A CRITICAL STUDY OF THE CHROMATOGRAPHIC SEPARATION OF CALCIFEROL FROM ERGOSTEROL

By

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AN ABSTRACT

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AN ABSTRACT

The chromatographic separation of synthetic mixtures of pure calciferol from pure ergosterol has been accomplished by using an ether or ether - hexane solvent and an alumina or superfiltrol adsorbent. Ether was found to be the best solvent and eluent for the accomplishment of the separation by means of an alumina adsorbent. When superfiltrol was used as the adsorbent, ether - hexane in the ratio of 1:5 parts by volume was found to be a very satisfactory solvent for the separation.

In obtaining a complete separation of the two compounds, the weight of the adsorbent and the height to which it was packed in the chromatographic tube were very important. Alumina gave a very good separation of the two compounds, the resultant form of each being pure and free from any other material. However, the lengthening of the column and the resulting broader banding of each compound within the column were found to be undesirable. Superfiltrol gave a very sharp separation much more quickly and on a very much shorter column. However, a conversion of the ergosterol into two irrelevant substances took place on the adsorbent and, while calciferol and ergosterol were obtained free from

each other, neither compound was found in the eluate completely free from one or both of the irrelevant materials. This observation lead to a pattern analysis of the conversion products and as a result, the eluate fractions could be analyzed by a system of simultaneous linear equations. Also, the concentration of calciferol or ergosterol in the total eluate, or any fraction thereof, could be determined exactly.

The relative and total concentrations of calciferol and ergosterol initially placed on either adsorbent had very little effect on the total calciferol recovery. However, both the separation of the two compounds and the total ergosterol recovery were influenced considerably by this property.

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INTRODUCTION

The study of the chromatographic separation of pure calciferol (vitamin D_2) from pure ergosterol has been of considerable interest in recent years. Perhaps through a more critical investigation of this separation further information can be found relative to the more complex separation of calciferol from irradiated ergosterol or related compounds.

In the course of a spectrophotometric investigation of an eluate, which had been obtained from a chromatogram in which the adsorbent, solvent, and eluent had been selected so as to retain the ergosterol and elute only the calciferol, it was observed that this eluate contained a substance foreign to the initial solution of pure calciferol and pure ergosterol. The presence of this foreign substance in the eluate has caused much speculation and further investigation.

Carlson[1] noted that when ethyl alcohol and diethyl ether rich solvents were passed through an adsorption column containing superfiltrol, the filtrate contained a substance, apparently eluted from the superfiltrol, which gave it a density appreciably greater than expected.

Concurrently, Baker[2], using the spectrophotometer

to analyze an eluate which was expected to contain only calciferol, observed that calciferol and another substance were present. Both had an absorption spectrum in the ultraviolet region. He concluded that this interfering substance was eluted from the superfiltrol and that a constant correction factor for it could be obtained by running a blank along with each sample. He also did some work with alcoholic solutions of pure ergosterol and calciferol and noticed that, using hexane - diethyl ether - ethyl alcohol as a solvent and eluent, ergosterol was adsorbed by activated bentonite clay and calciferol proceeded on through the column. However, he did no quantitative or even conclusive qualitative work along this line.

The study of the unknown substance which was thought to be eluted from the superfiltrol was further considered by Bullard[3]. She found that the method of carrying a correction factor blank along with each sample gave quantitative results. If the calciferol concentration in the original solution was sufficiently large, the correction factor was eliminated by adequate dilution of the resultant eluate. The solvent and eluent used for this work was n-hexane - diethyl ether - ethyl alcohol in the relationship of 50-10-1 respectively by volume. An examination of several similar binary and

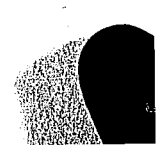
ternary solvents was made in an attempt to find a solvent which would eliminate the elution of this foreign material from the column and perhaps lead to an even more complete calciferol recovery.

More recently, in 1948, Pinkerton[4] found the ternary solvent of hexane - diethyl ether - alcohol to be more complex than necessary. A satisfactory separation of calciferol from ergosterol using superfiltrol as an adsorbent was accomplished by either a hexane - ether or benzene - ether solvent in the ratio of 5:1 respectively by volume. Ether seemed to be a very important constituent of every binary and ternary solvent examined. No satisfactory single solvent was found which would give the desired separation. In addition, a rather thorough study was made with respect to the separation of calciferol from ergosterol using superfiltrol and the binary solvent hexane - ether. The eluate was corrected for the interfering substance eluted from the superfiltrol by determining certain constant ratios between extinction values at two different wave lengths for both the interfering material and the calciferol.

The rates of migration of compounds on an adsorbent are influenced by the polarity of the solvent and eluent. Chen[5] studied the elution of vitamin D_2 and vitamin D_3 from an alumina column in which vitamin D_2 preceded

vitamin D_3 when a polar solvent was used, and as the polarity of the solvent decreased, the order of elution was reversed. Similarly, vitamins D could partially be separated from vitamin A and ergosterol on an alumina column. Using a superfiltrol column, she found that a hexane - diethyl ether solvent gave a better recovery of vitamins D than did a hexane - diethyl ether - ethyl alcohol solvent.

It is the purpose of this investigation to study the chromatographic separation of synthetic mixtures of pure calciferol from pure ergosterol using a diethyl ether or diethyl ether - hexane solvent and an alumina or superfiltrol adsorbent and to examine the interfering material in the eluate from a superfiltrol column.



MATERIALS AND EQUIPMENT

The calciferol used in this study was a pure synthetic crystalline vitamin D_2, each gram considered to contain a minimum of 40,000,000 U.S.P. units. This was obtained from the Special Markets Division of the Winthrop Chemical Company, Inc., New York, N. Y. and was used without further purification.

A good commercial grade of fine crystalline ergosterol was obtained from the Montrose Chemical Company, Newark, N. J. and was recrystallized from a mixture of one part of absolute ethyl alcohol to two parts of benzene by volume. The ergosterol crystals were filtered by suction, washed with methanol, and drying was completed in a vacuum at room temperature.

It was necessary to further purify a C.P. grade of absolute ethyl alcohol in order to use it as a solvent for the crystalline forms of calciferol and ergosterol. Twenty grams of potassium hydroxide and ten grams of silver nitrate were added to one liter of the alcohol and with occasional shaking this was allowed to stand for one week. The alcohol was decanted and distilled from all glass apparatus. The distillate was collected in 100 milliliter fractions and for use was required to transmit down to

230 millimicrons (transparency above 90 per cent) as measured on the spectrophotometer.

Appropriate amounts of sodium hydroxide and anhydrous sodium sulfite were added to the amount of anhydrous diethyl ether (C.P.) required for immediate use. After distillation from an all glass apparatus, the ether must transmit down to 230 millimicrons (transparency above 70 per cent) as measured on the spectrophotometer in order to be useable.

Skellysolve B, a commercial preparation of n-hexane, was purified by passing it through a large Tswett tube, 24 inches long and 1.5 inches in diameter, containing freshly activated silica gel. The percolate fractions to be used must transmit down to 230 millimicrons (transparency above 96 per cent) as measured on the spectrophotometer and must show no absorption due to benzene. The used silica gel may be repurified and reactivated by washing it free of hexane with distilled water, allowing it to air dry, and then further drying it in an oven at 250°C. for at least 24 hours.

The adsorbents used were activated alumina and superfiltrol. The alumina was the Alcoa brand, Grade F-20, manufactured by the Aluminum Ore Company, East St. Louis, Ill. The superfiltrol, SF 63, was a finely divided activated bentonite clay prepared by the Filtrol Corporation, Los Angeles, Calif.

Chromatographic tubes of different lengths were necessary when using the different adsorbents. For the investigation using alumina, the length of the adsorbent containing section of the tube was about 47 centimeters; and for the study using superfiltrol, the length of this section was 17 centimeters. In both cases, the diameter of the section of the tube which held the adsorbent was 7.5 millimeters and the diameter of the lower constricted portion of the tube was 3.7 millimeters.

When the volume of the eluate per fraction and the number of fractions required had been determined, they were collected identically with a Technicon Automatic Fraction Collector made by the Technicon Chromatography Corporation, New York, N. Y. The rate of elution could be controlled by varying the pressure at the top of the chromatographic column using a carbon dioxide source and an adjustable mercury manometer.

Absorption measurements were made with a Beckman Quartz Spectrophotometer, Model DU[6], made by the National Technical Laboratories, South Pasadena, Calif. The absorption cells were made of silica, the thickness of each being 1.000 ± 0.002 centimeter.

All computations were carried out using a completely automatic Fridén Ultra-matic Calculator made by the Fridén Calculating Machine Co., Inc., San Leandro, Calif.

EXPERIMENTAL PROCEDURE

From stock alcoholic solutions of calciferol and ergosterol, which were made up from the crystalline forms of the compounds, the desired quantities for each investigation were volumetrically taken. The stock solution of calciferol contained 0.0174 gram/100 milliliters of absolute ethyl alcohol, and that of ergosterol contained 0.0244 gram/100 milliliters of absolute ethyl alcohol.

Having determined the amount of calciferol or ergosterol or combination of these to be investigated, this quantity was taken from the stock solutions and using a warm water bath was taken to dryness under reduced pressure. The sample was then taken up in three milliliters of a hexane or ether - hexane solvent.

The adsorption column was prepared by placing a small wad of cotton at the top of the constricted portion of the chromatographic tube as a support for the adsorbent. The alumina was weighed out to the nearest gram and then while gently tapping around the sides of the tube, the adsorbent was introduced into the tube. The height to which the alumina came was then measured. If superfiltrol was used as the adsorbent, it was weighed out gram by gram. After the introduction of each gram into the tube, the superfiltrol was very

firmly packed with the aid of a large glass rod flattened on one end to a diameter just slightly less than that of the tube. The height of the adsorbent was then measured to help insure reproducible hand packing.

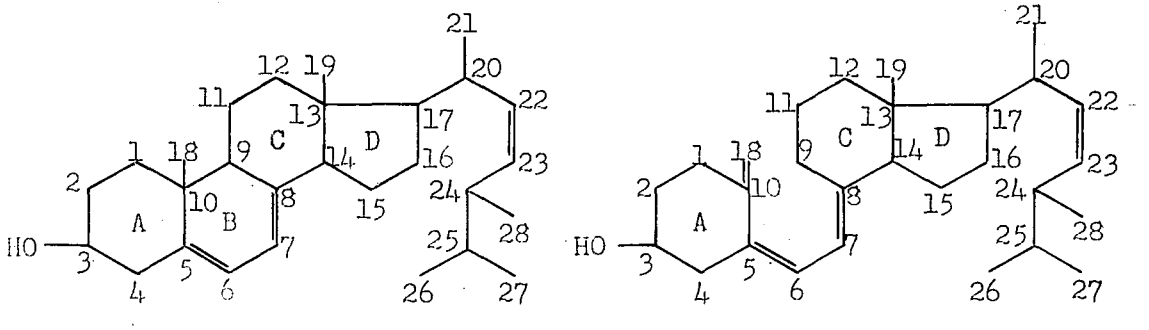
The chromatographic tube was then positioned in the fractionator. The column was washed with a definite amount of ether or ether - hexane solution which was determined by the type and amount of adsorbent. Just prior to the last portion of wash solution leaving the top of the adsorbent, the sample was introduced onto the column. The fractionator was immediately set into operation. The flask which had contained the sample solution was then rinsed with three milliliters of the previously used solvent, and as the last portion of the sample solution disappeared into the adsorbent, this rinse solution was added to the column. The developer, having been placed in a separatory funnel atop the chromatographic tube, was introduced onto the column as the rinse solution disappeared into the adsorbent. At no time after the initial wetting of the adsorbent with the wash solution was the top of the column ever permitted to become dry. The same glass dropping tip (a piece of the fractionator) was used for all experiments. This assured eluate drops of constant size.

When a sufficient number of fractions, having used

45 drops = 1 fraction ± 1 milliliter, had been collected, each was taken to dryness under reduced pressure and using a warm water bath. If a residue, due to a slight evaporation of the eluate as it collected on the dropping tip, had formed on the tip, it was removed with the developer, collected in a test tube, evaporated to dryness, and further treated the same way as any other sample. Each residue was volumetrically taken up in four milliliters of hexane and the absorption curve of the resultant solution determined on the spectrophotometer. A fraction was further diluted with hexane when its concentration was too great for the determination of its absorption spectrum to be made on the most accurate portion of the instrument. The extinction readings were taken at various intervals of one to ten millimicrons depending upon the positions of the maxima and minima of the eluate absorption spectrum.

DISCUSSION

Ergosterol has the empirical formula $C_{28}H_{44}O$, which has been established by careful analysis of derivatives containing hetero atoms. Calciferol, an isomer and derivative of ergosterol, also has the empirical formula $C_{28}H_{44}O$. However, the structural formulas of ergosterol and calciferol are different[7]. Ergosterol has an angular methyl group between rings A and B at carbon atom 10 and three double bonds, one in the side chain between carbon atoms 22 and 23 and two conjugate double bonds in the 5,6- and 7,8-positions. Calciferol has a methylene group on carbon atom 10 and hence four double bonds, one in the side chain between carbon atoms 22 and 23 and two double bonds, which are in conjugation with the double bond at the 7,8-position, in the 5,6- and 10,18-positions (see Figure 1).



Ergosterol Calciferol

Figure 1.

The presence of four double bonds, three of which are conjugate, in calciferol as compared to only three double bonds, two of which are conjugate, in ergosterol indicates that calciferol may be more polar than ergosterol. Since adsorption takes place most readily from non-polar solvents[8], calciferol, as compared with ergosterol, should be preferentially and more strongly adsorbed from a non-polar or slightly polar solvent. Desorption, the displacement of the adsorbed substance from the adsorbent, is done by an eluent which is more polar than the adsorbed substance. Thus, ergosterol, as compared with calciferol, should be more rapidly desorbed by a solvent more polar than itself. However, this was not found to be true when alumina or super-filtrol was used as the adsorbent and ether or ether - hexane was used as the solvent or eluent. Perhaps the vastly different steric configurations of the two compounds have altered the anticipated polar relationships between them or have made the more or less strongly adsorbed compound more available to the action of the eluent. Hence, polarity may only be a guide to the action taking place within an adsorbent column.

Solvents differ in polarity and adsorbents vary in activity. Considering the solvents and adsorbents used in this study, hexane, ether, and alcohol increase

in polarity and alumina is a less active adsorbent than superfiltrol[9].

The wetting and washing of the adsorbent column just prior to the introduction of the sample solution is done with the most polar solvent to be used in the course of the investigation. The sample is dissolved in a solvent of less or equal polarity and introduced onto the adsorbent. The development of the column and elution of the sample, or any fraction thereof, is carried out with a solution of equal or greater polarity than that previously used as the solvent for the sample.

The absorption spectra of calciferol and ergosterol vary with the solvent used[10,11]. Therefore, the absorption spectra of calciferol and ergosterol in hexane were determined over the range of 230 millimicrons to 300 millimicrons using the spectrophotometer (Figure 2). Throughout the investigation hexane was the solvent used for the final analysis because of its great transparency over the ultraviolet region and its straightforward and rapid method of purification.

THE USE OF ALUMINA AS AN ADSORBENT

The spatial configuration and forces existing among the atoms of calciferol and ergosterol evidently necessitate the use of quite active adsorbents in order to obtain a complete or even a partial separation of the

two compounds from a single solution.

Alumina is obtainable in a very pure state and is such that almost all of the irrelevant material remaining on the commercial preparation can be removed by prewashing the column with the proper organic solvent. Using various amounts of ether, depending upon the length of the column, as the prewash solvent, the irrelevant material in the eluate from the alumina column can be reduced to less than 0.002 extinction units at 264 millimicrons. To remove the possibility of any interference from a solvent residue, the solution in the reference cell was made up comparable to the sample solution from the column. That is, if a one milliliter sample was taken using ether as the eluent, evaporated to dryness, and the residue taken up in hexane, then one milliliter of ether was evaporated to dryness, the residue (if any) taken up in the same amount of hexane, and this was used as the reference solution for that sample.

Since the elimination of any interfering materials in the final analysis was quite complete, all of the resultant absorption curves were found to be either pure calciferol, pure ergosterol, or a linear combination of these. The combining of Lambert's and Beer's laws into a single absortpion equation gives

$$k_\lambda = \frac{\log (I_o/I)}{lc}$$

where k is the extinction coefficient at a given wave length, λ, I_o is the intensity of the transmitted light, I is the intensity of the incident light, l is the thickness of the solution, and c is the concentration of the solution. Using this fundamental relationship, the additivity of the absorption curves of two or more substances in solution is developed by Harris and Thimann[12]. If c_1 and c_2 are the unknown concentrations of two substances in a solution of one centimeter thickness, k_1 and k_2 their extinction coefficients at a wave length λ, k_1' and k_2' their extinction coefficients at some other wave length λ', and D and D' the densities, log (I_o/I), of the mixture at the two wave lengths, then

$$k_1 c_1 + k_2 c_2 = D$$

and

$$k_1' c_1 + k_2' c_2 = D'$$

therefore

$$c_1 = \frac{k_2 D' - k_2' D}{k_1' k_2 - k_1 k_2'}$$

and

$$c_2 = \frac{k_1 D' - k_1' D}{k_1 k_2' - k_1' k_2}$$

and the units of c can be determined and adjusted in accordance with k. As the critical points for the calculation of the content of each fraction, the maxima of calciferol at 264 millimicrons and that of ergosterol

at 281 millimicrons were used. Having found the content of each fraction to be a linear combination of calciferol and ergosterol and knowing the concentration of both compounds placed on the column, the relative and exact amounts of each can be computed.

Variation of Eluent Polarity

As the ether content (by volume) of the eluent is increased, the total amount of both calciferol and ergosterol eluted from the column is slightly increased, as can be seen from Table I. Hence, both compounds are a little more easily desorbed as the polarity of the eluent is increased. Calciferol is somewhat more completely desorbed from the column than is ergosterol. At the same time, the percent separation of the two compounds remains relatively constant. Therefore, the rates for which each compound is adsorbed-desorbed as it progresses downward on the adsorbent must be proportionately equal as the polarity of the solvent increases. Similar properties are observed when the length of the alumina column is varied. When the column is 7.8 centimeters long (by weight, 4 grams) and the eluent is composed of ether and hexane in equal parts by volume, 90% of the calciferol and 82% of the ergosterol are recovered while about 34% of the calciferol and 34% of the ergosterol are recovered

in the pure state. When the eluent is pure ether, 96% of the calciferol and 90% of the ergosterol are recovered while about 32% of the calciferol and 33% of the ergosterol are recovered in the pure state (see Figure 3). When the column is 15.6 centimeters long (by weight, 8 grams) and the eluent is composed of ether and hexane in the ratio of 7 to 3 parts by volume, 90% of the calciferol, about 48% of which is obtained in the pure state, and 83% of the ergosterol, about 46% of which is obtained in the pure state, are recovered. As the eluent is increased to pure ether, 94% of the calciferol, about 51% of which is obtained in the pure state, and 87% of the ergosterol, about 49% of which is obtained in the pure state, are recovered (see Figure 4). Thus, an increase in the length of the adsorbent column increases the percent separation of the calciferol from the ergosterol by quite an appreciable amount, but this is accomplished at the expense of the total amounts of calciferol and ergosterol recovered which show a slight decrease.

There is a change in the rate of elution of calciferol and ergosterol when the polarity of the eluent is altered, as would be expected (Table II). When ether is used as the eluent, the calciferol and ergosterol are eluted from the column more than twice as fast as when ether - hexane

in equal parts by volume, is used. At the same time, the volume of eluent necessary to elute the calciferol and ergosterol from the alumina is greatly increased as the ether content of the eluent is decreased. This increase in volume of eluent is accompanied by a broadening of the range in which the elution takes place and a concurrent increase in the number of fractions containing both calciferol and ergosterol. This band widening does not lend itself advantageously to a subsequent separation of the two compounds. Considering the 7.8 centimeter alumina column and the use of the hexane - ether eluent, in equal parts by volume, calciferol appears and terminates in fraction 37 and 60 respectively, while ergosterol is present in fractions 46 through 72. Hence, there are 15 fractions in which calciferol and ergosterol are together. When pure ether is the eluent used, calciferol appears in fractions 15 through 26 and ergosterol is found in fractions 20 through 35. Thus, there are only half as many fractions in which the two compounds appear simultaneously (Figure 5). Taking the 15.6 centimeter column for comparison, having used the ether - hexane eluent in the ratio of 7:3 parts by volume, calciferol is present in fractions 40 through 61 and ergosterol is found in fractions 51 through 78. This is an interval of 11

fractions in which both compounds occur. Using pure ether as the eluent, calciferol falls in fractions 29 through 42 and ergosterol occurs in fractions 37 through 56. This is an interval of 6 fractions in which both compounds are found (Figure 6). Therefore, for a column of given length, the increase in ether content of the eluent not only hastens the elution of both calciferol and ergosterol but lessens the interval in which both compounds are eluted. For a longer column, the compounds are eluted later, naturally, but the interval in which both appear is less or at least no greater than that for the shorter column.

Because there is little difference in the percent separation of the two compounds when an ether - hexane eluent is used and since there is a decided increase in the range in which both compounds appear together when ether - hexane is the eluent used, pure ether was selected as the eluent to be used for the further investigation of the change of column length and the effect of the change in calciferol and ergosterol concentration.

Variation of Column Length

Having observed the portion of eluate in which there is an overlap of the elution of calciferol and ergosterol to be 7 and 6 fractions for the columns of

7.8 and 15.6 centimeters in length respectively, it seems reasonable to investigate columns of longer length to see if this six fraction overlap can be removed. The elimination of a portion of the eluate where calciferol and ergosterol are eluted simultaneously will take place only when the rate of adsorption-desorption for one of the compounds is more rapid than the rate of widening of the range over which that compound is eluted. The various column lengths investigated are given in Table III and illustrated graphically in Figure 7. As the length of the column is increased, the number of fractions containing both compounds is slowly decreased and the total number of fractions required to elute each compound is increased. This latter property is unfavorable for keeping the initial calciferol and ergosterol concentrations, which are introduced onto columns of longer length, constant. Since the eluate becomes less and less concentrated per fraction, some of the extinction readings for these dilute fractions tend to fall below the range of maximum reliability of the spectrophotometer. However, when necessary, the results are corrected for this inherent error. A separation of calciferol from ergosterol was attained when the column length was about 44.6 centimeters (23 grams). The calciferol initially appeared in fraction 66 and terminated in fraction 86

and ergosterol was eluted in fractions 87 through 115. The range in which ergosterol was eluted being 8 fractions more than were required to elute calciferol.

It is not sufficient just to have obtained a separation of calciferol from ergosterol, but the completeness of the recovery of each compound should be considered. Figure 8 shows the percent of calciferol free from ergosterol as compared to the total percent of calciferol recovered as the length of the alumina adsorbent is changed. The percent of calciferol eluted free from ergosterol increases quite rapidly up to and including the 31.1 centimeter column. It then tends to level off. The total percent of calciferol recovered from the column declines very rapidly at first and then becomes almost linear between the 7.8 centimeter and 44.6 centimeter columns. The percent of ergosterol free from calciferol as compared to the total percent of ergosterol recovered as the column length is altered is shown in Figure 9. The curve showing the percent of ergosterol eluted free from calciferol is quite similar to that for the comparable calciferol curve. However, the pure ergosterol recovered from the columns of greater length than the 15.6 centimeter column increases considerably more slowly than did the pure calciferol recovered. The total percent of ergosterol recovered

declines more rapidly than does calciferol for columns of shorter length and then continues a linear declination, more rapid than calciferol, between the 7.8 centimeter and 44.6 centimeter columns. Considering the column on which the two compounds were separated, 86.5% of the calciferol and 76.2% of the ergosterol were recovered. It is quite possible that with the widening of the range of elution that is concurrent with the lengthening of the column, the calculated total recoveries of the two compounds are somewhat lower than the actual amounts recovered. However, further investigation shows this error to be less than two per cent.

Variation of Sample Concentration

The effect of varying the relative and total concentrations of calciferol and ergosterol to be introduced onto the alumina was studied using the 7.8 centimeter (Figure 10) and 15.6 centimeter (Figure 11) columns. Results from corresponding investigations of the two columns of different length are very comparable. This is particularly true for the total percent recovery of each compound; the main consistent difference being that the total recovery for each compound is slightly greater when using the shorter column. The parallelism between the results obtained from the two different length columns is less for the

percent separation of calciferol and ergosterol. However, the most consistent dissimilarity is that the elution of each compound when free from the other is considerably greater for the longer column. Using as a reference the basic calciferol (0.000260 gram per milliliter) and ergosterol (0.000488 gram per milliliter) concentrations and for comparison an equal concentration of calciferol with a double quantity of ergosterol, the separation of each is lessened on the longer column but the separation of calciferol is increased on the shorter column. Doubling the ergosterol concentration and keeping that of calciferol constant increases the separation of calciferol and decreases that of ergosterol on both columns. When both compounds are doubled, the calciferol and ergosterol separations are increased on the shorter column and decreased on the longer column. Keeping the reference concentration of calciferol the same, changing that of ergosterol to 0.000976 gram per milliliter, and comparing the results from this study with those when calciferol is doubled and ergosterol is unchanged, the separation of each compound is increased on the 15.6 centimeter column and that of ergosterol is decreased on the 7.8 centimeter column. Using the immediately preceding calciferol and ergosterol reference concentrations and making a comparison with

the results obtained when the calciferol concentration is doubled and that of ergosterol is constant, the separations of both compounds are increased on both columns. When the concentration of calciferol is doubled and that of ergosterol is unchanged and a comparison is made using the same amount of calciferol and doubling that of ergosterol, the calciferol separation is decreased while the ergosterol separation is increased on both columns. In general, as the ratio of the concentration of ergosterol to calciferol decreases, the percent of calciferol obtained free from ergosterol increases. However, the previously stated facts do not lend themselves to any other general conclusions.

THE USE OF SUPERFILTROL AS AN ADSORBENT

The commercial preparation of superfiltrol, one of the most active adsorbents, contains impurities which are soluble in the organic solvents used and have absorption in the ultraviolet region under investigation. The majority of this irrelevant material is removed from the adsorbent by prewashing the column with the proper organic solvent. However, these impurities are seemingly never completely removed from the superfiltrol and, beyond a certain amount of prewashing, tend to approach a repetitious concentration and absorption spectrum which are apparent in the analysis of successive equivalent

fractions of eluate. The nearly linear absorption spectrum of this irrelevant substance lends itself well to the mathematical treatment, or a modified form of the analysis, presented by Morton and Stubbs[13]. Their correction for an interfering substance, which accompanies a compound whose absorption spectrum in the pure state is known, is entirely general and relative to any linear absorbing material.

A few survey investigations, using calciferol and ergosterol in combination on superfiltrol columns of various lengths, indicated very positively that an irrelevant material, whose ultraviolet absorption spectrum was not at all linear, was appearing in the eluate. However, because of smaller total and relative concentrations of the two compounds and the practice of terminating the development of the chromatogram at the point where only calciferol and seemingly nothing else had been eluted, this nonlinear interfering substance has not previously been of much concern. Thus, the Morton-Stubbs correction alone is no longer applicable; and because equilibrium between solution and adsorbent is not instantaneous and from observation the effect of diffusion (nonsharp banding) is far from negligible, the theory of chromatographic analysis given by Wilson[14] offers little information relative to the analysis of

the content of the eluate. The more recent kinetic theory of chromatography proposed by Thomas[15] is of no aid in the solution of the composition of the eluate because of the binary compound and solvent system used for this work. Hence, a different method of analysis must be found for the separation of calciferol from ergosterol when using superfiltrol as the adsorbent.

The eluate from a 4.6 centimeter (by weight, 2 grams) superfiltrol column on which 0.000260 gram of calciferol had initially been placed was carefully analyzed. Each fraction was checked for purity by a ratio test at no less than ten different wave lengths over the range of 230 millimicrons to 300 millimicrons. Having determined each fraction to contain only pure calciferol, the percent of calciferol recovered from the adsorbent was computed and found to be 97.6%. Therefore, the non-linear impurity found in the eluate when calciferol and ergosterol are placed on the column together is not likely to be due to a steric or chemical change in the calciferol caused by its contact with the superfiltrol.

Ergosterol was then initially placed on a column of superfiltrol and most of the eluate, which was collected fractionwise, did not have an absorption spectrum resembling that of ergosterol at all. Varying the ergosterol concentration from 0.000244 gram per

milliliter to 0.001952 gram per milliliter by multiples of two and using a 4.6 centimeter adsorbent column, a very consistent pattern of absorption curves was noted as being eluted from the column in each case. Further investigations, using these same concentrations of ergosterol and a 2.3 centimeter superfiltrol column, gave comparable patterns for the absorption spectra of the column eluate. The content of the eluate could then be classified into three distinct components: (1) a substance with a maximum at 282 millimicrons, (2) a substance with a very distinct maximum at 250 millimicrons, and (3) the pure ergosterol unaltered. Applying a ratio test, similar to that used previously to determine the purity of fractions containing calciferol, to several successive eluate fractions which appeared to contain only the substance with a maximum at 282 millimicrons or the substance with a maximum at 250 millimicrons, a very consistent absorption spectrum, which differed only in magnitude from fraction to fraction, was found for each of the two substances (see Figure 12). Henceforth, the material with a maximum at 282 millimicrons shall be called substance 282 and that with a maximum at 250 millimicrons shall be called substance 250. Thus, the nonlinear irrelevant material in the eluate from a column of superfiltrol

initially containing only pure calciferol and ergosterol is very likely due to a conversion of some of the ergosterol, caused by its contact with the adsorbent in the presence of the solvent, into at least two different substances.

A substance with a maximum at 250 millimicrons was reported by Kimball[16], who, in separating the products of irradiated ergosterol chromatographically, noted its appearance just prior to the elution of the remaining nonirradiated ergosterol. At first, the substance was thought to be toxisterol, but upon chromatographing ergosterol in the absence of calciferol and any other products of irradiation and still obtaining a maximum of some substance at 250 millimicrons, this idea was eliminated because toxisterol presupposes the presence of calciferol[17]. Several investigations were carried out relative to whether or not the interfering material could be a product from the irradiation process, a reaction of the eluent with the adsorbent, a reaction of the eluent with the ergosterol, an impurity present in the ergosterol, or a result from some decomposition reaction of the ergosterol on the superfiltrol column itself. Kimball concluded that the substance with a maximum at 250 millimicrons could only be from some reaction between the ergosterol and the adsorbent.

Computations

Having isolated representative absorption spectra for two substances, which are apparently obtained from a decomposition of pure ergosterol which is caused by contact with superfiltrol, the additivity of the extinction curves, which was discussed in detail for two compounds when using an alumina adsorbent, should be able to be extended to four substances involving the use of a system of four simultaneous linear equations in four unknowns. If deviations from this theory are significant when the length of the superfiltrol column is varied, the total and relative concentrations of calciferol and ergosterol initially placed on the column are varied, or the relative composition of the eluent is altered, then either the absorption spectra for the products obtained from the decomposition of ergosterol are incorrect or ergosterol in combination with calciferol is not changed into the form observed when ergosterol was chromatographed alone.

The four unknowns, which represent each of the four constants in the system of four simultaneous linear equations, are some form of a concentration constant, which depends on the other units in use in the equations. Let w, x, y, and z be the unknown concentration constants for calciferol, substance 282,

substance 250, and ergosterol respectively in a solution of one centimeter thickness. Let a, b, c, and d be their respective extinction coefficients at a wave length λ and a', b', c', and d' their respective extinction coefficients at a second wave length λ'. Then let a", b", c", and d" be their respective extinction coefficients at a third wave length λ'' and a"', b"', c"', and d"' their respective extinction coefficients at a fourth wave length λ'''. If D, D', D", and D"' are the respective densities, log (I_o/I), of the mixture of the four substances at each of the four wave lengths, then

$$\begin{aligned}
&\text{at } \lambda && a\ w + b\ x + c\ y + d\ z = D \\
&\text{at } \lambda' && a'\ w + b'\ x + c'\ y + d'\ z = D' \\
&\text{at } \lambda'' && a''\ w + b''\ x + c''\ y + d''\ z = D'' \\
&\text{and at } \lambda''' && a'''\ w + b'''\ x + c'''\ y + d'''\ z = D'''
\end{aligned}$$

The critical wave lengths selected for use in the computation of w, x, y, and z were 250, 265, 281, and 293 millimicrons. Theoretically, extinction values observed at any four different wave lengths in the range of investigation could be used, but the maximum accuracy in the results will be obtained by using wave lengths where a critical maximum (or minimum) extinction of each component is most readily altered by a foreign

substance. Whenever possible, the wave lengths should be spaced at reasonable intervals and selected where the extinction values have been read from the most accurate settings of the spectrophotometer.

Solutions for the four simultaneous equations were found by using a method of addition and subtraction which is compiled by Dwyer[18]. For n equations in n unknowns, whose matrix of coefficients is nonsymmetric, this method of solution requires only $\frac{n(n+3)}{2}$ rows and has the additional advantages of having less chance for numerical error, less recording, and being easier to calculate, more accurate, less time consuming, and more compact than other methods of solution. When only three substances were present in the eluate, the system of equations was reduced to three and the fourth equation, involving the observed density for the mixture at 293 millimicrons, was omitted. When only two substances appeared in the eluate, the equation involving the density observed at the wave length suspected to be the least critical was eliminated.

For 0.000260 gram of calciferol and 0.000488 gram of ergosterol placed on a 4.6 centimeter column of superfiltrol, the seventh fraction of eluate was selected as an illustration of the agreement between the observed absorption spectrum and that calculated from the

assumption of the presence of pure calciferol, substance 282, and substance 250 (see Table IV). The concentration constants were found to be 1.0510 for calciferol, 0.1373 for substance 282, and 0.0046 for substance 250. The computed extinction curve has been broken down into the absorption due to calciferol and that due to substance 282 and substance 250. The graphic representation of this data and analysis is given in Figure 13. The graphs of the observed and computed extinction curves are very nearly coincident. The variations between these two curves are within the limits of experimental error and are not significantly different. The relative composition of this fraction at 281 millimicrons is about 89.5% calciferol, 10.4% substance 282, and 0.2% substance 250. The calciferol in this fraction is 28.3% of the total amount of calciferol initially placed on the column.

Assuming the presence of pure calciferol, substance 282, substance 250, and pure ergosterol in the fourth fraction of the eluate from a 1.1^{+} centimeter superfiltrol column, an example of the marked similarity between the observed and computed absorption spectra is given in Table V. The quantities of calciferol and ergosterol originally placed on the column were the same as before. The concentration constants were found to be

0.9429 for calciferol, 0.2323 for substance 282, 0.1346 for substance 250, and 0.6449 for ergosterol. The computed extinction values have been given separately for the absorption due to calciferol, that due to ergosterol, and that due to substance 282 and substance 250. In fact, the absorption spectrum due to one or any combination of these four substances can very easily be found. Figure 14 shows the graphs of the observed and computed extinction curves are again very nearly coincident. As before, the deviations of the computed values from the observed values are not sufficiently large to make the two curves significantly different. It is interesting to note the difference between the extinction curve due to the presence of only pure calciferol and ergosterol, Curve D, and the observed extinction curve for the four components, Curve F. There isn't any possible combination of calciferol and ergosterol alone that could give a resultant absorption spectrum with a maximum at 262 millimicrons. Thus, this eluate fraction must be contaminated, and the introduction of the absorption spectrum due to substance 282 and substance 250 seems to account for this maximum, and other variations, very well. The relative composition of this fraction at 281 millimicrons is about 46.5% calciferol, 10.2% substance 282, 3.2% substance 250, and

40.0% ergosterol. The amounts of calciferol and ergosterol, based upon the initial quantities placed on the column, in this fraction are 25.3% and 12.9% respectively.

All of the observed and computed absorption curves are in at least as close agreement as the two preceding examples for the entire investigation and for all fractions.

Since all of the conditions for each group of investigations are kept constant except the one for which a variation is being studied, an error inherent to these conditions is thought to result from the fact that a few (from one to three) of the initial and final fractions may be carrying very small quantities of calciferol and/or ergosterol and are discarded because of very low extinction readings at the critical points. For each fraction concerned, this error has been found to be less than 0.5% of the initial quantity of either compound placed on the column.

Variation of Column Prewash

The contaminents in the commercial preparation of superfiltrol are removed by prewashing the packed column with the most polar solvent, developer, or eluent to be used during the investigation. The quantity of ether - hexane, in the ratio of 1:5 parts by volume, prewash

solution required to reduce these impurities to a minimum and that necessary to indicate partial deactivation of the adsorbent are quite different. Having found 4 milliliters of this ether - hexane solvent to be the minimum volume of prewash solution that would satisfactorily free a 4.6 centimeter superfiltrol column from impurities, prewash solutions of 16 milliliters and 30 milliliters of this same ether - hexane concentration were investigated. In each case, the initial amount of calciferol placed on the adsorbent was 0.000260 gram and that of ergosterol was 0.000488 gram. As is evident from Figure 15, there is essentially no difference between the results obtained from prewashing the adsorbent with 4 milliliters of solvent and those obtained from having prewashed the column with 16 milliliters of solvent. However, when the column was prewashed with 30 milliliters of solvent, the amount of calciferol recovered was very slightly increased while the amount of ergosterol eluted from the column was quite decidedly increased to 3.5%. The presence of this quantity of ergosterol in the eluate may be incidental but is more likely due to a slight deactivation of the superfiltrol which was caused by the large volume of ether - hexane which came in contact with it.

There isn't any difference in the position of the

initial appearance of calciferol, substance 282, and substance 250 in the eluate for the three different prewash solutions. However, the fractions in which these components terminate vary within one or two milliliters (see Figure 16). Ergosterol is found in more fractions and terminates later from the column which was prewashed with 30 milliliters of ether - hexane.

As would be expected, the relative composition of the total extinction for all fractions for each investigation differs only slightly with respect to each component at 281 millimicrons (see Figure 17). Ergosterol has the most variable positions due to its appearance, disappearance, and reappearance from the columns which were prewashed with 4, 16, and 30 milliliters of solvent respectively.

Variation of Eluent Polarity

For the studies relative to the variation of the composition of the eluent, 0.000260 gram of calciferol and 0.000488 gram of ergosterol were introduced onto a 4.6 centimeter superfiltrol column. Three different ether - hexane combinations were considered. These were in the ratios of 1:1, 1:3, and 1:5 parts of ether to hexane by volume. As the ratio of ether to hexane is decreased, the total amount of calciferol recovered from

the column varies between 99.6% and 98.7%. Simultaneously, the recovery of ergosterol from the column decreases from 80.1% to about 1.3%. Figure 18 shows the consistently high recovery of calciferol, which seems to be independent of the ether content of the eluent, and the rapid decline of the ergosterol recovery as the polarity of the eluent is decreased. However, when 100% hexane was used as the eluent, neither compound was desorbed or recovered in the collection of over 60 fractions.

Although the recovery of each compound is important, the separation of the two compounds is also very important. The initial and terminal fractions in which each of the four components are found are shown in Figure 19 for each eluent variation. When the eluent is composed of ether and hexane in equal parts by volume, the elution of all four substances begins and ends quite quickly, but there are no fractions in which any one substance is found alone. As the polarity of the eluent is decreased, the volume in which the elution of all components takes place is increased. When the ether - hexane eluent ratio is 1:3, the first three fractions are free of substance 282. Very little ergosterol is eluted from the adsorbent when ether - hexane in the ratio of 1:5 is used as the eluent, as was mentioned

previously. This small percentage of ergosterol is found only in the last two fractions; and hence, all preceding fractions contain calciferol free from ergosterol. Unfortunately, the decomposition products of ergosterol are found in each fraction.

When the eluent is ether - hexane, 1:1, relatively little of the extinction value for the total eluate for each investigation is devoted to substance 282 and substance 250 at 281 millimicrons. However, as the polarity of the eluent is decreased, substance 250 and especially substance 282 obtain more and more prominence in the composition of the eluate. At the same time, the position of ergosterol decreases to almost zero, as would be expected, since its desorption is tending to zero, and the gain in position of calciferol is probably at the expense of ergosterol (Figure 20). It is quite evident that the relative ether - hexane content of the eluent has a direct influence on the conversion of ergosterol into substance 282 and substance 250. This conversion must also result in part from the volume of eluent necessary to elute the compounds from the column and/or the length of time and subsequent adsorbent-adsorbate-eluent contact.

Variation of Column Length

By changing the amount of superfiltrol placed in a

chromatographic tube of constant diameter, the height to which the adsorbent will rise is variable. In fact, for all practical purposes, the variation in height is a linear function of the weight. Since previous work has shown an almost complete separation of calciferol from ergosterol using a 4.6 centimeter column, columns of both shorter and longer lengths were studied. The eluent chosen for this work was ether - hexane in the ratio of 1:5 parts by volume, and the amounts of calciferol and ergosterol initially placed onto each column of super-filtrol were 0.000260 gram and 0.000488 gram respectively. The patterns resulting from this work are very clearly shown in Figures 21-30. Each figure is composed so as to present the absorption spectra of several residues.. Each residue is from a fraction of the same number and from a column of different length. The length of these columns vary from 1.1^{+} centimeters (1/2 gram) to 7.0 centimeters (3 grams) in intervals of about 1.2 centi-meters (1/2 gram). In all cases, the extinction values are those for which each fraction residue was volumet-rically taken up in 4 milliliters of hexane. The first fraction for each run is not given because none of the substances were eluted in any investigation. For the columns of shorter lengths, 1.1^{+} centimeters and 2.3 centimeters, the calciferol is eluted quite quickly

in the first few fractions. In fact, most of the calciferol, which is free from ergosterol, is eluted before the elution of the calciferol from the longer columns is even started (see Figures 21-23). As the elution of the shorter columns tends to completion, the distortion of the calciferol curve to that of ergosterol and substance 250 is very evident (Figures 24-27). Substance 250 seems to gain in prominence and then taper back to an absorption spectrum similar to that of pure ergosterol, as can be seen in Figures 25-29. For the longer columns, 5.8 centimeters and 7.0 centimeters, there is little visual evidence of any very prominent quantity of calciferol in the eluate before the sixth fraction, Figure 25. Prior to this fraction the majority of the eluate appears to be composed of substance 282. Calciferol is eluted very heavily from these longer columns in fractions 7-9. Lesser amounts of calciferol and the beginning of a slight distortion of the calciferol curve are evident in Figure 29. The absorption spectra for the remaining fractions eluted from the 7.0 centimeter column are shown in Figure 30. A larger extinction scale is used for this graph in order to show the distortion more clearly. Although the contribution, in terms of concentration, of substance 250 to the resultant absorption spectra may be small, its

presence is very evident. As the elution of substance 250 tends to completion, the spectral patterns, which may be partially due to some imputities still being released from the adsorbent, have a tendency to return to nearly linear spectra. The eluate fractions from the columns of intermediate lengths, 3.4+ centimeters and 4.6 centimeters, follow patterns between those for the shorter and longer column lengths.

A compilation of the total recovery of calciferol and ergosterol from each column of different length is shown in Figure 31. For each column, the calciferol recovery, seemingly independent of the column length, is consistently above 98%. On the other hand, the ergosterol recovery declines steadily from 28.7% from the 1.1+ centimeter column to 0.0% from the 5.8 centimeter column. Ergosterol is absent from the eluate from the 7.0 centimeter column also.

Regardless of the fact that several of the curves in the figures for the survey of the various column lengths appear to contain only pure calciferol, further mathematical investigation shows almost all of these fractions to be contaminated with varying portions of substance 282 and substance 250. Figure 32 presents in a compact form the initial and terminal fractions in which each of the components appears for each run. As

the length of the column is increased, the number of fractions required to free the superfiltrol of its contents is increased. This increase is not nearly as great, however, as it was when alumina was used as the adsorbent. For an increase in the length of the superfiltrol column, each component is delayed in its initial appearance and terminates two or three fractions later than for the immediately preceding shorter column. Substance 282 and substance 250 accompany or precede the elution of calciferol. As the column is lengthened, these two interfering substances precede the calciferol by an ever increasing number of fractions. There are no fractions which are free from either calciferol or ergosterol that are also free from both substance 282 and substance 250.

Substance 282 and substance 250 cannot be analyzed on a total percent recovery basis such as that used previously for calciferol and ergosterol (see Figure 31). This would require a knowledge of the amount of ergosterol retained on the column, in the converted and unconverted forms, as well as some additional information about the two products of conversion. However, the relative composition of each complete run, or any fraction thereof, for any defined wave length, say 281 millimicrons, can be computed (Figure 33). Since the amount of ergosterol

eluted from the column as the length is increased goes to zero, the relative position of ergosterol in the composition of the eluate will go to zero. Hence, with an increase in column length, calciferol, substance 282, and substance 250 should be present in the relative composition of the eluate to a greater degree. The extinction values for the eluate for each run decrease slightly and the concentration constants for substance 282 and substance 250 increase slightly as the length of the column is increased. Hence, the conversion of ergosterol into these two components appears to be a function of the column length. However, for columns of length greater than 4.6 centimeters, from which ergosterol is no longer eluted, the extinction values and concentration constants tend to become constant. Therefore, the conversion of ergosterol must be a function of the length of the column and the amount of pure ergosterol available for desorption and/or elution from the column.

Variation of Sample Concentration

To study the effect of the change of concentration upon the separation of calciferol from ergosterol, two different length columns of superfiltrol containing various quantities of each compound were investigated. The column of 2.3 centimeters in length was selected for

further study because there had definitely been proven (by a previous investigation) that some unconverted ergosterol had been eluted from it. The effect of halving and doubling the initial quantity of ergosterol (0.000488 gram) and keeping that of calciferol (0.000260 gram) constant was studied (see Figure 34). The total calciferol recovery remained above 99% until the run in which the amount of ergosterol was doubled. Here the calciferol recovery dropped to about 93%. This sudden decline in recovery can probably be attributed to some molecular entanglement between the calciferol and ergosterol at the surface of the superfiltrol particles since the ergosterol was initially present in a relatively large amount. The total recovery (in percent) of ergosterol is somewhat more variable. However, the actual gain in amount of pure ergosterol eluted from the column increases steadily from 0.000055 gram to 0.000075 gram to 0.000189 gram for quantities initially placed on the column of 0.000244 gram, 0.000488 gram, and 0.000976 gram respectively.

As the ergosterol concentration is increased, the four constituents appear in the eluate a little earlier and terminate slightly later (Figure 35). Thus, the volume of eluent necessary for their elution is greater.

The relative composition (Figure 36) of the eluate

for each run at 281 millimicrons is in agreement with the fact that the quantity of eluted ergosterol increases as the ratio of ergosterol to calciferol is increased. Simultaneously, the contribution of substance 282 and substance 250 to the total extinction value is greater. This is to be expected since these two substances are a result of a conversion of some of the ergosterol. The contribution of calciferol to the total absorption spectrum will naturally decline.

As a comparison for the above work, a similar survey was made on a column of 4.6 centimeters in length. This length of column was selected because there was very little ergosterol eluted from it as determined from a previous study in which the initial amounts of calciferol and ergosterol were 0.000260 gram and 0.000488 gram respectively. For this study the quantity of ergosterol was halved, doubled, and quadrupled while that of calciferol was held constant. As the original quantity of ergosterol on the column is increased from 0.000244 gram to 0.001952 gram, the total calciferol recovery decreases from 100% to 93% (see Figure 37). This decrease is almost linear, depending on the method of graphing. In comparison, the ergosterol recovery increases from 0% to 13%. This is somewhat more significant than the percentages seem to indicate since the

amount of ergosterol recovered increases from 0.000000 gram to 0.000006 gram to 0.000020 gram to 0.000263 gram for initial quantities of ergosterol of 0.000244 gram, 0.000488 gram, 0.000976 gram, and 0.001956 gram respectively.

The position of the initial appearance of the four components in the eluate is very comparable to that for the 2.3 centimeter column. However, the termination of the elution of each component is somewhat more variable (see Figure 38). As compared to the 2.3 centimeter column, the volume of eluent required to free the column of the constituents will be greater because of the increase in column length. Perhaps the earlier termination of the constituents from the column containing 0.000488 gram of ergosterol as compared to the column containing 0.000244 gram of ergosterol can partially be attributed to the earlier elution of substance 282 and substance 250. The earlier appearance of ergosterol from the column containing 0.000976 gram of ergosterol is quite in line with the parallel situation for the 2.3 centimeter column. The return of ergosterol to a later initial appearance from the column which contained 0.001952 gram of ergosterol is quite likely due to the large quantity of ergosterol which has forced the conversion products to appear still earlier and retained

calciferol for a later fraction. The increased banding is undoubtedly due to the increase in ergosterol concentration.

The relative composition of the totality of each run at 281 millimicrons is shown in Figure 39. The pattern for each component is almost identical to that for the comparable conditions on the shorter column. Hence, as the ratio of the amount of ergosterol to calciferol is increased, ergosterol, substance 282, and substance 250 increase while calciferol decreases in prominence in the relative composition of the eluate.

SUMMARY

The separation of calciferol from ergosterol using an ether or ether - hexane solvent and an alumina or superfiltrol adsorbent has been presented. Ether was found to be the best solvent and eluent for the accomplishment of the separation by means of an alumina adsorbent. When superfiltrol was used as the adsorbent, ether - hexane in the ratio of 1:5 parts by volume was found to be a very satisfactory solvent for the separation.

In obtaining a complete separation of the two compounds, the weight of the adsorbent and the height to which it was packed in the chromatographic tube were very important. Alumina gave a very good separation of the two compounds, the resultant forn of each being pure and free from any other material. However, the lengthening of the column and the resulting broader banding of each compound within the column were found to be undesirable. Superfiltrol gave a very sharp separation much more quickly and on a very much shorter column. However, a conversion of the ergosterol into two irrelevant substances took place on the adsorbent and, while calciferol and ergosterol were obtained free from each other, neither compound was found in the eluate

completely free from one or both of the irrelevant materials. This observation lead to a pattern analysis of the conversion products and as a result, the eluate fractions could be analyzed by a system of simultaneous linear equations. Also, the concentration of calciferol or ergosterol in the total eluate, or any fraction thereof, could be determined exactly.

The relative and total concentrations of calciferol and ergosterol initially placed on either adsorbent had very little effect on the total calciferol recovery. However, both the separation of the two compounds and the total ergosterol recovery were influenced considerably by this property.

TABLE I

Fig.	Alumina	Eluent		Recovery			
		Ether	Hexane	Cal. free from Erg.	Erg. free from Cal.	Total Cal.	Total Erg.
	(cm.)	(parts by vol.)		(%)	(%)	(%)	(%)
3	7.8	50	50	34.0	34.2	90.3	81.5
		70	30	34.5	37.6	93.4	85.2
		80	20	33.8	38.8	94.7	88.0
		100	0	32.4	33.1	96.2	90.1
4	15.6	70	30	48.4	45.7	90.1	82.9
		80	20	47.1	44.7	92.8	85.2
		100	0	51.6	49.0	94.3	87.2

Initial calciferol concentration: 0.000260 g./ml.
Initial ergosterol concentration: 0.000488 g./ml.

TABLE II

Fig.	Alumina	Eluent		Eluate				Fractions Containing		
		Ether	Hexane	Initial Fraction Containing Calciferol	Initial Fraction Containing Ergosterol	Final Fraction Containing Calciferol	Final Fraction Containing Ergosterol	Cal. Only	Erg. Only	Cal. and Erg.
	(cm.)	(parts by vol.)		(number)				(number)		
5	7.8	50	50	37	46	60	72	9	12	15
		70	30	24	30	40	50	6	10	11
		80	20	19	25	32	41	6	9	8
		100	0	15	20	26	35	5	9	7
6	15.6	70	30	40	51	61	78	11	17	11
		80	20	34	43	51	65	9	14	9
		100	0	29	37	42	56	8	14	6

Initial calciferol concentration: 0.000260 g./ml.
Initial ergosterol concentration: 0.000488 g./ml.

TABLE III

Fig.	Alumina		Eluate				Fractions Containing		
			Initial Fraction Containing Calciferol	Initial Fraction Containing Ergosterol	Final Fraction Containing Calciferol	Final Fraction Containing Ergosterol	Cal. Only	Erg. Only	Cal. and Erg.
	(g.)	(cm.)	(number)				(number)		
7	2	3.9	9	12	19	24	3	5	8
	4	7.8	15	20	26	35	5	9	7
	8	15.6	29	37	42	56	8	14	6
	16	31.1	49	63	65	86	14	21	3
	22	42.7	64	84	84	110	20	26	1
	23	44.6	66	87	86	115	21	29	0

Eluent: Diethyl ether
Initial calciferol concentration: 0.000260 g./ml.
Initial ergosterol concentration: 0.000488 g./ml.

TABLE IV

Superfiltrol: 4.6 centimeters
Initial calciferol concentration: 0.000260 g./ml.
Initial ergosterol concentration: 0.000488 g./ml.
Fraction: #7

Wave Length (m mu)	Correction	Extinction Calciferol	Computed	Observed
230	0.038	0.462	0.500	0.520
240	0.038	0.592	0.630	0.632
250	0.040	0.736	0.776	0.776
260	0.043	0.845	0.888	0.893
262	0.045	0.858	0.903	0.906
264	0.046	0.862	0.908	0.909
265	0.047	0.861	0.908	0.908
266	0.048	0.854	0.902	0.904
270	0.054	0.812	0.866	0.866
271	0.055	0.795	0.850	0.852
272	0.055	0.777	0.832	0.835
276	0.057	0.688	0.745	0.741
280	0.063	0.577	0.640	0.636
281	0.065	0.545	0.610	0.610
282	0.065	0.516	0.581	0.580
289	0.053	0.324	0.377	0.380
293	0.049	0.230	0.280	0.282
300	0.033	0.111	0.145	0.150

$w = 1.0510$ $y = 0.0046$

$x = 0.1373$

TABLE V

Superfiltrol: 1.1^{+} centimeters
Initial calciferol concentration: 0.000260 g./ml.
Initial ergosterol concentration: 0.000488 g./ml.
Fraction: #4

Wave Length (m mu)	Extinction Correction	Calciferol	Ergosterol	Computed	Observed
230	0.217	0.414	0.076	0.707	0.730
240	0.263	0.531	0.081	0.876	0.872
250	0.315	0.661	0.144	1.120	1.120
260	0.262	0.758	0.272	1.292	1.294
262	0.250	0.770	0.283	1.303	1.300
264	0.229	0.773	0.286	1.288	1.288
265	0.214	0.772	0.296	1.282	1.282
266	0.200	0.766	0.310	1.276	1.278
270	0.153	0.729	0.397	1.279	1.278
271	0.146	0.714	0.398	1.258	1.260
272	0.139	0.697	0.389	1.225	1.224
276	0.130	0.618	0.339	1.086	1.086
280	0.140	0.518	0.410	1.068	1.068
281	0.142	0.489	0.421	1.052	1.052
282	0.142	0.463	0.416	1.021	1.018
289	0.113	0.290	0.221	0.623	0.620
293	0.106	0.206	0.244	0.556	0.556
300	0.072	0.100	0.050	0.221	0.230

$w = 0.9429$ $y = 0.1346$

$x = 0.2323$ $z = 0.6449$

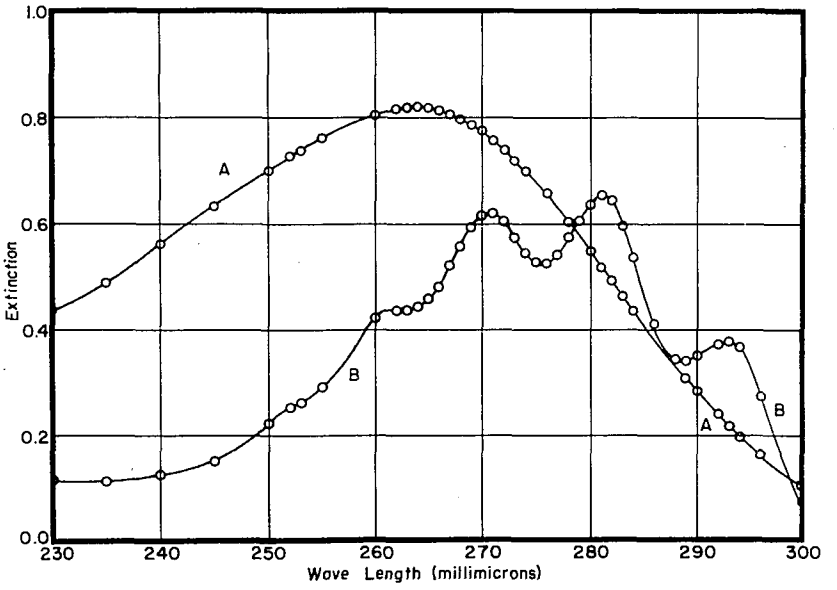


Figure 2. Absorption spectra of calciferol and ergosterol in hexane.
A—Calciferol (0.0000175 g./ml.)
B—Ergosterol (0 0000244 g./ml.)

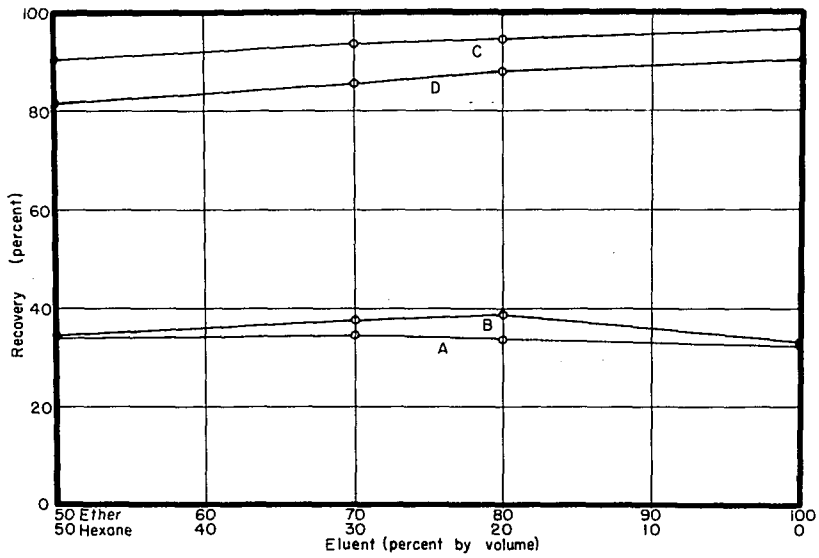


Figure 3. Recovery of calciferol and ergosterol from a 7.8 centimeter alumina column.
A—Pure calciferol
B—Pure ergosterol
C—Total calciferol
D—Total ergosterol

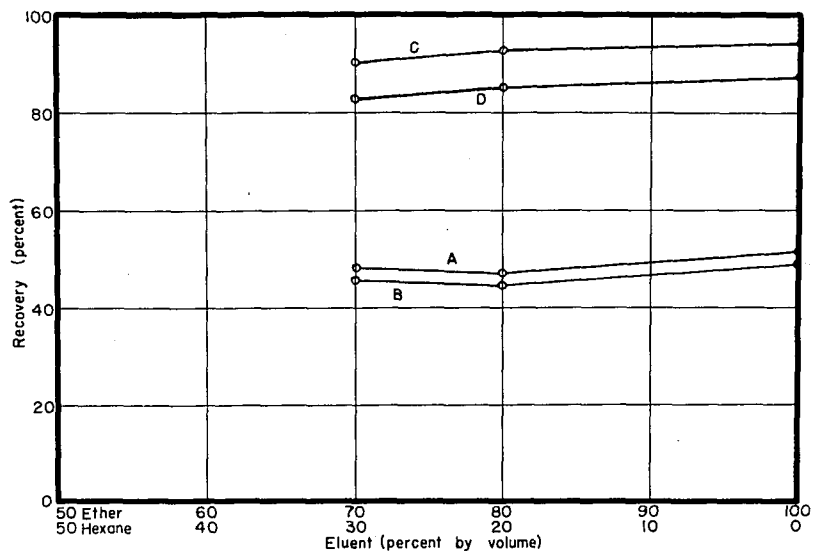


Figure 4. Recovery of calciferol and ergosterol from a 15.6 centimeter alumina column.
A—Pure calciferol
B—Pure ergosterol
C—Total calciferol
D—Total ergosterol

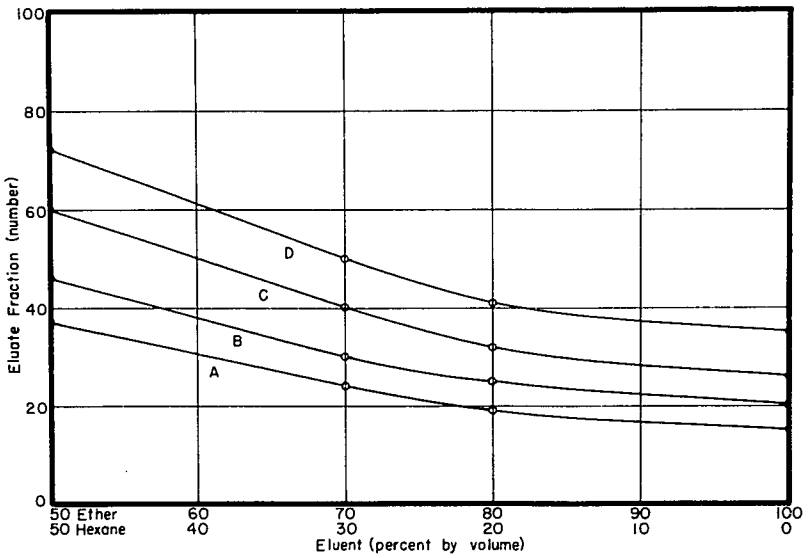


Figure 5. Elution of calciferol and ergosterol from a 7.8 centimeter alumina column.
A – Initial fraction containing calciferol
B – Initial fraction containing ergosterol
C – Final fraction containing calciferol
D – Final fraction containing ergosterol

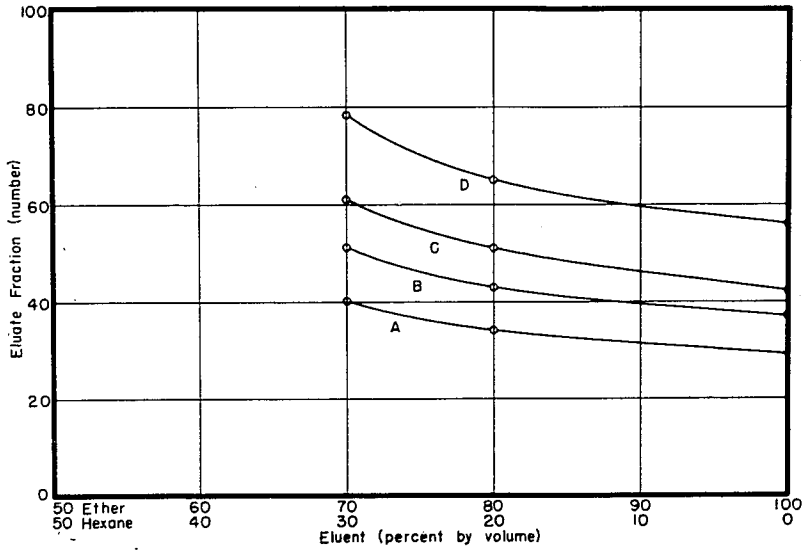


Figure 6. Elution of calciferol and ergosterol from a 15.6 centimeter alumina column.
A – Initial fraction containing calciferol
B – Initial fraction containing ergosterol
C – Final fraction containing calciferol
D – Final fraction containing ergosterol

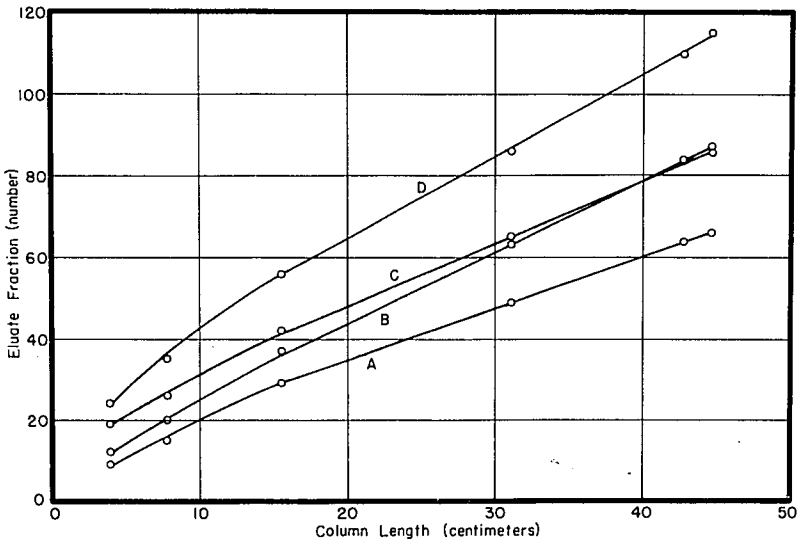


Figure 7. Elution of calciferol and ergosterol using a pure ether eluent.
A—Initial fraction containing calciferol
B—Initial fraction containing ergosterol
C—Final fraction containing calciferol
D—Final fraction containing ergosterol

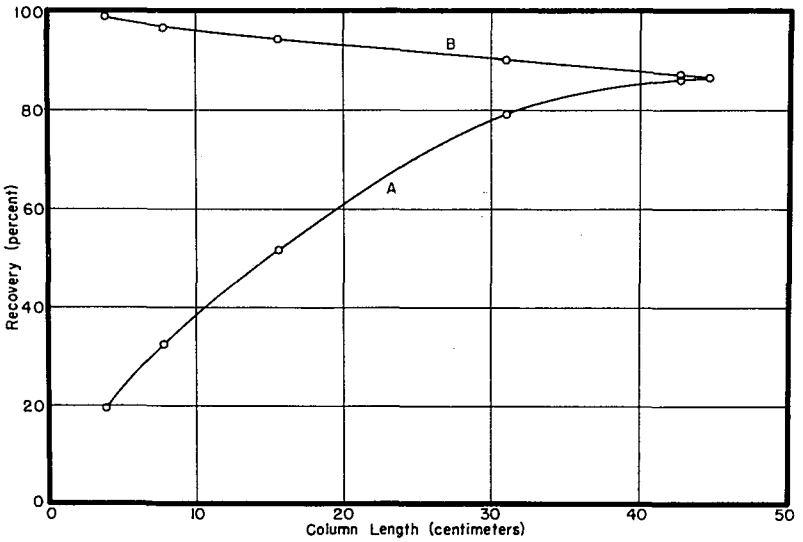


Figure 8. Recovery of calciferol using a pure ether eluent.
A—Pure calciferol
B—Total calciferol

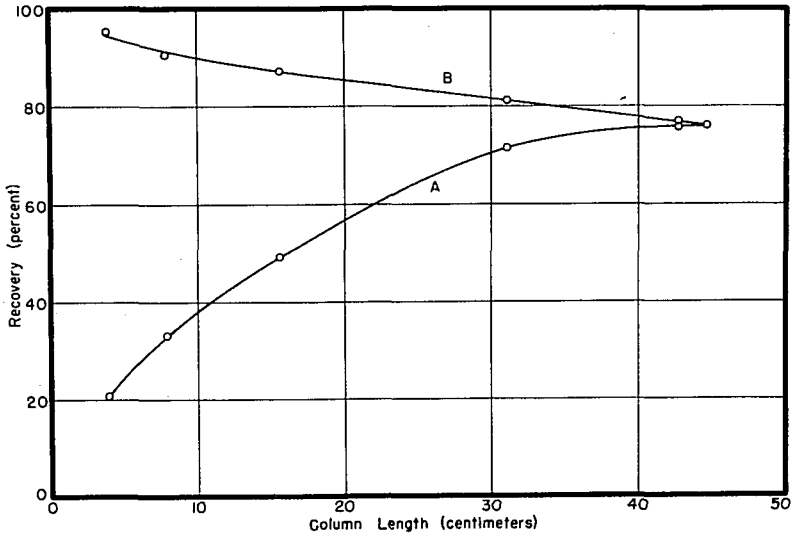


Figure 9. Recovery of ergosterol using a pure ether eluent.
A—Pure ergosterol
B—Total ergosterol

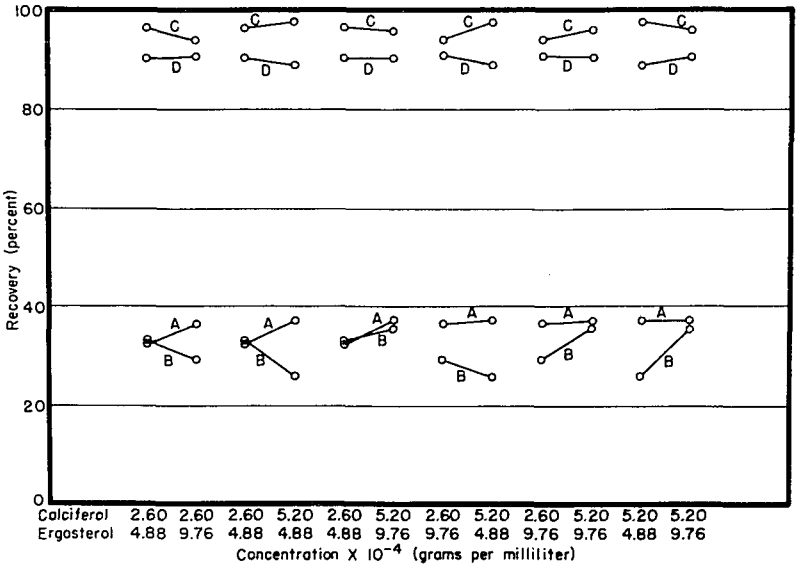


Figure 10. Recovery of calciferol and ergosterol from a 7.8 centimeter alumina column.
A – Pure calciferol
B – Pure ergosterol
C – Total calciferol
D – Total ergosterol

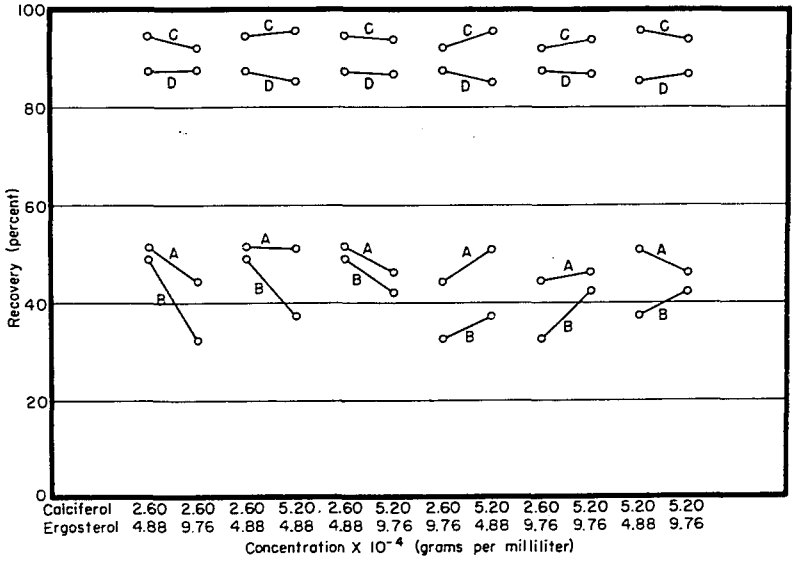


Figure 11. Recovery of calciferol and ergosterol from a 15.6 centimeter alumina column.
A – Pure calciferol
B – Pure ergosterol
C – Total calciferol
D – Total ergosterol

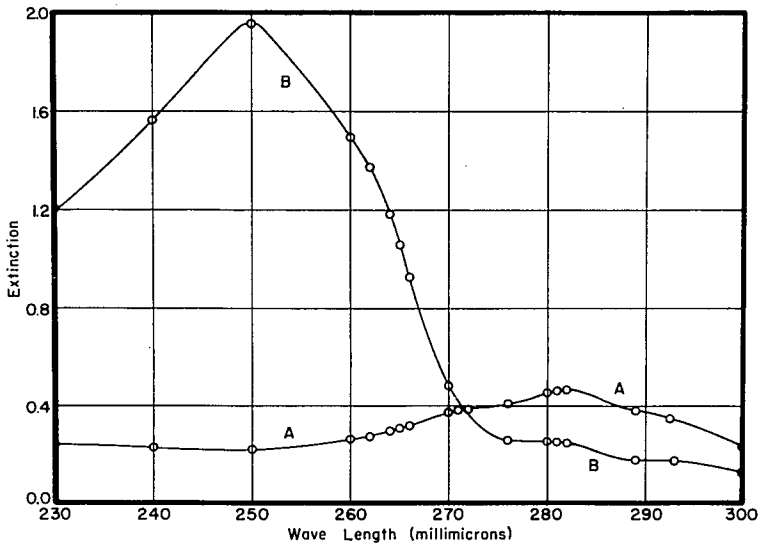


Figure 12. Absorption spectra of substance 282 and substance 250 in hexane.
A – Substance 282
B – Substance 250

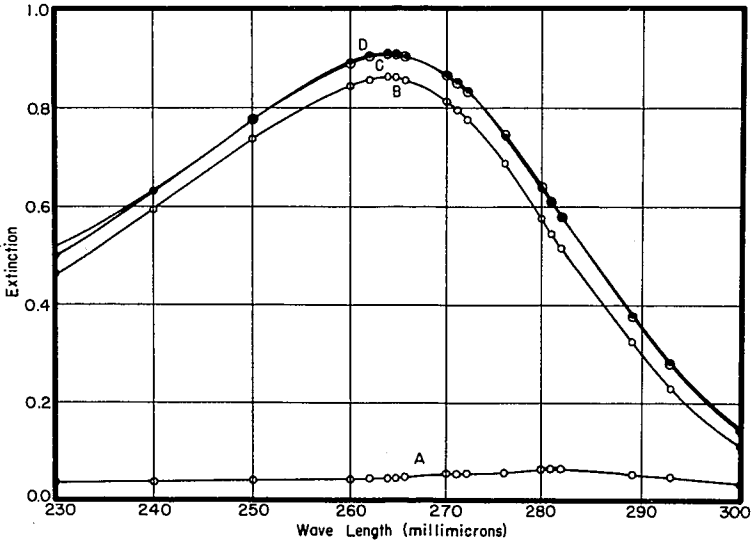


Figure 13. Seventh fraction of eluate from a 4.6 centimeter superfiltrol column.
A—Computed absorption spectra due to substance 282 and substance 250
B—Computed absorption spectra due to pure calciferol
C—Resultant computed absorption spectra — curve A + curve B (O)
D—Observed absorption spectra in hexane (•)

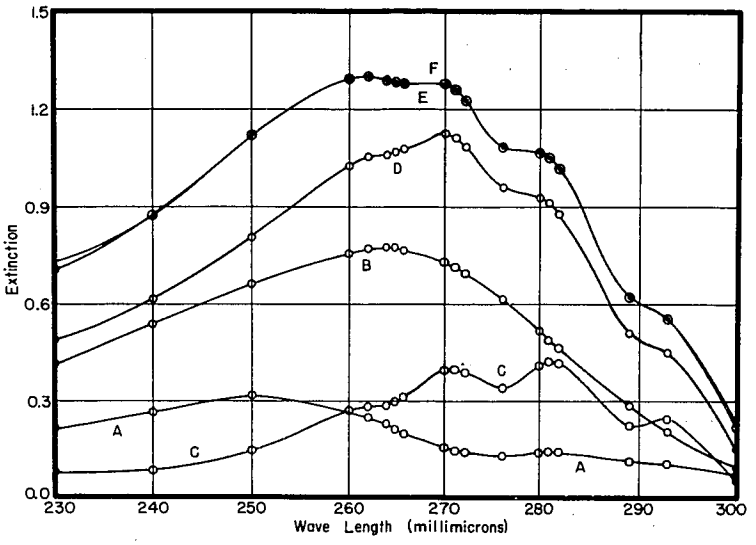


Figure 14. Fourth fraction of eluate from a 1.1+ centimeter superfiltrol column.
A—Computed absorption spectra due to substance 282 and substance 250
B—Computed absorption spectra due to pure calciferol
C—Computed absorption spectra due to pure ergosterol
D—Computed absorption spectra due to pure calciferol and pure ergosterol — curve B + curve C
E—Resultant computed absorption spectra — curve A + curve B + curve C (O)
F—Observed absorption spectra in hexane (•)

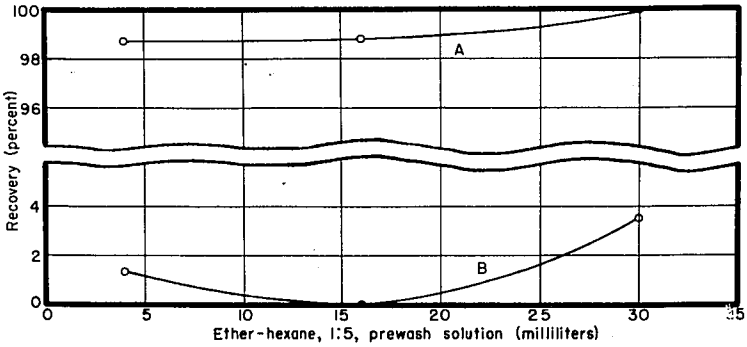


Figure 15. Recovery of calciferol and ergosterol from a 4.6 centimeter superfiltrol column.
A–Total calciferol
B–Total ergosterol

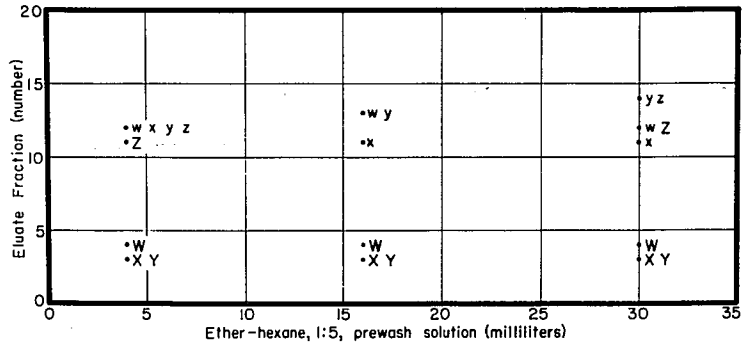


Figure 16. Elution of calciferol, substance 282, substance 250, and ergosterol from a 4.6 centimeter superfiltrol column.
W–Initial fraction containing calciferol
X–Initial fraction containing substance 282
Y–Initial fraction containing substance 250
Z–Initial fraction containing ergosterol
w–Final fraction containing calciferol
x–Final fraction containing substance 282
y–Final fraction containing substance 250
z–Final fraction containing ergosterol

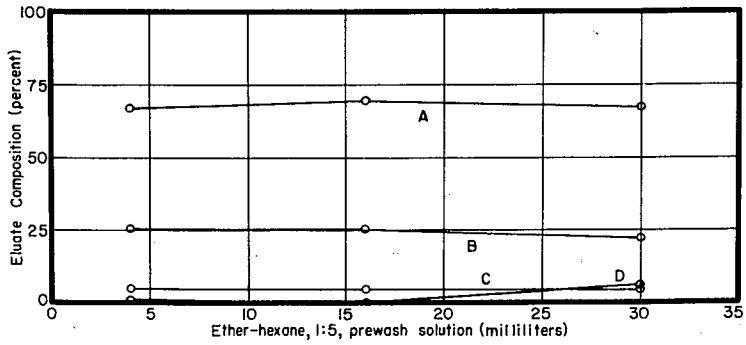


Figure 17. Relative composition of total eluate from a 4.6 centimeter superfiltrol column at 281 millimicrons.
A–Calciferol
B–Substance 282
C–Substance 250
D–Ergosterol

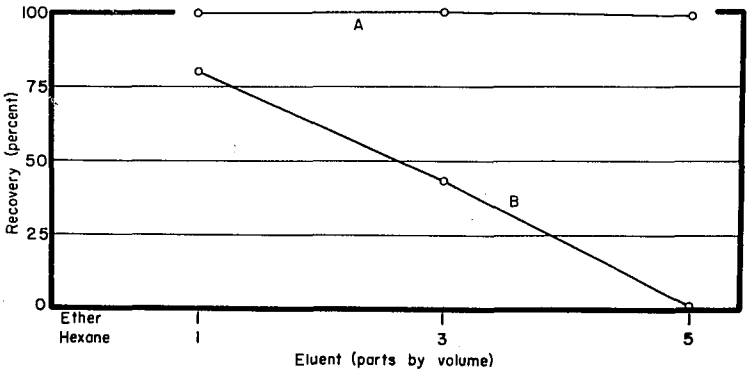


Figure 18. Recovery of calciferol and ergosterol from a 4.6 centimeter superfiltrol column.
A—Total calciferol
B—Total ergosterol

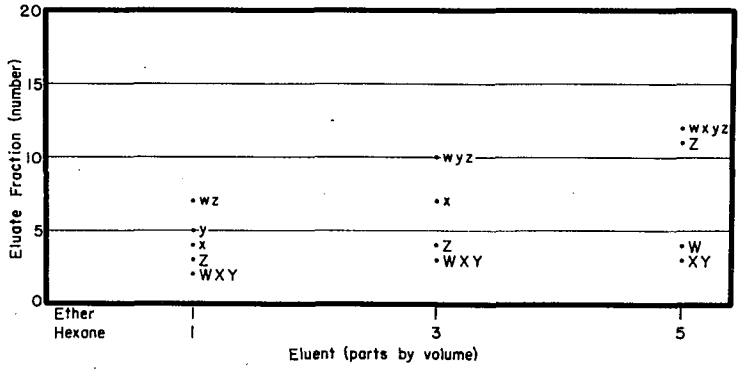


Figure 19. Elution of calciferol, substance 282, substance 250, and ergosterol from a 4.6 centimeter superfiltrol column.
W—Initial fraction containing calciferol
X—Initial fraction containing substance 282
Y—Initial fraction containing substance 250
Z—Initial fraction containing ergosterol
w—Final fraction containing calciferol
x—Final fraction containing substance 282
y—Final fraction containing substance 250
z—Final fraction containing ergosterol

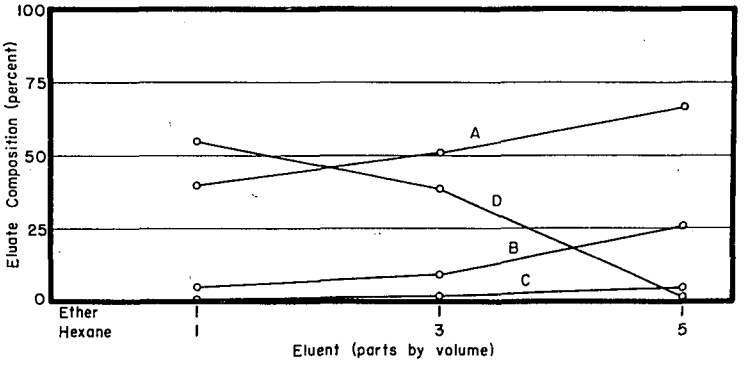


Figure 20. Relative composition of total eluate from a 4.6 centimeter superfiltrol column at 281 millimicrons.
A—Calciferol
B—Substance 282
C—Substance 250
D—Ergosterol

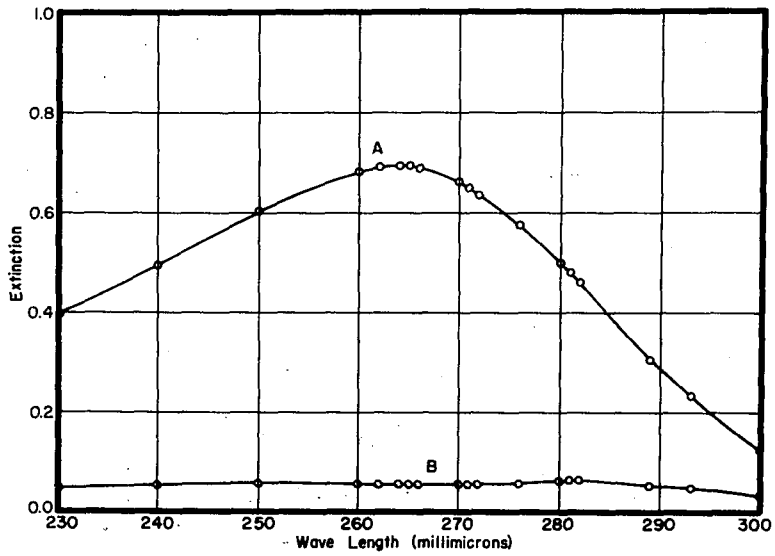


Figure 21. Absorption spectra of second fraction of eluate in hexane.
A–1.1+ centimeter superfiltrol column
B–2.3 centimeter superfiltrol column

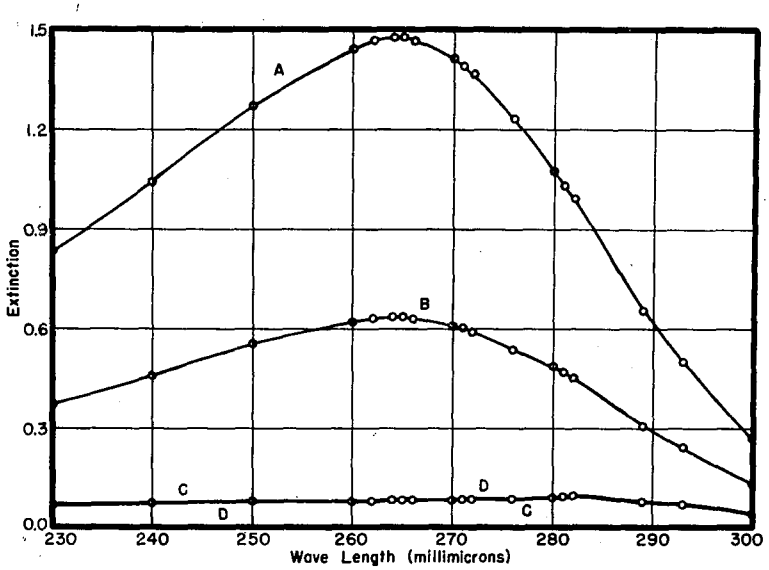


Figure 22. Absorption spectra of third fraction of eluate in hexane.
A–1.1+ centimeter superfiltrol column
B–2.3 centimeter superfiltrol column
C–3.4+ centimeter superfiltrol column
D–4.6 centimeter superfiltrol column

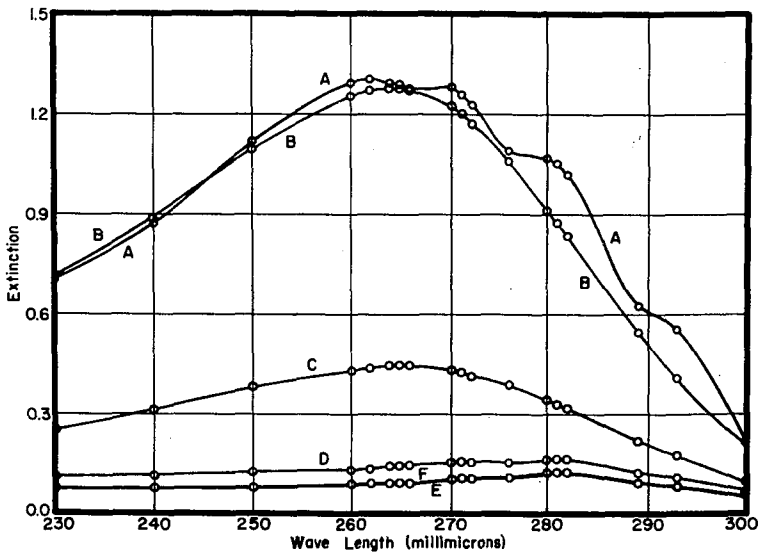


Figure 23. Absorption spectra of fourth fraction of eluate in hexane.
A – 1.1+ centimeter superfiltrol column
B – 2.3 centimeter superfiltrol column
C – 3.4+ centimeter superfiltrol column
D – 4.6 centimeter superfiltrol column
E – 5.8 centimeter superfiltrol column
F – 7.0 centimeter superfiltrol column

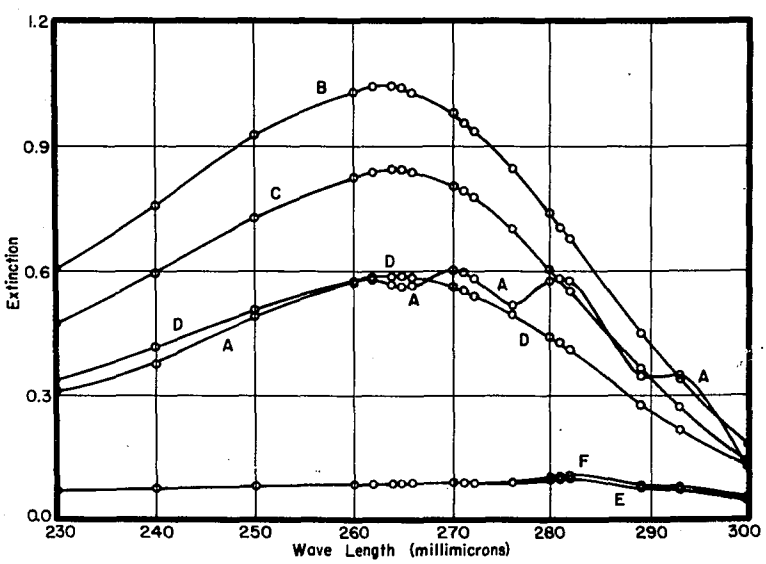


Figure 24. Absorption spectra of fifth fraction of eluate in hexane.
A – 1.1+ centimeter superfiltrol column
B – 2.3 centimeter superfiltrol column
C – 3.4+ centimeter superfiltrol column
D – 4.6 centimeter superfiltrol column
E – 5.8 centimeter superfiltrol column
F – 7.0 centimeter superfiltrol column

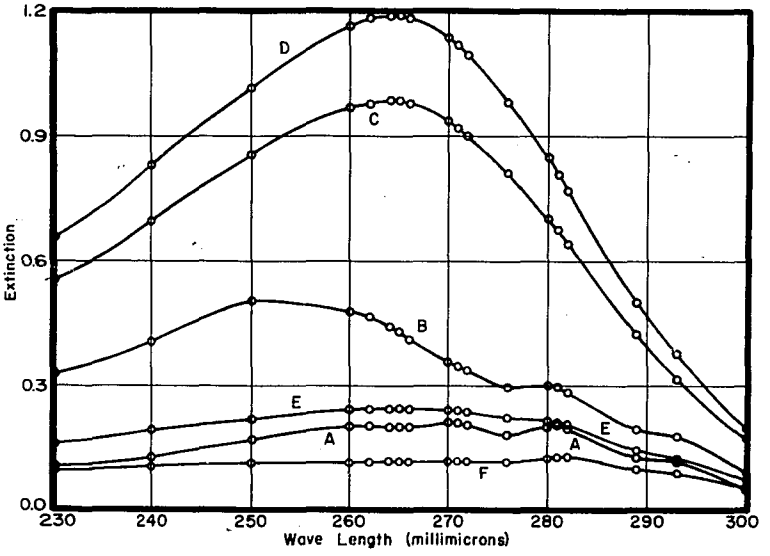


Figure 25. Absorption spectra of sixth fraction of eluate in hexane.
A–1.1+ centimeter superfiltrol column
B–2.3 centimeter superfiltrol column
C–3.4+ centimeter superfiltrol column
D–4.6 centimeter superfiltrol column
E–5.8 centimeter superfiltrol column
F–7.0 centimeter superfiltrol column

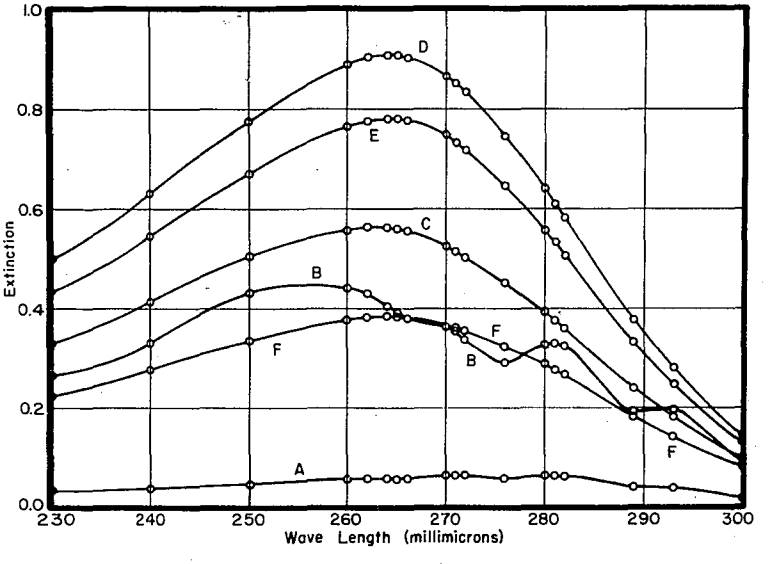


Figure 26. Absorption spectra of seventh fraction of eluate in hexane.
A–1.1+ centimeter superfiltrol column
B–2.3 centimeter superfiltrol column
C–3.4+ centimeter superfiltrol column
D–4.6 centimeter superfiltrol column
E–5.8 centimeter superfiltrol column
F–7.0 centimeter superfiltrol column

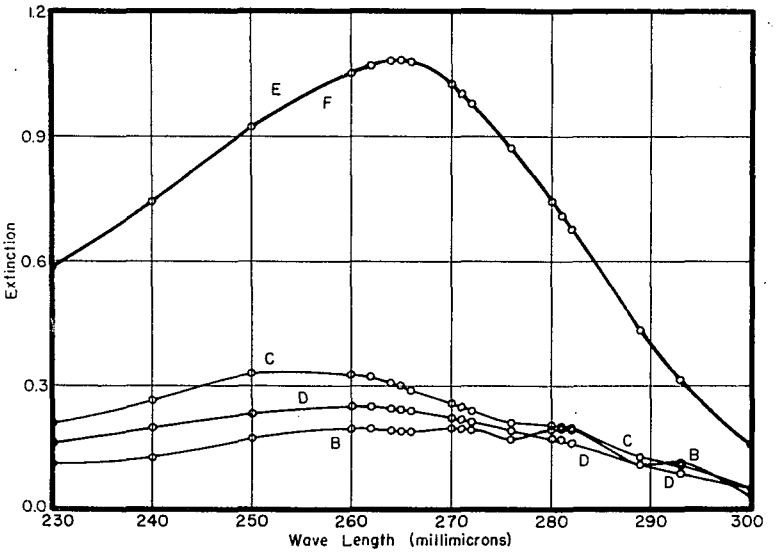


Figure 27. Absorption spectra of eighth fraction of eluate in hexane.
B – 2.3 centimeter superfiltrol column
C – 3.4+ centimeter superfiltrol column
D – 4.6 centimeter superfiltrol column
E – 5.8 centimeter superfiltrol column
F – 7.0 centimeter superfiltrol column

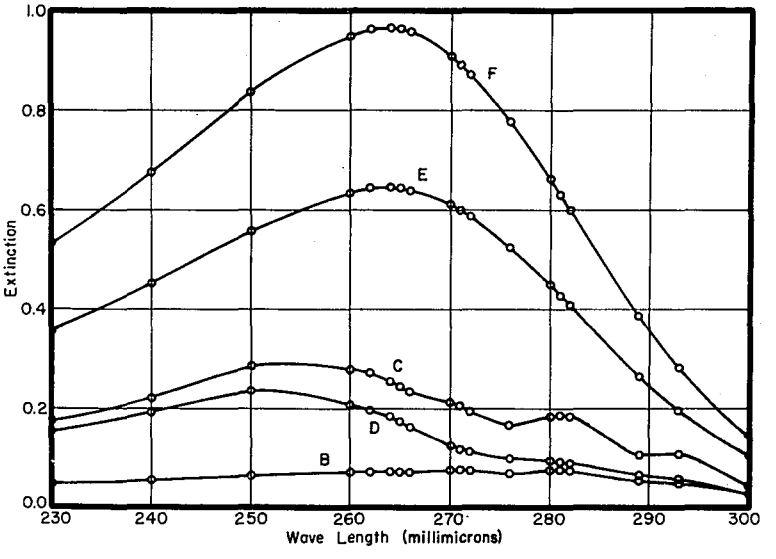


Figure 28. Absorption spectra of ninth fraction of eluate in hexane.
B – 2.3 centimeter superfiltrol column
C – 3.4+ centimeter superfiltrol column
D – 4.6 centimeter superfiltrol column
E – 5.8 centimeter superfiltrol column
F – 7.0 centimeter superfiltrol column

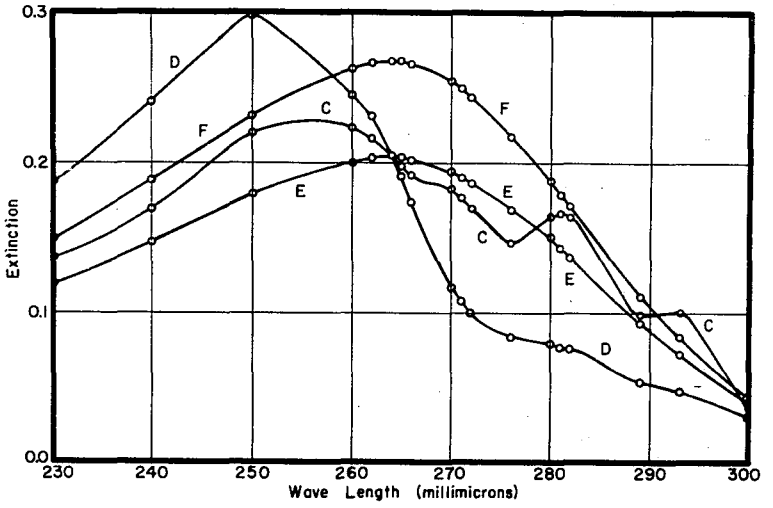


Figure 29. Absorption spectra of tenth fraction of eluate in hexane.
C – 3.4+ centimeter superfiltrol column
D – 4.6 centimeter superfiltrol column
E – 5.8 centimeter superfiltrol column
F – 7.0 centimeter superfiltrol column

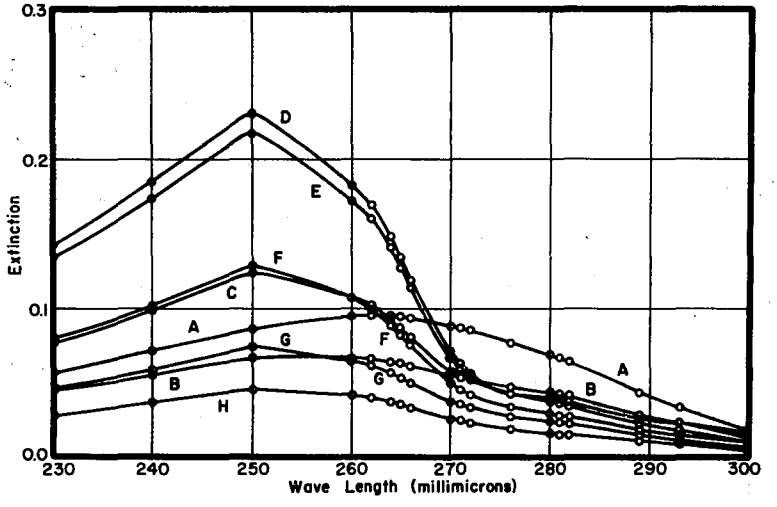


Figure 30. Absorption spectra of eluate in hexane from a 7.0 centimeter superfiltrol column.
A – Eleventh fraction
B – Twelfth fraction
C – Thirteenth fraction
D – Fourteenth fraction
E – Fifteenth fraction
F – Sixteenth fraction
G – Seventeenth fraction
H – Eighteenth fraction

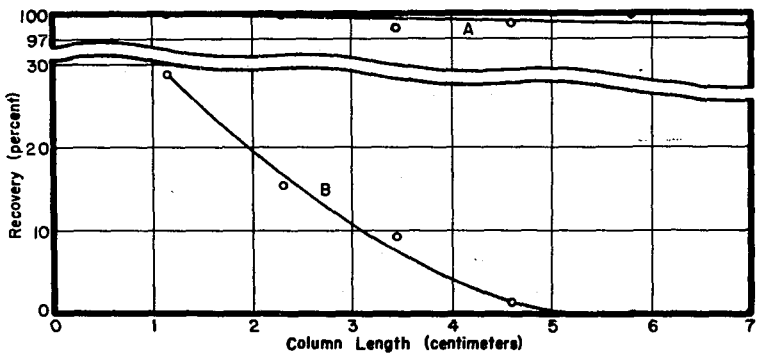


Figure 31. Recovery of calciferol and ergosterol using an ether–hexane, 1:5, eluent.
A–Total calciferol
B–Total ergosterol

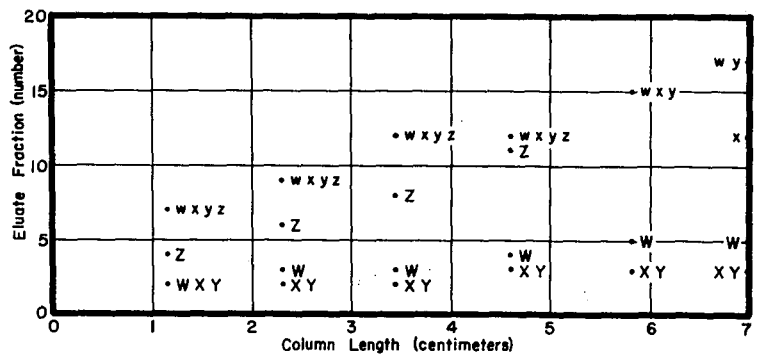


Figure 32. Elution of calciferol, substance 282, substance 250, and ergosterol using an ether–hexane, 1:5, eluent.
W–Initial fraction containing calciferol
X–Initial fraction containing substance 282
Y–Initial fraction containing substance 250
Z–Initial fraction containing ergosterol
w–Final fraction containing calciferol
x–Final fraction containing substance 282
y–Final fraction containing substance 250
z–Final fraction containing ergosterol

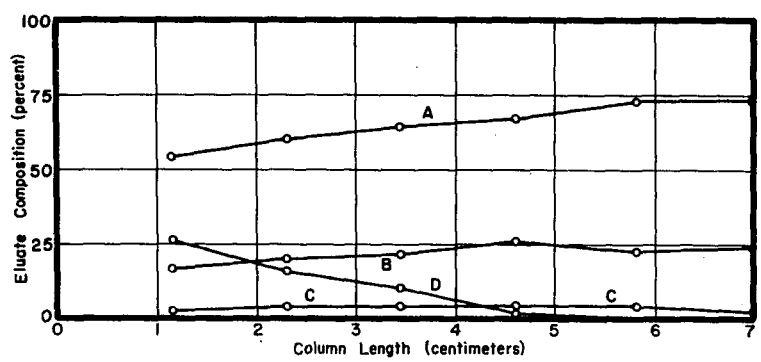


Figure 33. Relative composition of total eluate using an ether–hexane, 1:5, eluent at 281 millimicrons.
A–Calciferol
B–Substance 282
C–Substance 250
D–Ergosterol

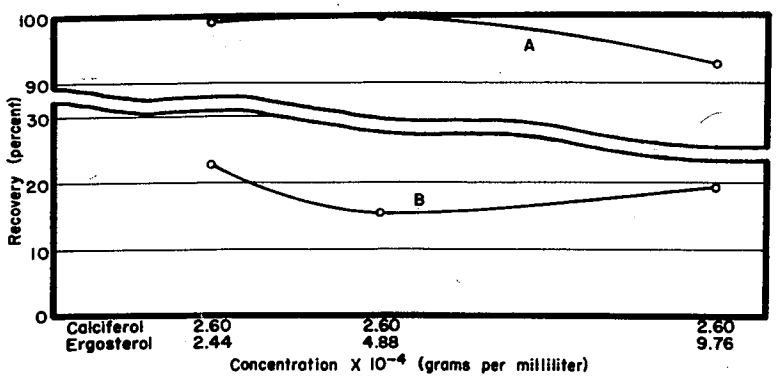


Figure 34. Recovery of calciferol and ergosterol from a 2.3 centimeter superfiltrol column.
A—Total calciferol
B—Total ergosterol

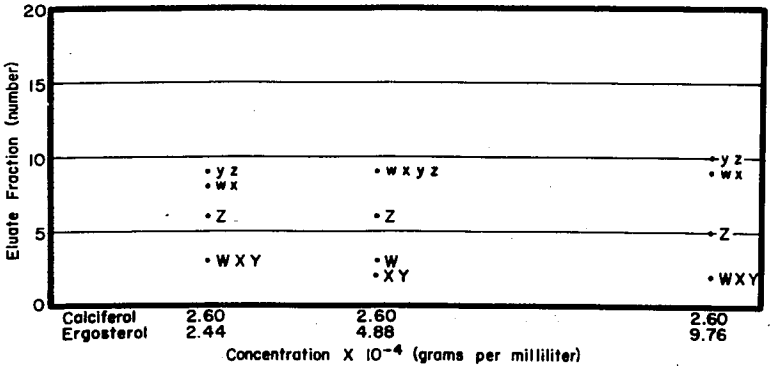


Figure 35. Elution of calciferol, substance 282, substance 250, and ergosterol from a 2.3 centimeter superfiltrol column.
W—Initial fraction containing calciferol
X—Initial fraction containing substance 282
Y—Initial fraction containing substance 250
Z—Initial fraction containing ergosterol
w—Final fraction containing calciferol
x—Final fraction containing substance 282
y—Final fraction containing substance 250
z—Final fraction containing ergosterol

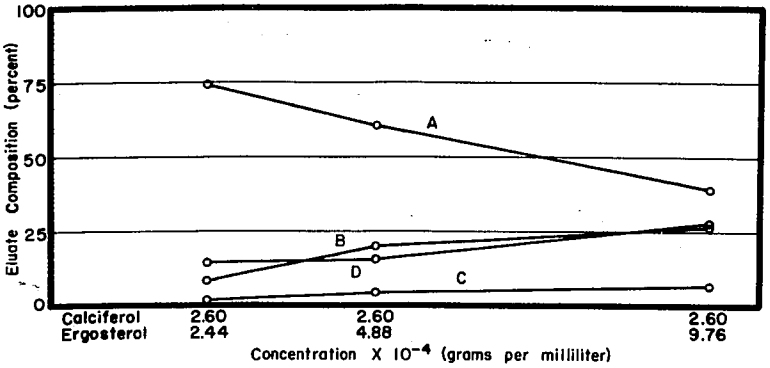


Figure 36. Relative composition of total eluate from a 2.3 centimeter superfiltrol column at 281 millimicrons.
A—Calciferol
B—Substance 282
C—Substance 250
D—Ergosterol

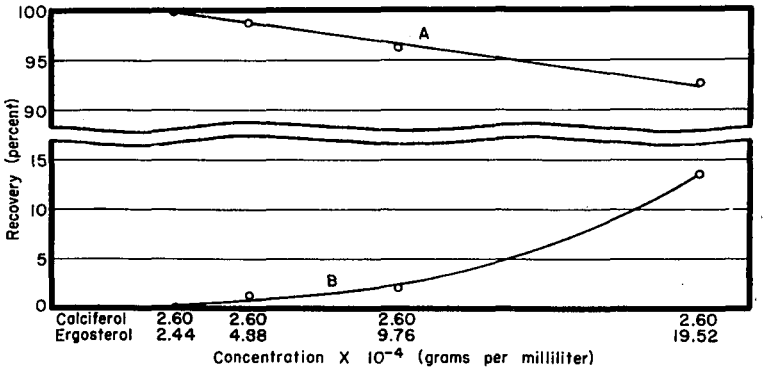


Figure 37. Recovery of calciferol and ergosterol from a 4.6 centimeter superfiltrol column.
A—Total calciferol
B—Total ergosterol

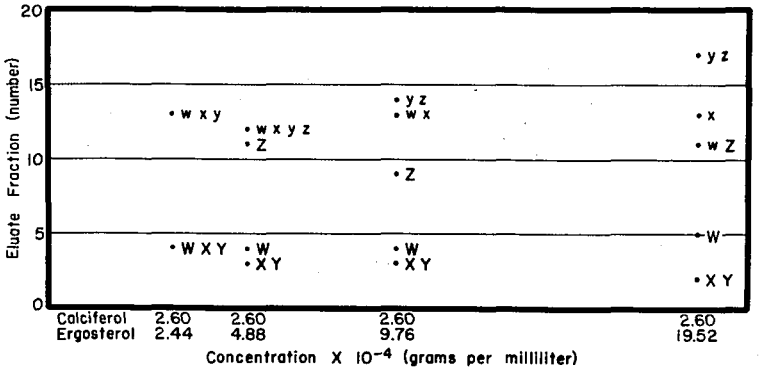


Figure 38. Elution of calciferol, substance 282, substance 250, and ergosterol from a 4.6 centimeter superfiltrol column.
W—Initial fraction containing calciferol
X—Initial fraction containing substance 282
Y—Initial fraction containing substance 250
Z—Initial fraction containing ergosterol
w—Final fraction containing calciferol
x—Final fraction containing substance 282
y—Final fraction containing substance 250
z—Final fraction containing ergosterol

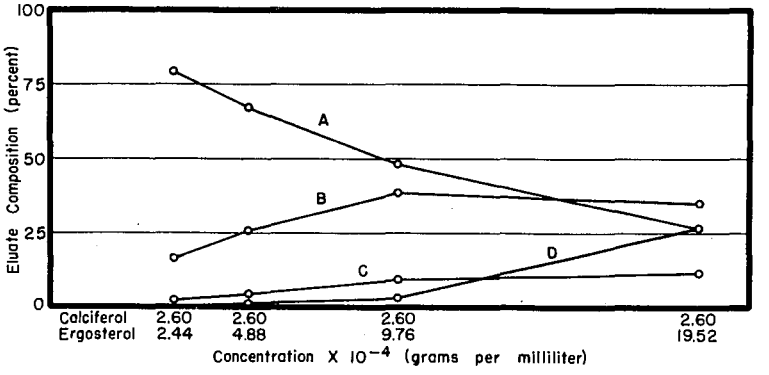


Figure 39. Relative composition of total eluate from a 4.6 centimeter superfiltrol column at 281 millimicrons.
A—Calciferol
B—Substance 282
C—Substance 250
D—Ergosterol

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