PART 1

A MODIFIED PHYSICAL-CHEMICAL METHOD FOR THE DETERMINATION OF VITAMINS D IN FISH LIVER OILS

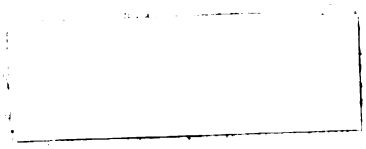
PART 2

STUDIES IN THE CHROMATOGRAPHY OF IRRADIATED ERGOSTEROL ON ACTIVATED SUPERFILTROL

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

Jacob Mitchell Hage

1943



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PART II

STUDIES IN THE CHROMATOGRAPHY OF IRRADIATED ERGOSTEROL ON ACTIVATED SUPERFILTROL

by

Jacob Mitchell Hage

A THESIS

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PART I

A Modified Physical-Chemical Method for the Determination of Vitamins D in Fish Liver Oils

A physical-chemical method for determining vitamins D in fish liver oils has been developed by Ewing and Kingsley (5). The authors based their procedure upon a two-step chromatographic treatment where E(1 percent, 1 cm.) is determined first for the combined vitamins D and sterols, and second for the separated sterols. By difference, the value for the vitamins D is obtained.

The second step in the above chromatographic treatment not only requires a considerable amount of time, but unless the procedure is very carefully followed, the E(1 percent, 1 cm.) for the sterols is likely to be high. From a critical study of this second chromatographic treatment, it has been concluded that errors are due to rapidity of drawing solution through column and also cracking of the packed superfiltrol. This would tend to cause incomplete removal of the vitamins D from sterols thereby giving high values for the E(1 percent, 1 cm.) of the sterols.

To avoid the above difficulties and to simplify and shorten the entire procedure, the following treatment is suggested to replace the original second chromatographic treatment:

Referring to the procedure of Ewing and Kingsley (5), page 303, column two, at the end of paragraph five, we have

the following sentence: "The sample at this point contains vitamins D and sterols". For the procedure after that sentence, substitute the following:

Procedure.--To correct for the absorption at 500 mμ due to the sterols present, another 1 ml. aliquot of the chloroform solution of vitamins D and sterols is evaporated to dryness in a 125 ml. erlenmeyer and then taken up in 25 ml. of benzene-skellysolve (2:1). To this is added directly a definite quantity of superfiltrol (a 5/8" test tube filled to a depth of one inch) and the mixture is allowed to stand for thirty minutes with frequent swirling. The superfiltrol is then filtered off, collecting the filtrate in a 125 ml. erlenmeyer. The flask should be rinsed twice with 10 ml. portions of benzene-skellysolve, adding each portion after the preceding portion has gone through. These portions should be added so as to wash down the sides of the filter paper. The filtrate is then evaporated to dryness under reduced pressure, and the residue taken up in 1 ml. of chloroform. To this is added 10 ml. of the antimony trichloride reagent. Using a 2 cm. absorption cell, the extinction is determined at 500 mμ exactly three minutes after mixing.

From the two extinction values, the E(1 percent, 1 cm.) is calculated for vitamins D and sterols combined, and for sterols alone. The difference between these two values gives the E(1 percent, 1 cm.) for the vitamins D in the original sample. This value multiplied by the factor 19300 gives the

potency in U. S. P. Units of vitamins D per gram of oil.

Experimental Determination of Time Necessary for Complete Separation of Vitamins D from Sterols.--It has been found that Vitamins D_2 and D_3 are very rapidly and completely adsorbed by activated superfiltrol from a solution in benzene-skellysolve (2:1), whereas the sterols which are present in natural fish liver oils in small amounts are adsorbed very slowly and to a small extent. To measure the time necessary for complete removal of vitamins D from sterols in a benzene-skellysolve (2:1) solution with activated superfiltrol, reference oil 47761 was used. A sample of this oil was run through the procedure up to the point where the modification for determination of E(1 percent, 1 cm.) for sterols present replaces the older second chromatographic treatment. We have at this stage 10 ml. of chloroform solution containing vitamins D and sterols. A series of 1 ml. aliquots of this solution are taken and run through the above modified procedure. However, instead of swirling frequently for thirty minutes, time intervals of one, five, ten, twenty, thirty, and sixty minutes were used. They were then filtered and evaporated to dryness under reduced pressure. The extinction for each sample was then determined as above. The results are shown in Table I. These values when plotted, Fig. 1, show very clearly the rapid removal of vitamins D and the leveling out of the curve at an extinction value due to the sterols present.

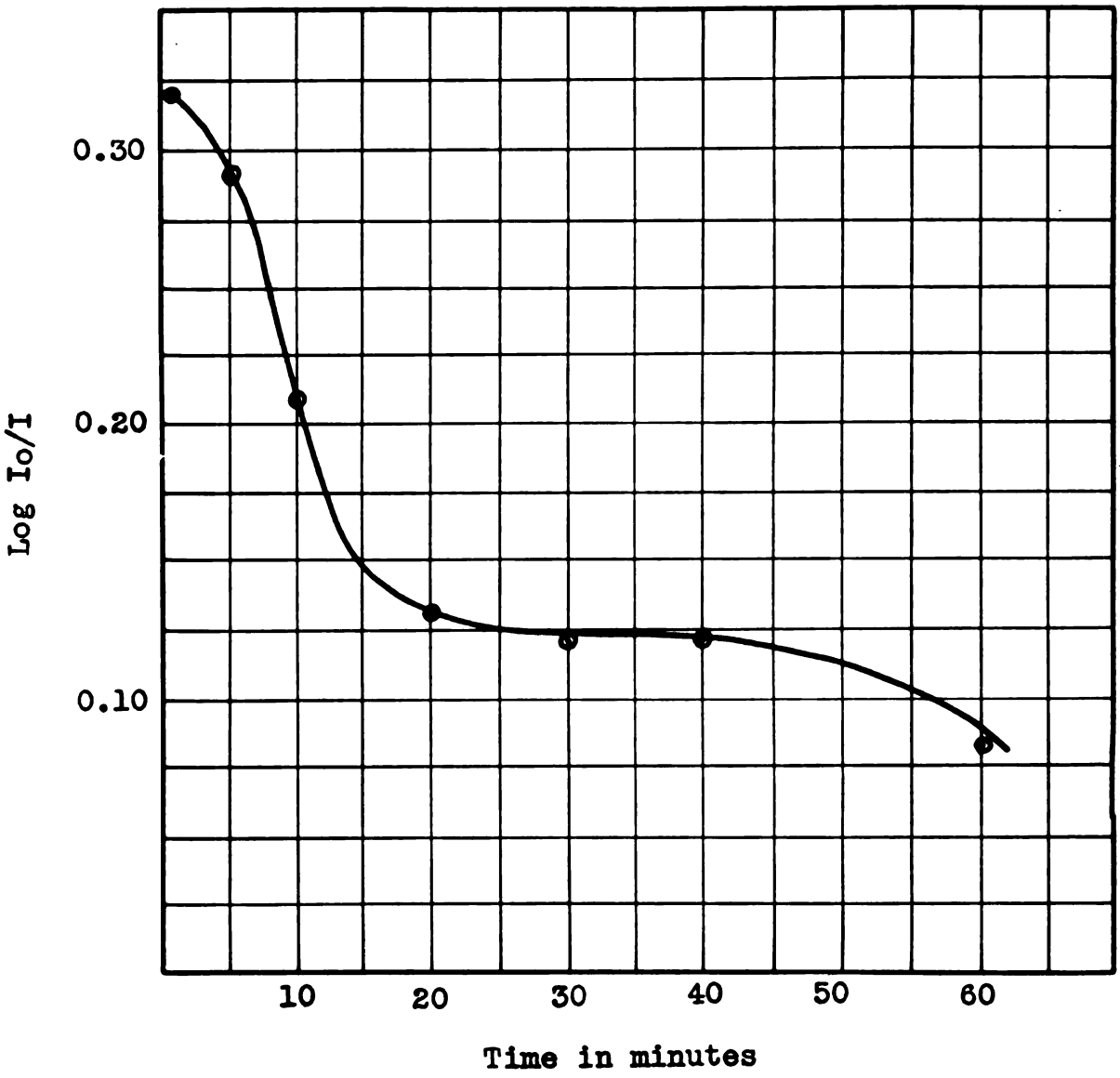


Fig.1.--Adsorption of reference oil 47761 from a solution in benzene-skellysolve (2:1) upon activated superfiltrol.

Table I

Adsorption of Reference Oil 47761 upon Activated Superfiltrol from Benzene-Skellysolve (2:1)

Time (Min.)	1	5	10	20	30	40	60
Log I_o/I	.32	.29	.21	.13	.12	.12	.08

Since an extinction value in the neighborhood of .14 has been considered correct for the sterols present in reference oil #47761, the time necessary for complete removal of vitamins D from sterols in ordinary fish liver oils has been set at thirty minutes. If, instead of swirling by hand, a device was used whereby the swirling could be done mechanically, this time period could be shortened.

Experimental Results.—The method was applied to the following types of fish liver oils (Tables II and III):

A High vitamin D fish liver oils

B Vitamin D liver oil concentrates

C Vitamin D liver oil distillates

The results in Tables II and III are self explanatory and show that the modified procedure is capable of reproducibility, if the details are followed exactly.

A fairly good agreement is indicated between the modified physical-chemical procedure and the bioassay. The modified

procedure also agrees very well with the original procedure. For those results which are somewhat lower than the results of Kingsley and Ewing (5), it is believed that a slight deteriorating of the oil has taken place. No oils of a potency less than 10,000 U. S. P. Units per gram were run. The time saved in using this modification on a set of four runs is approximately two hours. This makes it possible to carry through such a set of runs in an eight-hour day.

Peroxides in Ether.--Ether as obtained commercially very often contains peroxides. Among the simpler of these substances are ethyl hydrogen peroxide, $C_2H_5 \cdot O \cdot O \cdot H$, a colorless liquid; diethyl peroxide, $C_2H_5 \cdot O \cdot O \cdot C_2H_5$, a liquid boiling at 65°C; acetone peroxide, $(C_3H_6O_2)_2$, boiling at 132°C and triacetone peroxide, $(C_3H_6O_2)_3$, melting at 97°C. Many of these compounds are explosive.

Ether is used in the above procedure for making the developer, skellysolve-ether-ethyl alcohol (50-10-1), and for eluting. For proper developing of the columns, it is very important that the ether be free of peroxides or the bands will be dull in color and not very sharp.

Peroxides normally present in ordinary ether give a definite color reaction with antimony trichloride reagent. If the concentration of these peroxides is fairly high, they will be carried through the column of activated superfiltrol and will remain with the vitamins D and sterols. This is especially true in the case of the 25 mls. of ether used for

eluting after the first chromatograph. This ether passes through a column of activated superfiltrol which is only a few centimeters long and, therefore, any peroxides present will be carried through. The results in case this happens will invariably be high. It is recommended that highly purified ether be used throughout and that it be tested daily by dissolving a few crystals of potassium iodide in 1% starch solution and adding a few milliliters of the ether. There will be a starch-iodine reaction if peroxides are present. No reliance can be placed on the superfiltrol to remove peroxides. It has been shown that peroxides were present in the solutions with vitamins D and sterols after the first chromatograph when using impure ether.

Ethyl Alcohol Present in Chloroform.--Ethyl alcohol is used as a preservative in chloroform. It was, therefore, believed worth while to check the effect of alcohol in the chloroform used for the preparation of antimony trichloride reagent. For this purpose, chloroform which had been washed ten times with water to remove alcohol, dried over anhydrous sodium sulfate and doubly distilled was used. Antimony trichloride reagent was prepared using this chloroform and various amounts of ethyl alcohol was added to 25 ml. portions of this reagent. Results show that ethyl alcohol in the reagent tends to lower the extinction value for vitamins D as well as sterols. These results are shown in Fig. II for reference oil #47761. The aliquots taken were run using the above

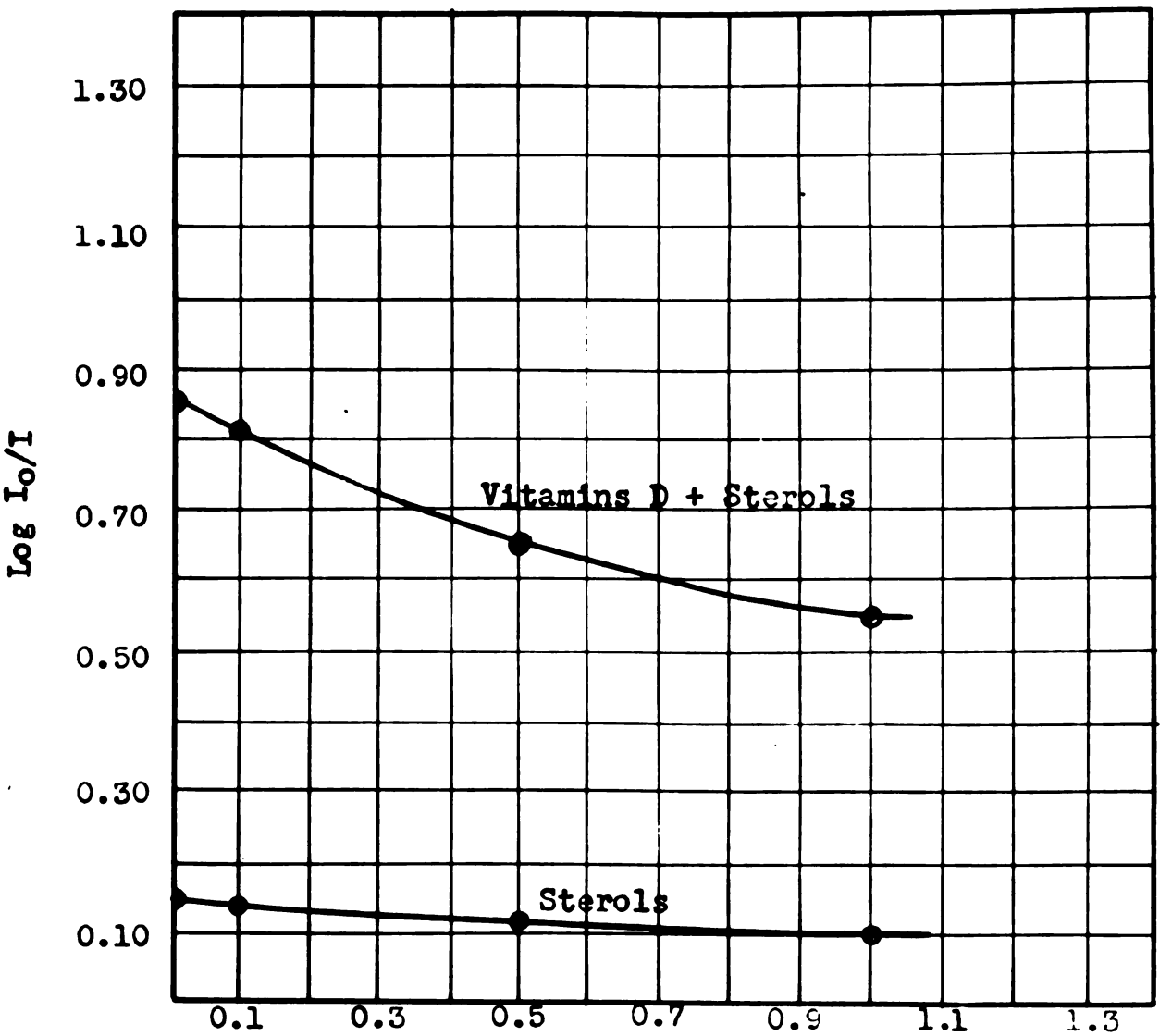


Fig. 2.--Effect of alcohol in the antimony trichloride reagent upon the extinction for vitamins D plus sterols and for sterols in reference oil 47761.

prepared reagents. In reagent containing ethyl alcohol, hydrogen chloride is liberated indicating the following reaction:

$$CH_3-\overset{\overset{O}{\|}}{C}-Cl + C_2H_5OH \longrightarrow CH_3-\overset{\overset{O}{\|}}{C}-OC_2H_5 + HCl$$

Decomposed Chloroform.--Chloroform which does not contain alcohol tends to decompose, forming phosgene. The presence of phosgene can be detected by odor or testing with starch-iodide solution. Shaking with activated charcoal tends to remove phosgene from the chloroform. This treatment must, therefore, be used daily. Contaminated chloroform of this type tends to give low results for both sterols and vitamins D. No extensive study has been made of this.

Modified Reagent.--A reagent which has been treated with metallic zinc, tin or antimony to remove pentavalent antimony has been suggested by Nield, Russell and Zimmerli (7). This reagent is claimed to shorten the initial reaction period and to cause less variation in color. Use of a reagent treated with zinc upon samples of cholesterol tends to confirm this claim. No directions for the preparation of this reagent was given so the following is suggested: Dissolve 18 g. CP antimony trichloride in 100 mls. of freshly purified chloroform. After completely dissolved, add 5 g. of zinc dust. Allow to stand for thirty minutes, shaking at intervals. The solution must be protected from the air. Filter into reagent bottle, add 2 mls. of acetyl chloride and allow to stand thirty minutes before use.

Discussion.--The chromatogram of the non-saponifiable portion of fish liver oils upon activated superfiltrol from a solution in skellysolve-ether-ethyl alcohol (50-10-1) is very interesting. A few seconds after adding the solution, a deep blue band develops about one-half centimeter from the surface of the superfiltrol. As the solution continues to pass through the column, a green band forms directly under the blue band and is separated from it by a very dark, narrow, greenish-blue band. The intensity of color and degree of banding depends, somewhat, on the nature of the fish liver oil, but they are all of the same general character. In the case of concentrates and distillates, the banding is not as intense, although they are similar to those of the natural fish liver oils. Upon developing the above chromatogram with skellysolve-ether-ethyl alcohol (50-10-1), the blue and green portions change to a deep gray and the narrow, greenish-blue band proceeds slowly down the column. The blue eventually fades out, leaving the band olive green. This band is formed whether Sudan III is added or not. No cases have been observed where it does not develop. It is, therefore, believed that the use of Sudan III in the determination of natural fish liver oils, concentrates and distillates by the above procedure is unnecessary.

It has been shown that peroxides in the ether will cause the E(1 percent, 1 cm.) of the vitamins D plus sterols as well as that of the sterols to be high. Since a difference is taken, it often happens that the final result comes out very well.

However, the amount of peroxide in the aliquot taken for the sterol correction may be lowered by the succeeding treatment and, in that case, the error will not cancel out. Experiments by R. B. Young (9/22/42, 9/8/42, 10/5/42, etc.) show the effects of peroxides in the ether. He, however, maintains in all cases that this is due to moisture from incomplete drying or poor antimony trichloride reagent. Time studies show that after fifteen minutes of drying at 50°C and under reduced pressure all the residues obtained in the above procedure show no change in E(1 percent, 1 cm.) upon further drying.

Reagent may cause some change in E(1 percent, 1 cm.) values from day to day, but these would probably be small fluctuations and not consistently high as is the case with peroxide contamination of the ether.

It is of interest to also point out that R. B. Young (7/7/42, 8/5/42, 9/9/42) frequently encountered E(1 percent, 1 cm.) values for the sterol correction using the old second chromatographic procedure which were high. He maintained again that this was either due to moisture because of incomplete drying or poor reagent. It is much more probable that vitamins D were coming through the column as explained above causing high sterol corrections.

If E(1 percent, 1 cm.) values are high for vitamins D plus sterols, as well as sterols, it must be assumed that some contaminent (peroxides) is causing the trouble. If, on the

other hand, the E(1 percent, 1 cm.) is normal for vitamins D plus sterols, and high for sterols, it must be assumed that vitamins D are not being removed completely from the sterols.

Table II

Comparative Vitamins D Values of Fish Liver Oils

by Physical-Chemical and Biological Methods

Sample No.	Weight of Sample, Grams		1% E 1 cm. 500 mμ	Calcd. U.S.P. Units/g.	Biological Method U.S.P. Units/g.	Diff. %	Ewing & Kingsley (5)
		A.	High Vitamin D Fish Liver Oil				
47761	1		0.79	15,250			
			0.80	15,450			
			0.77	14,875			
			0.78	15,050			
			0.77	14,875			
			0.77	14,875			
			0.78	15,050			
			0.77	14,875			
			0.76	14,675			
			0.77	14,875			
			0.77	14,875			
			0.78	15,050			
			0.77	14,875			
		Av.	0.775	14,975	15,000	-0.17%	15,000
p6846	.8		0.99	19,100			
			0.99	19,100			
			1.03	19,875			
			1.02	19,675			
		Av.	1.01	19,500	20,000	-2.5%	20,000

Table II (cont'd.)

Sample No.	Weight of Sample, Grams		1% E 1 cm. 500 mμ	Calcd. U.S.P. Units/g.	Biological Method U.S.P. Units/g.	Diff. %	Ewing & Kingsley (5)
41860	1		0.79	15,250			
			0.77	14,875			
		Av.	0.78	15,000	16,000	-6.2%	15,400
57481	1		0.70	13,500			
			0.73	14,100			
		Av.	0.715	13,725	16,500	-16.7%	14,800
65221	1		0.71	13,700			
			0.71	13,700			
		Av.	0.71	13,700	14,000	-2.1%	13,550
76892	1		0.80	15,450			
			0.81	15,625			
		Av.	0.805	15,525	16,000	-3.0%	15,450
28283	.8		0.60	11,575			
			0.62	11,975			
		Av.	0.61	11,775	11,500	+2.4%	
29263	.8		0.67	12,925			
			0.63	12,150			
		Av.	0.65	12,550	13,500	-7.7%	

Table II (cont'd.)

Sample No.	Weight of Sample, Grams		1% E 1 cm. 500mμ	Calcd. U.S.P. Units/g.	Biological Method U.S.P. Units/g.	Diff. %	Ewing & Kingsley (5)
21273	.8		0.65	12,550			
			0.60	11,575			
		Av.	0.625	12,050	12,000	+0.42%	
29653	.8		0.47	9,075			
			0.48	9,275			
		Av.	0.475	9,175	11,500	-21.8%	
Hg 1	.8		0.85	16,400			
			0.82	15,825			
			0.85	16,400			
			0.81	15,625			
			0.81	15,625			
		Av.	0.83	16,025	17,500?	-8.5%	
65231	1		0.77	14,875	14,000	+6.2%	13,000
	B. Vitamin D Fish Liver Oil Concentrates						
44470	.07		10.7	206,500			
			11.0	212,300			
		Av.	10.85	209,400	240,000	-12%	233,500
26693	.3		2.38	45,925			
			2.27	43,800			
		Av.	2.32	44,775	45,000	-0.5%	

Table II (cont'd.)

Sample No.	Weight of Sample, Grams	1% E 1 cm. 500 mμ	Calcd. U.S.P. Units/g.	Biological Method U.S.P. Units/g.	Diff. %	Ewing & Kingsley (5)
	C. Vitamin D Fish Liver Oil Distillates					
44080	.5	1.54	29,725			
		1.52	29,325			
		Av. 1.53	29,525	31,000	-5%	29,700

Table III

Results of D. H. Baker Using Modified Procedure

Sample No.	Weight of Sample, Grams		1% E 1 cm. 500 mμ	Calcd. U.S.P. Units/g.	Biological Method U.S.P. Units/g.	Diff. %
47761	1		0.748	14,400		
			0.737	14,200		
			0.771	14,900		
		Av.	0.752	14,500	15,000	-3.3%
Hg 1	.8		0.853	16,400		
			0.840	16,200		
		Av.	0.846	16,300	17,500?	-6.9%
p6846	.8		0.99	19,100		
			0.99	19,100		
		Av.	0.99	19,100	20,000	-4.5%

PART II

Studies in the Chromatography of Irradiated Ergosterol on Activated Superfiltrol

Chromatography is the process of resolution of mixtures by adsorption on Tswett columns. The principle objectives attainable through use of chromatography are (1) as listed:

(1) Resolution of mixtures

(2) Determination of homogeneity of chemical substances

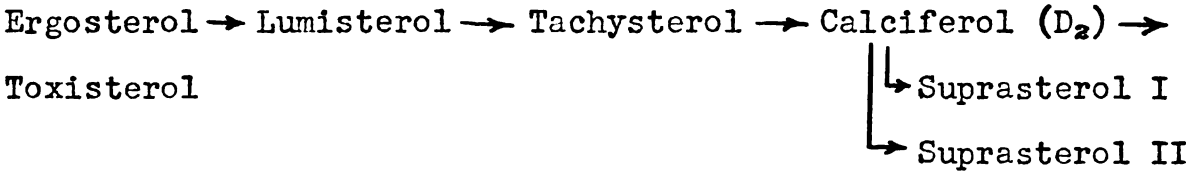
(3) Purification of substances

(4) Quantitative separation of one or more substances from complex mixtures

(5) Determination of molecular structure

The following work is an attempt to apply some of these uses to irradiated ergosterol in vegetable oil.

Irradiation of ergosterol in solution with ultraviolet light leads to the formation of the anti-rachitic Vitamin D_2 (calciferol). The photo chemical reaction which takes place has been the subject of extensive investigation and it is now believed that a series of reactions take place according to the following scheme (2):



Investigation seems to indicate that the lumisterol is

formed which in turn is converted into tachysterol and this is converted into calciferol (3). Therefore, upon irradiating ergosterol a mixture results which contains all or various amounts of the above substances.

Apparently, no chromatographic work has been done on irradiated ergosterol. Esters of lumisterol have been purified by filtration of their solutions through a column of alumina (4). The most successful methods of separating the constituents of irradiated ergosterol have so far been purely chemical, and this, of course, has not been quantitative.

The application of the physico-chemical procedure of Ewing and Kingsley (5) to irradiated ergosterol dissolved in corn oil have not been very satisfactory. Using this method, the results are very often low.

Equipment and Reagents.--The equipment and reagents used were the same as used in the work by Ewing and Kingsley (5). The ether, however, was dried over anhydrous sodium sulfate instead of phosphorus pentoxide before adding sodium.

Experimental Procedures

In the procedure developed for natural fish liver oils (5), activated superfiltrol was used as the adsorbent and a skellysolve-ether-ethyl alcohol (50-10-1) mixture was used as solvent and developer. In order to correlate results with the original procedure, it was decided to use the same adsorbent and developer. The term "irradiated ergosterols"

refers to solutions of irradiated ergosterol in vegetable oil.

Procedure A.--The sample of the oil is weighed out and dissolved in 10 ml. of purified chloroform. A 1 ml. aliquot of this solution is taken and to it is added 10 ml. of antimony trichloride reagent. The mixture is swirled for thirty seconds. Exactly three minutes after adding reagent, the extinction is measured on the Bausch and Lomb visual photometer, using a 1 cm. cell. This value of log. I_o/I is converted to E(1 percent, 1 cm.). This is multiplied by the factor 19300 to convert to D units per gram of sample. The above factor was determined experimentally from work on Vitamin D_3. However, since Vitamin D_2 gives the same maxima at 500 mμ as D_3 with antimony trichloride reagent, no error should be introduced by using this factor.

Procedure B.--The sample is weighed out and to this is added 10 ml. of N/2 alcoholic potassium hydroxide and the resulting mixture is kept at 70°C for one hour with frequent swirling. After this saponification treatment, the non-saponified residue is extracted with anhydrous-peroxide free ether, using the same procedure as outlined by Kingsley and Ewing (5). The resulting ether solution was evaporated to dryness under reduced pressure and the residue taken up in 10 ml. of purified chloroform and the extinction measured as in Procedure A.

Procedure C.--The sample of oil is weighed out and this is dissolved in 5 ml. of skellysolve-ether-ethyl alcohol (50-10-1). This is added very carefully to a chromatograph column containing superfiltrol which has been previously wet with 10 ml. of the above solvent. The flask is then rinsed with 5 ml. more of the solvent and this is added to the column just before the previous 5 ml. disappears. A pressure differential of 6 cm. of Hg is maintained up to this point. The column is then developed with 35 ml. of the above solvent at a pressure differential of 8 to 10 cm. of Hg. As the column develops a faint band will be noticed moving down the column. After developing, the material down to 2 mm. below this band is removed and discarded. The remaining portion is eluted with 25 ml. of purified ether. The resulting filtrate is evaporated to dryness and taken up in 10 ml. purified chloroform. The extinction is measured as in Procedure A.

Procedure D.--This is Procedure B and C combined. The oil is first saponified and then run through the chromatograph. The extinction is measured as in Procedure A.

Procedure E.--This is Procedure C except that the column is cut down to 2 ml. above faint band. This material is repacked into column after removal of band. The column is then eluted with 25 ml. of purified ether. The extinction is measured as in Procedure A.

Procedure F.--Ewing and Kingsley (5).

The following oils were used in this work:

3772 Vit. D_2 standard; 1 gm = 200,000 Du/gm (32,000,000 Du/gm)

12722 " " " " " " " " "

66701 Irradiated ergosterol in corn oil, Parke, Davis & Co.

45120 " " " " " " " "

78272 " " " " " " " "

15153 " " " " " " " "

13192 " " " " " " " "

84742 " " " " " " " "

26593 " " in sesame oil, " " "

G " 7-dehydro-cholesterol in corn oil, National Oil Products Co.

F " ergosterol in corn oil, National Oil Products Co.

B3494 " " in corn oil, Gelatin Products Co.

An examination of Table I reveals the following facts:

In the majority of cases irradiated ergosterols give results by Procedure A which are lower than the bioassay. In some cases, however, the results are higher. There is no correlation between deviations from the bioassay. If the bioassays are correct to within fifteen percent, the following conclusions seem to be indicated:

(a) In the case of oils which give high results with Procedure A, there must be additional substances present which

give a color reaction with antimony trichloride reagent. Lumisterol, tachysterol, etc., are known to give a reaction with the reagent.

(b) In the case of low results some of the calciferol seems to be in a form which does not allow it to enter into the color reaction. It is quite possible that this unavailable calciferol is tied up as an addition compound with lumisterol (6). Saponification with N/2 alcoholic potassium hydroxide fails to break any such addition compound.

(c) Upon chromatographing an irradiated ergosterol according to Procedure C, there is some holdback of calciferol in the column. This is not true for the calciferol standards which are made by dissolving pure calciferol in corn oil. Hence, again, it seems to be indicated that calciferol is tied up in an unavailable form in irradiated ergosterols. The material held back can be washed out of the column with ether as is shown in Procedure E. It is of interest to notice that Procedures A, B, and C give the same result with calciferol standard 3772, whereas Procedure D causes a lowering of about twenty-five percent in this same oil. Apparently, saponification causes calciferol to be held back on the chromatograph column.

(d) It is also of interest to observe that over the course of several months, the value of Oil F by Procedure A decreased from 636,000 to 440,000 Du/gm. This could be due to decomposition or conversion of calciferol into an

unavailable form such as an addition compound with lumisterol.

(e) From this work it follows that a successful method for the determination of calciferol in irradiated ergosterols must involve the following steps:

(1) Conversion of unavailable calciferol to free calciferol

(2) Separation of free calciferol from interfering substances such as lumisterol, etc.

Special Studies

The Nature of the Chromatogram.--A solution of irradiated ergosterol in vegetable oil forms a very simple visible chromatogram when taken up in skellysolve-ether-ethyl alcohol (50-10-1) and passed through a column of superfiltrol.

Shortly after adding the solution, a very faint orange band forms about 1 cm. from the surface of the superfiltrol. This band slowly progresses down the column upon developing with the above solvent. Under a pressure differential of 8 cm. of mercury, this band moves 3 cm. with 35 ml. of developer. No other bands are visible and the same chromatogram is obtained when the oil is saponified and extracted with ether before chromatographing. The band in the latter case, however, seems to be somewhat sharper and more intense. If the ether content of the developer is increased, no band is formed. If the ether content is decreased, the band moves down the column much slower. With a decrease in ether content, an increase in alcohol causes the band to move somewhat faster.

By examining aliquot portions of the filtrate as it comes through the superfiltrol column, it appears that nothing comes through until the last 10 ml. of the 35 ml. of developer passes through the superfiltrol. This seems to indicate further banding which is, however, invisible. Examination under ultraviolet radiation shows no such banding.

Identification of Above Band.--Since pure solutions of calciferol show only an extremely faint band of the above type, it was suspected that the band formed was either residual ergosterol or one of the intermediate substances. Upon passing a solution of pure ergosterol in skellysolve-ether-ethyl alcohol (50-10-1) through a column of superfiltrol, the band described above again formed. The faint band was cut out in some instances of runs made on irradiated ergosterol and the material eluted with ether. Upon filtering and evaporating off the ether, the residue gave a pink color reaction which is characteriè of ergosterol.

To further confirm this work varying amounts of pure ergosterol was added to samples of the calciferol standard 3772. These were then chromatographed as in Procedure C. The band formed in the sample containing no added ergosterol was extremely faint. In the samples containing ergosterol, the sharpness and density of the band noticeably increased with increased concentration of ergosterol. The resulting filtrates were evaporated to dryness, taken up in 10 ml. of chloroform and E(1 percent, 1 cm.) determined as in Procedure A.

Table II shows that there is no increase in the E(1 percent, 1 cm.) due to ergosterol coming through the column. It is doubtful if a quantitative separation is possible if the concentration of ergosterol is much higher than in Table II. Attempts to remove ergosterol by use of digitonin were not very successful.

Adsorption of Calciferol on Superfiltrol from a Solution in Benzene Skellysolve (2:1)

For this work a calciferol solution in skellysolve was used (General Mills). One milliliter of this solution upon evaporating to dryness gave an extinction of $E = \log I_o/I = .42$ by Procedure A. One milliliter samples of this solution were evaporated to dryness and taken up in 25 ml. of benzene-skellysolve (2:1). This was shaken with approximately five grams of superfiltrol for various periods of time and then filtered, evaporated to dryness and the extinction determined as in Procedure A. Table III shows the variation of $\log I_o/I$ with time. The results are also shown in Fig. 1.

Table II

Effect of Added Ergosterol to Calciferol Standard

Oil No.		E(1%, 1 cm.)	Du/g.
.07 g. #3772		.60	181,400
.07 g. "	+ .0002 g. ergosterol	.61	183,400
.07 g. "	+ .0004 g. "	.60	181,400
.07 g. "	+ .001 g. "	.60	181,400

Table III

Adsorption of Calciferol on Activated Superfiltrol

Time (min.)	0	5	10	20
Extinction (log I_o/I)	.42	.03	.02	.00

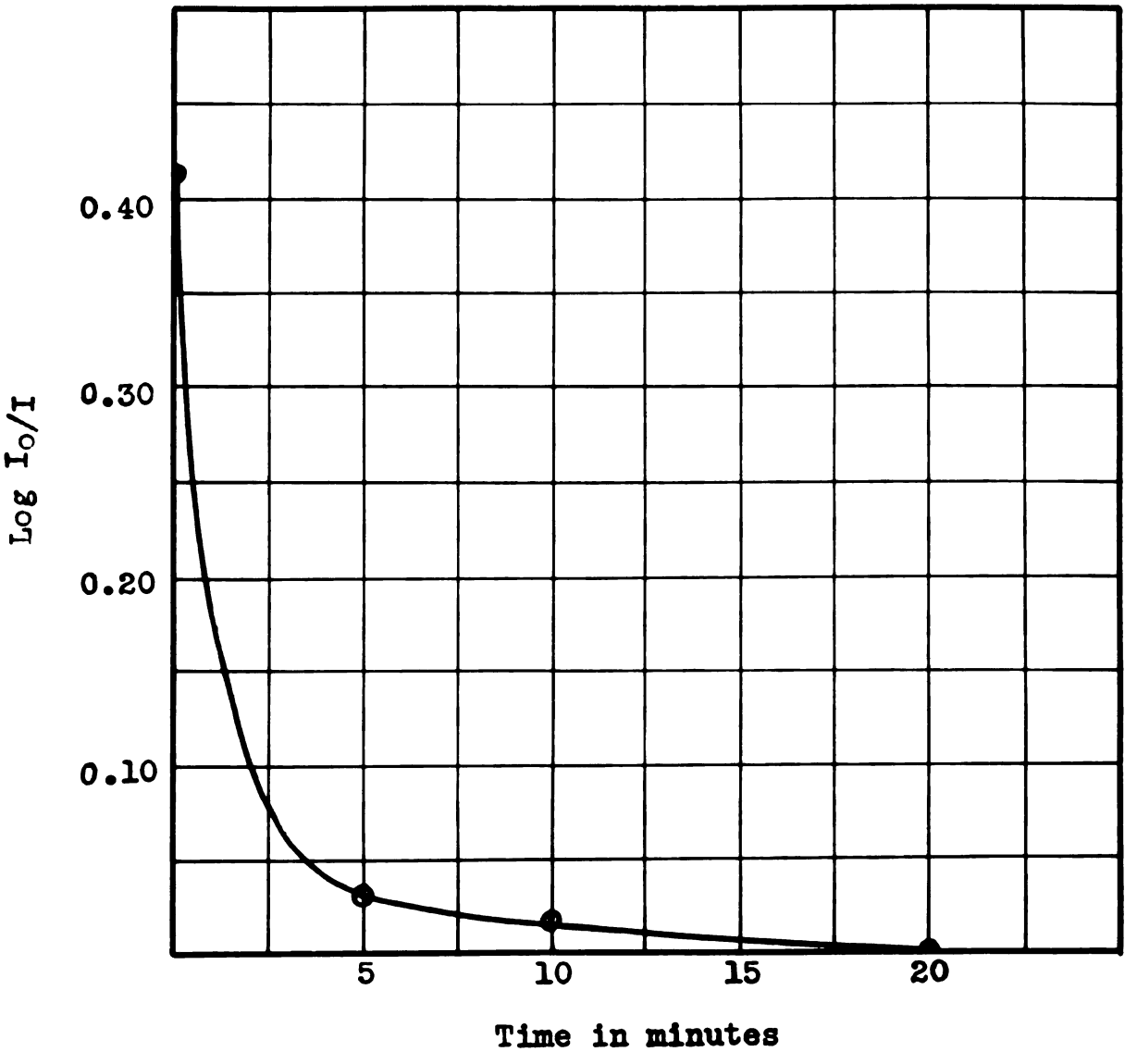


Fig. 1.--Adsorption of calciferol from a solution in benzene-skellysolve (2:1) upon activated superfiltrol.

Table I

Procedures A, B, C, D, E, F Applied to the Calciferol Standards and Irradiated Ergosterols

		Procedure					
Oil	Bioassay	A	B	C	D	E	F
3772	200,000	191,000	193,000	191,000	150,500	181,400	108,300
		187,200	187,200	191,000	148,600	181,400	
		191,000	191,000	191,000	144,700		
		191,000	187,200	196,800			
12722	200,000	208,400	187,200	118,300		191,000	
		208,400	196,800			191,000	
		206,500					
		206,500					
66701	250,000						140,000
							146,000
							142,000
45120	250,000	169,800	142,800	138,900			
78272	225,000	196,000	169,800	81,800		136,400	135,900
		191,000					127,400
15153		196,900	145,500	131,600		150,500	
		196,900					
		179,000					

Table I (cont'd.)

		Procedure					
Oil	Bioassay	A	B	C	D	E	F
13192	250,000	166,000	163,900	84,900		169,800	
		187,200	165,980			142,000	
		179,100				187,200	
		187,200					
84742	275,000	242,600	203,200	90,900		145,520	114,600
		227,500		90,900		187,200	116,800
		221,900					
26593	400,000	663,900	507,600	349,700		530,750	
		629,200	480,600	349,700		530,750	
		663,900	528,800	304,900			
		636,900					
		636,900					
G	225,000	152,500	154,400	144,250			78,600
		152,500	154,400				108,100
		151,500					
F	440,000	636,900	509,500	368,600	260,500		343,500
		592,500	528,500	368,600			326,200
		579,000	528,500	360,900			
		579,000	523,000	368,600			

Table I (cont'd.)

		Procedure					
Oil	Bioassay	A	B	C	D	E	F
		528,800					
		523,000					
		523,000					
		440,000					
B3494	450,000	700,600	654,300	496,000	389,900		
		667,800	565,500	565,200			
		735,300		565,200			
		743,000		579,000			

SUMMARY

Part I.--A modified procedure for determining vitamins D in fish liver oils has been developed and applied to a number of oils. By the use of this modification, the original procedure is greatly simplified and shortened.

Various causes of errors in the use of the procedure are discussed.

Part II.--In the majority of cases, the calciferol in irradiated ergosterols is in an unavailable form for determination by the use of antimony trichloride reagent. Saponification and chromatographing fails to convert the unavailable calciferol to an available form.

The nature of the irradiated ergosterol chromatogram from a solution in skellysolve-ether-ethyl alcohol (50-10-1) upon activated superfiltrol has been investigated and the one band formed has been shown to be due to ergosterol.

Separation of ergosterol from samples of calciferol in oil by chromatography has been accomplished.

The rate of adsorption of calciferol in benzene skellysolve (2:1) has been investigated and shown to be very rapid and complete.

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Pt.1: A modified physical-chemical method for the determination of vitamins D in fish liver oils;
Pt.2: Studies in the chromatography of irradiated ergosterol on activated superfiltrol.