1970) have claimed that their intestinal peak 4B, which corresponds to our peak V chromatographically, is biologically more active than 25-HCC in intestinal calcium transport *in vivo*. Ponchon and DeLuca (1969, 1970), however, failed to confirm this high degree of biological activity for peak V. The rapidity of appearance of these polar metabolites in the target tissues after ^{3}H-labeled 25-HCC administration indicates that they nevertheless are important to a complete understanding of the mechanism of vitamin D action. Continued experiments in this laboratory have shown that at least some of these polar metabolites appear in blood making their isolation from the plasma of pigs given large doses of vitamin D_3 feasible.

In previous work in this laboratory, the peak V fraction of blood has been resolved into at least three major components (Va, Vb, and Vc). The quantitatively major metabolite (Va) has already been identified as 21,25-DHCC (Suda *et al.*, 1970b). Another (major Vc; Vc,3) has now been isolated in pure form using isolation techniques similar to those used for the 21,25-DHCC.

The identification of Vc,3 as 25,26-DHCC was more straightforward than that of Va as 21,25-DHCC. A molecular ion of 416 demonstrated the addition of two oxygen atoms to the vitamin D_3 structure. The oxygen atoms were clearly on the side chain since loss of the side chains from both the parent vitamin and the metabolite gave identical *m/e* 271 fragments. One of these had to be a tertiary hydroxyl since it did not form an acetate but did form a trimethylsilyl ether. A loss of 18 and 31 from the molecular ion suggests a CH_2OH grouping. This must be adjacent to a tertiary hydroxyl. This limits the possibilities to the 20,21 and to the 25,26 positions. The 25,26 position of the added hydroxyls was clearly established by the mass spectra of the tritrimethylsilyl ether derivatives as described in the Results section, especially the *m/e* at 219 resulting from the cleavage of the C-24–C-25 bond.

The addition of a hydroxyl to either the 21 or the 26 position of 25-HCC has led to a decrease in the biological activity of this circulating active form of vitamin D (Suda *et al.*, 1970b, and present report). It is especially interesting that the 21 hydroxylation yields a compound preferentially active on bone while the 26 hydroxylation yields a compound which has only significant activity in the intestine. These observations may at least be important in designing chemical synthesis of vitamin D analogs with specific activities on bone or intestine. Biologically, it is difficult to assess the importance of either the 21,25-DHCC or the 25,26-DHCC especially since their origins are not clear. They may be functionally important in the tissue where they are generated, or they may be intermediates on the route to inactivation. These questions are currently under investigation.

Acknowledgment

We thank Dr. E. J. Briskey, Department of Meat and Animal Science, for the pigs and use of swine facilities.

References

Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968a), *Biochemistry 7*, 3317.

Blunt, J. W., Tanaka, Y., and DeLuca, H. F. (1968b), *Proc. Nat. Acad. Sci. U. S. 61*, 1503.

Cousins, R. J., DeLuca, H. F., Suda, T., Chen, T., and Tanaka, Y. (1970a), *Biochemistry 9*, 1453.

Cousins, R. J., DeLuca, H. F., and Gray, R. (1970b), *Biochemistry 9*, 3649.

DeLuca, H. F. (1969), *Amer. J. Clin. Nutr. 22*, 412.

Haussler, M. R., Myrtle, J. F., and Norman, A. W. (1968), *J. Biol. Chem. 243*, 4055.

Imrie, M. H., Neville, P. F., Snellgrove, A. W., and DeLuca, H. F. (1967), *Arch. Biochem. Biophys. 120*, 52.

Lawson, D. E. M., Wilson, P. W., and Kodicek, E. (1969), *Biochem. J. 115*, 269.

Myrtle, J. F., Haussler, M. R., and Norman, A. W. (1970), *J. Biol. Chem. 245*, 1190.

Olson, E. B., and DeLuca, H. F. (1969), *Science 165*, 405.

Ponchon, G., and DeLuca, H. F. (1969), *J. Nutr. 99*, 157.

Ponchon, G., DeLuca, H. F., and Suda, T. (1970), *Arch. Biochem. Biophys.* (in press).

Suda, T., DeLuca, H. F., Schnoes, H. K., and Blunt, J. W. (1969), *Biochemistry 8*, 3515.

Suda, T., DeLuca, H. F., and Tanaka, Y. (1970a), *J. Nutr. 100*, 1049.

Suda, T., DeLuca, H. F., Schnoes, H. K., Ponchon, G., Tanaka, Y., and Holick, M. F. (1970b), *Biochemistry 9*, 2917.

Trummel, C., Raisz, L. G., Blunt, J. W., and DeLuca, H. F. (1969), *Science 163*, 1450.

U. S. Pharmacopoeia (1955), 14th Revision, Easton, Pa., Mack Publishing Co., p 889.