The Synthesis of [1,2-^{3}H]Vitamin D_3 and the Tissue Localization of a 0.25-μg (10 IU) Dose per Rat*

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ABSTRACT: [1,2-^{3}H]vitamin D_3 has been synthesized chemically from [1,2-^{3}H]cholesteryl benzoate. This preparation is radiochemically pure, is fully biologically active, and has a specific activity (26,000 dpm/IU) high enough to permit experiments with 5–10 IU of vitamin D/rat.

Specific localization of tritium in bone and intestine following an injection of 10.7 IU of this preparation intravenously has been noted. Liver first accumulates much of the radioactivity (within 15 min) which declines rapidly. All other tissues reach a maximum at 4 hr and decline slowly. The skeleton, muscle, and intestinal contents contain most of the radioactivity but when expressed as disintegrations per minute per unit weight of cells, the skeleton and intestinal mucosa contain the highest concentration of radioactivity.

One of the approaches to the mechanism of vitamin D action taken in this laboratory has been a study of possible metabolically active forms of vitamin D (Norman *et al.*, 1964) and its subcellular location (Norman and DeLuca, 1964). This approach was made possible by the preparation of tritium-labeled vitamins D_2 and D_3 of moderate specific activity (Norman and DeLuca, 1963a). These preparations were much more radioactive than those commercially available[1] or that had been studied by Kodicek (1955) and by Blumberg *et al.* (1960) which allowed experiments to be carried out with 100–500 IU of vitamin D/rat (Norman and DeLuca, 1963a). Using them it was possible to demonstrate for the first time biologically active metabolites of vitamin D in kidney and intestine (Norman *et al.*, 1964) and the subcellular distribution of vitamin D could also be studied (Norman and DeLuca, 1964). However, because 10 IU or less is considered a physiological dose of vitamin D/rat (Carlson and Lindquist, 1955), it was clear that even these experiments were subject to the criticism that doses used were large and would reflect largely storage of vitamin D. In addition, the previous preparations were randomly labeled which also limits their usefulness. In any case, it soon became clear that for critical experiments relating to the mechanism of vitamin D action, a more highly radioactive vitamin D labeled in specific positions was essential. This has been accomplished in two ways: the synthesis of [1,2-^{3}H]vitamin D_3 and [22,23-^{3}H]vitamin D_4 (H. F. DeLuca and M. Weller, in preparation). In this paper the synthesis of the former is described, its radiochemical purity and biological activity are established, and the time course tritium distribution among tissues of the rat following an intravenous injection of 10.7 IU of the [1,2-^{3}H]vitamin D_3 is described.

Methods and Results

General Procedure. Radioactive vitamin D_3 was synthesized from specifically labeled cholesterol. The 7-dehydrocholesteryl benzoate was first purified by crystallization and, following ultraviolet irradiation, the vitamin D_3 was isolated and purified by silicic acid column chromatography. Absorption spectra were recorded with a Cary 11 recording spectrophotometer. The following molar extinction coefficients (in ethanol) were used: 7-dehydrocholesterol, λ 281 mμ (ϵ 10,920), vitamin D_3 λ 264 mμ (ϵ 18,200) (Huber *et al.*, 1945), and previtamin D_3 λ 262 mμ (ϵ 9000) (Fieser and Fieser, 1959). All radioactivity measurements were made in a Packard Tricarb liquid scintillation counter, Model 3000, and automatic external standardization was used to determine efficiency (Packard Instrument Co., La-Grange, Ill.). Radioactive samples soluble in toluene were counted in scintillator solution A which consisted of 2 g of PPO[2] (2,5-diphenyloxazole) and 100 mg of dimethyl-POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene]/l. of toluene while the water samples from combustions of tritium-labeled samples were counted in scintillator solution B consisting of 50 mg of dimethyl-POPOP, 4 g of PPO, 200 ml of absolute ethyl alcohol, and 800 ml of toluene.

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[1] [4-^{14}C]Vitamin D_3 (2.2 μc/mg) and [7-^{3}H]vitamin D_3 (0.14 μc/mg), N. V. Philips-Duphar, Amsterdam-2, Holland.

[2] Abbreviations used: PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; tlc, thin layer chromatography.

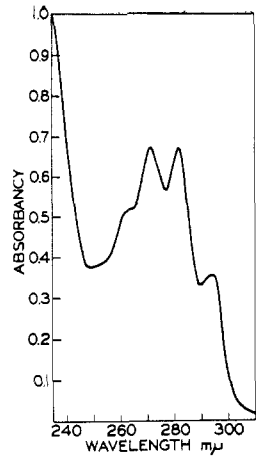

FIGURE 1: Spectrum of twice-crystallized synthetic [1,2-H^3]dehydrocholesteryl benzoate in absolute ethanol.

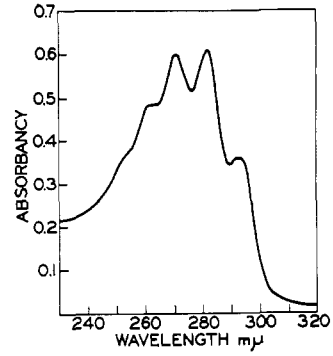

FIGURE 2: Spectrum of synthetic [1,2-^{3}H]7-dehydrocholesterol in absolute ethanol.

Preparation of 7-Dehydrocholesteryl [1,2-^{3}H]Benzoate. 7-Dehydrocholesteryl benzoate was synthesized according to a method described by Hunziker and Müllner (1958) from cholesteryl benzoate specifically labeled in the 1 and 2 positions with tritium (New England Nuclear Corp., Boston, Mass.).

Approximately 1 g of [1,2-^{3}H]cholesteryl benzoate (500 mc) was dissolved in 6 ml of distilled benzene and 6 ml of distilled Skellysolve B (a petroleum fraction which boils at 67°) prior to the addition of 0.40 g of powdered 5,5-dibromodimethylhydantoin (Aldrich Chemical Co., Milwaukee, Wis.). This solution was refluxed at 72° in a constant temperature water bath. After refluxing for 10 min, the mixture was allowed to cool to room temperature before being filtered through a Hirsch funnel into a 50-ml round-bottom flask. The residue was discarded after being washed with two 3-ml portions of ice-cold Skellysolve B. The solvents were removed at a temperature no greater than 40° in a rotary flash evaporator under water pump vacuum. The residue containing the brominated derivative was then dissolved in 3 ml of dry xylol. Another 50-ml round-bottom flask, containing 1 ml of trimethyl phosphite (Distillation Products Industries, Rochester, N. Y.) in 4 ml of dry xylol, was attached to a reflux condenser and heated to 125° in an oil bath. The brominated derivative was added dropwise to the trimethyl phosphite solution after the temperature reached 125°. Two 1-ml portions of dry xylol were used as rinses. It is important that the addition be completed in <10 min without cooling the mixture. After refluxing for 90 min, the reaction mixture was allowed to cool and the solvent was removed by vacuum distillation at 80°. The residue was dissolved in as little hot acetone as was necessary for complete solution. Hot methanol was added dropwise until the solution became cloudy. Crystallization was allowed to proceed overnight at −10°. Recrystallization was carried out with acetone–methanol (1:1). The yield from this synthesis was approximately 45%.

The spectra of the [1,2-^{3}H]7-dehydrocholesteryl benzoate (181 mc/mmole) is shown in Figure 1 and indicated at least 99% purity. The radioactive 7-dehydrocholesteryl benzoate was then dissolved in 15 ml of 10% methanolic KOH and refluxed for 30 min. Nitrogen gas was constantly bubbled through the solution. Water (20 ml) was added after the solution had cooled to room temperature and the 7-dehydrocholesterol extracted with four 20-ml portions of diethyl ether. The ether solution was washed three times with water. The yield is approximately 90% and the ultraviolet spectra of the [1,2-^{3}H]7-dehydrocholesterol (180 mc/mmole) is shown in Figure 2.

Irradiation of 7-Dehydrocholesterol. The irradiation of the vitamin D_3 precursor was conducted according to the method described by Norman and DeLuca (1963a) under an atmosphere of N_2. [1,2^{3}H]7-Dehydrocholesterol (50 mg) was dissolved in 50 ml of N_2 saturated, peroxide-free diethyl ether in a 250-ml round-bottom quartz flask connected to a reflux condenser. A capillary tube inserted through the bore of the condenser was used to bubble N_2 gas through the solution during irradiation. The solution was exposed to ultraviolet light from a Model 52303 Hanovia Alpine sun lamp (Hanovia Lamp Division, Newark, N. J.) at a distance of 15 cm for 8 min. The yield of vitamin D was approximately 10–15%.

Purification of Vitamin D_3. The radioactive vitamin D_3 was isolated from the irradiated provitamin solution by column chromatography with silicic acid (Bio-Rad silicic acid, HA, minus 325 mesh, California Corp. for Biochemical Research, Los Angeles, Calif.) essentially according to the procedure of Norman and DeLuca (1963b). One of the major problems in the purification of vitamin D was the separation of it from previtamin D_3. Thin layer and conventional monobore column chromatography were adequate for separating the vitamin from its precursor and irradiation by-products, and tlc was adequate for separating previtamin D and vitamin D in microgram quantities, but both methods were inadequate to separate mg quantities of these isomers. Good separation of previtamin D_3 and the vitamin as well as by-products was achieved through

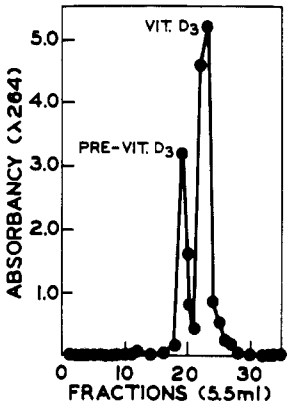

FIGURE 3: Silicic acid column chromatography of synthetic [^{3}H]previtamin D_3 and [^{3}H]vitamin D_3. A four-stage multibore column (60 cm) was packed with 14 g of silicic acid and developed with a 300-ml solvent gradient of 0–30% diethyl ether in Skellysolve B. Absorbancy at 264 mμ was measured in a recording spectrophotometer.

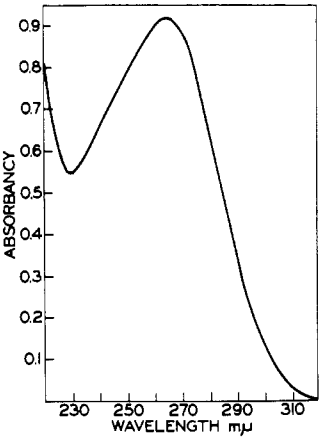

FIGURE 4: Spectrum of synthetic [1,2-^{3}H]vitamin D_3 in absolute ethanol.

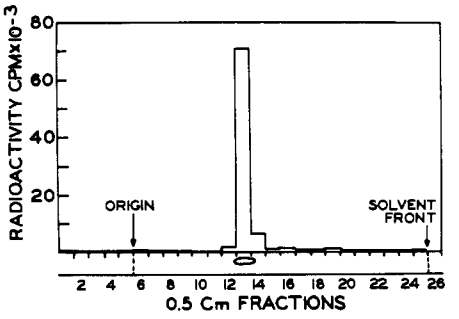

FIGURE 5: Silica gel thin layer chromatography of [1,2-^{3}H]vitamin D_3 and commercial crystalline vitamin D_3. The two vitamins were placed at the origin approximately 2 cm apart and the chromatogram developed with 10% (v/v) acetone in Skellysolve B. The commercial vitamin D_3 was detected with 0.2% $KMnO_4$ in 1% $NaHCO_3$ while radioactivity was measured in successive 0.5-cm segments of the chromatogram by liquid scintillation counting.

the use of silicic acid in a four-stage multibore column constructed according to the description given by Fischer and Kabara (1964).

A slurry was made of 14 g of heat-activated silicic acid and Skellysolve B which was poured into 60-cm water-jacketed multibore columns. The sample was introduced in 10 ml of Skellysolve B and allowed to enter the absorbant. The adsorbed samples were eluted with a 300-ml hyperbolic gradient (0–30% diethyl ether in Skellysolve B) produced by connecting a holding chamber containing 300 ml of 50% diethyl ether in Skellysolve B to a 250-ml mixing chamber containing Skellysolve B (Norman *et al.*, 1964). Fractions (10 ml) were collected and monitored spectrophotometrically with the Cary 11 recording spectrophotometer. Those tubes containing previtamin D_3 (262- mμ peak absorption) were pooled and stored at 4° under N_2 for 3 weeks to allow further conversion to the vitamin (Legrand and Mathieu, 1957). Only those tubes producing a perfect vitamin D (264- mμ peak absorption) spectrum were pooled and labeled as such. Typical separation of vitamin D_3 and previtamin D_3 by this method is shown in Figure 3. Doubtful fractions were pooled with the previtamin D for further chromatography. The vitamin D_3 fractions from the columns were dried under N_2, at temperatures below 30°, dissolved in redistilled benzene, and stored in brown bottles in a deep freeze.

Radiochemical Purity and Bioassay. A sample of the benzene solution estimated to contain approximately 1.5 mg of the radioactive vitamin D_3 was placed in a 100-ml volumetric flask, dried under N_2 at temperatures below 30°, and brought to volume with redistilled ethanol. A sample of this solution was used for ultraviolet analysis to determine the exact concentration of the vitamin present. The ultraviolet spectrum is shown in Figure 4. Three 1-ml samples of the ethanol solution were pipetted into three 250-ml volumetric flasks, each was brought to volume with ethanol and three 1-ml samples were taken from each flask for counting. Efficiency was determined using internal standard.

A sample calculated to contain 1 μg of vitamin D_3 on the basis of the ultraviolet analysis was taken from the first ethanol solution, dried under N_2, and dissolved in 2 ml of cottonseed oil (Wesson) for bioassay at the Wisconsin Alumni Research Foundation by the line-test assay method (U. S. Pharmacopeia, 1955). The data from these analyses indicated the radioactive vitamin D_3 had full biological potency with a molar specific activity of 180 mc/mmole or 26,000 dpm/IU (0.025 μg/IU).

Radiochemical purity was further verified by thin layer chromatography on silica gel (Figure 5). Commercial and a sample of the radioactive vitamin D_3

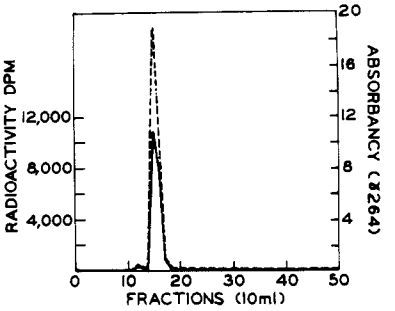

FIGURE 6: Silicic acid column cochromatography of commercial, crystalline vitamin D_3 and [1,2-^{3}H]vitamin D_3. A four-stage multibore column (60 cm) was packed with 14 g of silicic acid, and developed with a 300-ml solvent gradient of 0–30% diethyl ether in Skellysolve B followed by 100 ml of methanol. Approximately 0.012 μg of [1,2-^{3}H]vitamin D_3 was cochromatographed with approximately 2 mg of nonradioactive commercial vitamin D_3. Absorbancy in ethanol was measured with a recording spectrophotometer at 264 mμ. Radioactivity was measured by liquid scintillation counting. Absorbancy, ——; radioactivity, ———.

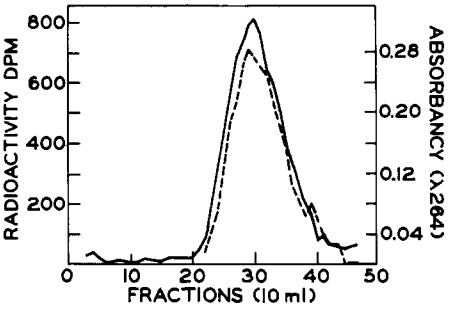

FIGURE 7: Partition cochromatography of anthenic and [1,2-^{3}H]vitamin D_3 on Fluoropak 80. A 1 × 12 mm column was packed to a height of 80 cm with Fluoropak 80 impregnated with isooctane (stationary phase). Approximately 0.012 μg of [1,2-^{3}H]vitamin D_3 and 2 mg of nonradioactive vitamin D_3 was applied to the column which was developed with 90% (v/v) methanol in water (saturated with isooctane). Absorbancy in ethanol was measured with a spectrophotometer at 264 mμ. Radioactivity was measured by liquid scintillation counting. Absorbancy, ·····; radioactivity, ———.

in diethyl ether were applied to heat-activated plates and developed in a solvent consisting of 10% (v/v) acetone in Skellysolve B. The commercial vitamin was located and marked with the aid of an ultraviolet light while the radioactive vitamin was located by scraping off successive 0.5-cm segments of the absorbent with a microscope slide. The scrapings were put in counting vials containing scintillation solution A. Approximately 96% of the radioactivity coincided exactly with the spot produced by the commercial vitamin D_3 as indicated by Figure 5.

Cochromatography of the [1,2-^{3}H]vitamin D_3 with authentic vitamin D_3 on silicic acid columns (Figure 6) demonstrated that the radioactivity in all cases corresponded with optical density at 264 mμ, again demonstrating radiochemical purity.

Still another system, namely reversed phase columns (Chen *et al.*, 1963), also verified the radiochemical purity of the isolated [1,2-^{3}H]vitamin D_3 (Figure 7).

Tissue Distribution in Rats. The methods used to study tissue distribution were essentially those described by Norman and DeLuca (1963a) except the radioactive vitamin was given intravenously *via* the jugular vein rather than by oral dosage. Male Holtzman rats maintained in individual cages with food and water *ad libitum* were used throughout this experiment. The vitamin D deficient ration fed to the rats for 3 weeks prior to beginning the study was diet 11 (0.4% calcium and 0.30% phosphorus) as described by Guroff *et al.* (1963). The [1,2-^{3}H]vitamin D_3 was prepared for injection by dissolving it in a small amount of acetone prior to adding an appropriate volume of 0.1% Tween-20 (Atlas Chemical Industries, Inc., Wilmington, Del.) in 0.9% NaCl. The acetone was evaporated off by bubbling N_2 through the solution. The dose given each rat was 10.7 IU of vitamin D in 0.2 ml of Tween–NaCl solution. After being dosed, each animal was placed in a wire cage equipped for trapping the feces and urine. Blood was collected from each rat at the time of sacrifice, mixed with heparin, and stored at −25° along with the other tissues dissected at that time. Two to four animals were used for each time period.

The samples to be combusted were placed in preweighed bags made of 1-in. dialysis tubing and lyophilized. After drying, the samples were weighed and combusted according to the method of Kelly *et al.* (1961) and Norman and DeLuca (1963a). The water vapor produced by combustion was frozen onto the bottom of the flask when it was placed in a Dry Ice–acetone bath and collected in 20 ml of counting solution B. Counting solution B (18 ml) was pipetted directly from the flask into counting vials and counted. Tissues such as the heart, kidney, lung, and spleen were combusted intact. The liver of each animal was mixed with enough water to produce a total volume of 15 ml, homogenized, and 1-ml samples were taken for drying and combusting. Samples (1 ml) of whole blood were also dried and combusted. The small intestine was split, washed free of its contents in 0.9% NaCl, and divided into four approximately equal sections for combustion. The large intestine (colon and caecum) was emptied of its contents in the same manner but combusted as one sample. Samples were taken of the small intestinal contents for combustion while the large intestinal contents were pooled with the feces before sampling and combusting. The values presented for bone are an extrapolation based on the data obtained from the combustion of the femur. All calculations were made by means of a digital computer (International Business Machine 1604).

TABLE I: Tissue Localization of Radioactivity Following an Intrajugular Dose of [1,2-^{3}H]Vitamin D_3[g].

Tissue	4 hr after Dose		24 hr after Dose		48 hr after Dose	
	% of Dose	Rel Incorp[a]	% of Dose	Rel Incorp[a]	% of Dose	Rel Incorp[a]
Spleen	1.04 ± 0.17	3620 ± 292	0.48 ± 0.006	1755 ± 306	0.49 ± 0.08	1540 ± 198
Lung	1.72 ± 0.49	2739 ± 732	1.03 ± 0.219	1443 ± 232	0.79 ± 0.20	926 ± 174
Heart	0.71 ± 0.20	1409 ± 353	0.64 ± 0.19	1100 ± 181	0.33 ± 0.026	624 ± 72
Intestine (large)	0.35 ± 0.03	813 ± 80	0.77 ± 0.19	1531 ± 467	0.91 ± 0.127	1847 ± 338
Intestine (small)	1.73 ± 0.07	2305 ± 502	1.66 ± 0.28	1736 ± 587	2.36 ± 0.70	1737 ± 322
Intestinal mucosa[b]	...	7113	...	5290	...	5366
Kidney	2.35 ± 0.19	2201 ± 441	3.22 ± 0.52	2577 ± 699	2.73 ± 0.966	2215 ± 643
Blood[c]	12.92 ± 1.96	2472 ± 413	7.84 ± 0.20	1391 ± 30	5.07 ± 0.79	936 ± 163
Muscle[d]	8.61 ± 2.75	244 ± 70	8.25 ± 2.56	213 ± 74	6.98 ± 1.64	181 ± 52
Liver	28.59 ± 2.04	5958 ± 893	9.16 ± 0.12	1339 ± 34	6.41 ± 1.48	1139 ± 349
Bone[e]	26.78 ± 3.8	501 ± 73	20.25 ± 2.0	340 ± 46	16.28 ± 2.3	272 ± 30
Bone cells[f]	...	12,525	...	8500	...	6800
Small intestinal contents	5.62 ± 1.05	2831 ± 545	4.64 ± 0.46	1660 ± 112	2.93 ± 0.64	1207 ± 475
Large intestinal contents + feces	3.17 ± 0.38	1010 ± 277	14.43 ± 1.46	2594 ± 52	16.11 ± 3.98	3176 ± 475
Total %	93.6		72.4		61.4	

[a] Relative incorporation expressed as disintegrations/min per 100-mg dry wt. [b] Calculated on the assumption that small intestine is made up of 30% mucosa and 70% muscle, connective tissue, etc. (on dry weight basis) and that smooth muscle has the same relative incorporation as skeletal muscle. [c] This assumes blood is 6% of body weight. [d] This assumes muscle is 30% of body weight. [e] This assumes skeleton is 15% of body weight. [f] This assumes bone is 4% cells on a dry weight basis and all vitamin D is located in these cells. Activity expressed as per 100-mg dry wt of bone cells. [g] Rats were fed a vitamin D deficient diet for 3 weeks before being used. Each deficient rat received an intrajugular injection of 10.7 IU of [1,2-^{3}H]vitamin D_3 in 0.2 ml of Tween–NaCl solution. Values ± the standard deviation are averages of three rats at 4 hr, two rats at 24 hr, and four rats at 48 hr.

The results of the 4-, 24-, and 48-hr distribution of radioactivity found in the various tissues of the rat are presented in Table I. The two categories containing the largest percentage of the dose during the 24- and 48-hr time interval are bone and the large intestinal contents (plus feces). During the 4-hr period, however, liver, bone, and blood took up the greater percentage of the dose. It should be reemphasized that the large intestinal contents value is an accumulative value since the feces excreted over these periods went into making up these samples. Interestingly when bone is corrected for noncellular material and the intestine is corrected for muscle, they have the highest concentration of radioactivity at all time periods examined. At 4 hr, liver and spleen are also very radioactive while at 24 and 48 hr the kidney and large intestinal contents are also high.

The distribution of radioactivity with respect to time in several key tissues is shown in Table II. Most of the tritium label was accumulated in the liver in early time periods and declined quite rapidly for the first 9 hr. Blood values remained relatively constant during the first 9 hr before showing a slight decline. Bone, small intestine, and kidney, on the other hand, increased in label during the first 9 hr and then leveled off somewhat. It is curious that the intestinal contents contained some of the label during the first 15 min after injection.

The cumulative per cent recovery of the radioactivity from all of the tissues examined *vs.* time was of some interest. Approximately 93% of the dose was accounted for in the tissues examined 4 hr after injection while only 61% could be accounted for after 48 hr.

Discussion

This communication describes for the first time a preparation of specifically labeled vitamin D radioactive enough to permit tracer experiments with truly nonstorage doses of the vitamin. The most highly labeled vitamin D previously described was that from this laboratory prepared by the tritiation of the respective provitamins. Even those preparations required that 100–500 IU doses be given to a rat to permit adequate detection and measurement of the radioactivity in the tissues (Norman and DeLuca, 1963a). According to Carlson and Lindquist (1955), 10 IU of vitamin D_3 is a normal physiological dose for rats allowing maximum intestinal absorption of calcium but less than maximum resorption of bone. Kodicek (1955) and Blumberg *et al.* (1960) with their radioactive vitamin D preparations were forced to use even higher doses of vitamin D (as high as 40,000 IU/rat). It is therefore obvious that a serious criticism of all previous work

TABLE II: Tissue Radioactivity *vs.* Time after an Intrajugular Dose of [1,2-^{3}H]Vitamin D_3.[a]

Time after Dose (hr)	Rel Incorp as dpm/100 mg dry wt of Tissue				
	Liver	Bone[b]	Blood	Kidney	Small Intestine
0.25	9,058	7,650	2,528	861	523
1.5	6,717	8,975	2,497	1,366	1,589
4	5,958	12,525	2,472	2,201	2,305
9	3,701	10,475	1,959	2,861	1,821
24	1,339	8,500	1,591	2,577	1,736
30	1,344	7,625	1,339	2,572	1,872
48	1,139	6,800	937	2,215	1,737

[a] Rats were fed a vitamin D deficient diet for 3 weeks prior to receiving an injection of 10.7 IU of [1,2-^{3}H]-vitamin D_3 in 0.2 ml of Tween–NaCl solution. The values are averages of the following number of rats per time period: two at 24 hr, three at 4 and 9 hr, *r* at 0.25, 1.5, 30, and 48 hr. [b] This assumes 4% cells on a dry weight basis.

with radioactive vitamin D is that mainly storage or nonfunctional vitamin D was being studied. With the present vitamin D preparation, this criticism is clearly avoided. Experiments with as little as 5 IU of the [1,2-^{3}H]vitamin D_3/rat have been carried out.

It was quite surprising that despite the low doses of [1,2-^{3}H]vitamin D_3 used in the present experiments (10.7 IU/rat) the per cent tissue distribution of radioactivity was similar to that observed by Norman and DeLuca (1963a) with a 500-IU dose. In that study, for example, 17% of the dose was found in the skeleton after 24 hr while 20% was found there after 24 hr in the present low dose study.

With regard to localization or selective accumulation, however, the most meaningful data is the relative incorporation or dpm/100 mg of tissue. It is instructive that muscle accumulates a high per cent of the dose but when expressed as relative incorporation (dpm/100 mg of tissue) it is rather low in concentration of radioactivity. Bone, on the other hand, is composed largely of noncellular material. If the relative incorporation data are corrected for ash, collagen, and other noncellular elements on the assumption that the vitamin is found only in the cells, then bone or skeletal tissue was the most radioactive. Similarly, if small intestine is corrected for the large proportion of muscle (assuming intestinal smooth muscle contains the same amount of radioactivity as skeletal muscle) it too contains a rather high relative incorporation. These two organs are certainly the targets of vitamin D action (Zull *et al.*, 1965) and it is interesting to find that they accumulate more vitamin D tritium than do all other tissues. The kidney also accumulated relatively larger amounts of radioactivity although not to the same degree as intestinal mucosa and bone.

Maximal uptake of tritium was seen 4–9 hr in most tissue including bone and intestine, but not liver. These results coincide well with the lag of 4–12 hr (Harrison and Harrison, 1960) between the time of vitamin D dosage and its earliest physiological effect in rats. The liver accumulated 44% of the dose at 15 min and then declined rapidly up to 9 hr. Considering the amounts found in the liver and the rate of decline during the early time periods it is quite possible that the entire dose accumulated there in the first few minutes after injection. This curious behavior was noted with larger doses of vitamin D (Norman and DeLuca, 1963a) and may be related to the possible role of the liver in the conversion of vitamin D to a metabolically active form or to the need for a transport system for vitamin D or its active form which might be produced by the liver.

The low per cent recovery observed after the first 4 hr was probably due to significant uptake of the vitamin by tissues not studied such as skin, adipose tissue, and brain and to the metabolic breakdown and elimination of the vitamin or tritium by routes other than feces. When one considers the loss of radioactivity from the various tissues between 24 and 48 hr (Table I) it becomes apparent that all tissues are declining at about the same rate. An extrapolation of these loses during this 24-hr period indicates the animal would have only 6% of the dose left by the fifth day after injection (this is excluding the amounts present in the large intestinal contents).

The chemistry of the synthesis of vitamin D_3 used in this paper is relatively well known. Of particular importance is the formation of the bromocholesteryl benzoate. The use of *N*,*N*-dibromo-5,5-dimethylhydantoin was of great importance simply because the *N*-bromosuccinimide reagent usually used in this type of reaction failed to produce the desired product in reasonable yield in our hands. Others have had similar difficulty with the *N*-bromosuccinimide reaction with cholesterol derivatives. The dehydrohalogenation step was best carried out with trimethyl phosphite. This material gave excellent yields provided (a) the temperature was maintained above 125° and (b) that the trimethyl phosphite is always present in excess. For that reason, the brominated derivative is added to the trimethyl phosphite with agitation. Fieser and Fieser (1959) believe that the mild reducing properties of the trimethyl phosphite protects the air-sensitive products thereby ensuring high yields.

The separation of previtamin D_3 from vitamin D_3 merits comment. When applied in microgram quantities these two compounds are easily separated on thin layer silicic acid, but silicic acid columns as described earlier (Norman and DeLuca, 1963b) were unable to achieve separation. The use of the multibore column of Fischer and Kabara (1964) proved to be a most useful innovation for this separation (Figure 3). This column retains the capacity of the larger columns but gives resolution approaching that of smaller bore columns.

Finally it might be mentioned that the synthetic [^{3}H]vitamin D_3 is completely biologically active by the

line test assay method and is radiochemically pure as revealed by at least three chromatographic methods. Purity is also revealed by ultraviolet spectral analysis of the vitamin and provitamin. Its specific activity (26,000 dpm/IU) is sufficiently high to be exceedingly useful for experiments on the mechanism of vitamin D action and is specifically labeled (1,2 position) to be very useful in metabolic experiments. It is hoped that this preparation will provide an important tool to be used in our quest of the mechanism of action and metabolism of vitamin D.

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The Reactions of Cations with Aqueous Dispersions of Phosphatidic Acid. Determination of Stability Constants*

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ABSTRACT: The reactions of univalent and divalent cations with aqueous dispersions of phosphatidic acid (PA) produced by ultrasonic radiation were studied by measurements of the H^+ ion released. Apparent stability constants (K') were calculated from equivalents of tetramethylammonium hydroxide (TMAOH) needed to maintain constant pH on addition of alkaline earth metal chlorides ($MeCl_2$) or alkali metal chlorides (MeCl). Titration curves gave concentrations of ionic forms of PA available for reactions $Me^{2+} + PA^{2-} = MePA$; $Me^+ + PA^{2-} = MePA^-$, and $HPA^- = PA^{2-} + H^+$. Assuming absence of specific reaction with TMA^+, values of K' for formation of CaPA, MgPA, $LiPA^-$, $NaPA^-$, and KPA^- are estimated as 1.6×10^4, 0.97×10^4, 17, 16, and 9, respectively. These values agree with those calculated from drop in pH on adding salt and in the case of CaPA from turbidimetric measurement.

The importance of the reactions of univalent and divalent cations in aqueous media with acidic lipids in biological tissues is abundantly clear (Ansell and Hawthorne, 1964). It is believed that determining the constants for the association of cations with these compounds will aid in understanding their characteristics in a manner analogous to the success attained in the study of the solutions of metal chelates (Dwyer and Mellor, 1964). In addition, this provides an opportunity for elucidating the reactions of ions with colloidal aggregates.

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