CHROMATOGRAPHIC SEPARATION OF VITAMINS D

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This is to certify that the

thesis entitled

"Chromatographic Separation
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Fu-ho Chen

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CHROMATOGRAPHIC SEPARATION OF VITAMINS D

by

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I. INTRODUCTION

The chromatographic adsorption method for the separation of certain substances was originated by the Russian botanist M. Tswett in 1906. He discovered that the pigments in the extracts of green leaves form a series of green and yellow bands in a certain sequence or order when a solution of the mixture was filtered through a glass tube filled with precipitated chalk. Moreover he found that complete separation of the pigments from one another could be effected only by eluting the adsorbed substances with fresh solvent or with mixtures of solvents. The process by which the different bands can be washed down the column at different rates has been called developing. Further study has been made by many scientists. Zechmeister and Cholnoky (21) and Strain (16) wrote treatises on chromatography. Theories have been developed by Wilson (20) and by Martin and Synge (11). And the measurement of a quantitative nature has been made by Cassidy (4, 5). Upon these basic discoveries and theories, it has been concluded that the efficiency of a chromatographic separation was related very closely to the nature of the adsorbent, adsorbed material, the solvent and the physical conditions in the column.

Many attempts have been made to separate mixtures of similar or dissimilar substances from each other by the chromatographic method. Vitamin A was removed from sterol and Vitamin D by Walff (19), Ritsert (14), Marcussen (10), and Ewing et al (9).

The separation of Vitamin D from the irradiated solutions of provitamins and natural oil was completed by Miller (12), De-Witt and

Sullivan (7), and Powell (13). The separation of pure Vitamin D from pure ergosterol had been also studied by Baker (1) and Ballard (2).

The purpose of this investigation was to study the factors which may increase the efficiency of separation of Vitamin D_2 from Vitamin D_3; also of separation of these from the mixture containing Vitamin A and ergosterol.

II. EXPERIMENTAL

1. Treatment of Solvents and Materials

Diethyl ether: A commercially good grade of anhydrous diethyl ether was distilled in the presence of sodium hydroxide and sodium sulfite to remove any water and peroxide which might have been present. The transmittance of the distillate should have been greater than 60% at 230 mμ.

n-Hexane: The commercial product of Skelly-solve was purified for n-hexane. The purification was accomplished by chromatographing the solvent through an activated silica gel column packed to a height about 2/3 m. with an effective inner diameter of 4 cm. The transmittance of the eluted hexane should be greater than 92% at 230 mμ.

Ethyl alcohol: To each liter of anhydrous ethyl alcohol 20 grams of potassium hydroxide (solution effected by first dissolving into a minimum amount of water), and 10 grams of finely powdered silver nitrate were added. The mixture was shaken in an amber bottle and allowed to stand one week before distilling. The transmittance of the distillate should have been greater than 99% at 265 mμ.

Silica gel: It must have been activated at approximately 250°C for at least four hours. After it had been used once, it could be reactivated by washing in a Buchner with a small amount of distilled water until the odor of hexane was no longer noticeable.

It was dried at room temperature and reactivated according to the above procedure.

Stock solutions of the testing materials: A very pure grade of Vitamin D_2 (calciferol), Vitamin D_3, Vitamin A ester of acetate and also a very pure commercial grade of ergosterol were made up in alcholic solution with definite concentrations.

Alumina: The activated alumina used is "Grade A and mesh minus 80". It was the product of the Aluminum Ore Company.

Superfiltrol: It was finely divided activated bentonite clay, obtained from the Filtrol Corporation.

It was very important that all the solvents and solutions used should be free from any trace of benzene and other impurities which had high absorption value in the ultra-violet range and might have interfered with the absorption curves of the substances that were determined in this experiment.

2. Procedures

The rate of migration of Vitamin D_2 and Vitamin D_3 as determined by the concentration (extinction) of the fractions from a chromatographic column: The chromatographic column was made of pyrex with an inner diameter about 7 mm. in which 4 grams of alumina was packed to a height of 8 cm. The method of preparation was similar to that used by Ewing et al (8). The column was fixed to the fraction collector which was connected to an

aspirator for 1 cm. mercury pressure. Then it was prewashed with 10 ml. of diethyl ether.

Three ml. of the alcoholic stock solution that contained 0.00026 g of Vitamin D_2 and 0.000253 g of Vitamin D_3 (about 10,000 units of each) was evaporated to dryness on a hot water bath at about 70°C under a reduced pressure. The residue was taken up in 3 ml. of the chromatographic developing solution which was made up of various proportion of hexane and ether, or hexane and alcohol. The solution was added and followed by another 3 ml. portion of the solvent for rinsing the flask on to the column. The column was not allowed to become dry at any time which might have destroyed the equilibrium between the phases of adsorbent, solute and solvent and then broken the band. Further additions of the same solvent was made to elute the adsorbed substance. Collected the eluent into 3 ml. fractions and ~~having~~ diluted ~~it with~~ to 4 ml. for determination of the absorption curves on a Beckmann spectrophotometer Model D U.

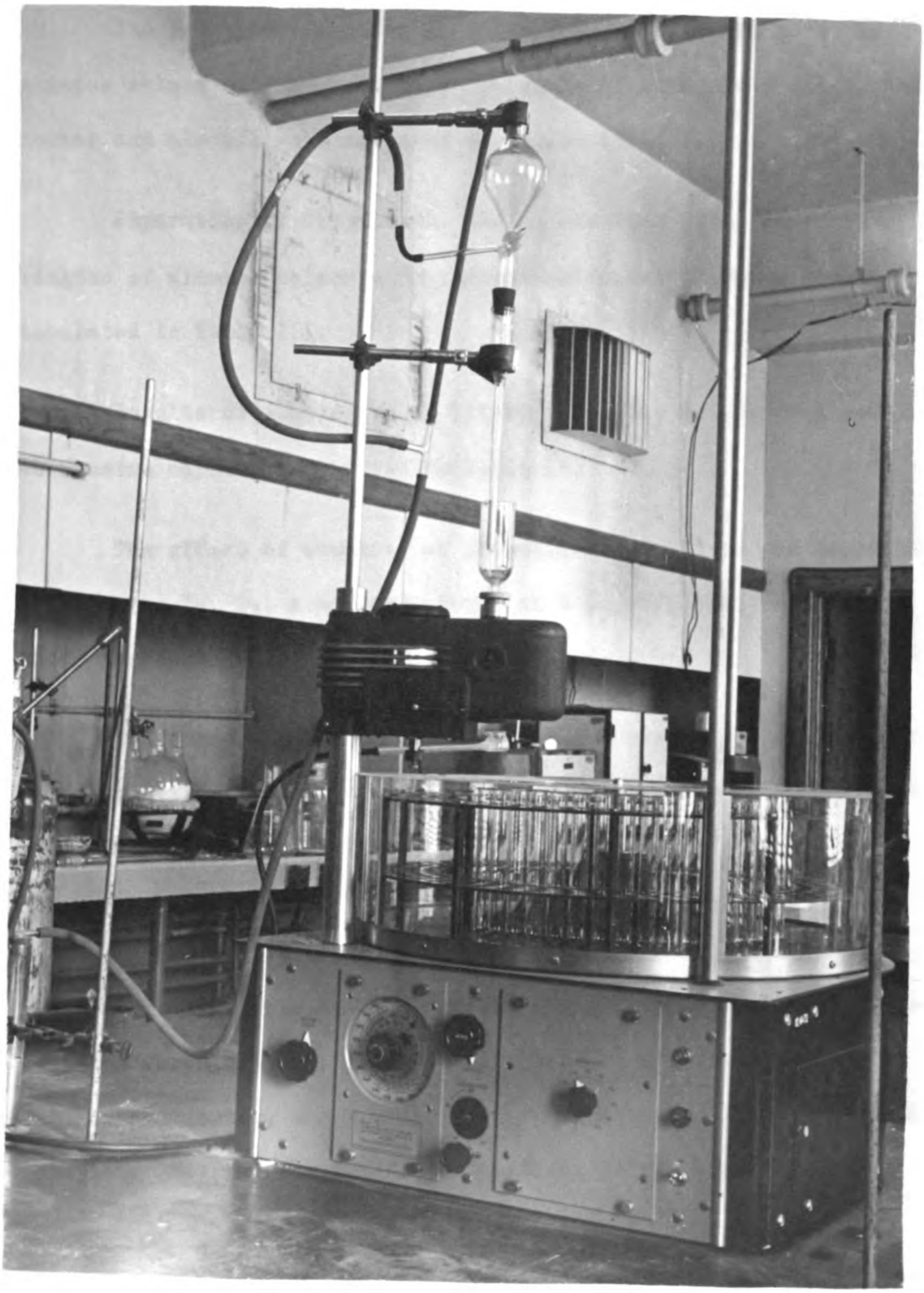
Separation of Vitamin D_2 and Vitamin D_3: Mixture of equal amounts of Vitamin D_2 and Vitamin D_3 (0.000261 g and 0.000253 g, respectively) was taken up in 3 ml. of developing solvent and chromatographed through the alumina column. The experiment was carried out following the procedure given in the above material. For a longer column, more diethyl ether was used for prewashing and the quantity increased with the same proportion as the increase of the length of the column.

Chromatographic separation of Vitamins D_2, D_3, A, and ergosterol:

By alumina column: -- The column must have been prepared as described in the above. It must have been mounted on the automatic fraction collector as shown in the picture on next page; then prewashed with 10 ml. of diethyl ether. A carbon dioxide tank was attached to furnish 1 cm. mercury pressure. The mixture of the alcoholic stock solution of 0.000174 g. of Vitamin D_2, 0.000169 g. of Vitamin D_3, 0.000199 g. of Vitamin A, and 0.000108 g. of ergosterol was evaporated to dryness and residue was taken up in 3 ml. pure hexane, then followed by 3 ml. hexane for rinsing the flask. The column was developed with 20 ml. of hexane and eluted with the mixed solvent of hexane and ether. The eluent was collected in 1 ml. fractions and diluted to 4 ml. for determination of the absorption curves on a Beckmann spectrophotmeter.

By superfiltrol: -- The chromatographic column was packed with superfiltrol to a height of 8 cm., then prewashed with a certain amount of the mixture solvent which was used for developing.

The residue from the stock solutions was taken up in the mixed solvent and the column was developed with the same solvent. The procedures employed were the same as used in the above. Since through the superfiltrol column, the solution passed rather slowly, a 10 cm. mercury pressure was required.



III. RESULTS

The rates of migration of Vitamin D_2 and Vitamin D_3 on an alumina column with various mixed solvents of hexane and ether, and hexane and alcohol were compared in Tables I and II.

Separation of Vitamins D_2 and D_3 had been tried on various lengths of alumina column with various solvents. Results were tabulated in Table III.

Results of separation of Vitamins D_2, D_3, A, and ergosterol on an alumina column were listed in Table IV.

The effect of quantity of prewashing solvent on the separation of Vitamins D_2, D_3, A and ergosterol on a superfiltrol column were shown in Table V.

The percentage of recovery of the adsorbed substance could be calculated from the additivity (2) of the extinction of each fraction of the eluent. Use was made of the equation

$$E(1\%, \text{1cm.}) = \frac{\log I_o/I}{lc}$$

where E(1%, 1cm.) was the extinction of 1 g. solute in 100 ml. solution measured through a cell of 1 cm. thickness, l was the thickness of the cell in centimeter, and c was the concentration in grams per 100 ml. solution. Having rearranged and modified the equation

$$C = \frac{\Sigma(\log I_o/I)}{E(1\%, \text{1cm.})l}$$

where $\Sigma(\log I_o/I)$ was the summation of the extinctions of the fractions

at a certain wave length and E(1%, 1cm.) was a constant for a given substance in a given solvent at the given wave length.

TABLE I: Extinction at 265 mμ. of Fractions of Eluent from Chromatographing Pure Vitamins D_2 and D_3 with Solvents of Mixture of Hexane (H) and Ether (E).

No. of Fraction	Vol. of eluent recov. (ml.)	H - E, 10-90		H - E, 20-80		H - E, 30-70		H - E, 40-60		H - E, 50-50		H - E, 60-40		H - E, 70-30	
		D_2	D_3	D_2	D_3	D_2	D_3	D_2	D_3	D_2	D_3	D_2	D_3	D_2	D_3
1	3	0.036													
2	6	1.747	0.204	0.338		0.115									
3	9	1.093	1.473	1.908	0.088	0.484	0.061	0.042		0.022					
4	12	0.106	0.995	0.594	0.810	1.745	0.149	0.130	0.083	0.064	0.041				
5	15		0.244	0.091	0.895	0.412	0.511	0.402	0.171	0.145	0.078	0.055	0.053		
6	18		0.087	0.021	0.542	0.087	0.795	0.960	0.613	0.298	0.122	0.086	0.086	0.008	0.022
7	21		0.039		0.305	0.027	0.573	0.725	0.805	0.540	0.349	0.251	0.202	0.032	0.047
8	24				0.152		0.332	0.305	0.543	0.675	0.603	0.470	0.423	0.034	0.050
9	27				0.082		0.201	0.156	0.324	0.530	0.618	0.524	0.555	0.042	0.124
10	30				0.047		0.124	0.098	0.175	0.354	0.481	0.433	0.480	0.085	0.308
11	33						0.072	0.070	0.098	0.195	0.276	0.328	0.362	0.230	0.486
12	36							0.044	0.058	0.091	0.163	0.242	0.252	0.419	0.544
13	39								0.029	0.058	0.091	0.168	0.179	0.550	0.450
14	42									0.025	0.051	0.112	0.117	0.506	0.352
15	45										0.029	0.079	0.087	0.368	0.270
16	48											0.051	0.059	0.262	0.182
17	51											0.032	0.037	0.178	0.105
18	54											0.020	0.020	0.117	0.082
19	57													0.070	0.052
20	60													0.059	0.031
21	63													0.029	

TABLE II: Extinction at 265 mμ. of Fraction of Eluent from Chromatographing Pure Vitamins D_2 and D_3 with Solvents of Mixture of Hexane and Alcohol.

No. of Fraction	Vol. of eluent recov.(ml.)	4% Alcohol in Hexane		2% Alcohol in Hexane		1% Alcohol		0.5% Alc.		0.25% Alc.	
		D_2	D_3	D_2	D_3	D_2	D_3	D_2	D_3	D_2	D_3
1	2										
2	4										
3	6	1.343									
4	8	0.454	0.392	0.014							
5	10	0.131	1.370	0.545	0.014						
6	12	0.015	0.150	0.772	0.745	0.161					
7	14			0.331	0.622	0.522	0.206				
8	16			0.162	0.412	0.338	0.616				
9	18			0.103	0.033	0.167	0.410				
10	20			0.035		0.128	0.315				
11	22					0.100	0.132				
12	24					0.064	0.052				
13	26										
14	28										
15	30										
16	32								0.217		
17	34								0.525		
18	36							0.241	0.343		
19	38							0.363	0.201		
20	40							0.422	0.188		
21	42							0.301			
22	44							0.097			
23	46							0.072			
24	48										
25	50										
26	52										0.106
27	54										0.147
28	56									0.079	0.155
29	58									0.444	0.180
30	60									0.393	0.170
31	62									0.278	0.128
32	64									0.194	0.121
33	66									0.111	00.117
34	68									0.079	0.063
35	70										0.050

*Fractions in 2 ml. portions.

TABLE III: Results of Separation of Pure Vitamin D_2 and Vitamin D_3 with Various Solvents and Column.

Adsorbent	Length of column (cm.)	Solvent	Result
Alumina "A"	8	hexane-ether (30-70)	no separation
Alumina "A"	40	hexane-ether (30-70)	no separation
Alumina "A"	8	hexane-ether (20-80)	no separation
Alumina "A"	24	hexane-ether (20-80)	no separation
Alumina "A"	8	5% alcohol in hexane	no separation
Magnisol	8	hexane-ether (90-10)	no separation
Alumina (E = 20)	24	hexane-ether (20-80)	no separation

TABLE IV: Separation of Vitamins D from Vitamin A and Ergosterol Using Alumina Column.

Length of column (cm.)	Solvent	% of Vitamins D recovered
8	2.5% alcohol in hexane	0
2.5	1.0% alcohol in hexane	0
8	hexane-ether (60-40)	9
8	hexane-ether (50-50)	25
8	hexane-ether (1-2)	28
8	hexane-ether (10-90)	41

TABLE V: Separation of Vitamins D from Vitamin A and Ergosterol Using Superfiltrol Column of 8 cm. Length with Different Quantity of Prewashing Solvent.

Solvent	Vol. of prewashing solvent (ml.)	% of Vitamins D recovered
hexane-ether-alcohol (50-10-1)	30	0
hexane-ether-alcohol (50-10-1)	15	67
hexane-ether-alcohol (50-10-1)	6	90
hexane-ether-alcohol (50-10-1)	3	87
hexane-ether (50-10)	30	94
hexane-ether (50-10)	15	95

IV. DISCUSSIONS

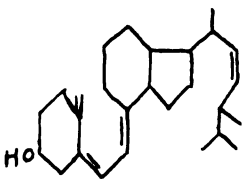
Effect of polarity of solvent on the rate of migration of Vitamin D_2 and Vitamin D_3 on an alumina column:

From the results obtained with Tables I and II, the extinction at 265 mμ. of each 3 ml. fraction was found. In Figures I and II the extinction at 265 mμ. of each fraction was plotted against the volume of eluent recovered, and the points on the curves were located at the end of each fraction. The curves showed the relationship that with the solvents containing higher concentration of ether and alcohol, samples would pass through the column with less quantity of eluent; also the extinction of the fractions around the maxima was greater than those with the solvents containing less ether and alcohol. Furthermore, comparing the curves of Vitamin D_2 and Vitamin D_3 in the same solvent, it could be noted that Vitamin D_2 will pass through the column earlier than Vitamin D_3 if solvent used was relatively rich in ether and in alcohol. The difference between the rates decreased as the concentration of ether and alcohol in hexane decreased; until at a certain concentration, the above order reversed. This phenomenon might be explained by the following theories.

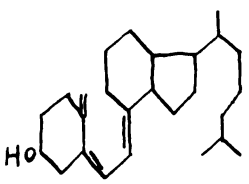
It was observed that the rate of migration of a solute through an adsorption column was a function of the adsorption isotherm. The adsorption sequence was also a function of the isotherms of the solutes (4, 5, 6). According to the theories of Strain (16), the more polar the substance, the stronger it was adsorbed and the higher the band was formed on the column. But the sequence or adsorption order of the bands was not the same under all circumstances. It varied with the

solvent and the adsorbent, with the kinds of substances adsorbed, and with the conditions in the column. It could also change with the alteration of one or more factors such as the adsorbent, the solvent, the temperature, the concentration of solutes, the presence of an impurity, and the hydrogen ion concentration (17).

In examining the structural formula of Vitamin D_2 and Vitamin D_3,



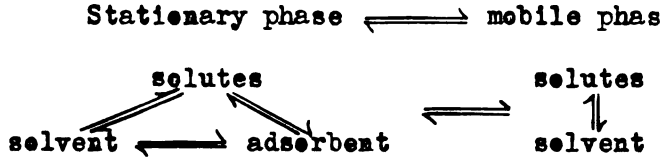
Vitamin D_2



Vitamin D_3

they were found to differ only by a double bond and a methyl group on the side chain. The presence of the double bond in a compound increased the polarity. Thus Vitamin D_2 should be more polar than Vitamin D_3 and would form a band above the latter. Since both of the bands formed by Vitamins D_2 and D_3 were colorless, it was not possible to follow the order or sequence visually. Nevertheless Vitamin D_2 did appear in the eluent earlier than Vitamin D_3 in solvents containing higher percentages of ether or alcohol in hexane. So it seemed that in the presence of ether or alcohol it might have influenced either the relative positions or the rates of migration of Vitamin D_2 and Vitamin D_3, or both. Compared with hexane, ether and alcohol were more polar in nature. Then polarity of the solvent could be responsible for the fact that the more polar the substance to be adsorbed, the greater would be the rate of migration in a more polar solvent. The desorption is carried on by the displacement of the adsorbed substance on

the adsorbent by the solvent which was more polar than the adsorbed substance (16) and the band formation was due to the equilibrium between the phases (18) as follows:



The rate of migration of the adsorbed substance on the column possibly could have been increased by increasing the affinity between the solvent and the adsorbed substance.

Effect of the solvent used for introducing the sample:

Hydrocarbons such as n-hexane and isoctane were considered as non polar compounds which possess little or no eluting power on a chromatographic column. If the sample was introduced by a non polar solvent, the effect of elution during adsorption might have been eliminated and the band formed would have been sharper. In other words it needed less quantity of solvent to elute it. This effect was noted by comparing the curves in Figures III and IV. Vitamins D_2 or D_3 introduced by pure hexane or isoctance were more concentrated in the eluent than those introduced by the developing solvent. This effect might have facilitated the separation of the mixture. In separating Vitamins D_2 and D_3 from ergosterol on an alumina column, better results were obtained by introducing the mixture in pure hexane instead of using the developing solvent, as shown in Figures V and VI. Further study was necessary before this proposal could be adopted.

Separation of Vitamins D:

Based on the previous results obtained in this experiment, Vitamin D_2 might have been completely or partially separated from Vitamin D_3, by choosing a solvent which caused a greater difference between the rates of migration of the two and a column which could increase the distance between the bands as they reached the bottom of the column. So a more polar solvent and a longer column were chosen for this purpose. Unfortunately, the curves obtained by plotting extinction at 265 mμ of each fraction against volume of eluent showed only one maximum which suggested that the two substances might form only one band eluted together or the two bands formed could be very close and overlapping. Moreover, the absorption curves of Vitamins D_2 and D_3 were too much alike to determine whether the eluent was a pure substance or a mixture. The results did not indicate separation.

Vitamins D_2 and D_3 could be separated quantitatively from Vitamin A and ergosterol by a superfiltrol column, (Table V). Vitamin A was adsorbed on the column and ergosterol could be eluted afterward. By an alumina column, Vitamin A could also be removed from the rest while ergosterol followed Vitamins D too closely and caused overlapping. Figures V, VI and VII showed that Vitamins D came out in the earlier fractions of the eluent and ergosterol in the latter portions. The mixture of the three appeared in the middle portions and the percentage of ergosterol contained in the mixtures increased as the number of fractions increased.

Effect of prewashing of superfiltrol column:

Prewashing affected the affinity between adsorbent and the adsorbed substances. It was probably connected with the water contact of the adsorbent, (15). Alcohol, because of its structural similarity, could replace water in the adsorbent and deactivated the latter. Ether, because it could dissolve water less readily than alcohol, gave no appreciable effect on prewashing. When a superfiltrol column was prewashed by 30 ml. of hexane-ether-alcohol (50-10-1), it lost its adsorption power. Then Vitamins D_2 and D_3 and ergosterol eluted together from the column in the first portion (Figure VIII). Nevertheless, Vitamin A still remained in the column. When it was prewashed with 30 ml. of hexane-ether (50-10), this effect was not noted (Figure XI).

The purpose of prewashing was to remove the residue contained in the adsorbent (13). From an 8 cm. superfiltrol column, the residue washed off showed the absorption curve as given in Figure XIII. During washing, a yellow band was formed on the column which migrated down with further prewashing. If 30 ml. of the solvent, hexane-ether-alcohol (50-10-1), was added, it was enough to wash the band from the column and destroy the adsorbility of the column. The yellow band had been collected and its absorption curve made as in Figure XIII. If alcohol was not present in the solvent, the yellow band did not appear.

In the solvent of hexane-ether-alcohol (50-10-1), the yellow band did change into blue color when the samples were introduced. Also the absorption curve showed the difference (Figure IX, curve No. 8). Whether the blue band was the combination of the substance in the yellow band and the adsorbing material added or the decomposed substance

formed on the column, could not be concluded from this experiment.

From the results listed in Table V, the trial of prewashing the superfiltrol column with 6 ml. of the developing solvent hexane-ether-alcohol (50-10-1) gave the higher percentage of recovery of Vitamins D_2 and D_3 from the mixture. When 3 ml. of the solvent was used which was about sufficient to wet the column, the earlier fractions of the eluent were contaminated with the residue washed from the column. When 15 ml. of the solvent was used, the latter fractions of the eluent contained the blue band (Figure IX). In both cases the percentage of recovery of pure Vitamins D_2 and D_3 was decreased. However, the developing solvent hexane-ether (50-10) which did not contain alcohol, gave better results than that containing alcohol (Figures VIII, IX, X, XI AND XII). In Figure XII the solid lines represented these fractions containing pure Vitamins D while the broken lines represented those contaminated with impurities.

Effect of different grade of alumina on the rate of migration:

Alumina of Grade (E = 20) had also been used for chromatographing pure Vitamin D_2, with the procedures as described in (III, 1). When hexane-ether (30-70) was used as the developing solvent, Vitamin D_2, in this case, would not appear until 50 ml. of eluent had passed. It also required more solvent to elute the adsorbed substance that alumina grade "A" did. Furthermore, the extinctions in each corresponding fraction were less. In other words, it could be considered that the band formed was wider. Nevertheless, it still possessed the same property that the increase of the ether content in hexane increased the rate of migra-

tion and also narrowed the band.

Evaporation of stock solution of Vitamins D:

In evaporating the alcoholic stock solution of Vitamin D_2 and D_3 on a hot water bath, care was necessary. Preferably, the solutions should have been evaporated to dryness within as short a time interval as possible. If the time for evaporation was too long, a yellow oily residue would be the result instead of white crystals in the flask. This substance would pass through the chromatographing alumina column without being adsorbed and would show an absorption curve as in Figure XIV. Whether this was a thermally decomposed product or a product formed in some other manner had not been further investigated.

V. SUMMARY

1. The rates of migration of Vitamin D_2 and Vitamin D_3 on an alumina column were influenced by the polarity of the developing solvent. They were decreased as the polarity of the solvent decreased. Comparing the rates of migration of Vitamin D_2 and Vitamin D_3 in the same solvent, that of Vitamin D_2 was greater than Vitamin D_3 in the more polar solvent. The order was reversed as the polarity of solvent decreased.

2. A sharper band formed if the substance was introduced by a non-polar solvent than by a more polar one on an alumina column.

3. Separation of Vitamin D_2 from Vitamin D_3 did not seem possible with solvent hexane-ether and hexane-alcohol using an alumina column.

4. Vitamins D could be separated from Vitamin A and ergosterol quantitatively by means of a superfiltrol column and partially by an alumina column.

5. Solvent of hexane and ether (50-10) gave better recovery of pure Vitamins D than solvent of hexane, ether and alcohol (50-10-1) from a chromatographic superfiltrol column.

6. The adsorbability of superfiltrol was affected by the prewashing solvent containing alcohol, thus having influenced the percentage of recovery of pure Vitamins D. Using a larger quantity of this prewashing solvent it deactivated superfiltrol and caused no separation of Vitamins D and ergosterol.

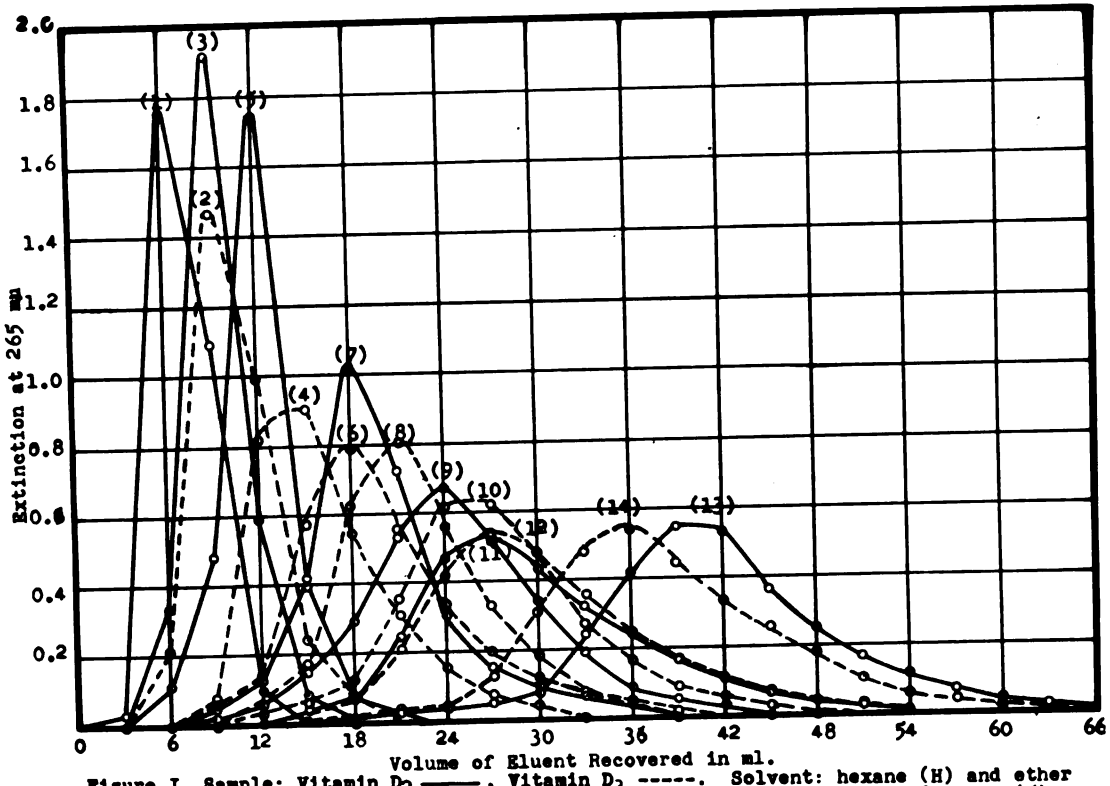


Figure I. Sample: Vitamin D_2 ———, Vitamin D_3 -----. Solvent: hexane (H) and ether (E) mixture in various proportions; (1)&(2). 10-90, H-E; (3)&(4). 20-80, H-E; (5)&(6). 30-70, H-E; (7)&(8). 40-60, H-E; (9)&(10). 50-50, H-E; (11)&(12). 60-40, H-E; (13)&(14). 70-30, H-E.

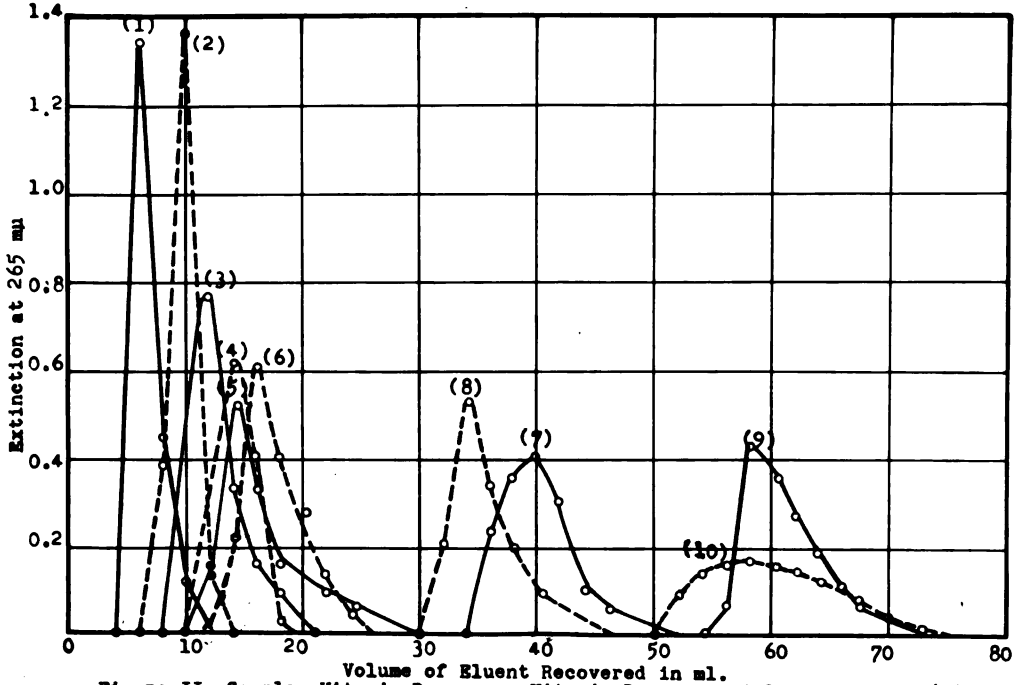


Figure II. Sample: Vitamin D_2 ———, Vitamin D_3-----. Solvent: hexane (H) and alcohol (A) in various proportions; (1)&(2). 4% A in H; (3)&(4). 2% A in H; (5)&(6). 1% A in H; (7)&(8). 0.5% A in H; (9)&(10). 0.25% A in H.

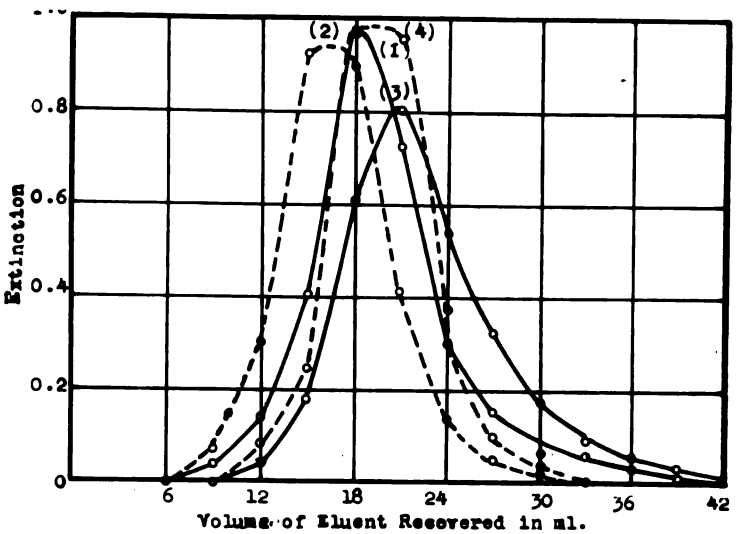


Figure III. Solvent used, hexane-ether (40-60); Sample introduced in mixed solvent ——, and in pure hexane -----. (1)&(2), Vitamin D_2; (3)&(4), Vitamin D_3.

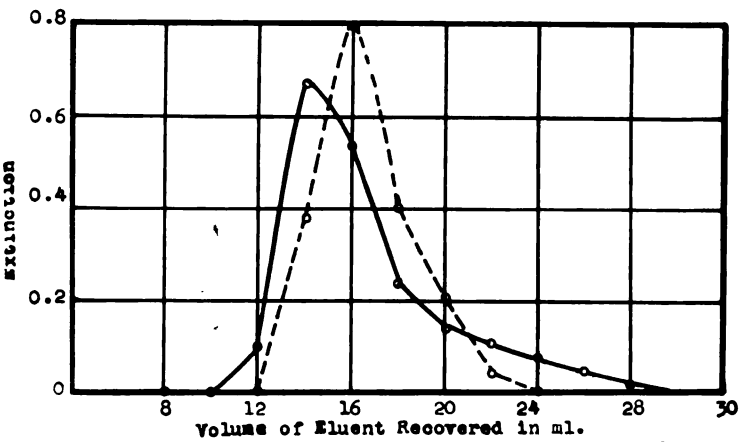


Figure IV. Solvent used, 1.5% alcohol in isoctane; Vitamin D_2 introduced in mixed solvent ——, and in pure isoctane -----.

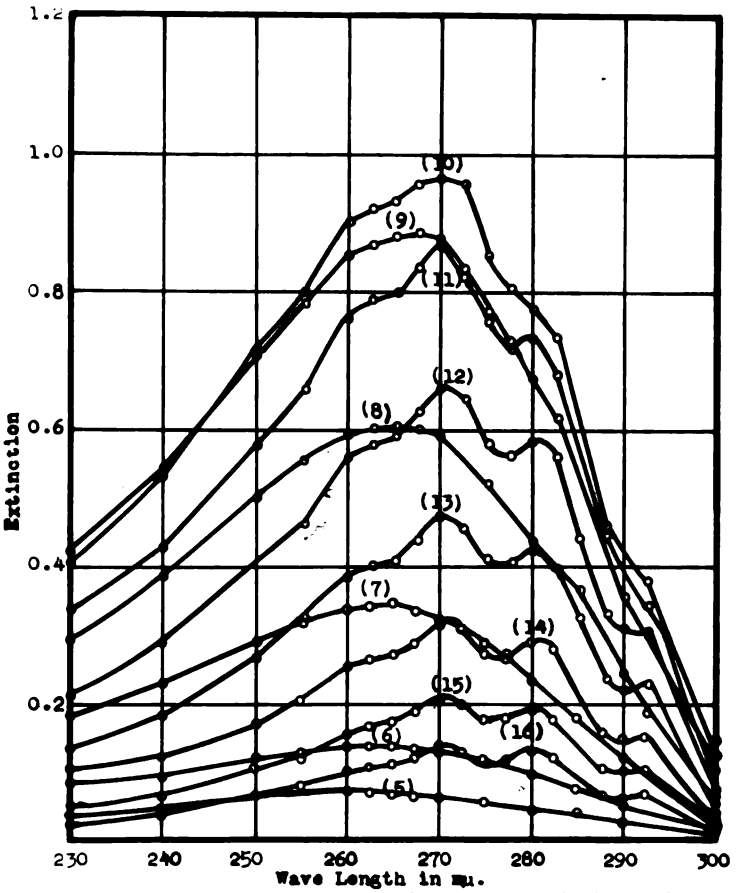


Figure V. Absorption curves of fractions of eluent from chromatographing a mixture Vitamins D_2, D_3, A, and ergosterol on alumina column with solvent hexane-ether (50-50). Sample introduced in mixed solvent.

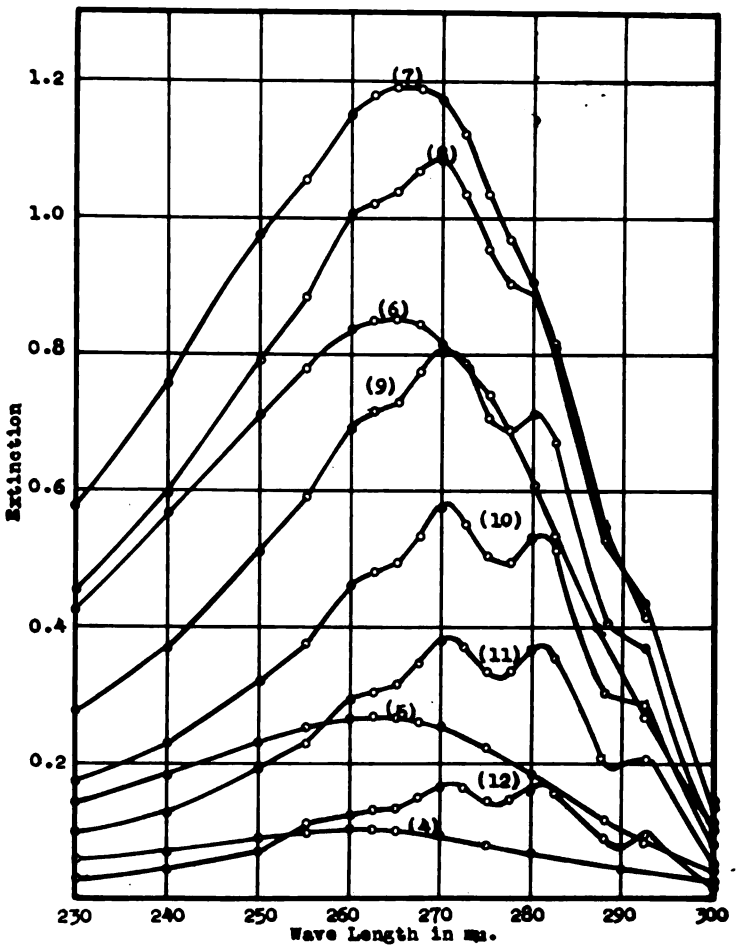


Figure VI. Absorption curves of fractions of eluent from chromatographing a mixture Vitamins D_2, D_3, A, and ergosterol on alumina column with solvent hexane-ether (50-50). Sample introduced in pure hexane.

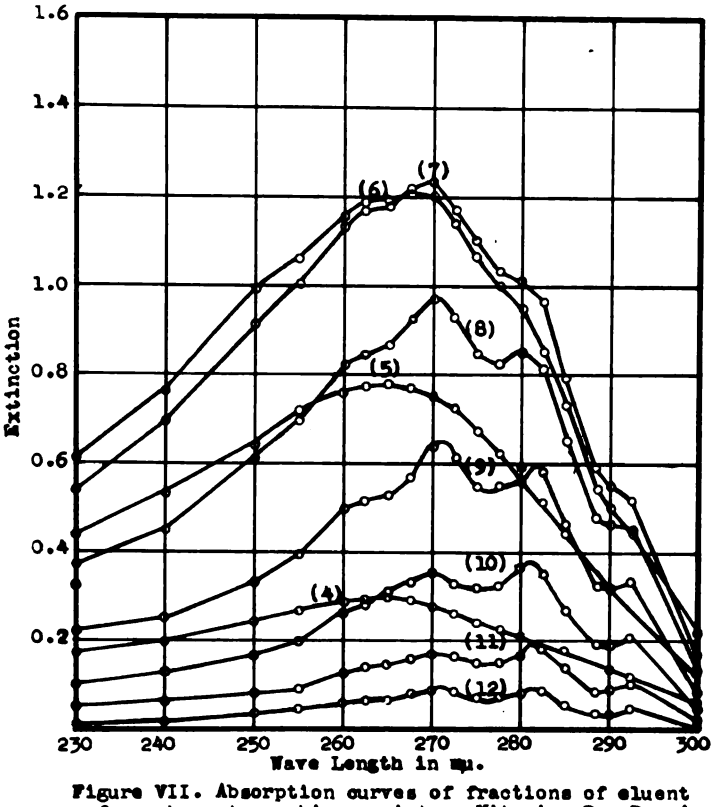


Figure VII. Absorption curves of fractions of eluent from chromatographing a mixture Vitamins D_2, D_3, A, and ergosterol on alumina column with solvent hexane-ether (1-2). Sample introduced in pure hexane.

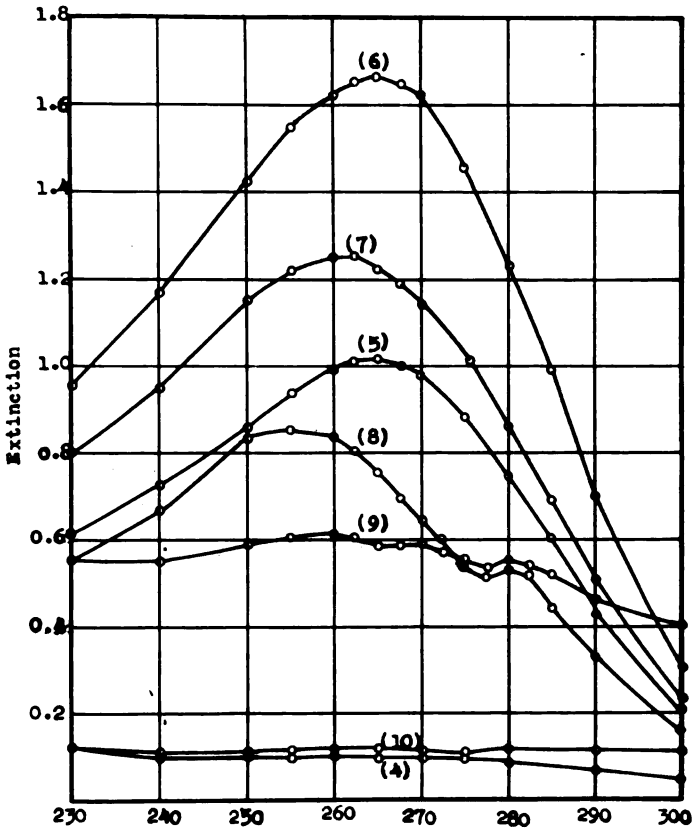


Figure IX. Absorption curves of fractions of eluent from chromatographing a mixture Vitamins D_2, D_3, A, and ergosterol on superfiltrol column with solvent hexane-ether-alcohol (50-10-1). Used 15 ml. of solvent for prewashing.

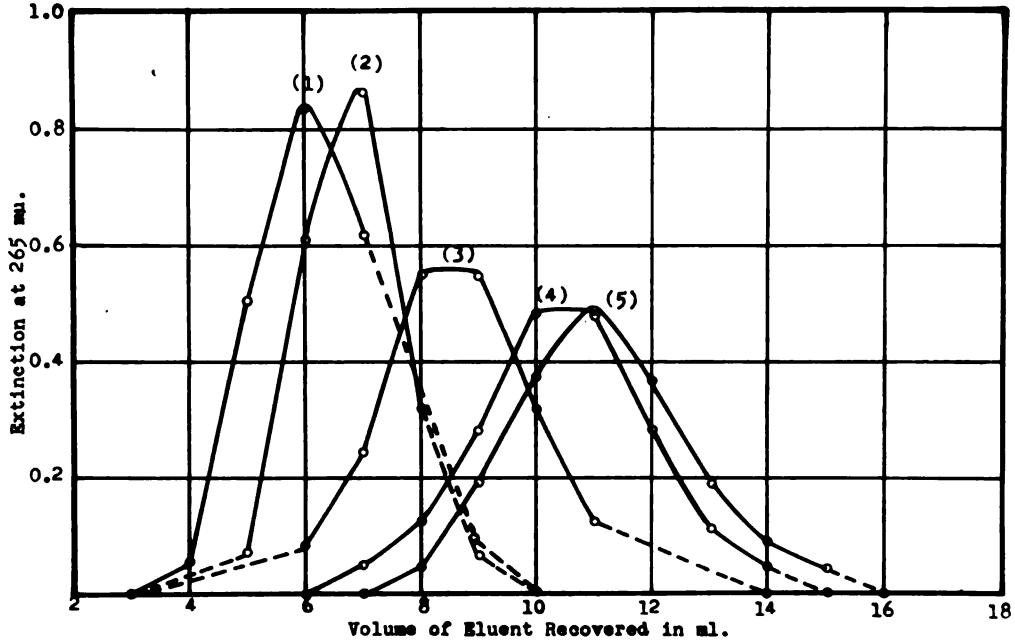


Figure XII. Vitamins D recovered from chromatographing mixture on superfiltrol column with various prewashing solvent: (1). 15 ml., H-E-A (50-10-1); (2). 6 ml., H-E-A (50-10-1); (3). 3 ml., H-E-A (50-10-1); (4). 15 ml., H-E (50-10); (5). 30 ml., H-E (50-10).

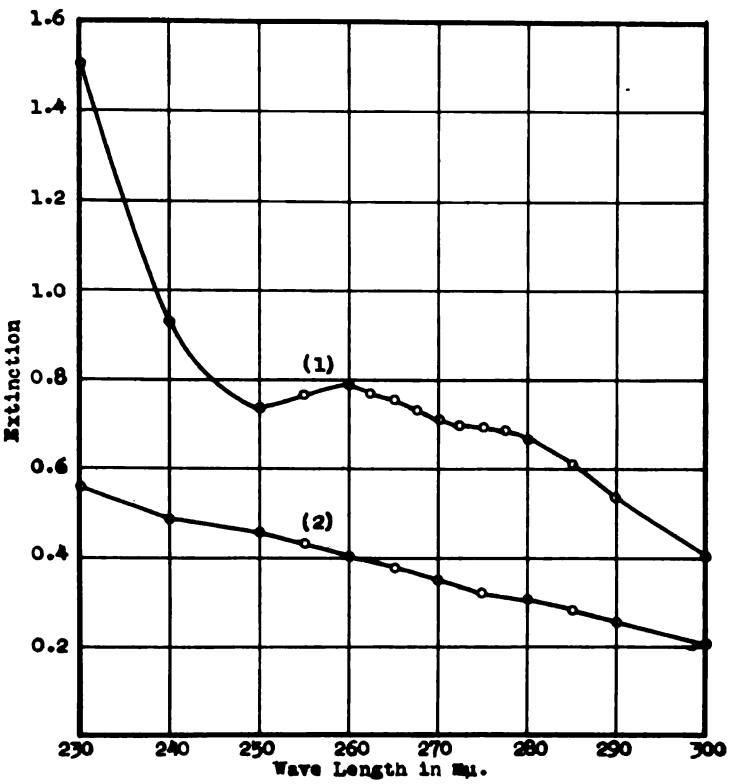


Figure XIII. (I) Absorption curve of prewashing solvent from superfiltrol column. (2) Absorption curve of the yellow band on superfiltrol column.

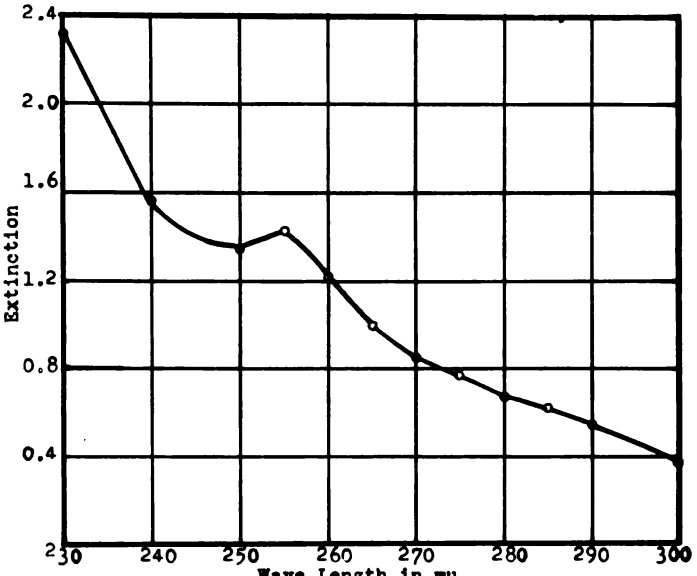


Figure XIV. Absorption curve of the yellow residue obtained from evaporating alcohollic Vitamins D solution.

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