

SPECTROPHOTOMETRIC
DETERMINATION OF PROGESTERONE

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This is to certify that the

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OF
PROGESTERONE

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INTRODUCTION AND REVIEW OF LITERATURE

For a number of years attempts have been made, with some success, to demonstrate the presence of the corpus luteum hormone, progesterone, in various tissues and fluids of animals, including the human. The assay for this material depends upon the production of progestational proliferation in the endometrium of the rabbit. Corner and Allen in 1929 (1) showed that this effect could be produced by extracts of the corpus luteum of sow ovaries. Clauberg and associates in 1933 (2) were able to produce the uterine response by using extracts of human ovaries. In the same year Mazer and Goldstein (3) reported the detection of this material in a 6 months human placenta. Other workers have either confirmed this (1934-1935) (Fels (4), Ehrhardt (5), Adler, de Fremery and Tausk (6), Lankeren (7), and Ehrhardt and Hagena (8)), or failed to do so in earlier attempts (1931-1933) (Phillips (9), Fels (10), de Fremery, Lucks and Tausk (11), Ehrhardt and Weigel (12), and Portman (13)).

Attempts to extract this substance from urine met with failure until, in 1935, Loewe and Voss (14) succeeded in obtaining one Clauberg rabbit unit from 20 liters of human urine collected during pregnancy or the last half of the menstrual cycle.

In working with blood, Clauberg and his associates (2) (1933) used up to 335 ml. of human blood and were unable to show the presence of the hormone. In 1936, Bloch (15) extracted as much as 500 ml. of human pregnancy blood, obtaining negative results. He also extracted 1.0, 3.0, 4.5, 6.0, 8.0, and 12.0 liters of blood from sows in which the reproductive history was unknown. Of these the smaller amounts were negative whereas the extracts from 8 liters and 12 liters showed the presence of 0.5 to 0.7 rabbit unit (one unit being the amount which suffices, when divided into 5 daily doses, to change the uterus of a doe weighing 3 to 4 kilograms, to a state representing the 8th day of normal pregnancy (1)).

Since biological assays at best have the variability of individual animals to contend with, and since the biological effect might be due to other substances, the search for a different method of determining the presence of progesterone was believed to be worth while. The use of absorption spectra has proved useful in working with numerous biologically important materials, and it was found that progesterone has an absorption maximum at 240 millimicrons (in absolute alcohol) (16). The curve is single-peaked and the maximum falls at a wave-length which is well separated from the maxima of most other compounds.

With these ideas in mind, the hope that the presence of progesterone might be detected in solutions and in blood

by spectrophotometric means, led to this study.

Due to the speculative nature of the problem various procedures were tried. The following outline will serve to indicate the order of development of the work. These will be described in detail under "Experimental Procedures".

- A. Spectrophotometer operation; graphing procedure
- B. Preliminary absorption curves
 - 1. Hexane
 - 2. Alcohol
 - 3. Water
- C. Diluted blood plasma
- D. Chromatographic studies
- E. Petroleum ether extractions
 - 1. With a separatory funnel
 - a. From aqueous solutions of progesterone
 - b. From plasma after precipitation of proteins
 - c. From plasma without precipitation of the proteins
 - (1) Synthetic mixtures
 - (2) Normal cows and calves
 - 2. With a continuous liquid-liquid extractor
 - a. From aqueous solutions of progesterone
 - b. From the blood plasma of a cow injected with progesterone
 - c. From hemolyzed blood of a cow injected with progesterone

EXPERIMENTAL PROCEDURES

SPECTROPHOTOMETER TECHNIQUE AND GRAPHING PROCEDURE

In all cases the final determinations were made on a Beckman Quartz Spectrophotometer, Model DU, using the same pair of 1 cm. square fused silica cells throughout. One of the cells was always used for the solvent or blank, the other for the unknown, and during this study these cells were used exclusively for **this purpose**. Each cell was placed in the carrier with the same face toward the light source on each trial. One of these cells had a light path length of 1.002 cm. The other was 1.003 cm. long. For the reason that the cells were always used in the same way, and since differences in the transmission of the two cells, when applied to actual data, gave results differing only slightly from the observed readings, cell corrections have been omitted. Cleaning of the cells was accomplished by immersion in chromic acid cleaning solution for about 30 seconds, then rinsing thoroughly with distilled water. **Following this**, they were rinsed 3 times with the solvent being used, the blank was filled with solvent, and the second cell was rinsed twice with the unknown and then filled.

The light source was a hydrogen discharge lamp. **as** it was necessary to change this tube during the work, the

effects of such changes are discussed below.

The sensitivity control was used at the counter-clockwise limit.

The absorptions, in percent transmission, were recorded for wave lengths between 208 and 300 millimicrons. The lower limit was determined by the solvent and the hydrogen lamp. In general, determinations were made every 2 to 5 millimicrons except at the maximum, when readings were taken for each millimicron. These percent transmissions (%T) were transformed to $\log I_0/I$ (or E) by the use of a chart prepared for that purpose. In the above expression, I_0 represents the intensity of the light entering the cell, and I represents the intensity of the light emerging. E is the extinction due to the dissolved substances.

In plotting the data, E was plotted against wave lengths in millimicrons in most cases. The concentration curves were prepared by plotting the E values against the various concentrations at the wave length where maximum absorption occurred.

PRELIMINARY ABSORPTION CURVES

Hexane. Preliminary trials showed that hexane (from petroleum, practical, Eastman Kodak Co.) absorbed all the light with wave lengths below 240 millimicrons. It was

therefore discarded in favor of absolute alcohol.

Absolute Alcohol. Crystalline progesterone* to the amount of 12.5 milligrams was dissolved in 100 milliliters of absolute alcohol. This was not used until six months later when 5 milliliters of this solution was diluted with 95 milliliters of absolute alcohol to make a solution containing 0.00625 mg./ml. Measurements of the absorption of this solution were made with the spectrophotometer 2 days following the dilution and again on the 4th, 8th, 13th, 21st, and 28th days. The results, presented in Graph No. 1 in the section entitled "Results and Discussion", indicated that the curves were highly variable as to wave length of maximum absorption and also as to the degree of absorption.

A fresh stock solution was made up by dissolving 10 milligrams of crystalline progesterone in 100 milliliters of absolute alcohol. Five milliliters of this was diluted with 95 ml. of alcohol to make a solution containing 0.005 mg./ml. The absorption of this solution was measured using absolute alcohol as a blank and was repeated on the 4th, 7th, 13th, 14th, twice on the 19th, and on the 20th day. Graph No. 2 shows the results. The curves are as variable as those in Graph No. 1.

Thirteen days after the above stock solution (0.1 mg./ml.) was prepared, a new dilution was made by adding

*The progesterone used in these studies was obtained from the Schering Corporation. Melting point = 128-129°C. Optical rotation = +174.0.

95 ml. of absolute alcohol to 5 ml. of the stock solution. This resulted in an alcoholic solution containing 0.005 mg./ml. as before. The absorption was measured as previously described on the same day the dilution was made and also on the following day to determine if similar results could be obtained. These curves (Graph No. 3) showed that determinations made on successive days using fresh solution were also variable.

Later in the study, occasion arose to again make up a stock solution by dissolving 5 mg. of progesterone in 100 ml. of absolute alcohol (= 0.05 mg./ml.) and to dilute this to make, among others, a concentration of 0.005 mg./ml. The results obtained with this solution are presented in Graph No. 4, and pertinent data from Graphs 1, 2, 3, and 4 are shown in Graph No. 5. All of these illustrated a lack of relationship between the extinction, the absorption maximum, the time since making the solution, and the slit-width.

Distilled Water. Due to the dissimilarity of the results obtained with alcohol, and because it was desired finally to work with blood, curves were made using distilled water as the solvent.

An attempt was made to dissolve 10 mg. of progesterone in 100 ml. of distilled water. The crystals did not dissolve completely. With this solution (0.1 mg./ml.) the absorption was measured on the 2nd day, twice on the 3rd, once on the 4th and 8th, and twice on the 9th day following its

preparation. In addition, it was diluted 1:1 to make 0.05 mg./ml. and analyzed on the 1st, 2nd, and 4th days after dilution. A further dilution of the original stock solution to 0.005 mg./ml. was made and absorption measured. All of these results are plotted in Graph No. 6. The curves showed less variability than the alcoholic curves.

A new aqueous stock solution was made by putting 10 mg. of progesterone into 4 ml. of absolute alcohol (in which it dissolved almost completely) and then diluting to 250 ml. with distilled water. The absorption of this solution (0.04 mg./ml.) was measured once on the next day and twice on the succeeding day. Further dilutions and determinations were made, the results of which are given in Graph No. 7. This graph indicated that dissolving progesterone in a small amount of alcohol facilitates its solution in water.

The third and a fourth aqueous stock solutions were made as follows: 10 mg. of progesterone was dissolved in 5 ml. absolute alcohol (all appearing to dissolve). Then for Stock III distilled water q.s. 500 ml. was added. For Stock IV the alcoholic solution was added to sufficient water to make 500 ml. Each of these (0.02 mg./ml.) was diluted to make concentrations of 0.018, 0.016, 0.014, 0.012, 0.010, 0.008, 0.006, 0.004, and 0.002 mg./ml. In addition Stock IV was diluted further to 0.001 and 0.0005 mg./ml. The absorption of all of these dilutions was then measured 3 or 4 times at and near the maximum of 248 millimicrons.

From these a concentration curve was constructed (Graph No. 8).

DILUTED BLOOD PLASMA

To determine if progesterone could be detected spectrophotometrically in the presence of blood plasma the following animals from the experimental dairy herd were bled (coagulation was prevented by bleeding into flasks containing 0.5 gram of sodium citrate crystals per 100 ml. of blood):

	Blood <u>in ml.</u>	Plasma <u>in ml.</u>
C574 (calf)	100	47
A 33 (non-pregnant cow)	120	80
A 27 (non-pregnant cow)	170	105
A 26 (pregnant cow)	190	105

The plasma was obtained by centrifugation.

It was necessary to dilute the plasma in order to allow light to pass through it. Dilutions of 5 ml. of plasma with 95 ml. of distilled water were made using plasma from C574 and A33. In addition the A33 diluted plasma was diluted again 5:95 to obtain a 0.25% plasma solution in distilled water. The absorption data from these dilutions, using distilled water as a blank, are shown in Graph No. 9. Curves for 0.25% plasma from the other animals did not differ enough from this one to allow them to be plotted. Later in the work occasion arose to collect blood from a number of

animals. The plasma from these was diluted to make 0.4% plasma solutions. All of these gave curves very similar to the single example in Graph No. 9.

Progesterone to the amount of 10.75 ng. was dissolved in 2 ml. of absolute alcohol. Of this, 0.2 ml. was added to 99.8 ml. of A27 plasma which had been diluted 5:95 and this in turn 5:95 to make 0.25% plasma in distilled water as above. This made a 0.001075% progesterone in 0.25% plasma solution. Graph No. 10 shows the absorption curve of this solution as compared with a curve obtained with the 0.25% A27 plasma without added progesterone. An alteration of the curve at and near the maximum of 248 millimicrons is evident.

The results of similarly prepared solutions containing 0.0005375%, 0.001075%, and 0.00215% progesterone in 0.25% of A27 plasma are shown in Graph No. 11. The estimations of the progesterone present were found to be in agreement with the amount added to the solutions.

A comparison of the 0.0005375% and the 0.001075% solutions with 0.25% plasma as a blank is given in Graph No. 12.

CHROMATOGRAPHIC STUDIES

Although very few substances can be adsorbed from an aqueous solution, it was thought that this should be determined. It was found that in running water through alumina

(grade 7-20, mesh 80-200, Aluminum Ore Co.) a cloudy filtrate resulted unless the column (2 cm. x 20 cm.) was first prepared by running water through it. Hence a series of steps was devised, as follows:

<u>Amount in ml.</u>	<u>Material</u>	<u>Filtrate</u>	<u>Spectrophotometer sample number</u>
50	Distilled water	Cloudy	
50	" "	"	
50	" "	"	
50	" "	"	
50	" "	Less cloudy	
50	" "	Quite clear	I
50	" "	Quite clear	II
100	0.01 mg./ml. progesterone in distilled water	Quite clear	III
50	Distilled water	Quite clear	IV
50	Distilled water	Clear	V

Results in Graph No. 13 proved that alumina did not adsorb progesterone from aqueous solution.

PETROLEUM ETHER EXTRACTIONS

From Aqueous Solutions. With a Separatory Funnel.

Inasmuch as it appeared that the progesterone in blood would have to be concentrated, extraction with some organic solvent was indicated. Consequently 100 ml. of the 0.01 mg./ml. progesterone in water solution, made up 5 weeks earlier, was extracted with 100 ml. of petroleum ether (Baker's C. P. Special, boiling point 20°-40° C.) for 5 minutes. The water layer was saved and the ether layer was distilled off. The residue was taken up in 100 ml. of distilled water. After transferring the aqueous solution to another flask, 1 ml. of

absolute alcohol was added to the distilling flask, shaken and added to 99 ml. of water. This was done to establish whether there was any residue not taken up by the water alone. The absorption was determined on the various solutions using water as the blank for the aqueous systems and petroleum ether for the others. Graph No. 14 illustrates that petroleum ether removed progesterone from aqueous solutions.

In order to determine the character of the extraction products of water only, a second series was made up as follows: 100 ml. of petroleum ether was distilled, and the absorption curve for the distillate determined. The amount of 100 ml. of distilled water was extracted with 100 ml. of petroleum ether in a separatory funnel for 5 minutes. The water layer and the ether layer were run on the spectrophotometer. The same procedure was applied to 100 ml. of 0.01 mg./ml. progesterone solution. The absorption of the water layer was determined. The ether layer was evaporated to dryness, the residue being dissolved in 2 ml. of absolute alcohol and this added to 98 ml. of water. The absorption curves of the latter and the ether distillate were determined. Graph No. 15 revealed, as did No. 14, that petroleum ether extracts progesterone from aqueous solutions.

To recheck the above procedures, a third series was developed. The amount of 100 ml. of the progesterone solution (0.01 mg./ml.) was extracted for 10 minutes with 100 ml.

of petroleum ether. The ether layer was evaporated to dryness over a water-bath, 2 ml. of absolute alcohol was added to the residue, shaken, and this then added to 98 ml. of distilled water. The flask was rinsed with the resulting solution to take up the residue more completely. In an attempt to dissolve any remaining residue 2 ml. of alcohol was again shaken in the flask and added to 98 ml. of water. As a control on this latter procedure a clean flask was subjected to the same procedure. Absorption curves for these various products are presented in Graph No. 16, adding further evidence that petroleum ether extracts progesterone.

From Plasma after Precipitation of the Proteins. It was believed that if the proteins could be precipitated from the plasma without removing the progesterone that this would allow the latter to show up better. Two experiments were carried out to see if trichloroacetic acid would accomplish this. In the first, to 100 ml. of mixed plasma (obtained from cows A26, A27, and A33 about 10 weeks previously) was added 1 mg. of progesterone dissolved in 0.5 ml. of alcohol. This plasma was added to 1000 ml. of 10% trichloroacetic acid slowly with stirring. After standing 10 minutes, the mixture was filtered. This yielded 975 ml. of filtrate, which was diluted to 1000 ml. The amount of 200 ml. was extracted with 100 ml. of petroleum ether for 10 minutes. The residue left from evaporating the ether layer to dryness was taken up in 0.5 ml. of alcohol and 49.5 ml. of water. A control

was prepared in the same fashion omitting the added progesterone. Graph No. 17 shows the absorption curves.

The second experiment was similar but a proportionately larger amount of progesterone was used. One mg. of progesterone was dissolved in 0.5 ml. of alcohol and added to 10 ml. of the mixed plasma used above. A control 10 ml. sample of plasma was mixed with 0.5 ml. of alcohol only. To each of these, 100 ml. of 10% trichloroacetic acid was added as before. After standing 10 minutes and filtering, each filtrate was diluted to 100 ml. Eighty ml. of each was extracted with 80 ml. of petroleum ether for 10 minutes. Sixty ml. of the ether layer from each was evaporated to dryness, and the residue was taken up in 0.5 ml. of alcohol and 49.5 ml. of water. Graph No. 18 shows the curves for the filtrates, the water layers, the ether layers, and the redissolved residues. Graphs 17 and 18 indicated that progesterone could not be detected in the presence of plasma when the proteins have been precipitated by trichloroacetic acid.

From Plasma without Precipitation of the Proteins.
Synthetic Mixtures. It being possible that petroleum ether could efficiently extract progesterone from plasma in the presence of proteins, the following was tried. One mg. of progesterone was dissolved in 0.5 ml. of alcohol and added to 10 ml. of the plasma. As a control, 0.5 ml. of alcohol was added to 10 ml. of the plasma. Each was extracted for 5

minutes with 20 ml. of petroleum ether. A sludgy ether layer resulted, but it cleared considerably upon standing 25 minutes. The ether layer was filtered to remove the remaining emulsion. Each was diluted 1 ml. to 19 of petroleum ether and was then run against petroleum ether. Graph No. 19 shows that progesterone may be removed from plasma by petroleum ether extraction and establishes the absorption maximum at 230 millimicrons.

Normal Cows and Calves. On the possibility that a difference could be shown between the plasmas of cows in various stages of reproductive activity, it was decided to make extracts of the plasma and/or serum of a number of animals. Chart I indicates the procedure. All samples were separated into plasma and cells by centrifugation. The absorption curves of the ether layers (Graph No. 20) showed no significant differences between animals.

With a Continuous Extractor. From Aqueous Solutions. In an attempt to minimize the emulsion formed by the ether, a continuous liquid-liquid extractor was made. The apparatus is illustrated in Figure 1. The solution to be extracted (50 to 55 ml.) is introduced into the lower part of the extractor, the inner tube is inserted, and the condenser fitted to the top. One hundred ml. of solvent (petroleum ether) is placed in the flask and warmed to boiling over an electric coil. When it vaporizes enough to reach the condenser, it condenses and falls into the inner tube. As it

Animal no.*	Amt. bled, ml.	Cit- rated or not	Yield, plasma or serum	Amt. ex- tracted with 50 ml. ether	Time, min.	Time standing, hours	Ether layer, ml.	Water layer, ml.	Fil- tered	Remarks
A32	75	+	43.5	25	5	27	46.0	23	+	Bred 9 days before; considerable emul- sion formed.
A22 (1)100 (2)100		+	65.5 59.0	25 25	5 5	21 5	44.0 44.0	25 23	+	Bred 17 days before. Bred 17 days before.
A27	100	+	65	25	5	6	43.0	25	+	Bred 18 days before.
G568	100	+	55	25	5	3	40.0	22	+	14 months old male.
G571	116	+	58	25	12	1.5	41.0	23	+	14 months old male.
127	102	-	49	25	12	1.25	41.5	27	+	7 months pregnant.
127	110	+	61	25	12	1	43.0	27	+	7 months pregnant.
442	56	+	31	25	12	1	37.5	27	+	7 months pregnant.

*C indicates calf.

CHART I

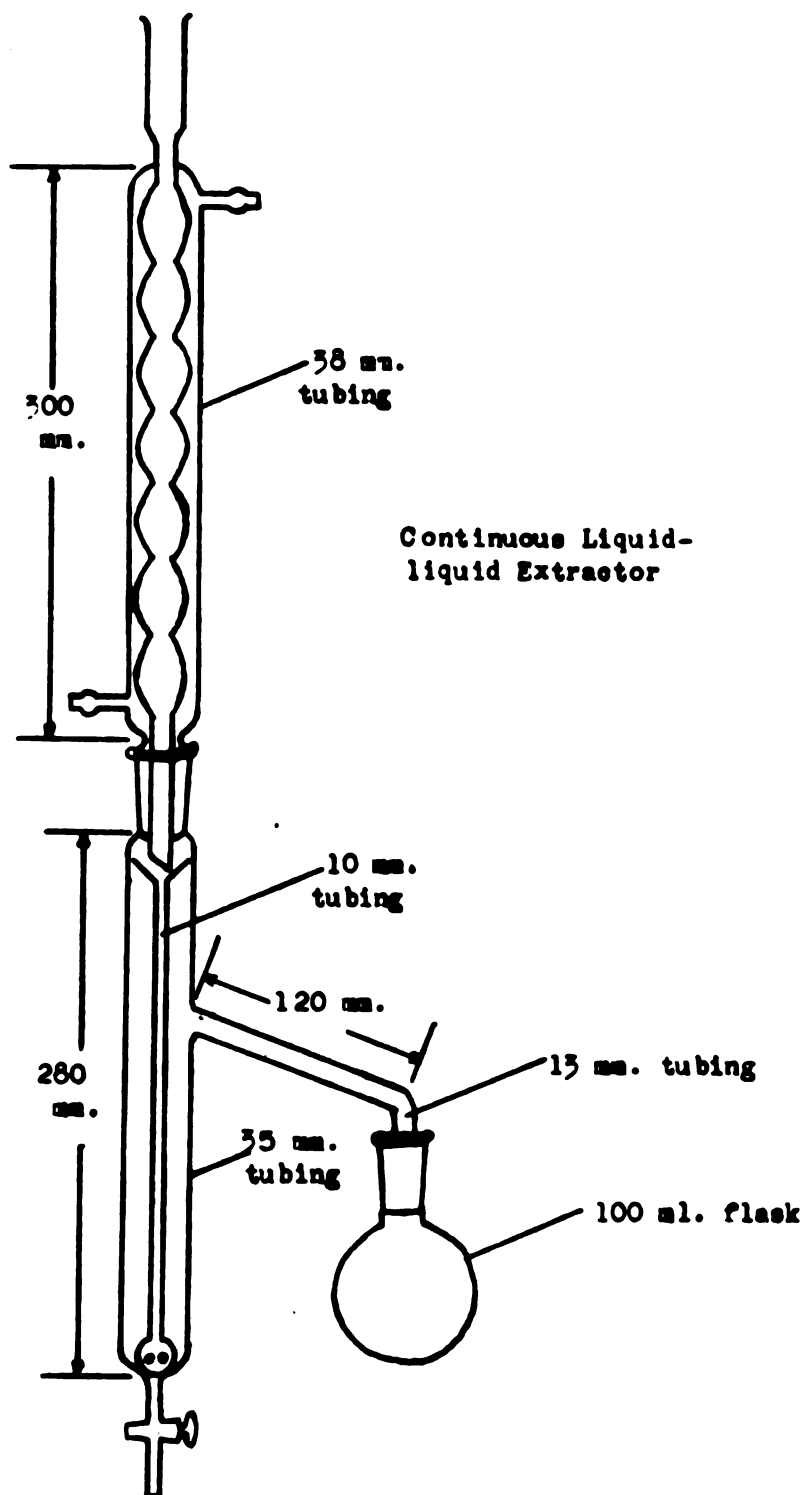


Figure 1
Scale: 1 mm. = 4 mm.

piles up there its weight forces solution down and out the holes in the bulb at the lower end of the tube. The solvent, being lighter than the aqueous layer, rises to the top and accumulates there. When it surpasses the level of the sloping side-arm it gravitates back into the flask. Thus the solvent circulates through the solution and has an opportunity to dissolve any soluble material.

The extractor was first used to remove some progesterone from 50 ml. of the 0.01 mg./ml. aqueous stock solution made up 3 1/2 months before. The extraction proceeded for 2 1/2 hours, when the ether in the extractor was added to the flask ether and the absorption curve determined using petroleum ether as a blank. Graph No. 21 shows that this extraction was at least partly successful.

It was necessary to start using a new bottle of petroleum ether. This was a Merck product, boiling point 30°-60°C. This was found to transmit no light between 230 and 260 millimicrons; so it was discarded.

A bottle of petroleum ether similar to the first was used with interesting results. The amount of 50 ml. of the 0.01 mg./ml. aqueous solution of progesterone was extracted with 100 ml. of this ether (hereafter called petroleum ether II) for 1 hour and 40 minutes. An equal amount of distilled water was used as a control. The extractor ether in both cases showed per cent transmissions above that of the pure solvent. The curves for the flask ether are shown

in Graph No. 22, along with a plotting of the slit-widths. The latter, being highly erratic, indicated that the petroleum ether differed from the first lot used (called petroleum ether I). Petroleum ether II was therefore discarded in favor of another lot, petroleum ether III. The labels of these three lots of Baker's C. P. Special Petroleum Ether, B. P. 20°-40°C. gave the following information:

	Lot I	Lot II	Lot III
Sp. Gr. at 15°/15°C.	0.650	abt. 0.62	abt. 0.62
Initial Boiling Point	31.5°C.	31.3°C.	30.0°C.
Maximum Boiling Point	35.0°C.	43.4°C.	35.2°C.
Nonvolatile matter	0.0002%	0.0004%	0.0002%
Sulfur compounds (as S)	0.000%	0.000%	0.000%
Heavy oils	0.000%	0.000%	0.000%

As some difficulty was encountered when working with petroleum ether in the spectrophotometer cells, due to rapid evaporation of the more volatile fractions, it was decided to evaporate the petroleum ether off and redissolve the residue in alcohol or water before determining the absorption curves. Fifty ml. of the old 0.01 mg./ml. stock solution was extracted with petroleum ether II for 1 hour. The residue, after evaporating the mixed flask and extractor ether, was dissolved in 0.5 ml. of absolute alcohol, and 49.5 ml. of water was added to it. A cloudy solution resulted, but the curves showed the extraction, when compared with the extraction of plain water, was nevertheless successful (Graph No. 23).

A new aqueous stock solution of progesterone was prepared as before by dissolving 10 mg. in 5 ml. of absolute

alcohol and adding this to distilled water to make a liter. A 50 ml. portion of this and a 50 ml. portion of plain water were extracted exactly as described in the preceding paragraph, except that the flask ether and the extractor ether were kept separate, and the alcoholic residue was added to the 49.5 ml. of water. This resulted in a very nearly clear solution. In making the absorption curves, the flask extract of progesterone solution was used against the flask extract of water as a blank, and the extractor ether extracts in a similar way. Graph No. 24 offered further proof of the ability of petroleum ether to extract progesterone.

From the Blood Plasma of a Cow Injected with Progesterone. It was decided at this stage to attempt to recover some injected progesterone from the blood stream of a cow. It was estimated that a 1000 pound cow would require 40 mg. of progesterone in order to have about 0.25 mg. in 200 ml. of blood, a quantity with which it was convenient to work. Therefore, 40 mg. was dissolved in 5 ml. of absolute alcohol and injected in that form as follows:

Blood was collected in 125 ml. flasks each containing 0.5 gm. of sodium citrate crystals.

Time	Approximate amount bled ml.	Sample no.	Remarks
4:24	100	1	From left jugular vein
4:25	100	2	From left jugular vein
4:29			40 mg. progesterone in 5 ml. absolute alcohol injected into right jugular vein
4:31	100	3	From left jugular vein
4:32	100	4	" " " "
4:35	100	5	" " " "
4:37	100	6	" " " "
4:40			Finished bleeding

Samples 3, 4, 5, and 6 represent continuous bleeding interrupted only by changing needles between samples 4 and 5. All samples were stored in a refrigerator over night.

The following day the plasma and the cells of each sample were separated by centrifugation with results as follows:

Sample no.	Total volume, ml.	Plasma volume, ml.	Remarks
1	108	71.0	
2	113	72.0	
3	110	72.5	
4	105	70.0	Red tinge, Due to hemol- ysis when needle was changed.
5	104	70.0	
6	109	74.5	

The plasma samples were stored in a refrigerator when not being used.

Two days later, the extracting of these samples was begun. Samples 1 and 2 were extracted with the same 100 ml. portion of petroleum ether II as were samples 3 and 4, and

also 5 and 6. The table shows the procedure:

Sample no.	Amt. of plasma ml.	Extraction time, hrs.	Amt. of ether ml.	Ether sample	Remarks
1	50	1.5	100	A	Large bubbles in ether layer. Let stand 2 hours 20 min.
1 2	16 30 }	1.7	100	A	Heated more slowly. Emulsification not so extensive.
2	35	1.5	100	A	Little emulsification
3	50	1.5	100	B	Little emulsification
3 4	17 32 }	1.5	100	B	Little emulsification
4	32	1.5	100	B	Little emulsification
5	50	1.5	100	C	Some emulsification
5 6	16 25 }	1.5	100	C	Some emulsification
6	42	1.5	100	C	Some emulsification

After evaporating the ether samples to dryness in a water-bath, each residue was dissolved in 0.5 ml. of absolute alcohol, and added to 24.5 ml. of distilled water. The first sample, A, resulted in a slightly cloudy solution; B was less cloudy than A; C was less cloudy than A but more cloudy than B. Using distilled water as a blank, the absorption curves (Graph No. 25) were found to be influenced considerably by the cloudiness and to indicate that progesterone was

not recovered.

The cloudiness described above might have been due to some ether reacting with the rubber stopper in the distilling flask; so an experiment was run as above, using 100 ml. of petroleum ether II and 38 ml. of a mixture of the aqueous solutions B and C above. The ether layer was evaporated to dryness without the use of a rubber stopper. When redissolved in 0.5 ml. of alcohol and 24.5 ml. of distilled water, a cloudy solution resulted, indicating that some non-volatile materials in the ether were forming an emulsion with water.

From Hemolyzed Blood of a Cow Injected with Progesterone. Bloch (15), in attempting to detect the presence of progesterone by biological means, used hemolyzed blood. To determine if hemolyzed blood could be used in the extractor, the following experiment was set up. All blood collections were made into 125 ml. Erlenmeyer flasks containing 0.5 gram of sodium citrate.

<u>Time</u>	<u>Approximate amt. bled, ml.</u>	<u>Sample no.</u>	<u>Remarks</u>
4:30	100	1	Flask contained 2 mg. of progesterone (and citrate). From left jugular vein.
4:31	100	2	From left jugular vein.
4:33	100	3	From left jugular vein.
4:35			75 mg. of progesterone in 10 ml. of absolute alcohol into right jugular vein
4:38	100	4	From left jugular vein
4:39	100	5	From left jugular vein
4:40	100	6	From left jugular vein
4:42	100	7	From left jugular vein
4:44			Finished bleeding

Samples 1, 2, and 3 represent continuous bleeding, as do 4, 5, 6, and 7. The blood was stored in the refrigerator.

The hemolyzing of this blood was accomplished by adding 200 ml. of distilled water to 100 ml. of blood. Then each resulting solution was extracted in the continuous extractor as outlined below.

Sample no.	Amount of hemolyzed blood, ml.	Extraction time, hours	Amt. of ether used, ml.	Ether lot no.	Ether sample
1	50	1.4	100	III	A
1	51	1.4			
1	56	1.5			
1	55	1.5			
1	55	1.5			
1	55	1.5	100	II	B
2	50	1.6			
2	50	1.5			
2	50	1.5			
2	50	1.5			
2	50	1.3			
2	50	1.4			
3	55	1.5	100	III	C
3	55	1.5			
3	55	1.5			
3	55	1.5			
3	52	1.5			
3	52	1.5			
4	150*	3.0	200*	III	D
4	150	3.5	200*	III	E
5	150*	3.0			
5	150	3.0	100	III	F
6	50	2.0			
6	50	1.5			
6	50	1.5			
6	50	1.5			
6	50	1.7			
6	50	1.5			

*A new, larger extractor was made and used for samples 4 and 5 but discarded for sample 6 as the results were not satisfactory.

All ether samples were evaporated to dryness over

steam. A 100 ml. portion of petroleum ether II and a like amount of petroleum ether III were evaporated to dryness over steam. The residues from A, B, C, petroleum ether II, and petroleum ether III were taken up in 25 ml. of absolute alcohol. Those from D and E were taken up in 15 ml. of absolute alcohol, and that from F in 20 ml. The absorption curves were determined using absolute alcohol in the solvent cell. Graph No. 26 showed that progesterone was partially recovered from hemolyzed blood when it had been added to the whole blood and that it was not recovered from the blood of a cow following intravenous injection.

To get an estimate of the amount of progesterone recovered from the blood to which it had been added, a concentration curve for progesterone in absolute alcohol was constructed. Five mg. of progesterone was dissolved in 100 ml. of absolute alcohol ($= 0.05 \text{ mg./ml.}$). This was diluted with alcohol to make 0.02 mg./ml. , 0.01 mg./ml. , 0.005 mg./ml. , 0.0025 mg./ml. , and 0.001 mg./ml. The absorption curves were determined on the same day using absolute alcohol as a blank. These curves were used in Graphs No. 27 and No. 28, which showed that progesterone in alcohol follows Beer's law in the concentration range used.

Absorption curves on the same alcoholic samples were made again 6 days later and showed the variations noted in Graph No. 5.

RESULTS AND DISCUSSION

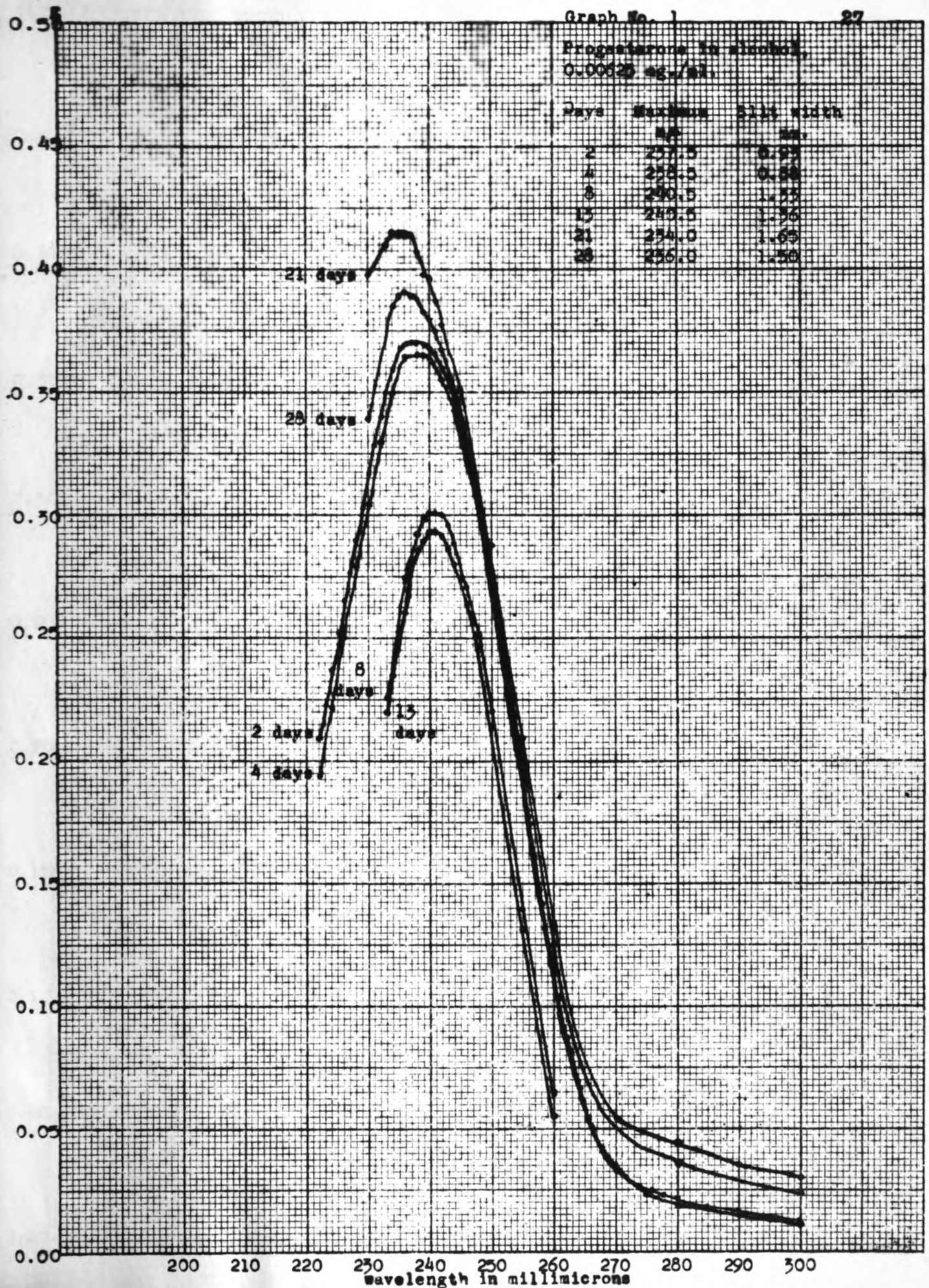
PRELIMINARY ABSORPTION CURVES

Absolute Alcohol. Graphs 1, 2, 3, and 4 indicate that alcoholic solutions of progesterone show spectrophotometric curves which are highly variable, both as to wave length of the maximum absorption and also as to the amount of absorption. It was noted that the results obtained on the day the solutions were prepared, were quite consistent, but after that there was no correlation between the curves and the time elapsed. It was apparent that the compound changed on standing, or that temperature or some other factor had objectionable effects. A combination of these effects is possible, and in spite of explanations, for the purpose of this problem, such variability was not to be desired. The variations in the slit-widths at the maximum are explained by the necessity of adjusting the hydrogen lamp in order to get maximum intensity from it. This change alone would affect the curves but other factors were obviously operating, as a change in slit-width did not always produce the same effect upon the curve. (See Graph No. 5.)

Graph No. 5 assembles pertinent data from the preceding graphs and illustrates the lack of definite correlation between the extinction, the wave length of maximum absorp-

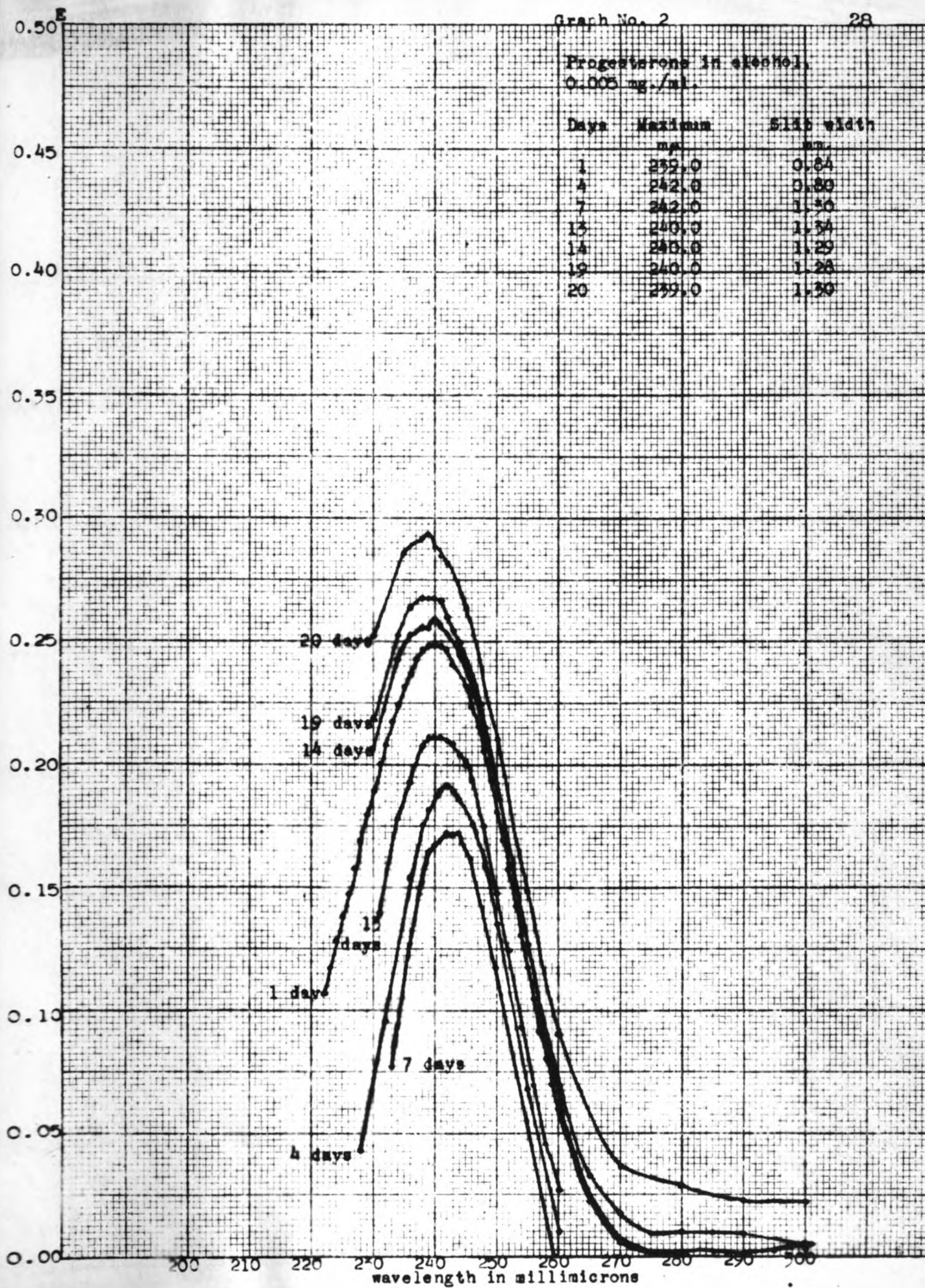
Progesterone in alcohol,
0.00525 mg./ml.

Days	Maximum mp.	Slit width mm.
2	237.5	0.95
4	238.5	0.95
8	240.5	1.55
15	240.5	1.55
21	244.0	1.65
28	246.0	1.50



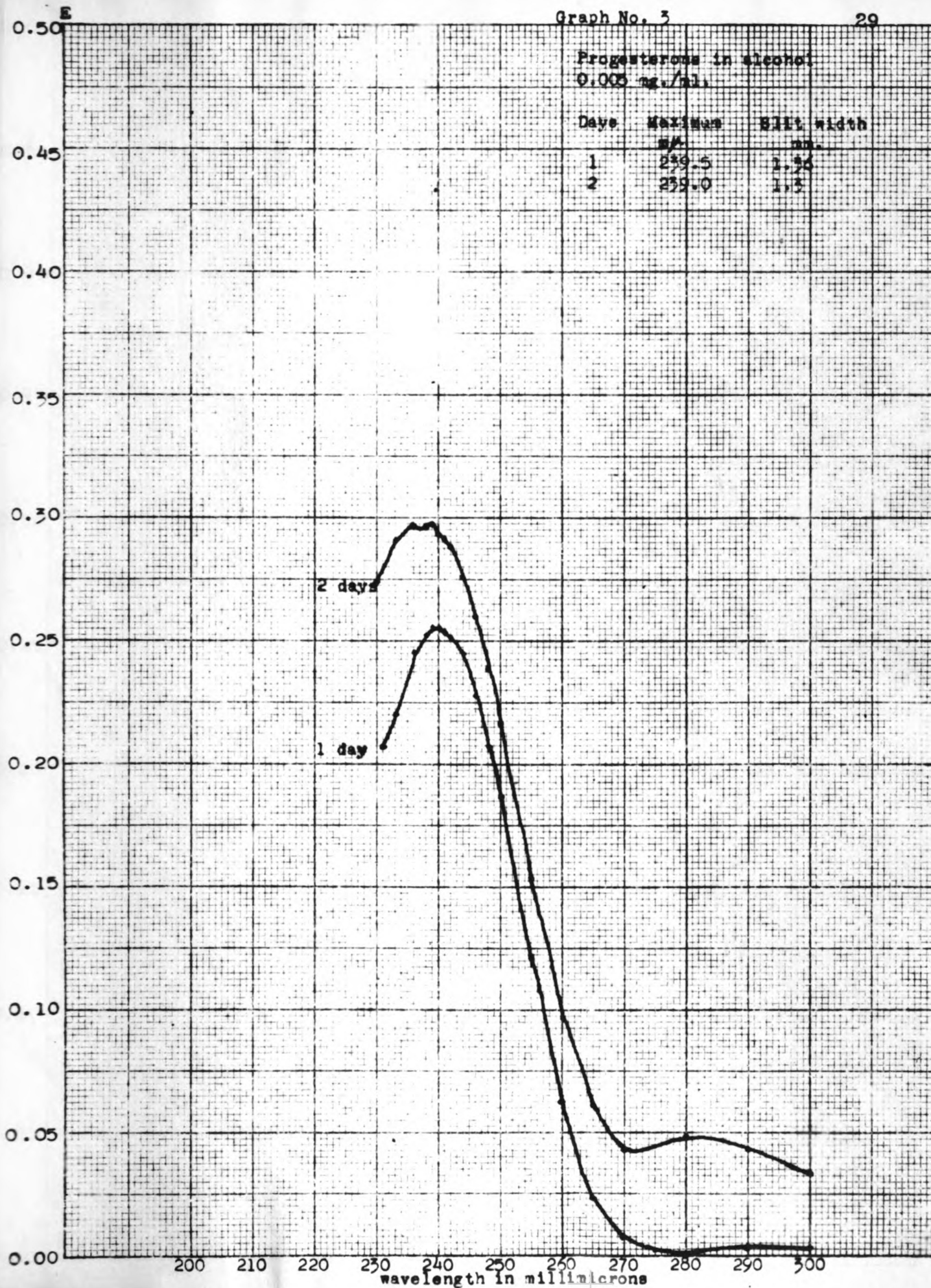
Progesterone in alcohol,
0.005 mg./ml.

Days	Maximum mm.	Slit width mm.
1	232.0	0.84
4	242.0	0.80
7	242.0	1.50
13	240.0	1.54
14	240.0	1.29
19	240.0	1.28
20	239.0	1.50



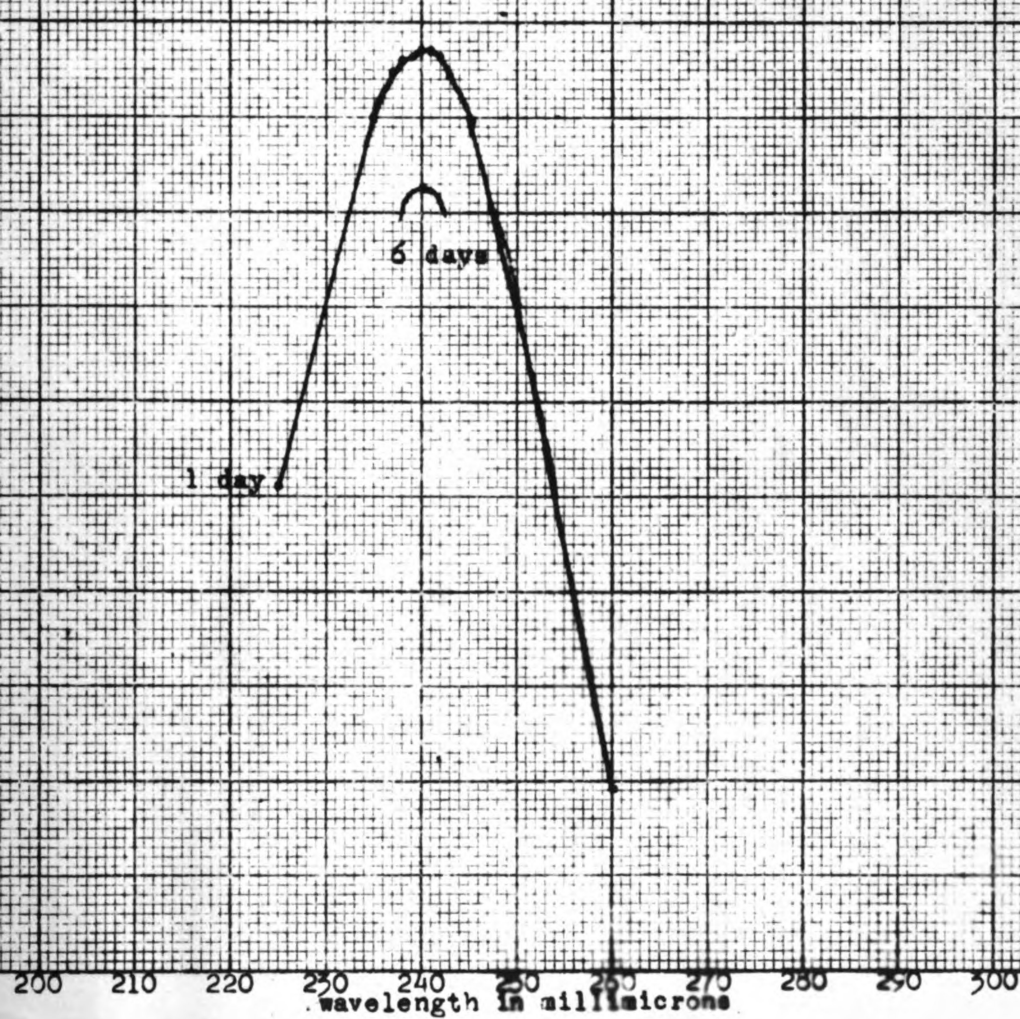
Progesterone in alcohol
0.005 mg./ml.

Days	Maximas mμ	Slit width mm.
1	239.5	1.36
2	239.0	1.3



Progesterone in alcohol
0.005 mg./ml.

Days	Maximum m μ	Slitwidth m μ
1	240.0	0.54
6	240.0	0.56



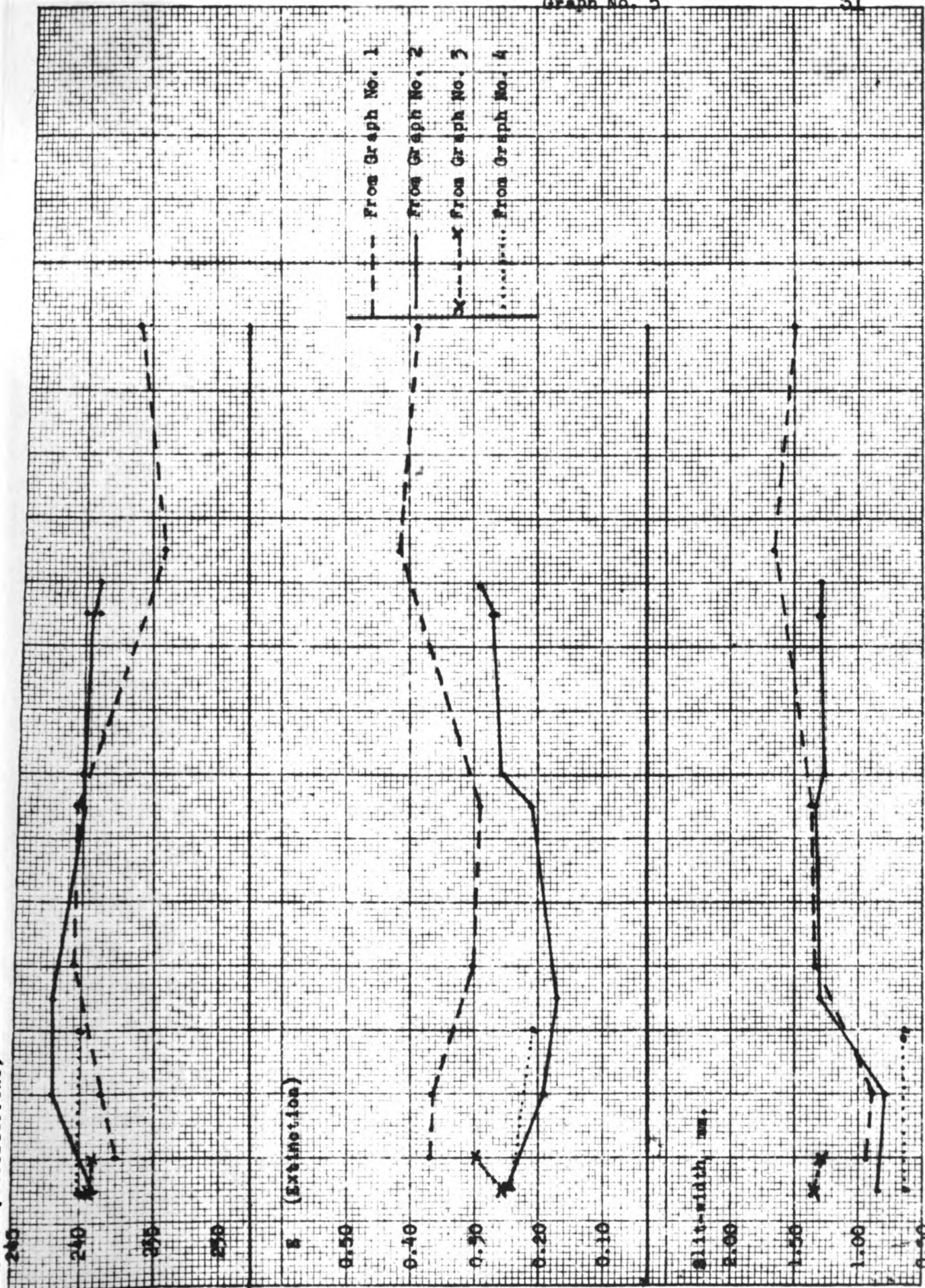
Max. (millimicrons)

E (Extinction)

Slit-width, mm.

Days

From Graph No. 1
From Graph No. 2
From Graph No. 3
From Graph No. 4

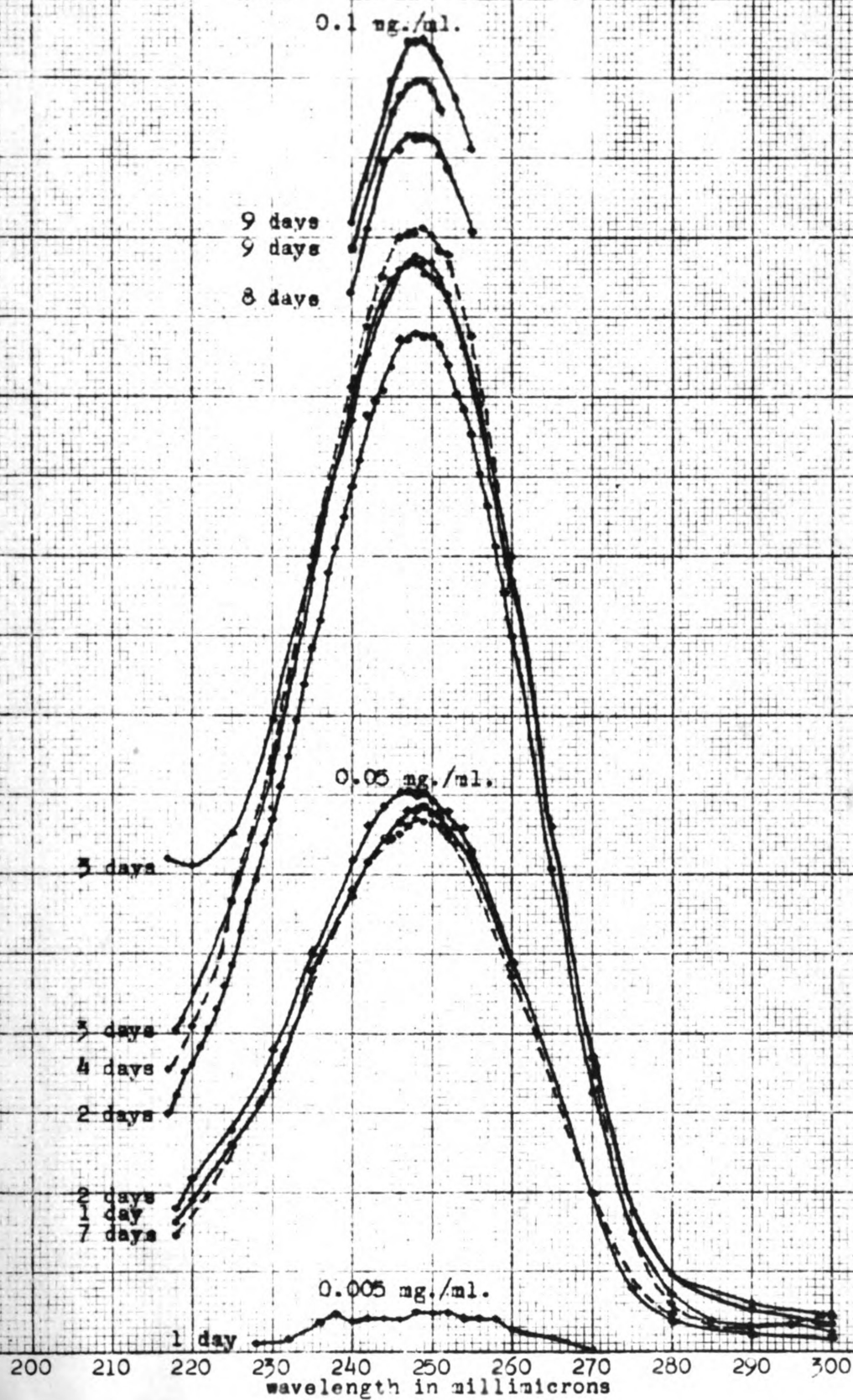


tion, the time since making the solution, and the slit-width. However, there seems to be a general tendency for: the extinction (E) to increase as the slit-width increases; the maximum to be lowered when the slit-width increases; the maximum to be lowered as the extinction is increased. None of these holds true in all cases.

Distilled Water. Graph No. 6 shows less variation between distilled water curves than between the alcoholic curves, and these variations may be explained in part by a factor which did not influence the alcohol curves. Progesterone is much less soluble in water than in alcohol and the upper curves in Graph 6 are from saturated solutions. The lower curves represent a half-saturated solution and might therefore be more constant, as they appear to be from the curves.

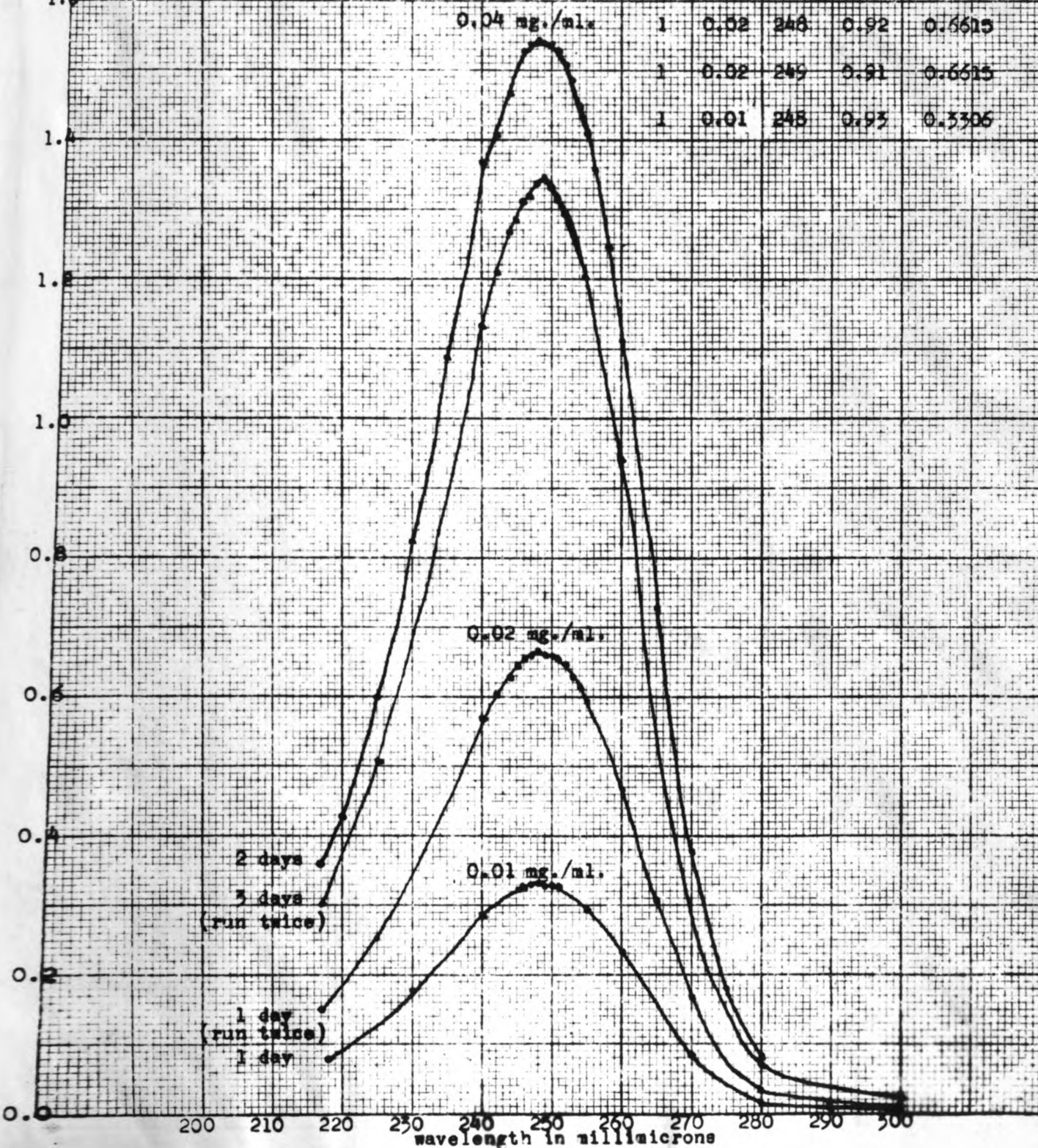
Graph 7 illustrates that preliminary dissolving of the progesterone in alcohol facilitates its solution in water. The greater E values indicate more progesterone in solution. There is a good correlation between the height of the curves and the concentration, with the exception of the curve for the 0.04 mg./ml. solution run on the second day. If all are run the same length of time after they are made up, satisfactory curves are obtained. This is shown in Graph No. 8 where E values are plotted against concentration. These determinations were made within 24 hours after the solutions were made up, and the results show a nearly

Progesterone in Distilled Water
Saturated solution and 1:1 dilution
of same.

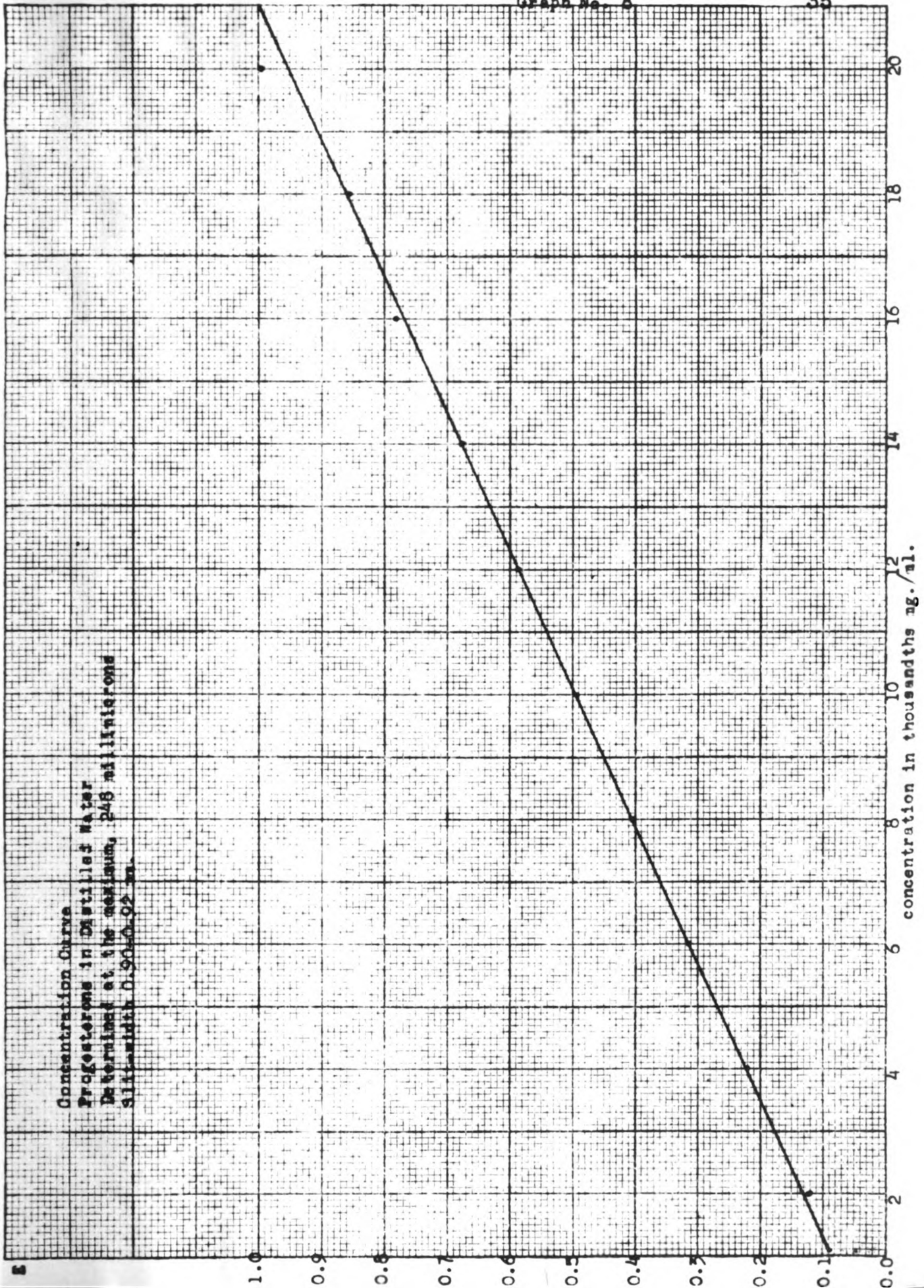


Progesterone in Distilled Water
0.04, 0.02, and 0.01 mg./ml.

Day	conc.	Max. mμ	SW mm.	E at Max.
2	0.04	248	0.92	1.5687
3	0.04	249	0.91	1.5450
3	0.04	248	0.92	1.5450
1	0.02	248	0.92	0.6615
1	0.02	249	0.91	0.6615
1	0.01	248	0.93	0.3306



Concentration Curve
Progesterone in Distilled Water
Determined at the minimum, 248 millimicrons
slit-width 0.90-0.92 mm.



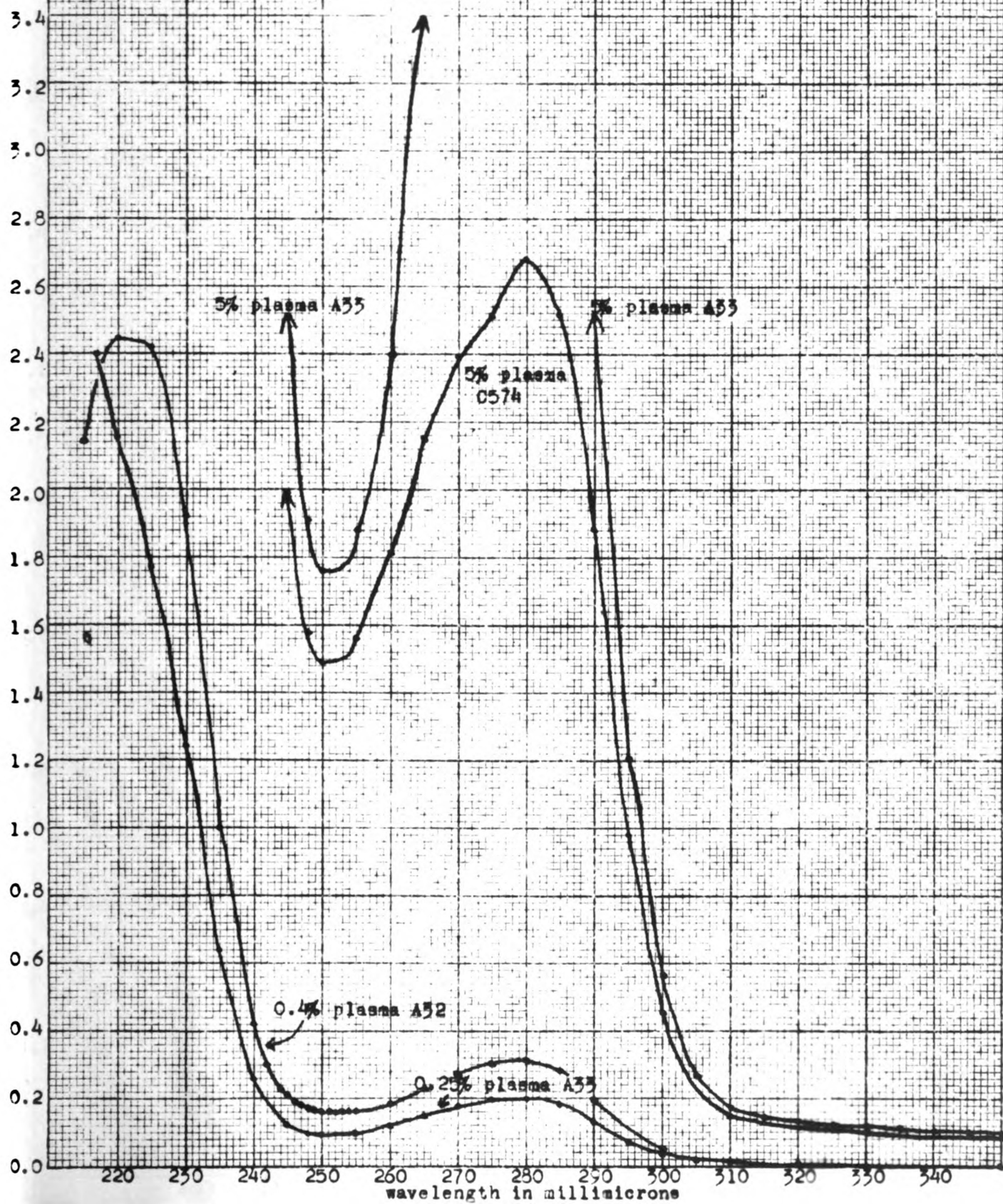
straight line. This was taken to mean that if progesterone could be obtained in an aqueous solution, an estimate of the concentration could be made.

DILUTED BLOOD PLASMA

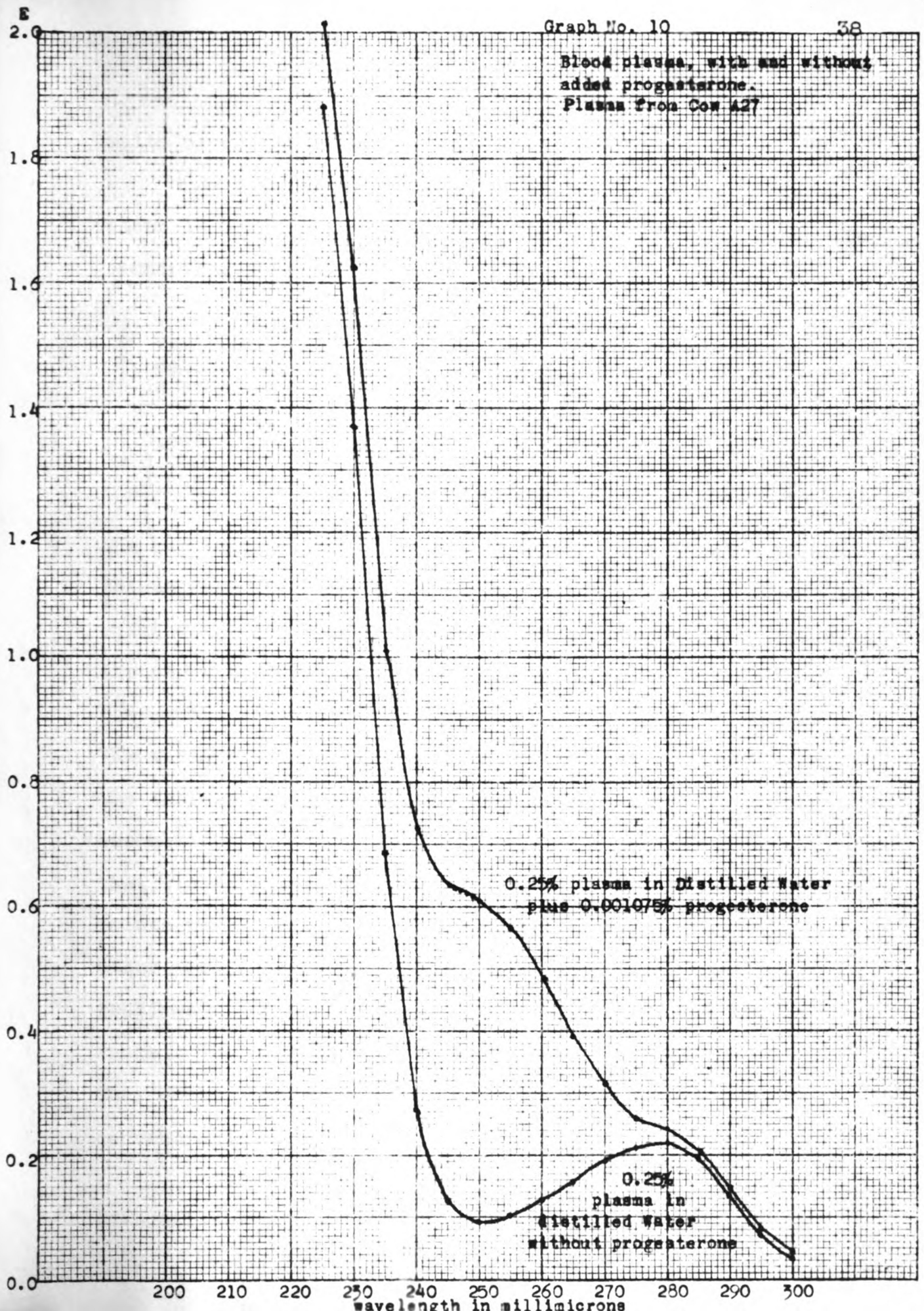
The protein present in blood plasma shows a maximum absorption at 280 millimicrons. Considerable dilution (1:400) is necessary to bring the extinction down to a suitable level. No difference was found in comparing the curves from different animals (Graph No. 9). A difference could hardly be expected in the presence of such dilution.

Progesterone added to this highly diluted plasma caused an alteration in the curve as shown in Graph No. 10. If the absorptions at wave length 248 millimicrons (the maximum for progesterone in water) are compared, the following is revealed. The extinction (E) of the progesterone-containing sample is 0.6200. From the concentration curve (Graph No. 8) this would represent 0.0127 mg./ml. if it were not for the fact that the diluted plasma alone shows some absorption at this wave length. This latter shows an extinction of 0.1000 which would subtract 0.0016 mg./ml. The difference is 0.0111 mg./ml. or 0.00111% progesterone. The actual amount added was in a percentage of 0.001075. While the above method of calculating may be open to criticism, it appears that the analysis of the mixture gave quantitative results for the progesterone added. (See Graph No. 12 for re-

Blood plasma, 5% in Distilled
Water and 0.25% in Distilled
Water. Also 0.4% in Distilled
Water.



Blood plasma, with and without
added progesterone.
Plasma from Cow 427



sults of running the solutions against a plasma blank.)

An extension of the work seen in Graph No. 10 is shown in Graph No. 11. Interesting data from this graph are summarized below:

Per cent progesterone in sample	Observed E at 248 m μ .	Per cent progesterone represented by E value	Calculated per cent progesterone
None	0.1045	0.00014	
0.0005375	0.3778	0.00074	0.000600
0.001075	0.5751	0.001175	0.001035
0.00215	1.0810	0.002200	0.002060

The per cent progesterone represented by the E value is obtained from the concentration curve (Graph No. 8). The calculated per cent progesterone is determined by subtracting the percent progesterone represented by the extinction of the sample containing no hormone (0.00014) from those for the various concentrations. Comparing the first column in the table with the last, the weakest solution is the only one noticeably out of line. This may be explained by noting that, in Graph No. 8, E values below 0.2 and above 0.7 give points which fall to one side of the straight line. In other words, very low or very high absorptions result in readings which are inconsistent with those in the optimum range. These deviations indicate that progesterone, like many other substances, does not adhere to Beer's law at all concentrations.

Graph No. 12, which compares these plasma solutions with diluted plasma in the blank cell, shows maxima at 248

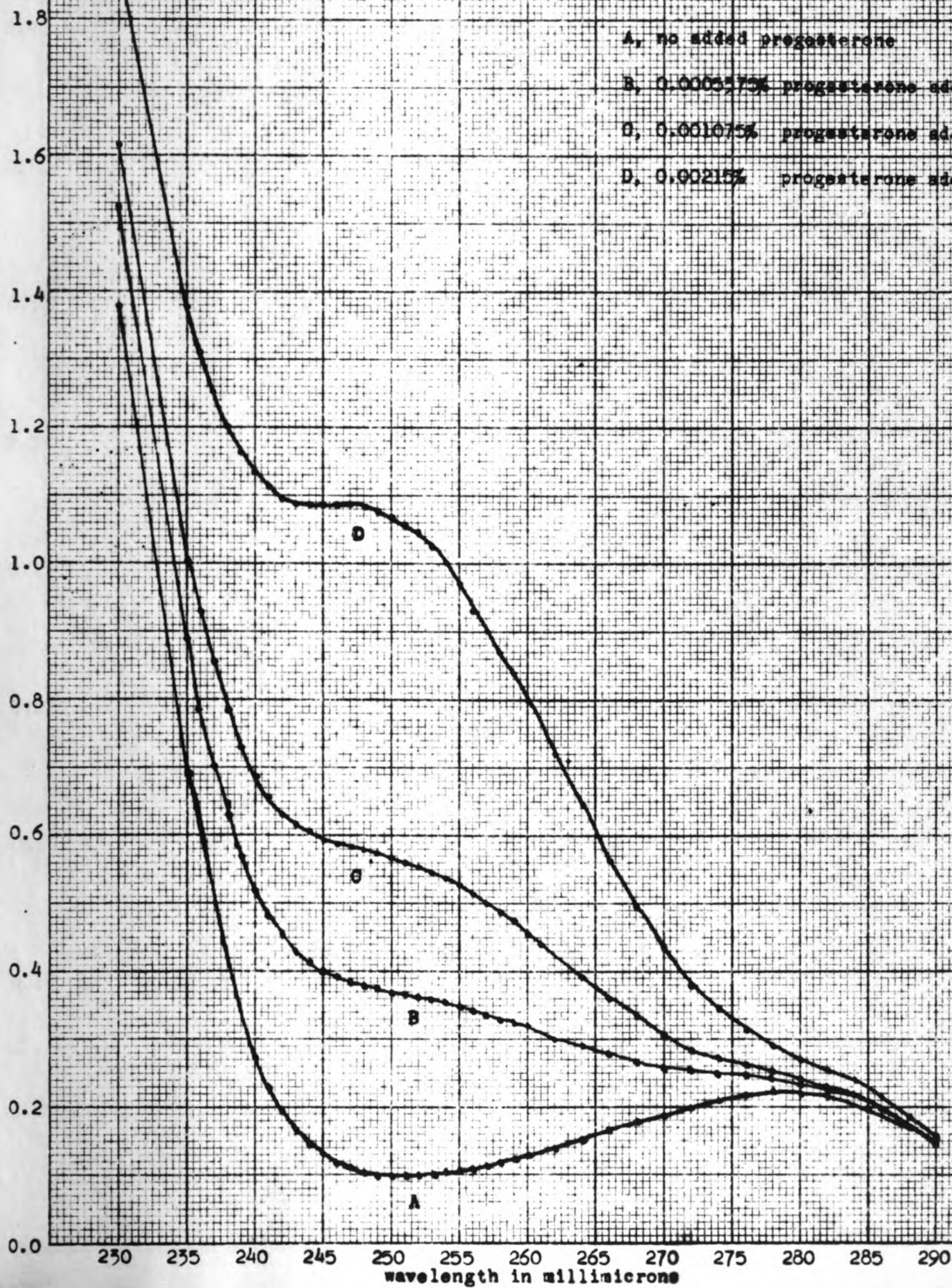
E

Graph No. 11

40

Blood plasma from Cee A27
diluted with Distilled Water to
make 0.2% plasma. Progesterone
added as follows:

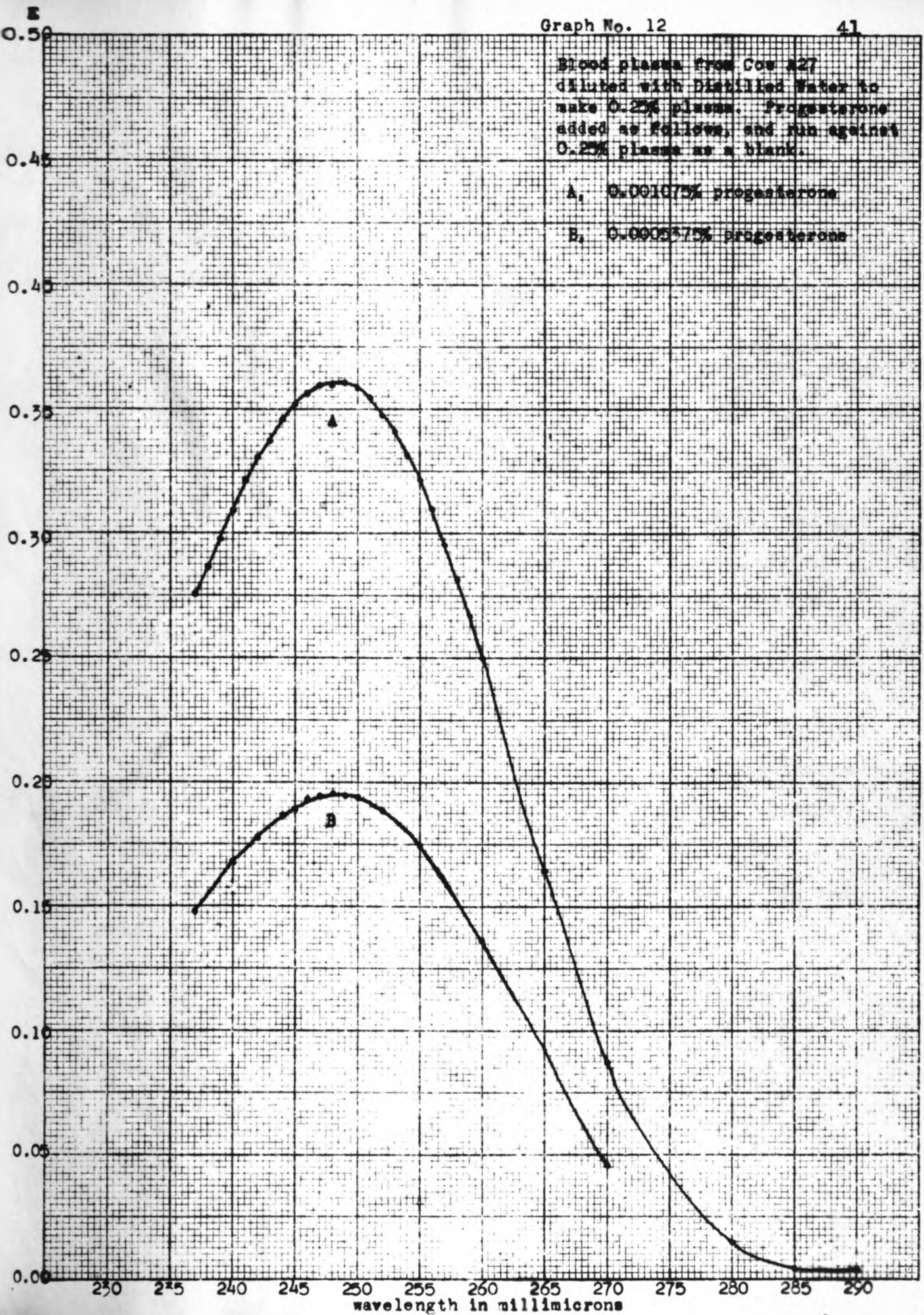
- A, no added progesterone
- B, 0.000575% progesterone added
- C, 0.001675% progesterone added
- D, 0.00215% progesterone added



Blood plasma from Cow #27
diluted with Distilled Water to
make 0.2% plasma. Progesterone
added as follows, and run against
0.2% plasma as a blank.

A. 0.001075% progesterone

B. 0.0005375% progesterone



millimicrons as expected, and no other peaks are encountered. However, the calculation of the amount of progesterone present, using the water concentration curve, gives results which are too low (35-36% below the actual values).

Per cent progesterone in sample	Observed E at 248 m μ .	Per cent progesterone represented by E value (from concentration curve)	Per cent deviation from progesterone in sample
0.0005375	0.1959	0.00034	-36
0.001075	0.3595	0.00070	-35

This indicates that a new concentration curve, using 0.25% plasma as a blank and dissolving the progesterone in the same, would be necessary if this type of study were to be extended.

CHROMATOGRAPHIC STUDIES

It is shown clearly in Graph No. 13 that progesterone is not adsorbed from aqueous solution by passing it through alumina. All is accounted for by estimating the amount which came through and comparing this with the amount in the solution at the start (1.0 mg.).

Sample	E at 248 m μ .	Progesterone calculated from concentration curve, mg./ml.	Volume of sample, ml.	Calculated progesterone in sample, mg.
III	0.3545	0.00685	100	0.6850
IV	0.3448	0.00667	50	0.3335
			Total	1.0185

Chromatographic studies.

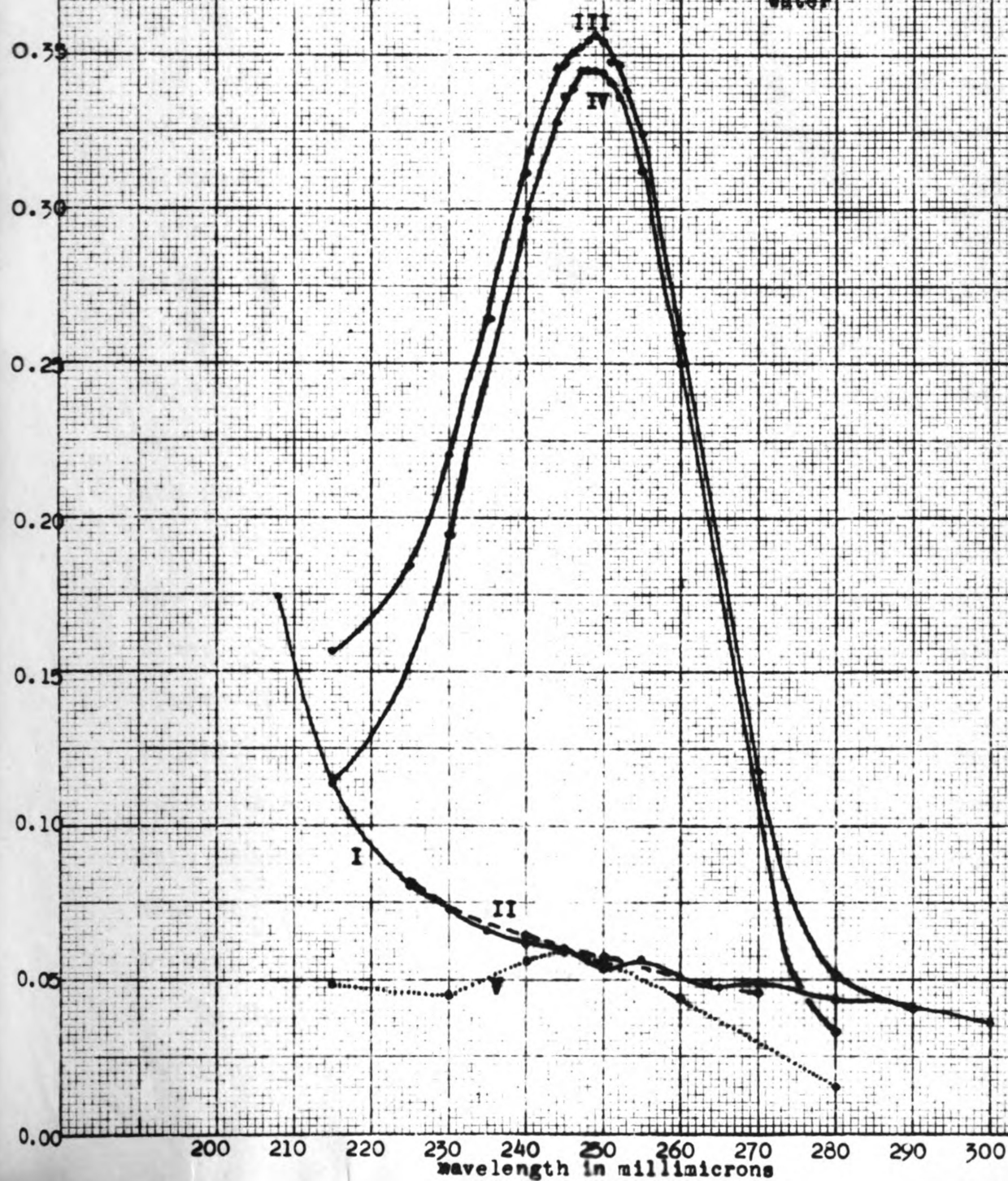
I Distilled water through alumina

II (---) Second sample of water through alumina

III Progesterone in water through alumina

IV First washing with water

V (---) Second washing with water

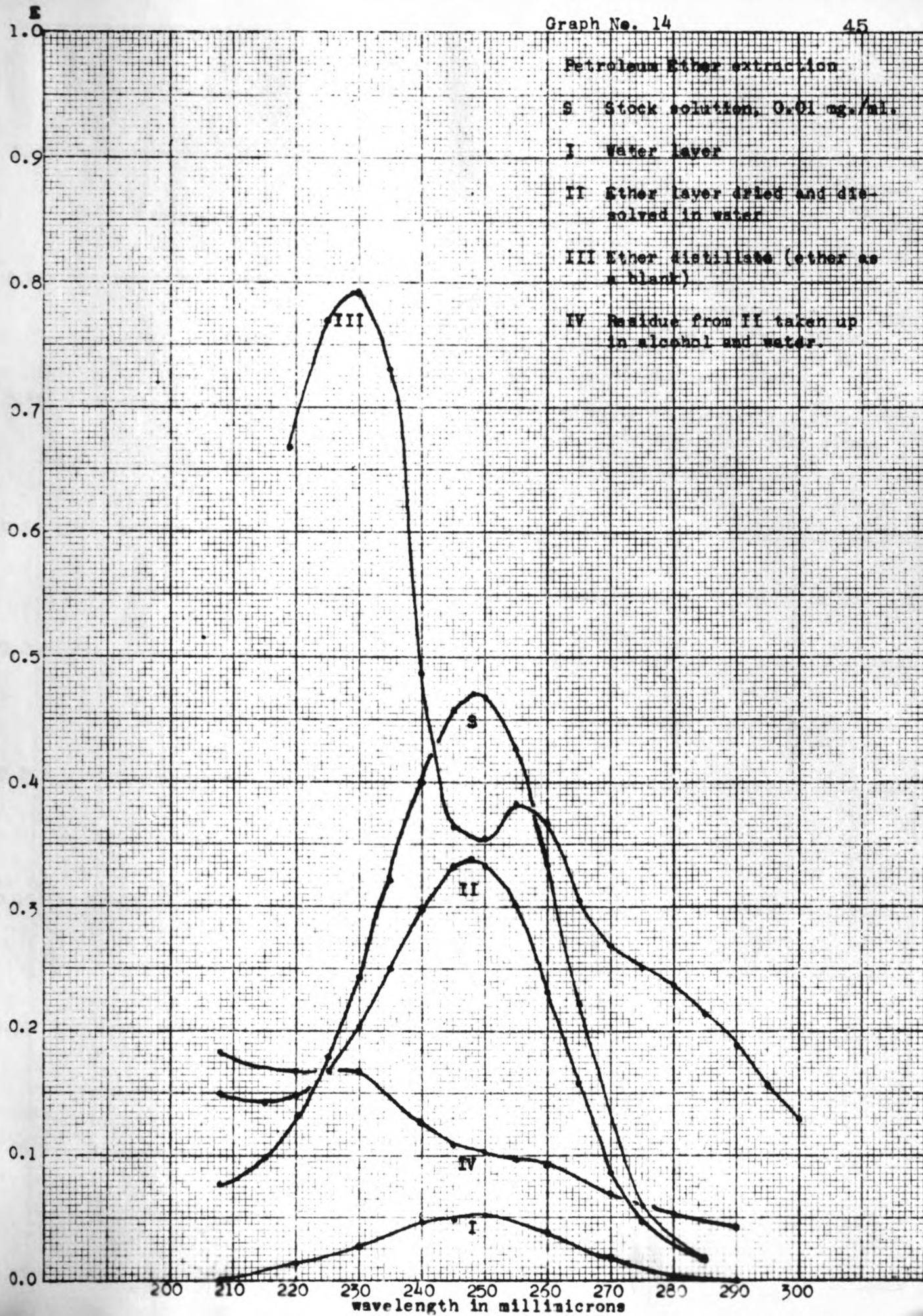


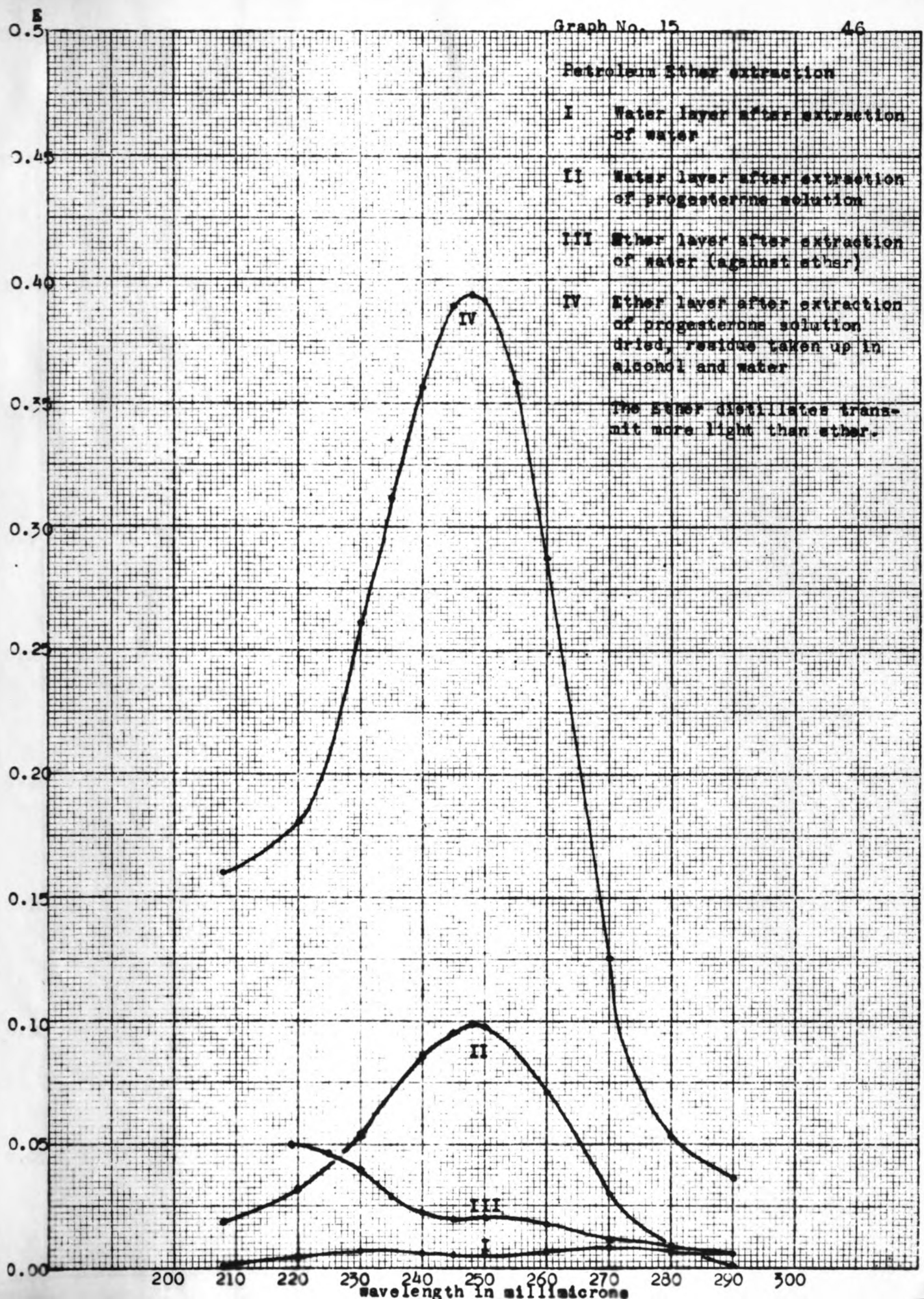
PETROLEUM ETHER EXTRACTIONSWith a Separatory Funnel. From aqueous solutions.

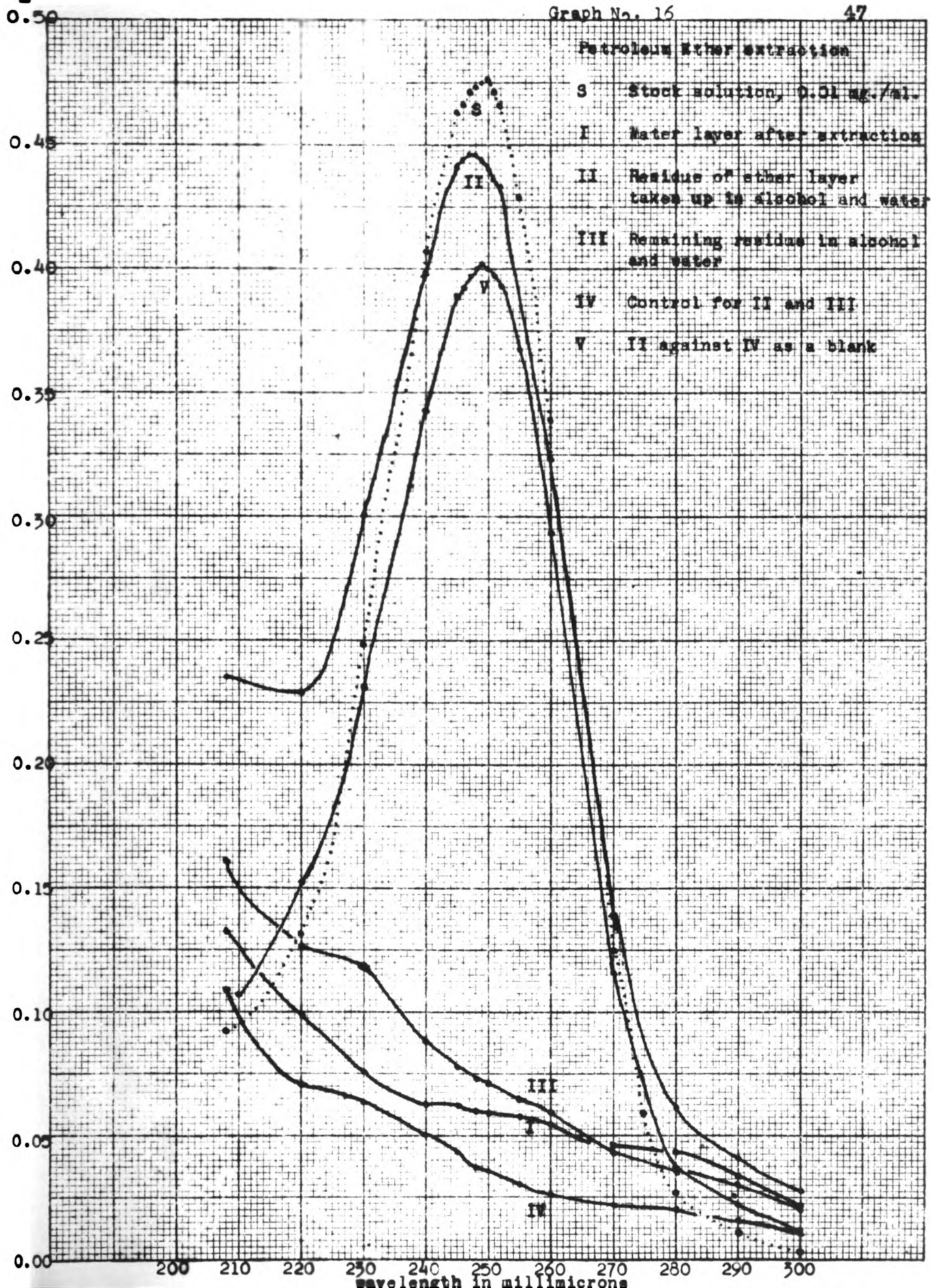
Graph No. 14 shows that petroleum ether removes progesterone from aqueous solutions. The stock solution (0.01 mg./ml.) was checked and showed a concentration, according to Graph No. 8, of 0.0095 mg./ml. The ether extract, after drying and dissolving in water (Curve II) contained 0.0065 mg./ml. or 68% of the original amount. The water layer (Curve I) accounted for 0.0005 mg./ml. or 5% of the original. The loss is quite large, but this work suggested that more complete extraction might be more satisfactory. It will also be noted that the distilled ether gives a characteristic type of curve. This will be considered more fully when comparing the different lots of petroleum ether (see Graphs 22 and 26).

Graph No. 15 likewise shows incomplete extraction of the progesterone, probably from too short an extraction time. The dissolved residue from the progesterone solution (Curve IV) has a calculated progesterone content of 0.00775 mg./ml. or 82% of the original amount. The water layer (Curve II) still contained 0.00125 mg./ml. or 13%. This accounts for 95% of the progesterone used. Curve I also indicates that water dissolves enough petroleum ether to alter its absorption slightly.

Rechecking the above gave the data for Graph No. 16. The stock solution was checked and was found to contain a







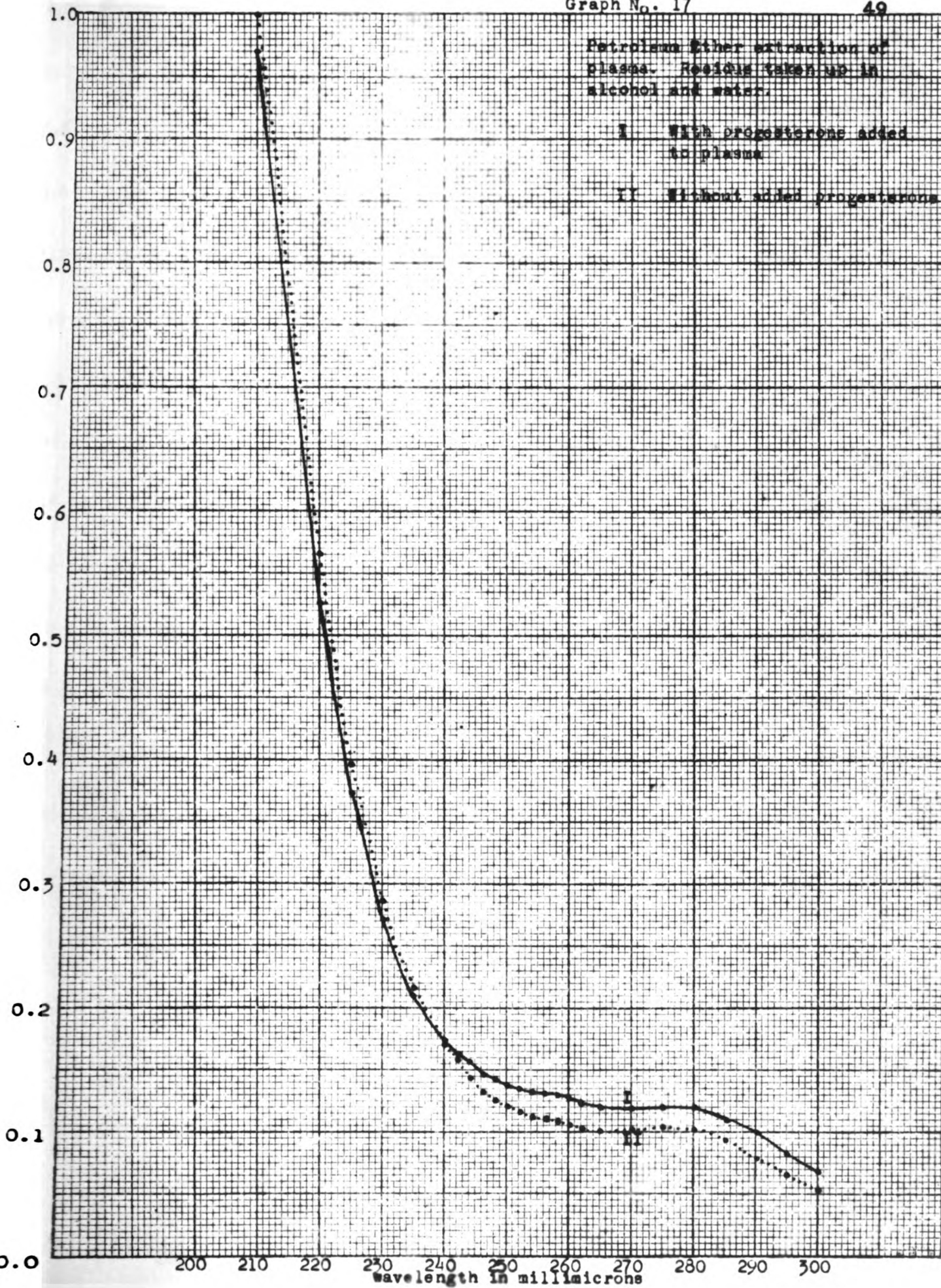
calculated progesterone level of 0.00955 mg./ml. (from concentration curve, Graph 8). The ether extraction residue (Curve II) when run against distilled water was calculated to contain 0.0089 mg./ml. or 93% of the original quantity. When compared with the control solution (alcohol and water from a clean flask) (Curve V), 0.00785 mg./ml. were found. This represents 82% of the total. The water layer after extraction (Curve I), the second attempt to take up the residue (Curve III), and the control consisting of 2 ml. of alcohol and 98 ml. of water rinsed in a clean flask (Curve IV), show no absorption maxima at the 248 millimicron level. Thus it is assumed that the extraction was quite complete and that the residue was quite completely taken up. However, it appears that 2% ethyl alcohol in water differs sufficiently from pure water to warrant setting up a concentration curve using this solution as a blank, although for comparative purposes, Graph No. 8 will be used in this thesis.

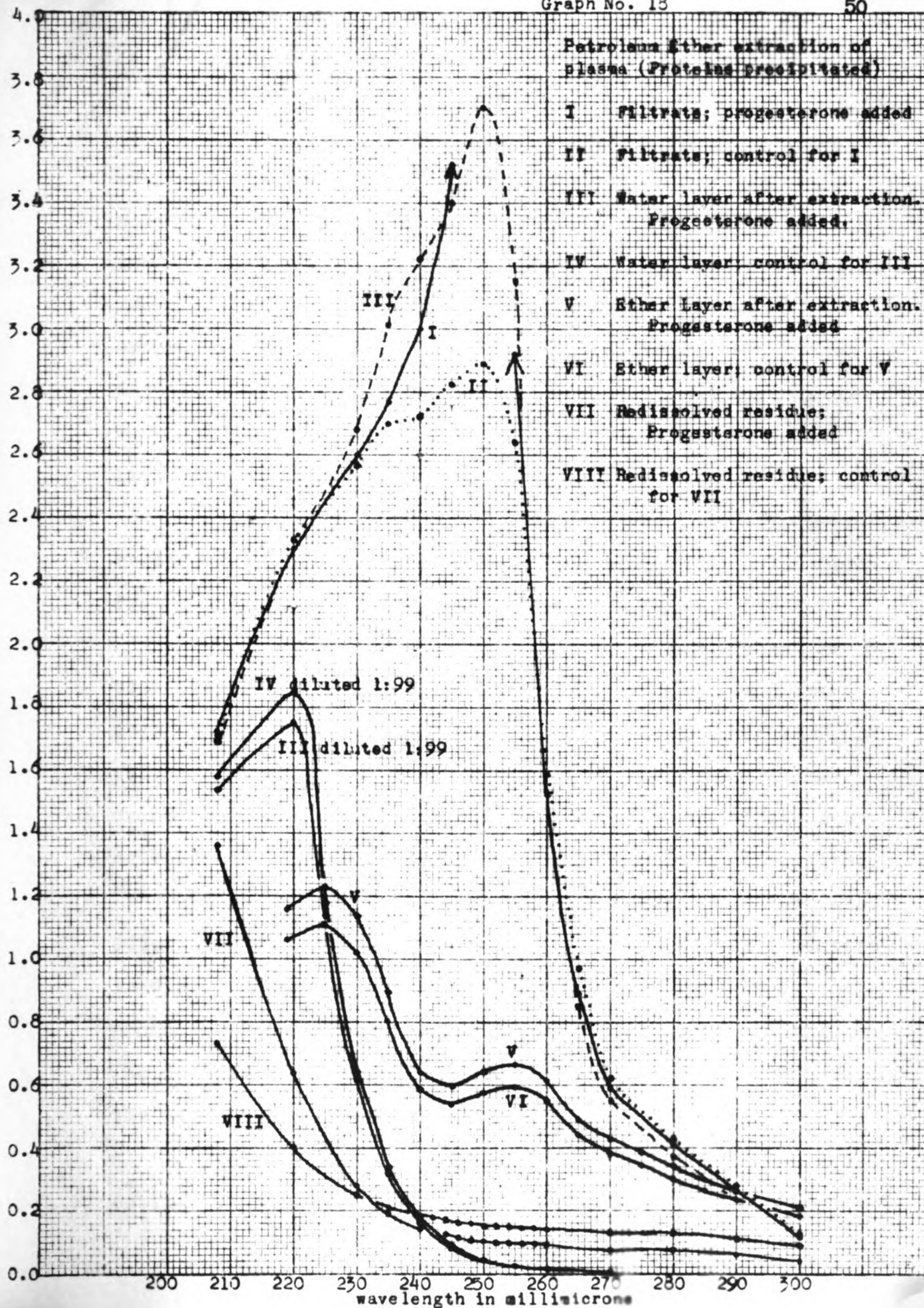
From Plasma after Precipitation of the Proteins. In Graph No. 17 and Graph No. 18 is shown that progesterone was not detected by the spectrophotometer in the presence of blood plasma in which the proteins had been precipitated by trichloroacetic acid. This may have been due to adsorption of the hormone on the surface of the protein particles. The great extinction in Curves I, II, and III of Graph 18 is presumably due to absorption of light by the trichloroacetic acid.

Petroleum Ether extraction of
plasma. Residue taken up in
alcohol and water.

I With progesterone added
to plasma

II Without added progesterone





From Plasma without Precipitation of the Proteins.

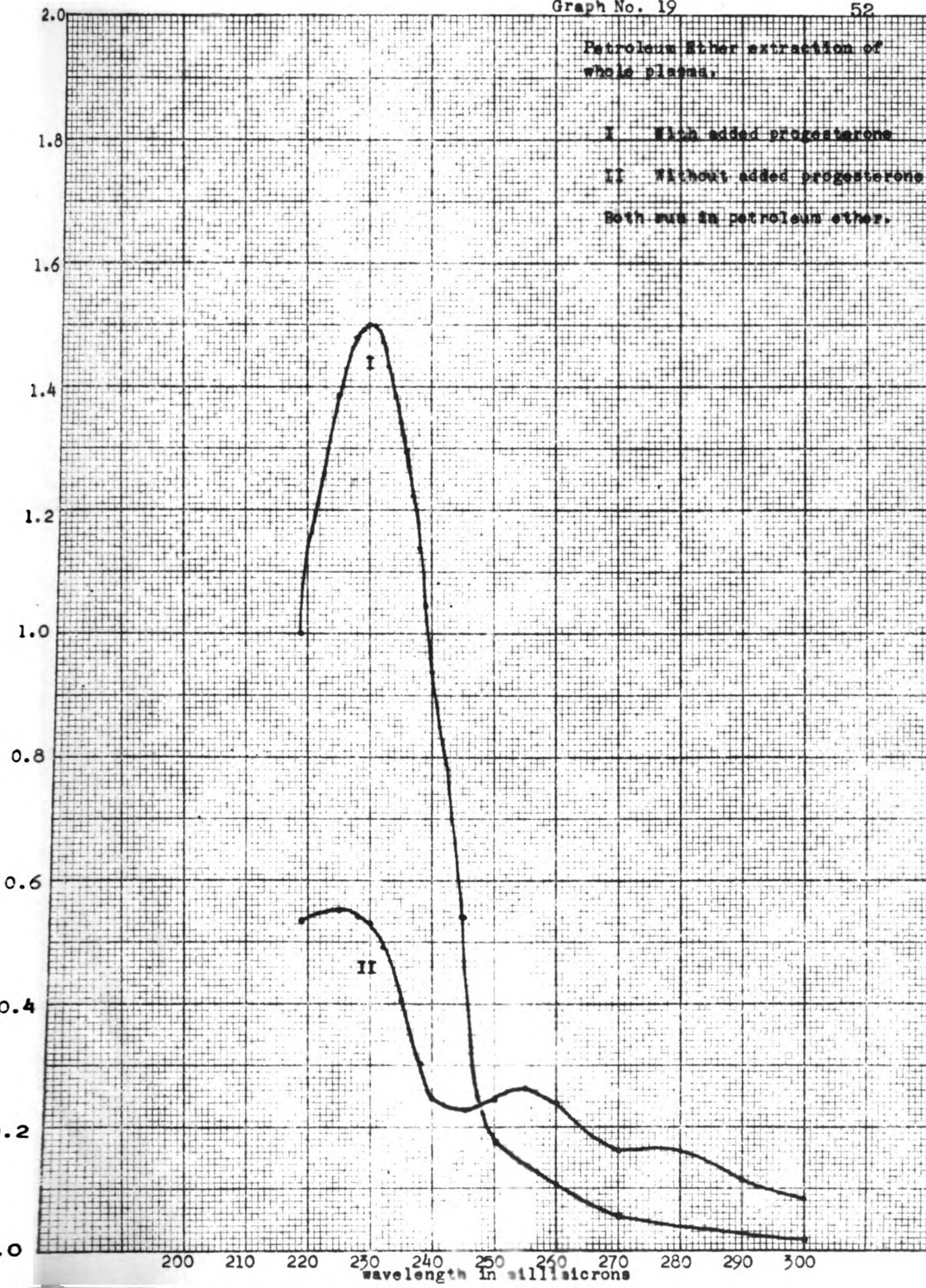
Graph No. 19 shows that progesterone may be removed from plasma by petroleum ether, and it also establishes the maximum for progesterone in petroleum ether at a wave length of 230 millimicrons. The maximum in alcohol is at 240 millimicrons, and in water it is at 248 millimicrons. From these data it appears most efficient to work with the hormone in aqueous solution as the maximum is not so near the lower limit of the spectrophotometer.

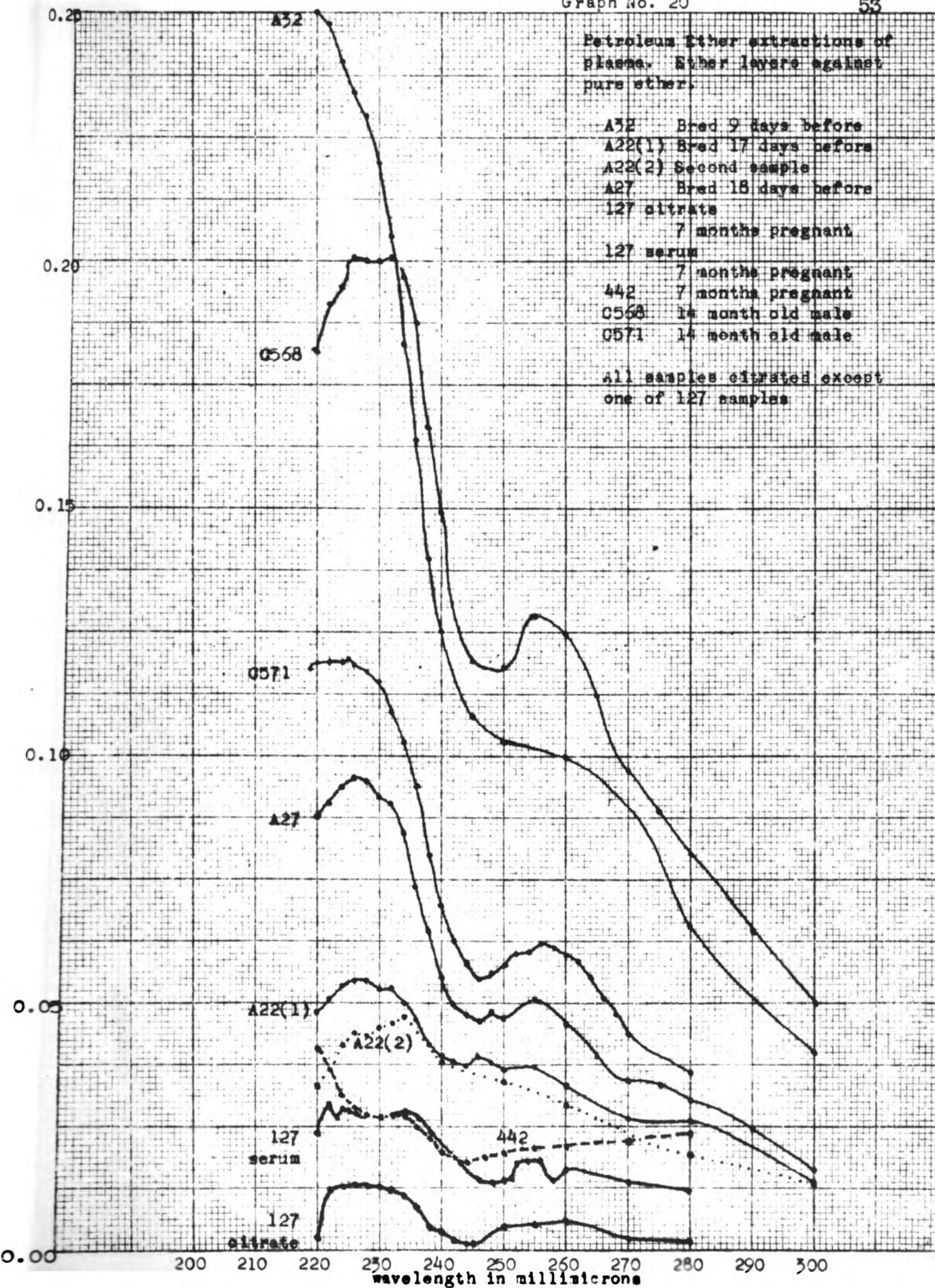
Normal Cows and Calves. These curves are petroleum ether curves, and the maximum for progesterone should therefore be at 230 millimicrons.

Graph No. 20 shows that there is no significant relation between the state of the reproductive cycle and the character of the absorption spectra. While many of the curves show a tendency for a peak at or near 230 millimicrons, this is a general characteristic of many of the ether extracts made in this study and should likely be discounted.

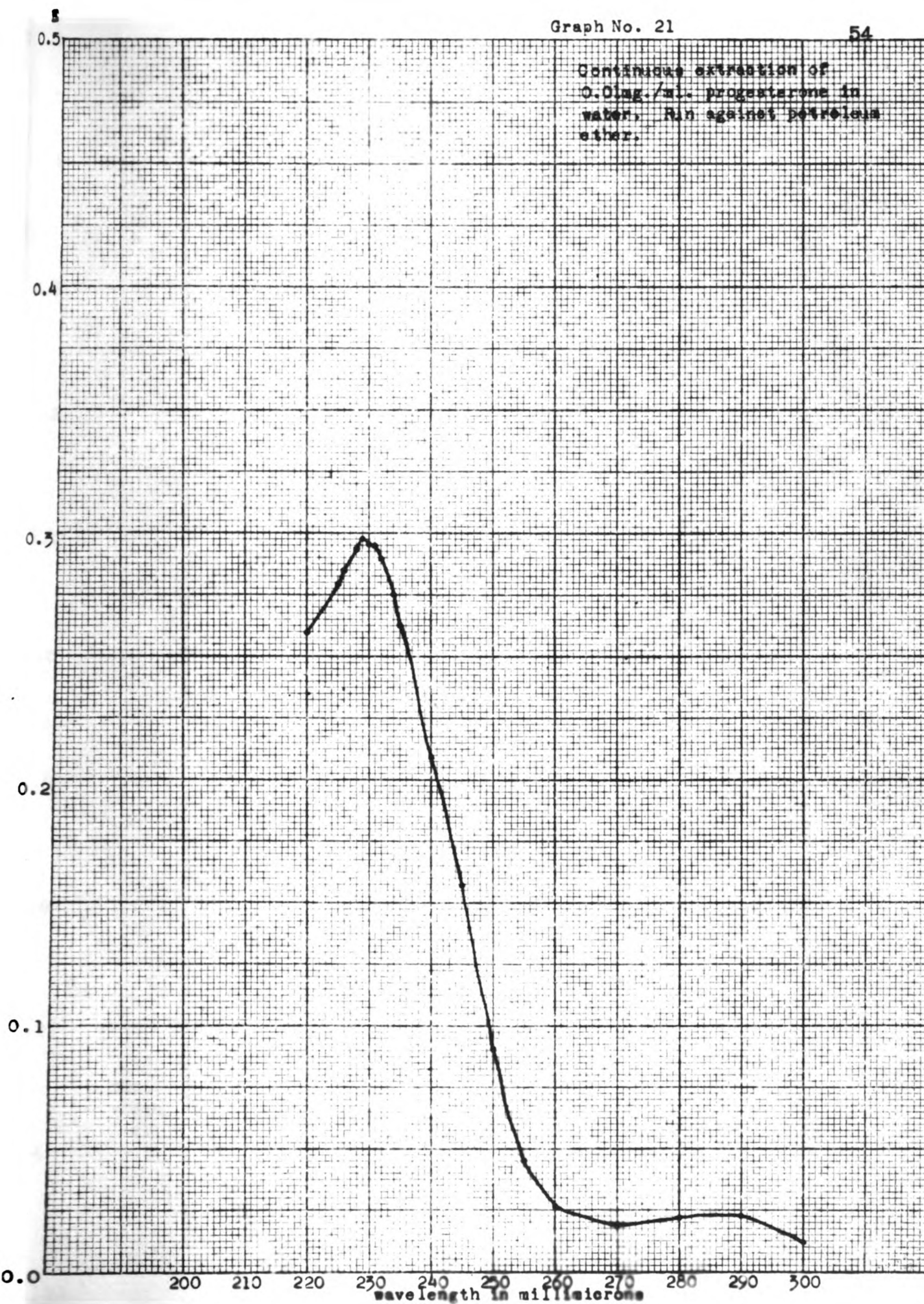
With a Continuous Liquid-Liquid Extractor. The first attempt to use this extractor gave results which are plotted in Graph No. 21, in which the absorption of the extraction product was determined, using petroleum ether as a blank. The definite peak at 230 millimicrons is taken to represent the progesterone which was extracted.

The data plotted in Graph No. 22 illustrate an interesting point in regard to the use of similar but not identi-





Continuous extraction of
0.01mg./ml. progesterone in
water. Run against petroleum
ether.



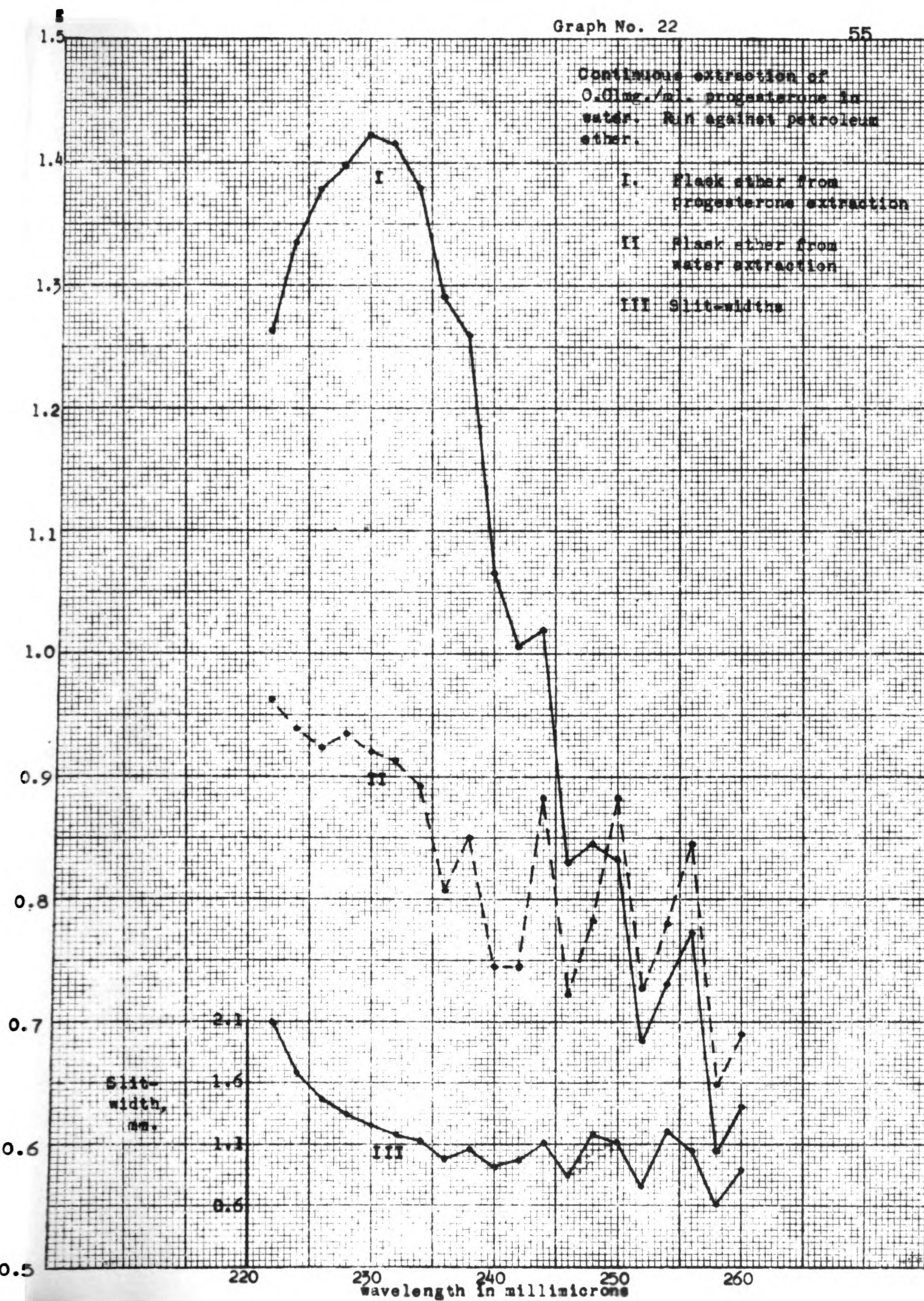


Continuous extraction of
0.01mg./ml. progesterone in
water. Run against petroleum
ether.

I. Blank ether from
progesterone extraction

II Blank ether from
water extraction

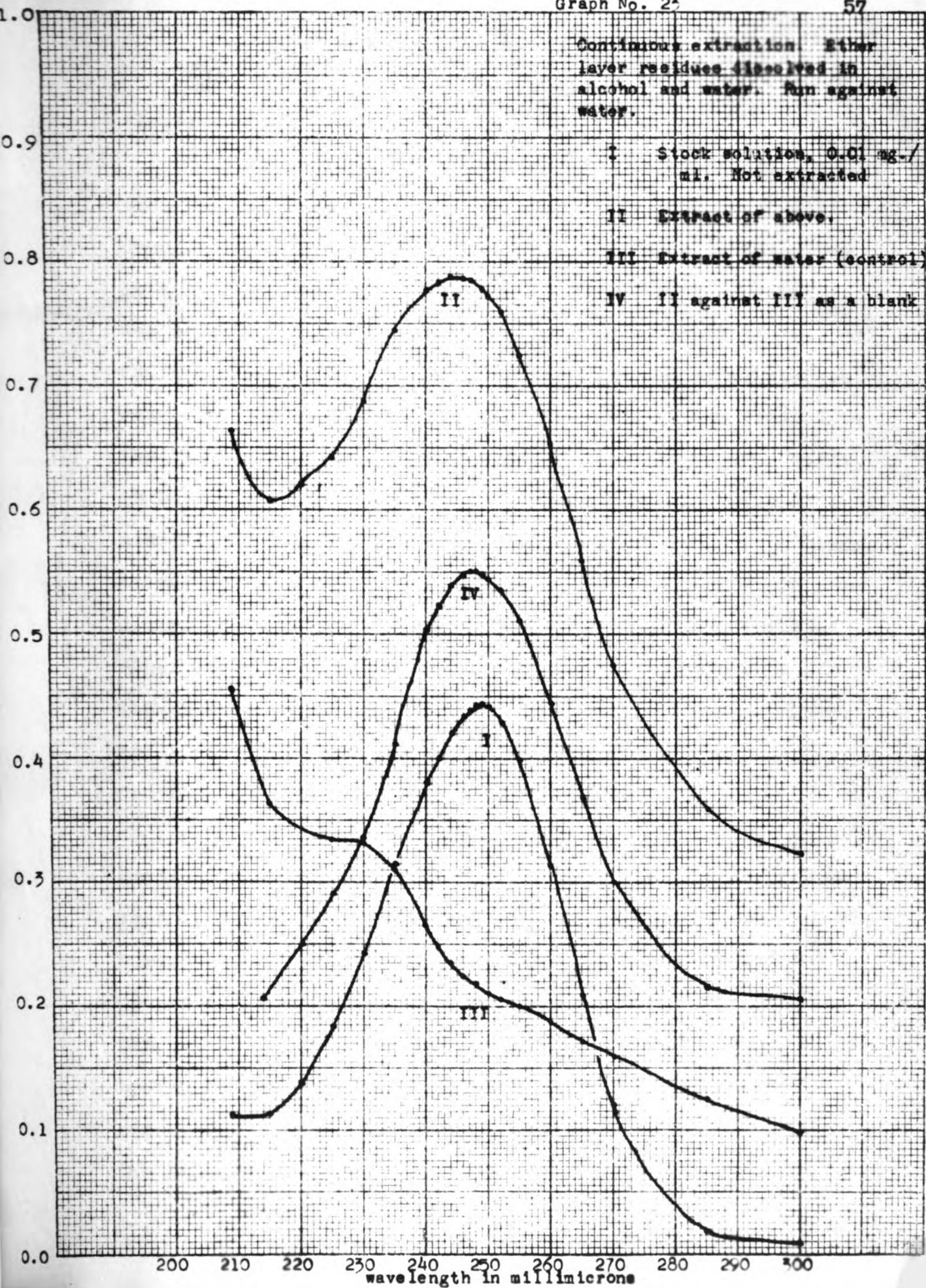
III Slit-widths



cal samples of petroleum ether. Petroleum ether II required unusual adjustments of the slit-width as seen in Curve III. All other solvents, including distilled water, absolute alcohol, and other lots of petroleum ether, required progressively decreasing slit-widths as the wave length increased. The effect of the erratic slit-widths when using petroleum ether II is observed in both Curves I and II of Graph 22. The extinction (E) tends to increase when the slit-width must be increased. However, the great absorption of light at 230 millimicrons in Curve I indicates that extraction of the progesterone was successful.

Comparison of the labels on the petroleum ether bottles indicates a difference not only in the boiling point range but in the nonvolatile matter (see page 19). Petroleum ether II contains twice as much non-volatile matter as either petroleum ether I or III. This probably accounts for the differences in the spectra of these solvents.

Graph No. 23 illustrates a further effect of this nonvolatile material in the ether. On evaporation and redissolving in alcohol and water, a cloudy solution resulted. This was expected to increase the extinction of light and accounts for the higher E value (Curve II) when compared with the stock solution (Curve I). Running the extract against the control (III) resulted in bringing the extinction (Curve IV) closer to that of the stock solution (I). This was due to the fact that the control (extract of distilled water



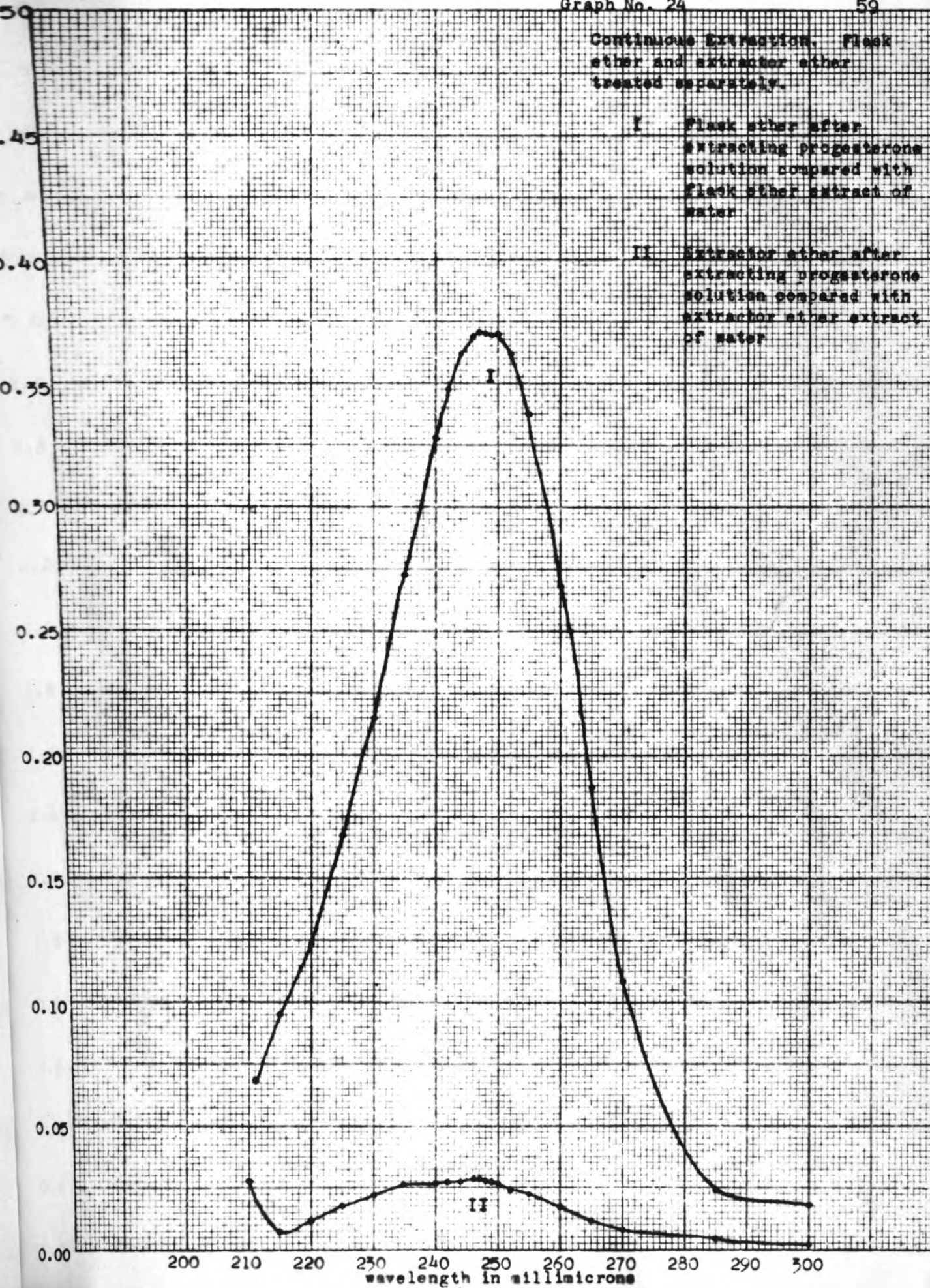
alone) was also cloudy.

Clearer solutions resulted when the alcoholic solution was added to water. Graph No. 24 shows that a small amount of the extracted progesterone is found in the ether left in the extractor (Curve II). The ether in the flask was calculated (from Graph 8) to contain 0.00725 mg./ml. or 72.5% of the total. The extractor ether was calculated to represent 0.0002 mg./ml. or 2%. It appears from these data that the extraction was not **complete**, but it must be borne in mind that the calculations were made on the basis of a concentration curve set up using distilled water as a blank. At any rate it appears that considerable extraction of progesterone from aqueous solutions by the use of petroleum ether is possible. It is also likely that extraction for a longer time would tend to make the recovery more complete and at the same time to cause the progesterone in the extractor ether to pass over into the flask.

From the Blood Plasma of a Cow Injected with Progesterone. In this study, the influence of cloudiness is seen again. There appear to be no significant differences in the curves (Graph No. 25) at the 248 millimicron level where progesterone would be expected to show up. Variations in the curves are most likely due to the cloudiness (due to petroleum ether II) as the most cloudy one (A) shows the greatest extinction and the least cloudy sample (B) shows the least extinction. It appears that the progesterone in-

Continuous Extraction. Fleck
ether and extractor ether
treated separately.

- I. Fleck ether after
extracting progesterone
solution compared with
Fleck ether extract of
water
- II. Extractor ether after
extracting progesterone
solution compared with
extractor ether extract
of water



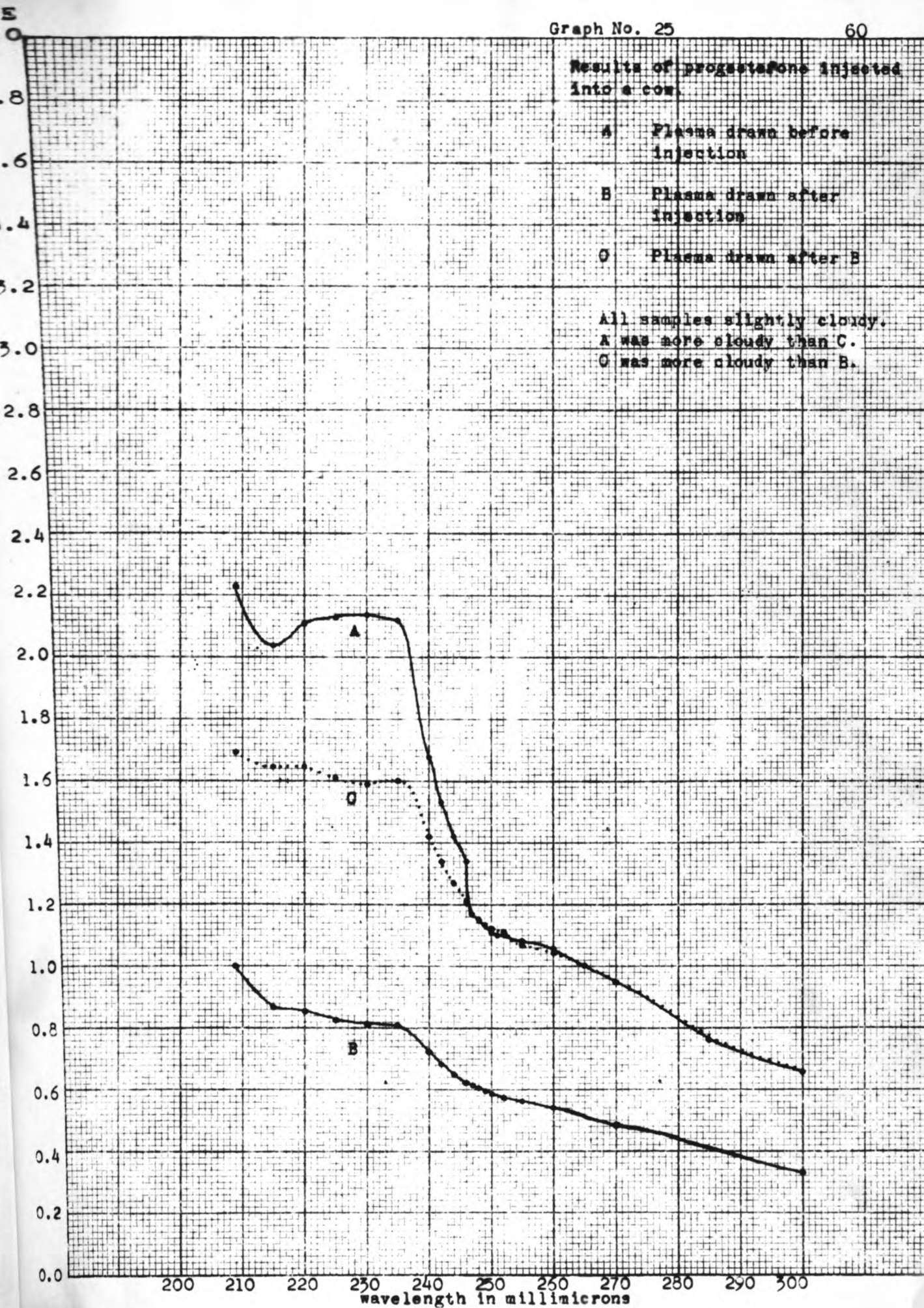
Results of progesterone injected
into a cow.A Plasma drawn before
injectionB Plasma drawn after
injection

C Plasma drawn after B

All samples slightly cloudy.

A was more cloudy than C.

C was more cloudy than B.



jected is not recoverable by this technique.

From Hemolyzed Blood of a Cow Injected with Progesterone. This graph (No. 26) illustrates many related points. Curve A shows that the technique will recover progesterone from hemolyzed blood when the hormone is added as the blood is drawn. The concentration (see Graphs 27 and 28 for a concentration curve for progesterone in absolute alcohol) calculated from the extinction (1.409) at the maximum, was 0.0278 mg./ml. As there were 25 ml. of solution, this represents a total of 0.695 mg. This is only 34.75% of the original 2 mg. This may be accounted for on the basis that all of the crystals may not have dissolved. Earlier studies demonstrated the difficulty of dissolving the hormone in aqueous solutions (see Graph No. 6).

In addition, Curves B and C show the difference between petroleum ether II and petroleum ether III. The former was used in preparing Curve B only and shows an absorption characteristic of an ether sample with a high nonvolatile matter content (see Curve II). In contrast, Curve C, representing an extract of blood prepared as in B except that petroleum ether III was used, reveals an entirely different curve which compares favorably with the pure solvent, dried and redissolved in alcohol (III).

Curves D and E show that the use of larger quantities of solvent (200 ml. in place of 100 ml.), causes an accumulation of nonvolatile material. This results in final curves

Results of progesterone injected into a cow.

A Alcoholic solution of dried ether III extract of blood drawn into flask containing progesterone crystals

B Same as A except extracted with Ether II and minus the added progesterone

C Same as B except extracted with Ether III

D* alcoholic solution of dried ether III extract of blood drawn after progesterone injected intravenously

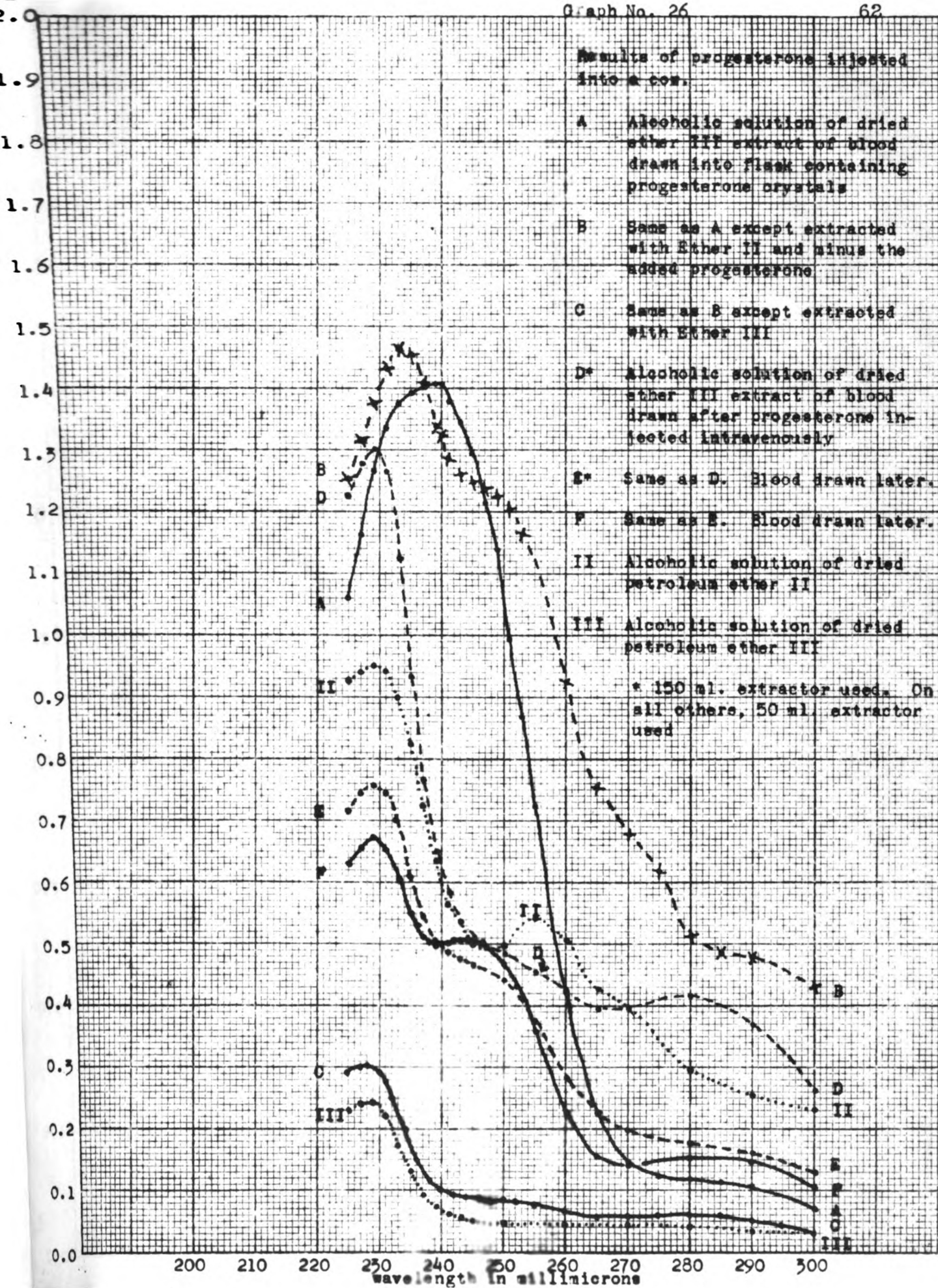
E* Same as D. Blood drawn later.

F Same as E. Blood drawn later.

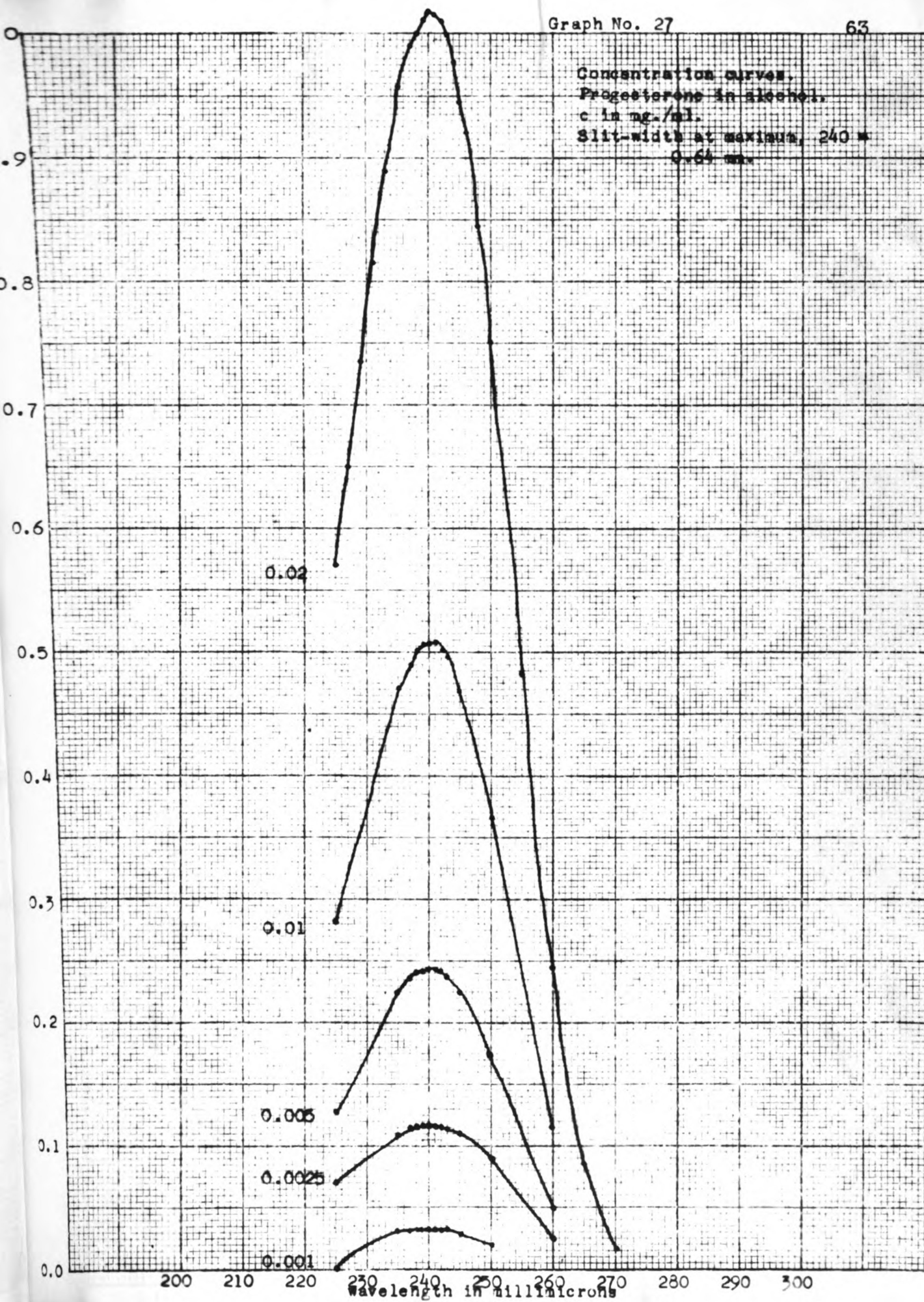
II Alcoholic solution of dried petroleum ether II

III Alcoholic solution of dried petroleum ether III

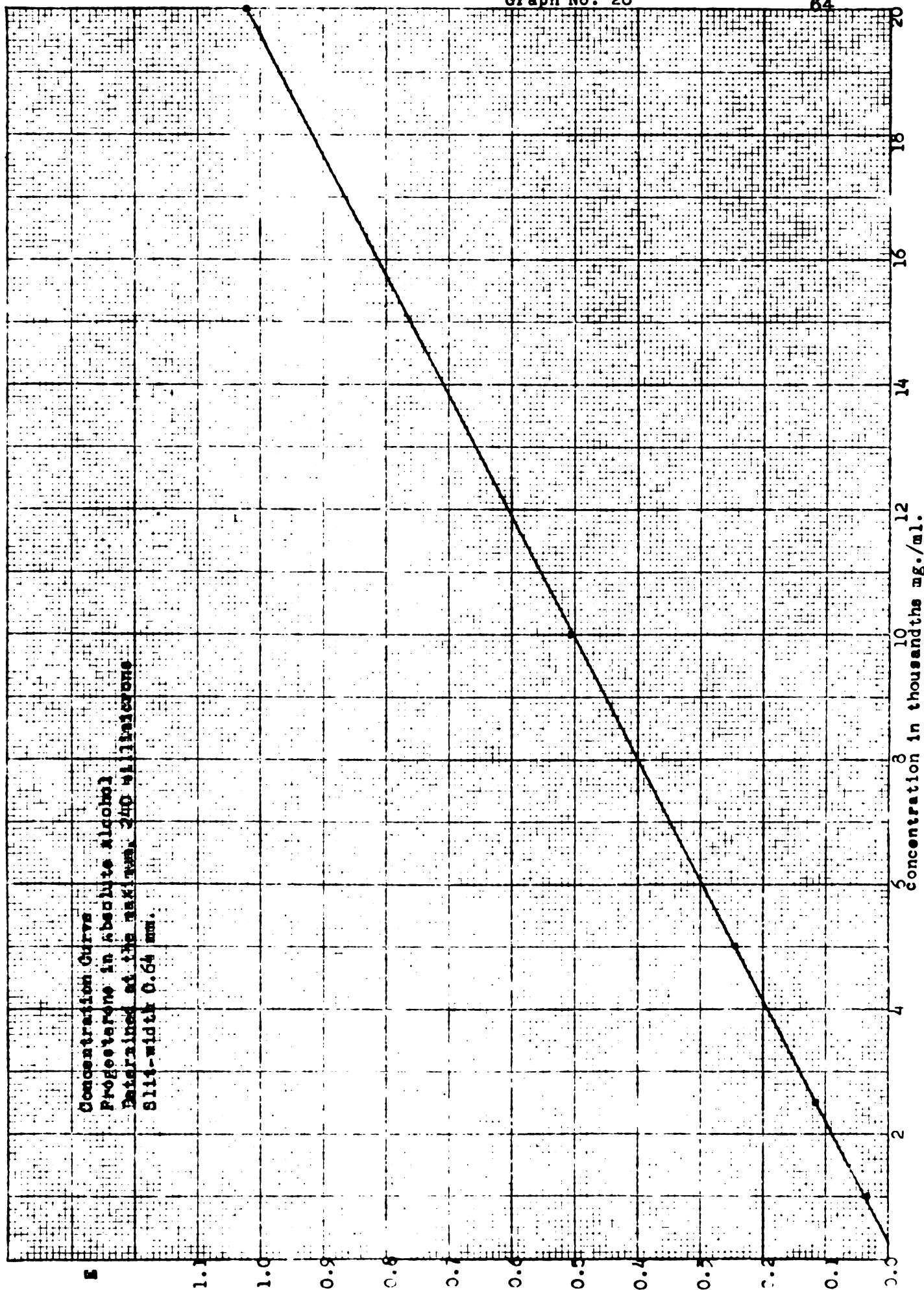
* 150 ml. extractor used. On all others, 50 ml. extractor used



Concentration curves.
Progesterone in alcohol.
c in mg./ml.
Slit-width at maximum, 240 m.
0.64 mm.



Concentration Curve
Progesterone in Absolute Alcohol
Determined at the maximum 340 millimicrons
Slit-width 0.64 mm.



similar to those from petroleum ether II. It is therefore objectionable to use the larger amounts.

Curve F is difficult to explain. It was expected to approximate Curve C, indicating the absence of progesterone or to be similar to Curve A with a maximum at 240 millimicrons, meaning that the injected hormone had been successfully extracted. While the result did not coincide with the first possibility, certainly there is no evidence to suggest that the blood as drawn contained any of the injected progesterone. It is concluded that the technique used was insufficiently effective or that progesterone is quickly removed from the circulation in some fashion. Possibilities which are suggested to explain the latter are: the hormone might be metabolized, for example, by the uterus to form pregnandiol (the latter may be conjugated with glucuronic acid in the liver); or it could simply be selectively absorbed by some tissue and thus removed from the circulation.

Many encouraging facts were learned from this study, but it is not possible to say at this time that the hormone progesterone can be demonstrated in the circulating blood by spectrophotometric means. It seems likely, however, that when a suitable extraction procedure is worked out, absorption spectra curves will be useful in measuring the progesterone quantitatively.

SUMMARY

1. Alcoholic solutions of progesterone resulted in spectrophotometric curves which varied considerably from day to day in regard to the wave length at which maximum absorption occurred and also in regard to the degree of absorption. These variations may be due to changes in the solutions, changes in the functioning of the apparatus, or both.

2. Fresh alcoholic solutions showed maximum absorption at a wave length of 239 to 240 millimicrons. This is in agreement with previously reported curves (16).

3. Aqueous solutions of progesterone were found to be more suitable for spectrophotometric measurements than alcoholic solutions.

4. The preparation of an aqueous solution was greatly facilitated by first dissolving the crystals in a small quantity of absolute alcohol.

5. The maximum absorption in aqueous solution was found to be at a wave length of 248 millimicrons.

6. Concentration curves for aqueous solutions of progesterone showed that this substance follows Beer's law at least **between** 0.004 mg./ml. and 0.018 mg./ml., that is, that absorption is closely proportional to the number of molecules in the light path. On either end of the above

range, the deviation may be caused by limitations of the spectrophotometer rather than by failure of the hormone solutions to follow Beer's law.

7. No significant difference could be detected between the curves obtained from the diluted blood plasma of different cows and calves. This is probably due largely to the high dilutions of plasma used for the determinations.

8. Crystalline progesterone added to diluted plasma showed characteristic absorption at 248 millimicrons. The amount calculated to be present, using concentration curves, coincided nicely with the amount added.

9. Progesterone was not adsorbed from aqueous solution by passage through alumina.

10. Progesterone was extracted from aqueous solutions, from blood plasma solutions, and from hemolyzed blood solutions, by the use of petroleum ether. The amount recovered ranged from 68 to 93%.

11. The absorption maximum of progesterone in petroleum ether was found to be 230 millimicrons.

12. Progesterone was not detected in the presence of blood plasma in which the proteins had been precipitated by trichloroacetic acid.

13. The absorption curves of petroleum ether extracts of the plasma of normal cows and calves did not show significant differences at the absorption maximum for progesterone.

14. Different lots of petroleum ether from the same source showed large variations in their absorption curves. This was apparently associated with the nonvolatile matter as the satisfactory samples contained 0.0002% while the unsatisfactory lot contained 0.0004%. This nonvolatile residue tended, in addition, to form an emulsion with water. This latter was minimized by taking the residue up in a small amount of absolute alcohol and adding this to water. Adding the water to the alcoholic solution of the residue resulted in an emulsion.

15. Progesterone injected into cows could not be detected spectrophotometrically in the petroleum ether extracts of the blood plasma or of the whole blood following hemolysis.

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