THE CHROMATOGRAPHIC SEPARATION OF IRRADIATED ERGOSTEROL

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Lillian Broch Kimball

1950

This is to certify that the

thesis entitled

"Chromatographic Separation
of Irradiated Ergosterol".

presented by

Lillian Kimball

has been accepted towards fulfillment
of the requirements for

M.S. degree in Physical Chemistry

Major professor

Date May 22, 1950

O-169

THE CHROMATOGRAPHIC SEPARATION OF IRRADIATED ERGOSTEROL

By

Lillian Broch Kimball

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

1950

ACKNOWLEDGMENT

The writer wishes to express her sincere appreciation to Doctor Dwight T. Ewing for the able guidance and helpful suggestions offered in the course of this work.

****
****
******
****
**
*

TABLE OF CONTENTS

PART I PAGE

THE CHROMATOGRAPHIC SEPARATION OF CALCIFEROL BY MEANS OF VARIOUS ADSORBENTS..... 1

MATERIAL AND EQUIPMENT..... 3

Ergosterol..... 3
Calciferol..... 3
Ether..... 3
Alcohol..... 4
Hexane..... 4
Silica gel..... 5
Chromatograph tubes..... 6
Irradiation source..... 6
Collection of fractions..... 6
Determination of absorption spectra..... 7
Purpose..... 7
Procedure..... 7

RESULTS..... 10

DISCUSSION..... 11

SUMMARY..... 13

LITERATURE CITED..... 18

PART II

THE CHROMATOGRAPHIC SEPARATION OF IRRADIATED ERGOSTEROL.... 19

Purpose..... 26
Procedure..... 27
Calculation of Irradiation Time Per Molecule..... 29

RESULTS..... 31

DISCUSSION..... 32

SUMMARY..... 38

LITERATURE CITED..... 47

LIST OF TABLES

TABLE PAGE

PART I

I. DATA FOR DIFFERENT ADSORBENTS USED..... 14

II. EXTINCTION VALUES AT 265 MU FOR FRACTIONS OF ELUANT RECOVERED FROM DIFFERENT ADSORBENTS..... 15

III. VOLUME OF ELUATE NECESSARY TO RECOVER CALCIFEROL FROM MAGNESOL AS RATIO OF ETHER TO HEXANE IN ELUANT IS VARIED..... 16

IV. EXTINCTION AT 265 MU FOR DIFFERENT FRACTIONS OF ELUATE RECOVERED FROM MAGNESOL COLUMN AS RATIO OF ETHER TO HEXANE IN ELUANT IS VARIED..... 17

PART II

I. DATA FOR RUNS WITH DIFFERENT IRRADIATION TIME..... 39

II. ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 1. 40

III. ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 2. 41

IV. ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 3. 42

V. ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN No. 4. 43

VI. ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 5. 44

VII. ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 6. 45

VIII. CALCULATION OF ABSORPTION SPECTRA FOR SUBSTANCE ELUTED WITH CALCIFEROL..... 46

PART I

PART I

THE CHROMATOGRAPHIC SEPARATION OF CALCIFEROL BY MEANS OF VARIOUS ADSORBENTS

Chromatographic processes are especially effective for the separation of certain mixtures and the isolation of certain components for purposes of identification. The chromatographic method was first used by Day in 1897 in work with Pennsylvania earth oil. He later noted that if crude oil were forced upward through a column of fuller's earth the sequence from top to bottom would be: saturated aliphatic hydrocarbons, then aromatics and unsaturated substances, and, finally, nitrogen and sulfur compounds (1).

The method was further developed and systematized by Tswett so he is usually considered to be the inventor of chromatography (2). Many modifications of the method have since been tried, some with astonishing success. The success of the chromatographic method is based on some difference in the affinity of the adsorbent toward the different components present in the mixture to be separated.

The affinity of an adsorbent for the adsorbate can be altered by varying the solvent and the preparation of the column. Components which cannot be separated using a particular adsorbent may oftentimes be separated by some other adsorbent which is more preferential in character.

The theory, limitations, and applications of the chromatographic process in general are fully explained in the works of Zechmeister (3) Strain (4) and others and so were not discussed in this paper.

The chromatographic technique has been applied in this laboratory to the separation of various vitamins from the impurities associated with their production.

Bullard (5) showed that ergosterol could be separated from calciferol using superfiltrol as an adsorbent and an eluant composed of a 50:10:1, ratio by volume of hexane, ether and ethyl alcohol.

Baker (6) investigated various adsorbents with regard to their use in the separation of vitamin D from the other components of fish oils and found that superfiltrol and possibly magnesia were suitable when a 50:10:1 ratio by volume of hexane, ether and alcohol was used as an eluant.

Powell (7) used chromatographic separations on superfiltrol for removing vitamin D_2 from ergosterol in the determination of the vitamin D content of various fish oils. He made use of the same eluant as Baker.

Chen (8) by varying the eluant used and changing the amount and type of solution used to prewash the column showed that the adsorption of vitamins A and D and ergosterol by alumina and superfiltrol could be altered.

MATERIAL AND EQUIPMENT

Since the materials and equipment used in both parts of this investigation were identical they will be described fully in Part I of this paper and will not be repeated in Part II.

Ergosterol:

The ergosterol was a good commercial grade received from Parke Davis and Company, lot F-103-48, and was prepared by the Montrose Chemical Company.

Calciferol:

The calciferol used was pure synthetic vitamin D in crystalline form, each gram containing a minimum of 40,000,000 U.S.P. units of crystalline D_2 and was obtained from the Winthrop Chemical Company, Inc.

From this calciferol a stock solution was made up containing 0.0166 grams of calciferol in each 200 ml. of ethyl alcohol. Another stock solution was made up containing 0.0174 g. of calciferol in each 200 ml. of solution. Three milliliters of one of the stock solutions were used for each run in Part I.

Ether:

A c.p. grade of anhydrous ethyl ether was distilled immediately prior to use over sodium hydroxide and sodium sulfite. Approximately 30 g. of sodium hydroxide and 30 g. of sodium sulfite were used for 500 ml. of ether. The distillate was collected and the absorption curve determined using the Beckman spectrophotometer.

In order that the ether be suitable for use it was necessary that it transmit down to 215 mu.

Alcohol:

A c.p. grade of ethyl alcohol was purified in the following manner: twenty g. of potassium hydroxide and 10 g. of silver nitrate were added to one liter of the alcohol and was allowed to stand for one week with occasional shaking. The alcohol was decanted from the flask and distilled. The distillate was collected in 100 ml. fractions and the absorption curves were determined on the Beckman spectrophotometer. Only those fractions which transmitted down to 220 mu were used. If upon addition to the ether, hexane mixture the alcohol showed that water was present (water gives a milky color to the mixture) the alcohol was further treated with amalgamated aluminum and allowed to stand for 24 hours before being redistilled.

Hexane:

Skellysolve B was purified by passing it through a column 24 inches in height and 1.5 inches in diameter containing activated silica gel. Fractions of about 100 ml. each were collected and the absorption curves determined on the Beckman spectrophotometer. Only those fractions which transmitted down to 215 mu, and which showed no absorption due to benzene were used. All other fractions were set aside and passed through the silica gel a second time. Approximately 300 ml. of hexane could be obtained from one column using 500 ml. of skellysolve.

The hexane was stored in a dark bottle and remained stable for several weeks.

In Part I of the investigation different ratios of hexane and ether were used as eluants.

In Part II the only eluant used with the exception of run No. 8 was a mixture containing a ratio by volume of 50 parts hexane, 10 parts ether and 1 part alcohol. This mixture will hereafter be referred to as 50:10:1.

Silica gel:

The silica gel used for purification of the skellysolve was P.A. 100 Refrigerant Grade purchased from the Davison Chemical Company and was suitable for use as purchased. Once used, the silica gel was re-activated by washing it in a Buchner funnel with distilled water until no odor of hexane remained. It was then air dried and then placed in an oven maintained at 250° for 24 hours. After removal from the oven the silica gel was kept in an air tight container until used.

The following adsorbents were used in the chromatographic process and were used without further treatment.

1. Alcoa Activated Alumina Grade E80 Mfd. by Aluminum Ore Co.
2. Granular Adsorptive Magnesia # 2652 California Chemical Co.
3. Magnesium Silicate 34 Lot No. 3.
4. Pacific Silicate Co. Ltd. Chemical Dept.
5. Silica Gel - finely divided.
6. Superfilterol - The superfiltrol used was a finely divided bentonite clay obtained from the Filtrol Corp.
7. Magnesol A

Chromatograph tubes:

The chromatographic tubes used in Part I had the following dimensions inside diameter upper portion 8.0 mm. inside diameter lower portion 4.0 mm., length, upper portion 17.0 cm., lower portion 8.5 cm. The upper portion of the chromatographic tube used in Part II was 21.5 cm. in length, the lower portion 11.0 cm. The inside diameter of the upper part of the tube was 17.7 mm. and the inside diameter of the lower portion was 4.4 mm.

Irradiation source:

For Part II of this investigation a 500 watt Hanovia low pressure mercury vapor lamp was used. This lamp operated on a current of 9 amps. The radiation was unfiltered.

A quartz ribbon cell with a thickness 0.21 mm. and volume 0.29 ml. was used as an irradiation chamber. The cell was arranged as shown in photograph (I) so that the cell was parallel to the mercury vapor lamp. The solution to be irradiated was poured into the reservoir, upper right in the photograph, and the irradiated mixture was collected in the flask, lower left.

Collection of fractions:

1. Throughout the first part of this investigation the fractions eluted from the chromatographic column were collected using the apparatus shown in photograph (II). The eluate from the column was allowed to drop into the vials contained in the suction flask. The vials could be moved by means of the handle at the top of the suction flask.

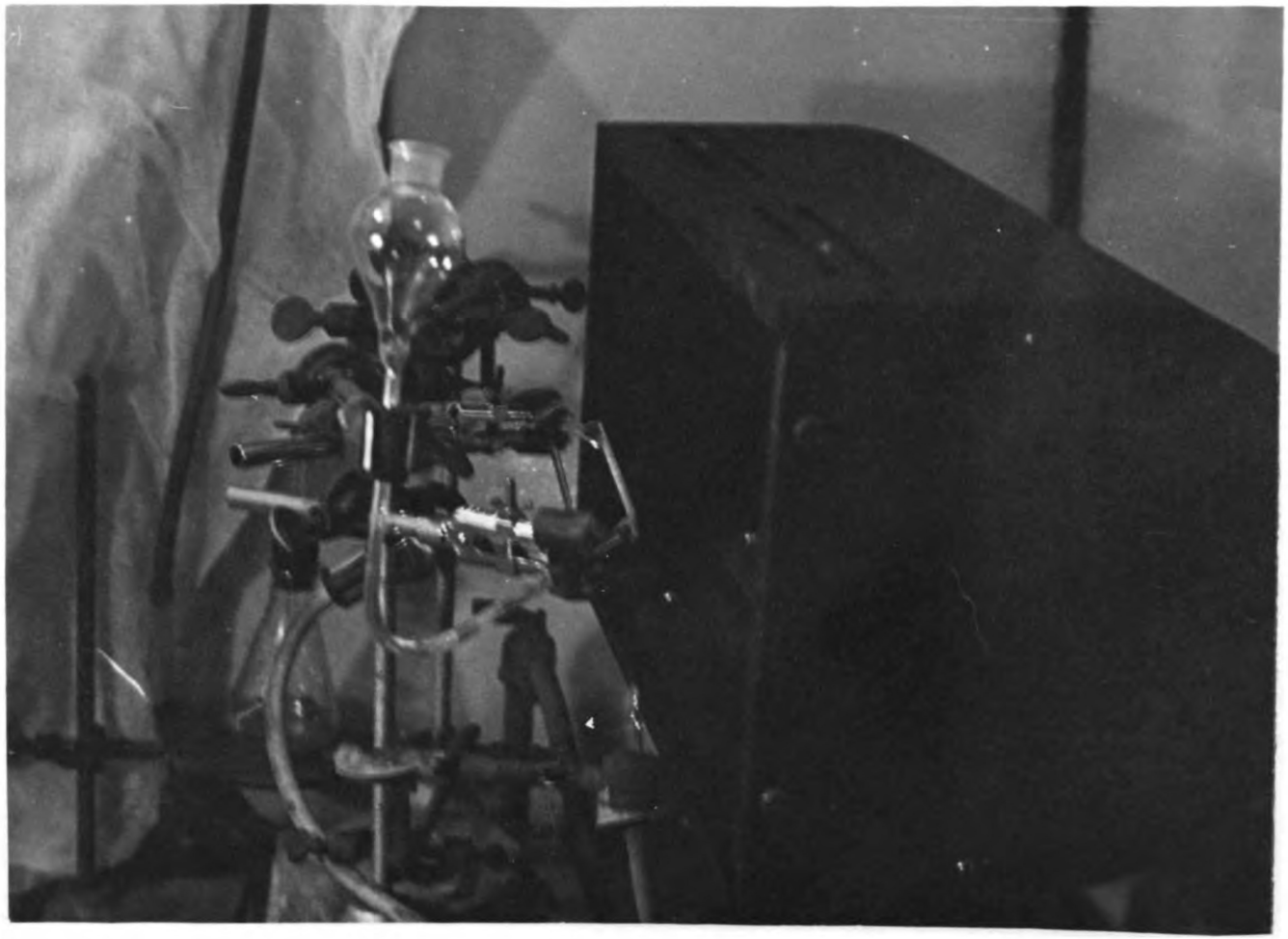


PLATE I

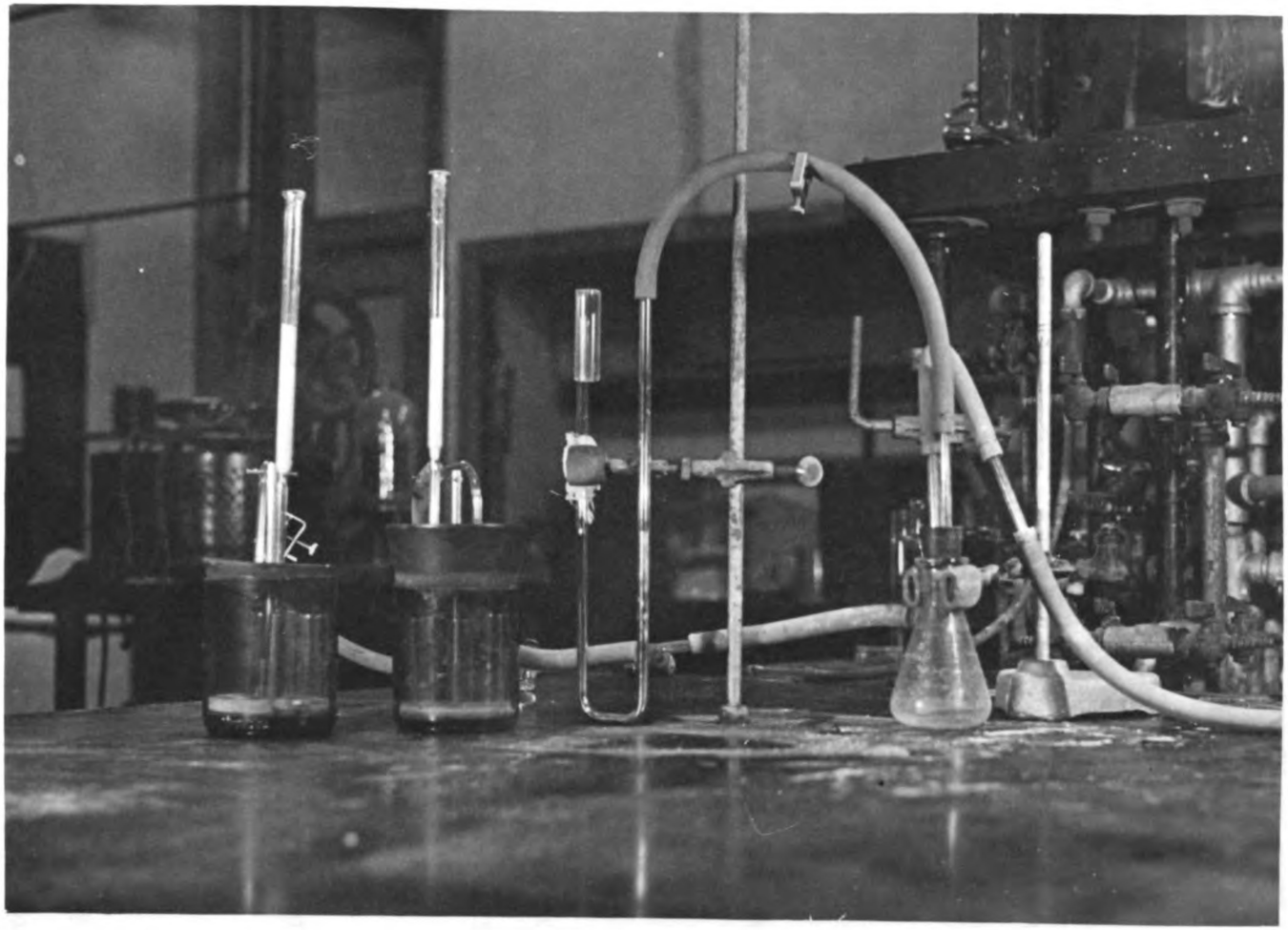


PLATE II

Suction was applied by means of a water aspirator connected with an open end manometer.

2. In the second part of this investigation fractions were collected using a Technicon Automatic Fractionator. The upper portion of this instrument is shown in photograph (III). The tubing from the top of the chromatographic column and the top of the separatory funnel leads to the carbon dioxide tank which supplied the necessary pressure and at the same time excluded air from the system during the chromatographic process.

Determination of absorption spectra:

All absorption curves were determined using a Beckman spectrophotometer equipped with quartz cells.

Purpose:

It was the purpose of this investigation to study the adsorption of calciferol by various adsorbents and to determine the possibility of effecting a chromatographic separation of calciferol from the other irradiation products of ergosterol. The absorption spectra of the fractions eluted was used as a means of determining the effectiveness of separation.

Procedure:

1. The chromatographic column was prepared in the usual manner using enough adsorbent so that the column when packed was 8 cm. in height. The apparatus was set up as shown in photograph (II).

PLATE III

2. Then 3 ml. of alcoholic stock solution of calciferol was pipetted into an erlenmeyer flask. The solution was evaporated to dryness over a water bath (temp. 60° C.) with the aid of suction. The residue was taken up in 3 ml. of eluant.

3. The chromatographic column was washed with 10 ml. of ether. At this time the water aspirator was adjusted so that a drop rate of one drop every three seconds was attained. The pressure differential was marked on the mercury manometer and was thereafter kept constant by adjustment of the water aspirator.

4. When the level of the ether in the chromatographic column dropped to a mm. of the top of the absorbent the calciferol solution was added and a clean vial was placed under the column by rotating the vial holder. The eluate from the prewash was discarded. All other vials were calibrated to hold 3 ml.

5. The flask was rinsed with 3 ml. of eluant. The resultant solution was added to the column when the level of the original calciferol solution dropped to a mm. of the top of the adsorbent.

6. When the level of the solution used for rinsing the flask reached a mm. of the top of the adsorbent the developer was added and the chromatogram developed until enough 3 ml. fractions had been collected to insure the complete elution of the calciferol from the column. The number of fractions necessary was ascertained for new adsorbents by making a survey run or by comparison with previous runs.

7. The vials containing the desired fractions were placed in a suction flask and evaporated to dryness over a water bath and the residue was taken up in 4 ml. of the eluant.

8. The absorption curves of the resulting solutions were determined on the Beckman spectrophotometer using 4 ml. of the eluant as a blank.

9. The extinctions of the various fractions were plotted as functions of the wave length.

RESULTS

The extinction at 265 mu is directly proportional to the amount of calciferol present in the fraction.

Table I shows the different adsorbents, the amount of adsorbent used, the pressure differential maintained, and the volume of eluant collected in the comparison of the adsorbents.

The data in Table II show the extinction values at 265 mu for the different fractions when different adsorbents were used. The curves obtained from this data are shown in Figure 1. The adsorbents for which the graphs are include are activated alumina E 80, magnesol, adsorptive powdered magnesia, and adsorptive gramular magnesia. In Table III the composition of the eluant is given when magnesol was used as an adsorbent.

Table IV shows the extinction values at 265 mu for the different fractions when magnesol was used as an adsorbent. Here the ratio of ether to hexane in the eluant was varied. The graphs obtained are shown in Figure 2. In these graphs the extinction value for the fraction corresponding to 0-3 ml. is plotted at 3 ml.

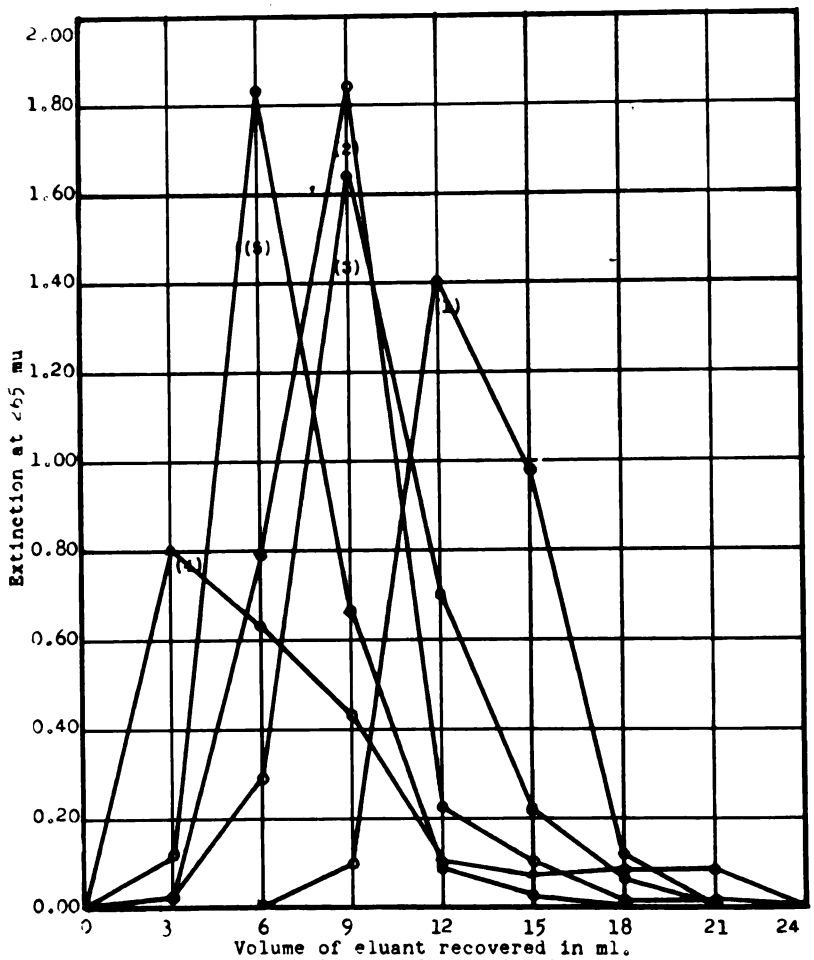


Figure 1. Rate of elution of calciferol from various adsorbents. Solvent 70:30 ether-hexane. Adsorbents (1) Activated alumina, (2) Magnesol, (3) Powdered magnesia, (4) Granular magnesia, (5) Magnesium silicate.

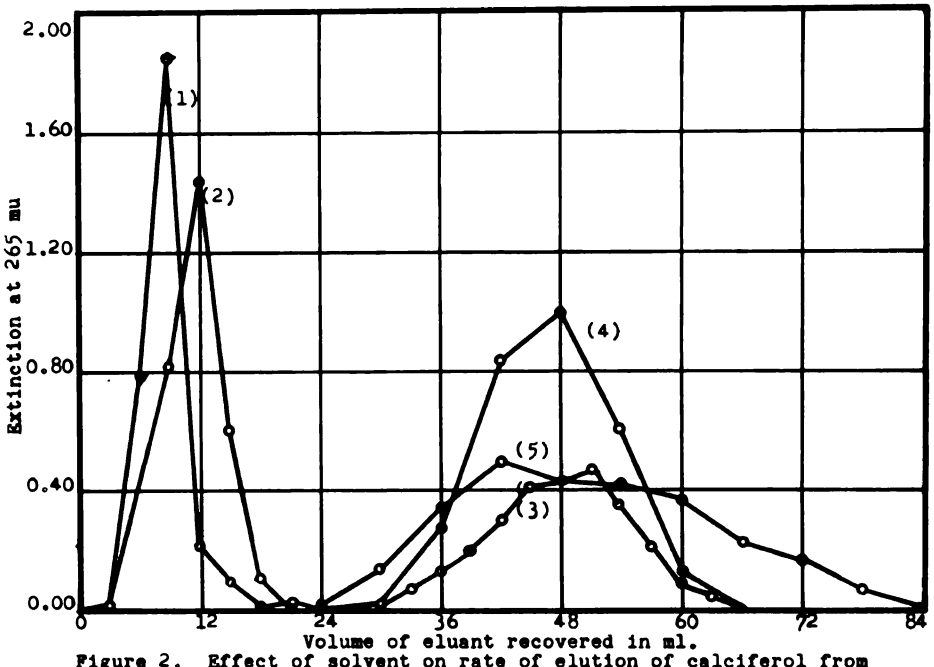


Figure 2. Effect of solvent on rate of elution of calciferol from magnesol. Solvent ether (E) and hexane (H). (1) 70:30 E-H, (2) 30:70 E-H, (3) 10:90 E-H, (4) 8:92 E-H, (5) 5:95 E-H.

DISCUSSION

Since the adsorption of calciferol by activated alumina has been investigated by others it was decided to work only with other adsorbents which might prove useful in the separation of D_2 and ergosterol.

A ratio by volume of 70 parts ether to 30 parts hexane was used as an eluant in comparing the various adsorbents since this ratio had been found by Chen (5) to give the most intense banding with activated alumina.

The silica gel investigated was found to be unsatisfactory due to the fact that it was so fine that it did not allow the passage of the eluant through the column. Adsorptive granular magnesia proved to be too coarse and the eluant ran through the tube too rapidly for quantitative results and so was not further investigated. Adsorptive powdered magnesia and magnesium silicate were so fine in texture that even with a pressure differential of 12 to 15 cm. of mercury the drop rate was exceedingly slow and in view of the fact that both showed little or no adsorption of calciferol they were not used further.

Figure 1 shows how the rate of elution of calciferol was affected by the different adsorbents. From the graph it can be seen that the adsorptive granular magnesia shows no adsorption of calciferol. It seems quite possible that this is also true of the magnesium silicate for the maximum extinction value occurs in the second fraction and the first fraction consists mainly of the wash ether which still remains on the column when the calciferol is added. Both the powdered magnesia

and the magnesol show some adsorption of calciferol but since the rate of elution from the powdered magnesia was extremely slow only the magnesol was tested further.

Figure 2 shows how the adsorption of calciferol is affected when the ratio of the hexane to the ether is changed. The results show that as the concentration of ether in the eluant decreased the adsorption of the calciferol increased and the rate of desorption decreased. Therefore the number of ml. eluted before the maximum extinction is reached increases and the adsorption band becomes wider.

When a solution containing a 70:30 ether, hexane ratio by volume was used as a developer the maximum extinction at 265 mu occurred when 9 ml. of eluate had been obtained. The calciferol eluted was contained in 18 ml. of eluate. However, when 5:95 ether, hexane ratio was used the maximum extinction at 265 mu occurs when 51 ml. have been eluted and the calciferol is contained in 36 ml. of the eluate.

Since the banding seemed sharpest using a 70:30 hexane, ether ratio by volume an attempt was made using this mixture to separate calciferol from ergosterol using magnesol as an adsorbent. The results showed that the calciferol was eluted from the column along with the ergosterol so a separation was impossible.

SUMMARY

1. Magnesium silicate and adsorptive powdered magnesia could not be used as adsorbents in the chromatographic process without the addition of some inert filler to increase the rate of elution.

2. Adsorptive granular magnesia and silica gel proved unsatisfactory adsorbents.

3. Magnesol can not be used as an adsorbent to effect a separation of calciferol and ergosterol.

4. As the concentration of ether in the eluant is decreased the adsorptive capacity of the adsorbent is increased and the width of the adsorption band is increased.

TABLE I

DATA FOR DIFFERENT ADSORBENTS USED

Eluant 70:30 ratio by volume of ether and hexane
Concentration of calciferol 0.0249 mg.

Run No.	Adsorbent	Wgt. Adsorbent g.	Pressure mm. Hg.	Total Vol. eluated ml.	Vol. Fraction ml.
1	Activated Alumina	4.80	1	24	3
2	Magnesol	1.33	12	18	3
3	Powdered Magnesia	2.00	12	18	3
4	Granular Magnesia	3.00	0	18	3
5	Magnesium Silicate*	1.84	15	15	3

* Eluant ratio by volume of 50:50, ether-hexane

TABLE II

EXTINCTION VALUES AT 265 MU FOR FRACTIONS OF ELUANT RECOVERED FROM DIFFERENT ADSORBENTS

No. of Fraction	Total Volume of eluant in ml.	Run No. 1	Run No. 2	Run No. 3	Run No. 4	Run No. 5
1	0-3	0.003	0.025	0.007	0.798	0.120
2	3-6	0.003	0.798	0.292	0.636	1.836
3	6-9	0.112	1.860	1.660	0.433	0.648
4	9-12	1.390	0.226	0.707	0.109	0.091
5	12-15	0.975	0.109	0.223	0.075	0.030
6	15-18	0.125	0.022	0.019	0.180	
7	18-21	0.049				
8	21-24	0.013				

TABLE III

VOLUME OF ELUATE NECESSARY TO RECOVER CALCIFEROL FROM MAGNESOL AS RATIO OF ETHER TO HEXANE IN ELUANT IS VARIED

Adsorbent Magnesol, 1.33 g.

Run	Parts Hexane by Volume	Parts Ether by Volume	Calciferol mg.	Total Vol. eluate ml.	Vol. Fraction ml.
A	30	70	0.0249	21	3
B	70	30	0.0261	21	3
C	90	10	0.0261	78	6
D	92	8	0.0261	66	6
E	95	5	0.0261	63	3

TABLE IV

EXTINCTION AT 265 MU FOR DIFFERENT FRACTIONS OF ELUATE RECOVERED FROM MAGNESOL COLUMN AS RATIO OF ETHER TO HEXANE IN ELUANT IS VARIED

Adsorbent Magnesol, 1.33 g.

Vol. of Eluate Collected ml.	Run A	Run B	Run C	Run D	Run E
0-3	0.025	0.096			
3-6	0.798	0.830			
6-9	1.86	1.45			
9-12	0.226	0.598			
12-15	0.109	0.114			
15-18	0.022				
18-21					
21-24			0.024		
24-27					
27-30			0.143	0.034	0.036
30-33					0.075
33-36			0.357	0.283	0.135
36-39					0.207
39-42			0.499	0.820	0.308
42-45					0.415
45-48			0.437	1.00	0.435
48-51					0.475
51-54			0.431	0.621	0.364
54-57					0.219
57-60			0.377	0.136	0.095
60-63					0.049
63-66			0.229		
66-69					
69-72			0.174		
72-75					
75-78			0.075		
78-81					

LITERATURE CITED

1. R. W. Miner, Ed.; Annals of the New York Academy of Sciences 49, 141-326 (1948)

2. R. W. Miner, Ibid.

3. L. Zschmeister, Principles and Practice of Chromatography, J. Wiley and Sons, N. Y. (1941)

4. H. H. Strain, Chromatographic Adsorption Analysis, Interscience Publishers, Inc. New York, N. Y. (1942)

5. L. J. Bullard, M. S. Thesis, Michigan State College (1945)

6. D. H. Baker, M. S. Thesis, Michigan State College (1944)

7. M. J. Powell, M. S. Thesis, Michigan State College (1946)

8. F. Chen, M. S. Thesis, Michigan State College (1950)

PART II

PART II

THE CHROMATOGRAPHIC SEPARATION OF IRRADIATED ERGOSTEROL

The study of ergosterol and its irradiation products has been the subject of a voluminous number of research papers, since Pohl (1) and Windaus (2) first reported, in 1927 that the impurity contained in sterols, which after irradiation possessed antirachitic properties, was ergosterol or a similar sterol. Ergosterol had been isolated in 1811 by Braconnot and was rediscovered by Tanret in 1889 (3) but the structure of the molecule was unknown.

After forming and irradiating various esters of ergosterol Windaus (4) found that the irradiated esters possessed no antirachitic properties. He found, that if the irradiated esters were reconverted to sterols that the sterols produced did possess antirachitic properties and he therefore concluded that the hydroxyl group present in the sterol was responsible for these properties and that irradiation affected ergosterol and its esters in the same manner.

Windaus also concluded from measurements of solutions of irradiated ergosterol that solvents pervious to the radiation had little effect upon the irradiation process (5). He found that the specific rotation of a solution of irradiated ergosterol varied directly with the time of irradiation, finally reaching a maximum positive value at which time the antirachitic properties of the solution also reached a maximum. With further irradiation the specific rotation of the solution decreased in magnitude finally becoming negative with the gradual

disappearance of antirachitic properties. If oxygen were excluded two isomers of ergosterol were formed, both containing three double bonds and one hydroxyl group, one with a positive and one with a negative specific rotation, the one latter isomer being the active one.(6)

In 1930 Windaus (7) reported that suprasterol 1. and 2., the products formed when ergosterol was irradiated with a mercury vapor lamp for 50 hours at 75° C. were stable to air. These two compounds were shown to have no absorption over 260 mu. They seemed to be formed simultaneously as neither one could be converted into the other by means of irradiation.

A toxic compound was reported by Windaus (8) to be present in irradiated ergosterol and was characterized by showing a maximum absorption in the region of 247 mu. At the same time a compound (later known as tachysterol) showing main absorption from 280 mu to 290 mu was postulated as originating from vitamin D.

The presence of the toxic compound was substantiated by Morton and others (9) who showed that after 150 minutes irradiation of an alcoholic solution of ergosterol the absorption spectra of the irradiated material showed a substance exhibiting a strong maximum at 247 mu. Up to this time all efforts to separate the irradiation products of ergosterol had been more or less unsuccessful. Windaus (10) had made futile attempts to separate the irradiation products of ergosterol by extraction from methyl alcohol and benzine solutions. The separations based on chemical reactions did not yield products sufficiently pure for quantitative work.

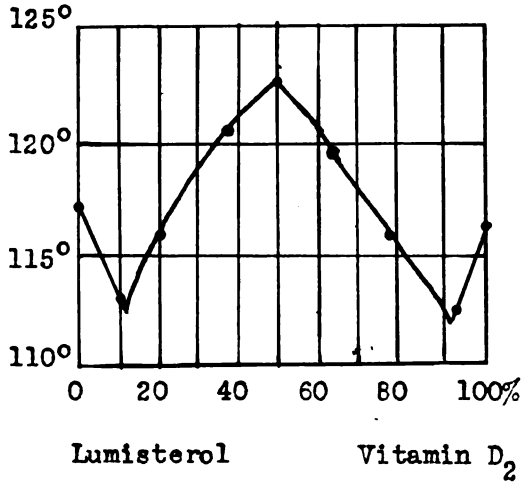
Windaus (11) believed that in the conversion of ergosterol into vitamin D by the photochemical process the molecular formula, the hydroxyl group, and the 3 double bonds remained unchanged and that the formation of vitamin D was due solely to some steric or structural rearrangement of the molecule which increased the spatial size and gave it a characteristic absorption maximum between 270 and 265 mu.

Vitamin D_1 was eventually separated (12) from irradiated ergosterol (40% conversion of the ergosterol used) by treating the irradiated mixture in Et_2O with citraconic anhydride for ten days and crystallizing the D_1 from acetone.

Vitamin D_1, was finally shown to be a molecular addition compound of lumisterol and vitamin D_2 (13). It was found that upon heating D_1 a short time with acetic anhydride and subsequent cooling lumisteryl-acetate crystallized out of the solution and the lumisterol could be recovered by saponification of the acetate, while the acetate of vitamin D_2 remained in solution. The lumisterol, an isomer of ergosterol, showed absorption maxima at 265 and 280 mu. Upon further irradiation with a magnesium spark lumisterol could be converted into vitamin D_2 but the reverse reaction could not be made to occur. Thus lumisterol was shown to be an intermediate between ergosterol and vitamin D_2.

Vitamin D_2 was then added to lumisterol in varying amounts and the melting points of the various mixtures was determined. The final melting point diagram shown below showed that when lumisterol and vitamin D_2 were added together in equal amounts a molecular addition

product was formed. The melting point of this compound and its absorption spectra coincided with those for vitamin D_1.



Lumisterol, tachysterol, and calciferol (14) were agreed to be the intermediates between ergosterol and the suprasterols but the order of conversion for some time remained doubtful. Setz (15) as a result of his investigations concerning the influence of different wave lengths of radiation on the conversion, stated that it appeared that light of longer wave lengths seemed to lead chiefly to lumisterol which could be converted only slowly to tachysterol and vitamin D_2. He reported that shorter wave lengths made it possible to skip the lumisterol stage with the immediate formation of tachysterol. Tachysterol is ten times more sensitive than ergosterol to radiation between 300 and 360 mu and the conversion to calciferol is rapid. With radiation from a mercury vapor lamp filtered through xylene which absorbed the greater portion of the radiation of wave lengths less than 280 mu he found that lumisterol and vitamin D_2 were formed in constant proportions. With radiation from magnesium light (chiefly between 278 and 280 mu)

practically no lumisterol was formed and the resulting products were mainly tachysterol and vitamin D_2.

Today it is generally agreed that the conversion takes place in the following manner (16).

Ergosterol ⟶ Lumisterol ⟶ Protachysterol ⟶ Tachysterol ⟶

Calciferol ⟶ Toxisterol

Calciferol ⟶ Suprasterol I.

Calciferol ⟶ Suprasterol II.

This is the sequence proposed by Dimroth (17) except that protachysterol has been added between lumisterol and tachysterol. Protachysterol has not been isolated in the pure form but spectroscopic studies of irradiated solutions show that an intermediate is formed in the irradiation process which on standing is converted into tachysterol.

This sequence was further substantiated by the investigations of Heilbron and Spring (18) which showed lumisterol to be tetracyclic in structure and tachysterol and calciferol tricyclic in nature.

The chemical structure of ergosterol and its irradiation products was for a long time a subject of much investigation. One of the first structures for ergosterol contained two six membered and two five membered rings with a single side chain of the formula $C_{11}H_{23}$, three double bonds, and one hydroxyl group (19). It was finally shown that ergosterol was a sterol because it yields γ-methyl-cyclo-penteno-phenanthrene (upon dehydrogenation with selenium) (20).

The reaction of ergosterol with ozone (21) yielding methyl isopropyl acetaldehyde shows the double bond on the side chain to be

between carbon atoms 22 and 23. The two double bonds in the ring are between carbons 5 and 6 and carbons 7 and 8. The attachment of the hydroxyl group to carbon atom three was shown by the oxidation of acetylated ergosterol with chromic acid (22). This position had been favored by Danielli and Adams (23) after measurements of the surface potential of films of ergosterol during irradiation. The changes in surface potential were surmised to be due to a change in the tilt of the hydroxyl group.

It is now believed that with the addition of radiant energy to the ergosterol molecule an activated molecule results which is lumisterol. With further addition of radiant energy the bond between carbon atoms 9 and 10 breaks giving rise to double bonds between carbon atoms 5 and 10, 6 and 7, 8 and 9 and tachysterol is formed. These bonds rapidly rearrange giving a more stable state which is calciferol. In this state the double bonds are located between carbons 5 and 6, 7 and 8, and 18 and 10. (24)

Thus the conversion is the direct result of absorption of radiant energy by the molecule. Various methods of irradiation have been investigated with ergosterol in the solid state in solution and in the vapor state (25,26,27). Jendrassek used a method whereby solid ergosterol in contact with a solution containing vitamin D or ergosterol was irradiated and the solution containing the ergosterol was led away by dialysis fresh solution being continuously added.(28)

Trufanov (29) believed that a 2% solution of ergosterol in benzine irradiated for four hours, at which time 50% of the ergosterol was

converted, gave the best result with regard to the production of D_2.

Bills, Honeywell, and Cox (30) after irradiating solutions of ergosterol in ether, cyclohexane, and alcohol with a mercury arc found that the same general absorption curves resulted. However, the potency of the ether solutions was about twice that of the other solutions. It now seems that the activation takes place more rapidly in ether solution.

Irradiation of solid ergosterol (31) has not proved successful because the vitamin D_2 is formed only on the surface of the crystals. With further irradiation the D_2 is decomposed before the provitamin in the middle of the crystal can be affected. The investigation of irradiation in the vapor state has not been sufficiently investigated to allow any definite conclusions as to the value of the process. The most efficient technical method now in use is the method whereby an ether solution flows into special quartz irradiation chamber built concentrically around a mercury vapor lamp (32).

The effect of temperature of the solution being irradiated upon the conversion was investigated by Webster and Bourdillon (33). They irradiated solutions of ergosterol at 77.8°, 30.6°, 1°, -18°, and approximately - 183° and - 195° C. They concluded there was very little change in the activity of products produced in any of the first four cases.

The wave lengths of light used for irradiation have been investigated at some length and it has been more or less agreed that wave

lengths between 275 and 300 mu produce the best yields of vitamin D with the smallest amount of by products.

Kon, Daniels and Steenbock (34) reported that for the 256, 265, 280 and 293 mu lines the quantity of radiant energy necessary to form an amount of vitamin D to cause demonstrable results in rats was 700 - 1000 ergs. The data obtained under the most careful conditions indicate that 7.5×10^{13} quanta will produce one U.S. Pharmacopoeia unit of Vitamin D. However in the active region the energy required depends upon the wave length.

In 1933 Bacharoch and others (35) proposed that the following E 1%, 1 cm. values for ergosterol and calciferol be adopted.

E 1%, 1 cm. for ergosterol at 281 mu not less than 320 and for calciferol at 265 and E 1%, 1 cm. not less than 470.

At the time of this investigation there were available no E 1%, 1 cm. values for any of the other products of the irradiation process. Though curves for all of the products (attributed to Brockmann) are published by Rosenberg (36) the original data was unavailable so that the curves could be used only in a qualitative manner.

Purpose:

In this part of the investigation an attempt was made to separate the irradiation products of ergosterol by chromatographing the crude irradiation mixture using superfiltrol as an adsorbent and a solution of 50:10:1 for developing the chromatogram.

A special quartz ribbon cell was used as an irradiation chamber and irradiation was carried out on ether solutions of ergosterol with a mercury vapor lamp.

The ether solutions were irradiated for different lengths of time and the resultant mixtures were chromatographed on superfiltrol. One milliliter fractions of eluate were collected and the absorption curves of the various fractions determined.

Procedure:

1. 0.25 g. of ergosterol was dissolved in 100 ml. of freshly distilled ether and cooled in an ice and salt mixture.

2. The ether solution was irradiated in the following manner:

   a. The mercury lamp was turned on until maximum light intensity was attained.

   b. The air jets focused on the cell were turned on to keep the air around the cell in circulation and thus cool the irradiation cell.

   c. The cell and reservoir were rinsed with 50 ml. of freshly distilled ether.

   d. The ether solution was poured into the reservoir and allowed to pass through the cell. The rate of flow was maintained a constant by regulation of the head of the solution in the reservoir.

3. The total irradiation time was measured with a stop watch.

4. For irradiation times longer than 12 minutes the solution was passed through the cell more than once.

5. The mercury vapor lamp was turned off.

6. One ml. of the irradiated solution was removed for absorption analysis.

7. The remaining solution was evaporated to dryness over a water bath (temp. 60° C.) with the aid of suction.

8. The residue was immediately taken up in 6 ml. of 50:10:1.

9. One tenth of a milliliter of this solution was reserved for analysis.

10. The remaining solution was chromatographed in the following manner on a column containing 12 grams of superfiltrol which had been prewashed with 50 ml. of 50:10:1.

a. When the level of prewash solution was about 1 mm. above the top of the column the solution to be chromatographed was added slowly to the column. (Note: If the adsorbent is disturbed the resulting bands will not be even.)

b. The flask was then rinsed with 3 ml. of 50:10:1 and this solution was added to the column.

c. When the level of the solution reached within a mm. above the top of the column the eluant was added to the column and elution was continued until the desired number of fractions were collected. (Note: in the early part of this investigation it was assumed that 30 fractions would be sufficient. It was later decided to increase the number of fractions collected until it was certain that the extinction of the last fractions were neglegible. This number turned out to be about 70.)

11. Each fraction consisted of 1 ml. of eluate or 43 drops.

12. The fractions were collected using a Technicon Automatic Fractionator.

13. The fractions were diluted to 5 ml. with 50:10:1 and the absorption curves for each fraction determined by means of a Beckman spectrophotometer. When necessary, fractions were further diluted so that the extinction values in the range from 400 mu to 220 mu did not exceed 0.900 extinction units.

14. The extinction values for the separate fractions were plotted as functions of the wave lengths.

Calculation of irradiation time per molecule:

The time necessary for the total ether solution to flow through the cell into the receiver was considered to be the total irradiation time. When it was necessary to pass the solution through the cell more than once (explained in the procedure) the total irradiation time was the sum of the times necessary for the solution to pass through the cell.

Example:in Run No. 1 the solution was passed through the cell once. The stop watch was started when the ether solution was poured into the reservoir and was stopped when the last of the solution had run into the receiver. This took 12 minutes 22.8 seconds and this is the total irradiation time.

In Run No. 2 the solution was passed through the cell twice. The first time the solution was passed through 11 minutes and 50 seconds elapsed. The solution passed through the cell the second

time in 11 minutes and 10 seconds. The sum of these two periods of time is 23 minutes. This is the total irradiation time.

In each run the volume of solution irradiated was 100 ml. The volume of the quartz irradiation cell was measured by Kirn (37) to be 0.29 ml. It was assumed that the rate of flow of solution through the cell was constant. In order for 100 ml. to flow through the cell it was necessary for the cell to empty 100/0.29 times. It was evident that each molecule would be in the cell for the length of time necessary for the cell to empty.

Thus the irradiation per molecule is obtained by dividing the total irradiation time by 100/0.29.

Example: Run No. 2

Total Irradiation time = 23 minutes

Irradiation per molecule $23 \div \frac{100}{0.29}$ = 0.062 minutes or 3.71 seconds

RESULTS

The pertinent data pertaining to each run has been tabulated and is shown in Table I.

The procedure for each run was the same except that for zero irradiation time the ergosterol was recrystallized from benzene and ethyl alcohol according to the method prescribed by Huber, Ewing, and Kriger (38).

The irradiation time for Run No. 1 was 12 minutes 22.8 seconds or 2.15 seconds per molecule.

Table II shows the extinction values of fractions 8, 10, 14, 16, 19, 22, 25, 28. The absorption curves for these fractions are graphed in Figure 1.

Run No. 2 was irradiated for a total of 23 minutes or 2.71 seconds per molecule. Table III gives the extinction values for some of the typical fractions obtained after chromatographing. The absorption curves for these fractions are graphed in Figure 2.

Table IV gives the extinction data for Run No. 3. The ether solution was irradiated for a total of 30 minutes and 35 seconds. This irradiation is the equivalent of 5.32 seconds per molecule. The absorption curves are graphed in Figure 3.

Tables V and VI give the extinction data for various fractions for Runs Nos. 4 and 5 which are irradiated 8.88 and 9.52 seconds per molecule respectively. The graphs for these runs are represented in Figures 4 and 5.

These graphs are only representative for the different runs for size does not permit reproducing them completely without foregoing legibility.

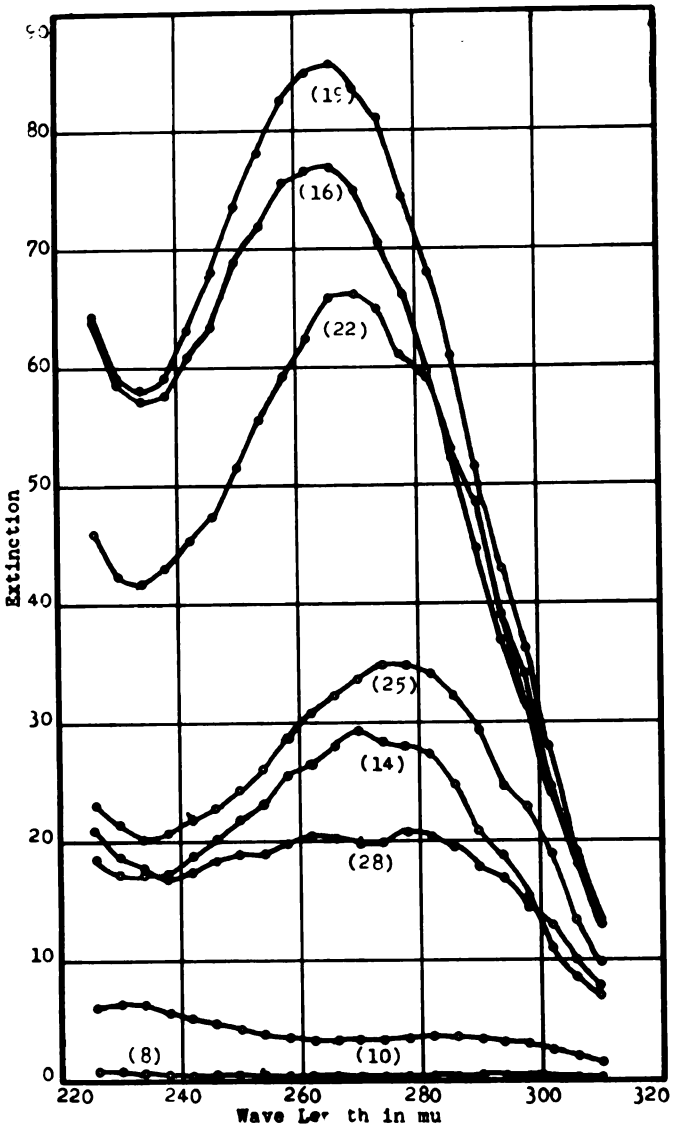


Figure 1. Irradiati[illegible]ve 2.15 sec. per molecule.

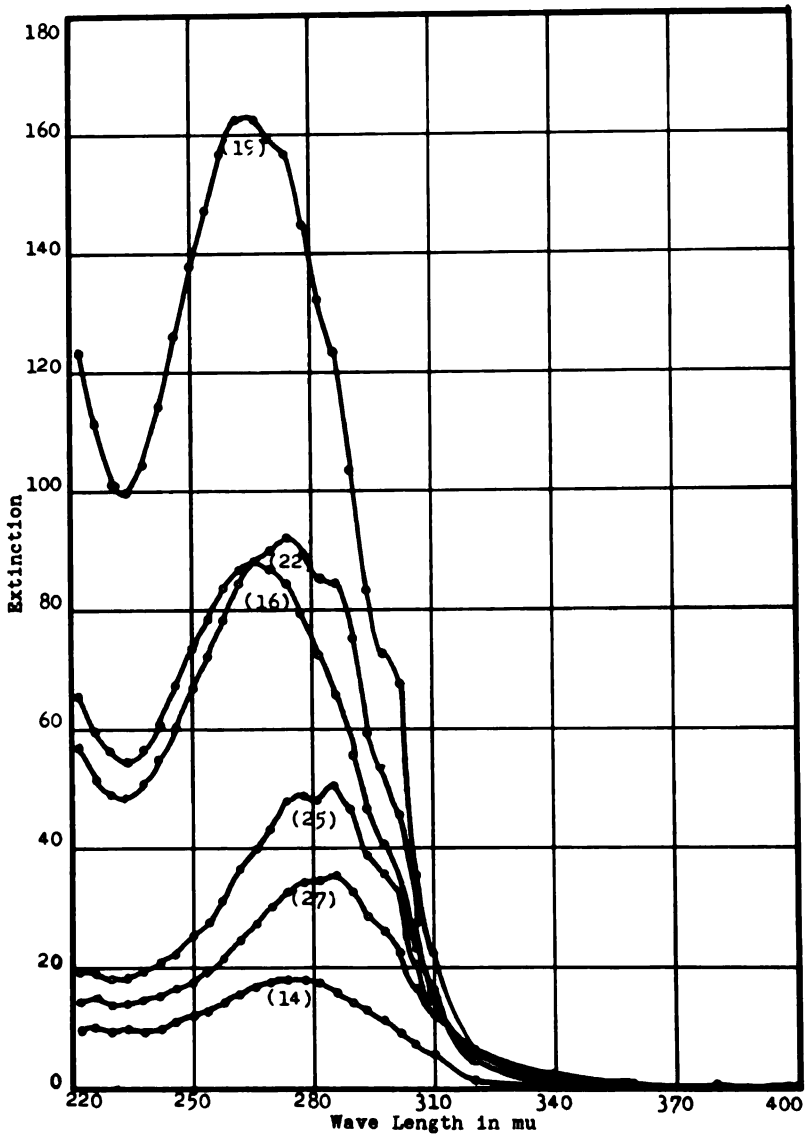


Figure 2. Irradiation Time 3.71 sec. per molecule.

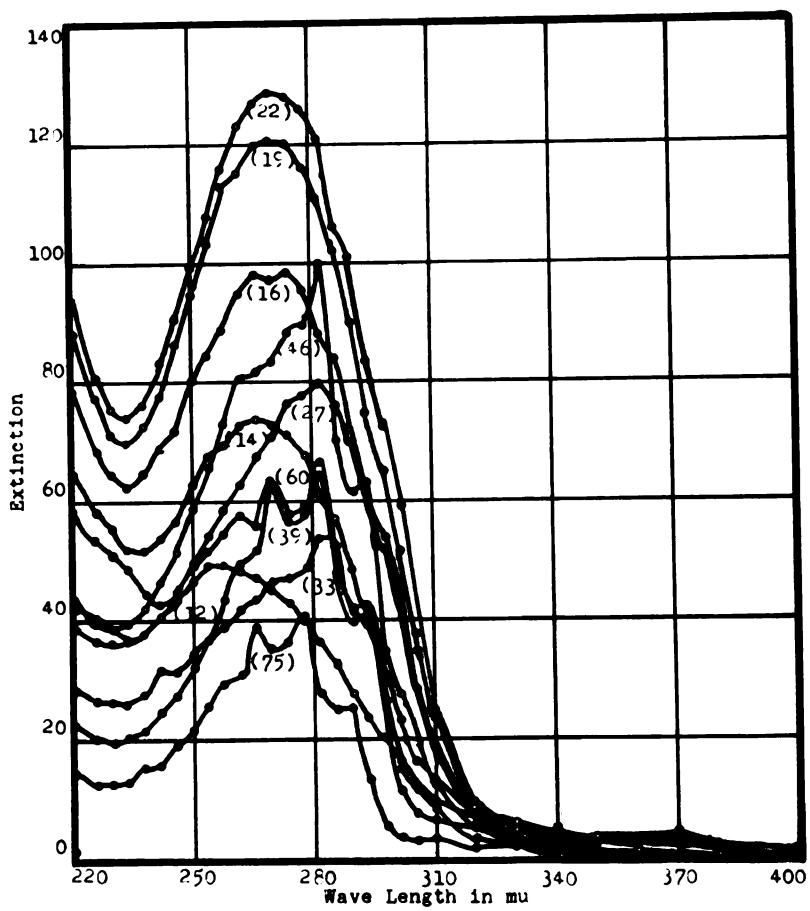


Figure 3. Irradiation Time 5.32 sec. per molecule.

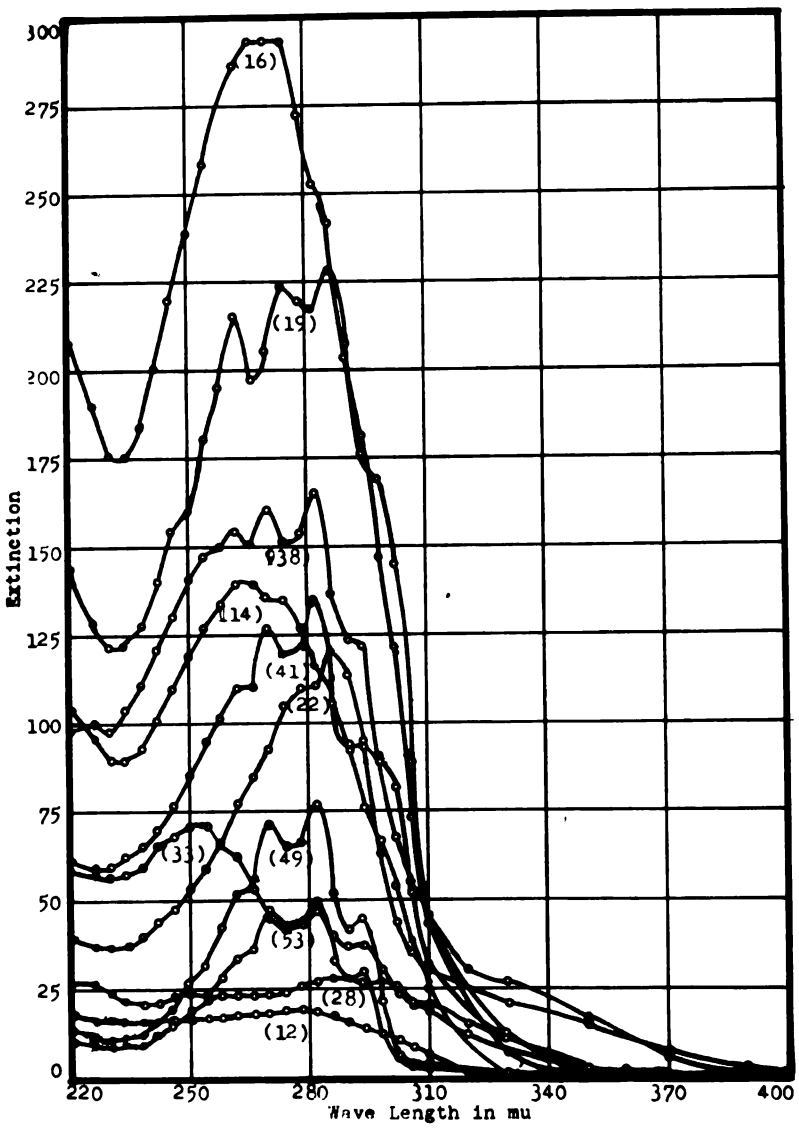


Figure 4. Irradiation Time 8.88 sec. per molecule.

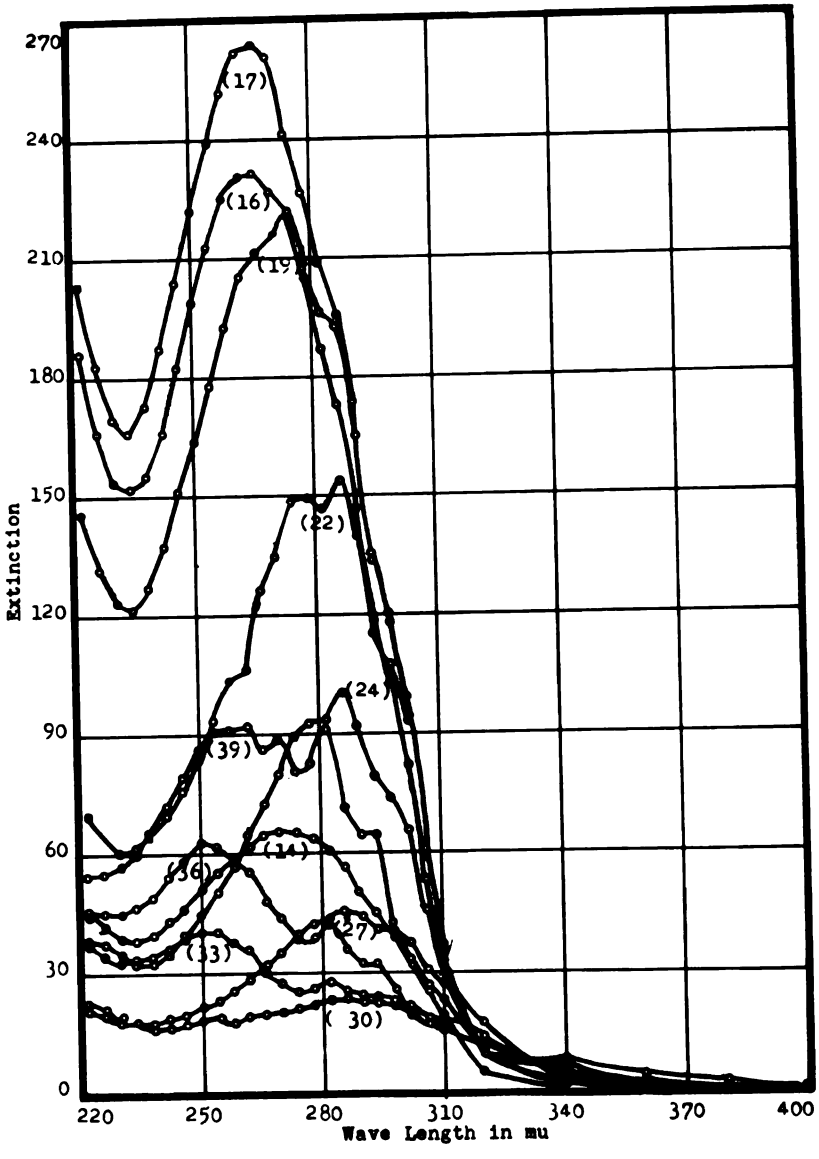


Figure 5. Irradiation Time 9.52 sec. per molecule.

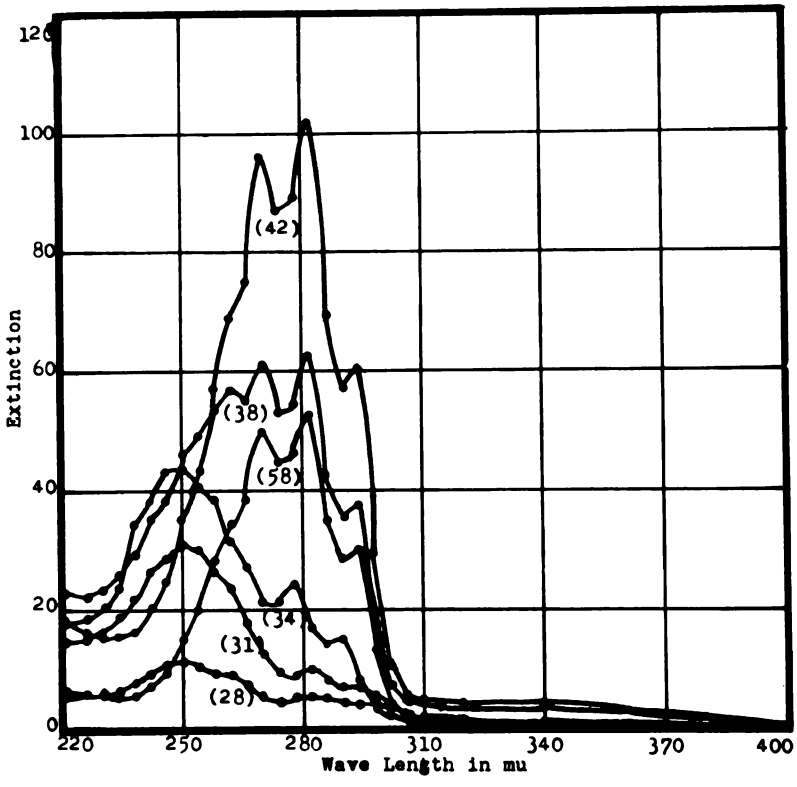


Figure 6. Irradiation Time Zero.

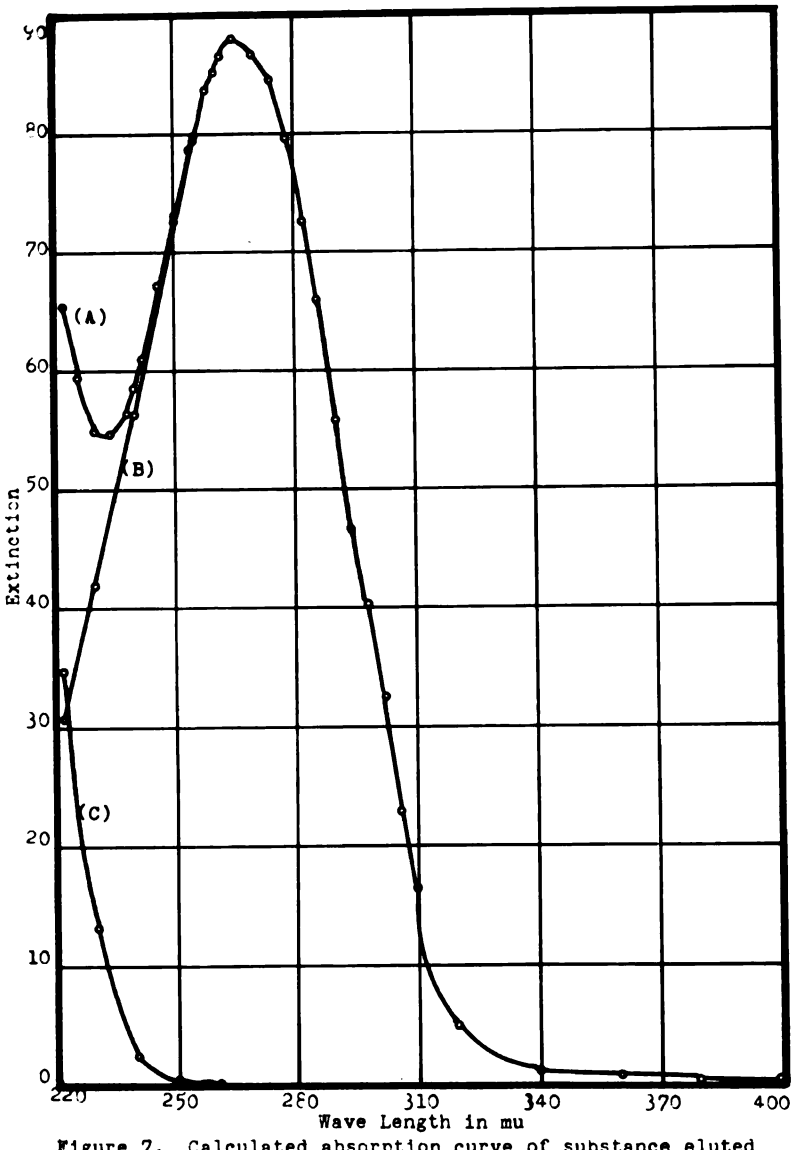


Figure 7. Calculated absorption curve of substance eluted with calciferol. (A) Absorption curve of fraction 16 Fig. 2 . (B) Curve if only calciferol were present, (C) A minus B.

DISCUSSION

The graphs shown in Figures 1-6 would seem to indicate that the amount of calciferol produced by the irradiation of ergosterol under the conditions herein described reaches a maximum when the irradiation time per molecule is approximately 8.88 seconds. The conditions of the experiment did not allow for closer adjustment of the time of irradiation.

It is difficult to state the above positively because as the irradiation time was increased the absorption spectra of the fractions eluted showed that the calciferol band was overlapping more and more with some other substance. Since the first sixteen or seventeen fractions eluted in each run show little or no distortion of the calciferol curve, between 230 and 300 mu it seems probable that calciferol could be separated from the interfering products by chromatographing fractions 17-25 a second time. In this way the curve of the second substance eluted from the column might be determined.

It seems evident that the increase in absorption of the various fractions in the range from about 232 mu (see Figures 1-5) down to 222 mu is caused by some product produced in the course of the irradiation because this increase is not characteristic of the curves obtained from the ergosterol which was not irradiated.

The only irradiation products of ergosterol which show this characteristic increase in absorption below 230 mu are the suprasterols. The extinction value for the suprasterols at 265 mu is zero. If it

is assumed that the extinction at 265 for fraction 16 in Run No. 2 is due only to the calciferol present in the fraction (see Figure 7) then the extinction values at other points between 265 and 222 mu can be calculated for the pure calciferol. This calculation has been shown in Table VIII. If the pure calciferol curve is subtracted from the experimental one curve C results. This curve compares favorably with the curve reported for suprasterol. The same calculations have been carried out for other fractions and the resulting curve has the same characteristics.

By comparison of the extinction values obtained for fraction sixteen in the different runs it can be noted that as the irradiation time is increased the distortion of the calciferol curve between 234 and 222 mu is increased. The increased distortion is due to an increase in concentration of the substance having an absorption spectra like that illustrated by curve C in Figure 7.

The fact that the concentration of this substance increased with irradiation time is also to be noted from the number of curves in each run showing this characteristic distortion. In Run No. 2 only the absorption spectra for fractions 9 to 26 show this distortion while in Run No. 4 the absorption spectra for all fractions from 12 through 66 show the same effect. This increase in concentration with irradiation time further supports the supposition that the increase in absorption from 234 to 222 mu is due to the presence of suprasterol for it is logical that as the time of irradiation is increased some of the calciferol would be decomposed to form suprasterols.

The rate of elution of the substance also supports the theory that it is suprasterol. According to theory, if all other variables are held constant those substances which contain the most rings should be eluted last but those having the greatest degree of unsaturation should be eluted last. Assuming the structure of suprasterol to contain two double bonds in a spiro-cyclo-pentane formula and one double bond in the side chain (39), and assuming of calciferol to have a ruptured cyclo-pentenophenanthrene structure containing three double bonds and a double bond in the side chain, it is evident that though calciferol is the most unsaturated, the suprasterol molecule contains more rings, thus the two molecules should migrate down the column at approximately same rate. This indeed proves to be the case.

Thus the presence of suprasterol along with calciferol in the first fractions eluted from the column seems fairly evident. Definite proof, however, has not been established since a complete separation of the two components has not been effected.

With an irradiation time of 51 minutes (see Figure 4, fraction 22) the calciferol band is overlapped by a band containing one or more other substances. The result is that the curves in this region show a maximum absorption at 286 mu. If a hypothetical calciferol curve (assuming high or low concentration of calciferol) is subtracted from such a curve a smooth curve is obtained having a maximum at 286 mu. Such a curve could not result from the addition of a lumisterol curve to a tachysterol curve or a toxisterol curve or any combination of these for both of the first two exhibit principal absorption at 280 mu while the absorption peak for toxisterol is at 248 mu.

Since similar fractions for the other runs show a similar maximum at 286 mu it is improbable that this absorption maximum is due to experimental error. Therefore, it seems evident that this absorption maximum is due to some intermediate formed in the conversion of ergosterol to calciferol which has not yet been isolated or to some error inherent in the method used.

In the runs where the number of fractions collected exceeded thirty it can be noted that fractions 30 through 40 show an absorption peak at about 250 mu. It was at first assumed that this peak was due to the presence of toxisterol. However, the run for zero irradiation time shows a similar series of curves.

The presence of calciferol is not evident from the absorption spectra of the unirradiated sample. Thus the maximum at 252 mu could not be due to toxisterol which presupposes the presence of calciferol.

Thus the substance causing this maximum could not be a product of the irradiation process but rather a result of a reaction of the eluant with the adsorbent, a reaction of the eluant with the ergosterol, an impurity present in the ergosterol, or some decomposition reaction of the ergosterol on the superfiltrol column itself.

All of the possibilities were investigated separately. First a run was made to determine whether the solvent was causing the elution from the absorbent of some substance showing maximum absorption at 252 mu. The column was packed in the usual manner and was washed with 50 ml. of 50:10:1. Then a solution of 50:10:1 was passed through the column and 66 one ml. fractions were collected. The absorption spectra

of each fraction was determined. The extinction values obtained were all so low, less than one extinction unit, that it was evident that the absorption maximum at 252 mu was not the result of an affect of the solvent on the adsorbent.

Next a run was made to determine whether or not the peak at 252 mu was caused by some affect of the alcohol on the ergosterol or the adsorbent. The column was perpared in the regular manner and 0.13 g. of ergosterol in 100 ml. of ether, evaporated to dryness, was taken up in 6 ml. of a solution containing 50 parts hexane and 10 parts ether and added to the column. Only half of the usual amount of ergosterol was used assuming 50% conversion would take place with irradiation under ideal conditions.

It was noted that in the chromatographic process when 50:10:1 was used as an eluant a blue band appeared about one third of the way down the column. As elution continued the band migrated down the column and was eluted when between 30 and 40 ml. of eluate had been collected. These fractions, 30-40 showed a yellow pigmentation.

A 50:10 hexane ether solution was used for elution. Seventy-six one ml. fractions were collected. During the chromatographic process it was noted that the blue band did not appear and the eluate showed no yellow color. The absorption spectra of each fraction was determined. The curves obtained still showed a maximum at 252 mu.

The possibility that the ergosterol was impure was next investigated. The ergosterol to be used was recrystallized from benzene and ethanol. Then 0.13 g. of ergosterol was dissolved in 100 ml. of ether,

evaporated to dryness, taken up in 6 ml. of 50:10:1, chromatographed in the regular manner and 70 fractions were collected. After the absorption spectra had been determined it was evident that the maximum at 252 mu was still present and had in no way been affected by the recrystallization process.

To determine whether decomposition of the ergosterol was occurring on the column a second run was made like the one above. When 138 ml. of eluate had been collected in 13 different portions the absorption spectra of the several portions were determined. The curves for the last three fractions were typical of pure ergosterol. These last three fractions, corresponding to the last 26 ml. of eluate were evaporated to dryness taken up six ml. of 50:10:1 and chromatographed in the regular manner. The absorption curves from the eluted fractions showed the same maximum at 252 mu as before.

Therefore, it can only be concluded that some reaction as yet undetermined occurs between the ergosterol and the adsorbents which is responsible for this maximum in the absorption curve.

The unconverted ergosterol is the last substance to be eluted from the column but it is not possible to recover the ergosterol in the pure state by this method due to the interference of the substances eluted with ergosterol in fractions 33 to 40. The ergosterol absorption curves substantiate the presence of a maximum occurring in the region of 330 mu as reported by Hogness but this was not investigated further.

SUMMARY

1. There is substantial evidence that suprasterol is eluted from the column along with calciferol in the first portion of the eluate.

2. It seems possible to separate calciferol from the ergosterol and its irradiation products other than the suprasterol by chromatographing part of the eluate a second time - approximately fractions 17 - 26.

3. The absorption maximum at 252 mu occurring in fractions 33 - 40 is not due to toxisterol as at first assumed but to some reaction between the ergosterol and the superfiltrol.

4. The substance causing the absorption maximum at 286 mu might be isolated by a second chromatographic process.

5. The maximum amount of calciferol seems to be produced with an irradiation time of 8.88 sec/molecule.

TABLE I

DATA FOR RUNS WITH DIFFERENT IRRADIATION TIME

Run No.	Total Irradiation Time	Total Vol. Eluate ml.	Irradiation per molecule calculated
1	12. min. 22.8 sec.	28	2.15 sec.
2	23 min.	27	3.71 sec.
3	30 min. 35 sec.	76	5.32 sec.
4	51 min.	78	8.88 sec.
5	54 min. 38.2 sec.	39	9.52 sec.
6	0	72	0.00

Concentration of ergosterol was 0.25 grams per 100 ml. of ether.
Volume of irradiation cell was 0.29 ml.
One ml. fractions were collected.
In Run No. 6 the concentration of ergosterol was 0.13 grams per 100 ml. of ether.

TABLE II

ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 1

Irradiation Time 2.15 sec./molecule

Wave Length in mu	Fraction 8	10	14	16	19	22	25	28
226	0.054	0.612	18.4	63.8	64.4	45.9	23.1	20.8
230	0.054	0.640	17.2	58.6	59.1	42.3	21.4	18.7
234	0.050	0.630	17.5	57.2	58.0	41.7	20.0	17.8
238	0.049	0.576	17.2	57.7	59.1	43.0	20.8	16.9
242	0.045	0.518	18.8	60.9	63.2	45.4	21.9	17.4
246	0.042	0.468	20.2	63.4	68.1	47.4	22.8	18.4
250	0.037	0.418	21.8	68.8	73.5	51.6	24.2	18.8
254	0.037	0.369	23.1	71.8	78.0	55.5	26.0	18.8
258	0.034	0.347	25.5	75.6	82.5	59.1	28.7	19.8
262	0.034	0.330	26.4	76.6	84.8	62.4	30.7	20.3
266	0.033	0.335	28.0	76.8	85.6	65.8	32.2	20.0
270	0.034	0.339	29.3	74.9	83.4	66.1	33.5	19.8
274	0.033	0.340	28.4	70.4	80.9	64.8	34.7	19.8
278	0.034	0.356	27.9	66.2	74.3	60.9	34.7	20.8
282	0.035	0.379	27.4	59.6	68.0	59.1	34.0	20.3
286	0.035	0.366	24.6	52.2	60.9	53.0	32.2	19.2
290	0.032	0.336	20.7	44.6	51.6	48.4	29.1	17.8
294	0.032	0.321	18.8	36.9	42.9	39.1	24.5	16.8
298	0.031	0.303	15.5	31.2	36.1	34.0	22.9	14.7
302	0.027	0.248	10.9	24.4	27.9	27.9	18.9	12.9
306	0.024	0.199	8.59	18.0	19.1	18.5	13.3	9.87
310	0.021	0.160	6.99	13.3	13.5	13.0	9.87	7.91

The extinction values in these tables were obtained in the following manner. For those fractions showing a maximum extinction of less than 1.00 in the range between 220 and 400 mu the extinction values were read directly using the Beckman spectrophotometer. All other fractions were diluted so that all the extinction values were between 0.400 and 0.900 and the extinction values were calculated by multiplying the values read on the instrument dial by the dilution factor.

TABLE III

ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 2

Irradiation Time 3.71 sec./molecule

Wave Length in mu	Fraction 14	16	19	22	25	27
222	9.93	65.4	124.	56.8	19.5	14.4
226	10.1	59.5	111.	51.7	19.1	14.9
230	9.45	52.2	101.	48.8	18.3	13.8
234	9.93	54.7	99.9	48.7	18.4	14.1
238	9.30	56.4	105.	51.0	19.2	14.6
242	9.93	61.0	114.	55.0	21.1	15.5
246	11.0	67.1	126	60.2	22.8	16.3
250	11.9	73.3	138.	66.8	25.6	17.8
254	12.5	78.6	147.	72.2	27.9	19.2
258	14.2	83.7	157.	78.2	31.3	21.7
262	15.3	86.5	163.	84.5	36.3	24.7
266	16.7	88.0	163.	88.1	39.8	27.3
270	17.5	86.7	160.	90.0	43.1	30.1
274	18.0	84.5	157.	92.7	47.8	32.6
278	18.0	79.5	145.	89.6	48.8	34.1
282	17.3	72.5	133.	85.2	48.1	34.6
286	16.0	65.8	124.	84.9	50.4	35.2
290	14.2	55.7	104.	75.3	46.5	32.9
294	13.0	46.7	83.4	59.3	38.8	28.8
298	11.3	40.4	72.9	53.3	35.7	26.3
302	9.15	32.4	57.9	45.2	31.2	22.8
306	7.45	22.9	35.7	27.3	20.6	16.3
310	5.67	16.4	22.5	14.6	12.1	11.8
320	1.55	4.18	6.51	4.49	5.58	6.51
340	0.620	1.24	1.71	1.08	1.86	2.64
360	0.620	0.930	1.24	0.465	0.930	1.39
380	0.465	0.620	1.08	0.155	0.775	1.08
400	0.465	0.465	.775	---	0.465	0.775

TABLE IV

ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 3

Irradiation Time 5.32 sec./molecule

Wave Length mu	Fraction 12	14	16	19	22	27	33	39	46	60	75
220	56.7	65.1	78.4	89.6	94.0	43.5	28.7	39.0	42.9	22.3	14.3
222	55.3	----	----	----	----	41.7	27.6	----	----	----	----
226	53.3	58.0	68.5	77.2	80.8	39.5	26.3	36.7	40.2	20.1	12.7
230	51.0	55.3	64.4	71.1	75.8	38.3	26.3	36.4	38.6	19.7	12.8
234	48.5	51.9	62.6	69.7	74.2	36.4	25.9	36.7	39.7	20.3	13.0
238	44.3	51.7	64.7	72.5	76.2	37.5	27.8	37.6	41.7	21.7	13.0
242	42.6	53.6	68.5	77.6	83.3	42.9	31.6	40.9	46.1	24.8	15.2
246	43.4	56.3	71.9	86.1	90.6	43.4	31.0	44.7	51.3	27.2	15.6
250	46.2	62.8	80.1	94.2	99.6	49.0	34.2	49.4	58.6	32.0	19.2
254	49.0	67.1	84.1	103.	108.	53.8	36.3	51.4	65.6	36.8	21.5
258	48.7	69.4	88.9	113.	116.	58.2	39.2	54.5	72.9	43.6	25.6
262	48.1	72.5	95.1	115.	123.	62.6	41.6	57.3	80.4	49.3	29.2
266	46.7	73.5	98.0	120.	127.	67.4	43.6	55.6	81.5	51.6	30.8
270	44.8	72.5	97.3	121.	129.	70.5	46.7	62.0	83.7	63.2	38.9
274	42.9	71.0	98.5	120.	128.	76.3	47.0	56.4	88.1	57.5	35.2
278	40.2	67.5	95.6	116.	126.	77.5	48.8	57.5	89.5	59.1	36.2
282	36.4	61.0	88.0	111.	121.	79.7	53.5	64.4	100.	66.7	40.9
286	32.9	56.9	84.3	102.	106.	84.0	52.2	47.7	70.1	47.1	27.8
290	27.7	48.2	72.0	90.0	101.	70.0	41.1	41.7	61.2	39.6	25.0
294	23.4	40.8	60.6	74.7	83.5	60.9	39.2	42.6	63.0	41.4	25.7
298	20.1	34.7	51.7	65.0	72.5	53.9	29.8	20.4	33.3	22.0	13.3
302	16.4	27.4	41.6	51.6	59.0	43.7	23.3	16.3	15.8	11.6	5.73
306	11.9	19.2	28.8	34.9	37.5	28.5	16.4	12.1	12.1	7.90	3.72
310	8.53	13.0	19.2	23.4	25.0	18.8	13.3	9.91	9.30	6.66	3.40
320	3.10	5.11	7.60	8.83	10.1	9.76	8.99	7.28	6.98	5.26	1.86
330	2.01	----	----	----	----	2.01	2.17	6.04	----	4.80	----
340	----	1.70	1.55	2.17	2.64	----	----	----	5.27	----	2.94
350	0.775	----	----	----	----	2.33	3.57	3.10	----	3.56	----
360	----	1.24	0.775	1.24	1.39	----	----	----	2.64	----	1.24
370	0.465	----	----	----	----	3.88	4.65	1.39	----	2.32	----
380	----	1.08	0.775	0.930	0.620	----	----	----	2.64	----	0.620
390	0.465	----	----	----	----	1.08	1.55	.930	----	1.39	----
400	----	0.930	0.465	0.155	.620	----	----	----	1.55	----	0.620

TABLE V

ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 4

Irradiation Time 8.88 sec./molecule

Wave Length mμ	Fraction 12	14	16	19	22	28	33	38	41	49	53
220	18.3	106.	210.	146.	39.5	26.4	58.4	98.5	61.8	11.9	10.8
226	16.3	96.1	190.	----	37.2	27.0	57.1	100.	59.6	11.3	9.30
230	16.1	90.0	176.	122.	36.9	23.7	56.7	98.2	59.6	11.0	8.99
234	16.3	89.7	176.	122.	37.8	21.7	57.6	104.	62.5	11.5	9.76
238	16.1	93.0	184.	128.	40.0	20.8	59.5	111.	65.3	12.9	9.76
242	16.1	101.	201.	140.	43.6	21.6	65.7	121.	70.1	15.8	12.4
246	16.3	110.	220.	155.	47.8	23.1	68.6	130.	76.7	19.9	15.0
250	16.9	119.	239.	160.	53.3	23.7	71.1	141.	85.7	27.0	19.2
254	17.2	127.	259.	180.	59.0	24.0	71.6	148.	95.2	32.6	22.0
258	17.7	134.	275.	196.	66.4	23.1	66.3	150.	102.	43.2	29.0
262	18.0	140.	287.	216.	77.8	23.7	62.4	155.	110.	51.8	33.8
266	18.4	140.	294.	198.	85.0	23.6	53.9	151.	111.	55.5	36.7
270	18.9	138.	294.	206.	92.4	23.7	48.0	161.	127.	71.6	46.0
274	19.2	135.	294.	224.	106.	24.5	42.9	152.	120.	65.6	42.1
278	19.5	127.	273.	220.	110.	25.4	43.7	154.	122.	67.5	43.2
282	19.2	117.	253.	219.	111.	28.1	47.1	166.	135.	77.4	49.9
286	17.7	109.	242.	229.	121.	28.5	40.3	137.	106.	51.9	33.6
290	16.6	92.6	204.	208.	114.	27.9	37.3	124.	94.0	43.1	28.2
294	14.7	76.5	182.	177.	94.9	27.1	37.7	122.	94.0	45.7	30.1
298	13.2	67.3	147.	169.	90.2	27.8	30.4	89.4	63.7	22.2	15.2
302	11.0	54.6	122.	146.	81.9	25.9	24.5	68.0	43.8	6.66	6.04
306	8.99	37.3	73.5	89.2	52.7	21.6	20.9	55.2	36.0	4.65	3.25
310	6.82	25.8	43.6	46.0	31.6	20.6	18.8	45.6	30.1	3.88	3.41
320	1.86	----	----	----	----	15.3	11.9	30.7	----	3.26	2.94
330	----	3.41	7.75	12.7	13.3	15.2	----	27.9	21.2	2.79	2.63
340	0.775	----	----	----	----	----	5.58	----	----	----	----
350		1.39	3.26	2.48	2.17	3.10	----	17.1	15.2	2.63	1.55
360	0.465	----	----	----	----	----	3.10	----	----	----	----
370		1.24	1.42	1.86	1.55	1.39	----	8.07	6.84	2.02	1.86
380	1.08	----	----	----	----	----	1.39	----	----	----	----
390		0.465	1.42	1.39	0.620	0.930	----	3.72	2.69	1.47	1.39
400	0.155	0.465	1.63	1.39	----	----	1.28	----	1.79	1.21	----

TABLE VI

ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN NO. 5

Irradiation Time 9.52 sec./molecule

Wave Length mμ	Fraction 14	16	17	19	22	24	27	30	33	36	39
222	45.4	186.	204.	146.	69.6	37.1	21.4	23.0	38.2	45.1	55.2
226	41.6	166.	183.	132.	64.2	34.5	20.0	21.7	37.7	45.3	55.2
230	38.5	154.	169.	123.	60.3	32.9	18.6	19.6	35.4	45.3	56.3
234	38.3	152.	166.	122.	61.2	33.2	18.1	17.5	33.2	46.4	60.0
238	39.2	155.	173.	127.	63.8	34.5	17.7	16.0	33.0	48.9	64.2
242	42.8	166.	187.	138.	69.2	36.9	18.5	16.7	36.3	54.8	71.8
246	46.5	183.	204.	151.	75.3	39.8	19.5	17.4	38.2	57.8	78.8
250	50.9	199.	222.	164.	84.0	44.4	21.3	18.1	40.5	62.7	86.6
254	55.2	213.	239.	178.	93.5	50.1	22.9	18.9	40.5	62.0	90.3
258	58.7	225.	252.	193.	104.	56.1	25.7	18.6	37.4	57.9	90.7
262	61.7	231.	262.	206.	116.	64.7	28.8	19.6	35.8	55.3	91.6
266	64.2	232.	264.	212.	126.	72.3	32.1	19.6	30.7	47.7	86.4
270	65.3	227.	261.	216.	136.	79.7	35.8	20.1	27.4	43.6	88.8
274	65.3	222.	241.	221.	149.	89.2	39.4	20.9	25.4	38.6	80.2
278	64.2	205.	226.	208.	149.	92.8	42.1	22.3	26.2	39.4	82.5
282	60.3	187.	209.	197.	147.	93.3	43.4	23.4	28.1	43.2	90.8
286	56.3	173.	196.	193.	154.	99.6	45.7	23.7	25.6	35.8	70.7
290	50.3	147.	166.	166.	140.	92.2	44.1	23.1	24.2	32.6	63.8
294	44.5	119.	135.	134.	115.	79.0	41.1	23.0	24.2	32.9	64.6
298	39.4	103.	118.	120.	107.	73.7	40.9	23.6	22.6	25.7	42.9
302	33.2	81.7	94.3	99.5	93.6	65.9	37.5	21.9	20.0	20.6	32.4
306	25.8	52.5	58.9	59.9	60.2	45.7	30.4	18.9	17.1	17.4	27.2
310	19.2	34.7	35.5	33.3	35.5	31.6	27.6	18.4	16.4	15.8	23.1
320	4.97	9.60	10.7	11.3	15.3	17.7	12.0	14.6	11.8	10.7	14.6
340	1.71	2.15	2.79	2.79	3.72	4.97	6.50	5.27	4.65	5.12	8.84
360	1.08	1.08	1.55	1.39	1.39	2.17	2.64	2.33	1.75	2.64	4.50
380	1.08	0.930	1.24	1.08	1.08	1.71	1.86	1.71	1.39	1.55	2.32
400	0.775	0.775	0.930	0.775	0.930	1.39	1.55	1.39	1.08	1.08	1.24

TABLE VII

ABSORPTION SPECTRA FOR TYPICAL FRACTIONS IN RUN No. 6

Irradiation Time Zero

Wave Length mμ	28	31	34	38	42	58
222	5.89	14.6	18.0	22.6	18.0	6.20
226	5.73	15.0	18.6	22.6	16.3	6.05
230	5.73	16.1	20.6	23.4	15.6	5.43
234	6.51	18.7	23.9	26.2	15.3	5.27
238	7.91	22.0	28.2	29.4	16.3	5.74
242	9.62	26.2	34.7	35.2	20.1	7.45
246	10.4	28.7	38.5	38.9	25.0	9.92
250	11.3	31.2	43.4	46.4	35.2	15.3
254	10.8	30.2	43.9	49.4	43.1	20.0
258	9.76	26.5	40.9	53.6	57.5	28.2
262	9.15	23.9	38.9	57.0	69.1	34.6
266	7.29	17.8	31.6	55.0	75.4	38.5
270	5.74	12.4	27.2	61.1	96.0	49.7
274	4.81	9.45	21.4	53.3	87.2	44.7
278	5.42	9.15	21.5	54.4	89.6	46.2
282	5.74	10.1	24.3	62.3	103.	52.7
286	5.27	8.22	17.0	42.5	69.4	34.8
290	4.65	7.13	14.4	35.6	57.2	28.7
294	4.65	7.28	15.0	37.8	60.6	30.4
298	4.19	5.42	8.37	19.2	29.6	13.4
302	2.95	3.72	3.72	7.60	10.8	3.57
306	1.86	2.64	2.33	4.80	5.73	1.39
310	1.86	2.33	2.02	4.34	4.96	0.930
320	1.39	1.55	1.24	4.03	4.81	0.930
340	0.930	1.08	0.620	3.88	5.27	0.775
360	----	0.775	0.310	----	3.41	0.310
380	0.775	0.465	----	1.71	2.17	0.310
400	0.775	0.465	----	1.39	1.24	0.310

TABLE VIII

CALCULATION OF ABSORPTION SPECTRA FOR SUBSTANCE ELUTED WITH CALCIFEROL

Wave Length Mu	A	B	C	D
222	----	65.4	30.6	34.8
230	0.158	55.	41.9	13.1
240	0.217	58.6	56.2	2.40
250	0.280	73.0	72.5	0.50
255	0.307	79.9	79.4	0.30
260	0.329	85.2	85.2	0.00
265	0.340	88.0	88.0	0.00

A = Extinction values obtained from a pure calciferol curve.
B = Extinction values for fraction 16, Figure 2.
C = Calculated extinction values if fraction 16 were pure calciferol.
D = Difference in extinction values B and C.

LITERATURE CITED

1. R. Pohl. Nachr. Ges. Wiss. Gottingen, Math.-physik. Flasse III, 142 (1927)

2. A. Windaus and A. Hess. Nachr. Ges. Wiss. Gottingen, Math.-physik. Klasse, III, 175 (1927)

3. C. Tauret. Compt. rend., 108, 98 (1889)

4. A. Windaus and O. Rygh. Nachr. Ges. Wiss. Gottingen, Math.-physik. Klasse 1928, No. 2, 206-16 (1928)

5. A. Windaus, K. Westphal, F. V. Werder, and O. Rygh., Nachr. Ges. Wiss. Gottingen, Math.-physik, Klasse 1929, 45-59 (1929)

6. A. Windaus, K. Westphal, F. V. Werder, and O. Rygh. Nachr. Ges. Wiss. Gottingen, Math.-physik, Klasse 1929, 45-59 (1929)

7. A. Windaus, J. Gaede, J. Koser and G. Stein. Ann. 483, 17-30 (1930)

8. A. Windaus. Nachr. Ges. Wiss. Gottingen, Math.-physik. Klasse 1930, No. 1, 36-57 (1930)

9. R. A. Morton, I. M. Heilbron and E. D. Kann. J. Chem. Soc. 1927, 2000-5

10. A. Windaus and E. Auhagen. Z. physiol. Chem. 197, 167-72 (1931)

11. A. Windaus. Proc. Roy. Soc. (London) B108, 568-75 (1931)

12. A. Windaus. A. Luttringhaus and M. Deppe. Ann. 489, 252-69 (1931)

13. A. Windaus. K. Dithmar and E. Fernholz. Ann. 493, 259-272 (1932)

14. A. Windaus, A. Luttringhaus and P. Busse. Nachr. Ges. Wiss. Gottingen Math.-physik. Klasse 1932, 150-9

15. P. Setz. Z. physiol. Chem. 215, 183 (1933)

16. H. R. Rosenberg. Vitamins, Interscience Pub. Inc., New York, N. Y. pp. 372-3 (1945)

17. K. Dimroth. Ber., 70, 1631-36 (1937)

18. I. M. Heilbron and F. S. Spring. Chem. and Ind., 1935, 795-7.

19. A. Windaus. Nachr. Ges. Wiss. Gottingen Math.-physik. Klasse 1929, 169-72

20. O. Diels and A. Karstens. Ann., 478, 129- (1930)

21. C. K. Chuang. Ann., 500, 270-80 (1933)

22. H. R. Rosenberg. Vitamins Interscience Publishers, Inc., New York, N. Y. (1945)

23. J. F. Danielli and N. K. Adam. Biochem. J., 28, 1583-91 (1934)

24. H. R. Rosenberg. Vitamins, Interscience Publishers, Inc., New York, N. Y. 372-3 (1945)

25. H. H. Beard, R. E. Burk, H. E. Thompson and H. Goldblatt. J. Biol. Chem., 96, 307 (1932)

26. F. A. Askew, R. B. Bourdillon and T. A. Webster. Biochem. J. 26, 814 (1932)

27. A. Windaus, K. Westphal, F. V. Werder and O. Rygh. Nachr. Ges. Wiss. Gottingen, Math.-physik. Klasse III, 45 (1929)

28. A. Jendrassek, Hung. 104,227, Apr. 14, 1931. C. A. 27, 1387

29. A. V. Trufanov. Khim Farm Prom 1933, 253-6. C. A. 28, 4108^{9}

30. C. E. Bills, E. M. Honeywell and W. M. Cox. J. Biol. Chem. 92, 601 (1931)

31. H. H. Beard, R. E. Burk, H. E. Thompson and H. Goldblatt, J. Biol. Chem., 96, 307 (1932)

32. F. Seitz. Darstellung von Vitamin praparaten, Leipzig, 1939, 50

33. T. A. Webster and R. B. Bourdillon. Biochem. J., 22, 1223 (1928)

34. S. Kon, F. Daniels, and H. Steenbock. J. A. C. S., 50, 2573 (1928)

35. A. L. Bacharach, E. L. Smith and M. M. Stevenson. Analyst, 58, 128-135 (1933)

36. H. R. Rosenberg. Vitamins, Interscience Publishers, Inc., New York, N.Y. (1945)

37. J. Kirn. Ph. D. Thesis Michigan State College (forthcoming)

38. W. Huber, G. W. Ewing and J. Kriger. J. A. C. S. 67, 609-17 (1945)

39. H. R. Rosenberg. Vitamins, Interscience Publishers Inc. (1945) p. 382