chloroform. To 100 cc. of this solution, 1 cc. of acetic anhydride or 2 cc. of acetyl chloride is added.

10) Alumina. Supplied by Scientific Research Institute Co. (KKl), 50 g. of which was wetted with 0.8 cc. of distilled water in an Erlenmayer flask, stoppered loosely with rubber, and heated at 100° for 15 minutes.

11) Solvents. Sufficiently purified to be suitable for spectroscopic and chromatographic purposes.

b) Apparatus and procedure. 1) Spectrophotometer. Beckman DU type for the ultraviolet absorption in ethanol and Beckman B type for colorimetry.

2) Color reaction. To 0.2 cc. of the $CHCl_3$ solution of a sample is added 3 cc. of the reagent in the room light or in the dark at a temperature regulated by a thermostat. The reactions in the dark are carried out in a test tube stood in a metal pipe stoppered with rubber at both ends to shut off the scattered light. After a given time the optical densities are measured in a glass-stoppered cell of about 3.5 cc. capacity and 1 cm. thick.

3) Chromatographic tube. 1.2×13 cm. for more than 100 mg. of samples and 0.7×12 cm. for about 10 mg. of samples. A slurry of alumina with small volume of petroleum benzine is poured into the tube, until height of alumina in the tube reaches 8~10 cm.

4) Development. Samples are dissolved in 1 to 2 cc. of petroleum benzine, poured slowly into the column, and developed with 10% acetone-petroleum benzine. Eluates are divided into fractions containing 1 to 3 cc. each and after evaporation of the solvent in each fraction optical densities are measured by the spectrophotometer just two minutes after the addition of the reagent to the residues.

Summary

1) It was ascertained that the color reaction of vitamin D with Nield's antimony trichloride reagent was not affected by the light and temperature.

2) Vitamin D containing some sterols was separated satisfactorily from vitamin A by liquid chromatography on alumina. The presence of two types of vitamin A alcohol was observed on the elution curve.

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46. **Tokunosuke Kanzawa and Hiroyuki Mima:** The Chemical Determination of Vitamin D*. II. The Separation of Vitamin D from Sterols and the Determination of Vitamin D in Fish Liver Oils.

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In the previous paper[1] the authors reported that a satisfactory separation of vitamin D from vitamin A was achieved by the chromatographic adsorption on alumina. The next problem is the elimination of the interfering sterols from vitamin D fraction. This is the most difficult problem in vitamin D assay, and no sufficiently reasonable method has been established. The methods of determining vitamin D by means of chromatograph proposed by Ewing, *et al.*[2] or maleic anhydride condensation by Fujita and Aoyama[3] are based upon the difference in the antimony trichloride color between the interfering sterols and vitamin D containing them and not directly upon vitamin D. The digitonin precipitation method proposed by Green[4] seems to be more effective in eliminating sterols than the others. However, these three methods have a chance to fail in collecting only the vitamin D fraction correctly after chromatographic separation, and to obtain wrong results interfered by vitamin A or other substances.

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* Reported at the Annual Meeting of Japan Vitamin Society on May 6, 1952.

1) Part I: This Bulletin, 1, 195(1953).

2) D. T. Ewing, G. V. Kingsley, R. A. Brown, A. D. Emmett: Ind. Eng. Chem. Anal. Ed., 15, 301 (1943). 3) A. Fujita, M. Aoyama: J. Biochem., 37, 113 (1950).

4) J. Green: Biochem. J., 49, 45 (1951).

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The authors attempted the separation of vitamin D from sterols by means of chromatography. A total of 9 mg. of the vitamin D fraction (Nos. 14~25) collected from the previous experiment[1] was developed by benzene on a 0.8×4 cm. column. Although vitamin D is separated to some extent from sterols, this method is not so favorable because of the difficulty in the preparation of alumina giving reproducible result and evaporation of the solvent.

Attempt was then made to determine vitamin D purified by the chromatographic method after the elimination of sterols. The order of the two steps in the separation is reversed, compared to the methods so far reported, so as to avoid the contamination of vitamin D with vitamin A or others due to the erroneous collection of eluates.

The bonito liver oil containing about 15,000 I.U. of vitamin D was saponified and the non-saponifiable fraction was dissolved in 72% ethanol as Green reported and added to a digitonin solution in the same solvent. After the precipitate was removed by filtration, the filtrate was extracted with carbon tetrachloride. The extract was evaporated, the residue was dissolved in petroleum benzine, and poured into a column of alumina. From the elution curve the apparent amount of vitamin D corresponding to the sum of optical densities over the vitamin D fraction was calculated.

Since the amounts of vitamin D obtained in this way show larger value than that by bioassay, the conditions of digitonin precipitation reaction were studied. Table I shows that the reduction of the apparent amount of vitamin D is dependent on the conditions of the digitonin precipitation reaction, i.e. the concentration of the non-saponifiable fraction and digitonin, in other words, the total volume of the solution, has a large effect on the precipitation reaction. The time of standing and the temperature also have some effect, but the variation of the apparent amount of vitamin D is observed even at the same standing time and the same temperature. This seems to be due to some other factors in the precipitation reaction. As the apparent amount of vitamin D approaches the bioassay value at a higher concentration, the volume of the solution was so adjusted as not to exceed 3 cc. by the use of the minimum quantity of the solvents necessary to wash the residue of the non-saponifiable fraction remaining undissolved in 72% ethanol. Moreover, taking care of the preparation of antimony trichloride reagent, vitamin D in three samples of fish liver oil was determined under condition of standing 1.5 hours in a room temperature. The results are shown in Table II.

TABLE I

Effect of Digitonin on the Elimination of Sterols from Vitamin D.

Concentration of		Time of standing and temperature	Apparent vitamin D. γ in 100 mg. oil
non-saponifiable fraction*	digitonin		
Without digitonin reaction			159.3
35.1 mg. in 10 cc.	50 mg. in 5 cc.	1 hr. room temp.	100.5
38.1 mg. in 10 cc.	〃	16 hrs. 〃	97.5
〃	〃	〃 〃	86.1
36.1 mg. in 5 cc.	25 mg. in 1 cc.	1 hr. 〃	77.4
〃	〃	16 hrs. 〃	66.2
37.0 mg. in 1 cc.	〃	1 hr. 〃	54.5
〃	〃	1 hr. −5°C	44.7
〃	〃	16 hrs. 〃	45.3
Value determined by bioassay			37.5

*Weight in mg. represents that of liver oil taken for saponification.

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Table II

Determination of Vitamin D in Fish Liver Oils.

	Bioassay (U.S.P. units)		Chemical determination	Mean
	Vitamin A	Vitamin D		
Skipjack	31,000	20,000	19,000, 23,600	21,300
Bonito	13,800	15,000	15,850, 17,470, 15,100	16,140
Skipjack	12,000	ca. 3,000	3,100, 3,950	3,525

From Table II it is seen that the chemical and biological assays are in good agreement and the coefficient of variation for chemical determinations is calculated as 7~17%. The recovery of vitamin D added to the non-saponifiable fraction of the bonito liver oil was found to be 95%. In these experiments the antimony trichloride color of vitamin D fraction shows no increase in intensity with time, and no tendency to become larger in absorption at 425 mμ than at 500 mμ. This indicates the elimination of cholesterol and other interfering sterols, at least to the extent negligible in colorimetry. Although the lower limit of the concentration to which this method may be applied has not been studied, it is concluded that the purpose of finding a direct method of analysis has been almost fulfilled.

The authors wish to thank Dr. Kuwada, head of our Research Laboratory, and Dr. Watanabe, our senior researcher, for their continued encouragements.

Experimental

The authors propose the following procedure as a method for the chemical determination of vitamin D.

Reagents. 1. 10% Potassium hydroxide solution in ethanol.

2. Ether. Washed with conc. H_2SO_4 and water, dried over Na_2SO_4, and distilled.

3. Ethanol. Commercial 94% ethanol boiled with $AgNO_3$ and NaOH for 5 hours, distilled, and diluted to 72% by volume.

4. Digitonin. Supplied by E. Merck Co., Germany.

5. Alumina. Supplied by the Scientific Research Institute Co. (KKl), 50 g. of which was wetted with 0.8 cc. of distilled water in an Erlenmayer flask, stoppered loosely with rubber, and heated at 100° for 15 minutes. It has the adsorptive power between No. 2 and No. 3 by Brockmann activity test and about 30 cc. of the solvent is needed to elute vitamin D.

6. Petroleum benzine, b.p. 50~80°. Boiled with $AgNO_3$, NaOH, and little water, distilled, dried over P_2O_5, and redistilled.

7. Acetone. Boiled with $KMnO_4$ for 12 hours and distilled. Dried over K_2CO_3 and redistilled.

8. Carbon tetrachloride. Washed successively with conc. H_2SO_4, 5% NaOH solution, and water. Dried over $CaCl_2$ and distilled.

9. Antimony trichloride reagent. For the purification of the components see part I.[1] 20 g. of $SbCl_3$ is dissolved in 100 cc. of chloroform and 2 cc. of acetyl chloride is added to the solution. The same as Nield's reagent. Freshly prepared when necessary.

Procedure

1. Saponification Weigh accurately 0.1~0.5 g. of fish liver oil in a glass-stoppered test tube or a test tube stoppered with rubber carrying a glass tube in order to pass an inert gas. Add 5 cc. of 10% ethanolic KOH to the oil and warm the solution in a water bath at 75° for 15 minutes. After cooling, wash the contents into a separating funnel with 5 cc. of water, and extract twice with 10 cc. of ether. Before shaking the funnel, substitute the air in the funnel with an inert gas. Wash ethereal extracts four times with water, evaporate in the inert gas stream, and dry up at the pump.

2. Digitonin precipitation reaction Dissolve the non-saponifiable fraction thus obtained in a volume of petroleum benzine. Take an aliquot of the solution, containing 10 to 15 γ of vitamin D into a small test tube and evaporate to dryness at the pump. Dissolve the residue in 1 cc. of 72% ethanol and warm for a few minutes. Should any substances remain undissolved, filter and wash them twice with 0.5 cc. of 72% ethanol. To the combined filtrates add 1 cc. of digitonin solution (20 mg./cc.) and allow to stand 1.5 hours at a room temperature. Filter the digitonides and wash twice with 1 cc. of 72% ethanol. Extract the combined filtrates twice with 5 cc. of carbon tetrachloride and combine the extracts. Filter and evaporate the filtrates on a water bath to dryness in an inert gas stream.

3. Chromatography The chromatographic tube consists of a glass tube about 15 cm. long

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and 0.7 cm. in internal diameter at the main part and 1.5 cm. in the upper part, equipped with a glass cock at the bottom. The lower end of this tube is combined with the measuring receiver by a rubber stopper. A side tube for suction is attached to the upper part of the receiver. At the bottom of the tube and upon the cock, glass wool is packed 5~6 mm. thick. A slurry of alumina in petroleum benzine is poured into the tube, until the height of the alumina reaches 7~8 cm. while the solvent flows down dropwise into the receiver.

Dissolve the residue from carbon tetrachloride solution in 1 cc. of petroleum benzine and pour it into the column before the solvent flow out entirely from alumina layer. As soon as the sample solution penetrates into alumina, add slowly the developing solvent, i.e. 10% acetone-petroleum benzine, into the tube so as to avoid the agitation of alumina layer. The filtrate from the column is fractionated into 2-cc. portions from the beginning of development and 20 fractions are sufficient for vitamin D to be eluted completely. Developing may be carried out with or without suction, but should be completed within 2 hours.

4. Vitamin D measurement. Evaporate each fractions to dryness at the pump, and to the residues add 0.2 cc. of chloroform and then 3 cc. of antimony trichloride reagent. Measure the optical densities at 500 mμ 2 minutes after the addition of the reagent. Calculate the weights of vitamin D in gamma from optical densities for each fractions, using a conversion factor, and sum them up over the vitamin D fraction. From this sum the amount of vitamin D in 1 g. of oil is calculated.

Summary

1) The optimum condition of digitonin precipitation reaction has been determined. The elimination of sterols seems to be satisfactory.

2) A method of the determination of vitamin D by means of the digitonin precipitation reaction followed by chromatography on alumina is proposed. This method applied to fish liver oils of medium potency results in good agreement with biological assays.

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47. **Shojiro Uyeo and Junji Koizumi:** Lycoris Alkaloids. XXV.[1] Studies on the Constitution of Lycoramine. (4).

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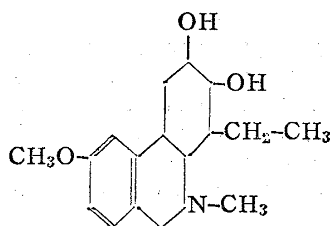
In the preceding paper[1] it was shown that catalytic reduction of lycoremine, a new alkaloid contained in *Lycoris radiata*, gave mainly lycoramine, a base first isolated from the same plant by Kondo, Tomimura, and Ishiwata[2]. Kondo and Ishiwata[3] assigned the formula $C_{17}H_{25}O_3N$ to lycoramine and subjected it to mild oxidation to obtain lycoramine lactam, which on distillation with zinc dust yielded 1-methylphenanthridine, and the skeleton of the molecule was established. Lycoramine lactam was then oxidized with chromic acid and yielded a ketone, which on further treatment with potassium permanganate furnished among others an *o*-dicarboxylic acid, "acid B", of the formula $C_{13}H_{13}O_6N$, m.p. 261~262°. The structure of the acid was proved by decarboxylation to the known 6-methoxy-N-methyldihydroisocarbostyril. This suggested that the rings A and B of lycoramine are represented by 6-methoxy-N-methyltetrahydroisoquinoline. Although Kondo and Ishiwata proposed, on the basis of this and other experimental findings, the structure (I) for lycoramine, they appear to have encountered some difficulties in the interpretation of the



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