The Preparation of H^3-Vitamins D_2 and D_3 and Their Localization in the Rat*

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A convenient procedure for the preparation of tritium-labeled vitamin D_2 and D_3 is described. The provitamins were exposed to tritium gas by the procedure of Wilzbach, irradiated with ultraviolet light, and isolated as the crystalline 3,5-dinitrobenzoates. The H^3-vitamin D_2, specific activity 3.2 mc/mmole, or 450 disintegrations per minute per IU, and H^3-vitamin D_3, specific activity 7.2 mc/mmole or 1050 dpm/IU, are fully biologically active and were shown to be radiochemically pure in at least three chromatographic systems. The distribution of a physiological dose of H^3-vitamin D_3 as a function of time has been studied in the various tissues of the rat. Evidence is presented for the involvement of the ileum in the absorption of vitamin D. The liver rapidly accumulated the largest amount of radioactivity whether the dose was administered orally or intracardially. No evidence was found for the involvement of the adrenals in the metabolism of vitamin D.

Despite its recognition as a vitamin for some 40 years, there exists a serious lag in our understanding of the metabolism and biochemical mechanism of action of vitamin D. One important factor contributing to this lag is the inability of current chemical tests to detect amounts of vitamin D normally encountered in biological systems. Investigators have relied on laborious and time-consuming biological assays which have at best a precision of only ± 20%. As a result, no thorough investigation has been made of the tissue distribution of a physiological dose of vitamin D. Cruickshank and Kodicek (1953) have found on administration of massive doses (40,000 IU) to a single rat that vitamin D could be found by biological assay in the liver, bones, and intestine. But only 25% of the dose could be accounted for. Further studies by Kodicek (1956) utilizing a dose of 40,000 IU/rat of low specific activity C^{14}-vitamin D_2 confirm these general observations.

An important question that cannot be answered through the use of the biological assay concerns the possible metabolism of vitamin D to a biochemically active form. It has been found that there is a definite delay of 4–12 hours in the rat (Schachter *et al.*, 1961) and 12–18 hours in the chicken (Sallis and Holdsworth, 1962a) between the time of oral administration of vitamin D and the observance of its earliest physiological effects. It is not known whether this delay is due to absorption and transportation of the vitamin to active sites or to a necessity for metabolic conversion to an active compound. It has been postulated by Sallis and Holdsworth (1962b) and Raoul and Gounelle (1958) that the adrenals may be involved in the conversion of vitamin D to an active form. This and many other important areas of investigation make clear the need for radioactive vitamin D of high specific activity.

Several preparations of C^{14}-vitamin D have been reported. The only specifically labeled preparation was made by Havinga and Botts (1954) starting with $Ba^{14}CO_3$ and 5-oxo-3,5-seco-A-nor-cholestane-3-carboxylic acid. Their final yield of vitamin D_3 after some seven to ten steps was only 4% of the original material and was of such low specific activity (7 mμc/mg) as to be of no value for biological studies. The preparation of randomly labeled C^{14}-vitamin D_2 has been carried out a number of times utilizing the biosynthetic preparation of C^{14}-ergosterol which is harvested from the yeast *Saccharomyces cerevisiae* (Kodicek, 1955), or from *Carpentiles brefeldianum* (Schaltegger, 1960) grown on C^{14}-acetate and C^{14}-glucose, respectively. But again the specific activities, 10 mμc/mg (Kodicek, 1960) of the isolated vitamin D_2, have been too low to allow adequate detection in the tissues following physiological doses of the vitamin.

It is the purpose of this paper to describe a relatively simple method for the preparation of H^3-vitamins D_2 and D_3 that are of high enough specific activity for physiological doses of 70–500 IU to be readily detected in the tissues. These preparations have been further used to study the distribution of vitamin D in tissues from the various organs of the rat.

Experimental and Results

General Procedures.—Ultraviolet absorption curves were determined on a Cary 11 recording spectrophotometer. Solutions of 1–2 mg in 100 ml of ethanol were employed for analysis. The following molar extinction coefficients were used (Huber *et al.*, 1945): ergosterol, $\lambda = 281$, $\epsilon = 11{,}900$; 7-dehydrocholesterol, $\lambda = 281$, $\epsilon = 10{,}920$; vitamin D_2, $\lambda = 264$, $\epsilon = 18{,}200$; vitamin D_3, $\lambda = 264$, $\epsilon = 18{,}200$; vitamin D_2-3,5-dinitrobenzoate, $\lambda = 264$, $\epsilon = 18{,}150$; vitamin D_3-3,5-dinitrobenzoate, $\lambda = 264$, $\epsilon = 17{,}750$.

Radioactivity measurements were made with a Packard Tricarb liquid scintillation counter, Model 314XE. Radioactivity measurements of tritium samples containing only organic solvents were made using a counting solution A which consisted of 100 mg dimethyl-POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene)[1] and 3.0 g of PPO (2,5-diphenyloxazole) per liter of toluene, A.R. (Herberg, 1960). The efficiency of measuring tritium was approximately 20%.

Tissue samples of up to 240 mg dry wt containing H^3-vitamin D were combusted according to the procedure of Kelly *et al.* (1961) utilizing a Thomas Ogg safety igniter (A. H. Thomas Co., Philadelphia). The resulting H^3–water vapor was frozen on the bottom of a 2-liter Erlenmeyer flask which was placed in a dry

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[1] Abbreviations used in this paper: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)-benzene.

[2] This was carried out by the New England Nuclear Corporation, Boston, Massachusetts.

[3] The irradiation was carried out at the Wisconsin Alumni Research Foundation, Madison, Wisconsin.

[4] Personal communication from Dr. P. Westerhof of Phillips-Duphar Company, Weesp, The Netherlands.

ice–acetone bath for 45 minutes. Twenty ml of counting solution B, consisting of 50 mg dimethyl POPOP, 4.0 g PPO, 200 ml absolute ethanol, and 800 ml of toluene, was added and the flask was allowed to stand in crushed ice for 45 minutes. Then 18 ml of the solution was withdrawn and assayed for radioactivity. The detection efficiency of the tritium, which was strongly dependent upon the amount of water present in the sample, varied between 7 and 13%. Recoveries of H^3-vitamin D_2 added to nonradioactive tissue samples ranged between 94 and 100%. Internal standards of H^3-toluene were added to all samples analyzed and the total disintegrations per minute (dpm) were calculated.

Preparation of Radioactive Precursor.—The radioactive ergosterol and 7-dehydrocholesterol-acetate were prepared essentially according to the procedure of Wilzbach (1957). One gram of ergosterol obtained from the Mann Chemical Co. (New York) was exposed to 3 curies of tritium at 250 mm Hg for 14 days and 3 g of 7-dehydrocholesterol-acetate obtained from the Aldrich Chemical Co. (Milwaukee, Wis.), was exposed to 15 curies of tritium at 660 mm Hg pressure for 14 days.[2] The compounds were dissolved in 30 ml of methanol and dioxane several times followed by vacuum distillation of the solvents to remove the exchangeable hydrogens. The purity of the ergosterol and 7-dehydrocholesterol-acetate, received from the New England Nuclear Corp., as measured by their ultraviolet absorption curves, was 80% and 66.1%, respectively.

The H^3-ergosterol was recrystallized once from hot methanol, A.R. The final product, 778 mg, had a melting point of 162–63°, was 98% pure by ultraviolet absorbance measurements, and had a specific activity of 11.9 μc/mg or 4.21 mc/mmole.

The H^3-7-dehydrocholesterol-acetate (3.0 g) was placed in 150 ml of fresh 5% KOH in methanol and refluxed under nitrogen for 30 minutes. Fifty ml of water was added to the methanol solution, and the flocculent white precipitate was collected and recrystallized in the dark from hot methanol. The final product, 2.11 g, had a melting point of 149–50°, was 99.9% pure by ultraviolet absorbancy measurements, and had a specific activity of 54 μc/mg or 20.7 mc/mmole.

Irradiation of Vitamin D Precursor.— One hundred ml peroxide-free diethyl ether containing 50 mg of H^3-ergosterol or H^3-7-dehydrocholesterol was placed in a 250-ml round-bottom quartz flask connected to a reflux condenser. This was exposed to ultraviolet light from a Model S-2303 Hanovia Alpine sun lamp (Hanovia Lamp Division, 100 Chestnut Street, Newark, N. J.) at a distance of 15 cm for 8 minutes. The concentration, time of exposure, and distance of the provitamin from the lamp were worked out in preliminary experiments to provide a maximum production of vitamin D.[3] A total of 1,950 mg of 7-dehydrocholesterol and 690 mg of ergosterol was irradiated.

After irradiation the diethyl ether was removed with a flash evaporator. The resulting solid irradiation mixture was dissolved in boiling methanol, 1 g/58 ml, and placed overnight in a freezer at −20° under nitrogen. The following day a precipitate of unconverted provitamin was removed and the methanol solution was evaporated with a stream of nitrogen to one-half its original volume. This solution again was placed at −20° overnight to insure complete removal of the provitamin. Approximately 112 mg of 7-dehydrocholesterol and 80 mg of ergosterol were recovered by this procedure.

After removal of the remaining methanol, 1 g of crude irradiation mixture was dissolved in 45 ml of benzene, A.R., containing 5.0 g of freshly distilled maleic anhydride. (Weight ratio of irradiation mixture to maleic anhydride should be 1:5.) Nitrogen was bubbled through this solution while it was heated at 70° for 30 minutes. Under these conditions approximately 95% of the tachysterol and 12% of the vitamin D present react with the maleic anhydride to form a Diels-Alder adduct. The golden yellow solution was poured into 30 ml of 12% KOH in methanol and allowed to stand 30 minutes.

This solution was transferred to a separatory funnel containing 100 ml distilled water, and shaken vigorously. After separation of the phases, the aqueous, maleic acid, and tachysterol–maleic adduct–containing layer was removed and washed four times with diethyl ether. The ether washings were added to the benzene, H^3-vitamin D–containing layer and concentrated to 40 ml.

The diethyl ether–benzene solution was extracted twice with 10 ml of 2% KOH in methanol to ensure removal of the maleic acid, and then extracted with 20-ml volumes of water until the water washings were a neutral *p*H. The organic layer was then dried over anhydrous $CaSO_4$, and evaporated with a stream of nitrogen to give a clear golden yellow oily product. Approximately 780 mg of the oil was obtained from the 7-dehydrocholesterol and 270 mg from the ergosterol irradiation mixtures.

An equal amount by weight of 3,5-dinitrobenzoyl chloride (Eastman Kodak Co., Rochester, New York) was added to the oil, and both were dissolved in 5–15 ml of anhydrous pyridine. The reaction was carried out in the dark under a nitrogen atmosphere at room temperature. After 3 days an equal volume of 10% $NaHCO_3$ was mixed with the pyridine solution and the mixture was allowed to stand for 45 minutes. The pyridine-$NaHCO_3$ solution was extracted with diethyl ether in 30-ml portions to remove the 3,5-dinitrobenzoate esters. The ether was washed successively with 3.6% HCl, 10% $NaHCO_3$, and water so that the final wash had a neutral *p*H. The resulting diethyl ether solution was dried over anhydrous $CaSO_4$ and evaporated with a stream of nitrogen. The ergosterol irradiation procedure yielded 350 mg of vitamin D_2-3,5-dinitrobenzoate and the 7-dehydrocholesterol irradiation procedure yielded 900 mg of the 3,5-dinitrobenzoate ester.

The 3,5-dinitrobenzoate esters were crystallized three times from hot methanol-acetone to a constant melting point and specific activity as shown in Table I. Ultimately from the crude vitamin D_2-3,5-dinitrobenzoate, 50 mg of a light golden yellow crystalline material, melting point 147–48°, and specific activity of 8.2 μc/mg or 450 dpm/IU (calculated as free vitamin D_2) was isolated. From the crude vitamin D_3-3,5-dinitrobenzoate, 130 mg of crystalline material, melting point

TABLE I

RECRYSTALLIZATION OF H^3-VITAMINS D_2- AND D_3-3,5-DINITROBENZOATES TO CONSTANT SPECIFIC ACTIVITY

	H^3-D_2[a] (dpm/unit)	H^3-D_3[a] (dpm/unit)
First recrystallization	440	1055
Second recrystallization	450	1045
Third recrystallization	445	1060

[a] The specific activity was calculated as disintegrations/minute/unit of nonesterified vitamin D. One international unit of vitamin D = 0.025 μg.

TABLE II
SUMMARY OF PREPARATION OF H^3-VITAMINS D_2 AND D_3

Step	Vitamin D_2[a]	Vitamin D_3[a]
1. Amount of provitamin exposed to tritium	0.97 g	3.0 g
2. Specific activity of provitamin after exposure to tritium gas	1,100 dpm/unit	25,000 dpm/unit
3. Purity of provitamin after exposure to tritium gas[b]	80%	66%
4. Purity of provitamin after crystallization[b]	98%	99.9%
5. Specific activity of crystallized provitamin	660 dpm/unit	3,000 dpm/unit
6. Amount of provitamin irradiated	690 mg	1930 mg
7. Amount provitamin recovered	80 mg	132 mg
8. Amount of 3,5-dinitrobenzoate ultimately isolated	50 mg	140 mg
9. Melting point of 3,5-dinitrobenzoate	147–48°	128–29°
10. Specific activity of vitamin D	450 dpm/unit	1,050 dpm/unit
11. Purity of 3,5-dinitrobenzoate[b]	98.6%	98.9%
12. Biological potency in the rat	40 IU/μg	40 IU/μg
13. Biological potency in the chicken	—	40 IU/μg
14. Radiochemical purity	Radiochemically pure in at least 3 chromatographic systems	

[a] One international unit (IU) of vitamin D_2 or D_3 is 0.025 μg. [b] As measured by ultraviolet absorbancy determinations.

128–29° and specific activity 18.9 μc/mg, or 1050 dpm/IU (calculated as free vitamin D_3) was isolated.

To define further the chemical purity of the isolated H^3-vitamins D_2 and D_3, an ultraviolet-absorption curve was determined on a known amount of the material. In both instances the H^3-vitamins D_2 and D_3 and their respective 3,5-dinitrobenzoate esters were found to be at least 99% pure.

Bioassay of H^3-Vitamins D_2 and D_3.—Milligram quantities of H^3-vitamins D_2- or D_3-3,5-dinitrobenzoate were placed in 15 ml of 10% KOH in methanol, A.R., and refluxed under nitrogen for 30 minutes. To this solution was added 20 ml of water. The resulting solution was extracted with 20-ml portions of diethyl ether. The combined ether solutions were washed once with 3.6% HCl and twice with water, dried over anhydrous $CaSO_4$, and diluted to 100 ml to serve as a stock solution. An aliquot of this solution was taken for ultraviolet analysis for the exact concentration of the vitamin present. An aliquot of the remainder was used for biological assay in the rat and in the chick. The analysis was generously carried out by Dr. Henry Scott and Mr. Lloyd Hein of the Wisconsin Alumni Research Foundation, Madison, Wis., according to general USP and AOAC procedures (U. S. Pharmacopeia 1950; Methods of Analysis, 1950). In all instances, the tritium-labeled vitamins D_2 and D_3 exhibited full biological potency in comparison with standard amounts of nonradioactive vitamins D_2 and D_3.

A summary of the preparation of H^3-vitamins D_2 and D_3 is presented in Table II.

Demonstration of Radiochemical Purity.—While attempting to prepare and purify radioactive vitamin D, the lack of suitable chromatographic systems for the isolation, identification, and demonstration of radiochemical purity became apparent. This was particularly evident when the isolation of vitamin D from a mixture resulting from the ultraviolet irradiation of ergosterol or 7-dehydrocholesterol was desired. Only the reversed phase paper chromatography system of Kodicek and Ashby (1954) demonstrated a potential usefulness in the problems at hand.

As a consequence two new methods for the chromatography of vitamin D and the other compounds produced by the ultraviolet irradiation of ergosterol or 7-dehydrocholesterol were developed. Both procedures are described in detail elsewhere (Norman and DeLuca, 1963). One involves thin-layer chromatography and is able to separate at least six compounds from such an ergosterol irradiation mixture, in comparison to the three compounds separated by Kodicek's system (1954). A summary of R_F values of some compounds pertinent to a demonstration of the radiochemical purity of the H^3-vitamins D_2 and D_3 is given in Table III. The other, a silicic acid–column chromatographic procedure, adequately separated mixtures of at least 25 mg of vitamin D and its parent irradiation mixture into at least four components.

A combination of the two new procedures and Kodicek's reversed-phase paper chromatography system (1954) were employed to demonstrate the radiochemical purity of the H^3-vitamins.

Reversed-phase paper strips were prepared by dipping Whatman No. 1 paper in a 15% (by volume) solution of liquid paraffin (Standard Oil Co.) in hexane for 20 minutes. The strips were air dried at room temperature.

Solutions of 10 μl of the H^3-vitamins D_2 and D_3 and standard commercial vitamins D_2 and D_3 (General Biochemicals, Cleveland, Ohio) were spotted on the reversed phase papers. Kodicek's solvent systems were utilized: *n*-propanol-methanol-water, 15:82:3, and 95% methanol in water. After an equilibration period of 1 hour the papers were developed by ascending chromatography until the solvent front had advanced approximately 30 cm.

After chromatography the spots were developed by dipping the paper strips in a 24% solution of $SbCl_3$ in chloroform and heating gently over a Bunsen burner. The radioactivity was located on the chromatogram after the strips had been cut into 0.5-cm seg-

TABLE III
R_F VALUES OF VITAMIN D AND RELATED COMPOUNDS FOLLOWING THIN-LAYER CHROMATOGRAPHY

Compound	R_F[a]
Vitamin D_2-3,5-dinitrobenzoate	0.96
Dihydrotachysterol	0.49
Coprostanol	0.44
Vitamins D_2 and D_3	0.37
Cholesterol	0.31
Δ-7-cholestenol	0.31
Ergosterol and 7-dehydrocholesterol	0.27

[a] The solvent system employed was 10% acetone in *n*-hexane, v/v. All compounds were crystalline and had the correct melting point.

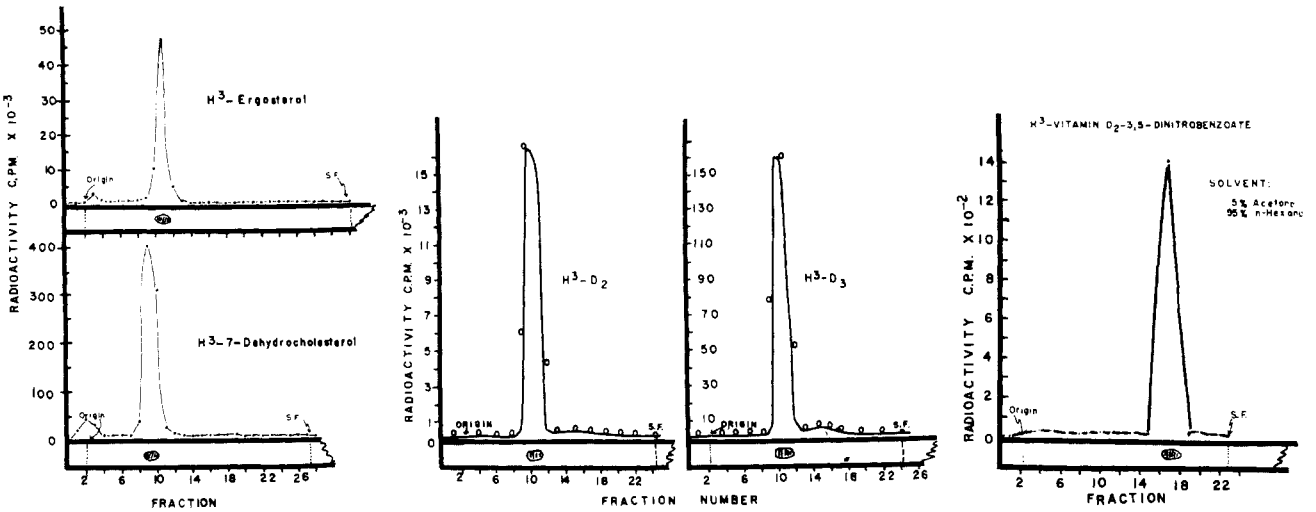

FIG. 1.—Silicic acid thin-layer chromatography of (a) H³-ergosterol and H³-7-dehydrocholesterol, solvent 10% acetone in *n*-hexane; (b) H³-vitamin D_2 and H³-vitamin D_3, solvent 10% acetone in *n*-hexane; and (c) H³-vitamin D_2-3,5-dinitrobenzoate, solvent 5% acetone in *n*-hexane. Approximately 5-10 μg of the compounds was placed at the origin initially. After chromatography the spots were developed with 0.2% $KMnO_4$ in 1% $NaHCO_3$.

ments and placed vertically in a 20-ml counting vial containing counting solution A.

Thin-layer chromatography apparatus and Cab-O-Sil silica gel (Research Specialties Company of Richmond, California) were used to prepare thin-layer silicic acid plates. Approximately 10–20 μg of the compounds in chloroform was applied to the silicic acid on the plates. The chromatograms were developed in a solvent consisting of 100% $CHCl_3$ or 10% v/v acetone in Skellysolve B according to the procedures described elsewhere (Norman and DeLuca, 1963). The radioactivity was located by scraping off successive 0.5-cm segments of the silicic acid with a microscope slide. These scrapings were then placed in a counting vial containing liquid scintillation counting solution A.

Columns, 58 × 1.5 cm, were prepared by slurrying 24.0 g of BioRad silicic acid, minus 325 mesh (California Corporation for Biochemical Research, Los Angeles) in redistilled petroleum ether (predominately *n*-hexane; boiling point 67–70°) as described previously (Norman and DeLuca, 1963). Samples were dissolved in petroleum ether and applied to the column. Elution was carried out with 10% diethyl ether in petroleum ether v/v. Ten-ml fractions were collected. Radioactivity was monitored in 1.0 ml aliquots. The absorbance at 264 mμ was measured in all fractions after the eluent solvent had been removed with a stream of nitrogen and replaced with 95% ethanol.

The H³-vitamins D_2 and D_3 were chromatographed individually and in combination with nonradioactive commercial vitamins D_2 and D_3 in the procedures previously described. In Figure 1 is presented a silicic acid thin-layer chromatogram of H³-ergosterol and H³-7-dehydrocholesterol, H³-vitamins D_2 and D_3, and H³-vitamin D_2-3,5-dinitrobenzoate. Figure 2 presents the reversed-phase paper chromatogram of H³-vitamin-D_3, and Figure 3 presents the chromatogram of H³-vitamin D_3 which was cochromatographed with commercial vitamin D_3 on a silicic acid column.

The H³-provitamins showed only one major peak of radioactivity which coincided exactly with the spot for ergosterol and 7-dehydrocholesterol. No radioactivity moved ahead of ergosterol or 7-dehydrocholesterol in the regions where H³-saturated compounds would migrate, e.g., cholesterol and Δ-7-cholestenol (see Table III). On the basis of these chromatograms, it was

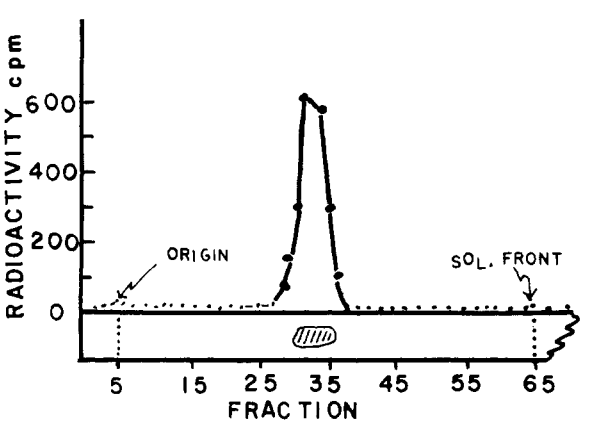

FIG. 2.—Reversed-phase paper chromatography of H³-vitamin D_3. The chromatogram was developed in a solvent of 95% MeOH. The spots were visualized by dipping the strips in a 24% solution of $SbCl_3$ in chloroform and heating gently over a Bunsen burner. Radioactivity was measured on successive 0.5-cm segments of the chromatogram by liquid scintillation counting techniques.

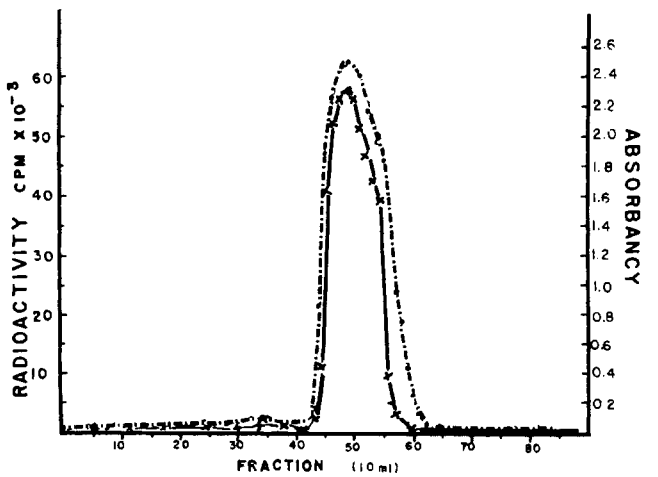

FIG. 3.—Silicic acid column chromatography of H³-vitamin D_3: Approximately 5-10 μg of H³-vitamin D_3 was cochromatographed with 3 mg of nonradioactive commercial vitamin D_3. The column (58 × 1.5 cm), which consisted of 24.0 g silicic acid, was developed with 10% diethyl ether in *n*-hexane v/v. One-ml aliquots of the fractions were taken for liquid scintillation measurement of tritium.

TABLE IV

FECAL EXCRETION OF AN ORAL DOSE OF H^3-VITAMIN D_2

The vitamin D-treated animals received 75 IU of vitamin D_2 three times weekly for 3 weeks before receiving the H^3-vitamin D_2. The vitamin D-deficient animals received no vitamin D prior to the dose of H^3-vitamin D_2. After oral administration of 100 IU of H^3-vitamin D_2, the feces were collected and frozen each day for 3 days. Each group consisted of nine animals, except the vitamin D-treated animals fed diet 24, a group consisting of six animals. The average weight of each group of animals was approximately 150 g. All values are averages ± the standard deviation.

	Vitamin D–treated	Vitamin D–deficient
	% Excretion	% Excretion
Diet 11 (0.45% Ca, 0.3% P)	46.7 ± 5.3[a]	36.5 ± 3.5[a]
Diet 24 (1.2% Ca, 0.1% P)	75.9 ± 13.6[a]	35.1 ± 5.9[a]

[a] The difference between the deficient and vitamin D-treated animals is highly significant, $p < 0.01$.

estimated that the provitamins were at least 90% radiochemically pure, and that the only radioactive contaminant present had an extremely low R_F value in the 10% acetone in *n*-hexane solvent system.

In all three chromatography systems, i.e., the reversed-phase paper, the thin-layer, and the silicic acid–column, the H^3-vitamins D_2 and D_3 showed only one compound which had the same R_F and which moved identically with the appropriate vitamin. Furthermore the same results were obtained when the 3,5-dinitrobenzoate esters of vitamins D_2 and D_3 were chromatographed in the thin-layer system utilizing a solvent of 5% acetone in *n*-hexane, v/v. The small contaminant of radioactivity which had a very low R_F value in the thin-layer chromatography of the provitamins was definitely absent. Finally, 96–100% of the radioactivity was always found to coincide exactly with the spot or peak.

Tissue Distribution. - Male rats of the Sprague Dawley strain were used in all experiments. They were maintained individually in hanging wire cages and given food and water *ad libitum*.

Prior to the determination of the tissue distribution of H^3-vitamin D_3, an experiment was carried out to determine which particular dietary conditions would permit the greatest absorption and retention of the H^3-vitamin D_3. Preliminary reports by Kodicek and Ashby (1959) indicated that rachitic rats were less able to absorb an oral dose of vitamin D than were normal rats.

Two semisynthetic diets were fed: Diet 11, a normal diet (0.4% calcium, 0.30% phosphorus), which does not induce rickets; and diet 24 (1.2% calcium, 0.1% phosphorus), which is rachitogenic. These diets were similar to those described by Steenbock and Herting (1955) and Guroff *et al.* (1963), except that vitamin-free casein plus 0.2% L-cystine replaced egg white as the protein source. Both diets and their nutritional effects on the rat are described in detail elsewhere (Steenbock and Herting, 1955; Guroff *et al.*, 1963). The animals fed each diet were divided into two groups. One group received 75 IU of nonradioactive vitamin D_2 per rat three times weekly for 3 weeks prior to the initiation of the experiment. The other group received no vitamin D.

On the first day of experiment, all animals received 100 IU of H^3-vitamin D_2 orally in 0.10 ml cottonseed oil (Wesson Oil). The animals were placed in individual metabolism cages, and the feces were collected and frozen each day for a 3-day period. Preliminary experiments indicated that after 3 days no significant amounts of radioactivity were excreted.

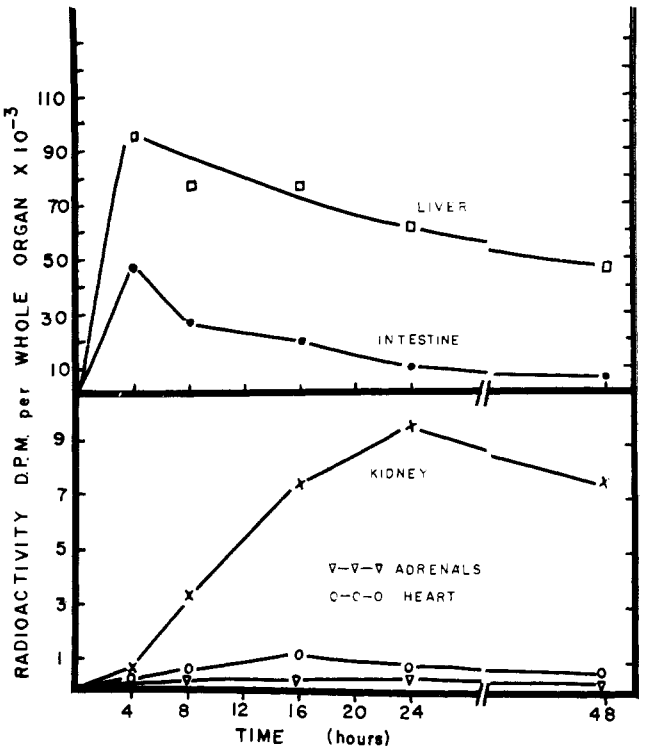

FIG. 4.—Distribution of radioactivity as a function of time after an oral dose of 500 IU of H^3-vitamin D_3: Tissue samples were collected and frozen for tritium analysis at 4, 8, 16, 24, and 48 hours after oral administration of 500 IU of H^3-vitamin D_3 (0.24 μc). Each point represents the average of six rats.

The radioactivity present in the feces samples was extracted with successive aliquots of Skellysolve B, 80% ethanol, and 50% ethanol while grinding with a mortar and pestle. The tritium present in the Skellysolve B was measured in counting solution A, and the ethanol extracts were measured in the counting solution of Bray (1960). The results in Table IV indicate that vitamin D–deficient rats, fed either the rachitogenic diet 24 or the normal diet 11, are able to absorb significantly greater amount of H^3-vitamin D than animals which had been pretreated for 3 weeks with nonradioactive vitamin D. From the point of view of radioactive vitamin D absorbed, vitamin D–deficient rats fed either diet would be equally suitable for a study of the distribution of H^3-vitamin D in the various organs. To avoid secondary complications due to calciumphosphate imbalance and rickets, the nonrachitogenic diet 11 was selected for use in all other experiments described in this paper.

For the distribution experiments, animals were fed diet 11 without vitamin D for 3 weeks. The H^3-vitamin D_3 (500 IU, 0.23 μc) was then administered orally in 0.10 ml cottonseed oil (Wesson Oil). After sacrificing the animals by decapitation, the tissues chosen for examination were washed free of adhering blood with 0.25 M sucrose, frozen, and stored at −25° until used for analysis. The whole small intestine prior to freezing was divided into three sections and the contents were thoroughly flushed out with Krebs bicarbonate buffer. The blood was mixed with a small amount of heparin before freezing. The femur, tibia, and fibula were cleaned of adhering tissue and split open so that the marrow could be removed before freezing.

TABLE V

LOCATION OF TRITIUM IN THE SMALL INTESTINE AFTER ORAL ADMINISTRATION OF H³-VITAMIN D_3

Five hundred IU of H³-vitamin D_3 was administered orally to rats fed the nonrachitogenic diet 11. At the time of sacrifice, the small intestine was divided into three equal-length segments. These were thoroughly washed with Krebs bicarbonate buffer to remove the intestinal contents. All numbers are the average ± the standard deviation. Each group consists of six animals.

Hours after Oral Dose	Duodenum		Jejunum		Ileum	
	% Dose	Relative Incorporation[a]	% Dose	Relative Incorporation[a]	% Dose	Relative Incorporation[a]
4	0.6 ± 0.2	1790 ± 670	1.9 ± 1.2	6590 ± 4600	8.4 ± 1.0	21,300 ± 6590
8	0.9 ± 0.4	2770 ± 770	1.5 ± 1.2	5030 ± 1290	3.6 ± 1.2	10,180 ± 2800
16	0.6 ± 0.1	1480 ± 320	1.4 ± 1.4	3980 ± 1630	1.7 ± 1.4	5,140 ± 700
24	0.5 ± 0.1	1190 ± 90	0.8 ± 0.2	2450 ± 570	1.3 ± 0.2	3,900 ± 740
48	0.4 ± 0.2	960 ± 360	0.5 ± 0.2	1400 ± 260	0.8 ± 0.2	2,730 ± 680

[a] Relative incorporation expressed as disintegrations/minute/100 mg dry wt of tissue.

The following whole organs were analyzed for radioactivity: intestine, kidney, brain, lung, adrenal, femur, tibia-fibula, epididymal fat pad, and heart. The kidneys were analyzed individually. A 30% water homogenate of the liver was made and analyzed in duplicate. Duplicate 0.5-ml blood samples from each rat were analyzed. Random samples of skin and the gluteus muscle were also analyzed for radioactivity. The analyses of duplicate samples were always within 5% of each other.

A time course study was made of the appearance of the radioactivity in several of the organs of interest. The results are presented in Figure 4 and Table V. Quite naturally the intestine contained the most radioactivity shortly after dosage. Concomitantly with the fall of radioactivity in the intestine, the radioactivity in the other organs rose gradually to a plateau at 16-24 hours and then fell slightly at 48 hours.

Table V demonstrates the relationship between the radioactivity in the three segments of the small intestine, the duodenum, jejunum, and ileum, and the time after dosage. It is apparent that the ileum contains the most radioactivity during time intervals when the H³-vitamin D would be absorbed. This is in agreement with the evidence presented by Kodicek (1960).

A summary of the distribution of radioactivity in the various organs of rats 24 hours after an oral dose of H³-vitamin D_3 is presented in Table VI. It is of interest that well over 90% of the administered dose of the vitamin was accounted for.

In view of the rapid accumulation at 4 hours by the liver of an orally administered dose of H³-vitamin D, short time studies were made of the tissue location of an intracardially administered dose of the radioactive vitamin. This should provide further evidence as to the involvement of the liver in the physiological action of vitamin D. Also these studies were made to examine Raoul's postulate that the adrenals are involved in the metabolism of vitamin D. Raoul found by an elaborate extraction procedure a difference in the ultraviolet absorbancy of the adrenal extracts between control animals and animals that had received a dose of 2000 IU of vitamin D_3 shortly before sacrifice (Raoul and Gounelle, 1958). Raoul claimed this as evidence for the involvement of the adrenals in vitamin D metabolism. In contrast, Kodicek *et al.* (1959) found that after 20 minutes the adrenals contained only 1% of the dose and the liver contained 50% of the dose of 2000 IU of intracardially injected C¹⁴-vitamin D_2.

Five hundred IU of H³-vitamin D_3 (0.23 μc) was suspended in 0.20 ml of serum from vitamin D–deficient rats. This sample was injected intracardially into vitamin D–deficient rats (fed the nonrachitogenic diet 11) under light ether anesthesia. The animals were sacrificed at 20, 40, and 60 minutes and at 8 and 24 hours. The adrenals, liver, kidney, and the small intestine were analyzed as described previously. Table VII presents a summary of the data obtained.

DISCUSSION

This report describes for the first time a detailed and inexpensive method of preparation of radioactive vitamin D that is of high enough specific activity for use in studies of the metabolism of physiological doses of the vitamin. Although the use of the Wilzbach method for labeling the vitamin D precursor may produce highly labeled nonbiologically active compounds, the subsequent irradiation and isolation of the crystalline vitamin D-3,5-dinitrobenzoate is essentially a self-

TABLE VI

DISTRIBUTION OF RADIOACTIVITY AFTER AN ORAL DOSE OF H³-VITAMIN D_3

Five hundred IU of H³-vitamin D_3 was administered to vitamin D–deficient animals fed the nonrachitogenic diet 11 for 3 weeks. Tissues were removed and frozen for analysis 24 hours after administration of the dose. All figures are the average of six rats ± the standard deviation.

Tissue	% of Administered Dose	Relative Incorporation (dpm/100 mg dry wt of tissue)
Kidneys	5.2 ± 0.9	9,900 ± 400
Adrenals	0.14 ± 0.03	4,450 ± 600
Heart	0.18 ± 0.02	840 ± 30
Liver	13.3 ± 2.3	2,910 ± 280
Blood[a]	3.6 ± 0.1	—
Brain	0.13 ± 0.03	390 ± 80
Lung	0.50 ± 0.10	760 ± 130
Intestine (small)	2.6 ± 0.2	2,400 ± 220
Epididymal fat pad	0.81 ± 0.20	1,450 ± 220
Muscle[b]	14.1	540 ± 270
Femur	0.17 ± 0.04	580 ± 170
Skeleton[c]	17.1	—
Feces	22.2 ± 4.0	—
Intestinal contents	13.3	—
Urine	1.1 ± 0.5	—
	94.4%	

[a] This assumes blood is 6% of body weight (0.50 ml of blood was analyzed in duplicate). [b] This assumes muscle is 30% of body weight (approx. 700 mg muscle was analyzed in triplicate). [c] This assumes skeleton is 15% of body weight (extrapolated on basis of amount of radioactivity in femur and tibia-fibula).

TABLE VII

LOCATION OF RADIOACTIVITY AFTER AN INTRACARDIAL DOSE OF H^3-VITAMIN D_3

Rats were fed the nonrachitogenic diet 11 for 3 weeks without receiving vitamin D. Five hundred IU of H^3-vitamin D_3 was administered intracardially in 0.20 ml of serum obtained from vitamin D–deficient rats. The values represent an average of four to six rats ± the standard deviation. The values for 20, 40, and 60 minutes are averages for six rats; those for 8 hours and 24 hours are averages of four rats.

Time after Dose	Adrenals		Kidneys		Liver	
	% Dose	Relative Incorporation[a]	% Dose	Relative Incorporation[a]	% Dose	Relative Incorporation[a]
20 minutes	0.11 ± 0.05	11,000 ± 4860	0.9 ± 0.4	2,070 ± 840	44.4 ± 26.8	12,300 ± 7290
40 minutes	0.05 ± 0.02	4,700 ± 1890	0.6 ± 0.3	1,270 ± 700	62.7 ± 11.9	16,900 ± 3880
60 minutes	0.11 ± 0.07	11,300 ± 5000	1.1 ± 0.8	2,000 ± 1600	61.0 ± 15.5	18,300 ± 5510
8 hours	0.20 ± 0.12	5,100 ± 3400	5.8 ± 1.5	9,760 ± 2800	9.1 ± 0.8	1,890 ± 350
24 hours	0.26 ± 0.06	4,750 ± 1820	7.4 ± 1.0	10,900 ± 540	7.4 ± 1.8	1,380 ± 103

Time after Dose	Small Intestine					
	Duodenum		Jejunum		Ileum	
	% Dose	Relative Incorporation[a]	% Dose	Relative Incorporation[a]	% Dose	Relative Incorporation[a]
8 hours	0.5 ± 0.2	1,570 ± 400	0.4 ± 0.1	1,890 ± 130	0.4 ± 0.1	2,100 ± 170
24 hours	0.5 ± 0.1	1,560 ± 40	0.5 ± 0.1	1,690 ± 150	0.5 ± 0.2	1,700 ± 60

[a] Relative incorporation expressed as disintegrations/minute/100 mg dry wt of tissue.

purification process. Only sterols with the 5–6, 7–8 double bond will be activated by ultraviolet light. Thus the irradiation and isolation discriminate strongly against the carry-through of any saturated, highly radioactive compounds. Also it should be noted that recrystallization of the provitamin before irradiation reduced the specific activity from 20 μc/mg and 460 μc/mg to 11.9 μc/mg and 54 μc/mg for ergosterol and 7-dehydrocholesterol, respectively. When these recrystallized provitamins were subjected to thin-layer chromatography (Fig. 1a) at least 90% of the radioactivity was present in the regions associated with ergosterol and 7-dehydrocholesterol. No evidence for the presence of highly radioactive cholesterol or Δ-7-cholestenol could be found. These compounds might be expected to be present if the tritium added across double bonds rather than exchanging with the hydrogens of the provitamins during the Wilzbach procedure. It is noteworthy that the specific activity of the vitamin D ultimately isolated was less than that of the provitamins which were irradiated, especially in view of the fact that the crystalline provitamins were better than 90% radiochemically pure in the chromatographic systems employed. A possible explanation lies in the fact that during the irradiation process a shift of the hydrogens in the B ring occurs. Thus a loss of further labile hydrogen and tritium atoms may occur.

The actual isolation of the H^3-vitamin D from the mixtures of compounds resulting from the ultraviolet irradiation of the provitamin is a modification of the procedures of Windaus *et al.* (1936) and Havinga and Botts (1954). Maleic anhydride was used to remove the tachysterol since the reaction time required is only 30 minutes rather than the 6 days necessary for the citraconic anhydride method of Windaus *et al.* (1936). Furthermore, preliminary experiments revealed that in 3 days a significant amount of the vitamin D in an irradiation mixture combined with citraconic anhydride.

That the vitamin D ultimately isolated contained virtually all the radioactivity was established by (1) reversed-phase paper chromatography of the H^3-vitamins D_2 and D_3, (2) thin-layer chromatography of H^3-vitamins D_2- and D_3-3,5-dinitrobenzoates, (3) thin-layer chromatography of the H^3-vitamins D_2 and D_3 in two solvent systems, and (4) column chromatography of the carrier-free H^3-vitamins D_2 and D_3 and cochromatography with commercial crystalline vitamin D. In all instances virtually all the radioactivity was associated with vitamin D. Additional data were obtained on the (1) purity of the 3,5-dinitrobenzoates as determined by ultraviolet absorbancy measurements, (2) recrystallization of the 3,5-dinitrobenzoates to a constant specific activity and melting point, and (3) the full biological activity (40,000 IU/mg) of the radioactive vitamin D preparations.

Several alternative methods for the preparation of radioactive vitamin D were attempted before the present procedures were adopted. The synthesis of vitamin D_4, 22–23 dihydrovitamin D_2, was considered initially to be the most fruitful method. The ergosterol–acetate–maleic anhydride adduct was prepared according to Imhoffen (1934) and then the 22–23 position was catalytically reduced with hydrogen and/or tritium with a palladium black catalyst. However, it was not possible to break the adduct as Imhoffen claimed (1934) so that reasonable amounts of the 22–23 dihydroergosterol could be obtained for irradiation and isolation of vitamin D_4. Other workers have encountered this problem also.[4]

The preparation of radioactive 7-dehydrocholesterol via the *N*-bromosuccinimide reaction with cholesterol (Kulkarni *et al.*, 1963), which was derived from radioactive cholesterol dibromide by dehalogenation with zinc dust, also failed because of poor yields. Other ways of preparing the radioactive provitamins involving biosynthetic means have already been explored by others (Kodicek, 1955; Schaltegger, 1960), but the specific activity achieved by this method makes the detection of physiological concentrations of vitamin D very difficult.

There are many possible uses of the tritium-labeled vitamin D that are already apparent. First, of paramount importance is the question of the active form of vitamin D. As pointed out earlier, there is a lag between the time of administration of vitamin D and its first observable physiological effects. This has led to the suggestion by Sallis and Holdsworth (1962b) and by Raoul and Gounelle (1958) that vitamin D is converted to an active form by the adrenals. Our data have failed to show a marked accumulation of radioactivity in the adrenals following either an oral or an intracardial dose of the vitamin. These agree with the observations of Kodicek *et al.* (1959). However,

this does not rule out a possible participation of the adrenals in the metabolic conversion of vitamin D. More plausible explanations of the lag in vitamin D action include: (1) slow conversion to a metabolically active form, (2) participation in some unknown induction process, and (3) the necessity for the accumulation of a minimal amount of vitamin D at the active sites for the initiation of an observable physiologic effect. All of these possibilities could be consistent with the present data.

It is of great interest to note that the liver accumulates 50–60% of the radioactivity after intracardial injection of H^3-vitamin D. These data, which agree with the preliminary report of Kodicek *et al.* (1959), also correlate nicely with the results shown in Figure 4 obtained after oral administration. In both instances, the liver accumulates the radioactivity more rapidly than do other organs. The radioactivity (per 100 mg dry wt) of the liver fell progressively from 5600 dpm/100 mg dry wt at 4 hours to 220 dpm/100 mg dry wt at 48 hours after an oral dose. Thus it would appear that this organ is involved in the early sequence of events in the physiological action of vitamin D. Whether this involvement is due to a "storage and release action" or to a metabolic conversion of vitamin D remains to be determined.

It appears that there is no impairment of vitamin D absorption in a vitamin D–deficient animal as was once supposed (Kodicek and Ashby, 1959). The vitamin D–deficient animals fed either a normal or a rachitogenic diet absorbed a larger proportion of the dose than the corresponding vitamin D–fed animals. If there is no impairment in absorption, then it might be expected that the deficient animals would have a greater capacity for utilization of the vitamin. The high excretion and extreme variability of the amount of excretion of radioactivity by animals fed diet 24 and vitamin D is most likely a reflection of the physiologic state of these animals. Vitamin D has long been known to inhibit growth of rats on such a high Ca diet (Steenbock and Herting, 1955). The development of urinary calculi in these animals is also a complicating feature (Schneider and Steenbock, 1940). It is difficult to rationalize the apparent disagreement with the work of Kodicek. However, his experiment involved extremely large doses (40,000 IU/rat) and crude corn rachitogenic diets which are known to be deficient in other important nutrients. The data obtained from H^3-vitamin D is in complete agreement with that of Blumberg *et al.* (1960) who found that rachitic animals excreted 55% and normal animals 75% of a dose of 15,000 IU of C^{14}-vitamin D_2.

The particularly high concentration of an oral dose of H^3-vitamin D_3 in the ileum at short time intervals may arise from absorption of vitamin D by that segment of intestine. If the vitamin were absorbed equally well in all the intestinal sections, one might expect equal relative incorporation/100 mg dry wt in all the sections. It is of interest that Benson *et al.* (1956) and Kremen *et al.* (1954) have shown that neutral fats and lipids are, for the most part, absorbed from the ileum. It is therefore reasonable that this would be the region of the intestine involved in vitamin D absorption. In this regard, it is of interest that Schachter *et al.* (1961) concluded from experiments with everted sacs of intestine that the duodenum actively transports calcium more readily than does the jejunum or ileum. Harrison and Harrison (1960) concluded that vitamin D promoted the diffusion of calcium along the entire length of the small intestine, and that any effect of vitamin D on the active transport of calcium was localized in the proximal portion of the intestine. Furthermore, we have shown that when H^3-vitamin D_3 was administered intracardially, all sections of the intestine (Table VI) accumulated H^3 radioactivity in equal amounts. This indicates that the high concentration of H^3-vitamin D_3 in the ileum following an oral dose is more likely a reflection of an "absorption" process.

Twenty-four hours after administration of a 500-IU dose was chosen as the most favorable time for a complete study of the distribution of H^3-vitamin D_3. By this time there was a readily measurable amount of radioactivity in all the tissues examined and the amount present had presumably reached a steady-state level (see Fig. 3). All the tissues normally associated with the action of vitamin D, i.e., the kidneys, liver, skeleton, and intestine, had accumulated a significant amount of the administered dose which was retained in the animal (Table VI). This is in general agreement with the data of Kodicek (Kodicek, 1956; Kodicek and Ashby, 1959; Kodicek *et al.*, 1959) obtained with a massive dose (40,000 IU/rat) and the work of Blumberg *et al.* (1960) who obtained distribution data from *Macacus rhesus* and rats given large doses of vitamin D (15,000 IU/100 g). The blood has a significant amount of the radioactivity. Whether this is a "storage form" of the vitamin, vitamin D in transit, or a breakdown product cannot be decided at this time. In addition radioactivity has been found in low levels in the lung, brain, heart, adrenals, adipose tissue, muscle, and skin. The physiological significance of this generalized distribution of radioactivity is as yet unknown. A small portion of radioactivity was excreted in the urine. Attempts to trap H^3-water from lyophilization of frozen urine failed, thus indicating that the tritium present in that fluid was organically bound.

Some of the data concerning the organ distribution of radioactivity have been calculated as relative incorporation per 100 mg dry wt (Tables V, VI, VII). This indicates the organs in which the radioactivity is present in highest concentration. As shown in Table VII, there is a general correlation between the relative incorporation/100 mg dry wt in the kidney, intestine, liver, and bone and their known involvement in the physiological action of vitamin D. The value for the femur is an extreme lower limit because of the large mass of inert bone present. The "high" value for the adrenals is more a reflection of their extremely low dry weight (6–8 mg) than of the presence of large amounts of radioactivity. The adrenals routinely contained only 20–40 cpm above background, which makes the apparent "high" relative incorporation per 100 mg dry wt highly questionable.

To define more clearly the role of the liver in the physiological action of vitamin D and to understand the significance of the vitamin's occurrence in the kidneys, intestine, bones, and the like, it is important to determine the subcellular location and chemical form of the radioactivity present in these tissues. Chromatography, coupled with biological assay, should provide fruitful knowledge as to whether vitamin D is converted to some metabolically active form. A more factual evaluation may then be made of the role of vitamin D in the transport of calcium and in other physiologic processes.

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Restoration of Photoreductase Activities in Acetone-extracted Chloroplasts by Plastoquinones and Tocopheryl-quinones

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Identification of a new member of the plastoquinone group in spinach chloroplasts brings the total number of quinones in these chloroplasts to eight. These include plastoquinones A, B, C, and D; vitamin K_1; and α-, β-, and γ-tocopheryl-quinones. Evidence is presented that each of these quinones function with a different pattern of specificity for restoration of photoreductase or for stimulation of photooxidase activities in acetone-extracted spinach chloroplasts.

As a result of studies of the lipids of spinach chloroplasts we have been able to identify eight quinones. These include plastoquinones A, B, C, and D; vitamin K_1; and α-, β-, and γ-tocopheryl-quinone. The presence of several related quinones in spinach chlorplasts raises a question as to their significance in electron transport function. It has previously been shown by Bishop (1959) and Krogmann and Olivero (1962) that plastoquinone A will restore ferricyanide reduction and photosynthetic phosphorylation in heptane-extracted chloroplasts. In this paper we should like to present evidence that the several plastoquinones and tocopheryl-quinones function at different sites in the electron transport system in chloroplasts. The plastoquinones have the same absorption maximum at 255 mμ, which indicates the same quinone chromophore. These can be separated by chromatographic procedures and they show different activities in restoration of electron transport functions in extracted chloroplasts. The isolation of plastoquinones A, B, and C (PQA, B, and C)[1] has been described previously (Kegel *et al.*, 1962). Plastoquinone D (PQD) is found in close association with plastoquinone C. The mixture of plastoquinones C and D is stable for several weeks in ethanol, but when the two quinones are separated they both become quite unstable under all conditions studied. Plastoquinone D is especially difficult to preserve for any length of time, and on standing either as an oil or in ethanol solution it is converted to another compound with a broad absorption maximum at 253 mμ. This degradation product of PQD still shows a decrease in absorbancy upon reduction with potassium borohydride, but there is no isosbestic point at shorter wavelengths which is characteristic of typical quinone spectra during reduction.

α-Tocopheryl-quinone (αTQ) is present in amounts comparable to PQC and PQD in spinach chloroplasts, whereas β- and γ-tocopheryl-quinones (β- and γTQ) are present in almost trace amounts (Henninger *et al.*, 1963). Again, each of these quinones shows different effects in electron transport function.

Vitamin K_1 has been recognized as a component of chloroplasts since the work of Dam (1942). We have recently isolated a purified sample of vitamin K_1 from spinach chloroplasts (Kegel and Crane, 1963). The only effects of vitamin K_1 on electron transport which we have observed are inhibitions.

The properties of the various plastoquinones and tocopheryl-quinones from chloroplasts are shown in Table I. The samples of PQC and PQD are the best

[1] Abbreviations used in this paper: PQ (combined with A, B, C, as PQA), plastoquinone; αTQ, α-tocopheryl-quinone; DCIP, 2,6-dichloroindophenol; PPNR, photosynthetic pyridine nucleotide reductase.