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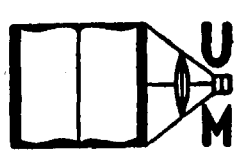

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STUDIES RELATING TO A PHYSICAL CHEMICAL METHOD OF VITAMINS D ASSAY

by

Robert Bruce Young

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INTRODUCTION

Numerous attempts have been made to quantitatively estimate vitamins D by chemical means. Much of this work was directed toward selective reactions, usually colorimetric, by which vitamins D could be determined in the presence of other substances. This search was complicated by the exceedingly complex mixture of vitamin D like substances and other organic material always present with the natural vitamin D.

One of the earlier methods, and probably the most specific for vitamins D, concerns the Tortelli-Jaffe color reaction (11). The test consists of layering an alcohol solution of the vitamin with a solution of bromine in chloroform. While the reaction is fairly specific, it is difficult in application and ergosterol interferes.

The color reactions of benzaldehyde with sterols and steroids on underlayering with concentrated sulphuric acid has been the object of investigations (12, 13). This reaction, however, is not specific.

An application of kinetic colorimetry was proposed by Raoul and Meunier (8) in which the difference in the speed of reaction of vitamins D and other sterols was used to estimate the vitamins. This method is inexact and the results are confused by mixtures and by the presence of vitamin A.

A colorimetric method, based on the reaction of vitamins D with antimony trichloride, proposed by Brockmann and Chen (5) seemed to offer the most promise. The reagent used was later modified by several investigators (1, 2, 7 and 8). This method, however, depends on the separation of vitamins D from most of the accompanying material. This latter was accomplished by Kingsley (4) and, using the reagent of Brockmann and Chen, modified by Nield, et al, (1), excellent results were obtained for about 80 natural fish oils.

The present investigation is, for the most part, concerned with the quantitative estimation of irradiated ergosterol by the method proposed by Kingsley.

Early work indicated that assay of samples of irradiated ergosterol by the physical chemical method resulted in values approximately half those obtained by biological assay. Accordingly it was decided to investigate thoroughly some of the factors influencing the physical chemical determination of vitamin D_2 and if possible to eliminate the above mentioned discrepancy.

APPARATUS AND MATERIALS

The chromatograph tubes used were Twsett columns made from pyrex test tubes 1.7 cm. in diameter and 14 cm. in length.

All extinction values, unless otherwise specified, were made on a visual type universal photometer equipped with a Martins Polarizing unit. The cells were 1 and 5 cm. matched sets with glass spacers and quartz end plates.

The photoelectric photometer used was a grating type and was equipped with 1 cm. open type cells and 1 and 5 cm. stoppered cells with glass spacers and Corex end plates.

The various solvents were purified as follows:

Skellysolve:

A commercial grade was intermittently shaken, with standing, with separate portions of concentrated sulphuric acid until a color was no longer imparted to the acid. It was then washed twice with a 10% sodium carbonate and 5% potassium permanganate solution. It was washed fifteen times

with distilled water, dried over sodium for 24 hours and distilled. The fraction boiling from 66° to 68° C. was collected.

Benzene:

A C.P. thiophene free grade of benzene was dried over sodium for 48 hours and distilled. The fraction distilling from 79.5° to 80.5° was collected.

Chloroform:

It was found that special precautions were necessary in the purification of chloroform and to keep it in the stable form. The small amount of ethyl alcohol, 0.5% to 2%, ordinarily present in commercial and C.P. grades of chloroform, appears to stabilize it for periods of several months. According to Nield, et al, (1) and Ritsert (2) even a small amount of either ethyl alcohol or water in the chloroform, reduces the sensitivity of the antimony trichloride-chloroform-acetyl chloride reagent, consequently both must be removed. The alcohol was removed by washing the C.P. grade of chloroform seven times

with equal volumes of distilled water, followed by drying over potassium carbonate (anhydrous) for 12 hours and fractionating. The fraction distilling at 61° was retained. This dry purified chloroform was unstable, however, and in from 4 to 7 days gave evidences of a breakdown, as shown by a positive starch-iodide test, and a white cloudiness with silver nitrate solution. When either of these tests was positive, the chloroform was discarded and in no case used more than four days after being distilled. Shaking the purified chloroform with activated carbon immediately after fractionating, often resulted in chloroform that was perfectly stable for periods of two weeks or longer. However, if the treatment with activated carbon was omitted, breakdown occured in from 4 to 7 days. When a sample of chloroform gave a positive starch-iodide test, shaking with activated carbon resulted in a chloroform giving a negative starch-iodide test. However, breakdown began again immediately and, since some time was required to

prepare the reagent, this chloroform was not used. Results obtained using chloroform giving evidences of decomposition are shown in Tables I and II and are discussed there.

Ethanol:

The ethyl alcohol used was a good grade of commercial absolute alcohol.

Ether:

A C.P. grade of ether was shaken for an hour each with two separate portions of a dilute ferrous sulfate solution. It was washed 8 to 10 times with distilled water, preliminarily dried over sodium sulfate and finally dried over night with an excess of sodium. It was then fractionated and the fraction distilling at 34° was collected. This ether was stored over solid ferrous sulfate in small glass stoppered bottles with a minimum of air space over the ether. Stored in this manner, the ether was stable (no detectable starch-iodide test or precipitate with silver nitrate solution) for as long as two weeks. Without the ferrous sulfate treatment peroxides

were detectable (starch-iodide test) in from 24 to 48 hours and with a considerable air space over the ether, this time was lessened. This property is further discussed under the heading "The Effect of Oxidation Products of Ether".

Antimony trichloride-chloroform-acetyl chloride reagent:

The purity of each of the chemicals used in the preparation of this reagent is extremely critical. This is particularly true of chloroform and has already been discussed. The antimony trichloride and acetyl chloride used were anhydrous reagent grades and care was taken to exclude water as both are decidedly hygroscopic.

The reagent was prepared by dissolving 18 g. of antimony trichloride in 100 ml. of chloroform at a temperature below 40°C., filtering the solution, and adding immediately 2 ml. of acetyl chloride. This reagent is good for at least four days but in these investigations was not used more than two days after preparation. The principal factor governing the usable life of the

reagent is the stability of the chloroform used.

STABILITY OF CALCIFEROL IN VARIOUS SOLVENTS

In order to determine whether calciferol was sufficiently stable in the various solvents used in this investigation, the following series of experiments were performed.

A stock solution was prepared containing 0.005 g. of calciferol in 100 ml. of purified skellysolve. This solution was divided into ten more or less equal parts, (the equality of division is unimportant here, since only the question of how the potency changed with time was considered) the skellysolve evaporated off and the residues dissolved in the following solvents: Purified chloroform + 0.5% absolute ethanol, purified chloroform, specially purified ether, commercial C.P. ether, anhydrous ether, commercial hexane, commercial skellysolve, absolute ethanol, purified skellysolve and anhydrous thiophene free benzene.

At each of four different intervals of time one ml. aliquots are taken from each of these

solutions, evaporated to dryness and each of the residues taken up in one ml. of purified chloroform. Ten ml. of antimony trichloride-chloroform reagent were added to each sample and log I_0/I read at 500 mu., after 3 minutes, on the visual photometer.

The above procedure was followed in detail for each of the following reagent solutions: Chloroform saturated with antimony trichloride, ~~18~~ g. of antimony trichloride in 100 ml. of chloroform, 18 g. of antimony trichloride in 100 ml. of chloroform plus 0.4 ml. of absolute ethanol, and a saturated solution of antimony trichloride in chloroform that had started to decompose.

Sampling and testing these calciferol solutions was discontinued after six days. In the physical chemical method of vitamins D assay no solution of the vitamins was allowed to stand longer than four hours.

The values recorded in Table I are log I_0/I values as read on the visual photometer and are as suitable as U.S.P. unit potencies for eval-

Table I. Stability Tests of Calciferol in Different Solvents Treated with Various Antimony Trichloride Reagents

Reagent	hours after prep.	$CHCl_3$ +0.5% C_2H_5OH	$CHCl_3$ pure	Ether specially purified	Ether C. P.	Ether anhydrous	Hexane Comm.	Skelly-solve	Skelly-solve purified	Ethanol absolute	Benzene pure, anhydrous
	0	.44	.46	.36	.46	.48	.42	.34	.42	.43	.40
$CHCl_3$ saturated with $SbCl_3$	24	.44	.53	.37	.48	.53	.46	.34	.44	.43	.38
	72	.47	.48	.36	.48	.49	.46	.36	.43	.43	.39
	144	.46	.49	.38	.46	.48	.43	.33	.42	.44	.38
	0	.47	.45	.40	.45	.43	.41	.32	.42	.42	.38
18g. $SbCl_3$ in 100 ml. $CHCl_3$	24	.45	.48	.38	.47	.49	.46	.36	.42	.40	.38
	72	.45	.50	.37	.48	.50	.44	.34	.43	.43	.40
	144	.47	.48	.38	.46	.48	.43	.34	.43	.41	.39
	0	.45	.45	.35	.45	.52	.43	.33	.39	.45	.41
18g. $SbCl_3$ in 100 ml. $CHCl_3$ +0.4% ethanol	24	.46	.49	.41	.43	.54	.47	.35	.42	.44	.40
	72	.47	.48	.39	.44	.49	.45	.35	.42	.43	.41
	144	.44	.47	.37	.45	.50	.44	.35	.41	.45	.41
	0	.08	.08	.12	.07	.18	.06	.10	.08	.08	.06
Partially decomposed $CHCl_3$ saturated with $SbCl_3$	24	.11	.33	.09	.08	.07	.07	.11	.12	.09	.06
	72	.13	.21	.09	.05	.12	.07	.17	.09	.11	.07
	144	.09	.19	.11	.08	.10	.08	.11	.14	.12	.05

The log I_o/I values in this table were read at 500 mu., after 3 minutes, on the visual photometer

uating stability.

It may be pointed out that the log I_0/I values for the same sample are reproducible to $\pm$ 0.02.

Conclusions drawn from the summarized data in Table I.

Calciferol is stable for at least six days at room temperature in each of the solvents tested.

No difference in the color reaction was observed by the use of a reagent prepared by saturating chloroform with antimony trichloride and one composed of 18 g. of antimony tirchloride in 100 ml. of chloroform. (Two ml. of acetyl chloride for each 100 ml. of chloroform were added to each reagent).

The addition of absolute ethanol, up to 0.4%, to the reagent makes no difference in the color reaction for calciferol. This agrees with the findings of Nield, et al, (1) who states that the presence of 0.3% ethanol has no effect on the sensitivity of the reagent but that 0.7%

ethanol in the reagent reduces its sensitivity.

Reagent prepared from partially decomposed chloroform has a large negative effect on the color reaction, causing errors as large as 75%.

STABILITY OF CHOLESTEROL IN VARIOUS SOLVENTS

Sterols are present in all natural vitamins D oils, consequently stability studies were made using cholesterol as a representative sterol. These studies are analagous to those described under "Stability of Calciferol in Various Solvents", each of the solvents and reagents being the same. The solutions contained approximately 0.2% cholesterol, since the extinction value is much smaller than that for calciferol. These measurements were made over a period of 20 days. The results are grouped in Table II.

Discussion of the results tabulated in Table II.

Cholesterol is quite stable in each of the solvents tested, no change being observed after 20 days at room temperature.

The addition of 0.4% ethanol to the reagent

Table II. Stability Tests of Cholesterol in Different Solvents Treated with Various Antimony Trichloride Reagents

Reagent	Hours after prep.	$CHCl_3$ +0.5% C_2H_5OH	$CHCl_3$ pure	Ether specially purified	Ether C. P.	Ether anhydrous	Skelly-solve purified	Hexane Comm.	Skelly-solve	Ethanol absolute	Benzene pure, anhydrous
$CHCl_3$ saturated with $SbCl_3$	0	.08	.09	.26	.11	.18	.13	.12	.12	.15	.10
	144	.07	.11	.28	.12	.17	.12	.12	.12	.14	.11
	480	.06	.10	.25	.12	.19	.12	.12	.13	.16	.11
18g. $SbCl_3$ in 100 ml. $CHCl_3$	0	.07	.11	.27	.11	.18	.12	.14	.13	.16	.11
	144	.07	.09	.28	.13	.16	.12	.12	.13	.14	.09
	480	.06	.09	.25	.13	.18	.11	.12	.12	.15	.11
18g. $SbCl_3$ in 100 ml. $CHCl_3$ +0.4% ethanol	0	.12	.16	.35	.19	.23	.18	.17	.18	.23	.19
	144	.11	.15	.36	.20	.24	.20	.20	.19	.25	.18
	480	.13	.16	.36	.21	.26	.19	.17	.18	.25	.16
Partially decomposed $CHCl_3$ saturated with $SbCl_3$	0	.67	.73	1.05	.59	1.16	.82	.71	.75	.85	.66
	144	.74	.76	1.26	.83	1.32	.93	.85	.91	.88	.78
	480	.71	.82	1.13	.79	1.33	.97	.88	.83	.93	.73

The log I_0/I values in this table were read at 500 mu., after 3 minutes, on the visual photometer.

makes no noticeable difference in the sensitivity of the reagent to cholesterol.

Reagent consisting of chloroform saturated with antimony trichloride produces a deeper color than reagents containing smaller amounts of antimony trichloride. Reagent saturated with antimony trichloride results in an increase in color depth of as much as 40% more than reagent containing 18 g. of antimony trichloride in 100 ml. of chloroform. This effect might be expected since the sterol color deepens on standing, becoming almost black in from 24 to 48 hours.

Reagent containing partially decomposed chloroform gives extremely high results, as much as a 500% increase, and obviously should not be used.

Both C.P. and anhydrous ether gave higher results than expected, although neither showed any change over the 20 day test period. A series of experiments were performed to check this effect and are described under the heading "The Effect of Oxidation Products of Ether".

STABILITY OF THE COLOR REACTION OF ANTIMONY TRICHLORIDE-CHLOROFORM REAGENT AND CALCIFEROL

The color reaction between the antimony trichloride-chloroform reagent and the vitamins D and other sterols has been investigated in several laboratories, Nield, et al, (1), Brockmann and Chen (5), and others (2), (3), (7) and (8), and is quite well standardized. These investigators, however, either used the vitamin in a pure state or mixed with substances giving no color with this reagent. In this investigation sterols are assumed to be present in all vitamins D samples and their effect must be evaluated and the proper corrections made to obtain the actual concentration of calciferol present.

The rates of change of the extinctions produced by adding antimony trichloride-chloroform reagent to chloroform solutions of calciferol and of chlosterol was determined on a photoelectric photometer. The concentrations of the cholesterol solutions were varied. The extinction readings for these concentrations are plotted against time

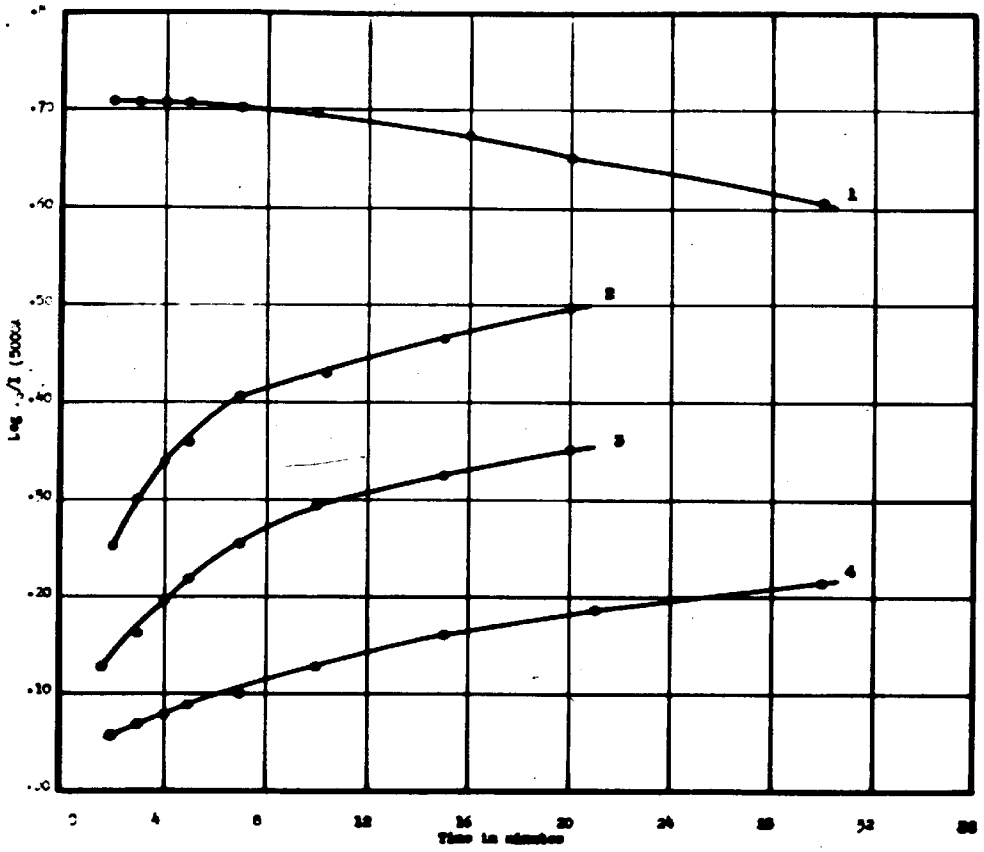

Fig. 1.—Rate of change of Log I_0/I of the color reactions of antimony trichloride-chloroform reagent with calciferol and cholesterol. 1, calciferol; 2, 0.05% cholesterol; 3, 0.1% cholesterol; 4, 0.2% cholesterol.

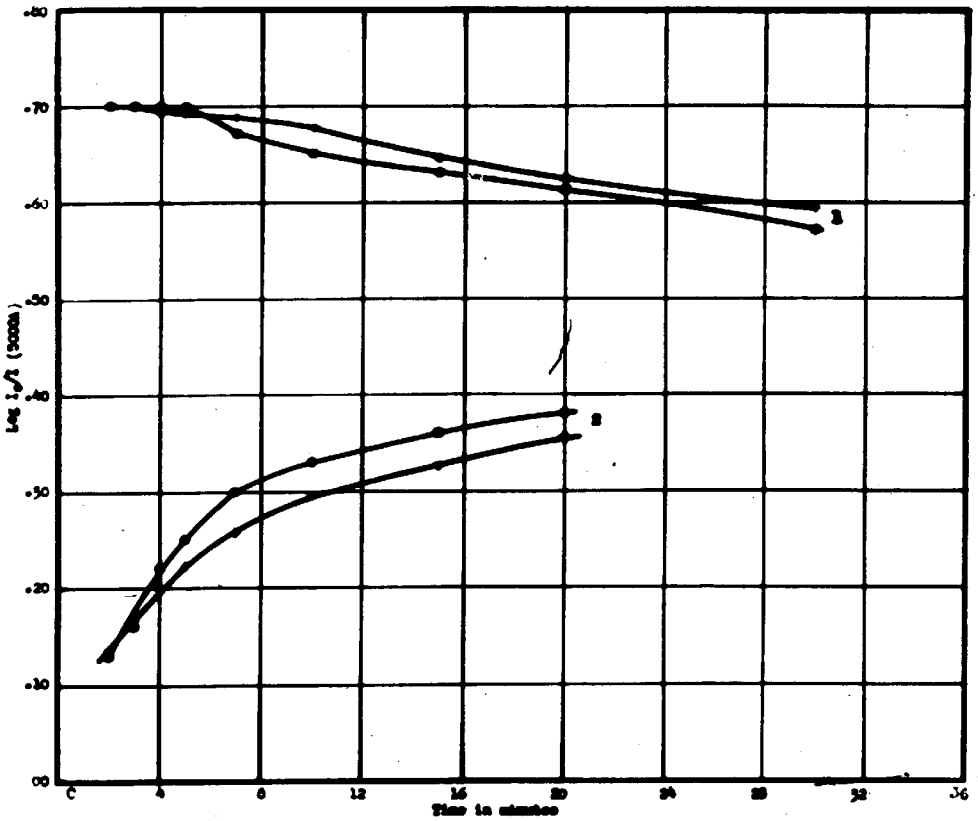

Fig. 2.—Rate of change of Log I_0/I of the color reactions of antimony trichloride-chloroform reagent with calciferol and cholesterol on two instruments: ⊙, Universal Spectrophotometer; ⊕, Photoelectric Spectrophotometer; 1, calciferol; 2, cholesterol.

on Figure I.

The rates of change of the same color reactions were also measured on the visual photometer. Figure II furnishes a comparison of the visual and of the photoelectric photometer, since it contains plots of the rates of change of the same color reactions measured on both photometers.

Conclusions

The red color for calciferol develops quickly to a maximum and is stable for 5 minutes, after which it fades slowly.

The cholesterol color deepens with time, changing from an extremely pale yellow to a deep red. This change is quite rapid during the first ten minutes, after which is slows slightly but still shows a definite trend.

Therefore, if the color reaction is to be applied to a solution containing both vitamins D and sterols or sterols alone, the time when readings are taken must be accurately controlled.

The extinction readings at 3 minutes of the 0.2%, 0.4% and the 0.8% cholesterol solutions are,

respectively, 0.06, 0.13 and 0.25 showing, within experimental error, the linearity of the response of the photoelectric cell in this region. If the response was not linear over the range used, a calibration curve would have to be run for each colored substance measured and corrected readings taken from this curve.

The visual and the photoelectric photometers are not strictly comparable, since differences of 9% and 3% were observed for the cholesterol and the calciferol curves, respectively. Moreover, the deviation for cholesterol was in the opposite direction from that of calciferol.

Effect of Diluting a Solution of Calciferol in Corn Oil With Corn Oil

A solution of calciferol in corn oil with a biological assay of 200,000 U. S. P. units per gram was chosen for this investigation. One gram aliquots of this solution were diluted by weight to 2, 4, 8 and 16 grams total weight with pure corn oil, thus reducing the calciferol content to

Table III

Physical Chemical Assay of Samples of Calciferol in Corn Oil Diluted with Corn Oil

Conc. g./100 ml.	E(1%. 1cm.) 500 mu.	Physical Chemical Method U.S.P. u./g.	Biological Method U.S.P. u./g.
0.0909	6.61	127,400	200,000
0.04545	3.25	62,650	100,000
0.02273	1.62	31,330	50,000
0.01136	0.81	15,660	25,000
0.00568	0.385	7,430	12,500

Table IV

Assay of Samples of Calciferol in Corn Oil Diluted with Corn Oil

Conc. g./100 ml.	E(1%, 1cm.) 500 mu.	Physical Chemical Method U.S.P. u./g.	Biological Method U.S.P. u./g.
0.0909	9.63	186,000	200,000
0.04545	4.84	93,450	100,000
0.02273	2.50	48,300	50,000
0.01136	1.27	24,420	25,000
0.00568	0.715	13,800	12,500

0.5, 0.025, 0.0125 and 0.00625 its original value. This series was run in duplicate by the method proposed by Kingsley (4) and the results are tabulated in Table III.

Since no vitamin A is present in corn oil and since corn oil has an E (1%, 1cm.) of less than 0.2, this series may also be run by dissolving the oil solution directly in chloroform. Aliquots of these chloroform solutions are then added to antimony trichloride-chloroform reagent and the extinction values measured at 500 mu, after 3 minutes. The results of such a series is shown in Table IV.

Discussion of Tables III and IV

In Table III, although there is a marked difference in the calculated potency and the biological potency of these solutions, the vitamin D concentration-potency ratio is the same in every case, within experimental error.

In Table IV the calculated potencies and the biological potencies are practically identical and the vitamin D concentration-potency ratios are

the same. Since unsaponified corn oil has an E (1%, 1cm.) of approximately 0.15, each dilution should give a value slightly greater than half the value of the preceding dilution. This is true in every case.

The data included in both Tables III and IV are in accord with Beers law, that is, the extinctions produced by the antimony trichloride-chloroform reagent are linear functions of the amount of calciferol present.

The Effect of Oxidation Products of Ether

To test the effect of the products of oxidations of ether on the color reaction for calciferol, the following experiments were performed.

Two samples of calciferol in corn oil #3772 were saponified. One was extracted with stock C. P. ether and the other with specially purified ether (ref. Apparatus and Materials). Both were evaporated to dryness, taken up in chloroform and aliquots reacted with antimony trichloride-chloroform reagent. Log I_0/I was read at 500 mu., after 3 minutes, on the visual photometer.

Extracted with C.P. stock ether----150,000 U.S.P. u./g.

" " purified " ----195,300 U.S.P. u./g.

Two samples of oil #3372 were dissolved, one in purified ether that had been standing for 4 months in a glass stoppered bottle and the other in purified ether prepared in the previous 4 hours. The ether was evaporated off, the samples taken up in chloroform, and the color reaction run. Log I_o/I was read at 500 mu., after 3 minutes, on the visual photometer.

Ether purified in last 4 hours--195,300 U.S.P. u./g.

Ether after standing 4 months---131,600 U.S.P. u./g.

Further, 30 ml. of purified ether from another bottle, that had been standing for 3 months, was placed in a clean dry flask and the ether evaporated off. A sample of oil #3772 was weighed into this flask and dissolved in chloroform. The color reaction was run with an aliquot of this solution and log I_o/I read at 500 mu., after 3 minutes, on the visual photometer. An assay of 142,200 U.S.P. units/g. resulted.

These data clearly show that the oxidation

products of ether have a definite large negative effect on the color produced by the reaction of calciferol and antimony trichloride-chloroform reagent, the apparent potency being reduced by as much as 35%. Moreover, the effect does not seem to be limited to solutions of the ether but also to a residue left by the evaporation of the ether.

Since this effect is not evident for natural oils, (the above mentioned tests are not applicable because of the presence of vitamin A) it was thought that chromatographing might filter out the interfering substance or substances. Accordingly, the following experiment was performed.

Two samples of oil #3772 were saponified, one being extracted with purified ether, and the other with stock C.P. ether that had, in the previous hour been run through a 4 cm. super-filtrol column. The ether was evaporated off, the residues dissolved in chloroform and a color reaction run with antimony trichloride-chloroform reagent on an aliquot of each solution.

Purified ether sample---------195,300 U.S.P. u./g.

Chromatographed ether sample--191,100 U.S.P. u./g. This indicates that running the ether through a superfiltrol column eliminates, for the most part, the interfering materials.

The latter experiment was twice repeated, using ether that had been put through superfiltrol columns 8 and 12 cm. in length. The results were the same, that is, a slight lowering from the potency obtained using purified ether.

The chromatographed ether, however, was very susceptible to further decomposition and within a few hours gave a definite starch-iodide test. Also, when ether that had been run through a superfiltrol column and then let stand for several hours was used to extract a sample of oil #3,772, a marked lowering in its potency was found. This indicates that further oxidation began immediately.

These results show that chromatographing removes most of the oxidation products already present in the ether but does not inhibit the formation of additional oxidation products.

As some ergosterol is present in all irrad-

iated ergosterols in oils and since sterols are present in all natural vitamins D oils, the effect of the oxidation products of ether on a common sterol was investigated. The cholesterol solutions listed in Table II were used.

A 5 ml. aliquot of the purified skellysolve solution, #6 of Table II, was evaporated to dryness, the residue taken up in stock C.P. ether, the ether evaporated off, and the residue dissolved in 5 ml. of chloroform. A 1 ml. aliquot of this solution was added to 10 ml. of antimony trichloride-chloroform reagent and the extinction read, after 3 minutes, at 500 mu. The value was 0.24, as compared with an extinction of 0.12 before the above treatment.

Another aliquot of purified skellysolve solution #6 was treated similarly, except it was redissolved in purified ether that had been standing for 4 months in a glass stoppered bottle. The extinction was 0.71, as compared to an original value of 0.12.

Another aliquot of the same solution was

treated similarly, except it was redissolved in purified ether prepared during the preceeding hour. The extinction was 0.13, compared to an original value of 0.12.

The same experiment was repeated in detail except that the residue was redissolved in stock C.P. ether that had been passed through a 4 cm. superfiltrol column during the preceeding hour. The extinction was 0.14.

These results clearly indicate that partially oxidized ether has the effect of greatly enhancing the extinction of the color produced by adding antimony trichloride-chloroform reagent to a cholesterol in chloroform solution.

The effects of partially oxidized ether on the color reactions of calciferol and of sterols with antimony trichloride-chloroform reagent are in opposite directions and, in a solution in which both were present, would tend to cancel. However, the concentrations would seldom be such that these errors would exactly counterbalance.

The ether used for extraction and for elution

of the chromatograph columns should be either purified by the method outlined under "Apparatus and Materials" or drawn through a 4 to 8 cm. superfiltrol column an hour or two before using. The latter is much the simpler procedure if any shortened method is to be used in the analysis of irradiated ergosterols. An example of such a shortened method consists of the following steps: Saponification, extraction with ether, evaporation of the ether, dissolving the residue in chloroform, and treating an aliquot of this solution with antimony trichloride-chloroform reagent. This method obviously could not be used if any vitamin A was present. If the oil is to be run through the entire analysis procedure, (4) the first chromatograph of the method would act to partially purify the ether.

Comparison of a Visual Photometer With a Photoelectric Photometer

A comparison was made of a photoelectric photometer and the visual photometer used in this work by measuring the extinction of the antimony

Table V

Comparative Extinctions on a Visual and on a Photoelectric Photometer

Type of Oil or Sample	Reacting Material	Extinction	
		Visual Photometer	Photoelectric Photometer
Calciferol	D(a)	0.70	0.66
Calciferol in olive oil	D	0.39	0.38
Cholesterol	S(b)	0.16	0.165
Irradiated ergosterol	D+S(c)	0.31	0.28
Calciferol	D	0.75	0.70
Calciferol	D	0.78	0.71
Irradiated ergosterol	S	0.02	0.02
Tuna liver oil conc.	S	0.13	0.13
Tuna liver oil conc.	S	0.14	0.13
Mixed high D oil	D+S	0.76	0.74
Mixed high D oil	D+S	0.78	0.75
Mixed high D oil	S	0.32	0.32
High D oil	S	0.36	0.37
Tuna liver oil conc.	D+S	0.76	0.74
Tuna liver oil conc.	D+S	0.62	0.57
Mixed fish liver oil	D+S	1.12	1.11
Mixed high D oil	D+S	0.75	0.76
D distillate	D+S	0.69	0.695
D distillate	D+S	0.95	0.97
D distillate	D+S	0.92	0.92

(a) D, vitamins D (b) S, sterols (c) D S, vitamins D + sterols

trichloride-chloroform reagent-vitamins D color reaction. All the measurements were made at 500 mu. The samples were chosen so that the extinction values covered a wide range and offered a representative comparison at the particular wave length used. Measurements were made on the photoelectric photometer using 1 cm. glass stoppered cells with both Corex and quartz end plates. Glass covered open type cells were first tried and found very difficult to use with this reagent due to the extremely rapid hydrolysis of antimony trichloride and the fact that the solution tends to creep over the edges of the cell.

To check for possible personal differences in the readings on the visual photometer, all except the first six values of Table V were checked independently by another investigator who had had long experience with this instrument. These values never differed by more than 0.02 and in most cases were the same. Extinction values for both instruments are listed in Table V.

Discussion of Table V.

In the lower range, the extinctions, as measured on each instrument, are the same. When the intensity of the color is increased, however, the readings are noticeably different. With a few exceptions, the extinctions read on the visual instrument were higher than those read on the photoelectric one. This is probably due to the fact that photoelectric cells in general do not respond linearily to increasing color intensity over a wide range and require a calibration curve for a particular color reaction. If such a calibration curve is made, the photoelectric photometer may be used interchangeably with the visual one. The visual instrument was used in these studies because the accuracy is well within the accuracy of the method and the cells used are much easier to handle.

Sensitivity of the Antimony Trichloride-Chloroform Reagent-Vitamins D Color Reaction

The question of the applicability of the chromatographic adsorption method for oils of

low vitamins D potency led to a determination of the lowest potency accurately measurable by means of the antimony trichloride-chloroform reagent.

The solutions for these measurements were prepared by successively diluting a solution of calciferol in purified skellysolve. One ml. aliquots of these solutions were evaporated to dryness, taken up in one ml. of chloroform, ten ml. of antimony trichloride-chloroform reagent added, and the extinctions determined on the visual photometer at 500 mu., after 3 minutes. These values are recorded in Table VI.

Table VI

Conc. (g./100 ml.)	log I_0/I, 3 min., 500 mu.	U.S.P. Units/ml.
0.00502	0.81	2,010
0.00251	0.39	1,005
0.00126	0.20	502
0.00063	0.10	251
0.00031	0.05	125
0.00016	0.02	63
0.00008	0.00	32

By the use of color comparison tubes, 7 mm. in diameter and filled to a height of 12 mm., the presence of 15 U.S.P. units of calciferol produced a detectable pink color with ten ml. of reagent. This slight color is difficult to measure accurately.

Discussion of Table VI

The color produced by the addition of a chloroform solution of 100 U.S.P. units of vitamins D to 10 ml. of antimony trichloride-chloroform reagent is accurately measurable on the visual photometer, and 50 U.S.P. units may be measured with fair accuracy. These results were obtained with cells 1 cm. in thickness. This lower limit of accurate measurement may be extended to 20 U.S.P. units if cells 5 cm. in thickness were used.

Physical Chemical Assay for Vitamins D

The chromatographic adsorption method as developed by Kingsley (4) gives excellent results for natural fish oils containing vitamins D. In

Table VII

Comparative Vitamin D Assay of Irradiated Ergosterol by Physical Chemical and by Biological Methods

Oil	Wt. (g.)	E(1%, 1cm.) 500 mu.	Calculated U.S.P. u./g. (a)		Biological method U.S.P. u./g. (b)	(b)-(a) / 44.8
	(A)	Irradiated Ergosterol in Corn Oil				
45120	0.1	6.93	133,000	(av.)	250,000	1.00
		6.82	131,600			
		7.91	152,800	138,000		
61691	0.1	5.83	123,200		200,000	0.92
		5.38	112,600			
				117,900		
78272	0.1	7.37	142,300		300,000	1.11
		7.70	148,600			
		7.70	148,600			
		8.21	158,400	149,500		
65751	0.1	6.06	116,800		250,000	1.16
		6.38	123,000	120,000		
		(B) Calciferol in Corn Oil				
3772	0.1	6.61	127,400		200,000	0.81
		6.61	127,400	127,400		
12722	0.1	5.94	114,700		200,000	1.00
		5.39	104,000	109,350		
	(C)	Irradiated Ergosterol in Halibut Liver Oil				
56391	0.1	7.59	146,500		225,000	0.83
		7.04	136,000	141,250		

Table VII (continued)

Oil	Wt. (g.)	E(1%, 1cm.) 500 mu.	Calculated U.S.P. u./g. (a)		Biological method U.S.P. u./g. (b)	$\frac{(b)-(a)}{44.8}$
66701	0.1	7.37	142,300		250,000	0.96
		7.37	142,300			
		6.82	131,600			
		7.59	146,500			
		7.65	147,700	142,000		
89052	0.1	7.59	146,500		250,000	0.92
		7.65	147,700	147,100		
89182	0.1	7.37	142,300		250,000	0.97
		7.26	140,100	141,200		
86632	0.1	7.26	140,100		250,000	1.02
		6.82	131,700	135,900		
			(D) Viosterol			
2242	1.0	0.121	2,340		3,800	0.79
		0.132	2,550	2,445		
62191	1.0	0.253	4,880		12,000	1.46
		0.176	3,400	4,140		

Table VIII

Comparative Vitamin D Assays of Fish Liver Oils by Physical Chemical and Biological Methods

Sample	Physical Chemical Method				Biological Method
	U.S.P. u./g. Author		U.S.P. u./g. Kingsley (4)		U.S.P. u./g.
44080	29,720 (av.)		29,300 (av.)		
	29,720	29,720	30,100	29,700	31,000
76902	12,300		12,400		
	12,300	12,300	12,900	12,650	12,000
P6846	18,500		19,700		
	18,050	18,275	20,300	20,000	20,000
V3428	1,133,000				
	1,119,000	1,126,000	1,262,000	1,220,000	1,200,000
77462	8,900		8,300		
	8,900	8,900	8,110	8,200	6,300
61211	13,160				
	11,470		7,720		
	11,470	12,030	6,950	7,335	12,000

Table IX

Effect of Saponification and Chromatographing on Irradiated Ergosterol in Corn Oil

Sample	Dissolved directly in Chloroform U.S.P. u./g.	Saponified only U.S.P. u./g.	Chromatographed only U.S.P. u./g.	Saponified and Chromatographed U.S.P. u./g.
45120	180,500	172,000	174,100	161,400
61691	172,000	160,000	157,100	152,600
78272	214,500	200,000	200,000	193,000
12722	191,100	183,200	186,900	166,400
3772	195,300	184,800	193,200	160,000

Table X

Summary: Determination of Conversion Factor

Oil No.	Type	Date	E(1%, 1cm.)	Bioassay
47761	Mixed D oil	9-22-42	0.80	15,000
			0.80	U.S.P.
		9-25-42	0.78	u./g.
			0.75	
		9-29-42	0.76	
			0.88*	
		9-30-42	0.76	
			0.72*	
		10- 1-42	0.76	
			0.76	
		10- 5-42	0.79	
			0.78	
		10-21-42	0.78	
			0.77	
		11-23-42	0.76	
		12- 2-42	0.77	
		12- 4-42	0.80	
			0.76	
		12-12-42	0.77	
		12-26-42	0.74	
		12-22-42	0.72*	
			0.75	
		1-8 -43	0.75	
		2-3 -43	0.77	
			0.75	
		Average E(1%, 1cm.)	0.770	

$$\text{Conversion Factor} = \frac{15{,}000}{0.77} = 19{,}480$$

This E(1%, 1cm.) value of 0.770 compares with a similar value of 0.779 obtained by Kingsley (4) in averaging 30 consecutive runs.

this section, this method was used for a physical chemical assay of both natural vitamins D oils and irradiated products.

According to Nield, et al, (1) the E(1%, 1cm.) for the natural vitamin, D_3, is the same as that for calciferol, D_2. This value is 1800. If this is true, the same conversion factor, 19,480 (Table X), may be used to convert E(1%, 1cm.) to U.S.P. units/g. for both vitamins D_2 and D_3.

A representative group of natural fish oils were run by the method developed by Kingsley and the results are listed in Table VIII. These results compare favorably with the bioassays (Parke Davis & Co.) and also with the values reported by Kingsley, using the same method.

A number of irradiated ergosterols in various oils also were run by this method and the results are grouped in Table VII. It will be noted that there is a decided difference between the physical chemical assay and the bioassay for each of the oils in this table. The average percentage deviation, obtained by taking the sum of the

percentage deviations and dividing by the total number of oils, is 44.8%.

The last column on the right of Table VII consists of values obtained by taking the difference between the bioassay and the corresponding physical chemical assay and dividing this difference by the average percentage deviation. This furnishes a method of comparing the various irradiated ergosterols. Thus, it is noted that six of the values fall within 8% of the average percentage deviation, while six of the remaining seven fall within 21%. Therefore, if the conversion factor was determined from one of these irradiated ergosterols, rather than from the reference oil #47761 (Table X), fair results would be obtained for the other irradiated ergosterols.

With the results of Table VII in mind, it was decided to further investigate certain steps of the chromatographic adsorption method as applied to solutions of irradiated ergosterol in oil.

Solutions of irradiated ergosterol in corn

oil were used for these investigations. Since such an oil contains no vitamin A and since corn oil has an E(1%, 1cm.) of less than 0.2, it may be dissolved directly in chloroform and an aliquot of this solution run with antimony trichloride -chloroform reagent. Thus an apparent loss or gain may be determined before and after each step of the procedure. The results of such a series of investigations appear in Table IX.

Reference of Table IX will show that a small loss, of from 4 to 7%, occurs during the time a sample of an irradiated ergosterol in corn oil is saponified, washed, and extracted with ether. If purified ether is used to extract the samples of oil #3772, calciferol in corn oil, no loss occurs. However, the loss is still evident for the irradiated ergosterols in corn oil.

Approximately the same percentage loss, from 3 to 7%, occurs if the samples are treated as follows: Dissolved directly in the developing solution (50-10-1, skellysolve, purified ether, and absolute ethanol, respectively), run through

a 6 cm. superfiltrol column, the column cut immediately below the orange band and eluted with ether, the mixed solvents evaporated off, the residue dissolved in purified chloroform and an aliquot of this solution run with antimony trichloride-chloroform reagent.

A combination of saponification and chromatographing results in a loss of from 8 to 12%. This is true for the samples of calciferol in corn oil as well as for those of irradiated ergosterol in corn oil.

Oil #3772 is a 160 times dilution of pure calciferol in corn oil. This calciferol gave a physical chemical assay value of 32,000,000 U.S.P. units/g., and on that basis oil #3772 should have a potency of 200,000 U.S.P. units/g. This value is almost reached by running an aliquot of a solution of this oil, dissolved directly in chloroform, with antimony trichloride-chloroform reagent. This indicates that the corn oil itself has no inhibiting effect on the color reaction. Corn oil has an E(1%,1cm.) of 0.17, and after

saponification this value drops to 0.06, thus acting only to increase the E(1%, 1cm.) very slightly.

Milas (9) and Clover (10) have shown that ethyl ether, and alkyl ethers in general, undergo spontaneous oxidation to form multiple oxidation products, the identity of which are in doubt. Hydrogen peroxide, however, was found in every partially oxidized ether solution. This oxidation is greatly accelerated by ultraviolet light, Milas finding very large amounts of oxidation products titratable with sodium thiosulphate, after an irradiation period of 80 hours. After a standing period of some weeks, every ether solution tested gave a strong starch-iodide test.

All of the irradiated ergosterol samples used in this investigation were irradiated in an ether solution and no attempt was made to purify the ether either before or after irradiation. In view of this fact and in view of the results found under "Effect of Oxidation Products of Ether", it was thought likely that the peroxides and/or

other oxidation products of ether were causing the physical chemical assay values to be lower than the corresponding bioassays.

As has been shown, passing ether itself through a 3 cm. superfiltrol column eliminates the interfering oxidation products. However, chromatographing an ether solution of an irradiated ergosterol has no effect other than a slight lowering of its vitamin D potency. Hence it was thought that a stronger treatment to eliminate the oxidation products might be necessary.

An ether solution of irradiated ergosterol in corn oil #3772 was successively shaken with 0.1N. ferrous sulphate, 0.1N. sodium thiosulphate, and 0.1N. ammonia solutions. It was then washed with distilled water, evaporated to dryness, and taken up in chloroform. An aliquot of this chloroform solution was added to antimony trichloride-chloroform reagent and, after 3 minutes, log I_0/I read at 500 mu. This treatment had no measurable effect on the log I_0/I value of the color reaction.

This procedure was repeated in detail for all the samples of irradiated ergosterol in corn oil listed in Table IX. No change in the vitamin D potency of any of these samples was observed, except a slight lowering in two cases, probably due to mechanical loss.

Also, an ether solution of a sample of each of the oils listed in Table IX was shaken with multiple portions of Fehlings solution, washed with distilled water, evaporated to dryness, and taken up in chloroform. Some of these samples, after being shaken with Fehlings solution, were also run through a 6 cm. superfiltrol column using a developer solution of 50-10-1, skellysolve, purified ether, absolute alcohol, respectively. An aliquot of each of the chloroform solutions was added to antimony trichloride-chloroform reagent and log I_o/I read at 500 mu., after 3 minutes. These log I_o/I values were slightly lower than those given by the same weight of sample dissolved directly in chloroform and were probably due to mechanical loss.

In the lower range, the extinctions, as measured on each instrument, are the same. When the intensity of the color is increased, however, the readings are noticeably different. With a few exceptions, the extinctions read on the visual instrument were higher than those read on the photoelectric one. This is probably due to the fact that photoelectric cells in general do not respond linearily to increasing color intensity over a wide range and require a calibration curve for a particular color reaction. If such a calibration curve is made, the photoelectric photometer may be used interchangeably with the visual one. The visual instrument was used in these studies because the accuracy is well within the accuracy of the method and the cells used are much easier to handle.

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Table VI

Conc. (g./100 ml.)	log I_o/I, 3 min., 500 mu.	U.S.P. Units/ml.
0.00502	0.81	2,010
0.00251	0.39	1,005
0.00126	0.20	502
0.00063	0.10	251
0.00031	0.05	125
0.00016	0.02	63
0.00008	0.00	32

By the use of color comparison tubes, 7 mm. in diameter and filled to a height of 12 mm., the presence of 15 U.S.P. units of calciferol produced a detectable pink color with ten ml. of reagent. This slight color is difficult to measure accurately.

Discussion of Table VI

The color produced by the addition of a chloroform solution of 100 U.S.P. units of vitamins D to 10 ml. of antimony trichloride-chloroform reagent is accurately measurable on the visual photometer, and 50 U.S.P. units may be measured with fair accuracy. These results were obtained with cells 1 cm. in thickness. This lower limit of accurate measurement may be extended to 20 U.S.P. units if cells 5 cm. in thickness were used.

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	(A)	Irradiated Ergosterol in Corn Oil				
45120	0.1	6.93	133,000	(av.)	250,000	1.00
		6.82	131,600			
		7.91	152,800	138,000		
61691	0.1	5.83	123,200		200,000	0.92
		5.38	112,600			
				117,900		
78272	0.1	7.37	142,300		300,000	1.11
		7.70	148,600			
		7.70	148,600			
		8.21	158,400	149,500		
65751	0.1	6.06	116,800		250,000	1.16
		6.38	123,000	120,000		
		(B) Calciferol in Corn Oil				
3772	0.1	6.61	127,400		200,000	0.81
		6.61	127,400	127,400		
12722	0.1	5.94	114,700		200,000	1.00
		5.39	104,000	109,350		
	(C)	Irradiated Ergosterol in Halibut Liver Oil				
56391	0.1	7.59	146,500		225,000	0.83
		7.04	136,000	141,250		

Table VII (continued)

Oil	Wt. (g.)	E(1%, 1cm.) 500 mu.	Calculated U.S.P. u./g. (a)		Biological method U.S.P. u./g. (b)	$\frac{(b)-(a)}{44.8}$
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		7.37	142,300			
		6.82	131,600			
		7.59	146,500			
		7.65	147,700	142,000		
89052	0.1	7.59	146,500		250,000	0.92
		7.65	147,700	147,100		
89182	0.1	7.37	142,300		250,000	0.97
		7.26	140,100	141,200		
86632	0.1	7.26	140,100		250,000	1.02
		6.82	131,700	135,900		
			(D) Viosterol			
2242	1.0	0.121	2,340		3,800	0.79
		0.132	2,550	2,445		
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Table VIII

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Sample	Physical Chemical Method U.S.P. u./g. Author		Physical Chemical Method U.S.P. u./g. Kingsley (4)		Biological Method U.S.P. u./g.
44080	29,720	(av.)	29,300	(av.)	
	29,720	29,720	30,100	29,700	31,000
76902	12,300		12,400		
	12,300	12,300	12,900	12,650	12,000
P6846	18,500		19,700		
	18,050	18,275	20,300	20,000	20,000
V3428	1,133,000				
	1,119,000	1,126,000	1,262,000	1,220,000	1,200,000
77462	8,900		8,300		
	8,900	8,900	8,110	8,200	6,300
61211	13,160				
	11,470		7,720		
	11,470	12,030	6,950	7,335	12,000

Table IX

Effect of Saponification and Chromatographing on Irradiated Ergosterol in Corn Oil

Sample	Dissolved directly in Chloroform	Saponified only U.S.P. u./g.	Chromatographed only U.S.P. u./g.	Saponified and Chromatographed U.S.P. u./g.
45120	180,500	172,000	174,100	161,400
61691	172,000	160,000	157,100	152,600
78272	214,500	200,000	200,000	193,000
12722	191,100	183,200	186,900	166,400
3772	195,300	184,800	193,200	160,000