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Sensitive Fluorescence Reaction for Vitamins D and Dihydrotachysterol[1]

PHILIP S. CHEN, JR., A. RAYMOND TEREPKA,[2] AND KEA LANE

From the Department of Radiation Biology, University of Rochester, School of Medicine and Dentistry, Rochester, New York

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Elucidation of the role of the vitamins D and related sterols in bone and mineral metabolism will require many further investigations, but such studies are hampered by a lack of quantitative chemical methods for analysis of vitamin D in biological samples. When using bioassay procedures based on healing of experimentally induced rickets in chicks or rats as an end point (3), estimations of human serum vitamin D concentrations range between 50–135 I.U./100 ml (10, 11) and average about 100 I.U./100 ml (equivalent to 2.5 μg/100 ml). More recent animal assays, e.g., in sheep (4), indicate even lower levels than those reported for man. Since available colorimetric reactions for vitamin D possess insufficient sensitivity for physiological quantities of the vitamin, we were stimulated to search among fluorescence methods which generally possess much greater sensitivity relative to spectrophotometric reactions.

Preliminary experiments confirmed that vitamins D_2 and D_3 and their 3,5-dinitrobenzoate esters were nonfluorescent in aqueous alcohol solutions at pH values between 1 and 14 over the entire spectral range between 200–800 mμ. It has been claimed (2) that histological distribution of vitamin D_2 in rats could be observed under the fluorescence microscope, but Kodicek suggested (6) that what was actually observed was fluorescence of the olive oil vehicle used for injection. We have, therefore, tested various reactions which might induce fluorescence by chemical pretreatment. A number of published colorimetric reactions for vitamin D produce fluorescence as well as color. The most sensitive reaction studied has been produced by acetic anhydride-sulfuric acid in trichloroethane, a reaction previously utilized in various versions for

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colorimetric or fluorometric (1) determination of cholesterol. With the spectrophotofluorometer, it was found that the optimal wavelengths of excitation and fluorescence were different for vitamins D, dihydrotachysterol, and cholesterol. Fluorescence characteristics of this acetic anhydride reaction are described in this communication.

EXPERIMENTAL

Apparatus and Reagents

An Aminco-Keirs spectrophosphorimeter equipped with fluorescence attachment was utilized as a spectrophotofluorometer (SPF). The arrangement (No. 2) of slit widths was chosen for resolution rather than maximum sensitivity, and the sensitivity potentiometer was set at midpoint (i.e., 25) on the photomultiplier microphotometer so that meter readings at various "meter multiplier" settings could be used to compare the sensitivities of different fluorometric reactions. Fluorescence intensities, when given in the text, are net meter readings in per cent of full scale recorded at meter multiplier setting of 003 or corrected to this setting. The photomultiplier slit was 1 mm.

EKC technical grade 1,1,2-trichloroethane was purified by passage through a column of silica gel (Davison, grade 12, 28-200 mesh). Acetic anhydride was redistilled with a Vigreux fractional distillation column. The fraction boiling between 136 and 140°C was collected. Other chemicals used were reagent grade.

Sterols used as standards were purchased from Nutritional Biochemicals Corp., Cleveland 28, Ohio, or Mann Research Laboratories, 136 Liberty Street, New York 6, New York. Ergosterol and 7-dehydrocholesterol required recrystallization from ethanol.

Reactions were conveniently carried out in 5-ml test tubes (15 × 60 mm) fitted with No. 13 glass stoppers (made by Blaessig Glass Specialties, 645 Atlantic Avenue, Rochester, New York).

Procedure

The sterol-containing sample was placed in a glass-stoppered test tube and the organic solvent removed under vacuum or with a fine stream of nitrogen. Two milliliters of 1:5 (20%) acetic anhydride-trichloroethane was added and the tube swirled to dissolve the sterol. After all the tubes were left standing for 15 min, 75 μl of concentrated sulfuric acid was added quickly with a Lang-Levy micropipet to each tube with gentle swirling of the contents, and the tubes were placed in the dark. Contents of the tubes were transferred to 1 × 1 cm SPF cuvette and read in the spectrophotofluorometer at 40–60 min after H_2SO_4 addition.

RESULTS

Fluorescence Characteristics of Acetic Anhydride Treatment

Wavelengths: A typical pair of excitation and fluorescence spectra for the reaction of acetic acid-H_2SO_4 in trichloroethane with vitamin D_3 are shown in Fig. 1, illustrating the observed peaks at 390 and 470 mμ.

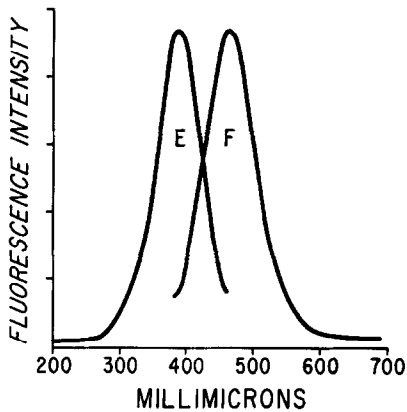

FIG. 1. Excitation-fluorescence spectra of vitamin D_3.

At these particular wavelength settings it was found that the provitamins ergosterol and 7-dehydrocholesterol and cholesterol also fluoresce but, as seen in Table 1, less intensely than did the vitamins D. On the

TABLE 1

FLUORESCENCE OF STEROLS WITH ACETIC ANHYDRIDE-SULFURIC ACID[a]

	Intensity at 390/470 mμ	Optimal wavelengths	Intensity
Vitamin D_2	42		
Ergosterol	10	475/510	25
Vitamin D_3	42		
7-Dehydrocholesterol	6	475/520	16
Cholesterol	6	350/415	16
Dihydrotachysterol	68	425/495	146

[a] All fluorescence intensity values given as: net meter readings (after blank subtraction) in per cent of full scale for 10 μg sterol. Meter multiplier 003.

other hand, dihydrotachysterol (DHT), another potent hypercalcemic sterol, showed more intense fluorescence than vitamin D_2 or D_3, even though its optimal settings were at 425/495 mμ. This is shown in the right part of Table 1 along with the optimal settings found for cholesterol

and the provitamins. DHT was the most intensely fluorescent of the sterols studied.

The intensity readings for vitamins D_2 and D_3 given in Table 1 will be noted to be lower than those mentioned below in the "Standard Curve" section. New lamp and power supply were installed prior to reading a complete standard curve for vitamin D_3. The light source obviously presents an important variable in comparing fluorescence intensities from one instrument to another.

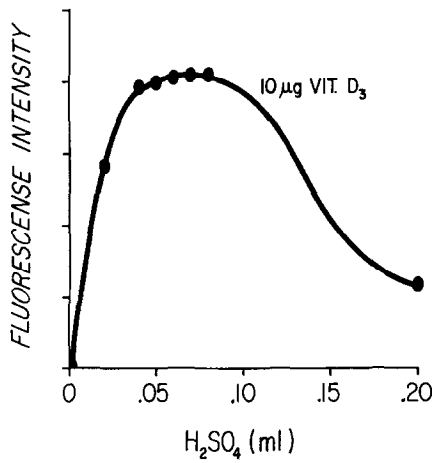

FIG. 2. Effect of sulfuric acid on fluorescence of vitamin D_3.

Volume of Sulfuric Acid: As shown in Fig. 2, the fluorescence of vitamin D_3 was relatively constant with added sulfuric acid volumes between 0.04–0.08 ml. Smaller or larger quantities of sulfuric acid resulted in a diminished sensitivity of the reaction.

Concentration of Acetic Anhydride in Trichloroethane: Using 0.075 ml concentrated H_2SO_4, a wide range of acetic anhydride concentration can be tolerated, but the standard curve of fluorescence intensity vs. vitamin D_3 concentration was linear over a wider range with the 20% concentration recommended, as can be deduced from Fig. 3.

Time of Reaction: The fluorescence intensity increases immediately upon addition of sulfuric acid and reaches a plateau extending from 40–60 min after addition, as seen in Fig. 4. The readings diminish gradually thereafter but samples can still be read after several hours if evaporation of solvent is prevented.

Purity of Solvents: The sensitivity of the reaction (i.e., fluorescence intensity) was relatively independent of solvent purity, but the use of purified solvents, especially redistilled acetic anhydride, reduced the

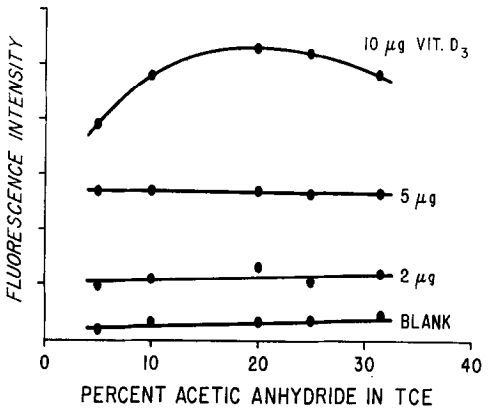

FIG. 3. Effect of acetic anhydride concentration on fluorescence.

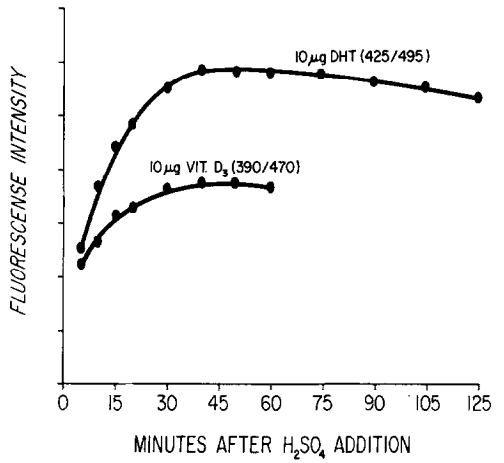

FIG. 4. Fluorescence of vitamin D_3 and dihydrotachysterol as a function of time.

values of blank fluorescence. It was not necessary to redistil the trichloroethane if first passed through the silica gel column as described, but shaking of the trichloroethane successively with concentrated H_2SO_4 and 10% KOH followed by distillation and passage through a silica gel column resulted in the lowest blank values.

Standard Curve: With our purest solvents, blank readings as low as 2.5–3.0 were obtained at the following settings of the SPF: excitation, 390 mμ; fluorescence, 470 mμ; meter multiplier, 003; slit arrangement, No. 2; sensitivity, 25; shutter slit, 1 mm. At these settings, the standard curve for vitamin D_3 was linear up to at least 10 μg, at which concentration meter readings of 100–114 were obtained. Thus the blank reading

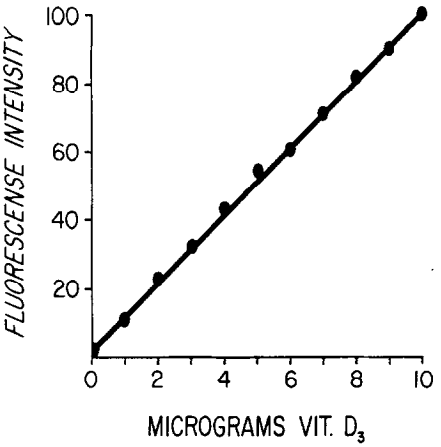

FIG. 5. Standard curve of vitamin D_3 fluorescence.

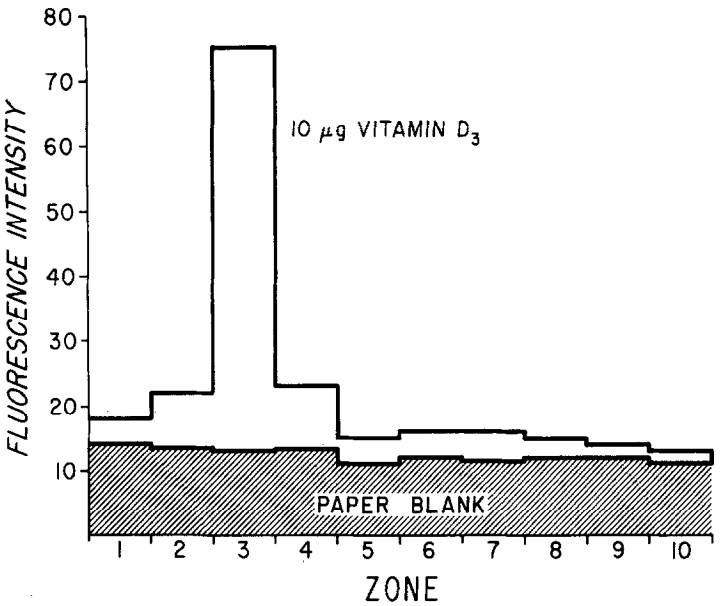

FIG. 6. Fluorescence reaction run on paper chromatographic eluates.

was equivalent to about 0.3–0.4 μg of vitamin D. A typical standard curve for vitamin D_3 is shown in Fig. 5.

Detection of Vitamin D_3 in Paper Chromatographic Eluates: In Fig. 6 is shown an application of the acetic anhydride-H_2SO_4 fluorescence reaction to the detection of vitamin D_3 in eluates from paper. Ten micro-

grams of crystalline vitamin D_3 was run for 17 hr in the Kodicek A reversed-phase system (7) and successive zones along the strip were eluted with 2 ml methanol for 30 min. Eluates were dried under a nitrogen stream and the acetic anhydride reaction run as described above. The net meter reading in zone 3 was equivalent to an over-all recovery of 68% when compared with fluorescence of 10 μg crystalline vitamin D_3 standard.

Survey of Possible Other Fluorescence Reactions of Vitamin D_3

Oxidation and Reduction of Vitamin D_3: An exhaustive study was not attempted, but vitamin D_3 was subjected to both oxidizing (0.2% chromic acid in glacial acetic acid and dried sterol) and reducing (ascorbic acid; 10% sodium hydrosulfite in 30% aqueous methanol) procedures. Neither of these processes resulted in formation of a fluorescent product from the vitamin.

Concentrated Strong Acids: To dried aliquots of 1, 5, and 10 μg vitamin D_3 were added 2 ml of 85% H_3PO_4, concentrated H_2SO_4, and fuming H_2SO_4 (20%). Phosphoric acid produced practically no fluorescence. The sensitivity of the sulfuric acid-induced fluorescence at 410/470 mμ was poor, 10 μg producing a net reading of only 9%, which was 1.5 times the blank value. Fuming H_2SO_4, while more sensitive (at 480/520 mμ) was deemed too corrosive a reagent for routine use (10 μg = 50% = 5 times blank).

1,3-Dichloro-2-propanol: This reagent (with acetyl chloride) was reported (8) to form with vitamins D_2 and D_3 a stable, colored complex absorbing at 625 mμ, with very little absorption by ergosterol, 7-dehydrocholesterol, or cholesterol complexes at the same wavelength. We used a 1% solution of acetyl chloride in 1,3-dichloro-2-propanol and found excitation/fluorescence maxima at 380/425 mμ. Sensitivities of the fluorescence reaction were poor. At 37°C, 10 μg = 27% (meter reading) = 2.7 times blank. The isomeric compound, 2,3-dichloro-1-propanol gave no fluorescence with vitamin D_3.

Aluminum Chloride: The reaction of vitamin D with $AlCl_3$ in the presence of pyrogallol in absolute ethanol has been reported (5) to give a relatively specific violet color. We found the absorption maximum to be 550 mμ, but there was no greater fluorescence of the vitamin D_3 solution than observed with the blank.

Trichloroacetic Acid: Jones, Wilkie, Morris, and Friedman (9) reported that 5 μg of vitamins D_2 and D_3 would demonstrate fluorescence with trichloroacetic acid having excitation/fluorescence wavelengths of 390/480 mμ but did not specify their experimental conditions in detail.

We observed maxima of 470/515 mμ using 20% trichloroacetic acid in dichloroethane with double the blank reading being given by 5 μg of vitamin D_3 at room temperature. At 40°C, 10 μg = 49% (meter reading) = 12 times blank.

Antimony Trichloride: This reagent has long been used as a standard colorimetric reagent for vitamin D in a number of modifications. With 25% $SbCl_3$ solutions, maximum fluorescence (380/415 mμ) was observed in dichloroethane with lesser sensitivity in chloroform and trichloroethane. For example, 10 μg vitamin D_3 gave meter readings of 38, 22, and 13% with the three solvents, respectively. Strangely, vitamin D_2 fluorescence at 380/415 mμ was much weaker than that of vitamin D_3, whereas both vitamins fluoresced equally at 430/460 mμ. Cholesterol and ergosterol exhibited peaks at 400/450 mμ.

COMMENT

The acetic anhydride-sulfuric acid induced fluorescence of vitamins D and dihydrotachysterol offers an extremely sensitive method of measurement for these sterols provided the preliminary isolation from biological samples is achieved. It is to these isolation techniques that we are currently devoting our attention. The numerous technical problems involved can be appreciated when one considers that the extraction scheme must: (*a*) release and extract the sterol from its physicochemical state in blood or tissue, primarily its lipid or protein-bound form, (*b*) separate the sterol from other related lipids including relatively enormous quantities (10^5 times greater in serum) of cholesterol, and (*c*) all the while maintaining experimental conditions (solvents, temperature, atmosphere) which will not result in chemical decomposition of the sterol in question.

At the present time, not all of these problems have been solved. If satisfactory saponification, solvent extraction, and chromatographic techniques can, however, be integrated properly with this sensitive fluorometric method, we believe that quantitative chemical determination of vitamins D and dihydrotachysterol in blood and tissues is feasible.

REFERENCES

1. Albers, R. W., and Lowry, O. H., *Anal. Chem.* **27**, 1829 (1955).
2. Araki, M., Chin, S., and Ryo, K., *J. Vitaminol. Kyoto* **3**, 61 (1957).
3. Bills, C. E., *in* "The Vitamins: Chemistry, Physiology, Pathology" (Sebrell, W. H., Jr., and Harris, R. S., eds.), Vol. II, p. 218. Academic Press, New York, 1954.
4. Dalgarno, A. C., Hill, R., and McDonald, I., *Brit. J. Nutr.* **16**, 91 (1962).
5. Halden, W., and Tzoni, H., *Nature* **137**, 909 (1936).
6. Kodicek, E., *Proc. Intern. Congr. Biochem., 4th, Vienna, 1958* **11**, 198 (1960).
7. Kodicek, E., and Ashby, D. R., *Biochem. J.* **57**, xii (1954).

8. SOBEL, A. E., MAYER, A. M., AND KRAMER, B., *Ind. Eng. Chem., Anal. Ed.* **17,** 160 (1945).
9. UDENFRIEND, S., "Fluorescence Assay in Biology and Medicine," p. 264. Academic Press, New York, 1962.
10. WARKANY, J., *Am. J. Diseases Children* **52,** 831 (1936).
11. WARKANY, J., AND MABON, H. E., *Am. J. Diseases Children* **60,** 606 (1940).