45. Tokunosuke Kanzawa and Hiroyuki Mima: The Chemical Determination of Vitamin D.* I. The Antimony Trichloride Reaction and the Separation of Vitamin D from Vitamin A.

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Since vitamin D in natural oil is generally accompanied with vitamin A and sterols, it must be determined by colorimetry or spectrophotometry after eliminating other interfering substances. Today this chemical assay is, however, still a troublesome problem, because the difficulties encountered at each stage, such as (1) the elimination of vitamin A, (2) the separation of vitamin D from sterols, and (3) the accurate determination of vitamin D with a sufficiently reproducible and specific reagent, have not been completely removed.

The various methods of separating vitamin D from vitamin A have been reported, as for example, chromatography using different adsorbents (Superfiltrol[1], a mixture of magnesia and Celite[2], alumina[3,4], and floridin earth[5]), elimination of vitamin A by condensation with maleic anhydride or with citraconic anhydride[4,6,7], or destruction of vitamin A by ultraviolet irradiation[7], but it seems from these works that the chromatographic method is the most effective.

For the separation of vitamin D from sterols the chromatographic method[1], freezing method[8], or digitonin precipitation reaction[5] has been used. However, this separation is the most difficult in the chemical determination of vitamin D and the difficulty has not completely been solved yet, although efforts have been concentrated on this problem.

The spectrometric determinations of vitamin D in both ultraviolet and infrared region[9] are of doubtful value unless purity is warranted. Of the reagents for colorimetry antimony trichloride reagent[10~13] is very sensitive but rather unstable, while others[14~16] are less sensitive.

The authors attempted to obtain a direct method of analysis by which vitamin D is separated as pure as possible and determined in a microquantity by colorimetry. In the present work, descriptions on antimony trichloride reactions will be shown first, as the reaction is fundamental in the analysis.

Antimony trichloride reactions. There have been many attempts made to improve the sensitivity and stability of the antimony trichloride reagents[10~14], among which Nield's reagent[14] containing acetyl chloride is the most sensitive. However, the effect of temperature and scattered light on the time change of the intensity of the color produced

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by Nield's reagent has not been shown. The authors carried out the factorial experiment with four factors, i.e. addition of acetic anhydride, scattered light, temperature, and time. By the analysis of variance it was clarified that the effects of acetic anhydride and the scattered light are highly significant. Three kinds of reagents were prepared, which were the solutions of antimony trichloride in chloroform, containing acetic anhydride, acetyl chloride and no other substances. These were added to the chloroform solution of vitamin D_2 of the same concentration, and the absorptions at 500 mμ were measured in a cell 1 cm. thick, after the mixtures were allowed to stand in the room light or in the dark at a given temperature. The results are shown in Figs. 1 and 2.

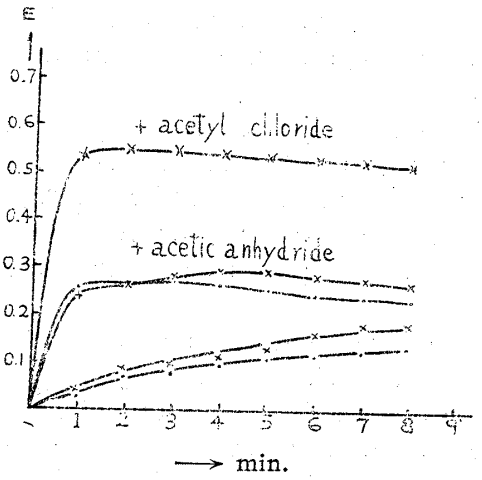

Fig. 1
Effect of Light on the $SbCl_3$ Color of Vitamin D_2 (9.4 γ) at 30°.
-×-×- in the dark.
-·-·- in room light.

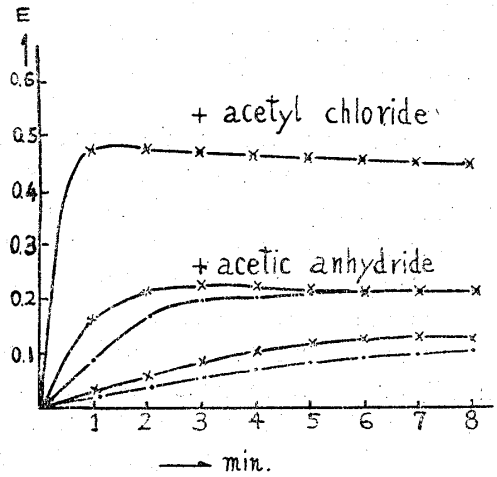

Fig. 2
Effect of Temperature on the $SbCl_3$ Color of Vitamin D_2 (8.2 γ) in room light.
-×-×- at 23°.
-·-·- at 7°.

From Fig. 1 it is evident that the Nield's reagent is not affected by light, although the others are considerably affected. Fig. 2 shows that in these temperature ranges the Nield's reagent gives almost constant absorption for the same concentration, but with the others, the values vary at different temperature. Consequently, it is concluded that Nield's reagent is the most suitable for colorimetry of vitamin D in both its sensitivity and reproducibility, but experiences have taught us that antimony trichloride and chloroform should be adequately purified and the reagent should be tested frequently by the standard solution of vitamin D.

With crystalline vitamin D_2 the optical densities were measured at different concentrations. The density readings are proportional to the concentrations in the range of 0~10 γ in 3.2 cc. of colored solution and the variances of readings are homogeneous in this concentration range. The conversion factor from density readings to concentration is 16.7. The color of cholesterol produced by the Nield's reagent has absorption maximum at 500 mμ increasing in intensity with time, and after thirty minutes the absorption at 425 mμ becomes greater than that at 500 mμ. These color changes are the same as that observed by Mueller[17]. Cholesterol has an optical density of 0.11 at 500 mμ at the concentration of 200 γ in 3.2 cc. after two minutes, and ergosterol has the same magnitude of density at 390 mμ.

Chromatographic separation. Fujita and Aoyama[4] reported the separation of vitamin D from vitamin A using alumina as the adsorbent and 10% acetone-petroleum benzine as

17) A. Mueller: J. Am. Chem. Soc., 71, 924(1949).

the developing solvent but showed no elution curve. In order to find the location of vitamin D fraction correctly, the elution curves of single and pure substances were observed by means of liquid chromatographic method. One-cc. portions of the eluates were taken successively and after evaporation of the solvent in vacuo the Nield's reagent was added and optical densities were measured. Vitamin A palmitate was eluted with maximum concentration at the fifth fraction. Vitamin A alcohol prepared by hydrolysis of the plamitate had two maxima in the elution curve, at No. 5 and No. 30 fractions. The former fraction showed ultraviolet absorption maximum at 328 mμ or 370 mμ and the latter at 328 mμ. Antimony trichloride colors of both fractions are blue. While the latter is assigned to vitamin A alcohol, the former may be due to the decomposition products of vitamin A, unsaponified vitamin A ester, or anhydrovitamin A. Vitamin D is eluted with maximum concentration at about No. 20 fraction, between the two types of vitamin A, and the recovery of vitamin D by the chromatographic procedure is 90 to 100%. Cholesterol and cholestenones were eluted in the same fraction as vitamin D, and ergosterol flowed out more slowly than vitamin A alcohol. It has been ascertained from these results that vitamin A is separated adequately from vitamin D. However, it should be noticed that in the existence of large amounts of vitamin A the separation is not so complete, and the best separation is attained at the quantity of 1,000 to 1,500 U.S.P. units of vitamin A and 600 I.U. of vitamin D.

In the case of non-saponifiable fraction of fish liver oil miscellaneous substances in addition to the above must be separated. Non-saponifiable fraction of the bonito liver oil (vitamin D=ca. 15,000 U.S.P. units, vitamin A=14,000 U.S.P. units) was developed by 10% acetone-petroleum benzine on a 1.2×13 cm. column. Eluates were divided into 3-cc. fractions and 0.3 cc. of each fraction was colored by Nield's reagent. The elution curve is shown in Fig. 3. The figures above the curve, marked with arrows, represent maximum wave length of the ultraviolet absorption of the substance in respective fraction.

The first portion of the eluates contains a yellow oily substance which shows absorption bands at 230 mμ and 255 mμ and its antimony trichloride color changes from blue to green (Fig. 4). This portion which flows down in a yellow band appears to contain the decomposition products of vitamin A, because the following fact has been observed in another experiment. When vitamin A alcohol is developed until it reaches the middle of the column, and left standing overnight, yellow pigment is formed at the location of vitamin A alcohol, and the pigment moves down in a yellow band by further development, being separated from vitamin A.

The second peak in the elution curve contains an oily substance which shows the absorption peak at 240 mμ and gives clear pink color of absorption maximum at 510 mμ by the reagent. This oily substance, which is not contained in the tuna liver oil, is considerably unstable and seems to be substances other than sterols (Fig. 4).

The third peak contains vitamin D and other sterols which give orange yellow color by the reagent. Fractions from Nos. 16~35 contain white crystalline powder which reaches maximum at No. 25 in the amount. The antimony trichloride color of the material in the No. 25 and No. 26 fractions is rather yellow and the intensity increases with time. The ultraviolet spectra show that the substances in Nos. 16, 20, and 25 fractions have all the same absorption peak at 265 mμ consistent with pure vitamin D, as shown in Fig. 5, although the ratio of the density at 265 mμ to that at 230 mμ is almost 1 for No. 25. From these observations it is evident that the latter part of vitamin D fractions is considerably contaminated with cholesterol and other sterols.

Next to the vitamin D fraction vitamin A alcohol is eluted. In this experiment the elution curve shows two peaks, both colored blue by the reagent. The absorption maxima of the ultraviolet spectra are found at 327 mμ for the substances in the No. 31 and at

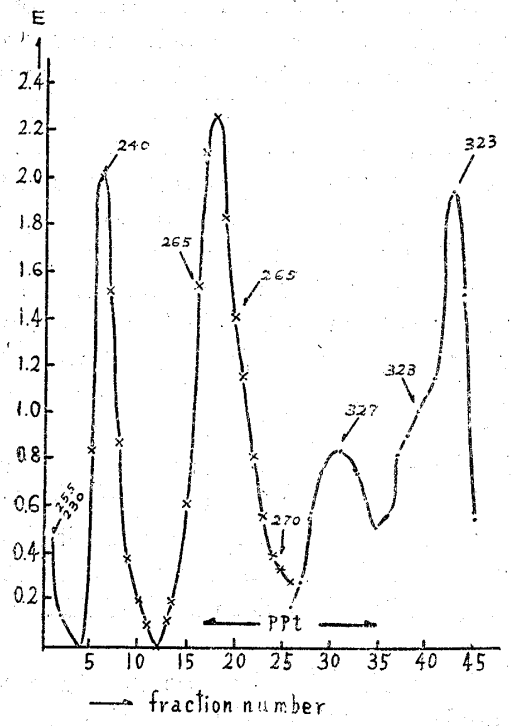

Fig. 3 Elution Curve of the Non-saponifiable Fraction of Bonito Liver Oil.
- · - · - measured at 610 mμ
-×-×- measured at 500 mμ

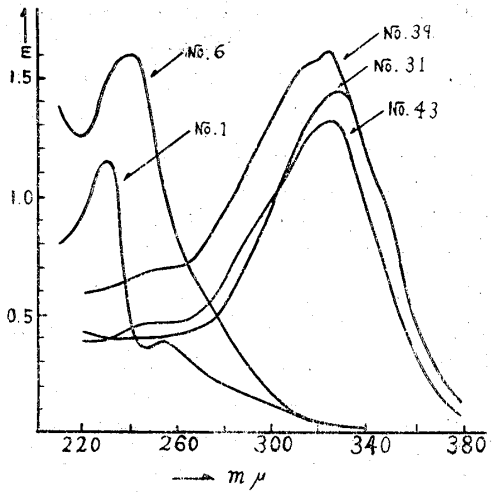

Fig. 4 Absorption Spectra of Vitamin A Fraction and Others.

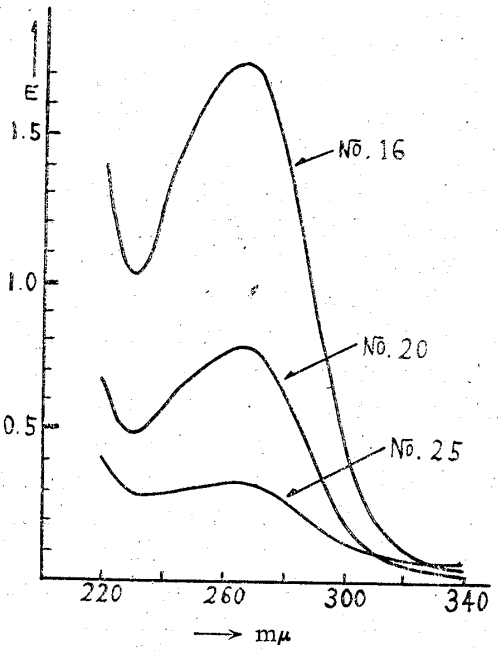

Fig. 5 Absorption Spectra of Vitamin D Fractions.

323 mμ for Nos. 39 and 43. Thus, it is concluded that this fraction contains vitamin A alcohol, and the two peaks might correspond to *cis*- (324 mμ) and *trans*- (328 mμ) vitamin A alcohols, respectively.

After vitamin D containing sterols has been separated satisfactorily from vitamin A, the next problem is the separation of vitamin D from sterols. This will be described in the succeeding paper.

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

Experimental

a) Materials. 1) Vitamin A palmitate. 1,800,000 U.S.P. units. Supplied by Merck Co.

2) Vitamin D, m.p. 114~115°. Supplied by Sterwin Chemical Co., U.S.A., and recrystallized from hydrated acetone.

3) Sterols. Cholesterol and ergosterol were purified by recrystallization.

4) Fish liver oils. Supplied by Ohki Pharmaceutical Co., Ltd.

5) Antimony trichloride. Distilled under a pressure of 20 mm. Hg. with glass joint distillation apparatus. The purified substance was stored in glass-stoppered flasks, each of 30~50 g. capacity, and pipetted out after melting.

6) Chloroform. Washed with 5% sodium hydroxide solution, six times with water, dried over P_2O_5, and distilled. Necessary amount was purified and used wthin one or two days.

7) Acetic anhydride, b.p. 140°. Redistilled.

8) Acetyl chloride, b.p. 51°. Redistilled.

9) Antimony trichloride reagents. 20 g. of antimony trichloride is dissolved in 100 cc. of

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. (KKI), 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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