I. THE ULTRAVIOLET ABSORPTION OF VITAMIN K_1 AND THE EFFECT OF LIGHT

II. THE QUANTITATIVE CHROMATOGRAPHIC DETERMINATION OF VITAMIN D IN FISH LIVER OILS

Thesis for the Degree of Ph. D.

MICHIGAN STATE COLLEGE

Frank Sargent Tomkins

1942

[redacted]

75.11

I. THE ULTRAVIOLET ABSORPTION OF VITAMIN K_1 AND THE EFFECT OF LIGHT

II. THE QUANTITATIVE CHROMATOGRAPHIC DETERMINATION OF VITAMIN D IN FISH LIVER OILS

by

Frank Sargent Tomkins

A THESIS

Presented to the Graduate School of Michigan State College of Agriculture and Applied Science in Partial Fulfillment of Requirements for the Degree of Doctor of Philosophy

Department of Chemistry

Michigan State College

1942

ACKNOWLEDGEMENT

The writer wishes to express his appreciation to Dr. D. T. Ewing for his help and guidance throughout this investigation; and to Parke-Davis and Company, without whose assistance the work would have been impossible.

I

THE ULTRAVIOLET ABSORPTION OF VITAMIN K_1 AND THE EFFECT OF LIGHT ON THE VITAMIN

The relationship between chemical structure and ultraviolet absorption spectra is nowhere illustrated better than in the study of vitamin K and related compounds. One generalization which we presented in a previous publication[1], namely that the vitamin K absorption curve presents a summation of the benzenoid and the quinoid components of the disubstituted naphthoquinone molecule, recently has been extended by Morton and Earlan[2] to the anthraquinone series. Because of the importance of this aspect we have reinvestigated the structure of the absorption curve of the vitamin and have studied in detail the influence of light and other factors such as the presence of acetic acid which sometimes is added as a stabilizer. It was hoped also that the results of this detailed study might clear up the controversy between Karrer[3][4] and Doisy[5][6] regarding the absorption coefficients of the pure vitamin. Another object of our work was to check the identity of the natural with the synthetic vitamin.

Apparatus and Materials

The samples of vitamin K_1, both natural and synthetic, used in this investigation were prepared by Doisy and his associates at the Saint Louis University School of Medicine. They were examined with a Bausch and Lomb medium quartz spectrograph and ultraviolet sector photometer, with a Hilger No. H-698 hydrogen discharge tube as the source of continuous ultraviolet light.

The hexane used as the solvent in this study was the Eastman "Practical" grade, which was purified and redistilled. The purification process consisted of five to ten shakings with 10% fuming sulphuric acid, two washings with 10% Na_2CO_3 solution, prolonged shaking with 5% $KMnO_4$-10%Na_2CO_3 solution, fifteen to twenty washings with distilled water, drying for twenty-four hours over calcium oxide, and distillation twice over freshly fused calcium chloride. The purified hexane boiled at 64.5-65.0°C, and its absorption spectrum between λ200 and λ800 mμ did not show the presence of impurities.

The source of irradiation for the study of the effect of ultraviolet light on the vitamin consisted of a General Electric No. H-4 mercury arc lamp, a condensing lens, a Cenco ultraviolet transmitting filter, and a Cenco infra-red absorbing filter. This combination transmitted only the λ365.5 and 366.3 mμ lines of mercury.

Bausch and Lomb 10mm. absorption cells with detachable quartz ends and monel metal fittings were selected for this investigation. The spectra were recorded on Eastman No. 40 plates, processed five minutes in Eastman Developer Formula No. D-19.

Experimental Part

Specimens of the vitamin weighing between one and two milligrams were dissolved in sufficient hexane to give a 0.0025% concentration on the weight-volume basis. Since in earlier work a trace of glacial acetic acid had been added to all specimens of the vitamin for preserving purposes and a question had arisen as to whether or not the acetic acid was affecting the absorption and should be removed, measurements were made both in the presence and absence of acetic acid to disclose this effect if present. It was found that the presence of acetic acid in amount equal to the weight of the vitamin had no noticeable influence on the absorption curve.

In studying the effect of ultraviolet light on the vitamin, the hexane solution was placed directly in the absorption cell and the spectrum of the unirradiated sample determined. The cell containing the solution was then exposed to the ultraviolet light generated as described above, at a distance of 30 cm., for a definite length of time, after which the cell was removed to the spectrograph and the absorption spectrum again determined.

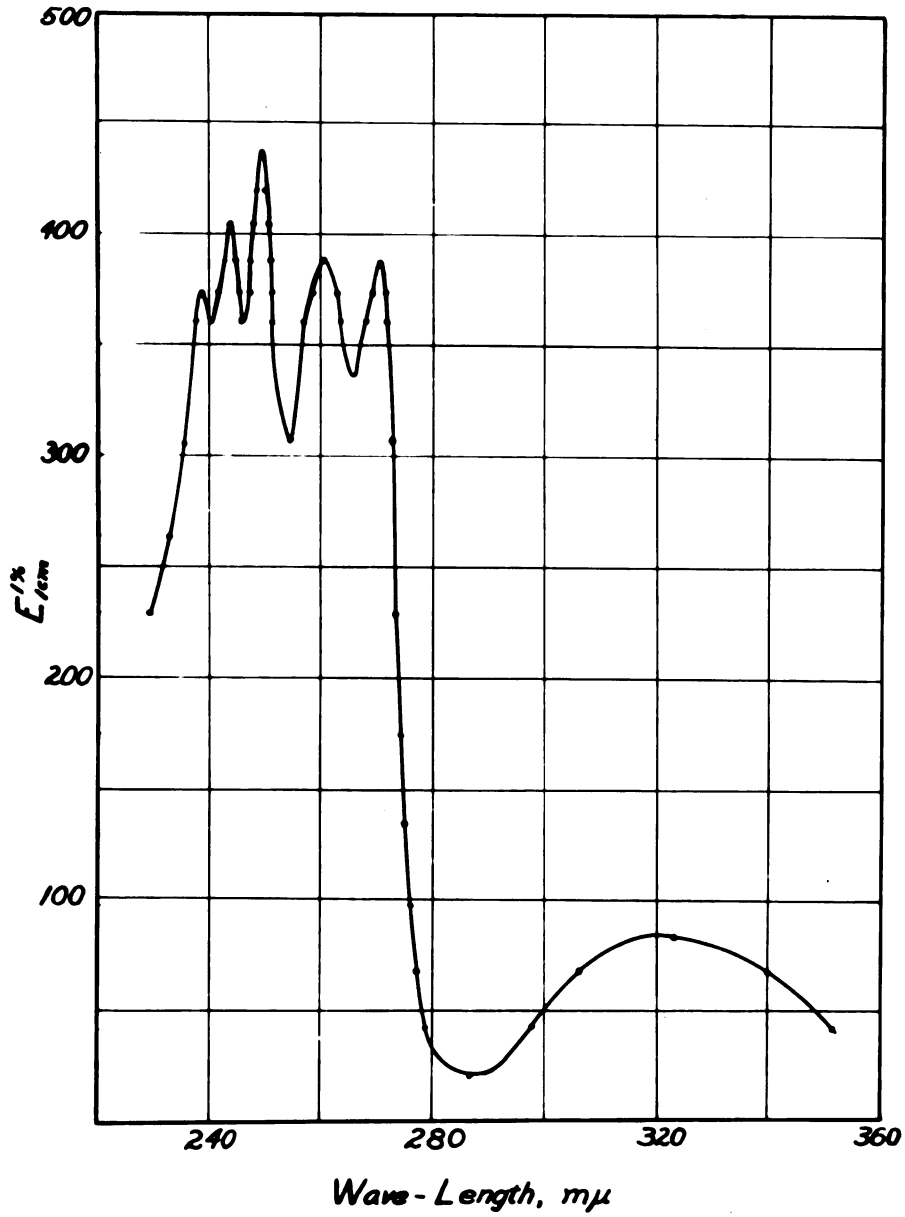

FIG. 1 ABSORPTION CURVE OF NATURAL VITAMIN K_1 IN HEXANE

Proceeding in this manner, a single sample served for a complete run, and errors involved in transferring volatile hexane solutions from one container to another were eliminated.

After samples of each solution were measured they were placed in tightly stoppered flasks, weighed, and stored in the dark. These samples were re-run from time to time to determine whether or not the vitamin decomposed upon standing in the dark.

Figure 1 presents the detailed structure of the absorption curve of the natural vitamin, and Figure 2 that of the synthetic product. It will be noticed that the curves are essentially identical. They differ from the curves previously published[1] in that they show a new maximum at λ239 mμ and a minimum at λ240 mμ. In both cases the highest maximum is found at λ249 mμ and has an extinction coefficient of 438. The extinction coefficients of the many samples of vitamin K_1, both natural and synthetic, which we have evaluated during the course of these investigations indicate that the $E^{1\%}_{1cm}$ of the pure vitamin is 435±5. This value is in good agreement with that of 425, which we previously reported for the synthetic product (Reference /1/ page 356). We feel that this value more accurately represents the extinction coefficient than that of 540 which we reported in the same publication (Reference /1/, page 350, Fig. 6).

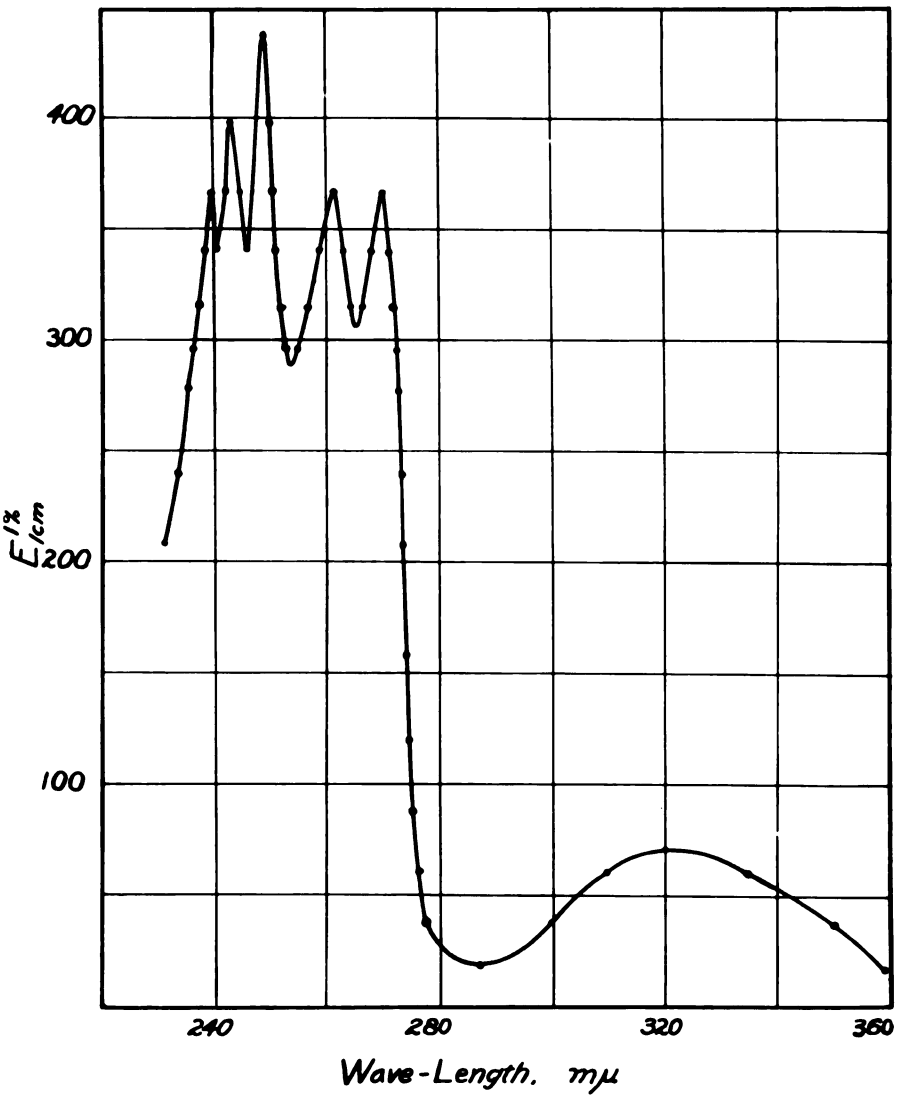

FIG. 2. ABSORPTION CURVE OF SYNTHETIC VITAMIN K_1 IN HEXANE.

It is known that vitamin K_1 is affected by light with loss of physiological activity and modification of the absorption spectrum. No systematic study of the effect of light has been reported and little or nothing is known concerning the chemical change that occurs in the structure of the vitamin when exposed to light. Accordingly, we have made a study of the progressive changes that occur when hexane solutions of the vitamin in a quartz container are exposed to ultraviolet light over definite time intervals.

Figure 3 shows nine absorption curves for a sample of natural vitamin K_1 in hexane solution in the absence of acetic acid which was exposed to λ365.5 and 366.3 mμ lines of mercury radiation, readings being taken at 0, 15, 30, 45, 60, 90, 135, 195, and 255 minutes, respectively. It will be noted that the exposure produces a gradual lowering of the maxima at λ239, 243, 249, 260, and 269 mμ, and a less pronounced decrease in the maximum at λ325 mμ, indicating a gradual decomposition of the vitamin.

Figure 4 shows a similar set of curves for a sample of synthetic vitamin K_1 containing a small amount of acetic acid, determined under identical conditions as those illustrated in Figure 3. The curves are essentially the same except for a slight stabilizing effect of the

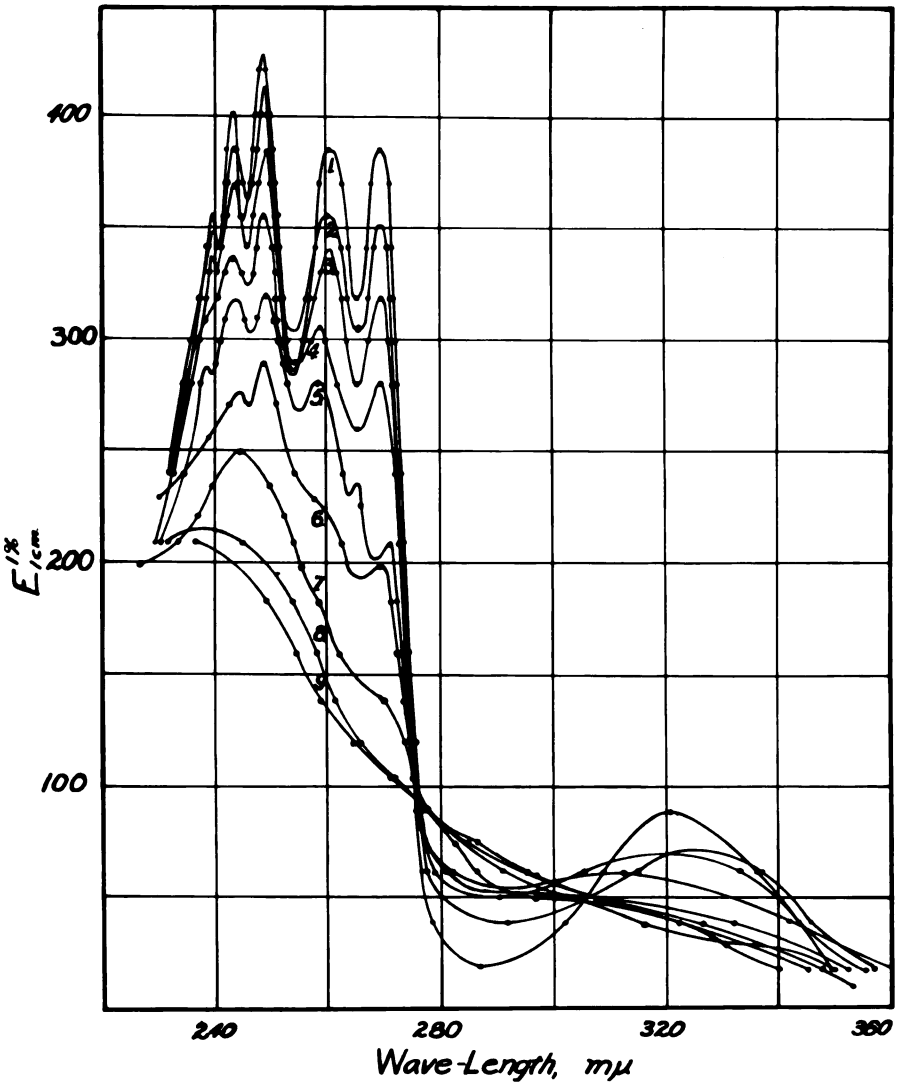

FIG. 3 ABSORPTION CURVES SHOWING THE EFFECT OF ULTRAVIOLET RADIATIONS ON NATURAL VITAMIN K_1 IN HEXANE

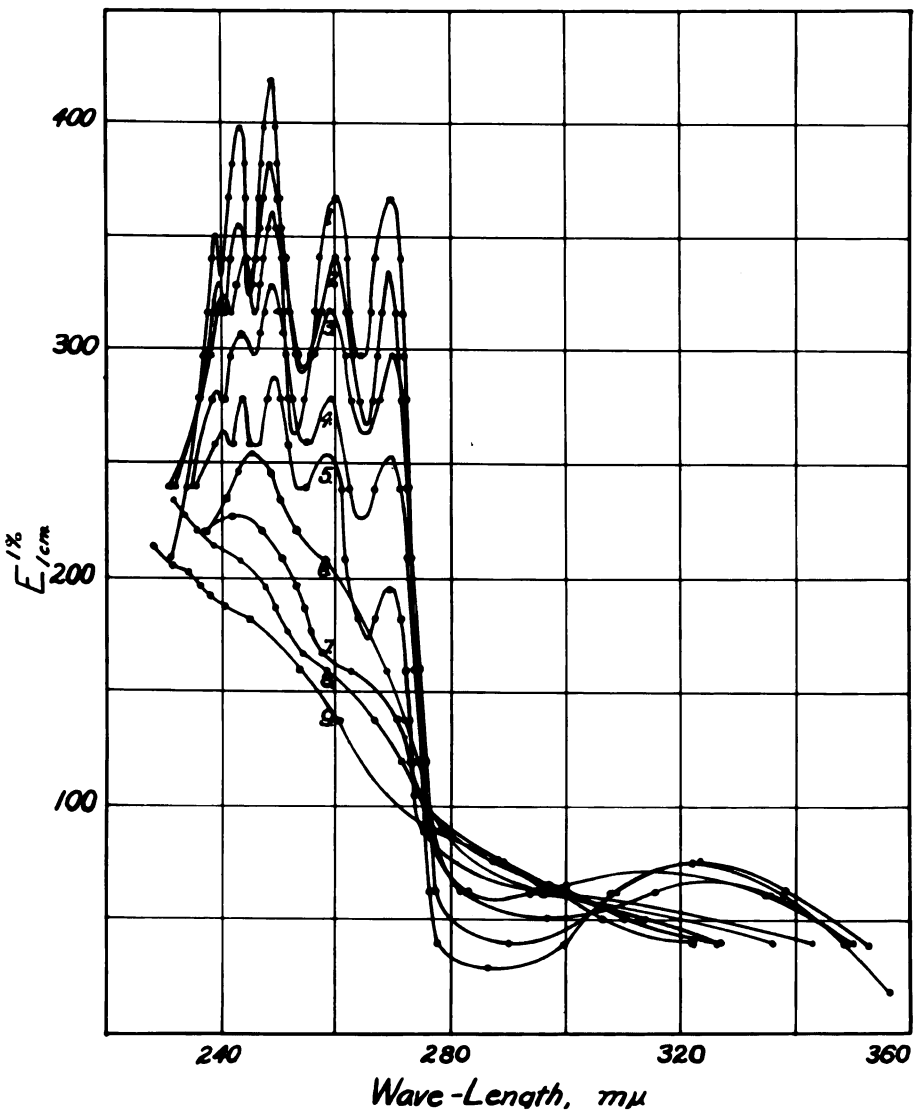

FIG. 4 ABSORPTION CURVES SHOWING THE EFFECT OF ULTRAVIOLET RADIATIONS ON SYNTHETIC VITAMIN K_1 IN HEXANE

acetic acid during the first few exposures.

In both of the above cases, the initial effect is most pronounced on the maxima at λ260 and λ269 mμ which we previously have shown to be associated with the quinone structure (Reference /1/, page 350). It follows, therefore that the point of attack is through the quinone grouping. In connection with these figures it is interesting to note that the nine curves intersect at approximately λ277 mμ, and that less definite iso-extinction coefficient points occur at λ230 mμ and λ305 mμ.

No attempt was made to correct for the effect of exposure of the sample to ultraviolet light from the hydrogen discharge tube during the exposure of the plate, because a series of runs made for the purpose showed that the effect of this light was negligible.

MacCorquodale, Binkley, et al.[7] report that vitamin K_2 is unstable when exposed to light, and curves were presented in our previous article showing the deterioration of the absorption curves of samples of vitamins K_1 and K_2 when exposed to diffuse daylight. In order to determine, if possible, the wave lengths or wave length of light causing this decomposition, samples of vitamin K_1 were exposed to various wave length regions from infra-red to ultra-violet. The results are shown in the following table.

Source of Radiation	Filters Used	Time	Effect
Nernst Glower	None	3 hrs.	Slight decomposition
" "	Zeiss R-30	" "	No effect
Tungsten Filament Lamp	Wratten "A"	45 min.	" "
" " "	Wratten "B"	" "	" "
" " "	Wratten "C"	" "	" "
Hg Arc	Cenco ultraviolet transmitting plus Cenco infra-red absorbing	15 min.	Decomposition
Diffuse daylight	None	2 hrs.	Decomposition

These data show that light radiations between λ400 and 800 mμ have no appreciable effect on the vitamin. The slight decomposition shown in the case of the Nernst glower with no filter was probably due to the ultraviolet light present in the radiation from the incandescent filament of the glower. Thus, the decomposition reported earlier as being due to the effect of visible light probably was due to the small amount of ultraviolet present in diffuse daylight.

We reported previously that vitamin K_1 was unstable in hexane solution in the dark. However, using specially purified hexane, we can now report that vitamin K_1

in dilute hexane solution is stable for periods up to five months when stored in the dark at room temperature.

Discussion

Since Karrer and Doisy first began publication on vitamin K_1 there has been a discrepancy between the two laboratories concerning the correct value for $E^{1\%}_{1cm}$ at λ249 mμ. In their first publication Dam et al.[8] gave a value of 280 and McKee et al.[5] a value of 385. In subsequent publications Karrer[3][4] has claimed that his vitamin preparation was pure and that 280 was the correct value for the extinction coefficient. In an effort to discern the cause of the discrepancy, we have made numerous measurements on both natural and synthetic vitamin K_1 samples which were prepared and supplied to us by Doisy. We find that the $E^{1\%}_{1cm}$ at λ249 mμ is 435±5 (log E_m = 4.29). This value is in good agreement with values reported by D. M. Bowen[9] (log E_m = 4.24-4.27) (in alcoholic solution) and T. J. Webb[9] (log E_m = 4.26) (alcoholic solution). We believe that this is the correct value for either pure natural or synthetic vitamin K_1 in hexane solution.

Of particular interest in connection with the controversy between the two laboratories is the fact that both groups agree on the values for $E^{1\%}_{1cm}$ for vitamin K_2[1][4] and for the diacetate of dihydrovitamin

K_1 (log E_m = 4.93)[1][4].

In view of our close agreement with other laboratories it is Karrer's responsibility to explain his low values for $E^{1\%}_{1cm}$ for vitamin K_1. From a structural point of view the chief difference between vitamin K_1 and vitamin K_2 is the size of the side-chain in the 3-position. Since the side-chain in each case is aliphatic and contains no conjugated double bonds, the absorption spectra for the two compounds would be expected to be quite similar. If the absorption is due to the naphthoquinone portion of the molecule and is not influenced by the size of the aliphatic side-chain in the 3-position, the $E^{1\%}_{1cm}$ values for the two vitamins should be inversely proportioned to their molecular weights and the log E_m values should be equal. The same reasoning holds true for the diacetates of vitamins K_1 and K_2. Actually this is the case. The log E_m values for vitamins K_1 and K_2 are 4.27 and 4.29 respectively, and the values for the corresponding diacetates are 4.93 and 4.93. It is significant that the molar extinction coefficients obtained for vitamins K_1 and K_2 agree well with values obtained by Tishler et al.[9] for three crystalline 2,3-dialkyl-1,4-naphthoquinones. These quantitative relationships are good evidence in support of the correctness of our values.

A comparison of the absorption curves previously published by us[1], as well as those now presented,

with that illustrated in the article by Dam, et al.[8] suggests that there might be a proportionate discrepancy between the heights of the respective extinction coefficients at λ249 mμ and λ325 mμ. As a matter of fact, the discrepancy is not serious. Karrer's curve was plotted as log $E^{1\%}_{1cm}$ versus wave-length, which tends to enhance the 325 m maximum, and was compared in this form with our curve which was plotted as $E^{1\%}_{1cm}$ versus wave-length. This latter method shows better the fine structure in the region λ239 mμ to λ270 mμ, but gives a less pronounced maximum at λ325 mμ.

An examination of the absorption curves showing the effect of ultraviolet light on solutions of vitamin K_1 gives a certain amount of information as to the actual chemical change involved. We have stated in the experimental part of this paper that the point of attack probably is through the quinone grouping, and may add that the λ325 mμ maximum which we previously associated with the ring structure changes more slowly than the rest of the curve.

2-Methyl-1,4-naphthoquinone upon exposure to light for long periods of time is decolorized and forms a polymer of known structure[10]. It is possible that a similar reaction occurs when vitamin K_1 is exposed to light.

Summary

1. A more careful examination of the absorption curve of vitamin K_1 shows the presence of a new maximum at λ239 mμ.

2. The $E^{1\%}_{1cm}$ of pure vitamin K_1 at λ249 mμ is 435±5.

3. Vitamin K_1 in hexane solution is stable upon standing in the dark at room temperature for as long as five months.

4. Vitamin K_1 in hexane solution is decomposed rapidly by the action of ultraviolet light, while visible and infra-red radiation have no effect. The point of attack probably is through the quinone group.

5. The presence of acetic acid has no noticeable effect on the absorption curve.

6. On the basis of the absorption values reported by Dam, Geiger, Glavind, Karrer, Rothschild, and Solomon, their product appears to have been 60 to 80 per cent pure.

****

References

(1) Ewing, D. T., Vandenbelt, J. M., and Kamm, Oliver, J. Biol. Chem. 131, 345 (1939).

(2) Morton, R. A. and Earlan, W. T., J. Chem. Soc. 159 (1941).

(3) Karrer, P. and Geiger, A., Helv. chim. Acta 22, 945 (1939).

(4) Karrer, P., Geiger, A., Legler, R., Ruegger, A., and Solomon, H., Helv. chim. Acta 22, 1464 (1939).

(5) McKee, R. W., Binkley, S. B., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., J. Amer. Chem. Soc. 61, 1295 (1939).

(6) Binkley, S. B., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., J. Biol. Chem. 130, 219 (1939).

(7) MacCorquodale, D. W., Binkley, S. B., McKee, R. W., Thayer, S. A., and Doisy, E. A., Proc. Soc. Exper. Biol. & Med. 40, 482 (1939).

(8) Dam, H., Geiger, A., Glavind, J., Karrer, P., Rothschild, E., and Solomon, H., Helv. chim. Acta 22, 310 (1939).

(9) Tishler, M., Fieser, L. F., and Wendler, N. L., J. Amer. Chem. Soc. 62, 1982 (1940).

(10) Rev. Acad. Cienc. 31, 617 (1934).

II

THE QUANTITATIVE CHROMATOGRAPHIC DETERMINATION OF VITAMIN D IN FISH LIVER OILS

The quantitative determination of vitamin D by chemical means has been the object of several investigations in recent years. Brockmann and Chen[1] reported that vitamins D_2 and D_3 when treated with a solution of antimony trichloride in chloroform give an orange-yellow color which soon reaches a maximum at λ500 mμ, and that the measurement of the absorption at this wavelength gives a measure of the amount of vitamin present.

This procedure has been reinvestigated and amplified by Marcussen[2], Nield, Russell, and Zimmerli[3], and Milas, Heggie, and Raynolds[4]. In each of these reports it has been shown that the Brockmann and Chen color reaction is applicable only where the vitamin is present in the pure form or where interfering biological materials have been reduced to very low concentrations. The most troublesome of these interfering materials are vitamin A, sterols, irradiation products of the sterols other than vitamin D, and the tocopherols. Among the methods suggested for separation of such materials were chromatographic adsorption, freezing out of sterols, and the treatment of the sample with maleic anhydride for the decomposition of vitamin A.

The method to be described here involves an improved chromatographic treatment to remove vitamin A, the removal of interfering sterols by freezing and precipitation with digitonin, and the measurement of the absorption at λ500 mμ produced when the purified sample is added to a solution of antimony trichloride in chloroform containing a small amount of acetyl chloride.

Apparatus and Materials

The extinction coefficient measurements were made on a Bausch and Lomb polarization type visual spectrophotometer. The absorption cells were Bausch and Lomb one centimeter cells with glass spacers, quartz end plates, and monel metal fittings. Adsorption tubes for the chromatographic procedure were made by sealing a 10 cm. length of 7 mm. pyrex tubing to the bottom of a 5/8"x 6" pyrex test tube.

The $SbCl_3$-$CHCl_3$-CH_3COCl reagent was prepared according to Nield et al[3], by dissolving 22g. of C.P. antimony trichloride in purified chloroform, diluting to 100 ml., and adding 2 ml. of redistilled acetyl chloride to the resulting solution. The chloroform for this reagent must be carefully purified and dried as follows: Shake repeatedly with distilled water to remove the alcohol, distill and discard the first quarter of the distillate, reflux for two hours over P_2O_5 and filter.

The filtrate is shaken with activated carbon to remove the small amount of phosgene formed during the refluxing with P_2O_5, filtered and the filtrate redistilled. The purified chloroform should be stored in the dark. This product is relatively unstable and the purification should be carried out on small lots.

The mixed solvent for the chromatographic procedure was prepared by adding 10 parts of C.P. anhydrous ethyl ether to 50 parts (by volume) of commercial hexane (Eastman Practical grade).

The ether was purified by washing repeatedly with distilled water, distilling, and redistilling over sodium.

The hexane was used as received.

The adsorbent was a fine grade of activated Bentonite clay or "Superfiltrol".

Samples of fish liver oils and vitamin D concentrate were supplied by Parke-Davis and Company.

Experimental

A sample of fish liver oil or concentrate to contain 20,000 to 80,000 vitamin D units was weighed, 10 ml. of N/2 alcoholic KOH added, and the sample heated for one hour on the steam bath. The resulting solution was cooled, 20 ml. of distilled water added, and the solution extracted three times with 25 ml. portions of ether. The combined ether extracts were washed with

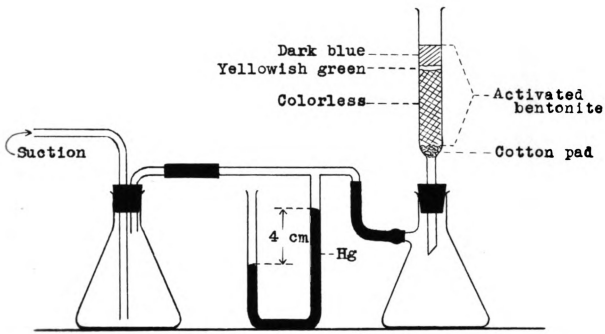

Fig. 1. SCHEMATIC DIAGRAM SHOWING CHROMATOGRAPH SET-UP AND APPEARANCE OF BANDS AT END OF RUN.

distilled water in a separatory funnel until the washings gave no reaction with phenolphthalein, dried over anhydrous sodium sulfate, and filtered.

The filtrate was evaporated to dryness under reduced pressure, the residue taken up in 10 ml. of absolute methanol, and 5 ml. of the resulting solution pipetted into a 15 ml. centrifuge tube. The centrifuge tube and metal holder were cooled to -15° C. in an acetone bath kept at this temperature by additions of dry ice. The cooled solution was centrifuged, the supernatant liquid poured off, the precipitate washed twice with 2 ml. portions of methanol--cooling and centrifuging after each addition--and the liquid and washings transferred to a second 15 ml. centrifuge tube.

To the resulting 9 ml. of solution was added 1 ml. of distilled water and 2 ml. of 2% digitonin in 90% methanol solution and the mixture allowed to stand at least two hours. At the end of this time the mixture was centrifuged and the precipitate washed twice with 2 ml. portions of 90% methanol; the washings were added to the bulk of the separated liquid. The solution was then evaporated to dryness under reduced pressure and the residue taken up in 5 ml. of the ether-hexane mixture. The resulting solution was chromatographed as follows:

The adsorption tube was prepared by placing a small cotton wad in the bottom of the tube and adding the adsorbent to a depth of about 50mm., tapping the tube

after each small addition to pack the adsorbent. Slight suction (4 cm. Hg) was applied to the tube and 5 ml. of the mixed solvent added to wet the adsorbent. The 5 ml. sample from the sterol separation was then added, followed by 5 ml. of the solvent used to rinse the flask, and finally 20 ml. of the solvent to develop the colored bands formed in the adsorption tube. Each addition of solvent was made before the liquid from the previous addition had quite disappeared, in order that drying out of the adsorbent be prevented. If the column becomes dry, further addition of the solvent destroys the bands and the sample is lost. During the washing of the column with the last 20 ml. of solvent a very narrow and sometimes rather indistinct yellowish-green band appears at the lower edge of the blue band formed by the vitamin A. This band provides a convenient reference point for the separation of the column later and should be marked just before the last of the wash solvent disappears. When the last of the liquid had gone through, the column was dried by pulling air through it for five to ten minutes and the upper portion of the column--to the mark previously made--removed with a bent spatula and discarded. This portion of the column contains all of the vitamin A. The remaining portion of the column was removed to a small erlenmeyer flask and the adsorption tube rinsed with 25 ml. of anhydrous ether which was added to the same flask. This ether-adsorbent mixture was shaken vigorously,

allowed to stand until the adsorbent had settled, and the supernatant liquid poured off through a coarse filter into a second erlenmeyer. This extraction was repeated with three additional 25 ml. portions of ether and the combined filtrate added to that from the adsorption process. This solution was evaporated to dryness under reduced pressure, taken up in 10 ml. purified chloroform, and 1 ml. of the resulting solution added to 10 ml. of the antimony trichloride reagent. The log I_o/I value of the resulting mixture at λ500 mμ was determined, using the Bausch and Lomb spectrophotometer, and the $E^{1\%}_{1cm}$ value calculated.

The procedure may be interrupted after the saponification and extraction, or after the extraction of the lower portion of the adsorption column, when the vitamin D active materials are in ether solution. Such a solution can stand overnight without measurable loss in potency.

Data and Results

Table I shows the values obtained on a series of seventeen fish liver oil and vitamin D concentrate samples by the procedure described above. The calculated potency values are on the basis of sample #44090 taken as a standard. In each case the $E^{1\%}_{1cm}$ value given in the table is the average value obtained from a set of two duplicate samples.

Sample	Type	$E^{1\%}_{1cm}$	Bio. potency DU/gm.	Calc. potency DU/gm.
44090	Vit. D Distillate	1.30	22,000	22,000
44080	Vit. D Distillate	1.74	31,000	29,500
47761	High vit. D Oil	1.01	15,000	17,100
41860	High vit. D Oil	.807	16,000	13,600
56021	High vit. D Oil	.670	13,000	11,300
55691	High vit. D Oil	.709	12,000	12,000
40090	High vit. D Oil	.912	20,000	15,400
18119	Bonita liver oil	1.09	36,000	18,400
55031	Vit. D Distillate	.859	18,500	14,600
44470	Tuna liver conc.	14.2	240,000	240,000
56961	Vit. D Distillate	1.05	20,000	17,800
57481	High vit. D Oil	.495	15,000	8,400
57951	Blue fin tuna oil	1.43	28,000	24,200
57971	Albacore liver oil	2.80	55,000	47,400
57991	Yellow fin tuna oil	.946	3,000	16,000
58011	Bonita liver oil	3.20	65,000	54,000
57381	Tuna liver conc.	15.1	260,000	255,000

Table I

Sample #18119 was in very poor physical condition --discolored and full of sediment and suspended particles-- which may account for the very low result obtained in this case. There is no obvious explanation for the low value obtained for sample #57481 or the high value for #57991; assuming that the biological value is correct as given.

The values obtained by the chromatographic procedure show good agreement with the biological values --with the exception of the instances cited above--and represent an appreciable saving in time and expense over the biological assay method.

Discussion

Vitamin D containing biological materials such as milk, butter, yeast, fish liver oils, etc., are exceedingly complex mixtures in which the vitamin D, although it may be very active biologically, represents but a minute fraction of the total amount of material present. At the same time the vitamin "D" in the given material may be any one of many vitamin D active substances, or a combination of two or more such substances.

Fish liver oil, for example, is a mixture of fatty acids, sterols, carotenoids, vitamin A, vitamin "D", and in some instances tocopherols; in which, as it has been shown by Brockmann and Busse[5], the characteristic antirachitic principle is vitamin D_3, the vitamin D_3 being accompanied by varying quantities of vitamin D_2. The problem of chemically determining the antirachitic potency of such a sample then, offers two possibilities. Either we must separate the vitamin D active principle from the bulk of the material sufficiently well that the interferences from these materials will be small, or we must find a reaction specific to the vitamin D active

substance which will work in the presence of other biological materials and be sensitive to the small amount of vitamin D present. Since the first alternative seemed to offer the most possibilities, it was along this line that the work was directed.

The antimony trichloride color reactions of vitamins D_2 and D_3 are identical, as first stated by Brockmann and Chen[1] and confirmed by Nield et al[3], and the improved reagent described by the latter in the same paper gives a greatly increased sensitivity to the test. The difficulty in the use of this reagent lies in the fact that vitamin A, certain sterols, and fatty acids, carotenoids, and tocopherols; all give color reactions which interfere with the vitamin D color. The vitamin A color is particularly intense and troublesome.

Milas, Heggie, and Raynolds[4] describe a method in which the non-saponifiable fraction of a fish liver oil is heated with maleic anhydride in 1,4 dioxane in order to destroy the vitamin A, carotenoids, and possibly 7-dehydrocholesterol; after which the treated non-saponifiable fraction is added to antimony trichloride solution and the $E^{1\%}_{1cm}$ at λ500 mμ taken as an indication of the amount of vitamin D present. Several samples were run using this method but consistent results could not be obtained. Upon further investigation it was found that, contrary to the statement of the authors, maleic anhydride in dioxane reacts with vitamin D under the

conditions recommended and results in the destruction of part of the vitamin D as well as the vitamin A. This was tested by running samples of irradiated ergosterol in corn oil, containing no vitamin A, with and without the maleic anhydride treatment. The treated samples gave lower $E^{1\%}_{1cm}$ values at λ500 mμ with antimony trichloride than did the untreated samples, as shown in the accompanying Table II.

Sample No.	Treatment	$E^{1\%}_{1cm}$
33209*	As received	7.71
"	Non-saponifiable fraction	8.12
"	Milas procedure using fresh maleic anhyd. and commercial dioxane	2.79
"	Milas procedure with Eastman Kodak Co. maleic anhyd. and commercial dioxane	3.76
"	Milas procedure with fresh maleic anhyd. and purified dioxane**	2.59
"	Heated 1 hr. with dioxane alone	7.52
"	Heated 1 hr. with dioxane and .2g maleic anhyd.	4.92
"(I_a)	Milas procedure	4.02
"(II_a)	Milas procedure	3.50
"(I_b)	Milas procedure plus 15 min. heating after second addition of KOH	4.87
"(II_b)	Milas procedure plus 15 min. heating after second addition of KOH	3.63

Table II

* Sample #33209 is irradiated ergosterol in corn oil.

** The dioxane used in these tests was purified according to: J. Amer. Chem. Soc. 58, 2264 (1936)

These results show that in any case in which the sample is heated with maleic anhydride, a breakdown of the vitamin D_2 occurs; and that the breakdown is not due to impurities in the dioxane. The last four samples were heated after the second addition of alcoholic KOH to determine whether the breakdown product could be converted back to the free vitamin. However, if the effect takes place it is very small--as shown by the very slight increase in $E^{1\%}_{1cm}$ value of the heated samples. This indicates that either the loss in vitamin D_2 upon heating with maleic anhydride is not due to esterification or that the vitamin D_2 esters are not easily hydrolyzed.

Attention was next directed toward chromatographic adsorption as a means of separating vitamin D from interfering materials. Marcussen[2] reported that an effective separation of vitamin A from vitamin D could be made by adsorbing the vitamin A from a heptane solution of the non-saponifiable fraction of a fish liver oil. Hydraffin K_4, an activated carbon, was used as the adsorbent. Since neither Hydraffin K_4 nor heptane was available, a series of tests were made using Norite A as the adsorbent and hexane as the solvent, following the Marcussen procedure in other respects. It was found that only a slight separation of the two vitamins was possible by this method--both vitamins being very strongly held by the Norite A. Slightly better separation was obtained when a small amount of ether was added to the

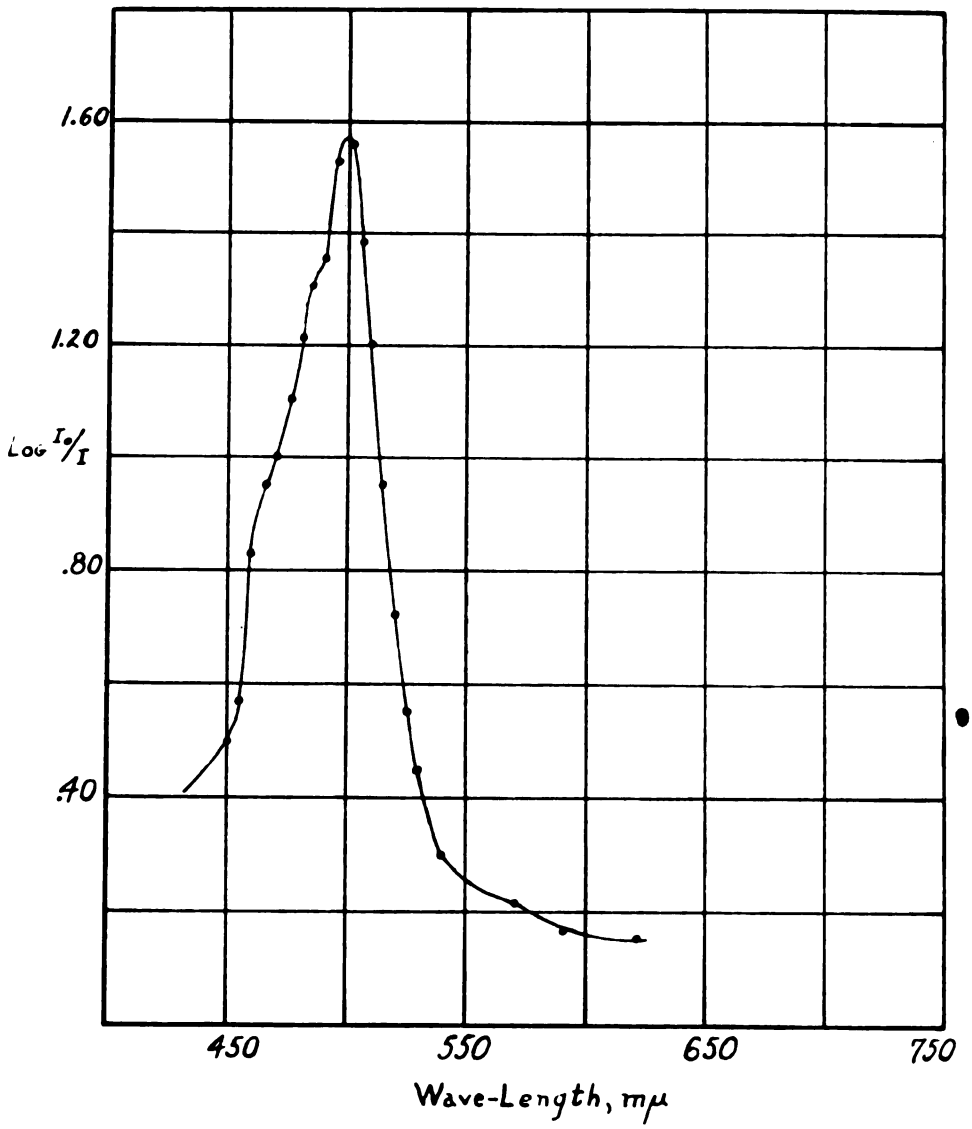

FIG. 2 ABSORPTION CURVE OF IRRADIATED ERGOSTEROL IN CHLOROFORM TREATED WITH $SbCl_3$ REAGENT

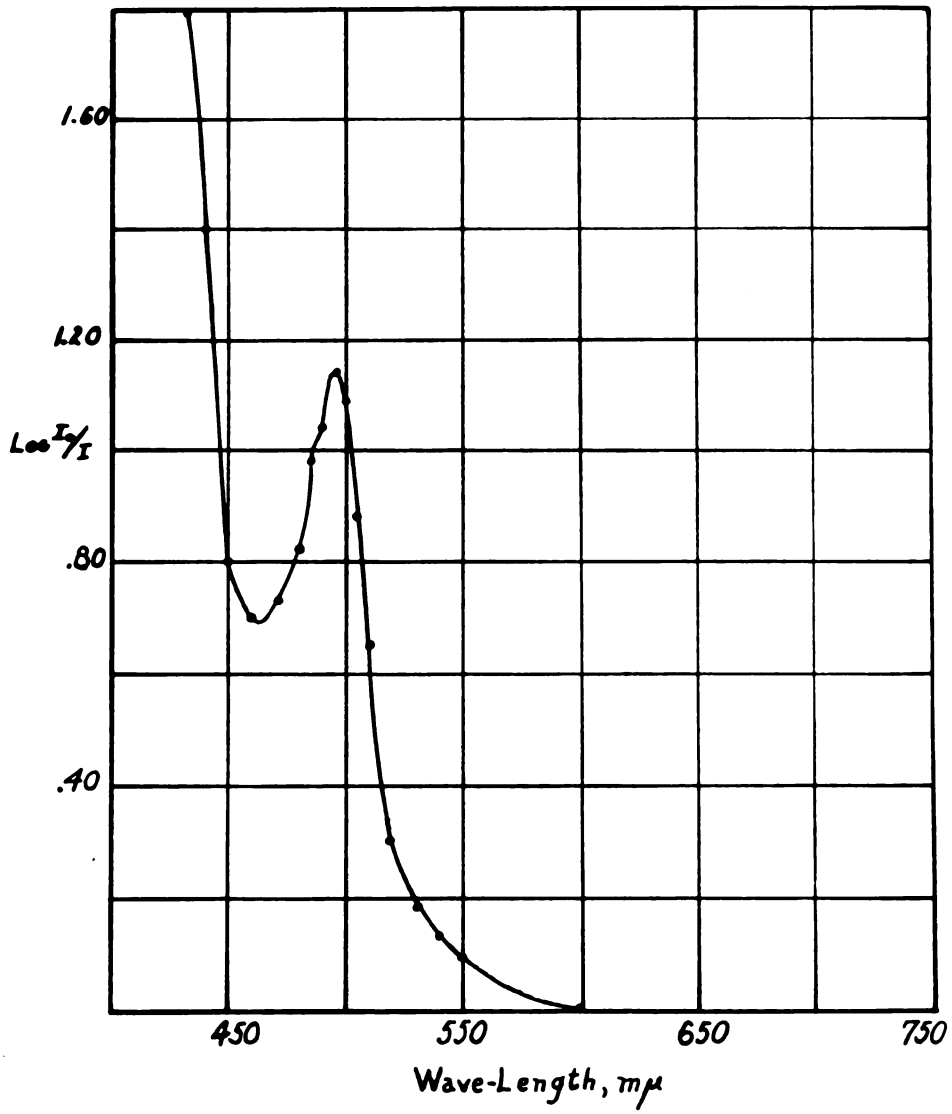

FIG. 3 ABSORPTION CURVE OF CHOLESTEROL IN CHLOROFORM TREATED WITH $SbCl_3$ REAGENT

hexane, but there was still too slight a difference in the adsorptive power of the Norite A, with respect to the two vitamins, to make the separation complete.

Several other adsorbent-mixed solvent combinations were investigated before the activated bentonite-ether-hexane combination was found. These included activated aluminum oxide, calcium hydroxide, dicalcium phosphate, zinc carbonate, and bentonite; chloroform, benzene, carbon tetrachloride, and hexane. Of the adsorbents, zinc carbonate, bentonite clay, and activated bentonite were the only ones which gave positive results. The action of the zinc carbonate was particularly interesting because of the large number of sharply defined bands which were visible in ultraviolet light after the non-saponifiable fraction of a fish liver oil had been run through a column of this material. No sharp separation of vitamins A and D was obtained with this adsorbent, but a more complete investigation of the bands produced would make an interesting problem. In each of the above trials the adsorption column was cut into sections, each section extracted with ether, the resulting solution evaporated to dryness and the residue taken up in chloroform, and antimony trichloride solution added. A qualitative examination of the absorption spectrum of the resulting solution, particularly in the regions λ620 and 500 mμ, gave an indication of the degree of separation of vitamins A and D.

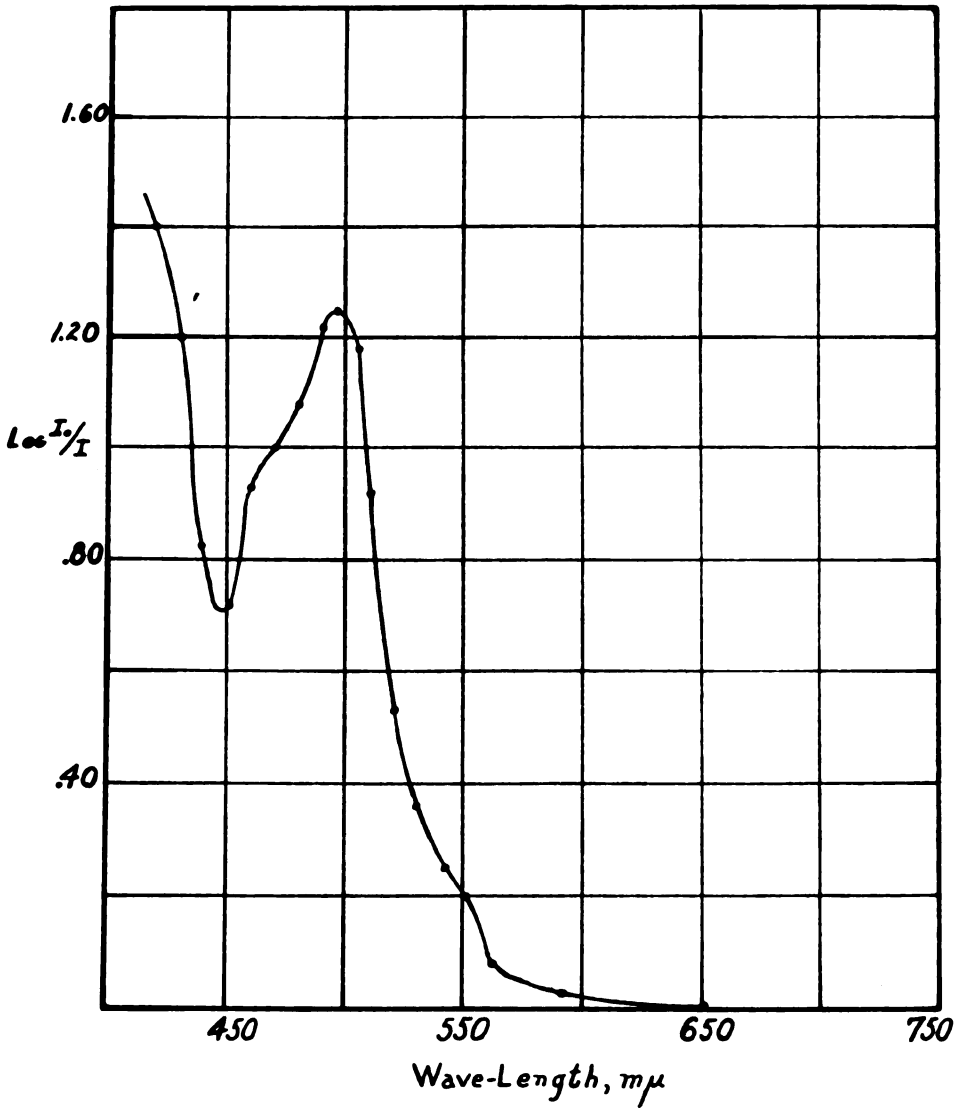

FIG. 4 ABSORPTION CURVE OF ADSORBATE FROM CHROMATOGRAPH COLUMN IN CHLOROFORM TREATED WITH $SbCl_3$ REAGENT

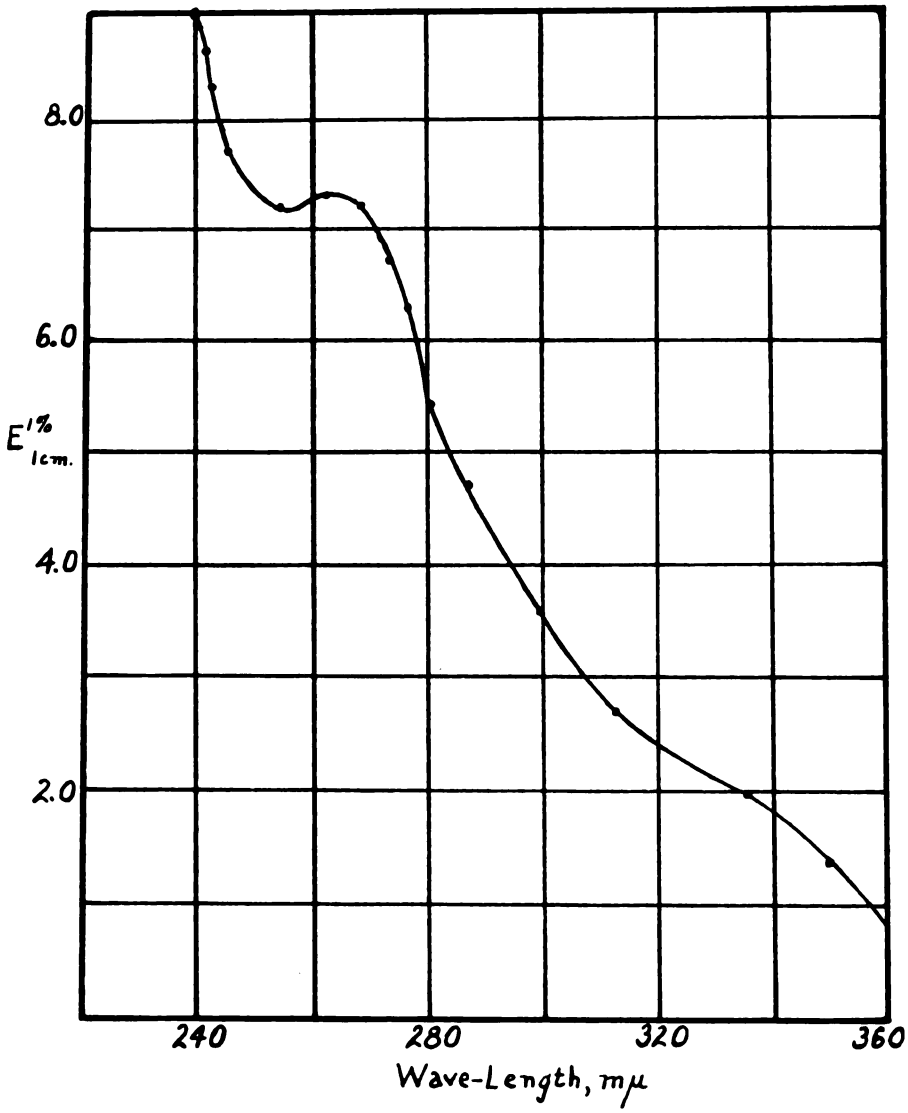

FIG. 5 ABSORPTION CURVE OF IRRADIATED ERGOSTEROL IN CORN OIL IN HEXANE

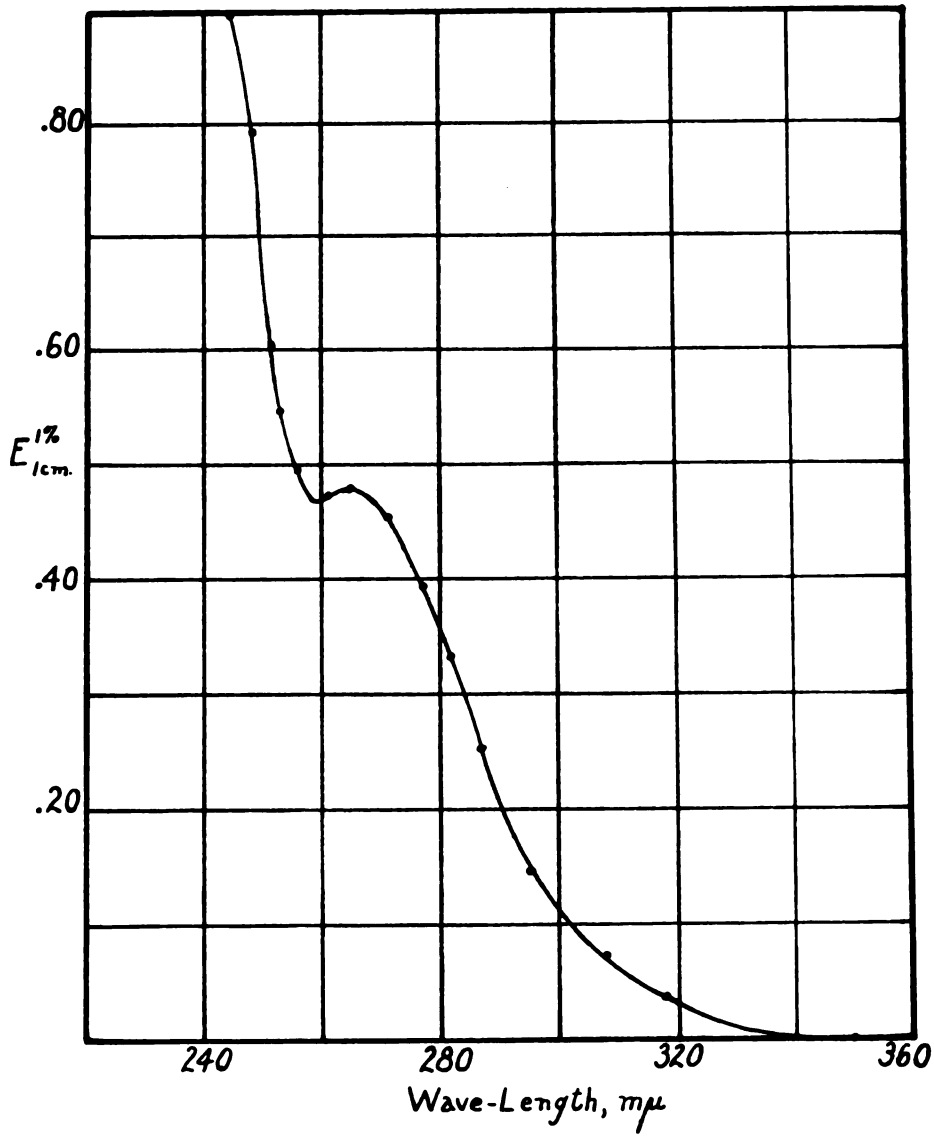

FIG. 6 ABSORPTION CURVE OF ADSORBATE FROM CHROMATOGRAPH COLUMN IN HEXANE

The first positive separation was obtained using bentonite as the adsorbent and a hexane solution of the saponified sample. With this combination a blue band was formed at the top of the adsorption column which when extracted and tested with antimony trichloride solution, showed very strong absorption at λ620 mμ. This was due to the presence of a large concentration of vitamin A. The lower portion of the column and the filtrate, when tested in the same way, showed strong absorption at λ500 mμ and very slight absorption at λ620 mμ. A better separation was obtained when a small amount of ether was added to the hexane used as the solvent. This was to be expected since the adsorption process had already shown the vitamin D to be less tenaciously held by the bentonite, and consequently the desorbing action of the ether would be more effective toward this vitamin. The final modification of the adsorption procedure was the substitution of activated bentonite for bentonite as the adsorbent. This is a finely divided product which results in more uniform adsorption columns.

Figure 2 shows the absorption spectrum of irradiated ergosterol (vitamin D_2), Figure 3 that of commercial cholesterol, and Figure 4 that of the adsorbate from the chromatograph column--all after treatment with antimony trichloride solution. It will be noted that Figure 4 is the type of curve which would be expected to result from a mixture of the pure vitamin D_2 and

cholesterol. This is consistent with the statement by earlier workers that sterols are among the interfering substances present in fish oils. Figure 5 and Figure 6 show the similarity between the ultraviolet absorption curves of the adsorbate from a chromatograph column and a solution of irradiated ergosterol in corn oil, in hexane.

Assuming that free sterols constitute the greater part of the interfering materials left in such an adsorbate, the next step was to find a means of removing these sterols--either before or after the chromatographic adsorption. Freezing out of the sterols from a methyl alcohol solution by cooling to -15° C. was tried, but it was found that the removal by this means was not complete. The method finally adopted combined the freezing process with a precipitation of the remaining sterols with digitonin--before the adsorption. Figure 7 is the absorption curve obtained from a sample of the same oil as was used for Figure 4, after the sterols had been removed by the above treatment. It shows a marked decrease in absorption in the region below 450 m , and more nearly matches the curve for the pure vitamin. It was this treatment that was incorporated into the final procedure.

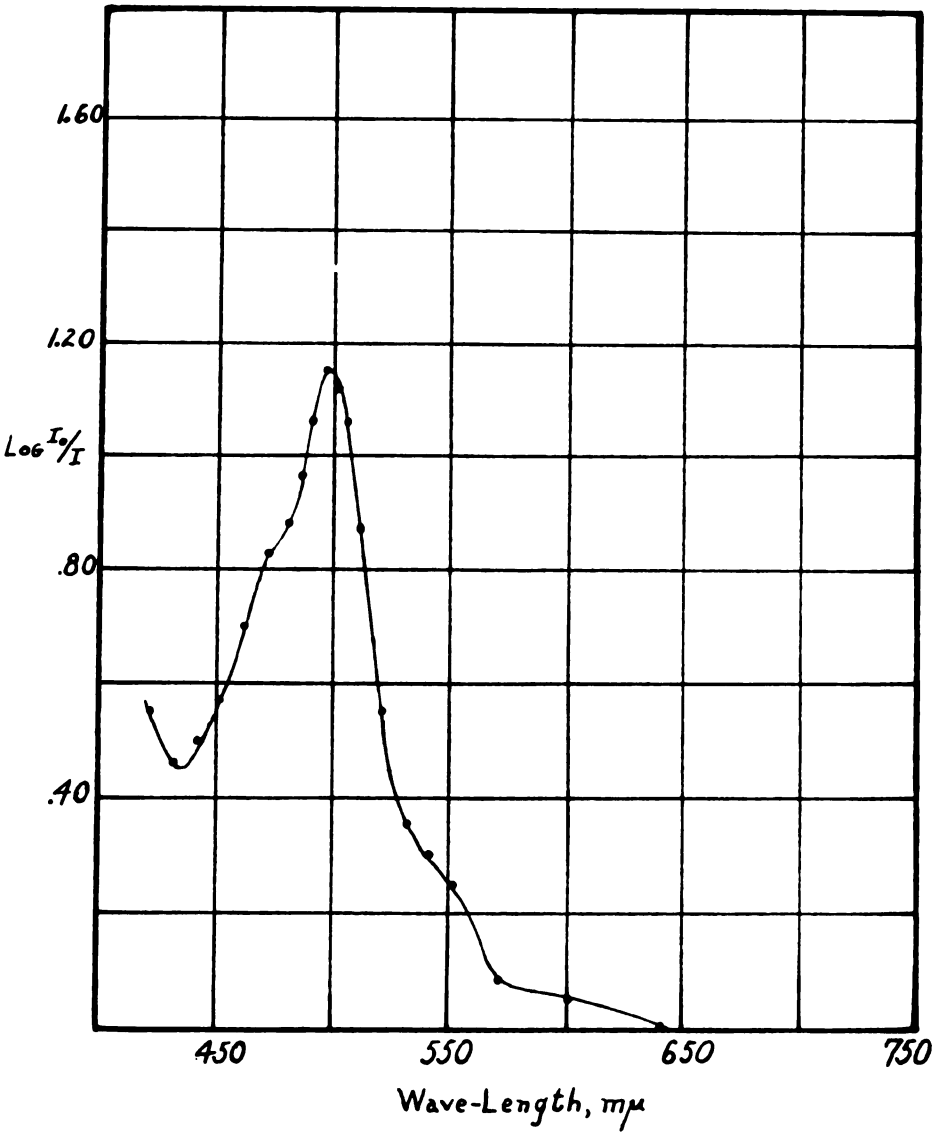

FIG. 7 ABSORPTION CURVE OF ADSORBATE FROM CHROMATOGRAPH COLUMN IN CHLOROFORM TREATED WITH $SbCl_3$ REAGENT. SAMPLE TREATED WITH DIGITONIN

Summary

1. A method for the quantitative determination of vitamin D in fish liver oils has been presented; in which the oil is saponified, cooled and treated with digitonin to remove sterols, chromatographed to separate the vitamin A and D, and the vitamin D determined by measuring the absorption at λ500 mμ produced when the sample is added to a solution of antimony trichloride in chloroform.

2. A table is presented showing the results obtained on a series of seventeen samples, using the procedure outlined above.

****

References

(1) Brockmann and Chen, Zeit. physiol. Chem. 241, 129-33 (1936)

(2) Marcussen, Dansk. Tids. Farm. 13, 141-159 (1939)

(3) Nield, Russell, and Zimmerli, J. Biol. Chem. 136, 73-79 (1940)

(4) Milas, Heggie, and Raynolds, J. Ind. Eng. Chem. Anal. Ed. 13, 227-231 (1941)

(5) Brockmann and Busse, Zeit. physiol. Chem. 256, 252 (1938)

T541 T658 146126

Tomkins

I. The ultraviolet absorption of vitamin K_1 and the effect of light. II. The quantitative chromatographic determination of vitamin D in fish liver oils.

Louis R. Baker	Nov 2 '5