Preparation of Tritio-Cholecalciferol

(Tritiated Vitamin D_3)

By C. T. PENG

Cholecalciferol was tritiated by exposure to tritium gas at −198° (liquid nitrogen). Pure tritiated vitamin D_3 with a specific activity of 216 μc./mg. was obtained following equilibration with solvents and purification by successive recrystallization from acetone at −80° and column chromatography on Florisil until a constant specific activity was achieved. The formation of radiation decomposition products was minimal because of using low temperature during tritiation. A possible explanation for this low temperature effect is given.

BIOLOGICALLY important compounds have frequently been randomly labeled with tritium by the Wilzbach gas exposure method (1). This method, however, has not been widely used with unsaturated compounds because tritium atoms add across the —C=C— bond giving rise to saturated products of extremely high specific activity (2) which are difficult to remove.

The addition reaction of tritium was first observed by Jones, *et al.* (2), with methyl oleate which, on tritiation, yielded methyl tritio-stearate as the only radioactive product. However, in other unsaturated compounds such as cholesterol, isobutylene (3), 1-hexene, 1,5-hexadiene (4), methyl linolenate (5), etc., substitution as well as addition of tritium occurred during the labeling process. While the mechanism of tritiation by Wilzbach method has been studied in homogeneous systems of methane-tritium (6), ethylene-tritium (7), and propane-tritium (8) and found to involve decay- and β-labeling, the mechanism of tritiation in heterogeneous systems remains obscure (9). Moreover, no systematic investigation has been made of the mechanism of hydrogenation of the —C=C— bond by exposure to tritium gas and the effect of changing the reaction parameters, *viz.*, tritium pressure, temperature coefficient, etc.

In this laboratory, tritio-cholecalciferol was prepared by exposure to tritium gas at −198°. This low temperature, as discussed later, minimizes molecular decomposition by radiation because of an enhancement of the "cage" effect and reduces the formation of radioactive impurities. Purification of the tritiated vitamin D_3 was effected by extraction, column chromatography, and recrystallization.

Received December 19, 1962, from the Radioactivity Research Center, School of Pharmacy, University of California Medical Center, San Francisco.

Accepted for publication February 26, 1963.

This investigation was supported in part by a grant-in-aid from the U. S. Public Health Service (C-3630), Cancer Research Funds of the University of California, and research funds of the Academic Senate of the University of California, San Francisco.

MATERIALS

All solvents used were reagent grade and were redistilled. Peroxide in ethyl ether was removed by passage through a column of basic alumina. Silica gel G, prepared for thin-layer chromatography according to Stahl (10), was obtained from E. Merck Co. Florisil, activated at 260°, was purchased from Floridin Co., Tallahassee, Fla.

EXPERIMENTAL

All manipulations involving tritiated vitamin D were carried out in an atmosphere of nitrogen and in low actinic glassware, or in glassware protected from light by wrapping in aluminum foil to minimize oxidation and photodecomposition.

Tritiation.—Approximately 500 mg. of vitamin D_3 (Mann Research Laboratories, New York, N. Y.) was exposed to 50 curies of tritium gas at a pressure of 130 mm. Hg at −198° (liquid nitrogen) for 39 days. The vitamin sample was introduced into the tritiation chamber shown in Fig. 1 through the open end *A* which was subsequently sealed. The sample-filled chamber was then attached at *T* to the pumping arm of a Toepler pump in a high vacuum line and evacuated to a pressure of 10^{-6} mm. Hg (11). Tritium gas, obtained from the Oak Ridge National Laboratory, was admitted without further purification into the tritiation chamber. After filling with the gas, the chamber was detached from the vacuum line by sealing off the capillary constriction at *D*.

The sealed tritiation chamber containing vitamin D_3 and tritium gas was immersed in liquid nitrogen (−198°) in a dewar flask and kept there for the duration of the exposure time.

To terminate the labeling process, the tritium gas, together with any radioactive gaseous products formed by radiation decomposition during exposure, was removed by breaking seal *B* of the tritiation chamber in the high vacuum line system.

Purification.—Purification of the tritiated vitamin D_3 consists of removing labile tritium activity and then eliminating radioactive impurities. Complete recovery at each step of purification was not attempted because it was more important that trace impurities of high specific activity be completely separated from the parent compound rather than high yields be obtained.

The unpurified tritiated vitamin D_3 was repeatedly dissolved in dry acetone and evaporated to dryness to remove labile tritium activities. The

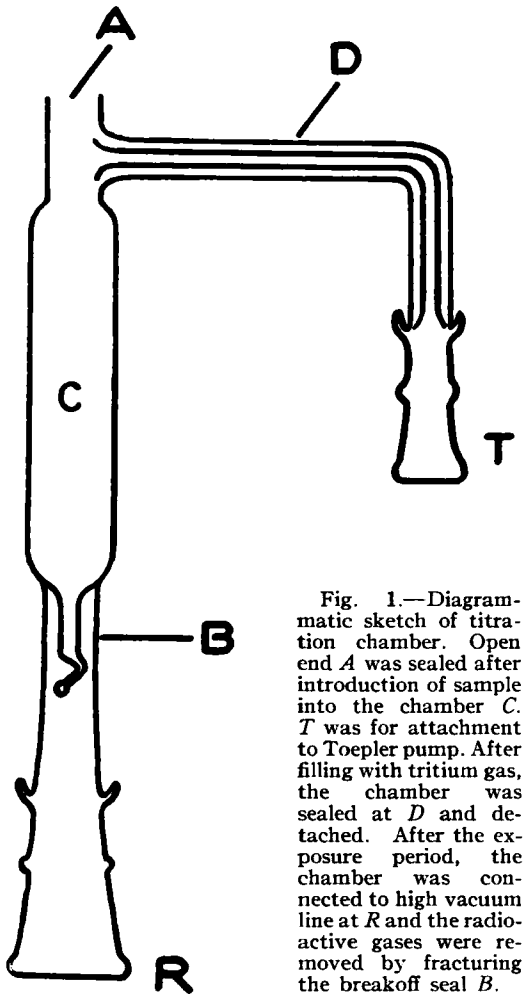

Fig. 1.—Diagrammatic sketch of titration chamber. Open end *A* was sealed after introduction of sample into the chamber *C*. *T* was for attachment to Toepler pump. After filling with tritium gas, the chamber was sealed at *D* and detached. After the exposure period, the chamber was connected to high vacuum line at *R* and the radioactive gases were removed by fracturing the breakoff seal *B*.

residue was recrystallized[1] from dry acetone at −80° to eliminate acetone soluble radioactive impurities. The recrystallized tritio-cholecalciferol (approximately 100 mg.) was dissolved in 150 ml. of peroxide-free ethyl ether and the ether solution was washed successively with three 50-ml. portions of ice-cold water to remove any hydroxylic protium exchangeable activities. The solution was dried with anhydrous sodium sulfate, filtered, and taken to dryness at room temperature in a rotatory evaporator. Whenever the residue was not completely clear, it was azeotropically dried by repeated evaporation with 20 ml. of a mixture of equal volumes of benzene and absolute ethanol. The clear residue was then taken up in 50 ml. of *n*-hexane for purification by column chromatography.

Approximately 10 to 15 mg. of the partially purified tritio-cholecalciferol in *n*-hexane was applied to a chromatographic column, 1.2 cm. in diameter and 15 cm. in height, prepared by pouring a slurry of 12 Gm. of 60 to 80-mesh Florisil in *n*-hexane into the chromatographic tube containing the same solvent. The column was kept at 15° by a water jacket to prevent solvent evaporation and thus eliminate formation of bubbles along the adsorbent bed. After loading, the column was rinsed with 100 ml. of *n*-hexane followed by elution with 400 ml. of 10% ethyl ether in *n*-hexane. The flow rate of the eluent was adjusted to 2 to 3 ml. per minute with a Teflon plugged stopcock at the lower end of the column. The eluent was collected in 10-ml. fractions. Concentration of tritio-cholecalciferol in the eluent was measured with Nield's reagent (12) against a known standard.

Purity of the tritiated cholecalciferol was ascertained by thin-layer chromatography on 250 μ-thick silica gel plates using a mixture of chloroform: methanol:water (10:50:10 by volume) as solvent. Color development was effected by spraying the plates with 20% antimony pentachloride in chloroform (13). Appearance of a pink color changing to dark brown indicated the presence of vitamin D_3.

The processes of recrystallization from acetone at −80° and column chromatography on Florisil were repeated until a constant specific activity was achieved.

Radioactivity Measurement.—Radioactivity of all samples was measured in a liquid scintillation spectrometer using 10 ml. of 0.3% diphenyloxazole in toluene as scintillator as previously reported (14). For samples in aqueous solution a scintillator consisted of 5 ml. of the above solution plus 5 ml. of a dioxane solution containing 7 Gm. diphenyloxazole, 50 mg. POPOP (2,2′-*p*-phenylenebis (5-phenyloxazole)), and 50 Gm. of naphthalene to 1 L. of purified dioxane.

The amount of the label in thin-layer chromatograms of tritio-cholecalciferol was determined by transferring the silica gel in narrow sections from the chromatogram into counting vials and assaying the radioactivity by liquid scintillation counting.

Absorption Spectrum.—The infrared spectra of unpurified tritio-cholecalciferol, nonradioactive vitamin D_3, and an unidentified decomposition product were obtained with a Beckman IR-5 infrared spectrophotometer using the KBr disk method. The ultraviolet spectra of these compounds were deter-

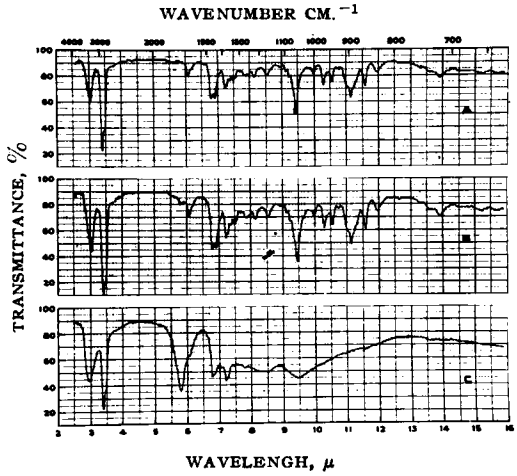

Fig. 2.—Infrared absorption spectra of cholecalciferol (*A*), unpurified tritio-cholecalciferol (*B*), and an unidentified decomposition product (*C*).

[1] The presence of moisture occasionally caused vitamin D_3 to separate as a viscous oil at this temperature in which case the supernate was removed by aspiration.

mined with a Cary model 11 recording spectrophotometer, using an ethanolic solution of 2 mg.% concentration.[2]

RESULTS AND DISCUSSION

The average β energy of tritium is 5.5 kev with a half-thickness value of 0.031 mg./cm.[2] in hydrogen (15) and a maximum range of 6 μ in water (16). At the prevailing pressure in the tritiation chamber, complete dissipation of the decay energy of tritium within the container is certain. The radiation dose to the cholecalciferol crystals, which occupied only a fraction of the total volume of the chamber, was estimated to be a minimum of approximately 2.4×10^7 rads. Apparently at $-198°$, this dose level did not cause excessive radiation damage as indicated by the identity of the infrared spectrum of the unpurified tritio-cholecalciferol (*B*) and that of an authentic nonradioactive standard (*A*) (Fig. 2). It should be noted, however, that the former contains a small absorption band at 5.8 μ, characteristic of carbonyl groups.

The purity of the tritio-cholecalciferol was ascertained by thin-layer chromatography on silica gel. The R_f value and the contour of the vitamin D_3 spot after solvent irrigation varied not only with the quantity of vitamin D_3 but also with the polarity of the solvent used for spotting. For instance, the spot obtained from a solution of vitamin D_3 in *n*-hexane or in petroleum ether (b.p. 40–60°) was more compact in shape and faster in movement as shown by an increased R_f value than that obtained from a solution in acetone, probably because of differences in ease of evaporation of the solvents and in the intrinsic solubility of vitamin D_3 in them. The R_f value of vitamin D_3 also varied inversely with the quantity of material being chromatographed on the thin-layer silica gel plate in the region of 5 to 40 mcg. regardless of the spotting solvent. In this concentration range, the vitamin D_3 spot tended to migrate nearer to the solvent front. At high concentrations ranging from 60 to 200 mcg., migration of the spot remained practically constant (R_f value: 0.651 ± 0.017 (S.D.) for hexane; 0.791 ± 0.012 (S.D.) for acetone). For routine assay, this uncertainty of the R_f value may be overcome by chromatographing spots of graded material concentration or by comparison with spots co-chromatographed with a known standard.

Distribution of tritium label in the unpurified tritio-cholecalciferol was studied on a 10-mg. sample. The results are shown in Table I. The labile tritium activity exchangeable with protium atoms on equilibration with acetone, water, and ether amounted to 73% of the initial activity. Purification by recrystallization from acetone at $-80°$ which was carried out with the bulk tritiated vitamin D_3 was neglected in this case because it would entail an excessive loss of material. Recovery of radioactivity from column chromatography on Florisil was about 71% although material recovery using a nonradioactive standard was 92%. Apparently the adsorbent at the top of the chromatographic column retained some radiation decomposition products as a yellowish band; this band was intensely radioactive and could not be eluted by acetone.

Radioactivity eluted by acetone represented approximately 53% of the label initially applied to the column. This acetone eluable fraction was found by means of thin-layer chromatography to coincide with a fast running spot which frequently appeared after long storage of known solutions of nonradioactive vitamin D_3 and which had a R_f value of approximately 1. The infrared spectrum of this compound was shown as *C* in Fig. 2. Loss of fine structure in the region of 7 to 14 μ and the presence of carbonyl stretching band at 5.8 μ, together with the minimal absorption in ultraviolet region of an ethanolic solution (2 mg.%) serve to indicate the absence of the tri-ene conjugation in the molecule. However, no attempt was made to identify this compound.

Tritio-cholecalciferol was eluted with 10% ether in *n*-hexane. The specific activity of the eluent fractions along the ascending and descending limbs of the band was found to be practically identical within experimental error, thus indicating the absence of radioactive congeners of high specific activity as possible contaminants. However, to insure a high degree of radiopurity, only the peak fractions of the tritio-cholecalciferol band were pooled for further purification. In this manner, by successive purification processes, including one recrystallization from acetone at $-80°$ and three chromatographic passages on Florisil column, a preparation of pure tritiated vitamin D_3 with a specific activity of 216 μc./mg. was obtained. The adequacy of this compound as a tracer was verified by biological studies (17) which yielded results similar to those found for nonradioactive vitamin D_3.

Condition of the adsorbant for column chromatography was found to be essential for successful separation of the tritio-cholecalciferol from its contaminants. Florisil activated at 260° was found to give better results than that deactivated with 6% water or neutral alumina (Brockmann activity III). The latter two adsorbants yielded vitamin D_3 fractions contaminated with trace of the fast-moving impurity described above, possibly due to artifact of decomposition on column. Purification of tritio-cholecalciferol by preparative thin-layer chromatography on silica gel is also feasible, provided that oxidation and photodecomposition are avoided.

TABLE I.—TRITIUM INCORPORATION AND DISTRIBUTION IN UNPURIFIED TRITIO-CHOLECALCIFEROL

		Tritium Activity
I.	Incorporation after exposure of 1.95×10^3 curie-days	2.7 mc./mg.
II.	Loss after equilibration with (%)	
	Acetone	66.6
	Water	5.6
	Ethyl ether	<1.0
III.	Distribution in chromatographic eluents (%)	
	n-Hexane	2.3
	10% Ether in *n*-hexane	15.3
	Acetone	53.4
	Activity remained on column (by subtraction)	29.0

[2] The measurement was performed by M. K. Hrenoff of the Spectrographic Laboratory, School of Pharmacy, University of California, San Francisco.

When a sample of vitamin D_3 was exposed to tritium gas at room temperature, no useful product was isolated, indicating that the use of low temperature (−198°) for labeling by exposure to tritium gas is of marked advantage since the formation of radioactive impurities is decreased. Because β labeling is due to formation of excited and ionized molecular species (18) which require energy of activation for subsequent chemical reactions, it is reasonable to postulate that at −198°, the small RT value in the Boltzmann factor reduces the probability of reaction. In addition, recombination of molecular ions is facilitated because their diffusion in solids is restricted as a result of an enhancement of the reaction cage effect at −198°.

The validity of the latter factor is supported by the work of Wenzel, *et al.* (19), who noted an increase in specific activity as well as fewer radioactive impurities after gas exposure labeling of charcoal-adsorbed compounds compared to the nonadsorbed control compounds. It is probable that either charcoal adsorption or low temperature (−198°) brings about a common effect, *i.e.*, a passive rigidity of molecular configuration and a restricted diffusion of molecular ions; both of these effects tend to diminish radiation decomposition and to increase substitution by tritium during exposure.

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Kinetics of the Metabolism of Acetaminophen by Humans

By EINO NELSON and TADASHI MORIOKA

The kinetics of the metabolism of acetaminophen to its sulfuric and glucuronic acid conjugates has been studied in normal adult humans. In post-absorptive and post-equilibrative times the elimination process was found to be first order with a mean half-life of 1.95 hours with a range of 1.62 to 2.83 hours in nine tests using a five-subject test panel. Previously published data on pain threshold elevation resulting from ingestion of acetaminophen were examined during times following maximum elevation. Decay of pain threshold elevation was shown to be an apparent first-order process with a half-life of 0.42 hours. This half-life was less than one-fourth the half-life for metabolism, indicating that poor correlation exists between pharmacological activity and body level of acetaminophen, even though a kinetic relationship may exist between these quantities.

THE ANALGESIC DRUG, acetaminophen (4′-hydroxyacetanilide), is known to be metabolized nearly completely after administration to humans. Information on the metabolic fate of this drug in humans has been summarized by Williams (1). About 3% of an oral dose is excreted unchanged in the urine, and most of the balance of a dose can be found in the urine conjugated with sulfuric and glucuronic acids, with the latter conjugate predominating. The conjugates are formed at the 4′-hydroxy position on the molecule. Apparently the kinetics of the metabolism of this substance have not been studied previously. This communication reports the results of a pharmacokinetic study of the metabolism and excretion of acetaminophen and discusses the results obtained in terms of the physiological and pharmacological factors involved.

Received December 19, 1962, from the Pharmacokinetics Laboratory, School of Pharmacy, State University of New York, Buffalo, and School of Pharmacy, University of California Medical Center, San Francisco.
Accepted for publication February 8, 1963.
Support in part by a grant-in-aid from the Lilly Research Laboratories, Indianapolis, Ind., is gratefully acknowledged.

EXPERIMENTAL

Subject and Test Procedure.—One-gram doses of drug grade acetaminophen (finely powdered by