A new chromatographic system for vitamin D_3 and its metabolites: resolution of a new vitamin D_3 metabolite

M. F. HOLICK and H. F. DeLUCA*

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT A simple yet powerful new chromatographic procedure for vitamin D_3 and its metabolites is described. Liquid–gel partition chromatography on Sephadex LH-20 using a solvent of various percentages of $CHCl_3$ in Skellysolve B (petroleum ether, bp 67–69°C) permits excellent resolution of vitamin D_3, 25-hydroxyvitamin D_3, and their more polar metabolites. Of special importance is the resolution of the metabolites of vitamin D_3 more polar than 25-hydroxycholecalciferol. Because of this resolution, a new metabolite of vitamin D_3 has been demonstrated in the plasma of rats and in the intestines of chicks given 100 IU of vitamin D_3-1,2-^{3}H.

SUPPLEMENTARY KEY WORDS Sephadex LH-20 · cholecalciferol · intestine · plasma · chick · rat · 25-hydroxycholecalciferol

Progress in liquid chromatographic methods for vitamin D and its metabolites has been limited to various procedures with Celite, silicic acid, and alumina (1–4). Recently, countercurrent distribution was employed for separating the more polar metabolites of vitamin D_3 (5). Celite liquid–liquid partition chromatography has been used effectively for the purification of polar vitamin D_3 metabolites (6–8) and serves as a powerful tool for separating various metabolites of the vitamin. This type of chromatography, however, is time-consuming, with recoveries which vary between 60 and 80%, and its use is limited to separating metabolites of vitamin D_3 that are similar in polarity.

Silicic acid chromatography of vitamin D_3 and its metabolites was first described by Norman and DeLuca (9) and later modified (1–3). Suda et al. (7) and Suda, DeLuca, Schnoes, Tanaka, and Holick (8) used a multibore silicic acid system (10) to resolve three metabolites from hog plasma that are more polar than 25-HCC. Although silicic acid chromatography is widely used for metabolism studies of vitamin D_3 and 25-HCC, this method has many disadvantages. The silicic acid column requires a gradient generating system, and after the chromatography is completed, the silicic acid bed must be discarded. The results reported in this paper also demonstrate that a number of metabolites more polar than 25-HCC cannot be resolved on silicic acid columns.

Sephadex gel chromatography of lipophilic compounds in organic solvents has been studied by many investigators using methyl ether, hydroxypropyl ether, and acetylated derivatives of various Sephadex products for gel permeation, liquid–liquid partition, and adsorption chromatography (11–13). Nyström and Sjövall (11) observed that the behavior of bile acids and steroids with mixed solvents on methylated Sephadex could not be explained by either liquid–liquid partition, adsorption, or gel filtration alone.

Van Baelen, Heyns, and De Moor (14) investigated the potential of Sephadex LH-20 for separating various urinary estrogen metabolites and noted that a use of different solvents or mixtures of solvents may render the procedure more suitable for practical application. Recently, Ellingboe, Nyström, and Sjövall (15) synthesized hydrophobic long-chain alkyl ethers of Sephadex and reported straight- and reversed-phase chromatography for various steroids, bile acids, and triglycerides.

Abbreviations: 25-HCC, 25-hydroxycholecalciferol; 21,25-DHCC, 21,25-dihydroxycholecalciferol; 25,26-DHCC, 25,26-dihydroxycholecalciferol; RVD, retention volume of vitamin D_3 metabolites relative to vitamin D_3.

* Address requests for reprints to H. F. DeLuca, Department of Biochemistry, University of Wisconsin, Madison, Wis. 53706.

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The present investigation was undertaken to evaluate gel chromatography as a means of resolving vitamin D metabolites that exhibit similar rates of migration by silicic acid chromatography. The use of Sephadex LH-20 with various mixed solvents as a rapid and efficient method for investigating vitamin D metabolism is described. A new metabolite of vitamin D_3 in intestinal lipid extracts from chicks and in the plasma of rats that received 100 IU of tritiated vitamin D_3 is also reported.

EXPERIMENTAL METHODS

All solvents were of analytical grade unless otherwise stipulated. Skellysolve B (predominantly *n*-hexane) was redistilled (67–69°C) before use. Reference compounds 21,25-dihydroxycholecalciferol (21,25-DHCC), 25,26-dihydroxycholecalciferol (25,26-DHCC), and hog plasma peak V_b were isolated as described previously (7, 8).

Sample Preparation

The plasma extracts were prepared according to the modified extraction procedure reported by Lund and DeLuca (16). Groups of four rats (weighing 400 g) fed a low vitamin D diet (17) were injected intraperitoneally with 100 IU of radiochemically pure vitamin D_3-1,2-^3H (specific activity 9.0×10^4 dpm/IU) in 0.02 ml of EtOH. 24 hr later, blood was collected either by cardiac puncture or decapitation, giving 36 ml of plasma after centrifugation. This was extracted with methanol–chloroform 2:1 (v/v) and prepared as previously described (7).

In other experiments chicks were maintained on a vitamin D-deficient diet for 4 wk. Under light ether anesthesia, groups of three chicks were injected in the wing vein with either 10 IU or 100 IU of vitamin D_3-1,2-^3H (specific activity 10,750 dpm/IU) in 0.05 ml of EtOH and sacrificed 24 hr later. The entire small intestine (each weighed ca. 3 g) from each group was pooled, washed with saline, and frozen at −20°C. The frozen tissue was ground in a meat grinder which had been previously cooled with dry ice. The tissue was homogenized in a Waring Blendor for 1 min in 150 ml of MeOH–$CHCl_3$ 2:1 (v/v). 100 ml of distilled H_2O and 50 ml $CHCl_3$ were added 2 hr later; this resulted in the separation of the phases. The chloroform phase was collected and washed with an equal volume of tap water and stored at 4°C overnight. The chloroform phase was then collected and evaporated to dryness with a flash evaporator. Each of the dried samples was redissolved in 0.2–1.0 ml of $CHCl_3$–Skellysolve B 65:35 (v/v).

Chromatography Columns

20 g of Sephadex LH-20, a hydroxypropyl ether derivative of Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), was slurried in 70 ml of the appropriate solvent ($CHCl_3$–Skellysolve B, either 3:7, 1:1, or 65:35 [v/v]). After 24 hr of equilibration, the slurry was poured into a 60×1.1 cm glass column containing 15 ml of solvent. The stopcock was opened at the same time the slurry was poured and it was allowed to settle by gravity with free solvent flow. The column was washed with at least 50 ml of solvent before the sample was applied. Air pressure of 1–2 lb was applied to the $CHCl_3$–Skellysolve B 65:35 columns in order to obtain a flow rate of 0.66 ml/min; the other two systems were run under gravity.

Silicic acid chromatography of the lipid extracts was carried out according to the procedure of Ponchon and DeLuca (1). Columns 60×1.1 cm were packed with 25 g of silicic acid (18) in Skellysolve B. The column was eluted with consecutive additions of 400 ml of 100% diethyl ether, 300 ml of 5% (v/v) methanol in diethyl ether, and 200 ml of 50% (v/v) methanol in diethyl ether to a holding chamber of an exponential gradient generating apparatus. The column was stripped with 200 ml of MeOH that was applied directly to the column. The constant volume mixing chamber contained 230 ml of Skellysolve B.

Measurement of Radioactivity

Radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb model 3375 equipped with an automatic external standard system. Samples 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 liter of toluene), and counted.

RESULTS

Chromatographic Properties

To avoid the problem of the Sephadex LH-20 floating in $CHCl_3$, a mixed solvent system was used. Although it has been reported that alkanes are too nonpolar for Sephadex LH-20 (15), when Skellysolve B was mixed with $CHCl_3$ in varying amounts it complemented the $CHCl_3$ by making the gel bed suitable for the chromatography. This lipophilic solvent also improved the chromatography of vitamin D_3 and its metabolites to the extent that resolution was generally superior to silicic acid chromatography.

In most of the experiments, vitamin D_3-1,2-^3H was used as a reference and the elution volumes of the other metabolites were expressed relative to it. The relative values for each of the metabolites on different batches and different quantities of Sephadex LH-20 were reproducible.

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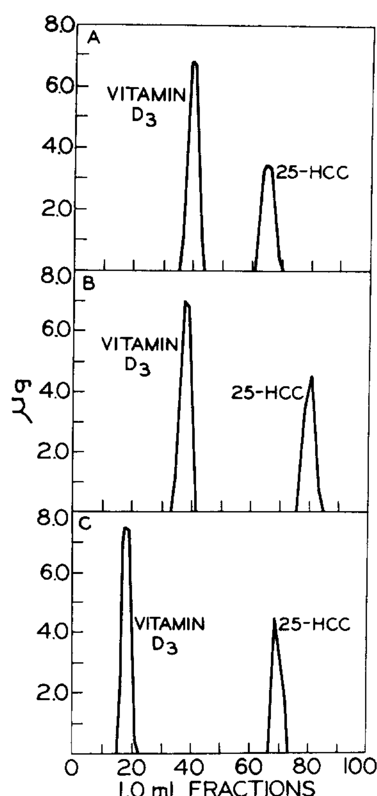

FIG. 1. Separation of vitamin D_3 and 25-HCC on Sephadex LH-20 in various solvent mixtures. Column: 1.1 × 60 cm containing 20 g of Sephadex LH-20. Solvent $CHCl_3$–Skellysolve B; (*A*) 65:35, (*B*) 1:1, (*C*) 3:7; flow rate, 0.66 ml/min.

Fig. 1 illustrates the effects of different solvent mixtures (i.e., $CHCl_3$ in Skellysolve B) on the Sephadex LH-20 separation of 25-HCC from vitamin D_3. The retention volume for 25-HCC relative to vitamin D_3 increased as the percentage of $CHCl_3$ in Skellysolve B decreased from 65% to 30%. It was noted, however, that as the amount of $CHCl_3$ decreased so did the swelling of the gel. This decreased the void volume of the column and elution volume for vitamin D_3. The $CHCl_3$–Skellysolve B 3:7 and 1:1 methods appeared to be the best systems for routine separations of vitamin D_3 and 25-HCC. Because the Sephadex LH-20 did not swell very well in $CHCl_3$–Skellysolve B 3:7 this system was not investigated further. $CHCl_3$–Skellysolve B 1:1 was a suitable solvent for Sephadex LH-20 and is the method of choice for purifying vitamin D_3 and 25-HCC. The capability of the $CHCl_3$–Skellysolve B 65:35 system in resolving the various vitamin D_3 metabolites in rat plasma as compared with that obtained with a silicic acid column is clearly demonstrated in Fig. 2. The peak *V* region, which appears to be homogeneous on silicic acid, separates into four distinct peaks, V_{t_1}, V_a, V_b, and V_c, on Sephadex LH-20. It should be noted that when the peak *V* fraction from silicic acid columns is chromatographed alone on LH-20, V_{t_1}, V_a, V_b, and V_c all appear in the chromatogram, illustrating that they are found in the *V* region of silicic acid. Peak *III* (vitamin D_3) and peak *IV* (25-HCC) appeared to be homogeneous in both systems. The compound(s) corresponding to the peak *VI* region of the silicic acid chromatogram was stripped off the Sephadex column with 100 ml of $CHCl_3$–Skellysolve B 7:3. Although the column swelled as the percent of $CHCl_3$ increased, the flowrate was kept constant by slightly increasing the air pressure. After the chromatography was completed, the column was regenerated by passing 100 ml of $CHCl_3$–Skellysolve B 65:35 through the column. The various peak *V* assignments for the Sephadex LH-20 chromatograms were determined by cochromatography of each of the Sephadex LH-20 peaks with 21,25-DHCC, 25,26-DHCC, and hog plasma peak V_b; they are summarized in Table 1.

A new metabolite(s) V_{t_1}, which is more polar than 25-HCC, was detected in rat plasma. This metabolite also appeared in the intestine from chicks given 100 IU of tritiated vitamin D_3, but could not be detected in the case of a 10-IU dose (see Fig. 3). Another metabolite that cochromatographed with 21,25-DHCC on three different chromatographic systems (i.e., Celite liquid–liquid partition (7), silicic acid (1), and Sephadex LH-20 with $CHCl_3$–Skellysolve B 65:35) also became evident in the intestinal extracts from chicks given 100 IU of tritiated vitamin D_3. Blood present in the intestine probably does not account for the appearance of these metabolites in that tissue extract. On silicic acid, however, all of the peak *V* metabolites seemed to comigrate as a single peak in the peak *V* region of the chromatogram (see Figs. 2 and 3).

Applications

The Sephadex LH-20 chromatographic systems described in this report offer several advantages in column

TABLE 1 ELUTION VOLUMES OF VITAMIN D_3 METABOLITES RELATIVE TO VITAMIN D_3 ON SEPHADEX LH-20 ($CHCl_3$–SKELLYSOLVE B 65:35)

	V_e*	RVD†
	ml	
Peak I	25	0.56
Peak III (vitamin D_3)	44	1.00
Peak IV (25-HCC)	65	1.47
Peak V_{t_1}	93	2.11
Peak V_a (21,25-DHCC)‡	152	3.45
Peak V_b	183	4.16
Peak V_c (25,26-DHCC)§	248	5.63
Peak V	254	5.77

* Elution volume.
† Retention volumes of vitamin D_3 metabolites relative to vitamin D_3.
‡ 21,25-dihydroxycholecalciferol.
§ 25,26-dihydroxycholecalciferol.

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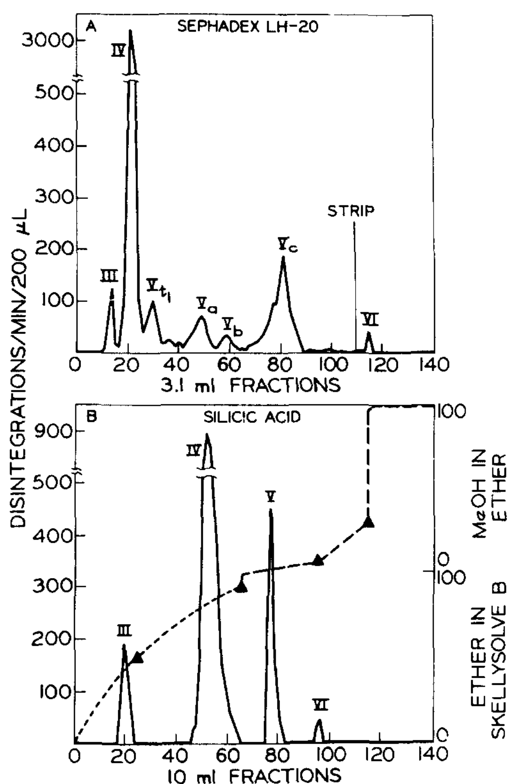

FIG. 2. Comparison of the chromatography of a lipid extract from plasma of rats that received 100 IU of vitamin D_3-1,2-^3H intraperitoneally 24 hr earlier. (*A*) Sephadex LH-20 (20 g) in $CHCl_3$–Skellysolve B 65:35, (*B*) silicic acid (25 g). (———) Radioactivity, (— — —) gradient. The Sephadex LH-20 column was stripped with 100 ml of $CHCl_3$–Skellysolve B 7:3.

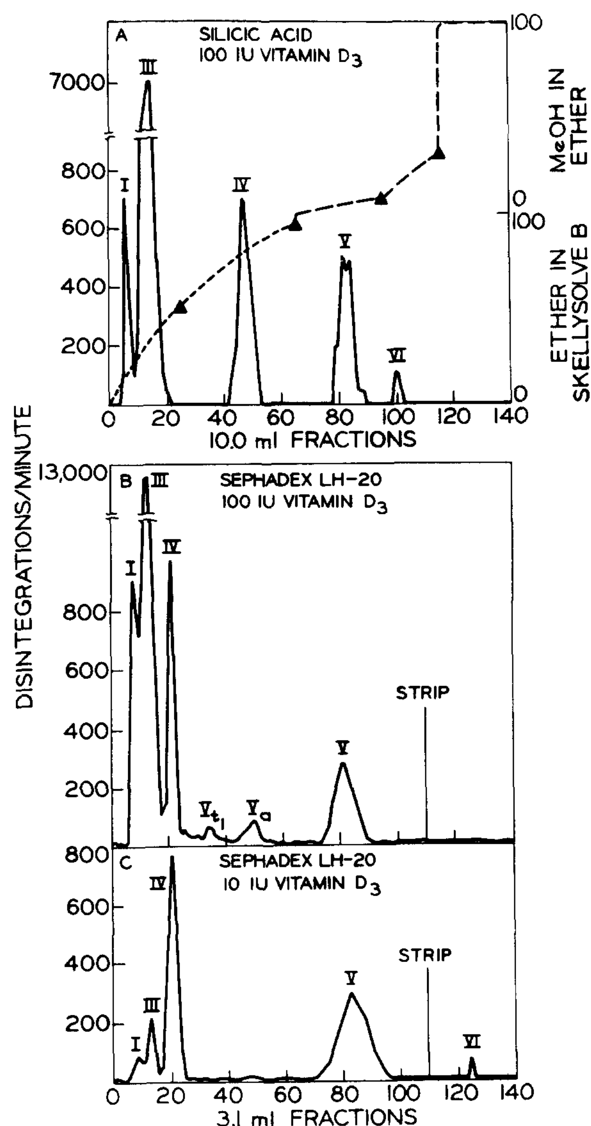

FIG. 3. Comparison of the chromatography of intestinal lipid extracts from groups of three chicks that received vitamin D_3-1,2-^3H in the wing vein 24 hr earlier. (*A*) Silicic acid chromatography of an intestinal lipid extract from chicks that received 100 IU of vitamin D_3-1,2-^3H; (*B*) Sephadex LH-20 ($CHCl_3$–Skellysolve B 65:35) chromatography of an intestinal lipid extract from chicks that received 100 IU of vitamin D_3-1,2-^3H; and (*C*) Sephadex LH-20 ($CHCl_3$–Skellysolve B 65:35) chromatography of an intestinal lipid extract from chicks that received 10 IU of vitamin D_3-1,2-^3H. The Sephadex LH-20 columns were stripped with 100 ml of $CHCl_3$–Skellysolve B 7:3.

chromatography of vitamin D_3 and its metabolites. They are inexpensive to run since only one solvent is used throughout the chromatography and the volume of solvent used is about 30% of that needed for silicic acid chromatography. Also, the Sephadex LH-20 columns are simple to prepare and, as is true with most Sephadex columns, the same column can be used repeatedly until resolution of the metabolites begins to be noticeably poor. The resolution of more polar metabolites of the parent vitamin is comparable to that obtained on a Celite liquid–liquid partition column (7), and the recoveries approximate 100% as contrasted with 60–80% for both Celite partition and silicic acid chromatography. The $CHCl_3$–Skellysolve B 1:1 system is extremely useful for purifying vitamin D_3, vitamin D_2, and 25-HCC, and the entire chromatography takes less than 2 hr.

Tissue extracts, especially those obtained from the intestine, contain a large amount of neutral fat, i.e., about 1 ml of yellow oil from three chick intestines. On the $CHCl_3$–Skellysolve B 65:35 Sephadex LH-20 system, most of the yellow lipid eluted at the apparent void volume of the column. This property makes it a likely candidate for the initial step in the purification of vitamin D_3 metabolites.

The $CHCl_3$–Skellysolve B 65:35 system is particularly useful for investigating the metabolism of vitamin D_3 and its 25-OH derivative. The resolution of the plasma peak *V*'s (Fig. 2) and intestinal peak *V*'s on Sephadex LH-20 was shown to surpass that obtained on silicic acid and is the method of choice for most of the vitamin D metabolism studies.

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DISCUSSION

In order to understand the mechanism of action of vitamin D in the various target tissues, many investigators have sought to understand its metabolism. Lund and DeLuca (16) were the first to determine by silicic acid chromatography that the tritiated parent vitamin D_3 was metabolized to at least five radioactive component fractions found in the various target tissues and blood of the rat. Similarly, Haussler, Myrtle, and Norman (2), Lawson, Wilson, and Kodicek (3), and Ponchon and DeLuca (1) have demonstrated via silicic acid chromatography the metabolism of vitamin D_3 to more polar compounds. This effort led to the discovery that vitamin D_3 must be metabolized enzymatically in the liver to its 25-OH derivative before it can be transported as one of the active circulating forms to the various target tissues (19–21). Cousins, DeLuca, Suda, Chen, and Tanaka (22) have suggested that meaningful studies of vitamin D_3 metabolism in target tissues must be carried out at the level of its 25-hydroxy derivative. They have shown by silicic acid chromatography that the polar metabolites of vitamin D_3 in the intestine, bone, and liver also originate from tritiated 25-HCC (22, 23).

Recently two metabolites of vitamin D_3 were isolated in pure form from hog plasma and identified as 21,25- (7) and 25,26-dihydroxy derivatives of the vitamin (8). The 21,25-dihydroxy derivative of the parent vitamin showed marked action on mobilization of bone mineral and was one-half as active as vitamin D_3 in curing rickets, while the 25,26-dihydroxy derivative showed an effect on intestinal calcium transport and no bone-mobilizing ability. On a straight bore silicic acid column these two metabolites are inseparable (Fig. 2), whereas on the Sephadex LH-20 $CHCl_3$–Skellysolve B 65:35) column they are clearly resolved. Similarly, the peak V from the intestinal extracts of chicks given 100 IU of vitamin D_3 appeared homogeneous on silicic acid (Fig. 3), while on Sephadex LH-20 ($CHCl_3$–Skellysolve B 65:35) it was resolved into three peaks, V_t, V_a, and V. It is interesting to note that only peak V was detected in intestinal extracts from chicks that received 10 IU of vitamin D_3.

These results offer interesting avenues of exploration for understanding the metabolism of vitamin D and one of its circulating active forms, 25-HCC, at the target tissue level.

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References

1. Ponchon, G., and H. F. DeLuca. 1969. Metabolites of vitamin D_3 and their biological activity. *J. Nutr.* **99:** 157–167.
2. Haussler, M. R., J. F. Myrtle, and A. W. Norman. 1968. The association of a metabolite of vitamin D_3 with intestinal mucosa chromatin *in vivo*. *J. Biol. Chem.* **243:** 4055–4064.
3. Lawson, D. E. M., P. W. Wilson, and E. Kodicek. 1969. Metabolism of vitamin D—a new cholecalciferol metabolite involving loss of hydrogen at C-1 in chick intestinal nuclei. *Biochem. J.* **115:** 269–277.
4. Chen, P. S., Jr., A. R. Terepka, K. Lane, and A. Marsh. 1965. Studies of the stability and extractability of vitamin D. *Anal. Biochem.* **10:** 421–434.
5. Haussler, M. R., E. T. Littledike, D. W. Boyce, and H. Rasmussen. 1970. Separation of vitamin D metabolites by countercurrent distribution. *Federation Proc.* **29:** 368. (Abstr.)
6. Blunt, J. W., H. F. DeLuca, and H. K. Schnoes. 1968. 25-Hydroxycholecalciferol. A biologically active metabolite of vitamin D_3. *Biochemistry.* **7:** 3317–3322.
7. Suda, T., H. F. DeLuca, H. K. Schnoes, G. Ponchon, Y. Tanaka, and M. F. Holick. 1970. 21,25-Dihydroxycholecalciferol. A metabolite of vitamin D_3 preferentially active on bone. *Biochemistry.* **9:** 2917–2922.
8. Suda, T., H. F. DeLuca, H. K. Schnoes, Y. Tanaka, and M. F. Holick. 1970. 25,26-Dihydroxycholecalciferol. A metabolite of vitamin D_3 with intestinal calcium transport activity. *Biochemistry.* **9:** 4776–4780.
9. Norman, A., W., and H. F. DeLuca. 1963. Chromatographic separation of mixtures of vitamin D_2, ergosterol and tachy-sterol$_2$. *Anal. Chem.* **35:** 1247–1252.
10. Fischer, G. A., and J. J. Kabara. 1964. Simple, multibore columns for superior fractionation of lipids. *Anal. Biochem.* **9:** 303–309.
11. Nyström, E., and J. Sjövall. 1965. Separation of lipids on methylated Sephadex. *Anal. Biochem.* **12:** 235–248.
12. Joustra, M., B. Söderqvist, and L. Fischer. 1967. Gel filtration in organic solvents. *J. Chromatogr.* **28:** 21–25.
13. Determann, H. 1964. Chromatographic separation on porous gels. *Angew. Chem. Int. Ed. Engl.* **3:** 608–617.
14. Van Baelen, H., W. Heyns, and P. De Moor. 1967. The separation of estrogens on Sephadex. *J. Chromatogr.* **30:** 226–227.
15. Ellingboe, J., E. Nyström, and J. Sjövall. 1970. Liquid–gel chromatography on lipophilic–hydrophobic Sephadex derivatives. *J. Lipid Res.* **11:** 266–273.
16. Lund, J., and H. F. DeLuca. 1966. Biologically active metabolite of vitamin D_3 from bone, liver, and blood serum. *J. Lipid Res.* **7:** 739–744.
17. Steenbock, H., and A. Black. 1925. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light. *J. Biol. Chem.* **64:** 263–298.
18. Hirsch, J., and E. H. Ahrens, Jr. 1958. The separation of complex lipid mixtures by the use of silicic acid chromatography. *J. Biol. Chem.* **233:** 311–320.
19. Horsting, M., and H. F. DeLuca. 1969. *In vitro* production of 25-hydroxycholecalciferol. *Biochem. Biophys. Res. Commun.* **36:** 251–256.
20. Ponchon, G., and H. F. DeLuca. 1969. The role of the liver in the metabolism of vitamin D. *J. Clin. Invest.* **48:** 1273–1279.

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21. Ponchon, G., A. L. Kennan, and H. F. DeLuca. 1969. "Activation" of vitamin D by liver. *J. Clin. Invest.* **48:** 2032–2037.
22. Cousins, R. J., H. F. DeLuca, T. Suda, T. Chen, and Y. Tanaka. 1970. Metabolism and subcellular location of 25-hydroxycholecalciferol in intestinal mucosa. *Biochemistry.* **9:** 1453–1459.
23. Cousins, R. J., H. F. DeLuca, and R. W. Gray. 1970. Metabolism of 25-hydroxycholecalciferol in target and nontarget tissues. *Biochemistry.* **9:** 3649–3652.

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