Part I: IRRADIATION OF SOLUTIONS OF ERGOSTEROL IN AN IMPROVED TYPE QUARTZ CELL

Part II: A CHROMATOGRAPHIC SEPARATION OF CALCIFEROL FROM IRRADIATED ERGOSTEROLS IN HIGHLY VOLATILE SOLVENTS

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Manly Joy Powell

1946

This is to certify that the

thesis entitled

Pt I Irradiation of Solutions of Ergosterol in an Improved Type Quartz Cell
Pt II A Chromatographic Separation of Calciferol from Irradiated Ergosterols in Highly Volatile Solvents

presented by

Manly Joy Powell

has been accepted towards fulfillment
of the requirements for

M.S. degree in Physical Chemistry

D T Ewing
Major professor

Date June 3, 1946

M-795

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PART II: A CHROMATOGRAPHIC SEPARATION OF CALCIFEROL FROM IRRADIATED ERGOSTEROLS IN HIGHLY VOLATILE SOLVENTS

by

Manly Joy Powell

A THESIS

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

MASTER OF SCIENCE

Department of Chemistry

Michigan State College

-1946-

ACKNOWLEDGMENT

The writer wishes to express his appreciation to Dr. D. T. Ewing for his helpful suggestions and guidance during the course of this investigation and to the Parke Davis Company for the fellowship making this work possible.

PART I

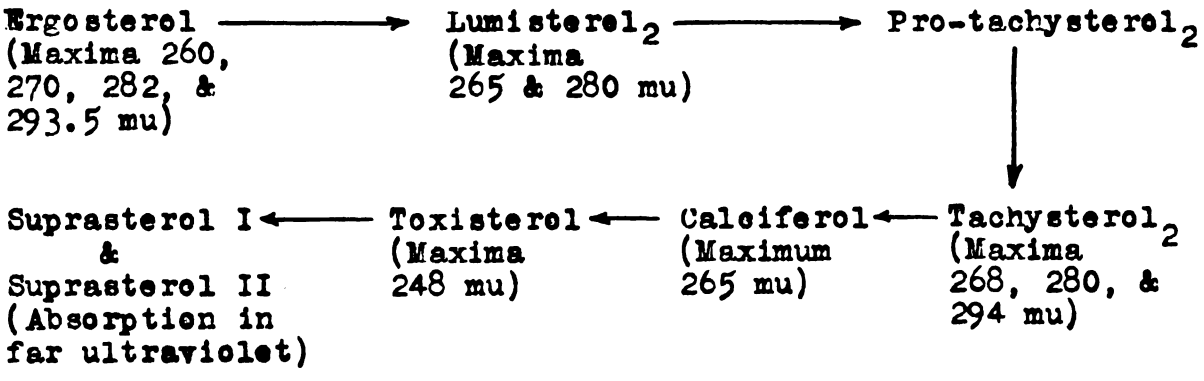
IRRADIATION OF SOLUTIONS OF ERGOSTEROL IN AN IMPROVED TYPE QUARTZ CELL

CONTENTS

I

Irradiation of Solutions of Ergosterol in an Improved Type Quartz Cell.

The conversion of ergosterol to calciferol (vitamin D_2) when activated either in the solid form or in solution with ultraviolet light has been a subject of research for several years. It has been quite generally accepted that during the course of this reaction several products are formed and that there is no equilibrium between these products. By actual isolation of the pure intermediate compounds and determining all the products obtained after irradiation of each isolated compound, the actual number of intermediates and their sequence of formation has been shown in general to be as follows:



The greater part of this work was done by Windaus and his co-workers.

The course of this reaction has been followed by many investigators using ultraviolet absorption curves of the irradiated solution.

Morton, Heilbron and Kamm (4) irradiated alcohol solutions of ergosterol using a quartz mercury vapor lamp and obtained absorption curves of the irradiated material at fifteen-minute intervals. They found that the bands exhibited by ergosterol practically disappeared after 90 to 150 minutes irradiation and a new absorption band at 247 mu appeared quite strongly.

Van Stolk, Dureuil and Hendebert (6) observed that when ergosterol was irradiated with a hydrogen lamp the first three absorption bands disappeared while the fourth (2608 A^0) increased in intensity. Two new bands also appeared at 2503 A^0 and 2405 A^0. These three bands did not disappear with further irradiation.

Bourdillon, Fischman, Jenkins, and Webster (2) concluded from their study of the absorption curves of irradiated ergosterols, as compared to the activity of the solutions, that three substances were produced in succession. One substance was thought to exhibit an absorption band at 280 mu twice as intense as that of ergosterol. A second substance having no antirachitic activity showed an absorption maxima at 240 mu while a third substance showed neither antirachitic activity nor marked absorption.

Kisch and Reiter (3) observed that when ergosterol was irradiated with the unfiltered light of a mercury arc lamp short periods of irradiation produced a material showing absorption maximas at 275 and 245 mu. Further irradiation led to new absorption bands at 270 and 290 mu.

The effect of using different solvents when irradiating solutions of ergosterol has also been studied. Bills, Honeywell and Cox (1) found that curves of the same general shape were obtained when irradiating ergosterol with a mercury arc in ether, cyclohexane, and alcohol. The rates of activation, however, were found to be considerably different. Alcohol solutions reached their maximum activity in 22.5 minutes while cyclohexane and ether reached their peak in 27 minutes and 252 minutes, respectively. The ether solutions showed a maximum cod liver oil coefficient of 710,000 while cyclohexane and alcohol solutions only exhibited coefficients of 330,000 and 250,000 respectively. They concluded that spectrographic changes of the solutions were no measure of antirachitic potency.

The temperature of the solution being irradiated has very little effect upon the activity of the resulting product. Webster and Bourdillon (7) irradiated solutions of ergosterol at temperatures of 77.8°, 30.6°, 1°, -18°, and approximately -183° and -195°C. They found that with the exception of the

last two temperatures, very little change in activity resulted in the product obtained.

The work of numerous other investigators has indicated that certain wave lengths of radiation are more efficient and desirable than others. However, since no attempt was made to filter out any part of the light used to activate the solutions in this investigation, work of this nature has been omitted from this review.

The main purpose of this investigation is to study by means of absorption curves the product obtained when irradiating ether solutions of ergosterol with unfiltered light from a mercury vapor lamp in an improved type quartz cell of industrial size. Some attempt will be made to correlate the curves obtained with activities of the solutions as determined by bio-assay as well as by the colorimetric method proposed by Nield, Russell and Zimmerli (5).

Experimental

Apparatus and Materials.

1. Ergosterol.-- A very pure grade of commercial ergosterol was used for these experiments. The absorption curves of these ergosterols in alcohol is shown in Figs. III and IV.

2. Ethyl Ether.-- A c.p. grade of anhydrous ethyl ether was used and was tested before each run to insure that it was free of peroxides.

3. Alcohol Mixture.-- For precipitating unchanged ergosterol from the irradiated solutions an alcohol mixture was used made up by volume of 902 parts absolute ethyl alcohol, 47 parts absolute metnanol, and 45 parts water.

4. Irradiation Unit.-- An improved type quartz cell was used for irradiating the solutions of ergosterol. Two types of set ups were used with a few variations. The two general types are shown in Figs. I and II. The setting on the variable transformer which furnished the power for lighting the mercury vapor lamp was maintained constant throughout these runs.

5. Spectrophotometer.-- All ultraviolet absorption curve measurements were made upon a Beckman quartz spectrophotometer.

Procedure.

Irradiation of Solutions.

A. Batch Operation.-- The ether solution of ergosterol (conc. 2.5 g. per liter) was placed in the cell and constantly agitated and forced back and forth through the cell by alternately raising and lowering the leveling bulbs shown in Fig. I. The rate at which the solution flows is controlled by means of the orifice shown.

The solution was irradiated for the desired length of time and then removed from the cell.

B. Continuous Type Operation.-- The solution to be irradiated was placed in the reservoir shown in Fig. II and the cell was filled by gravity feed. The length of time in the cell, and thus the irradiation time, was controlled by varying the rate of take-off either by use of an orifice in the line just entering the irradiation chamber or by placing a capillary tube in the take-off line.

The first 300 ml. taken off, as well as the solution remaining in the irradiation chamber at the end of the run, was not added to the product taken off during the run.

Treatment of Irradiated Solutions.

After taking a small sample (1-3 ml.) of the irradiated solution for absorption curve measurements, the ether was distilled off in an atmosphere of nitrogen or carbon dioxide and the residue taken up in the alcohol mixture used to precipitate out any unreacted ergosterol. 10 ml. of the alcohol mixture per 1 g. ergosterol present in the original solution represented by the residue is added. The solution is cooled over night in an icebox and the precipitated ergosterol is filtered off and washed with small portions of the cooled alcohol mixture. It is then allowed to dry in air and weighed. The filtrate is combined with the washings and made up to any desired volume by adding additional alcohol.

Colorimetric Method for Determining the Potency.

The potency of these irradiated solutions after unreacted ergosterol has been precipitated out is determined colorimetrically using the modified antimony trichloride reagent of Nield, Russell and Zimmerli (5). The preparation of the reagent and procedure used by this method is discussed in Part II of this paper.

Results

Pertinent data pertaining to each run made has been tabulated and is shown in Table I.

Batch Runs.-- Run 3 was made taking small samples of the irradiated solution every five minutes up to fifty minutes total irradiation time. The absorption curve of each sample in alcohol is shown in Fig. III.

Runs 17, 18, 19, and 20 were solutions irradiated for 10, 15, 20, and 25 minutes, respectively. Absorption curves of the solutions as taken from the irradiation chamber and also after precipitating out any unchanged ergosterol were made and are shown in Figs. IV and V.

Continuous Runs.-- The solutions of Runs 6-14 were irradiated for various lengths of time ranging from 9.5 to 28.8 minutes. Irradiation time for these runs was calculated from the rate the solution was taken off and the volume of the irradiation chamber (300 ml.) assuming that no mixing occurred while passing through the chamber.

Absorption curves of the solutions, both as taken from the chamber and also after removing any unreacted ergosterol, are shown in Figs. VI, VIII, and in Figs. VII and IX, respectively.

Physical-chemical potency values listed in Table I were run by the colorimetric method of Nield, Russell, and Zimmerli (5) and were taken from the second part of this paper. Per cent conversion to D_2 was calculated from these values relative to the amount of ergosterol present in the solution after the unreacted ergosterol was removed.

Discussion

The family of curves shown in Fig. III shows very clearly the change in the absorption curve of a solution of ergosterol at successive intervals of time throughout the period of irradiation. The absorption bands at 270, 282, and 293.5 mu exhibited by ergosterol gradually decrease with increasing irradiation time until they finally disappear. Then the solution shows only an absorption maxima in the neighborhood of 250 mu corresponding to that of toxisterol.

The absorption band of ergosterol at 260 mu behaves somewhat differently. The intensity of this band remains about the same for the first five minutes of irradiation but then reaches its maximum intensity at ten minutes. It then gradually decreases and at twenty-five minutes time increases again to a value not quite as high as that ex-

hibited after ten minutes irradiation. Further irradiation decreases the intensity until the band disappears.

Although the absorption curves of the irradiated solutions change considerably for different periods of irradiation, the per cent conversion to D_2 in both batch and continuous runs remain quite constant.

The conversion to D_2 ranges from 26.4% to 37.4% and averages 31.3% for the ten continuous runs made. Variation from the average is very small in most cases.

For the four batch runs the per cent conversion ranges from 26.1 to 28.9.

The varied results obtained for the continuous runs indicate that it would be difficult to obtain a solution having a desired type of absorption curve. This is probably due to the solution already irradiated mixing with the feed thus contributing toward non-uniformity of solutions irradiated for the same period of time.

Since the continuous runs gave such varied results with respect to types of absorption curves obtained for a certain period of irradiation, no conclusions can be drawn as to which irradiation time is most desirable.

Better control of irradiation is obtained by the batch type method.

The absorption curves of each batch run as taken from the irradiation chamber (Fig. IV) agree quite well with those of corresponding irradiation periods in the family of curves shown in Fig. III. Of the four batch runs made, the 20 minute run appears to be the most efficient. However, not enough runs of this type were made to draw any definite conclusions. The results from the continuous runs indicate that solutions having absorption curves corresponding to those of longer periods of irradiation than this contain larger amounts of D_2. A more extensive study would be necessary to draw more definite conclusions.

It is interesting to note the difference between the absorption curves for the irradiated solutions before and after any unreacted ergosterol has been removed. The presence of tachysterol in Run #17 (Fig. V) is indicated by the increased absorption bands exhibited at 280 and 290 mu. Upon further irradiation the intensity of these bands decrease and shift slightly toward the lower end of the spectrum as shown by Runs 18 and 19. This would be expected to occur as the tachysterol is converted to calciferol. The curve begins to level off and a wide absorption band from 250 to 290 mu exists indicating the formation of toxisterol along with calciferol. This is shown by the absorption curve of Run 20 (Fig. V).

The products of all these irradiation runs were dark yellow in color and an attempt to decolorize them was made. An alcohol solution of Run #17 was shaken with activated charcoal and allowed to sit overnight. The solution was completely decolorized.

The material adsorbed on the charcoal was desorbed by swirling with hexane. After filtering off the charcoal, a colorless solution was obtained having about the same absorption curve as the original solution. Thus, it appears that the coloring matter still remains adsorbed on the charcoal even after the hexane treatment.

Absorption curves of the original solution, the decolorized solution, and the material desorbed from the charcoal are shown in Fig. X.

Summary

1. Ether solutions of ergosterol (Conc. = 2.5g/liter) were irradiated with unfiltered light from a mercury vapor lamp, both as batch and as continuous runs, in an improved type quartz cell.

2. The per cent conversion of ergosterol to D_2 is practically constant and for moderate periods of irradiation is almost independent of the irradiation time.

3. For the batch type runs the progress of the reaction can be followed by use of absorption curves of the irradiated solutions.

4. For the continuous type runs, it is almost impossible to obtain reproducible results, with respect to the type of absorption curve obtained, for solutions irradiated the same length of time under the same conditions.

Key

A -- Leveling Bulbs
C--- Irradiation Chamber
I--- Mercury Vapor Lamp
O--- Orifice
W--- Water Cooling Jackets

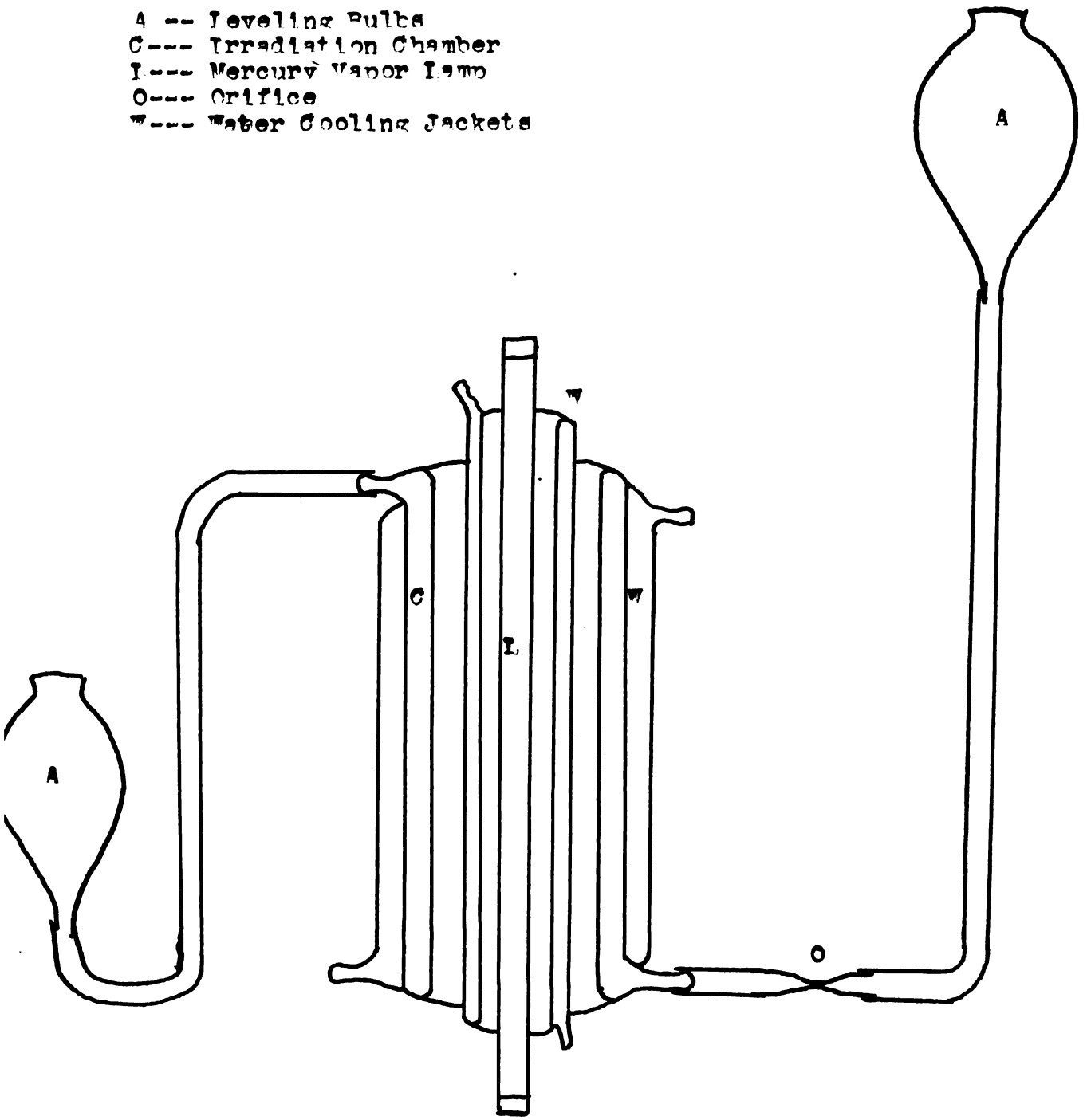


Fig. 1. Apparatus Used For Batch Type Runs.

Key

C--- Irradiation Chamber
I--- Mercury Vapor Lamp
O--- Orifice
R--- Reservoir
W--- Water Cooling Jackets

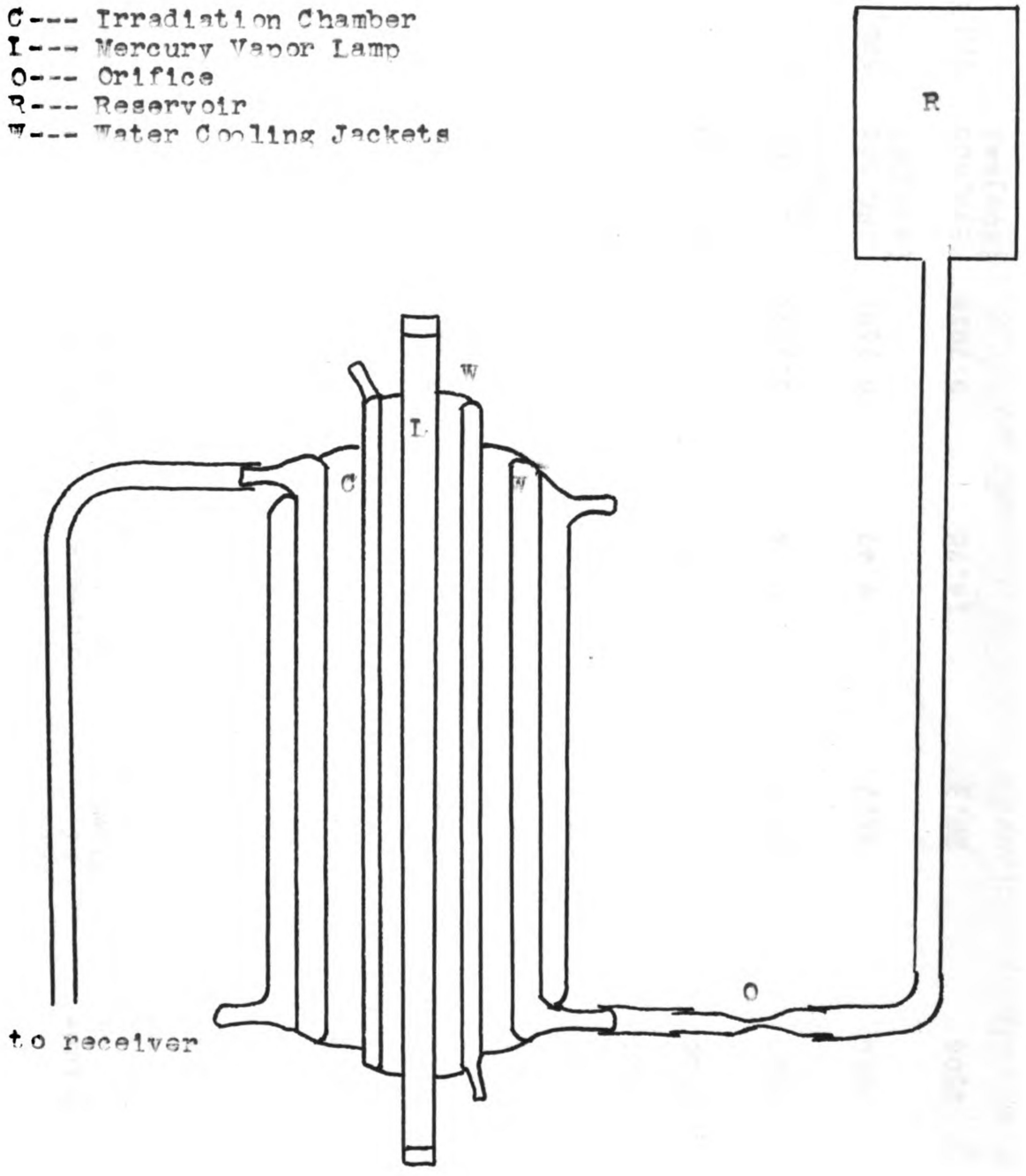


Fig. II. Apparatus Used For Continuous Type Runs

Table I. SUMMARIZED DATA OF IRRADIATION OF ERGOSTEROL RUNS

Run No.	Ergosterol Used	Volume Sol'n. Ran (ml.)	Rate of Flow (Ml./min.)	Irradiation time (min.)	Grams Ergosterol Recovered	Bio Assay D u/ml.	Phys. Chem. D u/ml.	% Conversion to D_2
3	440491	500	Batch	50 (Sample every 5 min.)				
6	43623 43653	3000	25.4	11.8	1.216		865,000	28.8
7	43643 43653	3000	23.2	12.93	1.024		685,000	26.4
8	34647	3000	10.4	28.8	0.499		751,000	37.2
9	34647	3000	15.6	19.2	1.2479		894,000	28.0
10	34647	2410	19.4	15.5	0.908		679,000	30.1
11	34647	2500	25.1	11.95	0.8418	520,000 (#66314)	530,000	31.7
12	34647	2500	30.8	9.74	0.9890	600,000 (#66324)	573,000	30.0
13	34647	2500	31.7	9.47	0.9587	480,000 (#66334)	520,000	32.6
14	34647	2500	20.3	14.78	0.7016	590,000 (#66344)	573,000	31.3

Table I. SUMMARIZED DATA OF IRRADIATION OF ERGOSTEROL RUNS (Cont'd.)

Run No.	Ergosterol Used	Volume Sol'n. Ran (ml.)	Rate of Flow (Ml./min.)	Irradiation time (min.)	Grams Ergosterol Recovered	Bio Assay D u/ml.	Phys. Chem. D u/ml.	% Conversion to D_2
15	34647	2500	15.c	19.2	0.456		508,000	37.4
17	34647	500	Batch	10.0	0.1526		487,000	27.8
18	34647	500	Batch	15.0	0.0154		514,000	26.1
19	34647	500	Batch	20.0	0.0143		570,000	28.9
20	34647	500	Batch	25.0	0.0172		514,000	26.1

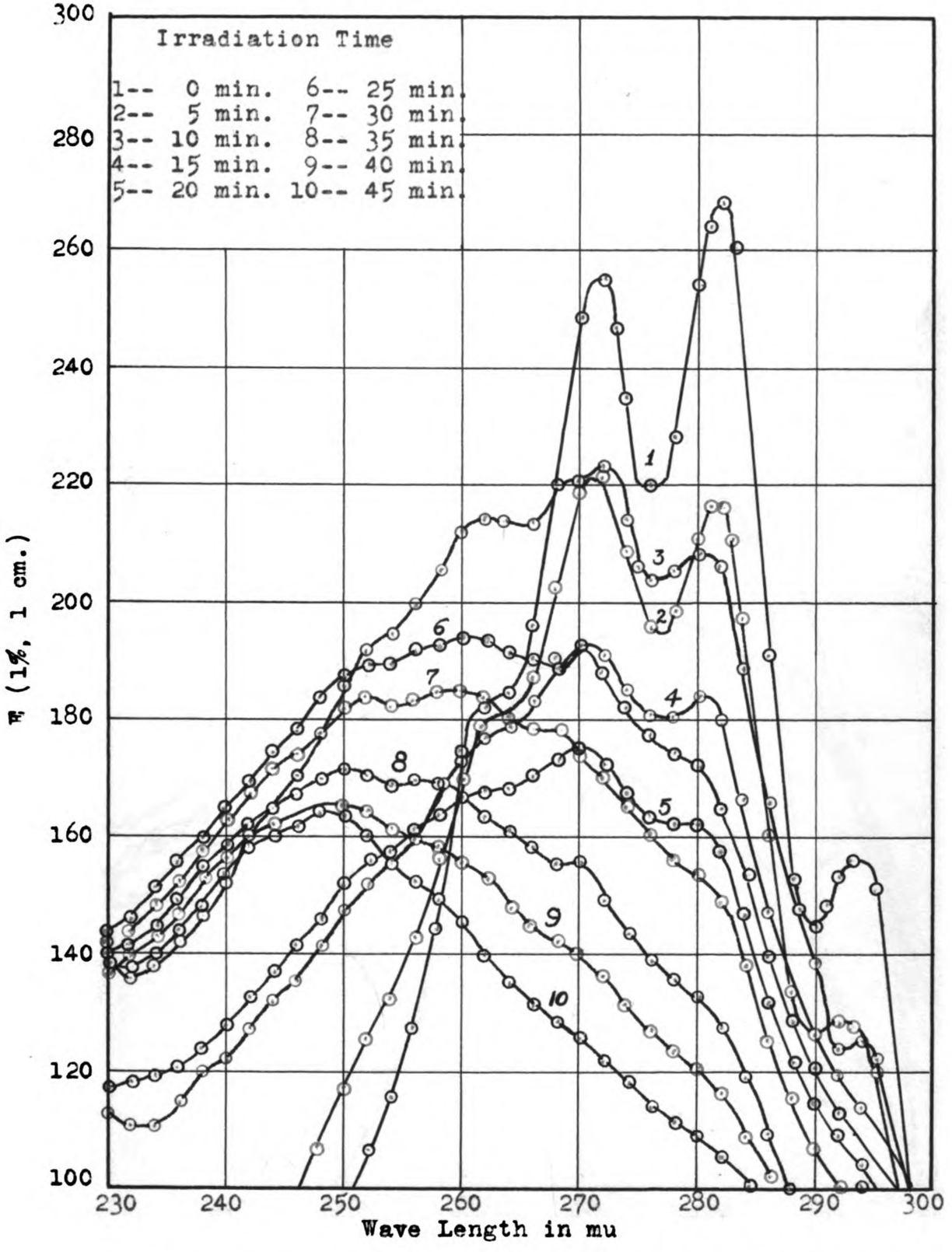


Fig. III.-- Absorption Curve of a Solution of Ergosterol at Successive Intervals of Time throughout the Irradiation Procedure (Run #3).

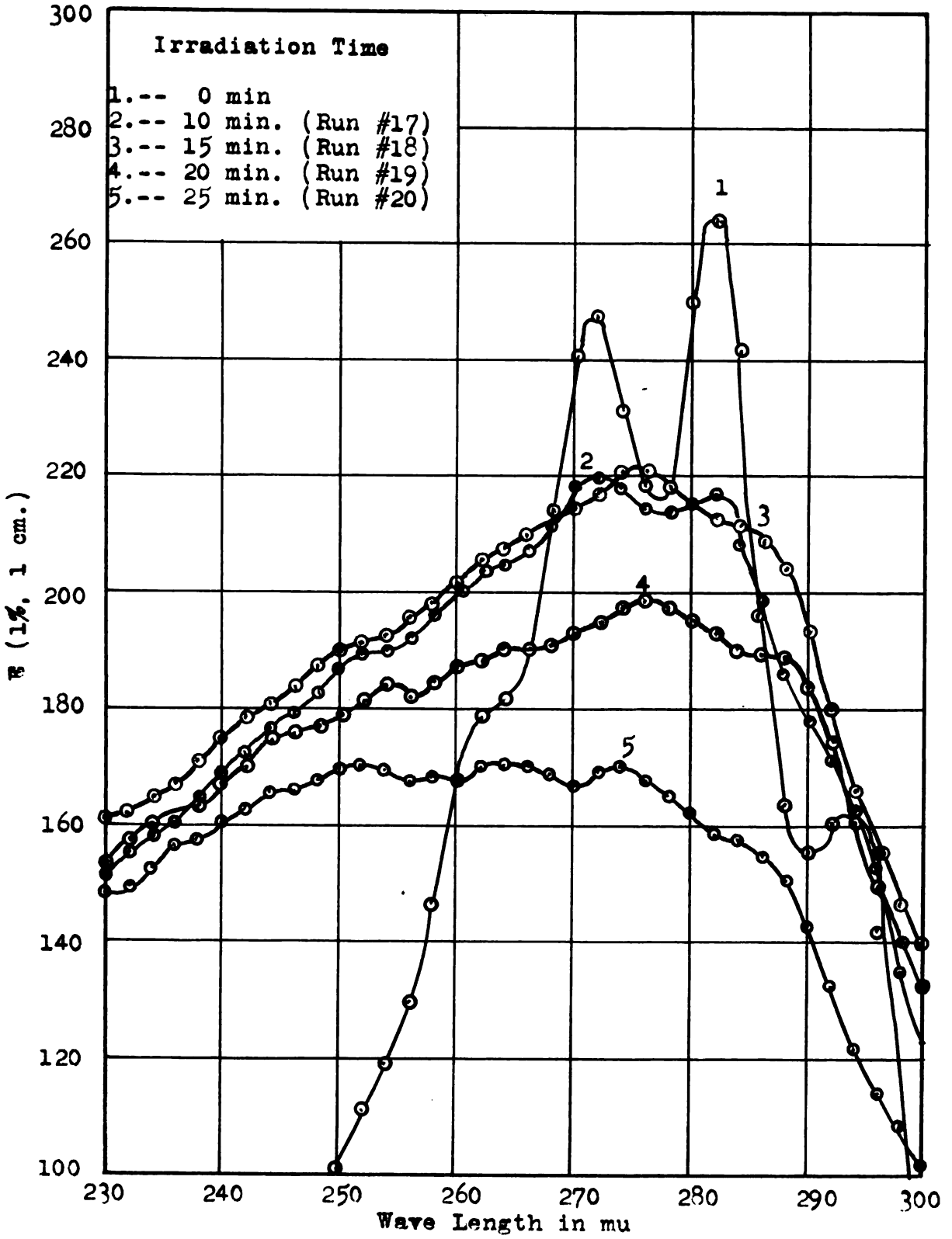


Fig. IV.-- Absorption Curves of Solutions Taken From Irradiation Chamber (Batch Runs).

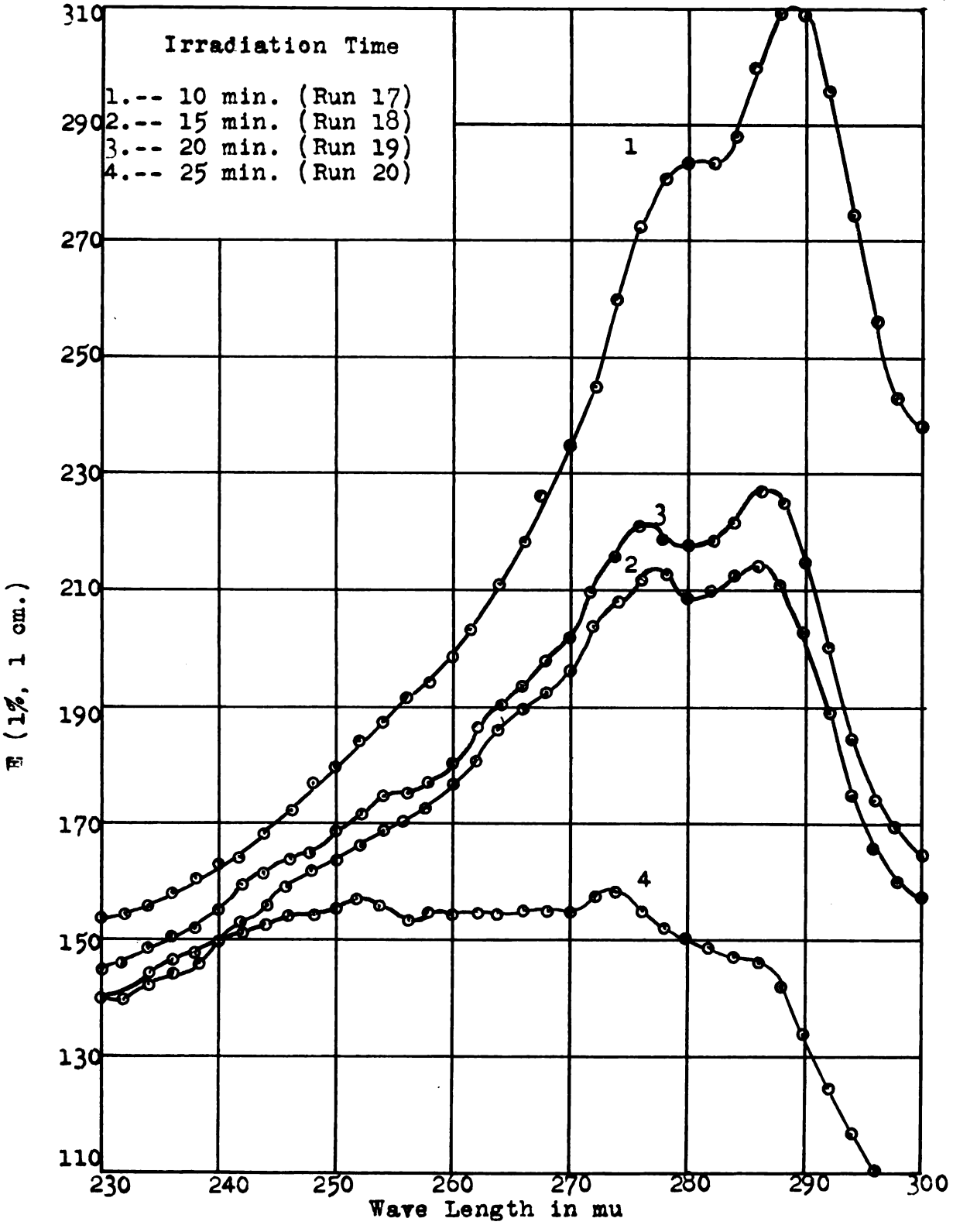


Fig. V.-- Absorption Curves of Irradiated Solutions After Ergosterol was Removed (Batch Runs).

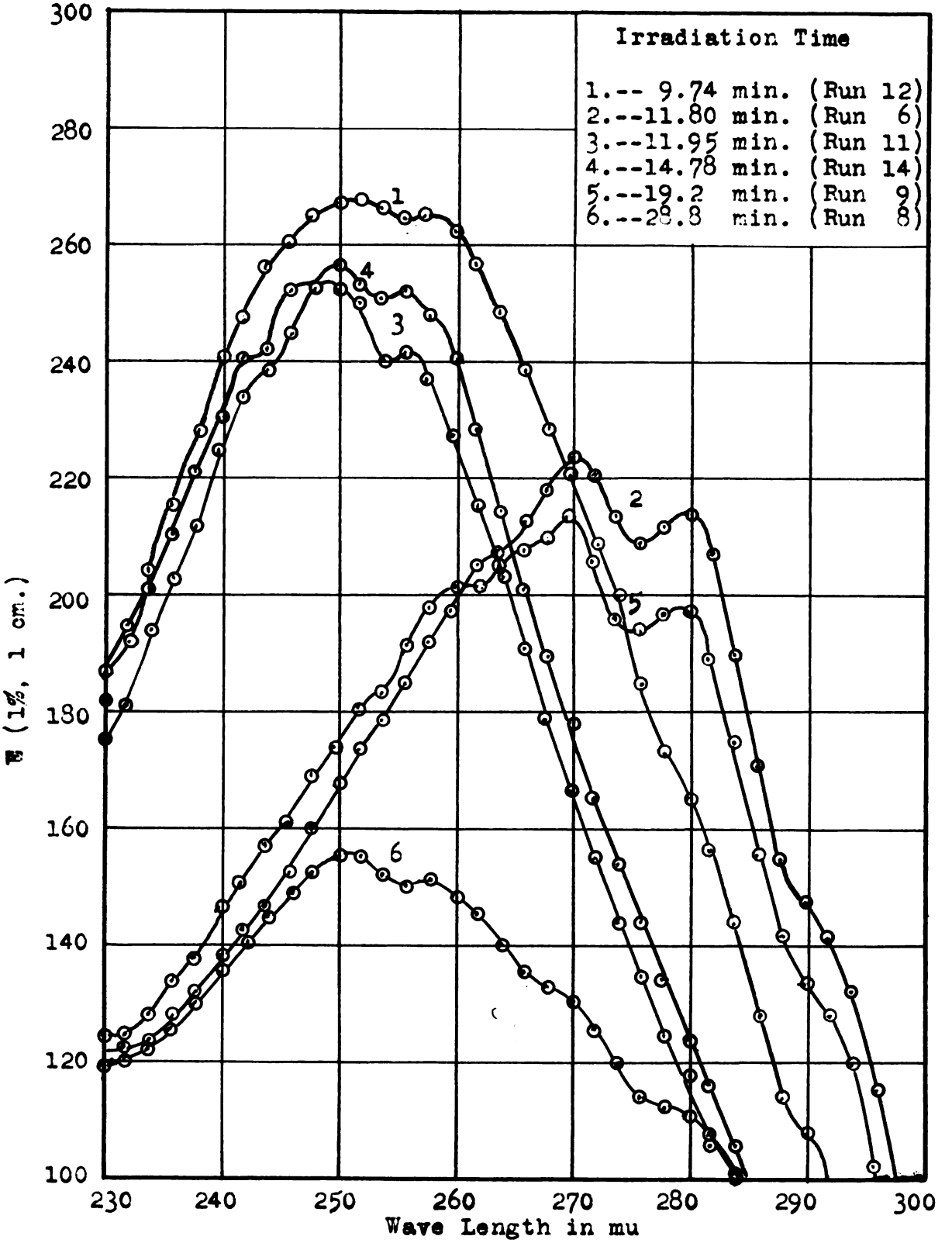


Fig. VI.-- Absorption Curves of Solutions Taken from Irradiation Chamber (Continuous Runs).

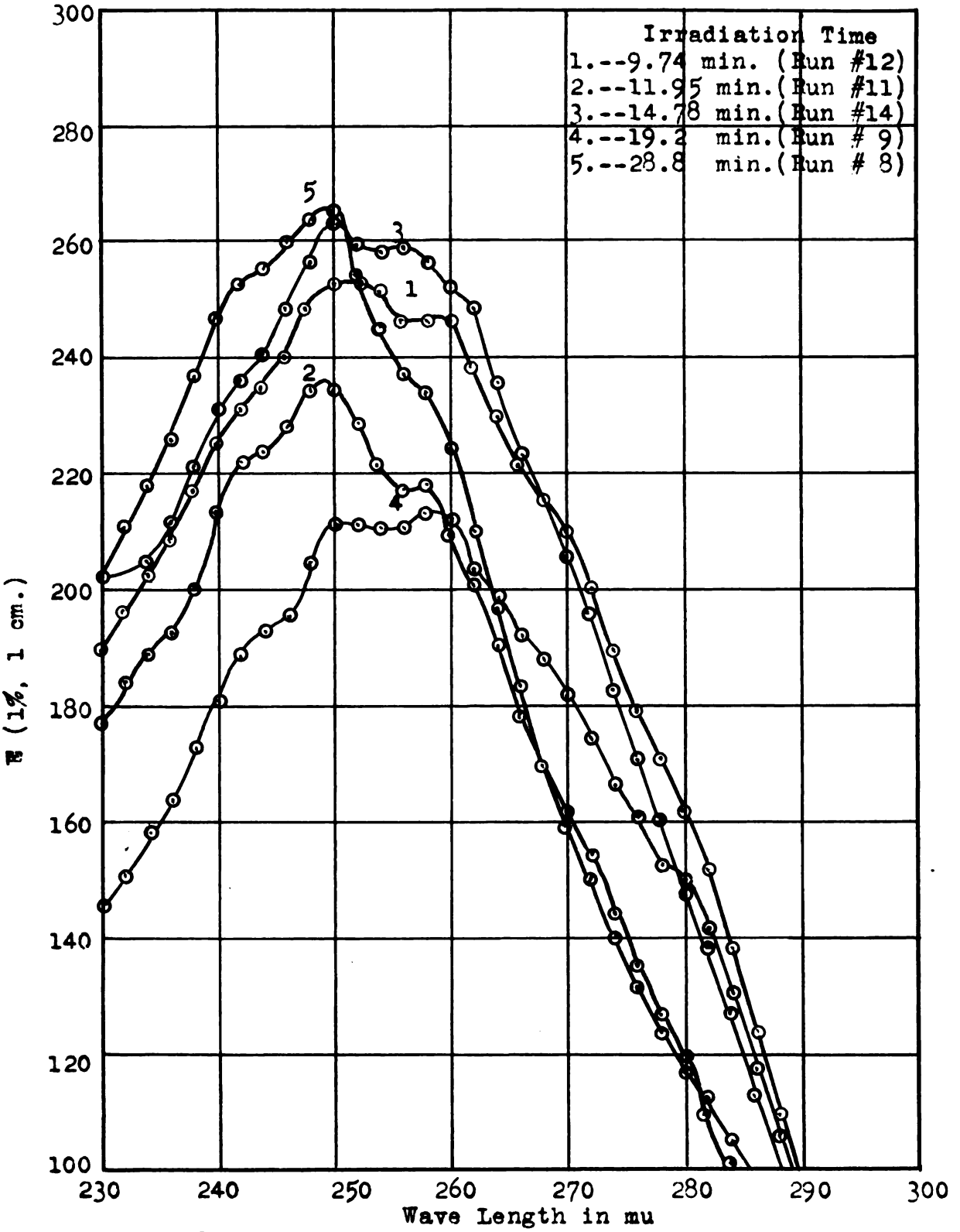


Fig. VII.-- Absorption Curves of Irradiated Solutions After Ergosterol was Removed (Continuous Runs).

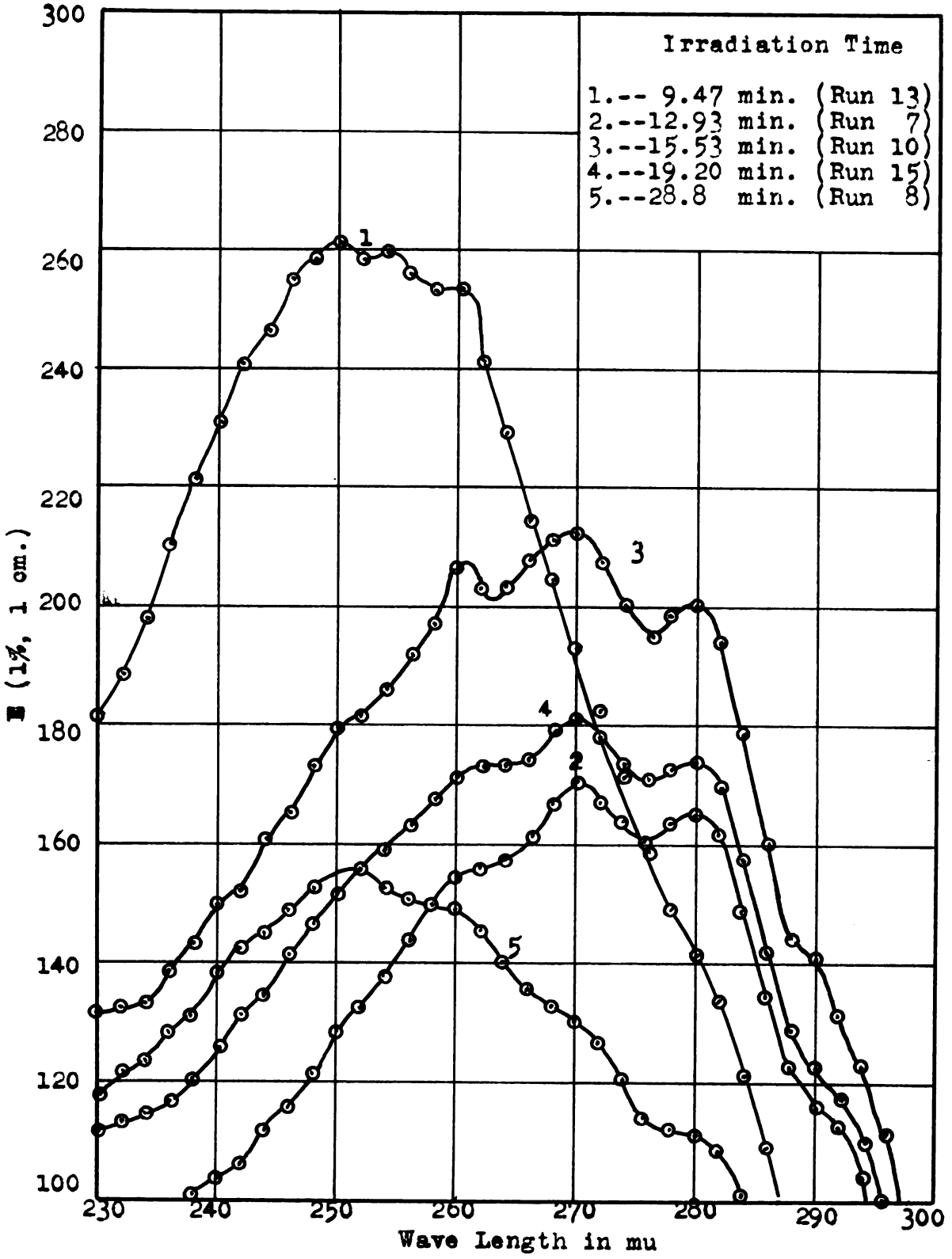


Fig. VIII.-- Absorption Curves of Solutions Taken from Irradiation Chamber (Continuous Runs).

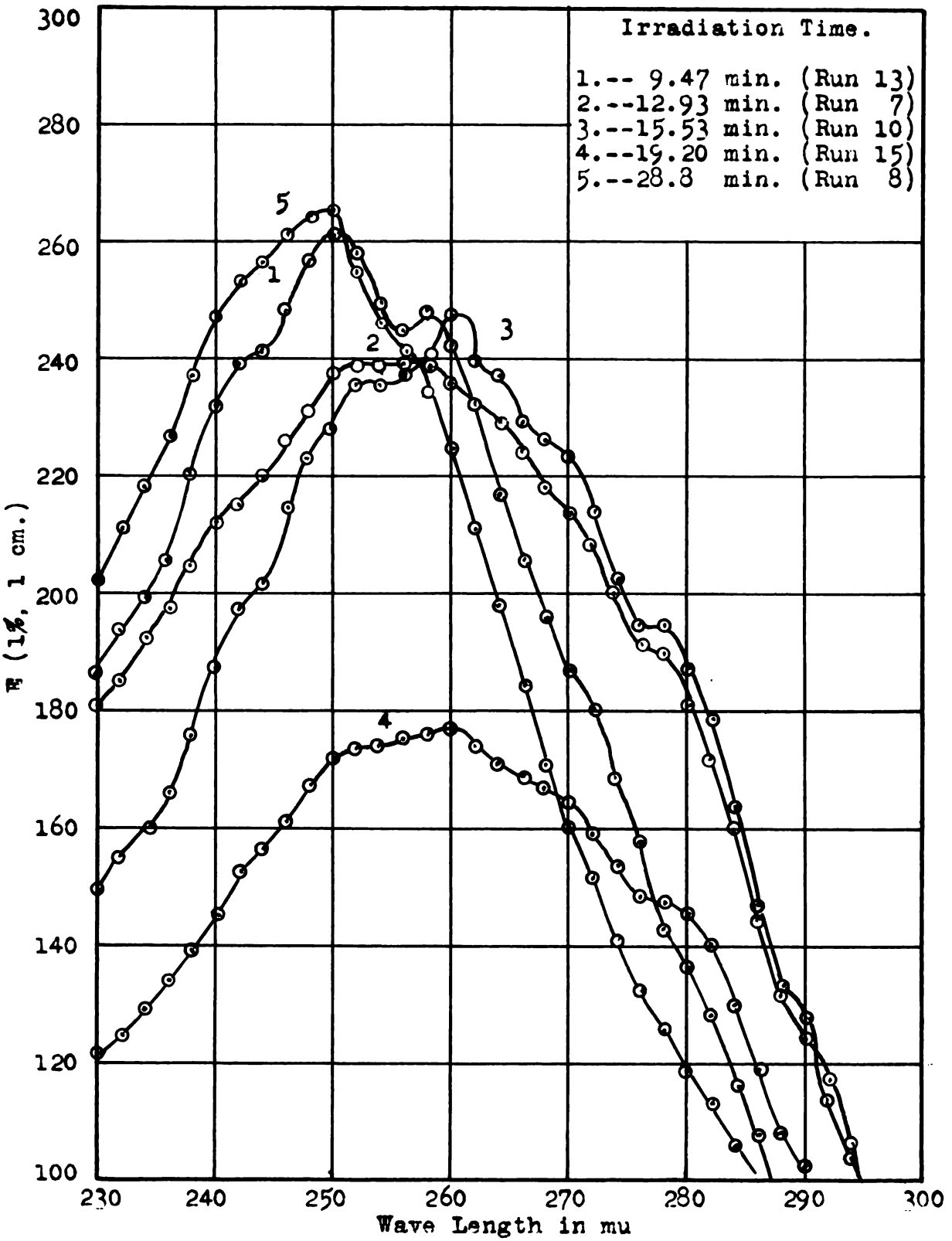


Fig. IX.-- Absorption Curves of Irradiated Solutions After Ergosterol was Removed (Continuous Runs).

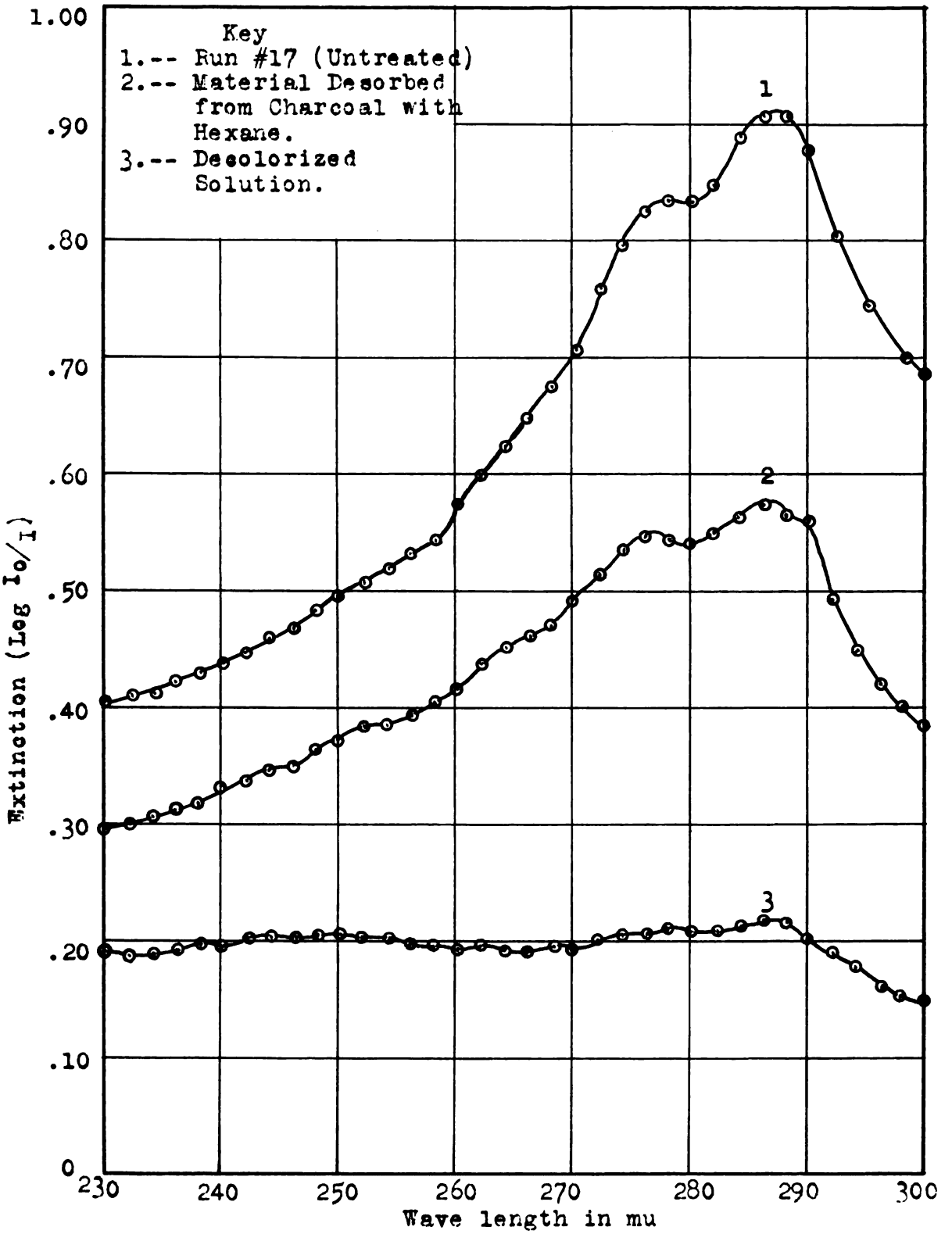


Fig. X.-- Absorption Curve of a Solution of Run #17 Before and After Decolorizing Treatment with Charcoal.

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PART II

A CHROMATOGRAPHIC SEPARATION OF CALCIFEROL FROM IRRADIATED ERGOSTEROLS IN HIGHLY VOLATILE SOLVENTS

CONTENTS

II

A Chromatographic Separation of Calciferol from Irradiated Ergosterols in Highly Volatile Solvents.

The problem of separating provitamins D from various sterols and vitamins D from natural oils or irradiated solutions of the previtamins is one which has been studied extensively and which lends itself readily to the principles of chromatographic adsorption.

The provitamin, ergosterol, has been separated successfully from cholesterol by several investigators using aluminum oxide as an adsorbent.

Winterstein and Stein (18) used a column of this adsorbent to separate these two components chromatographically and found colorless zones containing ergosterol in the upper part of the column and cholesterol in the lower. Benzine was used as the solvent.

Windaus and Stange (17) and Boer, Reerink, van Wijk and van Niekirk (2) obtained this same separation using different solvents. They used a mixture of benzene and petroleum ether and the former added a small amount of methyl alcohol when eluting the column.

Karrer and Nielson (10) separated ergosterol from cholesterol by means of the fluorescence produced by irradiation of the column, but Ladenburg, Fernholz and Wallis (11)

found that the fluorescence produced with ultraviolet light was weak and for more complex mixtures it was impossible to distinguish between the zones of the ultra-chromatogram. In order to produce visible zones to obtain a better separation they prepared the azobenzenemonocarboxylic acid esters of the sterols which gave visible adsorption zones and which could be hydrolyzed by simple saponification. Benzene was used as the solvent and a mixture of benzene and petroleum ether was used for developing the chromatogram. After studying various sterol mixtures they concluded that only those differing in the number of double bonds could be separated by this method.

The chromatographic separation of vitamins D from natural oils and irradiated solutions of provitamins is somewhat different because a different type of by-products must be eliminated. After isolating the unsaponifiable fraction in the natural occurring oils the chief impurities that must be removed consists of vitamin A, sterols, carotenoids and the tocopherols.

Brockmann (3) separated vitamin D from Tunny liver oil by chromatographic adsorption on aluminum oxide using benzene and methanol as solvents. An indicator having the same adsorption characteristics as the vitamin was added to mark the position of the adsorbed vitamin on the column.

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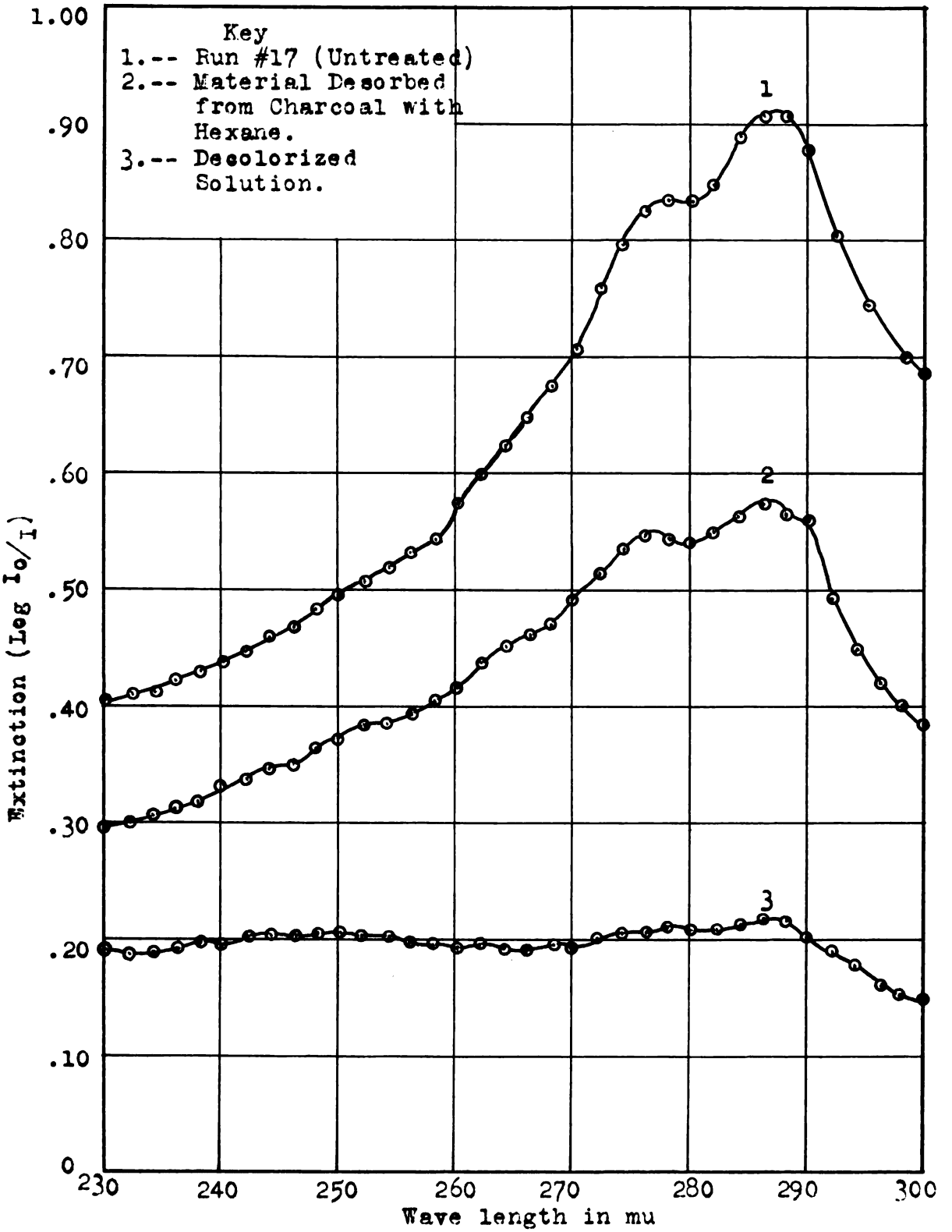
Wolff (19) separated vitamin A and carotenoids from the vitamins D in fish liver oil by chromatographic adsorption on Montana earth from benzene solution. Most of the sterols were removed by precipitation with digitonin.

Ritsert (15) removed vitamin A from fish liver oils by adsorption on aluminum oxide. A mixture of methyl alcohol, benzene, and petroleum ether was used as the solvent.

Marcussen (12) removed vitamin A, carotenoids, inactivated sterols and other substances from fish liver oils by use of a Tswett column filled with activated carbon (hydraffin K_4) as adsorbent and heptane as the solvent.

Ewing and Tomkins (8) separated vitamin A from the nonsaponifiable fraction of fish liver oils by chromatographic adsorption on superfiltrol from hexane-ether-alcohol solution. They removed sterols by using digitonin.

Ewing, Kingsley, Brown and Emmett (7) modified the procedure used by Ewing and Tomkins (8) by use of a two-step chromatographic treatment. In the first step vitamin A, carotenoids, and pigments were removed from the combined vitamins D and sterols by adsorption upon superfiltrol using a skellysolve-ether-alcohol solution as the solvent. The sterols were separated from vitamins D in the second step by use of a column of the same adsorbent but a benzene-skellysolve mixture for the solvent.



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Winterstein and Stein (18) used a column of this adsorbent to separate these two components chromatographically and found colorless zones containing ergosterol in the upper part of the column and cholesterol in the lower. Benzine was used as the solvent.

Windaus and Stange (17) and Boer, Reerink, van Wijk and van Niekirk (2) obtained this same separation using different solvents. They used a mixture of benzene and petroleum ether and the former added a small amount of methyl alcohol when eluting the column.

Karrer and Nielson (10) separated ergosterol from cholesterol by means of the fluorescence produced by irradiation of the column, but Ladenburg, Fernholz and Wallis (11)

found that the fluorescence produced with ultraviolet light was weak and for more complex mixtures it was impossible to distinguish between the zones of the ultra-chromatogram. In order to produce visible zones to obtain a better separation they prepared the azobenzenemonocarboxylic acid esters of the sterols which gave visible adsorption zones and which could be hydrolyzed by simple saponification. Benzene was used as the solvent and a mixture of benzene and petroleum ether was used for developing the chromatogram. After studying various sterol mixtures they concluded that only those differing in the number of double bonds could be separated by this method.

The chromatographic separation of vitamins D from natural oils and irradiated solutions of provitamins is somewhat different because a different type of by-products must be eliminated. After isolating the unsaponifiable fraction in the natural occurring oils the chief impurities that must be removed consists of vitamin A, sterols, caretenoids and the tocopherols.

Brockmann (3) separated vitamin D from Tunny liver oil by chromatographic adsorption on aluminum oxide using benzene and methanol as solvents. An indicator having the same adsorption characteristics as the vitamin was added to mark the position of the adsorbed vitamin on the column.

Wolff (19) separated vitamin A and carotenoids from the vitamins D in fish liver oil by chromatographic adsorption on Montana earth from benzene solution. Most of the sterols were removed by precipitation with digitonin.

Ritsert (15) removed vitamin A from fish liver oils by adsorption on aluminum oxide. A mixture of methyl alcohol, benzene, and petroleum ether was used as the solvent.

Marcussen (12) removed vitamin A, carotenoids, inactivated sterols and other substances from fish liver oils by use of a Tswett column filled with activated carbon (hydraffin K_4) as adsorbent and heptane as the solvent.

Ewing and Tomkins (8) separated vitamin A from the nonsaponifiable fraction of fish liver oils by chromatographic adsorption on superfiltrol from hexane-ether-alcohol solution. They removed sterols by using digitonin.

Ewing, Kingsley, Brown and Emmett (7) modified the procedure used by Ewing and Tomkins (8) by use of a two-step chromatographic treatment. In the first step vitamin A, carotenoids, and pigments were removed from the combined vitamins D and sterols by adsorption upon superfiltrol using a skellysolve-ether-alcohol solution as the solvent. The sterols were separated from vitamins D in the second step by use of a column of the same adsorbent but a benzene-skellysolve mixture for the solvent.

Hage (9) modified the second step of this procedure by swirling the benzene-skellysolve solution of vitamins D and sterols with superfiltrol instead of using a chromatographic column.

A few investigators have used, or attempted to use, chromatographic methods to isolate vitamins D from irradiated solutions of the provitamins.

Miller (13) separated vitamins D from both natural oils and irradiated provitamin solutions by adsorbing the vitamins from a benzene or ether solution using tri-calcium phosphate as an adsorbent.

DeWitt and Sullivan (6) also separated vitamins D from both natural oils and irradiated ergosterols by a chromatographic procedure. A column made up from a 1:1 mixture of Magnesia and diatomaceous earth was used as the adsorbent and petroleum ether as the solvent and developer. Separation was achieved by eluting separately the fluorescent zones observed on the column when irradiated with ultraviolet light.

Windaus, Schenck and von Werder (16) separated the antirachitically active component of irradiated 7-dehydro cholesterol by employing as part of the purification procedure a chromatographic adsorption step using aluminum oxide as the adsorbent.

Young (20) used the two-step chromatographic procedure developed by Ewing et al. (7) to determine if the method was applicable to irradiated ergosterols, but found that the results obtained varied.

The separation of pure calciferol (vitamin D_2) from pure ergosterol has also been studied.

Baker (1) found that when using superfiltrol as an adsorbent and a skellysolve-ethyl-alcohol mixture as a solvent, ergosterol was retained on the column of adsorbent while calciferol was carried through and was found in the percolate.

Bullard (4) studied this same separation more extensively.

From this review it seems possible that with the proper adsorbent and solvents the quantitative separation of vitamins D from the accompanying by-products existing in solutions of irradiated previtamins might be accomplished.

A modification of the chromatographic method used by Ewing et al. (7) is proposed by the author to separate calciferol quantitatively from the by-products obtained when irradiating ergosterol. The proposed method uses superfiltrol as the adsorbent and a hexane-ether-alcohol solution as the solvent both for dissolving the sample and for developing the chromatogram. The quantity and degree of purity of the calciferol collected in the percolate is determined from its corresponding

ultraviolet absorption curve. The possibility of using this method for determining the potency of irradiated ergosterols in volatile solvents is shown by a comparison of the results obtained by this method to those obtained using the modified antimony trichloride reagent of Nield, Russell, and Zimmerli (14).

Experimental

Equipment and Reagents.

1. Hexane.-- A commercial grade of hexane is redistilled to remove any residues. The distilled product should transmit to 210 mu.

2. Ethyl Ether.-- Anhydrous Ether (c.p.) is used as obtained. It should transmit to 219.5 mu.

3. Ethyl Alcohol.-- A c.p. grade of absolute alcohol is purified by distillation after first decanting it from a silver oxide precipitate prepared by adding 6 grams of potassium hydroxide and 3 grams silver nitrate per 2 liters of alcohol. Further purification is obtained by adding activated aluminum amalgam, decanting, and redistilling. The purified solvent should transmit to 215 mu.

4. Chromatographic Developing Solution.-- This solution is made up by volume of 50 parts hexane, 10 parts ethyl ether (abs.) and 1 part absolute ethyl alcohol.

5. Chloroform.-- Small amounts of alcohol are removed from chloroform (c.p.) by washing with an equal portion of water seven times. It is then dried by filtering it through anhydrous sodium sulphate, decanted and distilled, discarding the first and last ten per cent of the distillate.

6. Antimony Trichloride Reagent.-- This reagent is prepared fresh for each day's run. Eighteen (18) grams of antimony trichloride (c.p.) are dissolved in 100 ml. purified chloroform. After filtering this solution, two ml. redistilled acetyl chloride are then added.

7. Adsorption Columns (Small Scale).-- A 9 cm. column of superfiltrol is used and is prepared similar to the method used by Ewing et al. (7). The packed column must be prewashed with the chromatographic developing solution before adding the sample.

8. Adsorption Columns (Large Scale).-- Columns of superfiltrol having a diameter ranging from 2.8 cm. to 9.2 cm. and variable lengths were used. These were also prewashed with the chromatographic developing solution.

9. Spectrophotometers.-- For ultraviolet measurements a Beckman spectrophotometer (quartz) equipped with a special hydrogen discharge tube is used. A Bausch and Lomb visual spectrophotometer equipped with a Martin's polarizing unit and using 1 cm. glass cells is used for making measurements in the visual range.

Procedure.

Chromatographic Separations.

A. Analytical Scale Separation.-- A large enough volume of the solution of irradiated ergosterol to contain at least 75,000 units of vitamin D is transferred to a 125 ml. Erlenmeyer flask. The solvent is evaporated off using a hot water bath (about 50°C.) and suction. The dry residue is then taken up in 10 ml. of the chromatographic developing solution.

This solution is added to a 9 cm. column of superfiltrol which has been previously washed with enough of the developing solution such that the final percolate taken from the column shows very little absorption at 230 mu when measured against the original developing solution. The column is not permitted to become dry at any time. Any remaining residue in the flask is rinsed with 5 ml. of the developing solution and added immediately to the column.

A short-stemmed separatory funnel fitted to the tube is used to add the developing solution to the column drop by drop to develop the chromatogram. A 10 cm. pressure differential is maintained throughout the chromatographic procedure. Enough developing solution is added until the lowest visible band progresses to the bottom of the column.

The percolate from the column is then evaporated to dryness using suction and a hot water bath and the residue (vitamin D_2 fraction) is taken up in absolute alcohol. The extinction at 265 mu of this alcohol solution, or a dilution of this solution, is measured on the Beckman quartz spectrophotometer. The complete absorption curve of this alcoholic solution should have a maxima at 265 mu and be identical with that shown by pure calciferol.

If the potency of the original sample in D units per ml. is desired, it can be determined by the following relationship:

$$\text{D units per ml. of original sample} = 1/100 \times \text{Dil'n. of original sample} \times \text{Log } I_o/I \text{ of diluted sample @ 265 mu} \times 86{,}950$$

The factor 86,950 is a theoretical one obtained by dividing the literature value of the number of D units per gram of calciferol (40,000,000) by the E (1%, 1 cm.) of calciferol at 265 mu given as 460.

B. Larger Scale Separations.-- The procedure used in this part of the investigation differed from that used in the smaller analytical scale separation only in the amount of D units present in the original samples and in the size of the chromatograph column itself. Samples ranging from 15,000,000 units to 115,000,000 units of vitamin D present in the original sample were run through columns varying from 2.8 cm. to 9.2 cm. in diameter and 9 cm. to 14 cm. in length. In some instances the percolate from the columns was collected in fractions. A more thorough discussion of this procedure will be found later in this paper.

Colorimetric Method for Determining the Amount of Calciferol Present.

A large enough sample of the irradiated ergosterol solution to contain approximately 20,000 units of vitamin D is transferred to a 125 ml. Erlenmeyer flask and the solvent is evaporated off using suction and hot water bath. The residue is taken up in 10 ml. of purified chloroform and to a 1 ml. aliquot 10 ml. of the antimony trichloride reagent is added. The extinction at 500 mu is measured exactly three minutes after the

reagent is first added. The potency of the original solution can then be calculated from the $E_{1\%}^{1\ cm.}$ of this solution at 500 mu and by use of a factor determined by Ewing et al. (7).

$$\text{Potency in D units/g.} = E_{1\%,\ 1\ cm.}\ @\ 500\ mu \times 19{,}300$$

Results

Analytical Scale Separations.-- Seven of the irradiation runs made in part I of this paper, along with eight commercial irradiated ergosterols, were taken through this procedure.

The absorption curve of the original sample of Run #11, along with that of Run #11 after the chromatographic treatment, is shown in Fig. I. An absorption curve of pure calciferol is also plotted upon the same axes in order to make a comparison of the purified Run #11 with that of pure calciferol. The curves are very similar.

Potency values of each sample calculated from the absorption curves of the chromatographed sample are listed in Table I along with values obtained by the antimony trichloride colorimetric method. A few bioassays have also been listed for comparison.

Large Scale Separations.-- Attempts to separate calciferol from three of the runs made in part I of this paper and two commercial samples of irradiated ergosterol were made on a large scale. The amount of sample, size of column, calculated purity, and per cent recovery of D_2 present in the original sample has been tabulated in Table II.

The absorption curve of sample #3415 both before and after chromatographic treatment is shown in Fig. II.

A limited study of the visible bands found in the chromatograms was also made. Fig. IV shows the absorption curves of the material contained in various colored bands of the chromatogram from Run #14 (large scale).

Discussion

Various impurities are introduced by the chromatographic method but with the proper precautions can be practically eliminated.

A small amount of residue may exist in the solvents used and show absorption in the ultraviolet region. However, if the solvents are purified as described above, the residue present becomes negligible. The residue of 60 ml. of chromatographic developing solution taken up in 10 ml. alcohol only shows an extinction of .390 @ 265 mu. In almost every case the solution whose absorption curve is being determined will require enough dilution that the error due to the residue in the solvents is completely negligible.

The residue present in superfiltrol (which was observed by Carlson (5) and later corrected for by Baker (1) and Bullard (4) by subtracting a correction factor from the

extinction of the chromatographed material) can be more easily removed by simply prewashing the column of adsorbent with the developing solution until the wash solution shows very little absorption in the ultraviolet range when compared to the original developer. This eliminates the use of a factor which is probably dependent upon the amount of solvent put through the columns, degree of packing the adsorbent, and variations due to different lots of adsorbents.

The separation achieved by the analytical scale chromatographic procedure was very good. In every case the absorption curve of the chromatographed material was almost identical with that of calciferol.

In order to determine when all the vitamin D_2 had passed through the column, the percolate from sample #98952 was collected in fractions. The absorption curve of each fraction was then determined and is shown in Fig. III. The position of the visible bands when each fraction was taken, and the approximate volume of percolate in each fraction is found in Fig. IV.

From these results it appears that all the calciferol has passed through to the percolate just before the lowest visible band reaches the bottom of the column.

The separation obtained with the large scale chromatographic procedure varied. The chromatographed material from the larger diameter columns had absorption curves more nearly like that of calciferol than the original sample, but a smooth curve was not obtained. Best results were obtained when using the smaller diameter column. This is probably due to greater uniformity of packing and development of the chromatogram made possible by a smaller column.

Although the chromatographed material has an absorption curve identical with that of calciferol, the calculated percentage of D_2 in the material is only about 50%. This indicates that some other material which does not show absorption in the ultraviolet region is present. More work needs to be done on this phase of the separation.

A small amount of work has been done toward identifying the material contained in the visible bands of the chromatogram. Fig. IV shows the absorption curves of the material contained in the light blue band and the dark green band usually found in the chromatograms. The absorption maxima at 250 mu indicate that toxisterol may be held in these bands.

This phase of the separation was only lightly investigated. Use of fluorescent zones might help locate the positions of various products retained on the column of adsorbent.

Summary

1. A quantitative chromatographic separation of vitamin D_2 from irradiated ergosterols in volatile solvents was used employing superfiltrol as the adsorbent and a mixture of hexane, ethyl ether, and ethyl alcohol for the solvent and for developing the chromatogram.

2. The ultraviolet absorption curve of the chromatographed material is almost identical with that exhibited by pure calciferol.

3. The potency of these solutions, as calculated from the ultraviolet absorption curves of the chromatographed material, agree quite well, in general, with those values obtained by the antimony trichloride colorimetric method.

4. Larger scale application of this separation yields a product having an absorption curve similar to that of calciferol but of approximately 50% purity.

Table I. COMPARISON OF POTENCY VALUES OBTAINED FOR VARIOUS IRRADIATED ERGOSTEROLS BY THE CHROMATOGRAPHIC ULTRAVIOLET ABSORPTION CURVE METHOD WITH THOSE OBTAINED BY THE ANTIMONY TRICHLORIDE COLORIMETRIC METHOD.

	Potency by Chromatographic Ultraviolet Absorption Curve Method		Potency by Antimony Trichloride Colorimetric Method			Bio Assay
Sample No.	Dil'n./100 X Log I_o/I	Calculated D units/ml.	E (1%,1cm.)	Calculated D units/g.	Calculated D units/ml.	D units/ml.
Run 8	7.38	643,000	49.4	952,000	751,000	600,000
Run 9	13.23	1,150,000	63.2	1,134,000	894,000	
Run 10			44.9	867,000	679,000	
Run 11	6.56	569,000				
	6.27	544,000	34.9	672,000	530,000	520,000
Run 12	6.45	562,000	37.8	728,000	573,000	600,000
Run 13	6.09	529,000	34.2	660,000	520,000	480,000
Run 14	7.18	624,000	37.8	728,000	573,000	590,000
Run 15			33.5	646,000	508,000	
Run 17			31.8	614,000	487,000	
Run 18			33.7	649,000	514,000	

Table I. COMPARISON OF POTENCY VALUES OBTAINED FOR VARIOUS IRRADIATED ERGOSTEROLS BY THE CHROMATOGRAPHIC ULTRAVIOLET ABSORPTION CURVE METHOD WITH THOSE OBTAINED BY THE ANTIMONY TRICHLORIDE COLORIMETRIC METHOD. (Cont'd.)

	Potency by Chromatographic Ultraviolet Absorption Curve Method		Potency by Antimony Trichloride Colorimetric Method			Bio Assay
Sample No.	Dil'n./100 X Log I_o/I	Calculated D units/ml.	E (1%,1cm.)	Calculated D units/g.	Calculated D units/ml.	D units/ml.
Run 19			37.3	720,000	570,000	
Run 20			33.7	649,000	514,000	
84364	3.41	291,500	22.6	436,000	348,000	
96872	12.65	1,142,000	95.4	1,842,000	1,457,000	
82544	3.97	340,000				
97182	5.19	451,000	39.7	764,000	604,000	
82414	3.06	265,500	16.22	313,000	247,300	
97375	2.98	258,000	19.02	367,200	290,000	
97365	7.38	641,000				
AF113	1.03	88,200				

Table II. DATA AND RESULTS FOR LARGE SCALE CHROMATOGRAPHIC SEPARATIONS OF VITAMIN D_2 FROM IRRADIATED ERGOSTEROLS

Sample No.	Calculated D units in Original Sample	Size of Column Length in cm.	Size of Column Diameter in cm.	Wt. of residue in Percolate in g.	Calculated % D_2 in residue	% Recovery D_2 in original sample
Run #15	48,800,000	9	9.2	2.1097	38.5	66.7
#97375	131,200,000	9	9.2	5.0665	57.9	89.6
Run #14	42,975,000	9	2.8	----	----	
Run #13	39,000,000	14	2.8	----	----	
#3415	(100 ml.)	12	2.8	0.9312	40.9	

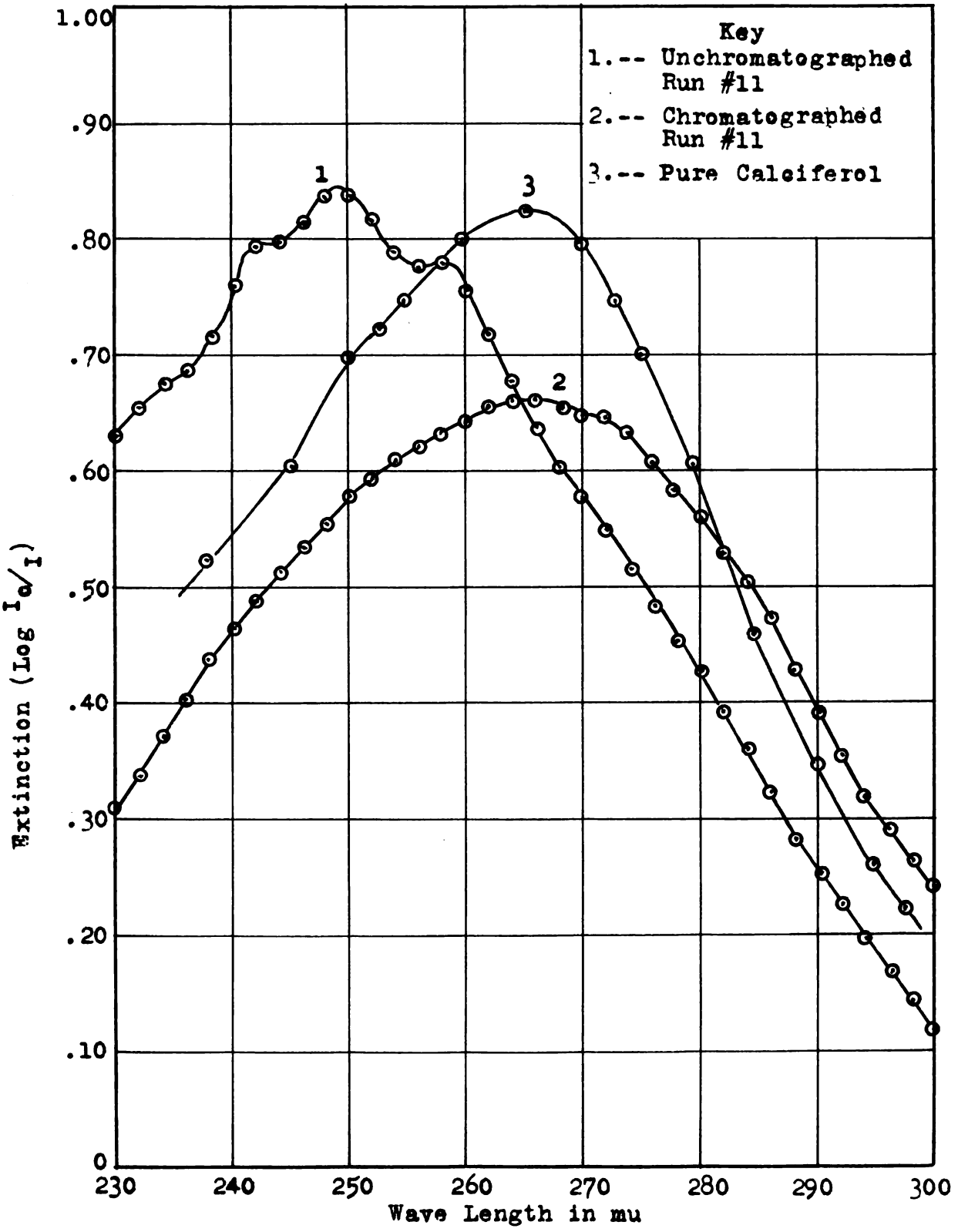


Fig. I.-- Absorption Curves of Run #11 Before and After Chromatographic Treatment (Analytical Scale).

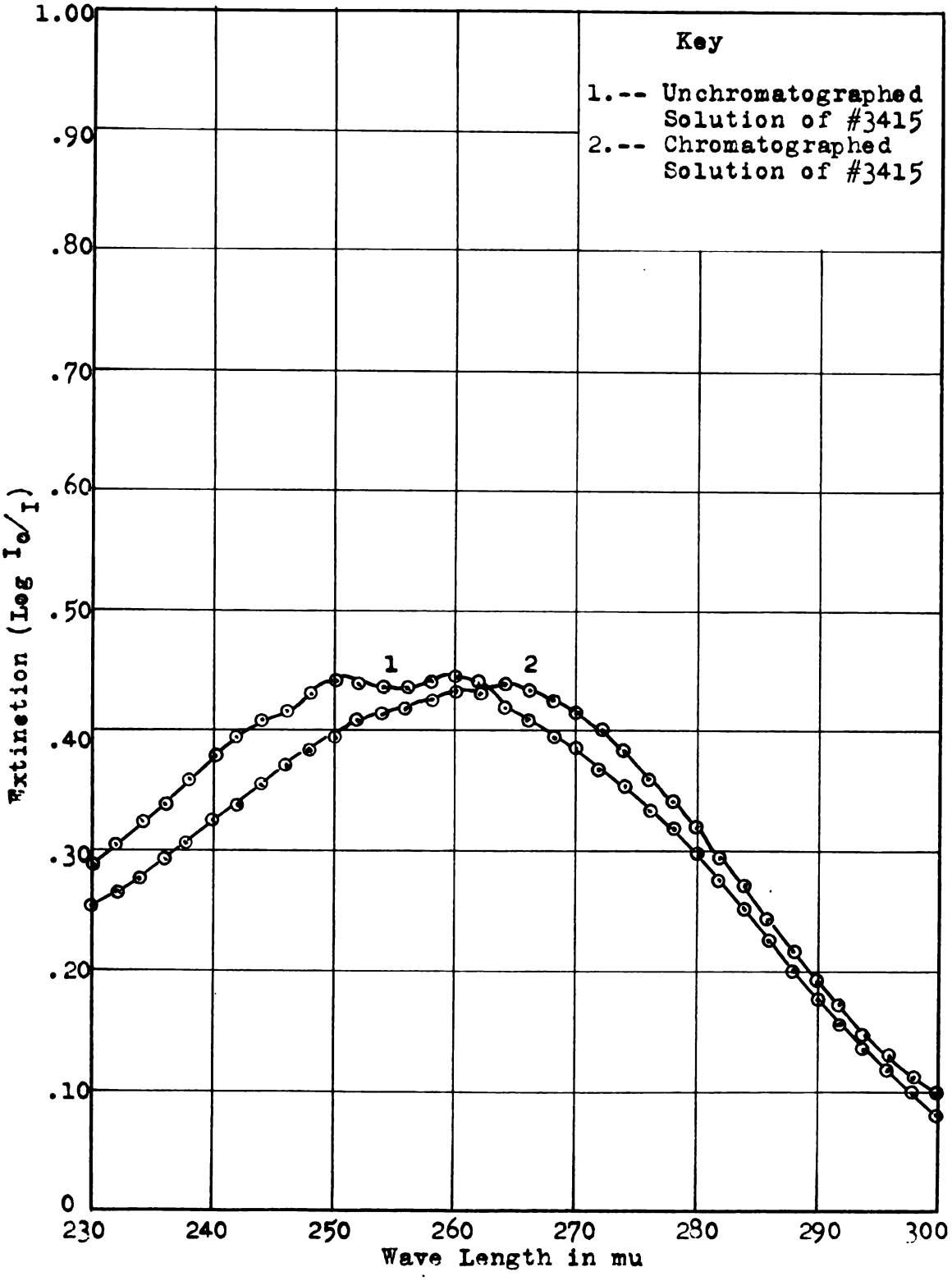


Fig. II.-- Absorption Curve of Commercial Sample #3415 Before and After Chromatographic Treatment (Large Scale).

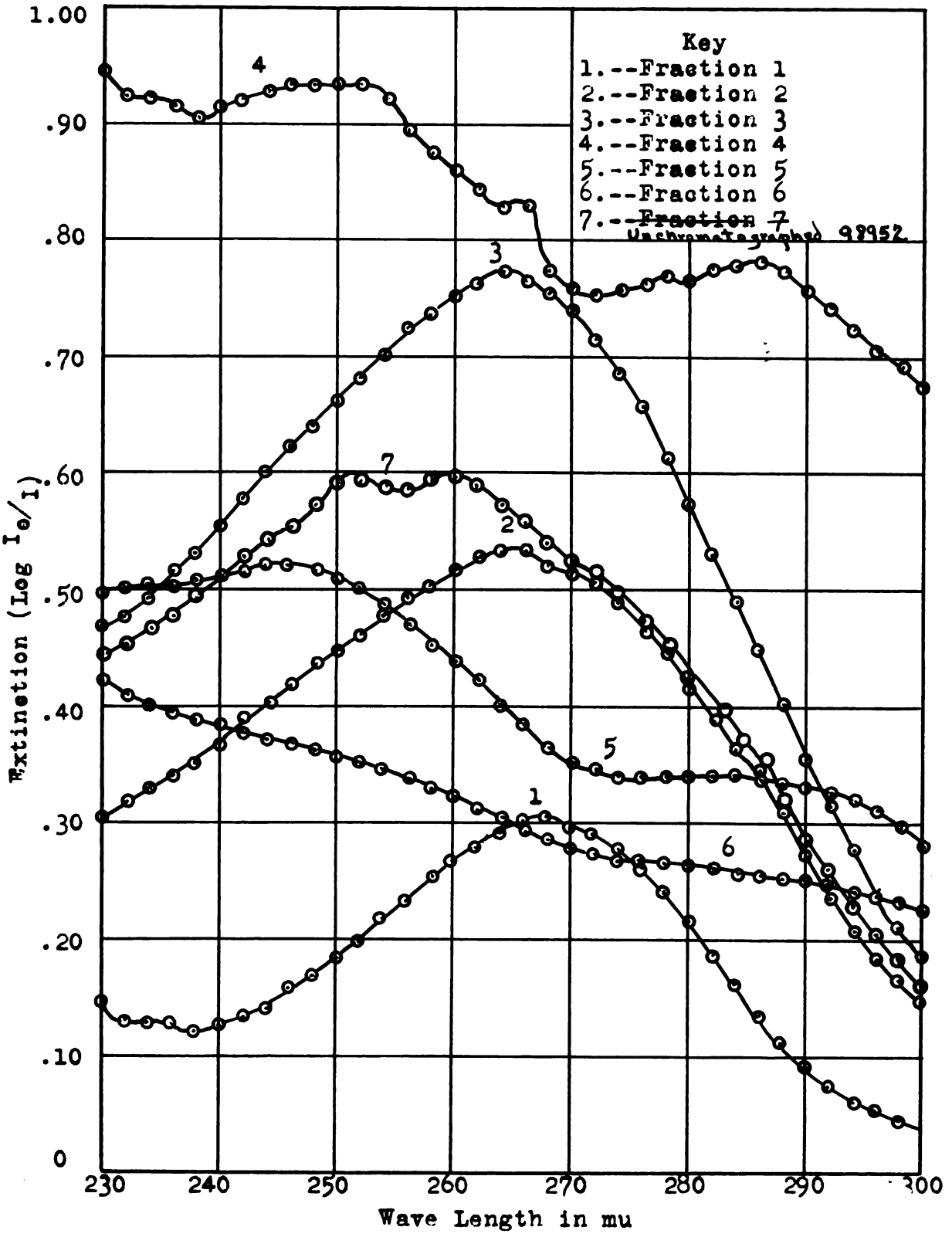


Fig. III.-- Absorption Curves of Various Fractions of Percolate Obtained Chromatographing Irradiated Ergosterol #98952 (Analytical Scale).

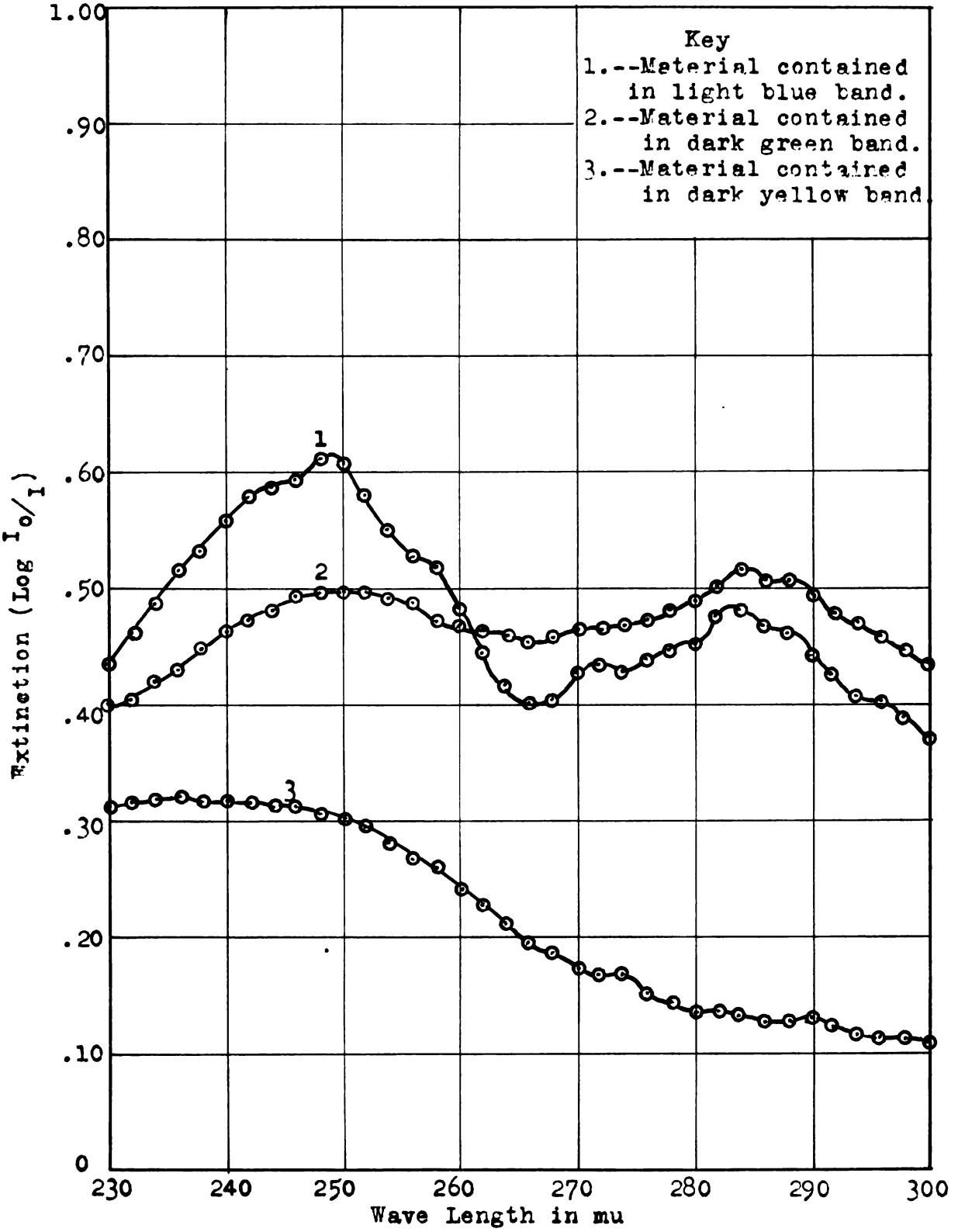
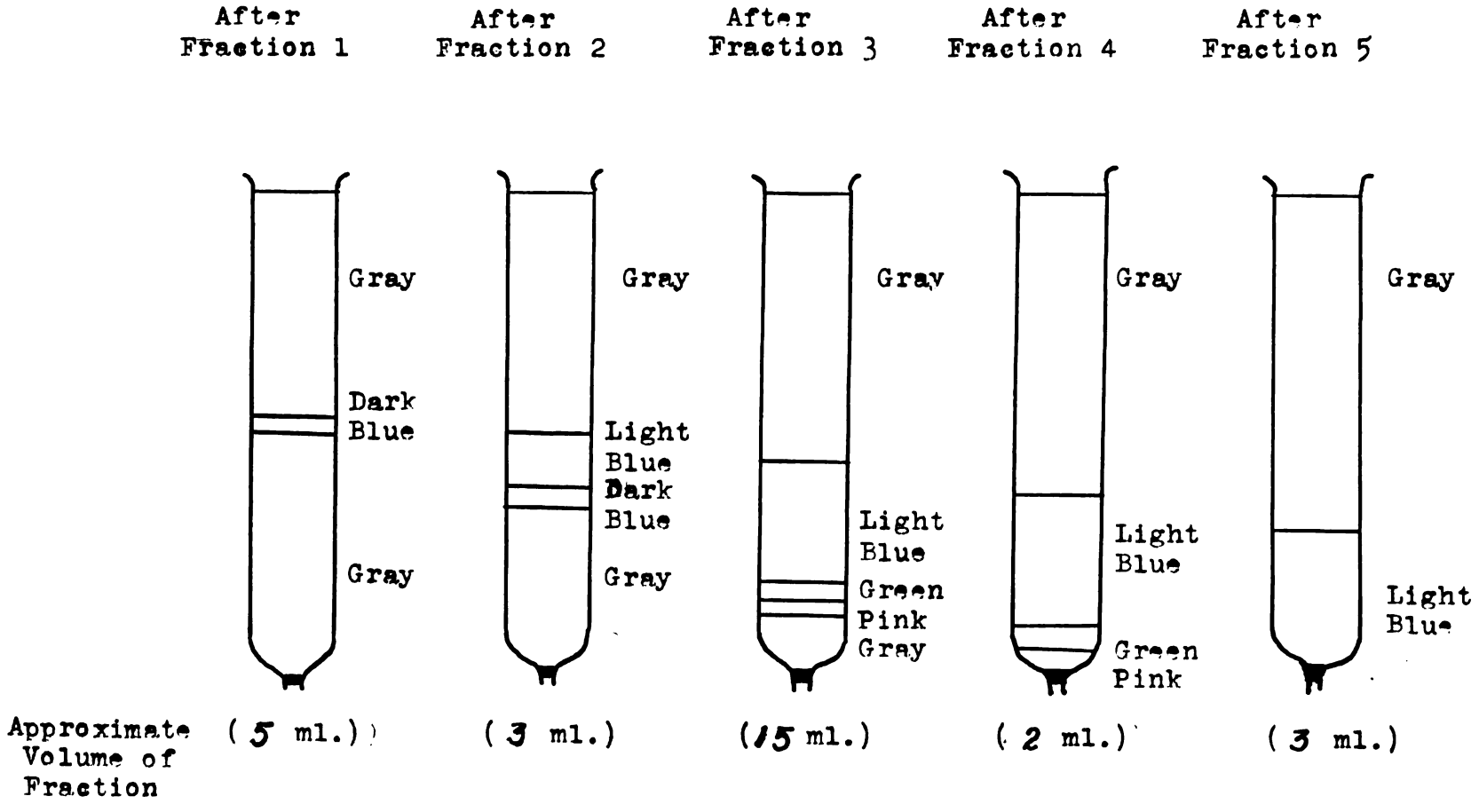


Fig. IV.-- Absorption Curves of Material Contained in Visible Bands of Chromatogram of Run #14 (Large Scale).

Fig. Appearance of Chromatogram After Each Fraction of Percolate from 98952 Was Taken.



Fraction 6 contains ether eluate of developed chromatogram.

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Irradiation of solutions of ergosterol in an improved type quartz cell.