# THE PREPARATION, SEPARATION, AND BIOLOGICAL EXAMINATION OF ESTROGENIC FACTOR OF RESIDUAL OVARY

by

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# INTRODUCTION

The recognition many years ago of the ovary as an organ of internal secretion, has been followed by numerous attempts to obtain extracts from ovarian tissue that, upon administration to female animals, would be effective in introducing characteristic changes in accessory sex organs. Beyond a doubt the greatest difficulty encountered by early workers was the lack of a rapid method of making quantitative determinations of the potency of their extracts. With the introduction of the vaginal smear technique, a roughly quantitative test for oestrus producing extracts became available, and improvements in methods of extraction and purification of the hormone immediately followed.

The term "oestrus" as used by Heape, from the review by Allen (1935), designates the restricted period of mating activity of female animals, the outstanding criterion being the female's sex urge or drive. Since this activity in many primates, such as the human, is not restricted to such brief periods this term is not used in connection with a menstrual cycle.

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## REVIEW OF LITERATURE

The need of a satisfactory method of following the recovery of estrogenic material was filled by the discovery of Long and Evans (1922) that the various stages of the oestrus cycle could be followed accurately by the examinations of changes in vaginal comtents, and the cessation of these changes after ovariectomy. This observation has provided an easy method of testing the oestrus producing activity of extracts.

Many studies have been made of the chemistry and

physiology of ovary tissue and components in recent years. One of the first active fractions, not contaminated by objectionable materials, was made by Dickens, Dodds, and Wright (1924). Extracts contaminated by excessive amounts of cholesterol were improved by the use of a 1% solution of digitonin as a precipitant for the cholesterol.

Marrian and Parkes (1930) observed that 200 times the amount of oestrus producing factor required to induce oestrus, is necessary to induce copulation in female castrates; therefore such amounts or more are undoubtedly produced by the normal animal during its cycle.

Allen (1935) demonstrated that estrogenic substances have no stimulating effects upon the ovary. Long continued injections of large amounts into immature animals may even retard the ovarian development.

Allen and Meyer (1935) have noted the fact that corpus luteum extracts which have been freed from oestrus producing factors, but which contain progestin, when injected in conjunction with cornifying amounts of estrogenic hormone to castrated mice, prevent both cornification and oestrus. When suitable doses are given, i.e. more rabbit units of progestin than rat units of estrogenic hormone, the cornifying properties of the estrogenic factor are completely suppressed and modifications characteristic of those seen during pregnancy result.

Marlow and Groetsema (1935) have pointed out that extracts made from the interstital tissue of sow ovaries produced precocious sexual development in immature rats and mice.

Marlow (1936) has observed that when 2.0 rat units (Gustavson) of residual ovarian extract were injected into normal female rats that there was no effect on the vaginal epithelium cells. The same dosage injected into adult female castrates caused continuous oestrus until 48 hours after cessation of injections. Blood serum from normal adult females that had been injected with the ovarian extracts caused oestrus when injected into castrates; while, blood serum from theelin treated normal females had no effect when injected into castrates.

Dorfman, Gallagher, and Koch (1935) have made an interesting observation upon the difference in uterine response of infantile rats to injections of pure, crystalline hormones. This observation is used as a method of identification of unknown extracts.

Wade and Doisy (1935), and Enery and Schwabe (1936) have observed that the frequent examination of the vaginal contents of rats by means of a cotton swab may, without the injection of an estrogenic hormone, produce full cornification of the vaginal epithelium. Individuals

may show a rhythmic return of oestrus when examined four times daily for fifteen days.

Sanders and Cole (1936) point out clearly the necessity of knowing the stage of sexual development of rats used in the study of response to gonadotropic materials, and that the difference in response of rats of the same age from different strains may explain the inconstancy of results reported by different observers.

MacCorquodale, Thayer, and Doisy (1935) report the isolation of a crystalline estrogenic product from the liquor folliculi of the sow ovary resembling, in all physical and chemical properties determined, dihydro-theelin. Its potency is about 4 to 8 times that of theelin as assayed upon the spayed female rat.

The recent report of Andrew and Fenger (1936) if duplicated by others will most certainly throw more light upon the estrogenic hormones of the ovary. They have been able to isolate from the entire sow ovary a crystalline substance possessing a melting point of 95.1 degrees C. and having a calculated molecular weight of 320, and with an empirical formula of $C_{20}H_{41}O_2N$. The potency of this newly isolated substance seems to be such that 0.00001 mg. when injected in one dose into the spayed female rat, produces positive oestrus after 96 hours and lasting for a

period of at least 24 hours before a gradual return to dioestrus.

In table 1 is given an outline of facts concerning the crystalline hormones associated with the production of oestrus. In table 2 is given an outline of the estrogenic factors recovered from various parts of the ovary.

Table 1. Properties of Estrogenic Hormones.

| Name | Mp. °C. | Formula emp. | Activity r.u. per mg. | Discovery |
|---|---|---|---|---|
| Theelin | 253 | $C_{18}H_{22}O_2$ | 8,000 | Doisy; Butendant. 1930. |
| Theelol | 273 | $C_{18}H_{24}O_3$ | 1,000 | Doisy; Butendant. 1931. |
| Dihydro-theelin | 170 | $C_{18}H_{24}O_2$ | 40,000 | MacCorquodale, Thayer, and Doisy. 1935. |
| ------- | 95.1 | $C_{20}H_{41}O_2N$ | 100,000 | Andrew and Fenger.1936. |

Table 2. Comparison of Yields of Estrogenic Factors from Ovary.

| Observer | Estrogenic content |
|---|---|
| Allen, Meyer (1933) | 100 r.u. per kg. ovary tissue |
| D'Amour, Gustavson (1933) | 950 r.u. per l. follicular fluid |
| D'Amour, Gustavson (1933) | 65 r.u. per kg. residual tissue |
| Ralls, Jordan (1926) | 1000 r.u. per l. follicular fl. |
| Thayer,Jordan,Doisy (1928) | 933 r.u. per l. follicular fluid |
| Thayer,Jordan,Doisy (1928) | 490 r.u. per kg. total ovary |
| Thayer,Jordan,Doisy (1928) | 167 r.u. per kg. residual tissue |
| Marlow, Groetsema (1935) | 276 r.u. per kg. fresh res. tis. |
| Sealey (1936) | 300 r.u. per kg. preserved tissue |

## MATERIALS AND METHODS

The ovaries used in this work were supplied by Dr. E. A. Doisy of the school of medicine, Saint Louis University. They had had the follicular fluid, as completely as possible, previously removed. The ovaries were received, stored in an iron container under alcohol.

The method of extraction was similar to that followed by Allen (1930) in his extraction of corpus luteum tissue. Two separate preparations were made using two separate solvents for extraction. First was used, 95% ethyl alcohol, next was used 95% ethyl alcohol made acid by the addition of 41 cc. concentrated HCl per liter, in an attempt to improve the yield of estrogenic factor recovered from the tissue.

Thayer, Jordan, and Doisy (1928) have stressed the importance of solvent purification. Occasionly it has been found that peroxides develop in the ethers used in the extractions, and inasmuch as the active concentrates are extremely sensitive to oxidizing agents it was thought advisable to redistill all ethyl ether and petroleum ether used in extractions. The petroleum ether was agitated with concentrated sulphuric acid previous to distillation. The solvents were ascertained to be peroxide free by testing with an aqueous solution of potassium iodide.

The first extraction was commenced Feb. 19, 1936. 1500 grams of the ground tissue were placed in a muslin bag. This was placed in the upper chamber of a Clarke-Bloor extractor. The tissue was extracted with 500 cc. portions of solvent at atmospheric pressure four times, each extraction lasting 1¼ to 1½ hours. The mass of tissue was kneaded after each extraction to break up any lumps of tissue that might have been formed, thus facilitating the penetration of the solvent fumes. In this way all of the lipid material was removed and none of the extracted material heated for more than 1½ hours at the boiling temperature of alcohol. The extraction alcohol contains considerable insoluble material which need not be removed until subsequent treatment. The alcoholic extracts are evaporated under vacuum, the boiling point rarely allowed to go above 35 degrees C. Each separate extract was evaporated down before the following extract portion was added. With this proceedure much of the tissue water removed in the first extraction is progressively removed from the residue (the third and fourth extracts were in essentially 95% alcohol.) This method avoids much of the foaming usually encountered when the extracts are combined and the alcohol content of the residue gets low. The residue following the evaporation of the fourth extract is de-

hydrated by the addition of 500 cc. absolute alcohol, which, in a like manner, is removed under vacuum. The final residue is extracted five times with peroxide free ethyl ether, 500 cc. being used the first time and 200 cc. for each succeeding extraction. The ether solutions are combined and the volume reduced to 150 cc. by vacuum distillation. To this solution is added four volumes of acetone together with 15cc. saturated alcoholic magnesium chloride. A heavy, flocculent precipitate is formed consisting principally of phospholipids and is removed by decantation. The precipitate is dissolved in ether and reprecipitated three times by the addition of four volumes acetone. After the second precipitation a fine white precipitate is formed consisting chiefly of sphingomyelin which does not dissolve in the ether.

The acetone-ether solutions are combined and the solvents removed by vacuum distillation on a steam bath. A heavy oily residue is obtained which may be assayed by incorporation of a portion in olive oil. The remaining residue may be safely stored in 95% alcohol without fear of appreciable activity loss for quite a long period of time.

At this point a small amount of the residue equivalent to 200 gm. of tissue was incorporated in olive oil

to make a total volume of 10 cc., 1 cc. being equivalent to 20 gm. tissue. The extract at this point is assayed; the preparation using neutral alcohol as a solvent yielded 0.137 rat units per gram of tissue and the preparation using acid alcohol as a solvent yielded 0.300 rat units per gram of tissue.

Removal of Neutral Fats

The stored alcoholic solution is evaporated under vacuum. The residue is shaken with 250 cc. hot methyl alcohol and placed in a refrigerator at -29 degrees C. for 24 hours and then centrifuged. The fatty precipitate is reshaken with 200 cc. hot methyl alcohol and along with the supernatant solution again chilled at -29 degrees C. Some additional fatty material separates from the supernatant liquid and is dissolved in the supernatant solution resulting after the second precipitation of fats. The precipitate from the second treatment is again agitated with 200 cc. hot methyl alcohol. The two solutions are again chilled at -29 degrees C. for 24 hours. The precipitate after the third chilling is free from active material and may be discarded. The precipitate carried through the second rechilling is dissolved in the supernatant solution and the solution chilled at -29 degrees C. for 24 hours. The solution is centrifuged and the fat

separated. When assayed it showed no activity, even after the equivalent of 100 gm. of tissue was injected.

All methyl alcohol solutions are combined and distilled in vacuum. The resulting residue may be assayed directly or stored in alcohol.

Separation of Active Factor

The residue after distillation of the methyl alcohol was taken up in petroleum ether (redistilled over concentrated sulphuric acid ) and washed with 2 volumes of .01 N NaOH solution. The ether layer is again washed with 2 volumes of the dilute alkali. The aqueous layer is shaken with $\frac{1}{2}$ volume of petroleum ether; this petroleum ether is washed with the second aqueous layer. The original petroleum ether is washed with a final alkali wash which remains alkaline to litmus. The original aqueous solution is again washed with $\frac{1}{2}$ volume of petroleum ether which in turn is used to wash the final aqueous solution used in washing the original petroleum ether solution. All petroleum ether solutions are combined and washed with one volume of .01 N HCl, the aqueous solution is combined with all previous aqueous solutions which are reserved for extraction. The ether solution is again washed with one volume of .01 NHCl and finally with one volume distilled water.

The petroleum ether solution is evaporated and found to be free from estrogenic principle in amounts equivalent

to 25 gm. original tissue. It is not discarded but is reserved for further examination, stored in 95% alcohol.

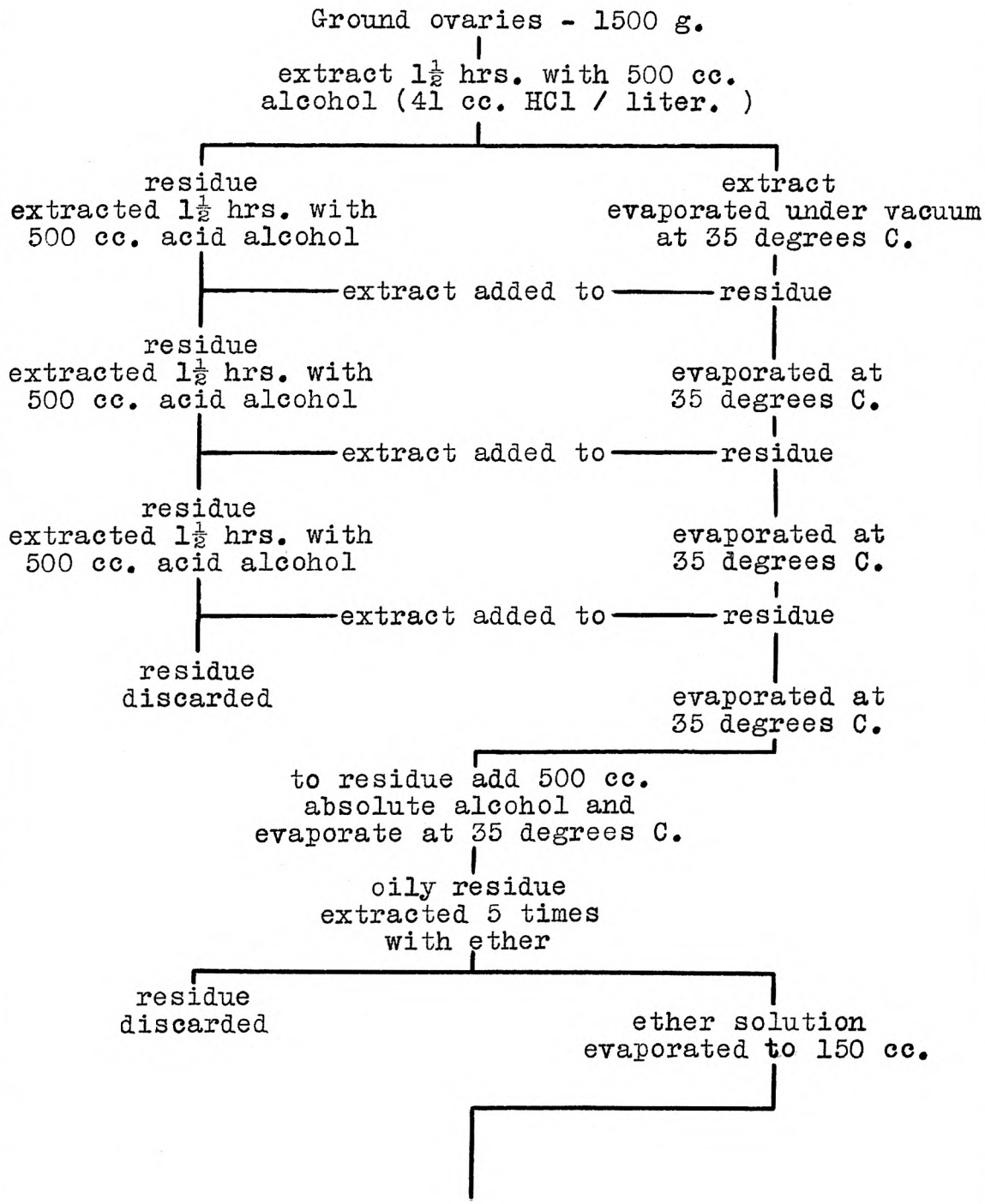
The aqueous solutions used in extraction of the petroleum ether fractions are all combined and made acid with HCl and extracted three times with ½ volumes of redistilled ethyl ether, the final aqueous solution is discarded.

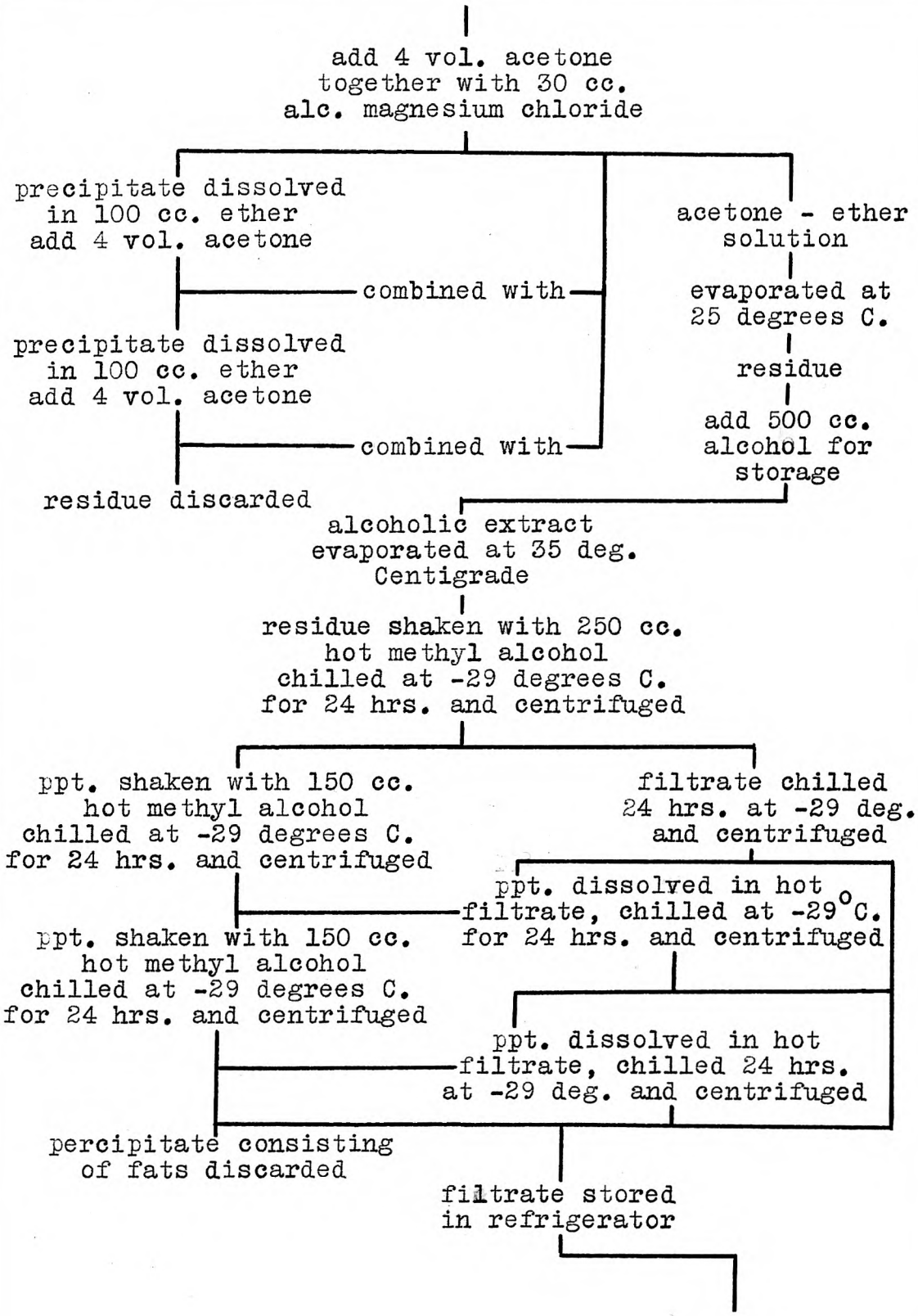
The ether solution is evaporated and the residue is leached with 200 cc. of .25 N NaOH; most of the residue goes into solution. The residue that remains may be discarded. The alkaline solution is extracted three times with 100 cc. volumes of ethyl ether. The ether is evaporated under vacuum and the residue taken up in 100 cc. absolute alcohol. The alkaline solution, after ether extraction, is made faintly acid with HCl and extracted with 100 cc. volumes of ethyl ether three times. The ether is evaporated and the residue taken up in 250 cc. absolute alcohol.

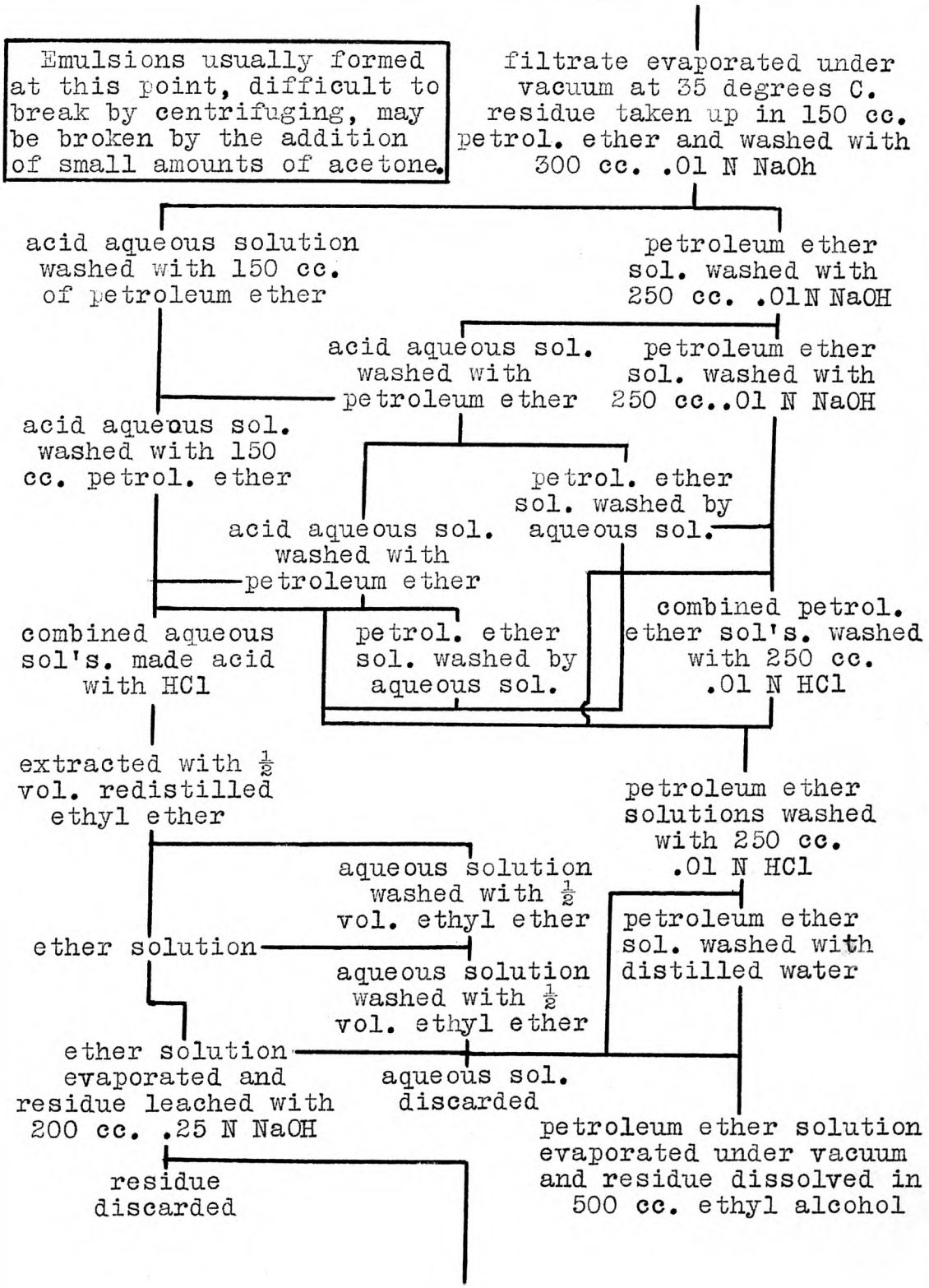
The ether solution residue before leaching with the .25 N NaOH was assayed and found to contain 0.275 rat units of estrogenic activity per gm. This represents a loss of 8.3% from the previous assay; the activity may have decreased due to deterioration during storage, but the small number of animals used in assay could easily account for such a small loss.

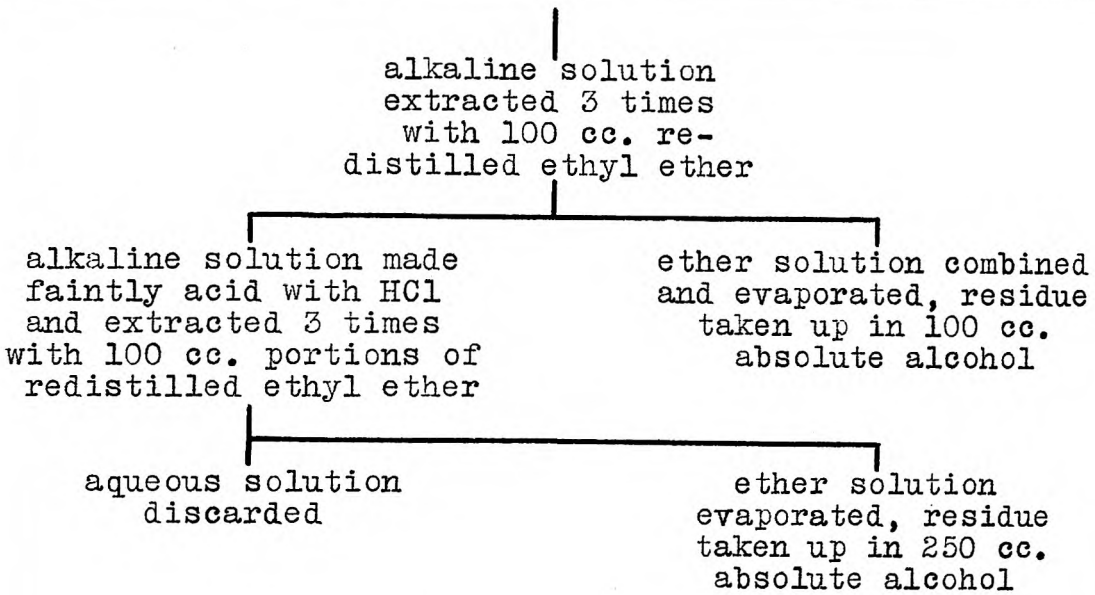
Schematic Diagram of Separation

of Estrogenic Factor from Residual Tissue




add 4 vol. acetone
together with 30 cc.
alc. magnesium chloride
precipitate dissolved
in 100 cc. ether
add 4 vol. acetone
combined with
precipitate dissolved
in 100 cc. ether
add 4 vol. acetone
combined with
residue discarded
acetone - ether
solution
evaporated at
25 degrees C.
residue
add 500 cc.
alcohol for
storage
alcoholic extract
evaporated at 35 deg.
Centigrade
residue shaken with 250 cc.
hot methyl alcohol
chilled at -29 degrees C.
for 24 hrs. and centrifuged
ppt. shaken with 150 cc.
hot methyl alcohol
chilled at -29 degrees C.
for 24 hrs. and centrifuged
filtrate chilled
24 hrs. at -29 deg.
and centrifuged
ppt. dissolved in hot
filtrate, chilled at -29°C.
for 24 hrs. and centrifuged
ppt. shaken with 150 cc.
hot methyl alcohol
chilled at -29 degrees C.
for 24 hrs. and centrifuged
ppt. dissolved in hot
filtrate, chilled 24 hrs.
at -29 deg. and centrifuged
percipitate consisting
of fats discarded
filtrate stored
in refrigerator


Emulsions usually formed at this point, difficult to break by centrifuging, may be broken by the addition of small amounts of acetone.
filtrate evaporated under vacuum at 35 degrees C. residue taken up in 150 cc. petrol. ether and washed with 300 cc. .01 N NaOh
acid aqueous solution washed with 150 cc. of petroleum ether
petroleum ether sol. washed with 250 cc. .01N NaOH
acid aqueous sol. washed with petroleum ether
petroleum ether sol. washed with 250 cc..01 N NaOH
acid aqueous sol. washed with 150 cc. petrol. ether
petrol. ether sol. washed by aqueous sol.
acid aqueous sol. washed with petroleum ether
combined petrol. ether sol's. washed with 250 cc. .01 N HCl
combined aqueous sol's. made acid with HCl
petrol. ether sol. washed by aqueous sol.
extracted with ½ vol. redistilled ethyl ether
petroleum ether solutions washed with 250 cc. .01 N HCl
aqueous solution washed with ½ vol. ethyl ether
petroleum ether sol. washed with distilled water
ether solution
aqueous solution washed with ½ vol. ethyl ether
ether solution evaporated and residue leached with 200 cc. .25 N NaOH
aqueous sol. discarded
petroleum ether solution evaporated under vacuum and residue dissolved in 500 cc. ethyl alcohol
residue discarded




## Biological Assay

The method of assay used throughout the experimental work has been that devised by Coward and Burn (1927). The animals were carefully selected young mature albino rats that had received bi-lateral ovariectomy two weeks before use. There were operated upon under ether anesthesia; septic precautions were taken. Only the rats giving a dioestrus smear for five consecutive days were used.

A qualitative of each fraction assayed was first made by single subcutaneous injections of a series of varying amounts of oil solution usually equivalent to, 1-3-5-8-10-20-50 and sometimes 100 grams of tissue. If more than the equivalent of 50 grams of tissue was necessary for an oestrus smear, the amount of estrogenic

factor was considered negligible.

The technic employed in making smears was that suggested by Loewe and Lange (1926). Vaginal smears were taken at time of injection and at 24-36-48-60 hour intervals, using a cotton swab made by wrapping a bit of cotton around the large end of a toothpick. The end of the swab is moistened with 0.9% NaCl solution and introduced into the vagina, and pressed out into approximately 0.2 cc. of the salt solution on a glass slide. The smears were examined under the low power of the microscope without staining. The determination of oestrus was taken as the complete disappearance of leucocytes from the field and their replacement by masses of non-nucleated epithelial cells, as described by Long and Evans (1922).

The proceedure used in the determination of the rat unit by the author was to inject into 10 rats an amount of estrogenic factor calculated to produce a positive response of all rats. The dosage was then decreased until a fraction of the animals showed no response. The amount which caused 50% of the rats to respond is taken as one rat unit.

The graph prepared by D'Amour and Gustavson (1930) may be quite successfully employed in making conversions, in event that the number responding are not equal to the number remaining in dioestrus.

## THE NATURE OF THE ESTROGENIC FACTOR

A quantitative biological study is necessary to differentiate among theelin, theelol, dihydro-theelin, and any other possible substance possesing estrogenic properties, in event that an insufficient amount is available for a chemical examination.

The estrogenic material used in this work was an extract prepared by H.W. Marlow in a manner similar to that used by the author; the alkali extraction step was not taken.

The method of examination has been similar to that followed by Dorfman, Gallagher, and Koch (1935). Infantile, littermate, female albino rats from our own stock were employed in this study. Injections of the active material in olive oil was started at 25 days of age and continued with a like amount injected each day for four more days. The animals were killed upon the 30 th. day of age. Body and uterine weights were recorded. The values given in plate I are rat units injected each day.

## DISCUSSION OF RESULTS

The outstanding feature of the experimental work was the finding of the active factor in the aqueous wash solution formerly discarded. After repeated activity losses, care was taken to check all possible points of loss.

From results obtained in the work it was found that about double the amount of estrogenic was extracted by the substitution of the acid alcohol for the neutral alcohol formerly used.

It was thought that a study of the biological effect of the ovarian extract, prepared in a similar way, upon the immature female rat would possibly throw light upon the nature of the estrogenic factor. The author regrets that due to the lack of time he has been unable to observe the effect of the refined extract upon the young animals; it is quite possible that factors may have been removed that would effect the result observed.

It is quite apparent that the results presented here are not conclusive;however, it does appear that the response to dihydro-theelin, by the infantile animals, is in obvious excess to that obtained from the crude extract. The response, as far as it was examined, most nearly resembled that to theelin (see plates I and II); however, Marlow (1936) observed a great difference in the effect of theelin and the residual ovarian extract upon the normal mature female rat.

## CONCLUSIONS

1. The estrogenic factor extracted from the residual ovarian tissue by acid alcohol was found to be soluble

in dilute alkali.

2. The use of acid alcohol instead of neutral alcohol greatly increases the amount of estrogenic material extracted from the tissue.

3. The crude extract quite likely contains an estrogenic factor other than the dihydro-theelin present in the follicular fluid.

4. It will be necessary to make a biological analysis of the purified extract, prepared in the way outlined, before definite conclusions concerning the nature of the estrogenic factor of the residual ovary may be made.

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PLATE I

Horns of uterus and ovaries of infantile female rats 30 days of age, that had previously recieved 5 daily injections of 0.5 rat unit (Gustavson) estrogenic hormone.

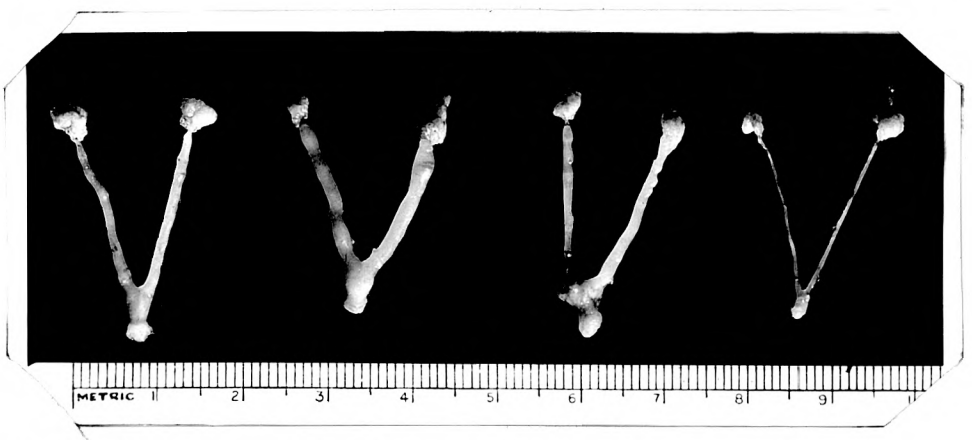


Fig. 1. Fig. 2. Fig. 3. Fig. 4.

Fig. 1. This animal recieved theelin.
Body weight- 35.0 gm. Uterus weight- 42.1 mg. Ratio- 1.21.

Fig. 2. This animal recieved dihydro-theelin.
Body weight- 30.0 gm. Uterus weight- 92.4 mg. Ratio- 3.08.

Fig. 3. This animal recieved residual ovary extract.
Body weight- 31.0 gm. Uterus weight- 49.9 mg. Ratio- 1.61.

Fig. 4. This animal recieved .25 cc. olive oil daily.
Body weight- 27.0 gm. Uterus weight- 10.3 mg. Ratio- 0.38.

PLATE II

Affect of Injections of Estrogenic Factors

on Infantile Rats 25 Days of Age.