ON THE METABOLISM OF RING B UNSATURATED ESTROGENS AND THE URINARY ESTROGENS IN THE NORMAL MENSTRUAL CYCLE

A Thesis

by

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GENERAL INTRODUCTION

Although the existence of the ovary had been well known to ancient physicians, the morphological details of this organ were first described by Regnerus de Graaf (1) in 1673. This Dutch physician described the structures now known as the "Graafian Follicles," which are visible on the ovarian surface during the fertile period of life. It was not until after primitive microscopes became available that the Russian anatomist Von Baer (1) saw and described the ovum, which is on the borderline of unaided visibility.

The elucidation of the ovary as an organ of internal secretion must be attributed to Knauer (2) and Halbon (3), who proved that ovarian transplants could renew the sexual cycle of spayed animals. Knauer (2) also concluded that apart from their function in production of ova, the female gonads play an important role in regulating estrus phenomena.

In the early nineteen hundreds attention was focused on the artificial production of estrus by the administration of ovarian extracts to spayed animals. (4,5). These early observations were not clear cut, due to the use of water or saline solutions as the extraction agent. Thus it was not until Iscovesco (6) in 1912, and Fellner (7) in 1913, substituted "fat" solvents in the process of extraction that well defined

positive results were obtained. By the use of "fat" solvents, Fellner (7) discovered that the human placenta yields extracts which are potent in producing estrus in castrated animals. This finding implicated the placenta in the metabolism of the estrogens.

Little progress was made in ovarian physiology and biochemistry in the fifteen years following Fellner's discovery mainly because of two factors. One factor was the First World War and the second was the lack of a rapid and accurate method for the assay of ovarian extracts. In that era most investigators used alterations in the uterus of immature or castrated animals as an indicator of estrogenic activity, and hence had to kill the animal for each test. This bio-assay procedure was slow, expensive and somewhat uncertain and little progress was made by its use. Research with ovarian hormones was therefore greatly facilitated when Allen and Doisy (8) introduced the vaginal smear bio-assay method. This simple and accurate method was the basis for further work on the purification of estrogenic principles, and acted as one of the greatest stimuli for the rapid development which occurred in the field of steroid hormones during the following quarter of a century.

After the introduction of the vaginal smear bio-assay method there was a great revival of interest in the ovarian hormones. Most of the investigations carried out in this

period were concerned with the preparation of purified extracts, first from ovaries (liquor folliculi) and then from human placentae, the latter being the more abundant and cheaper source. These investigations were expensive and time consuming, and although they yielded much valuable information about the methods of extraction of estrogenic hormones, they were largely displaced by the discovery of Aschheim and Zondek (9) of the great concentration of estrogenic material in the urine of pregnant women. This discovery should be regarded as one of the most important factors contributing to the isolation of the estrogenic hormones. Following this discovery many chemists fruitfully devoted their energies towards the isolation of the estrogens from human pregnancy urine. In the short period of time between 1929 and 1930, Doisy, Veler, and Thayer (10) reported the isolation of the first crystalline estrogen, and this was followed almost simultaneously by announcements of the isolation of the same crystalline compound by three other groups of investigators (11,12,13). Two years later Butenandt (14), and independently Marrian and Haslewood (15), proved the structure of this estrogen ($C_{18}H_{22}O_2$), which we now know as estrone. In 1930 Marrian (16) reported the isolation of another active estrogenic compound from human urine with properties differing from estrone. Butenandt and Hildebrandt (17) and Doisy (18) also isolated the same compound now known as estriol,

from human pregnancy urine and showed that it differed from estrone only by the elements of water.

The isolation of the estrogenic hormones in a pure state led to a rapidly increasing demand for their supply in quantities sufficiently large to enable a study of their physiological properties as well as their therapeutic value. This problem was solved when Zondek (19) in 1930 discovered that the urine of pregnant mares is a far richer source of estrogenic activity than human pregnancy urine. This discovery was followed by the isolation of estrone from mare's pregnancy urine by de Jongh, Kober, and Laqueur (20), and three new estrogenic compounds, equilin, equilenin and hippulin by Girard (21).

The years 1930-1938 saw rapid progress in the isolation and identification of hormonal principles. In fact almost every major hormone that we know today was isolated and its structure characterized in those eight years. This period also saw the discovery of synthetic estrogens by Cook, Dodds and Hewett (22). Following this meteoric progress in the isolation of the estrogens, attention was focused on their physiological action in the animal body. With the greater availability of crystalline sex hormones, many investigations of the metabolism of the estrogens, both from a clinical and biochemical approach, were reported. A number of excellent reviews of the metabolism of estrogenic hormones

have been published in recent years (23,24,25).

It is generally agreed that whether estrogens are supplied to the organism in physiological or massive doses, only about ten per cent of the administered dose is identifiable in the urine without alteration of the original ring system (25,26) and only minute traces have been demonstrated in the feces by bioassay (26). Such important questions as the fate of administered estrogens in the animal body and other pressing questions concerning their origin remained unanswered for many years.

It is only in very recent times that some insight has been gained into the metabolism of administered estrogens. This is largely due to the impetus gained from the advent of the technique of tagging molecules with isotopic carbon and deuterium. The discovery of carbon-14 by Ruben and Kamen (27) made it possible to synthesize steroid hormones containing stably bound radioactive carbon, for use in the study of their metabolic pathways. As early as 1947 methods were devised in this laboratory for introducing carbon-14 into a number of steroid hormones (28) and this culminated in the successful preparation of progesterone-3-C^{14}, -4-C^{14} and -21-C^{14} (29), desoxycorticosterone acetate-3-C^{14}, -4-C^{14} and -21-C^{14} (30), estrone-16-C^{14} and β-estradiol-16-C^{14} (31) in pure state and of high specific activity.

Preliminary investigations with estrone - 16- C^{14} in mice showed that 52 per cent of the administered radioactivity was excreted in the urine, feces and respiratory carbon dioxide (31). However, the mouse excretes estrogens at such a low level that chemical isolation of urinary metabolic products was precluded. These findings were nevertheless immediate proof of the valuable information that could be derived from metabolic studies using labeled estrone. To enable us to isolate the urinary metabolic products of administered estrone- 16 - C^{14}, an investigation of the metabolism of this hormone in the pregnant mare was initiated.

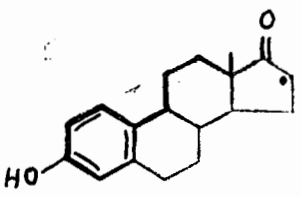
The work presented in the first part of this thesis describes the metabolism of the ring B unsaturated estrogens in the pregnant mare after administration of estrone - 16- C^{14} (Section A) and sodium acetate- 1-C^{14} (Section B).

It is generally agreed that both the biological and physiochemical methods now available for the assay of urinary estrogens are inadequate for the determination of the small amounts of estrogens present in normal female urine. Therefore, an investigation was initiated to ascertain whether the recent methods of paper chromatography would be sufficiently sensitive to identify the small amounts of estrogens present in such urines. The preliminary results obtained from this study will be presented in the second part of this thesis.

A. THE FAILURE OF ESTRONE TO BE CONVERTED TO EQUILIN AND EQUILENIN

1. Introduction and Discussion

After the discovery of Zondek (19) that mare's pregnancy urine was a rich source of estrogens, many investigations ensued to determine the chemical structure of the hormones present therein. Zondek (32) made the surprising disdovery that stallion's urine contained larger amounts of estrogens than did mare's pregnancy urine. However, this finding has been refuted by Doisy (33) and several other investigators. Indeed, Doisy (33) stated that there are wide individual variations in the excretion of estrogens by pregnant mares and stallions and that variations also exist in every mare at different stages of gestation. In 1932, Girard and his associates (34) found that aside from estrone, (I), the urine of pregnant mares contains two ring B unsaturated estrogens which he named equilin (II) and equilenin (III).



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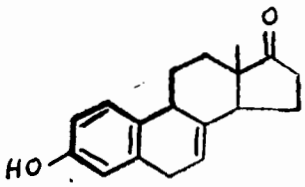

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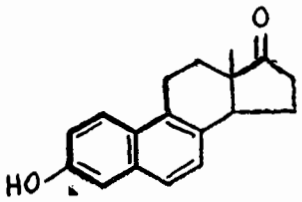


III

C' - designates the position of C^{14} in estrone-16-C^{14}(I).

Along with equilin (I) Girard et al. (34) found another compound which he named hippulin, but its presence in mare's pregnancy urine has never been confirmed. Equilenin (III) was characterised by Girard and his associates (34) as a β-naphthol because it formed an insoluble picrate and gave the specific color tests of naphthols. Complete chemical identification of equilin and equilenin was accomplished by Cohen, Cook and Hewett (35) in 1935.

Until 1944, mare's pregnancy urine was the only commercial source of estrone. As a result, pharmaceutical companies on this continent and in Europe processed huge volumes of mares' pregnancy urine for estrone and donated the remaining fractions of the urine to interested investigators. Because of the ready access to extracts of mares' pregnancy urine, it is not very surprising that such a great variety of steroids have been isolated from this source. A list of the steroids isolated from mare's pregnancy urine is presented in Table I.

TABLE I

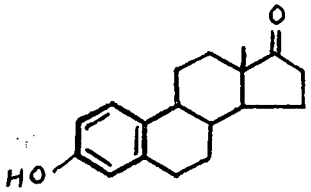
Steroids Isolated from Mare's Pregnancy Urine

	Name	Reference
	C_{18} Steroids.	
(I)	Estrone	(20)
(II)	Equilin	(34)
(III)	Equilenin	(34)
(IV)	Dihydroequilenin	(36)
(V)	3-Desoxyequilenin	(37)
(VI)	Estradiol-17α #	(36,38)
(VII)	Estradiol-17β #	(38)
(VIII)	Estra-5,7,9-triene-3-ol-17-one	(39)
(IX)	Compound 3	(36)
	C_{21} Steroids.	
	Pregnane-3α,20α-diol	(41)
	5α-Pregnane-3α,20α-diol	(41)
	5α-Pregnane-3β,20α-diol	(42)
	5α-Pregnane-3β,20β-diol	(43,44)
	5α-Pregnane-3β-ol-20-one	(45,46,47)
	Pregnane-3,20-dione	(45)
	5α-Pregnane-3,20-dione	(45)
	Pregn-5-ene-3β,20α-diol	(48)
	5α-Pregn-16-ene-3β-ol-20-one	(49)
	5α-Pregnane-3β,16α,20β-triol	(50)
	$C_{21}H_{34}O_2$	(47)
	C_{19} Steroids	
	Androstane-3β-ol-16-one	(45,47)
	Androst-5-ene-3β-ol-17-one	(47)
	$C_{19}H_{26}O_3$	(51,52)
	$C_{19}H_{30}O_2$	(47)
	$C_{19}H_{24}O_2$	(47)

The nomenclature employed is that of Fieser and Fieser (53).

Aside from the compounds listed in Table I a number of steroids of the urane series have been isolated from pregnant mare's urine by Marker and Rohrmann (54). These uranes have recently been shown to be 3,17a-substituted, 17-methyl-D-homo-androstanes by Klyne and Shoppee (55). The estrogens and some of the related steroids isolated from mare's pregnancy urine are formulated in Figure 1.

Figure 1.



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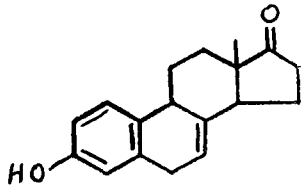

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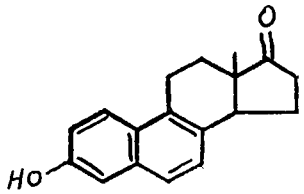

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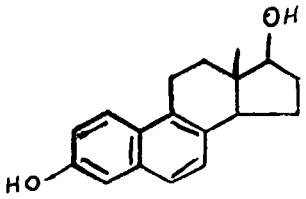

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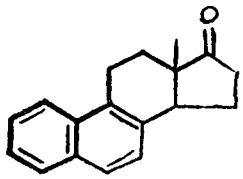

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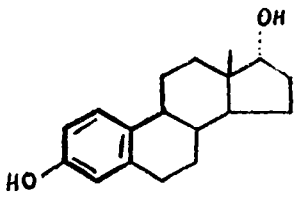

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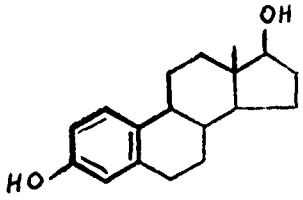

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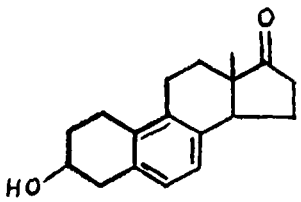

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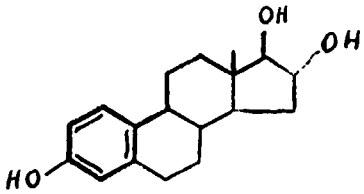
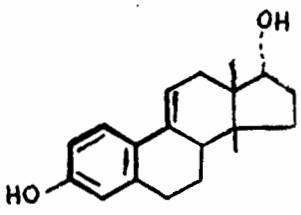


IX

The most abundant C_{18} steroids present in mare's pregnancy urine are estrone (I), equilin (II) and equilenin (III). The remaining C_{18} steroids listed in Table 1 have been isolated only in minute quantities. Estriol (IX) has never been isolated from equine urine but it has been isolated from human placenta (56), and from human female pregnancy urine (57,58). Dihydroequilenin (IV) was originally designated as the 17β epimer but Shoppee (59) has recently shown that it is dihydroequilenin-17α. Compound 3, originally isolated by Hirschman and Wintersteiner (36), from the "δ follicular hormone" of Schwenk and Hildebrandt (60), has recently been shown to be estra-1,3,5(10),9(11)-tetraene-14-iso-17α-ol (X) by Banes, Carol and Maenni (61). These authors suggested that Compound 3 is an artifact formed during acid hydrolysis of mare's pregnancy urine. Banes et al. (61) also claimed that estra-1,3,5(10),8(9)-tetraene-14-iso-17 -one (XI) is identical with hippulin previously isolated by Girard et al. (34) However, the evidence for this claim is not very conclusive since the isolated hippulin was not properly characterized and may be an artifact.



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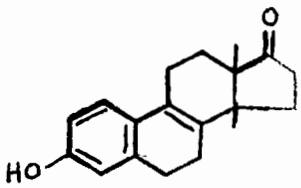


XI

Of the C_{21}-steroids, the only one whose presence in equine urine is questionable is pregnane-3α,20α-diol. Marker and his associates (41) have reported its presence in mare's urine, but other investigators have not been successful in isolating it (62). The compound $C_{21}H_{34}O_2$ and an epimer of it has been isolated by Oppenauer (47) from equine urine, but this work has not been confirmed. Oppenauer was unable to characterize these compounds but he suggested that they may be isomeric pregnanolones.

Of the C_{19}-steroids isolated from mare's urine, androstane-3β-ol-16-one(formerly known as Heard's oxyketone) has recently been synthesized by Huffman and Lott (63), Oppenauer (47), reported the isolation of androst-5-ene-3β-ol-17-one from equine urine, but this finding has not as yet been confirmed. Heard (51), and Jacobs and Laqueur (52) have isolated a compound of empirical formula $C_{19}H_{26}O_3$ from equine urine, and both groups of investigators provided evidence which suggests that this compound is a steroid lactone, but complete identification of it is still lacking. Finally Oppenauer (47) has isolated two compounds, $C_{19}H_{30}O_2$ and $C_{19}H_{24}O_2$, the structures of which are unknown.

At present, there are two theories concerning the metabolism of estrogens in the pregnant mare. The first theory was postulated by Girard and his associates (34c) on the basis of the following observations. These investigators found that as

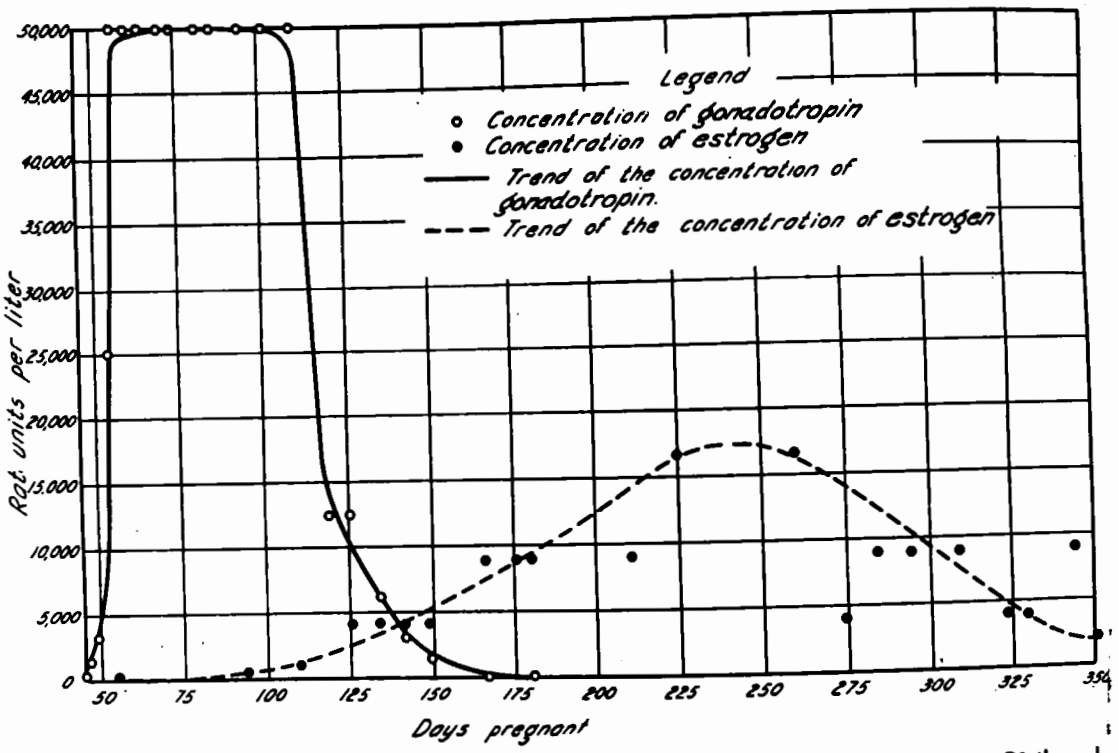
gestation in the mare progresses, the ratio of urinary estrone (I) to the more highly unsaturated compounds, equilin (II) and equilenin (III) decreases. From this finding the above-mentioned authors postulated that estrone is partially dehydrogenated to equilin and equilenin. This theory has also been put forth by Fieser and Fieser (53), who further pointed out that the phenomenon of dehydrogenation of estrone to equilin and equilenin may be a mechanism by which the pregnant mare partially inactivates estrone. Inhoffen et al. (64) found that the biological activity of estrone is 1.5 and 21 times that of equilin and equilenin respectively.

The second theory of estrogen metabolism in the pregnant mare was postulated by Doisy et al. (65) in 1942. Previously, Heard and Hoffman (39), isolated estra-5,7,9-triene-3-ol-17-one (VIII) from mare's pregnancy urine. This finding prompted Doisy et al. (65) to postulate that (VIII) is a likely intermediate in the production of estrogens from certain sterols by partial degradation. These authors rightly cited aromatization of saturated ring structures as a metabolic pathway more common than nuclear hydrogenation. According to Doisy's theory, equilin and equilenin are possible intermediates in the formation of estrone rather than products of its catabolism. The total synthesis of the benzenoid ring in the animal organism has never been demonstrated, but Dickens (66) has cited many examples of aromatization of saturated 6-Carbon ring derivatives.

The two theories presented above have not until now been tested experimentally and it is for this reason that we set out to determine whether estrone is converted to equilin and equilenin in the pregnant mare. To accomplish this aim, it was decided to administer estrone-16-C^{14} to a pregnant mare and to isolate estrone, equilin and equilenin from her urine. The relative abundance of C^{14} in the three isolated estrogens would determine whether estrone is converted to equilin and (or) equilenin.

The pregnant mare is the only animal which is known to excrete equilin and equilenin. Girard et al. (34c) found that equilenin can barely be detected in the urine of the pregnant mare on the 175th day of gestation, but it appears in appreciable quantities about the 200th day and is very abundant in the last months of pregnancy. Thus, the stage of pregnancy is very important when one desires to isolate equilin and equilenin.

Quantitative data of the estrogens excreted in the urine by the mare during pregnancy are not available in the literature. However, Cole and Saunder (67) and Cole and Hart (68) have published some qualitative results of the urinary estrogens determined throughout pregnancy in the mare. The data taken from Cole and Hart (68) is shown in Figure 2.



The concentration of gonadotropin in blood serum and of estrogen in the urine of mare C8 throughout pregnancy.

Fig. 2 Data taken from Cole and Hart (68).

From Figure 2 it is seen that estrogen excretion is maximal at about 240 days and steadily decreases in the last 100 days of pregnancy. In this study the estrogens were extracted with benzene after mild acid hydrolysis of the urine, and were determined with a crude bioassay procedure. As a result the data presented in Figure 2 must be considered only as qualitative.

Beall and Edson (69) have published data on the isolation of estrone from mare's pregnancy urine collected at monthly intervals throughout gestation. Their data is graphically presented in Figure 3.

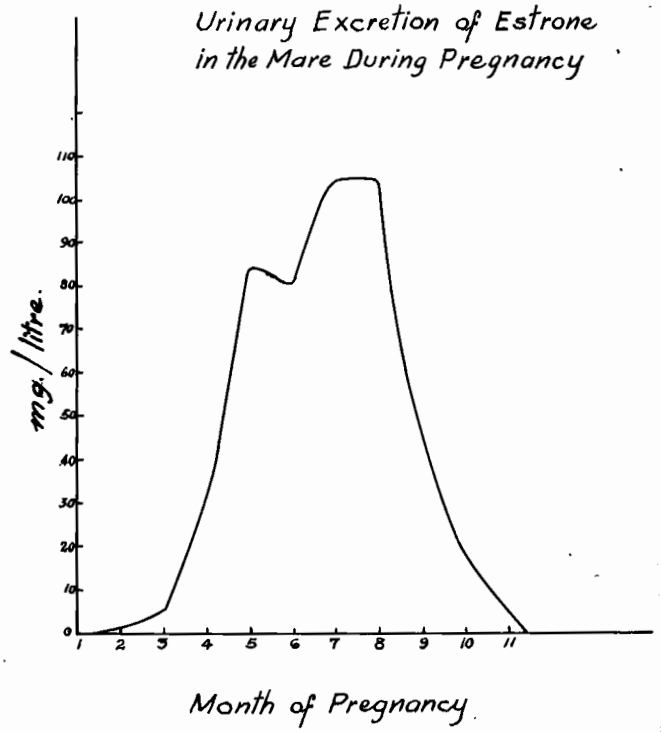


Fig. 3 Data taken from Beall and Edson (69).

From Fig. 3 it is seen that estrone excretion is at a maximum in the seventh to eight month of pregnancy but falls off sharply in the ninth to eleventh month. In the ninth month of pregnancy the urinary excretion of estrone was found to be about 40 mg. per litre. Such a quantity of estrone admits facile isolation.

From the data presented above, it is evident that the ninth month of pregnancy is the most suitable period for the isolation of estrone, equilin and equilenin from the urine of the pregnant mare. We were therefore guided by these facts in choosing a Clydesdale mare in her ninth month of gestation for this investigation.

The yield and purity of the isolated estrogens from mare's pregnancy urine depend to a large extent upon the methods of hydrolysis and extraction. In the early work on the isolation of estrogens from equine urine, huge volumes of urine were processed and as a result very little work was done to determine optimum conditions of hydrolysis and quantitative methods of extraction. In 1930, Zondek (19) first observed that acid hydrolysis increased the quantity of ether extractable estrogens from equine urine. The following year, Kober (70) developed a colorimetric method for the determination of estrogens. This method was modified by Cohen and Marrian (71) and was applied to the determination of the estrogens present in mare's urine.

The use of this colorimetric method greatly facilitated the development of better methods of hydrolysing urine and of extracting the estrogens. Beall and Marrian (72) found that heating acidified mare's pregnancy urine at 100°C for varying periods of time or autoclaving the acidified urine at 18 pounds pressure resulted in some destruction of estrone. These authors reported that the highest yield of extractable estrone was obtained when the urine was acidified to pH 1 and allowed to hydrolyse at room temperature for one or two weeks. This was essentially the method previously described by Curtis et al. (73). A systematic study of the hydrolysis of equine urine has been reported by Edson and Heard (74). These authors found that the optimal hydrolytic conditions are attained when the urine is acidified to pH 0.4-0.6 and allowed to stand at room temperature for four weeks.

Cohen and Marrian (75) found that 90% of the estrogens in equine pregnancy urine are excreted as water soluble, ether-insoluble conjugates and are physiologically relatively inert. Two years later, Schachter and Marrian (76) succeeded in isolating a small quantity of the potassium salt of estrone sulphate from mare's pregnancy urine. In recent years Klyne and his associates (77,78,79) have described the isolation from equine pregnancy urine of the sulphuric acid esters of 5α-pregn-16-en-3β-ol-20-one, uranediol and 5α-pregnane-3β-ol-20-one. A novel enzymatic hydrolysis of the conjugated estrogen

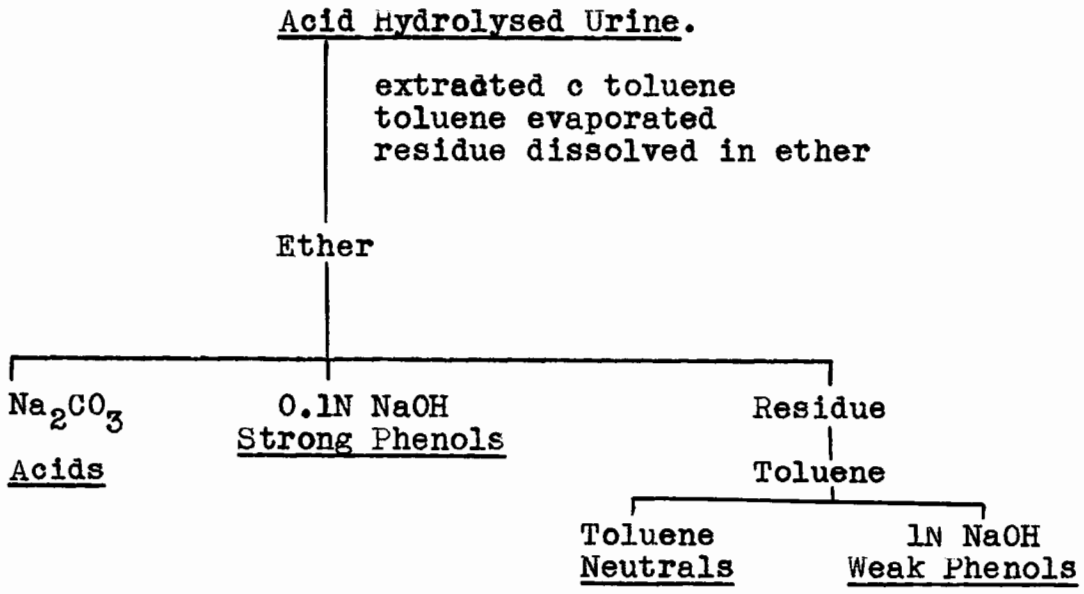
sulphates by a sulphatase was published by Cohen and Bates (80). These authors used the specific phenolsulfatase present in extracts of aspergillus oryzae to hydrolyse the estrogen conjugates and found that enzymatic hydrolysis was 20% more efficient than acid hydrolysis.

Thus it can be seen that there are a wide choice of methods for hydrolysing mare's pregnancy urine. The method of hydrolysis which one chooses is usually governed by what one desires to isolate from the urine. In this investigation we were primarily concerned with the isolation of estrone, equilin and equilenin and we therefore used the method of hydrolysis developed by Charles E.Frosst and Co. With this method the urine is acidified to pH 1 with concentrated hydrochloric acid, covered with an equal volume of toluene and refluxed for two hours. The toluene cover protects the urine from the destructive action of oxygen from the air, and removes the estrogens away from the hydrochloric acid as they are hydrolysed. Cohen and Bates (80) have reported that covering the urine with toluene during hydrolysis yields large amount of extractable estrogens.

In spite of the high concentration of estrogens in mare's pregnancy urine, very little has been published on its extraction and purification. Curtis et al. (73) used benzoic acid to absorb the estrogens from equine urine and on desorption obtained as much as 100 mg. of pure estrone per gallon of urine.

Beall and Marrian (72) published a procedure for the extraction of estrogens from mare's urine which has formed the basis for all the extraction procedures used since. These authors modified the procedure originally used by Cohen and Marrian (71) for the extraction and separation of estrone and estriol from human pregnancy urine, and applied it to the extraction of the estrogens from mare's urine. An outline of this extraction procedure is given below.

The Marrian Procedure.



Beall and Marrian (72) found that in the extraction procedure described above most of the estrone appears in the weak phenolic fraction along with some equilin. These authors were able to separate estrone and equilin contained in the weak

phenols by the difference in the solubilities of their mercury complexes. Beall and Edson (69) have modified the original Marrian procedure in order to get a more complete separation of the acids from the total phenols. These authors found that approximately 20% of the estrone enters the strong phenolic fraction along with most of the equilin and equilenin.

In the present investigation a modified version of the Marrian procedure was used to separate the toluene extract of the acid hydrolysed urine into acid, strong phenols, weak phenols and neutrals. It was realized that such a fractionation would entail a further separation of estrone, equilin and equilenin from the strong phenols.

Having discussed the methods used in hydrolysing and extracting mare's pregnancy urine, we shall now go on to describe the experimental conditions used in this investigation and to discuss the results obtained.

The solution used for the administration of the estrone-16 -C^{14} to the pregnant mare consisted of 24% ethyl alcohol-isotonic saline. Since estrone is not soluble in isotonic saline, ethyl alcohol was used to get the hormone into solution. By trial and error, it was found that a 24% ethyl alcohol-isotonic saline solution would hold about 10 mg. of estrone in solution and would not precipitate the blood proteins. Human plasma was used in these trial experiments.

The pregnant mare used in this investigation was kindly donated by Arnold Farms, Grenville, Quebec. The administration of the hormone, collection of excreta and the initial steps of hydrolysis and extraction were performed on the premises of Steroid Laboratories, Grenville, Quebec. As a result, the extracts of the urine and feces had to be concentrated to small volumes for transportation to our laboratory in Montreal.

A solution of 9.82mg. of estrone-16-C^{14}, containing 25.53×10^6 d./min.#, in 24% alcohol-isotonic saline was infused intrajugularly into a Clydesdale mare in the ninth month of pregnancy. Samples of blood were taken immediately after injection and 1.5 hours later. The blood, preserved in 3.2% trisodium citrate, was shipped to Montreal for analysis. A sample of saliva was taken 1.5 hours after injection and kept for subsequent analysis.

Urine and feces were collected for 72 hours following the injection of the labeled estrone. The urine was acidified to pH 3 immediately after collection, and stored in the cold with a cover of toluene until a sufficient quantity accumulated for hydrolysis. The feces were collected and preserved under a cover of chloroform until the end of the experiment.

- Disintegrations/minute.

An examination, by Dr. F.Peron, of the blood taken from the mare revealed that only the alcohol soluble fraction of the plasma contained detectable amounts of C^{14}. If we assume that the radioactivity found in the blood is due to estrone-16-C^{14} or some metabolite of it (i.e. α or β estradiol-16-C^{14}) then this finding confirms the earlier observations made by Haussler (81). This author found that small amounts of estrogens are closely associated with the proteins in mare's serum. In species other than Equus Caballus the circulating estrogens have been found to be closely associated with the plasma proteins (82,83).

The urine, acidified to pH 1 with hydrochloric acid and covered with an equal volume of toluene, was hydrolysed by refluxing for two hours. The hydrolysed urine, after cooling, was extracted with toluene and the toluene extract was partitioned into acids, strong phenols, weak phenols and neutrals by extracting with 5% and 10% Na_2CO_3, 0.1N NaOH and 1N NaOH respectively. In order to determine whether estrone-16-C^{14} gives rise to water soluble excretory metabolites, the urine remaining after hydrolysis and toluene extraction (aqueous urine) was reduced to a small volume and saved for future analysis.

The CO_2 evolved during the acidification and hydrolysis of a portion of the urine collected on the second day, was

trapped in sodium hydroxide and converted to $BaCO_3$. The $BaCO_3$ did not contain any detectable radioactivity. This result indicated that the D ring of estrone-16-C^{14} was not ruptured with the elimination of $C^{14}O_2$, since equine urine contains abundant quantities of acetate buffers which would have incorporated any radioactive carbon-dioxide had it been present.

The determination of C^{14} in crude extracts, such as those met with in this investigation, is a very vexing problem. The difficulties entailed in such determinations have been enumerated by Calvin et al. (84). In this laboratory, a method for the determination of C^{14} in crude fractions was devised by Yates (29). This method allows correction for the self-absorption errors inherent in the assay of crude extracts. When the procedure of Yates (29) was applied for the determination of the C^{14} content of the various fractions obtained in the extractions of the acid hydrolysed urine, the results obtained were as follows. Of the injected radioactivity 39.64% was recovered in the urine, and 74.70% of this activity was extractable with toluene. Of the radio-activity extractable with toluene, 1% was present in the acids, 68.2% in the strong phenols, and 28.7% in the weak phenols and 14.98% in the neutrals. The striking fact in these partitions was that 68.2% of radioactivity was removed by extraction with 0.1N NaOH (strong phenols.) The strong phenols thus contained about 20% of the injected radioactivity.

The feces, collected for 72 hours following the administration of the labeled estrone to the pregnant mare, was exhaustively extracted with chloroform and a portion of the fecal residue was retained for future examination. However, this chloroform extract did not contain any radioactivity when it was assayed with the method described by Yates (29). In order to eliminate the possibility that the radioactivity excreted in the feces was in a water soluble form, an aliquot of the fecal residue was extracted with water but this aqueous extract did not contain any C^{14}. Another possibility that had to be eliminated was that the chloroform extract of the feces contained very small amounts of C^{14} and that this low level of radioactivity could not be detected in the assay of the crude extracts. Therefore half of the chloroform extract of the feces was extracted by the procedure described for the fractionation of the toluene extract of the hydrolysed urine. In this fractionation, heavy emulsions were encountered and only a very crude separation into acids, strong phenols, weak phenols and neutrals was realized. These fractions were further purified by extraction with ethyl alcohol. When the alcoholic solutions were assayed for C^{14}, no radioactivity could be detected in them. It was therefore concluded that estrone-16 -C^{14} does not give rise to metabolic products which are excreted <u>via</u> the gastrointestinal tract.

The above findings are not in agreement with the observations published by Kemp and Pedersen-Bjergaard (85). These authors found that the feces of the pregnant mare contain sizable amounts of estrogens. However, the activity found in the feces was determined by bioassay and it is not altogether certain whether the values obtained were actually due to the known estrogens. There seems to be a sharp species difference in the metabolism of estrone-16-C^{14}. Thompson (31) subcutaneously injected five female mice with one milligram of estrone-16-C^{14} per animal. The pooled feces excreted during six days contained 20.45% of the injected radioactivity and the expired CO_2 accounted for approximately 10% of the injected dose. More recently, Budy (86) subcutaneously injected immature C.F. mice with 0.1 mg. of estrone-16-C^{14}. At 24 hours after injection, 10 to 20% of the injected activity appeared in the feces and the urine. However, there was no measurable radioactivity in the expired air. The expired $C^{14}O_2$ obtained by Thompson (31) may have been due to the relatively large quantity of estrone-16-C^{14} injected. The results obtained by Thompson (31) and Budy (86) clearly demonstrate that the rodent excretes a sizable quantity of administered estrone-16-C^{14} in the feces, whereas in the pregnant mare the excretion of radioactivity is confined to the urine.

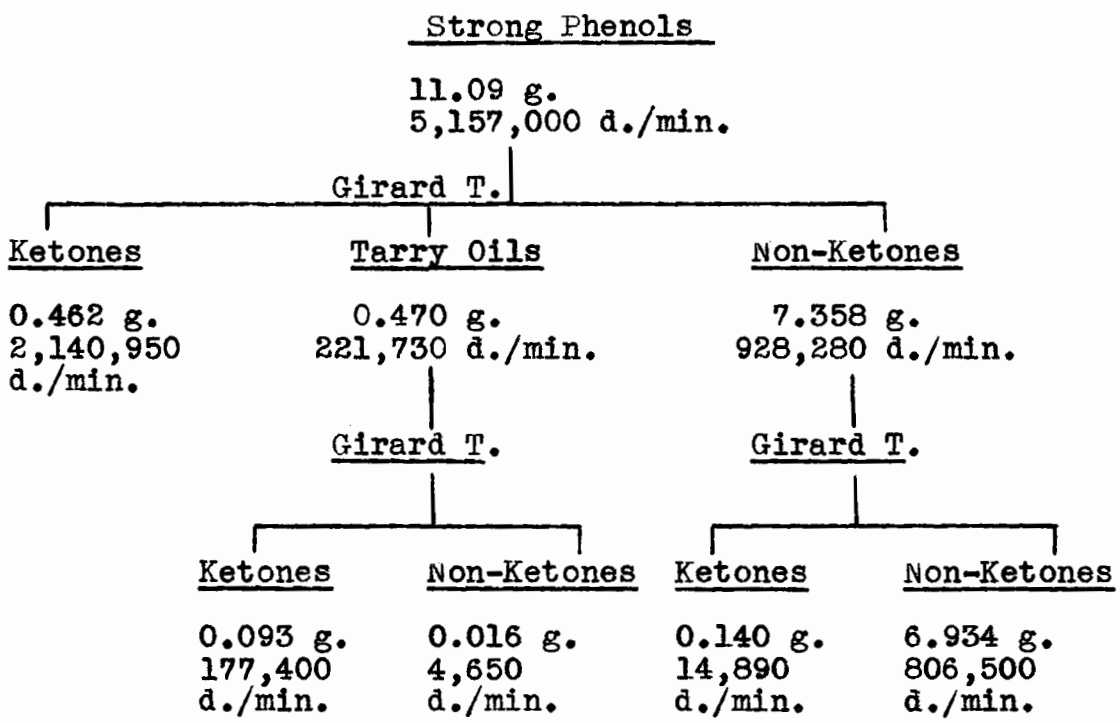
The alcohol extract of the saliva obtained 1.5 hours after injection of the estrone-16-C^{14} contained a small amount of radioactivity. This result demonstrated that at 1.5 hours after injection, the labeled estrone was well distributed in the animal body.

Since sizable amounts of estrone are excreted in the urine of the pregnant mare even in the ninth month of gestation, it was decided to determine the distribution of this hormone in the extraction procedure used in fractionating the acid hydrolysed urine. To accomplish this aim, estrone-16-C^{14} was added to mare's urine from which the estrogens had previously been removed, and this material was partitioned by the identical method used to extract the urine collected in this investigation. After assaying the fractions obtained, it was found that 80% of the estrone was extracted from the urine with toluene. Fractionation of this toluene extract showed that the acids, strong phenols, weak phenols and neutrals contained respectively 1.79%, 56%, 29.08% and 11.33% of the radioactivity originally present in the toluene extract. These results are in close agreement with the values obtained in the fractionation of the toluene extract from the urine collected after the administration of estrone-16-C^{14}. The close agreement between the two sets of data led us to believe that the major C^{14} labeled excretory product present in the urine of the pregnant mare after administration of estrone-16-C^{14} was unaltered estrone.

Mather (87), and Bachman and Pettit (88) have investigated the relative distribution of the estrogens among immiscible solvent pairs. However, these authors did not use any of the solvent pairs employed in this investigation. It was therefore of interest to us to determine the distribution of estrone -16-C^{14} between the solvent pairs used in the extraction of the urine collected after injection of the C^{14}-estrone. In these experiments the procedure described by Mather (87) was used to partition estrone-16-C^{14} between the immiscible solvent pairs. The results obtained from these partition studies are generally in agreement with the distribution of the radioactive material present in the urine of the pregnant mare used in this investigation.

During hydrolysis of urine collected from the pregnant mare large quantities of reddish-brown solids precipitated. This material was collected and an attempt was made to identify it. After crystallizing this material from 95% ethyl alcohol and removing colored impurities with activated charcoal, long colorless needles were obtained. These crystals proved to be non-radioactive hippuric acid. This result indicated that carbon atom-16 of estrone was not incorporated into the benzene ring or into the side chain of the isolated hippuric acid.

In 1936, Girard and Sandulesco (89) published a method for the separation of ketones from non-ketones present in urinary extracts, which has been widely used in the isolation of urinary metabolic products. These authors described a ketone coupling reagent, trimethylaminoacetohydrazide, $[(CH_3)_3 NCH_2CONHNH_2] Cl^-$ which forms a water soluble complex with ketones, thus making it possible to separate the ether soluble non-ketonic material. The ketones are then regenerated by acid hydrolysis of the Girard complex. This reagent is generally referred to as Girard's reagent T. Girard and Sandulesco (89) also found that a single treatment of a crude estrogen extract with this reagent does not effect a complete separation of ketones from non-ketones. These authors therefore recommended that the non-ketones should be subjected to a second Girard reaction ("reGirard") in order to further separate the ketones. In applying the Girard separation to the strong phenols, it was found that tarry oils separated at the interface between the ether solution containing the non-ketones and the aqueous phase containing the Girard complex. Girard made the same observation, but he combined these tarry oils with the ketonic fraction. In this investigation it was decided to determine whether these tarry oils were ketones or non-ketones by reacting them with Girard's reagent T. Hence, following Girard's instructions, the strong phenols were separated into ketones and non-ketones as outlined diagramatically below.



Total Ketones - 0.695 g.
2,332,840 d./min.

Total Non-Ketones - 6.95 g.
811,150 d./min.

From the above data it can be seen that there was a large loss, both in weight and in radioactivity, in the first Girard separation on the strong phenols. Assay of all the aqueous solutions resulting from this reaction only accounted for an additional 287,760 d./min. The only reasonable explanation for these large losses is that they may be due to the loss of radioactive volatile light oils. It is possible that in the process of distilling solvents from the urinary fraction and in reducing these fractions to dryness, these volatile oils are lost. Prior

to reacting the strong phenols with Girard's reagent T, an attempt was made to remove these oils by distillation under high vacuum. This procedure was not successful because of heavy bumping, even though precautions were taken to prevent this.

As a result the strong phenols were reacted with Girard reagent T in the presence of the volatile light oils, which may account for the large losses incurred in this reaction. It is of interest to note that the total ketones contained the major portion of the recovered radioactivity.

Because the ketonic strong phenols contained the major portion of the radioactivity, experiments were designed to separate the estrone, equilin and equilenin present in this fraction. Girard et al. (34c) found that equilenin can be separated from estrone and equilin by forming the insoluble, stable equilenin picrate. Under the conditions used by these investigators, estrone and equilin do not form stable picrates. It was therefore logical to try to isolate equilenin via its picrate.

In trial experiments it was found that 20 mg. of equilenin readily forms an insoluble picrate in 80% yield. However, when the ketonic strong phenols were dissolved in an alcoholic solution saturated with picric acid, equilenin picrate did not precipitate. Instead, a small amount of crystalline material separated which proved to be estrone. From this it was deduced

that the ketonic strong phenols contained either very small amounts of equilenin or relatively large amounts of estrone. Girard et al. (34c) found that equilenin cannot be separated from estrone by picrate formation in the cold, unless the ratio of equilenin to estrone is greater than 1 to 10. Thus equilenin picrate would not precipitate if the ketonic strong phenols contained large quantities of estrone.

Since equilenin picrate failed to precipitate, the ketonic strong phenols were extracted with 1% Na_2CO_3 in order to decompose any picrate which may have been in solution and to remove the residual picric acid. This treatment removed large amounts of colored impurities from the ketonic strong phenols without any appreciable loss of radioactivity.

Doisy et al. (90) found that equilenin is almost twice as soluble as estrone in ethyl alcohol at 30°C. In a preliminary experiment it was determined that equilin is also more soluble than estrone in ethyl alcohol. On the basis of these findings, it was decided to separate estrone from the ketonic strong phenols by fractional crystallization. By this method five crops of crystals weighing 40 mg. separated from the ketonic strong phenols. The mother liquors resulting from this procedure were kept for further analysis. The third crop was recrystallized from ethyl alcohol and the colorless needles obtained weighed 20 mg. These crystals had a melting point of 260-262°C, which was not depressed on admixture with authentic estrone. In ultraviolet light this material exhibited an ab-

sorption maximum at 280 mμ, and its ultraviolet spectrum in sulphuric acid was identical with the spectrum given by authentic estrone. This isolated estrone had a specific activity of 37,650 d./min./mg.

In this investigation the ultraviolet spectra in sulphuric acid (sulphuric acid chromogens) of estrone, equilin and equilenin yielded valuable information in establishing the identity of these estrogens.

Zaffaroni (91) first introduced this spectrophotometric technique for the identification of adrenocortical steroids. Since then, this method has been widely used for the identification of a large variety of steroids (92). It was found that estrone, equilin and equilenin give characteristic ultraviolet absorption spectra in sulphuric acid, and as a result this technique was used as an aid in the identification of all the compounds isolated in this investigation.

The mother liquors remaining after fractional crystallization of estrone from the ketonic strong phenols should contain some estrone as well as equilin and equilenin. In order to separate the three estrogens it was necessary to determine their relative concentration in this fraction. A search of the literature revealed that there is only one published method for the estimation of estrone, equilin and equilenin in mixtures. There are however two other methods

which should be mentioned here.

Marx and Sobotka (93) found a specific test for equilenin and its diol, based on the coupling reaction with p-nitro-benzeneazodimethoxyaniline (Fast-Black-Salt-K). This color reaction has not been developed into a quantitative method for the determination of equilenin. Umberger and Curtis (94) showed that a mixture of equilin and estrone can be analysed by heating in 90% sulphuric acid and measuring the colorimetric intensity at four different wave lengths. This empirical method has never been applied for the determination of urinary estrogens. Banes (95) has devised a colorimetric method for the determination of estrone, equilin and equilenin in mixtures, based on the colored complexes formed when equilin and equilenin are treated with dibromoquinonechloroimide (B.Q.C.). In this method, equilin and equilenin produce stable colored complexes when treated with dibromoquinonechloroimide, while estrone does not. Upon conversion of the three estrogens to their benzenesulfonates, only equilin yields a stable colored complex. Thus by difference it is possible to determine the concentrations of equilin and equilenin. Estrone is determined by the Haenni modification of the Kober reaction (95). This colorimetric method has been successfully used to determine the estrogen content of the ketonic fractions from mare's pregnancy urine. This three step reaction, which will be referred to here as the "B.Q.C. analysis," was originally very time consuming but recently Grant and Beall (96) have introduced to it some time saving modifications.

Trial determinations with the B.Q.C. method on a sample solution of pure estrogens gave good recoveries of estrone, equilin and equilenin. Therefore this colorimetric method was used to analyse the mother liquors obtained from the ketonic strong phenols after removal of estrone. The results obtained showed that these mother liquors contained 15 mg. of equilin, 11.25 mg. of equilenin and 37.5 mg. of estrone. From these results it was evident that the pregnant mare used in this investigation excreted very small quantities of equilin and equilenin and relatively large amounts of estrone. Our next problem was to find a method of separating these three estrogens.

In 1935, Duschinsky and Lederer (97) described a procedure for the separation of estrone and equilenin by absorption chromatography. These authors used crude estrogen extracts from mare's pregnancy urine and adsorbed them from benzene on columns of calcium oxide or aluminum oxide. After eluting with benzene, the course of chromatography was followed by observing the different pigments formed along the length of the column. From the calcium oxide column it was possible to isolate estrone; but the aluminum oxide column yielded both estrone and equilenin. No equilin was isolated by either method. The estrogens thus separated were impure and could only be purified by numerous recrystallizations from alcohol. In view of the small quantities of the three estrogens present in the mother liquors of the ketonic strong phenols, no attempt

was made to use the above method to separate them. Veitch and Milone (98) found that estrone and equilenin can be separated by chromatography of their 2,4-dinitrophenylhydrazones on alumina. This method has never been applied for the separation of the estrogens present in extracts of mare's pregnancy urine. Banes, Carol and Haenni (99) have succeeded in separating a large number of isomeric estrogens with a novel method of partition chromatography. These investigators adsorbed the estrogens on a column of Celite previously impregnated with 0.4N NaOH (stationary phase) and fractionally eluted them with benzene (mobile phase). This method of chromatography has been applied for the resolution of isoequilin A into its component ketosteroids (100). Among the components of isoequilin A there were also found small amounts of equilin and equilenin which were eluted from the Celite column. Equilin was the first to be eluted and equilenin separated in the final fractions. In a private communication, Dr. J.Carol stated that from his experience in separating closely related estrogens, equilenin should readily separate from estrone and equilin on the Celite column, but that equilin would separate from estrone only on rechromatography of the zone where the two overlap.

We therefore set out to test this method of chromatography with mixtures of pure estrone, equilin and equilenin. The unesterified B.Q.C. reaction was used to analyse the fractions eluted.

As was previously mentioned, this reaction is only sensitive to equilin and equilenin and as a result the fractions eluted containing pure estrone can be readily detected. In trial experiments using the pure estrogens, it was found that the best separation of estrone, equilin and equilenin was obtained when 10 ml. portions of benzene were eluted. These trial experiments showed that on the average pure estrone is eluted from the Celite column in the first nine fractions. The next ten fractions contained the zone of overlapping of estrone and equilin while pure equilenin was eluted in the last six fractions. When the eluates containing a mixture of equilin plus estrone were rechromatographed on the Celite column, only 38% of the equilin separated in pure form. Thus there was always a portion of the equilin which remained in admixture with estrone. On the basis of the above results it was concluded that this method of Celite chromatography was capable of effecting a separation of urinary estrone, equilin and equilenin.

A number of refinements were introduced in the chromatography of the mother liquors from the ketonic strong phenols because it was known that this fraction contained highly radioactive material. It was thus possible to analyse each fraction eluted from the Celite column for both its B.Q.C. colorimetric content, and its C^{14} content. This made it possible to follow the estrone eluted from the column using the following procedure. In the B.Q.C. procedure, the colored complexes of

equilin and equilenin were dissolved in chloroform and this solution served for the determination of the optical density. Prior to reading the optical density, the chloroformic solutions were washed with 20 ml. of 10% sodium hydroxide. This treatment removed brown by-products formed from the excess B.Q.C. reagent during the course of the reaction. Since estrone does not react with the B.Q.C. reagent it is free to distribute itself between the alkaline phase and the chloroform phase. In a separate experiment it was found that under the conditions employed in the B.Q.C. reaction, the partition coefficient of estrone between chloroform and 10% sodium hydroxide is 3.0. Because the specific activity of the estrone isolated is 37,650 d./min./mg., only very minute quantities of it need enter the alkaline phase to be detected. Therefore the alkali washes of the chloroform solutions were acidified and the estrone present was extracted with ether. The ethereal solutions were evaporated and the residues were dissolved in ethanol and assayed for radioactivity. This procedure made it possible to detect the zone where estrone and equilin overlapped and the fractions in which pure equilin was eluted.

The mother liquors from the ketonic strong phenols obtained after fractional crystallization of the estrone were chromatographed on Celite previously impregnated with 0.4N NaOH. Each eluted fraction was analysed by the methods described above. Chromatography of these mother liquors on Celite yielded

fractions containing pure estrone and pure equilenin. In addition, a small amount of equilin was eluted. However, the major portion of the equilin was eluted in admixture with estrone. These latter fractions were rechromatographed on Celite and an additional amount of equilin separated from the estrone. B.Q.C. analysis showed that the equilenin eluates from the first Celite column contained 3.92 mg. of material, while the amount of material present in the combined equilin eluates from both chromatograms was 7.89 mg. From the first chromatogram 8 mg. of estrone (m.p. 256-258°C, specific activity 37,620 d./min./mg.) were isolated and characterized.

The equilenin eluates (3.92 mg. by B.Q.C. analysis) from the first chromatogram could not be induced to crystallize because of the large amounts of impurities present in this fraction. On the basis of the colorimetric assay and total radioactivity present in this fraction, the specific activity of the B.Q.C. positive material was calculated to be 413 d./min./mg. That equilenin was present in this fraction was revealed by the absorption peak it gave at 340 mu. Equilenin is the only estrogen having an absorption peak in this region of the ultraviolet spectrum. (36). The ultraviolet spectrum in sulphuric acid given by the equilenin eluates corresponded closely to that obtained with authentic equilenin. It was therefore concluded that the equilenin eluates did indeed contain equilenin.

The combined equilin eluates from both Celite chromatograms consisted of oily material which could not be induced to crystallize from either ethyl acetate, 95% ethanol or 80% aqueous methanol. Beall and Edson (69) found that 80% aqueous methanol is a very effective solvent for crystallizing equilin, but these authors had very large quantities of the hormone to work with, whereas here we were dealing with milligram amounts. In view of the fact that the equilin eluates failed to crystallize, an attempt was made to separate the steroids from the impurities in this fraction by distillation under high vacuum. Fractional distillation at low pressures as a method of purifying crude urinary estrogens was first introduced by Herrmann (101) in 1915. In subsequent years this technique has been widely used to purify urinary estrogens.

Sublimation of the equilin eluates under high vacuum yielded 6.42 mg. of semi-crystallized material. This material crystallized from ethyl acetate to give 2.28 mg. of brown crystals, melting at 238-242°C. These crystals showed a depression in melting point on admixture with authentic equilin but the melting point was not depressed on admixture with authentic equilenin. The ultraviolet spectrum of this crystalline material corresponded closely with the spectrum of equilenin published by Dirscherl and Hannusch (102) and by Hirschmann and Wintersteiner (36). In particular this material showed an absorption maximum at 340 mμ., which is located in a range where

no other estrogen but equilenin absorbs light. From the extinction coefficient at 340 mμ. it was calculated that the crystalline material in the equilin eluates was 83% equilenin. The ultraviolet spectrum of this crystalline material in sulphuric acid corresponded closely to the spectrum obtained with authentic equilenin, except the region 320-380 mμ. In this region, equilin exhibits its maximum absorption. It was therefore concluded that the equilin eluates contained equilenin contaminated with a small amount of equilin. Zaffaroni (92) found that when two steroids are subjected to treatment with sulphuric acid, the resulting absorption spectrum is identical with the curve obtained by integration of the spectra of the two substances. This is in agreement with the observations made in this laboratory. The specific activity of this equilenin (83% pure) was found to be 380 d./min./mg. Since the specific activity of the isolated estrone was 37,650 d./min./mg., contamination of the equilenin with 1% of this estrone was sufficient to account for the specific activity of the former. In order to obtain confirmatory evidence for the identity of the isolated estrogens, attempts were made to find a method of paper chromatography that would separate estrone, equilin and equilenin.

To our knowledge, there are only two published methods for the separation of estrone, equilin and equilenin on paper. Although there are a number of systems that will separate estrone, estradiol-17β and estriol on paper, it does not necessarily follow that these chromatographic systems will separate estrone,

equilin and equilenin. The first method for the separation of estrone, equilin, and equilenin was described by Heftman (103). He found that the estrogens when diazotized with Fast-Black-Salt-K, can be readily separated by a number of solvent systems. This method could not be used to advantage in this investigation because we were not dealing with highly purified estrogens. Boscott (104) has recently published a novel method for separating estrone, equilin and equilenin on paper strips. He impregnated Whatman No. 3 paper strips with a saturated solution of sodium p-toluenesulphonate and used toluene equilibrated with the latter as a mobile phase. In our hands this method was effective in separating estrone, equilin and equilenin. The R_f values obtained were 0.55 for estrone, 0.25 for equilin and 0.10 for equilenin. Using this method of chromatography it was found that the material present in equilenin eluates exhibited the same R_f value as equilenin and travelled with equilenin in mixed chromatography. The crystalline material isolated from the equilin eluates, when chromatographed with this method also exhibited the R_f value of equilenin and travelled with the latter in mixed chromatography. However this method of chromatography was abandoned because it gave inconsistent results and the separated estrogens could not readily be eluted from the paper strips.

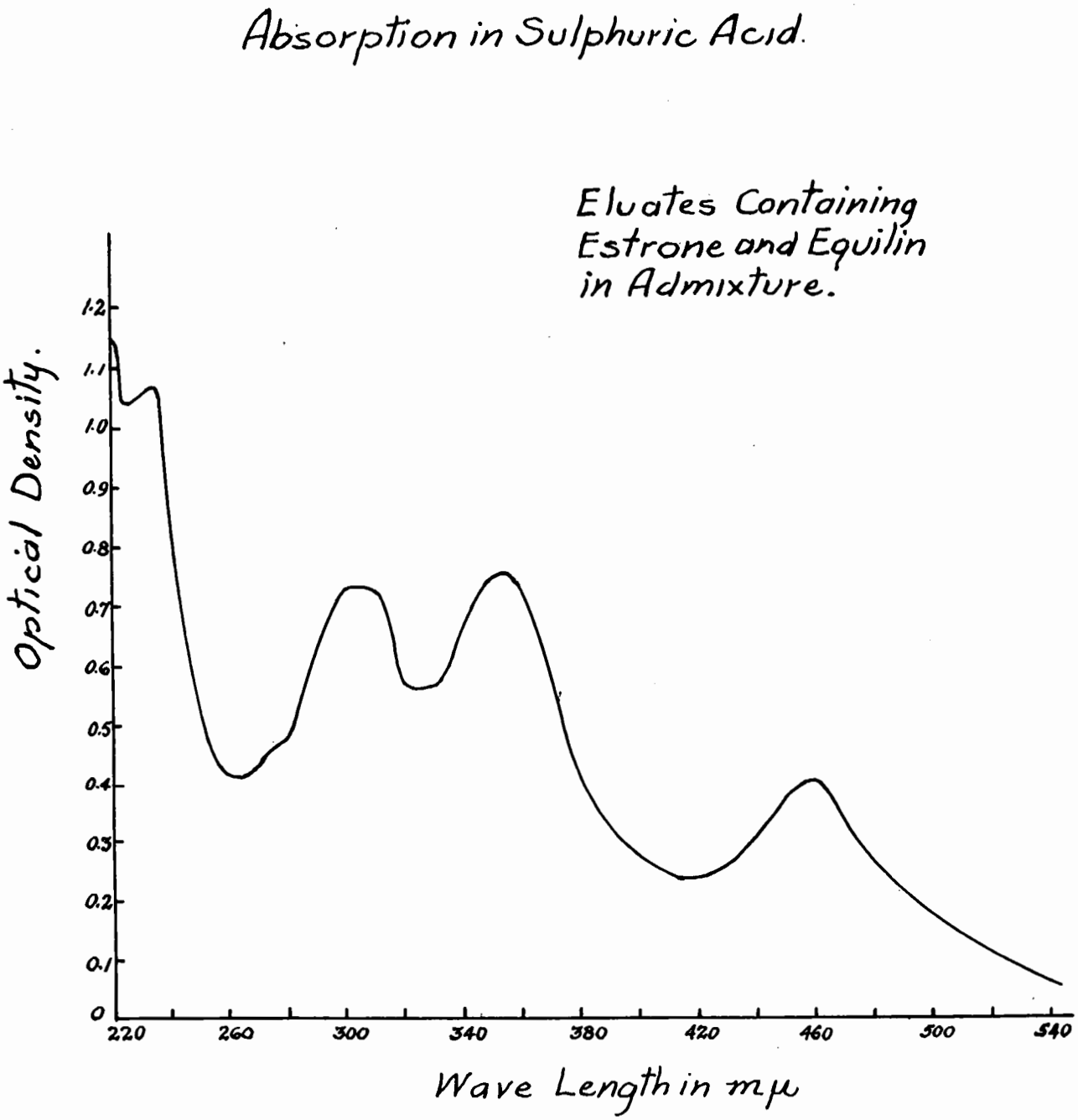
Paper strips impregnated with sodium hydroxide were not effective in separating the estrogens, although Celite impreg-

nated with sodium hydroxide does separate estrone, equilin and equilenin. Likewise, paper strips impregnated with propylene glycol as a stationary phase and using toluene or benzene as the mobile phase was not effective in separating estrone, equilin and equilenin. With this system estrone can be separated from equilenin but not from equilin. This method has been successfully used by Burton, Zaffaroni and Keutmann (105) for the separation of adrenocortical steroids on paper.

The chromatography system which was most effective in separating estrone, equilin and equilenin was o-dichlorobenzene-formamide-methanol (1:1). This system has recently been described by Axelrod (106), who used it for the separation of estrone, estradiol-17β, and estriol. The R_f values found for estrone, equilin, and equilenin were 7.4, 5.5, and 4.0, respectively. With this system it was possible to elute the estrogens from the paper strips with absolute methanol and to do mixed chromatograms. Using this system it was again found that the equilenin eluates contained equilenin and that the crystalline material isolated from the equilin eluates was equilenin. It was previously shown that there is always a zone on the Celite chromatogram where equilin and estrone overlap. When the fractions corresponding to this zone were chromatographed, using the above system, it was found that equilin was not completely separated from estrone. This is

probably due to the large amounts of estrone left in this fraction, although some had been previously removed by crystallization. When an aliquot of this fraction was dissolved in sulphuric acid, and the absorption spectrum of the solution was determined, the resulting curve (Fig. 4) was identical with the curve that is obtained by integrating the spectra of estrone and equilin. Thus, it was established that equilin was present in the eluates from the Celite chromatogram, but its isolation has not as yet been accomplished.

Figure 4


Absorption in Sulphuric Acid.
Eluates Containing Estrone and Equilin in Admixture.
Optical Density.
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220
260
300
340
380
420
460
500
540
Wave Length in mμ

In conclusion it should be stated that estrone of relatively high specific activity was isolated from the urine of the pregnant mare injected with estrone-16-C^{14}. Equilenin of very low specific activity was isolated from the same urine. There is some evidence which suggests that equilin was converted to equilenin on sublimation of the impure equilin eluates obtained from the Celite chromatogram. It has been shown that estrone is not appreciably converted to the ring B unsaturated estrogens, since the isolated equilenin contained about 1% of the radioactivity present in the isolated estrone. Thus, if 64 µg. of equilenin are assayed for C^{14}, there need only be 0.64 µg. of estrone present to give the radioactivity observed for equilenin. It may very well be that the radioactivity observed in the isolated equilenin was due to estrone.

From the results presented above it can be safely stated that estrone is not converted to equilenin and it is probably not converted to equilin. In view of these findings, Girard's theory (34c) concerning the conversion of estrone to equilin and equilenin is no longer valid. These negative results however do not reveal much concerning the validity of the theory postulated by Doisy et al. (65). To test this theory, C^{14} labeled equilin and equilenin would have to be administered to a pregnant mare in order to ascertain whether these ring B unsaturated estrogens are converted to estrone.

2. Experimental

All melting points were taken on a Fisher-Johns melting point apparatus, standardized with reference samples supplied by Arthur H.Thomas and Company. All values are corrected.

C^{14} determinations were recorded on a Nuclear Instrument and Chemical Corporation 64-scaler unit working in conjunction with a Nuclear Instrument and Chemical Corporation Windowless Q-gas flow counter, or a Tracerlab three stage windowless Q-gas flow counter. A standard plate was counted with all samples assayed. The radioactivity of all samples was determined to within 2% probable error (107,108) and this observed radioactivity was corrected for background radiations. Specific activities were expressed as disintegrations/minute/milligram (d./min./mg.)

Pure crystalline compounds were counted as infinitely thin plates (84). All crude extracts were counted according to the procedure described by Yates (29). In the single instance where C^{14} was assayed in the form of $BaCO_3$, an infinitely thick plate was made according to the method described by Calvin et al. (84).

The ultraviolet spectra of steroids in sulphuric acid were done according to the instruction given by Zaffaroni (91). The

spectra were recorded on a model D.U. Beckman spectrophotometer equipped with a photomultiplier.

Unless otherwise stated, U.V. spectra were recorded in 95% ethanol using the Beckman spectrophotometer.

The diethyl ether used in all extraction procedures was rendered peroxide free by the method described by Venning (109).

The undenatured 95% ethyl alcohol was purified first by refluxing for one hour with 5% zinc dust and 5% KOH, and then by distillation.

Authentic samples of equilin (m.p. 236-239°C, $[\alpha]_D^{20}$ + 309° (1% dioxane)) and equilenin (m.p. 255-256°C, $[\alpha]_D^{20}$ + 86° (1% dioxane)) were kindly donated by Dr. D.Beall, of Ayerst McKenna and Harrison Ltd., Montreal, Quebec. Estrone, (m.p. 258-260°C, $[\alpha]_D^{20}$ − 159° (1% dioxane)) was obtained from the Charles E.Frosst Co., Montreal, Quebec.

(a) Solution of Estrone-16-C^{14} (I) for Injection.

Since estrone is insoluble in isotonic (0.9%) saline, experiments were designed to determine the minimum quantity of alcohol necessary to keep estrone in solution in the presence of saline. By trial and error, it was found that the slow addition of 130 to 135 ml. of saline did not precipitate the estrone (ca. 10 mg.) dissolved in 40 ml. of ethyl alcohol. Accordingly, 9.82 mg. of estrone-16-C^{14}, containing 25.53 x 10^6 d./min., were dissolved in 42 ml. of absolute ethanol, and to this solution was added dropwise and with stirring 130 ml. of isotonic saline. The saline was added slowly because once the estrone is precipitated it cannot be induced to redissolve. The 24% alcohol saline solution containing the C^{14}-estrone was autoclaved and transferred to a blood infusion apparatus.

(b) Administration of Estrone-16-C^{14} to the Mare.

The solution containing the C^{14}-estrone was infused into the jugular vein of a Clydesdale mare in her ninth month of gestation. The time of infusion was two minutes.

(c) Collection of Blood, Urine and Feces.

Fifty ml. of blood was collected from the jugular vein of the mare immediately after injection, and 1100 ml. was collected 1.5 hours after injection. The blood was preserved

with 3.2% trisodium citrate and shipped to Montreal in containers surrounded with ice.

By means of a rubber tube attached to the vulva of the mare, urine was collected for 72 hours following injection of the C^{14}-estrone. Each sample of urine collected was acidified to pH 3 with 6 N HCl, covered with a layer of toluene, and stored in the cold. The total volume of urine was 13.5 litres.

Feces were collected for 72 hours following the administration of the labeled estrone and preserved under a cover of chloroform.

(d) Fractionation and C^{14} Assay of the Blood.

The blood was assayed and fractionated by Dr. F.Peron in this laboratory. The experiments described here were of a preliminary nature and the data obtained should be considered as qualitative. The sample of blood taken from the pregnant mare immediately after injection of the labeled estrone did not contain detectable amounts of C^{14}. The second sample of blood (1100 ml.), taken 1.5 hours after injection of the C^{14}-estrone, was separated into cells and plasma (720 ml.) by centrifugation. A 50 ml. aliquot of this plasma was treated with sodium tungstate and copper sulfate and the precipitated proteins were removed by centrifugation. The supernatant was extracted five times with an equal volume of ether, and the combined ether extracts taken to dryness. The ether extracts

were made up to a known volume and an aliquot was assayed for radioactivity. There was no detectable radioactivity in these extracts.

The precipitated proteins were extracted three times with twice their volume of 95% ethanol. The alcoholic extracts were reduced to a small volume and diluted to 10 ml. in a volumetric flask. One ml. aliquots were plated and assayed for radioactivity. The count obtained was 43 d./min. above background. Therefore the plasma proteins obtained from 1100 ml. of blood contained 6,192 d./min.

(e) Hydrolysis of Urine.

When 5 litres of urine had been collected, it was acidified to pH 1 with concentrated HCl, covered with an equal volume of toluene, and the mixture hydrolysed by refluxing for two hours. During the hydrolysis of half the urine collected on the second day, the CO_2 evolved from acidification of the carbonate buffers was trapped in 250 ml. of 1 N NaOH. The CO_2 was converted to $BaCO_3$ by the addition of 10.9 g. of NH_4Cl and 122 g. of $BaCl_2$ to the 1 N NaOH solution. The precipitated $BaCO_3$ was centrifuged, washed twice with 20 ml. portions of H_2O and twice with 20 ml. portions of ethyl alcohol. After drying at 120°C for 2 hours, the $BaCO_3$ weighed 23.96 g. An aliquot of this $BaCO_3$ was plated as an alcohol slurry on an aluminum planchet and, after evaporation of the alcohol, was counted. The $BaCO_3$ did not contain any detectable C^{14}.

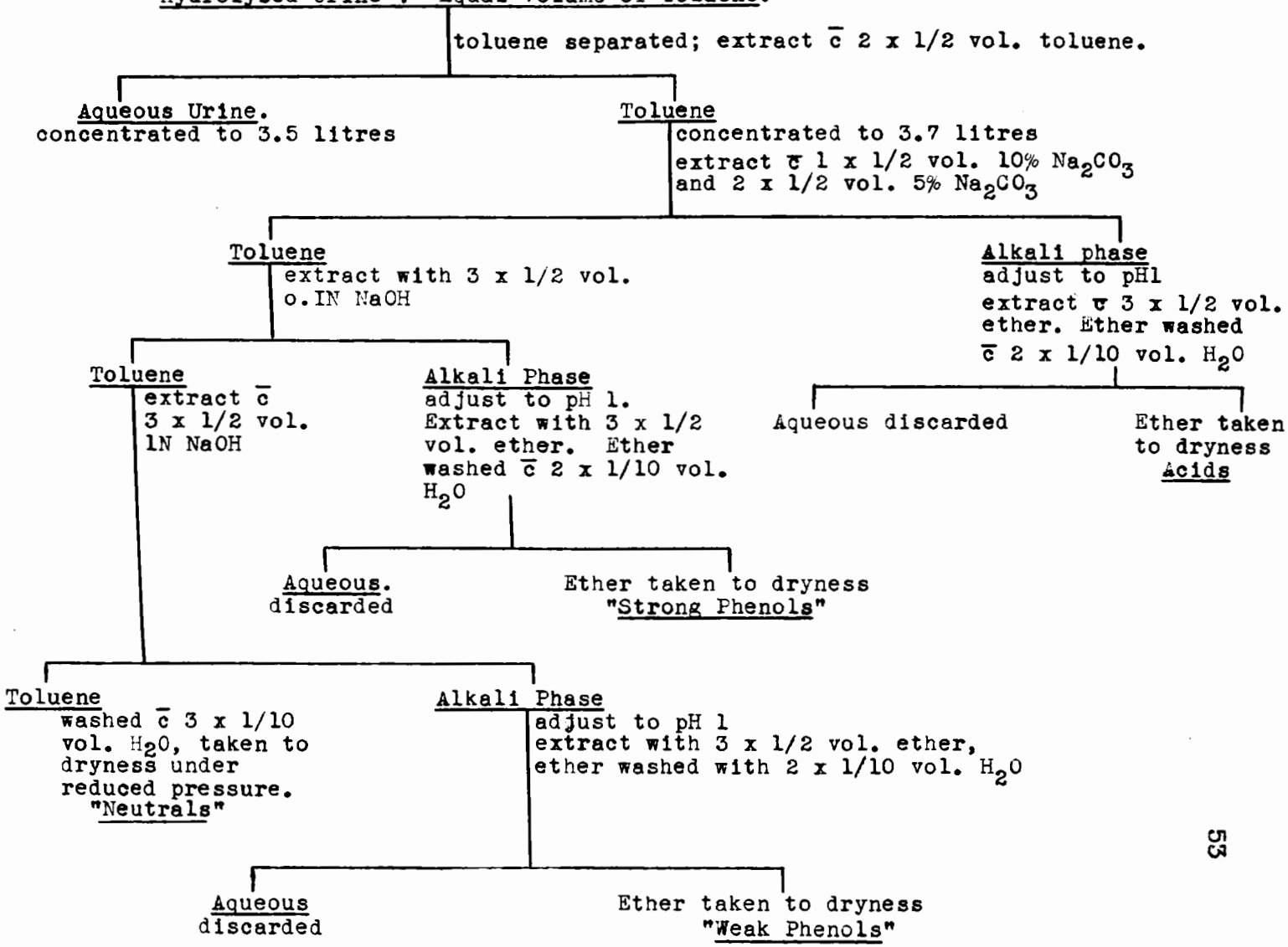
(f) Fractionation of the Urine.

After hydrolysis of the urine, the toluene layer was separated and the urine was extracted twice with a half volume of toluene. The toluene extracts were concentrated to 3.7 litres on the steam bath in vacuo and an aliquot was removed for assay of its C^{14} content. A scheme of the complete extraction procedure used is shown in Figure 5, p. 53.

The aqueous urine was filtered with the aid of suction and the solid residue was saved for further analysis. The filtrate was concentrated on the steam bath in vacuo to 3.5 litres. Each alkali phase was back extracted with 1 x 1/2 volume of toluene, and the toluene washes were pooled for further extraction. The acids, strong phenols, weak phenols, and neutrals were dried overnight in a vacuum dessicator, weighed, dissolved in absolute methanol and made up to a known volume in volumetric flasks. The strong phenols could not be dried to a viscous oil and, as a result, their true weight could not be obtained.

Figure 5.

Fractionation of the Urine.



(g) C^{14} Assay of the Excreta.

The results derived from the C^{14} assay, by the method of Yates (29), of the fraction resulting from the extraction of the urine are tabulated below. An aliquot of the aqueous urine was first neutralized and then assayed for C^{14}, same method.

Table II

C^{14} Assay of Excreta.

Fraction	Weight in gm.	Radioactivity in d./min. x 10^6	% of injected dose.
Injected dose		25.53	
Aqueous urine		2.566	10.04
Chloroform extract of feces		0.00	-----
Toluene extract of urine		7.571	29.60

			% of Toluene extract
Acids	16.335	0.076	1.001
Strong phenols	11.109	5.157	68.20
Weak phenols	0.351	2.167	28.70
Neutrals	7.026	1.131	14.98
Total		8.531	112.9

It is to be noted that of the injected dose, 39.64% of the radioactivity was recovered in the urine, of which 74.70% was toluene extractible after acid hydrolysis.

(h) Fractionation and C^{14} Assay of the Feces.

The feces, which were preserved in chloroform during the 72 hours collection period, were separated by filtration on a Buchner drum. The residual material was shaken three times with an equal weight of chloroform and the chloroform was separated by filtration. There was a slightly oily residue (191 lbs.) remaining after this treatment. In all, 70 gallons of chloroform were used. The chloroform extracts were reduced to a volume of 7 litres by distillation in a 10 gallon still. When this extract was assayed for C^{14}, using the method of Yates (29), no radioactivity was found.

350 g. of extracted fecal residue was leached three times, in a Waring blendor, with 100 ml. portions of water. The aqueous extract was reduced to a small volume in vacuo and an aliquot was removed for C^{14} assay, but no radioactivity could be detected. Likewise, the residue left from this leaching process, did not contain any C^{14}.

Half of the chloroform extract (3.5 L.) of the feces was subjected to the same fractionation procedure outlined in Figure 5. An attempt was thus made to separate the fecal extracts into acids, "strong phenols", "weak phenols", and neutrals. Here, however, heavy emulsions were encountered at each stage of the separation and, as a result, only a very crude fractionation was realized. The fractions separated

contained large quantities of residual material, and were therefore purified by extraction with alcohol. The alcoholic solutions were centrifuged and adjusted to known volumes in volumetric flasks. Aliquots were removed for C^{14} assay; no detectable radioactivity was found.

(i) C^{14} Assay of Saliva.

A sample of saliva was taken 1.5 hours after injection by absorption on cotton swabs. The cotton swabs were extracted with ethyl alcohol and the alcohol extract was concentrated to a small volume under reduced pressure. A small aliquot of the alcoholic solution was assayed for radioactivity. It was found that the saliva contained about 625 d./min.

(j) Distribution of Estrone-16-C^{14} (I) in the Fractionation Scheme. (Figure 5).

To 100 ml. of aqueous urine buffered with sodium acetate-acetic acid buffer (10 ml.) was added 5 ml. of an alcoholic solution of estrone-16-C^{14} containing 1,121,500 d./min. A second solution of aqueous urine was similarly prepared and it contained 1,015,800 d./min. Both solutions were treated in exactly the same manner. To the aqueous urine was added 100 ml. of toluene and the estrone-16-C^{14} was extracted by the same procedure outlined in Figure 5. The acids, strong phenols, weak phenols, and neutrals were assayed for C^{14} by

the procedure described by Yates (29). The results obtained from the fractionation are shown in Table III.

Table III

C^{14} Assay of Estrone-16-C^{14} (I) in Various Fractions.

Fraction	Estrone-16-C^{14} Recovered in d./min.	Average % Recovery from Toluene.
Aqueous urine	A- 1,121,500 B- 1,015,800	
Toluene extract	A- 859,000 B- 847,800	
Acids	A- 15,400 B- 15,100	1.79
Strong phenols	A- 482,650 B- 478,400	56.00
Weak phenols	A- 250,000 B- 246,500	29.08
Neutrals	A- 101,430 B- 92,540	11.33
Total	A- 849,480 B- 832,540	99.20

It is seen from Table III that toluene extracts only 80% of the estrone-16-C^{14} from the urine. This is in agreement with the values obtained in the actual fractionation of the acid hydrolysed urine (Table II).

(k) Partition of Estrone-16-C^{14} between Immiscible Solvent Pairs.

The partition of estrone-16-C^{14} between immiscible solvent pairs was carried out according to the procedure described by Mather (87). In these experiments, however, 50 ml. portions of both organic and aqueous phases were used in place of the 100 ml. portions used by Mather.

A standard alcohol solution of estrone-16-C^{14} containing 3,013 d./min./ml. was used in all of these experiments. Five ml. of the standard solution in a 50 ml. Erlenmeyer flask was taken to dryness at 60°C, under a stream of nitrogen. The residue was usually dissolved in a small volume of the alkaline phase (except in the case of 1 N HCl) and was transferred to a separatory funnel with the aid of both the alkaline and organic phases. Care was taken to use not more than 50 ml. of both phases. The two phases were shaken vigorously for 10 minutes and were allowed to stand for five minutes in order to separate. The organic phase was taken to dryness under reduced pressure, and the residue was dissolved in absolute methanol and diluted to 10 ml. in a volumetric flask. The alkaline layer was acidified with 3 N HCl and extracted three times with 25 ml. portions of peroxide free ether. The ether solution was taken to dryness on the steam bath, dissolved in absolute methanol and diluted to 10 ml. in a volumetric flask. The alcoholic

solutions of the residues from both phases were assayed for C^{14} by the method of Yates (29). The results obtained are shown in Table IV. These experiments were carried out in duplicate and on the average, the error incurred was 5%.

Table IV

Partition of Estrone-16-C^{14} between Immiscible Solvent Pairs

Solvent Pairs		
Aqueous Phase 50 ml. (a)	Organic Phase 50 ml. (b)	Fraction of total estrone in (b) after equilibration. %
0.1 N NaOH	Toluene	52.3
1N NaOH	Toluene	17.6
5% Na_2CO_3	Toluene	99.0
10% Na_2CO_3	Toluene	95.0
10% NaOH	Chloroform	16.9
1N HCl	Ethyl Ether	92.0

(1) Isolation of Hippuric Acid.

The reddish-brown solid which precipitated during hydrolysis of the urine was collected by filtration, dried in the oven at 100°C and dissolved in 95% alcohol. Most of the material was alcohol soluble (large volumes needed). Crystallization from alcohol yielded light brown needles (m.p. 174-180°C). This material was purified by treatment with animal

charcoal and crystallization from ethyl alcohol. The m.p. of this purified material was 186-189°C. The melting point of this material is very close to that reported for hippuric acid. On admixture with authentic hippuric acid, there was no depression in m.p. The isolated hippuric acid was counted as an infinitely thin plate (36μg/cm.^{2}); it did not contain any C^{14}.

(m) Fractionation of the Strong Phenols into Ketonic and Non-Ketonic Portions.

The separation of the strong phenols into ketonic and non-ketonic fractions was carried out following the method described by Girard and Sandulesco (89). The Girard reaction will be described here in detail. In all other instances where this reaction is encountered, only the quantities of the reagents used and results obtained will be given.

(n) Reagents Used.

Absolute ethanol was prepared from commercial absolute ethanol (99.8%) by the method described by Manske (110).

Girard's Reagent T. (trimethylaminoacetohydrazide) was recrystallized from absolute alcohol and stored in a vacuum desiccator over $CaCl_2$.

The glacial acetic acid used was purified by refluxing with 5% $KMnO_4$ for one hour and then collecting the fraction

distilling at 114-115°C. The acetic acid was titrated against NaOH (ca. 2 N) and this alkali was used to neutralize the acid in the Girard reaction.

Wherever necessary, the pH of the solutions was adjusted using the Beckman pH meter.

Before applying the Girard reaction to the strong phenols, an attempt was made to remove the light volatile oils under high vacuum. However, all efforts met with failure because of bumping, in spite of the precaution (boiling chips, glass wool) taken to avoid it. Therefore the separation was carried out in the presence of these light oils.

The strong phenols (11.09 g. of oils, containing 5,157,000 d./min.) were dissolved in 200 ml. of absolute alcohol and to the solution was added 10 g. of Girard's reagent T and 20 ml. of glacial acetic acid.# The mixture was refluxed for one hour and then poured into 1800 g. of an ice water mixture## containing sufficient NaOH to neutralize the acetic acid present. The pH of the solution was quickly adjusted to ca. pH 6.8 (range 6.5-6.0) with the use of dilute acid or alkali and was extracted with 500 ml. of peroxide free ether.

In this reaction, 5 to 10% Girard's Reagent T and 10% glacial acetic acid in absolute ethanol was used throughout.

The amount of ice-water added was calculated to give a final concentration of not more than 10% alcohol.

Tarry oils separated at the interface between the ether and the aqueous phase. These brown tars were not soluble in ether but were soluble in ethyl alcohol and acetone. The tarry oils (0.47g.) were dissolved in alcohol and the alcoholic solution was reduced to dryness in vacuo.

The pooled ether extracts were washed with 3 x 200 ml. of water and these water washes were combined with the residual aqueous solution. The ether was removed by distillation on the water bath, and the last portion was evaporated in vacuo. The residue thus obtained constituted the non-ketones, and weighed 7.358 g. The aqueous solution containing the water soluble Girard complex of the ketones was adjusted to pH 1 with 6 N H_2SO_4 and allowed to hydrolyse at room temperature for 1 hour. This aqueous solution was then extracted with 4 x 525 ml. portions of peroxide free ether and the pooled ether extracts were washed with 3 x 200 ml. portions of water. The ether solution was reduced to dryness on the water bath, and suction was applied at the end of the distillation to prevent overheating of the residual ketones. The weight of this ketonic fraction was 0.462 g.

All the crude urinary extracts were assayed for their C^{14} content as follows. The extracts were diluted to a definite volume with absolute methanol, calculated to give a final concentration of about 1.80-2.0 mg./ml. Two one ml. aliquots were

removed for assay by the procedure described by Yates (29). Following this method, the values obtained for the ketones, non-ketones and tarry oils were 2,140,950; 221,730: and 928,280 d./min. respectively.

(o) Girard Separation of the Tarry Oils.

The tarry oils (0.470 g., 221,730 d./min.) were dissolved in 50 ml. of absolute ethyl alcohol and to this solution was added 5.01 g. of Girard reagent T plus 5 ml. of glacial acetic acid. After the mixture was refluxed for one hour, it was poured into 450 g. of ice-water, containing sufficient NaOH to neutralize the acetic acid present. The non-ketones were separated from the ketones as described above. The amounts of ketones and non-ketones were 0.093 g. and 0.016 g. respectively. However, the ketones contained 177,400 d./min. (80% yield), whereas the non-ketones only contained 4,650 d./min. (2% yield).

(p) Girard reaction on the Non-Ketones.

The non-ketones (7.358 g., 928,280 d./min.) were dissolved in 150 ml. of absolute alcohol and to this solution was added 7.4 g. of Girard reagent T plus 15 ml. of glacial acetic acid. The mixture was refluxed for one hour and then poured into 1,350 g. of an ice-water mixture containing sufficient NaOH to neutralize the acetic acid present. Separation into ketones and non-ketones was carried out as described above. The weight

of ketones and non-ketones was 0.140 g. and 6.934 g. respectively. Assay for C^{14} showed that the ketones contained 14,890 d./min., while the non-ketones contained 806,500 d./min.

The combined ketones weighed 0.695 g. and contained 2,332,840 d./min., while the combined non-ketones weighed 6.95 g. and contained 811,150 d./min.

(q) Attempt to form Equilenin Picrate.

In trial experiments 5 ml. of a saturated alcoholic solution of picric acid was pipetted into centrifuge tubes containing 5, 10, 15, 20 and 25 mg. of equilenin. The tubes were warmed on a steam bath to dissolve the equilenin and were left overnight at room temperature to allow the picrate to crystallize. The tube containing 20 mg. of equilenin yielded 29.80 mg. (80% yield) of equilenin picrate. This quantity of picrate could be isolated with great ease. The picrate melted at 208-212°C which compares favorably with the value 205-208°C quoted by Girard et al.(34c).

The combined ketonic strong phenols (0.695 g., 2,332,840 d./min.) were dissolved in alcohol saturated with picric acid (20 ml.) and set aside overnight at room temperature to crystallize. The crystals which separated were filtered and washed with cold 95% ethanol and dried overnight in a vacuum desiccator. These crystals melted at 248-250°C and the m.p.

was not depressed on admixture with authentic estrone. (m.p. 258-260°C). The weight of these crystals was 3.4 mg.

The mother liquors, containing the bulk of the ketonic strong phenols and possibly some equilenin picrate and free picric acid in solution, were concentrated to dryness in vacuo at 60°C. The yellow residue was dissolved in 100 ml. of peroxide free ether and the ether solution was washed first with 6 x 25 ml. portions of 1% Na_2CO_3 and then with 3 x 10 ml. portions of water. The combined aqueous washes were back extracted with 2 x 25 ml. portions of ether. The combined ether solution was reduced almost to dryness by distillation of the ether on the steam bath. The last portions of ether were removed with the aid of suction. The residual ketones were dried by azeotropic distillation with dry benzene, weighed, and then diluted to a known volume with methanol. The weight of the ketonic strong phenols was now 0.265 g. and it contained 2,314,540 d./min.

(r) Fractional Crystallization of Estrone.

The ketonic strong phenols contained in a 50 ml. centrifuge tube were dissolved in 25 ml. of absolute ethyl alcohol and allowed to stand at room temperature overnight. The small needles which precipitated were separated by centrifugation and then were washed with a small quantity of absolute ethyl alcohol.

By this procedure, 5 crops of crystals weighing a total of 40 mg. were separated from the ketonic strong phenols. Crop 3 (24 mg.) was recrystallized from absolute methanol and yielded 20 mg. of colorless crystals. After drying overnight in a vacuum desiccator this material melted at 260-268°C, and on admixture with authentic estrone (m.p. 258-260°C) the m.p. obtained was 259-262°C. In ultraviolet light this material exhibited only one absorption maximum at 281 mμ (log ε 3.44). The U.V. absorption spectrum in sulfuric acid of this substance was identical with that obtained with authentic estrone (Fig. 6, p. 67). From the above evidence, the substance isolated was judged to be pure estrone. The isolated estrone had a specific activity of 37,650 d./min./mg. when counted as an infinitely thin plate (4 μg./cm^2.).

(s) Colorimetric Determination of Estrone, Equilin and Equilenin.

The colorimetric methods used were originally described by Banes (95) and lated modified by Grant and Beall (96).

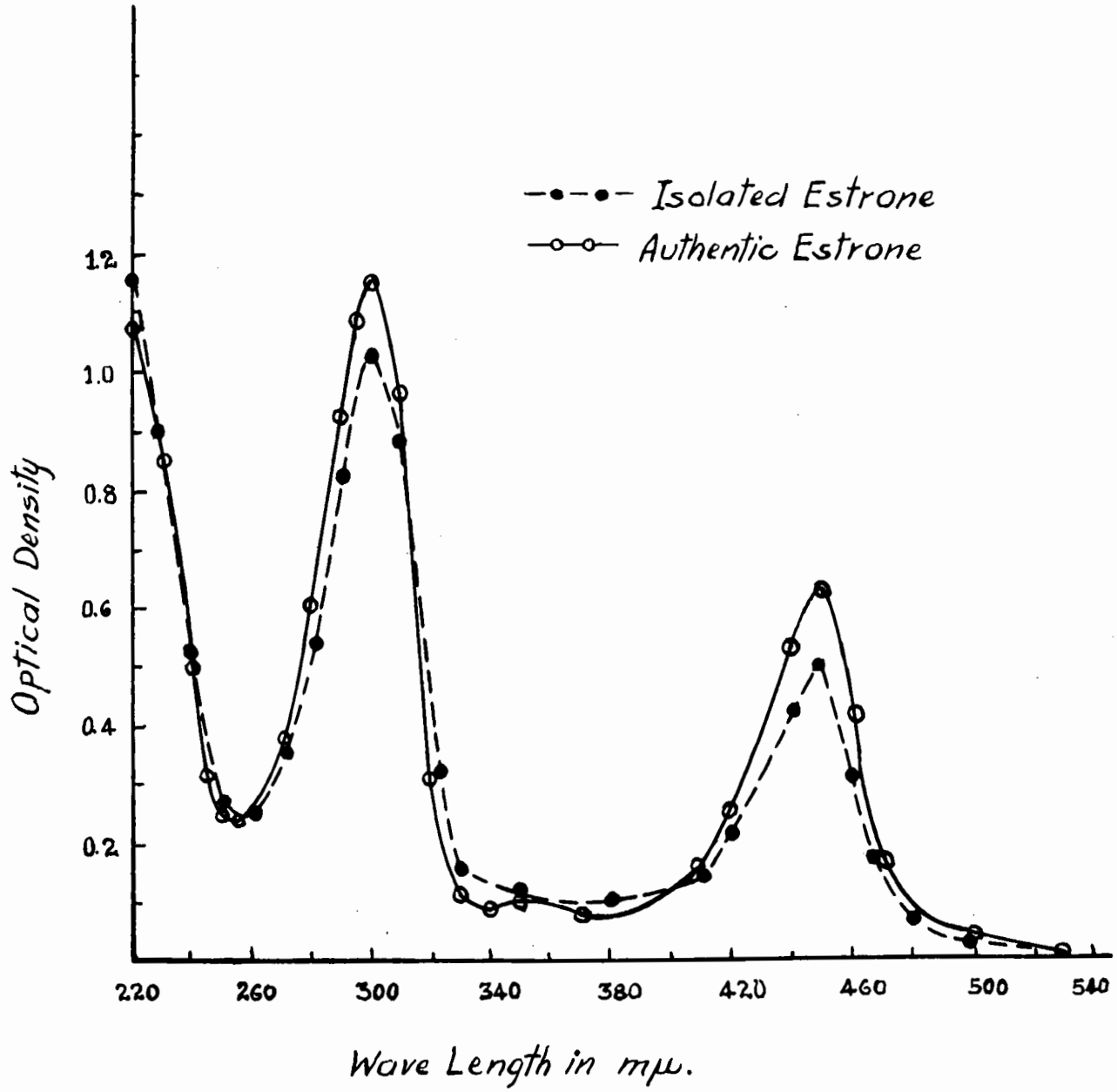
Reagents and Apparatus:

Benzensulfonylchloride: Reagent grade benzenesulfonylchloride was further purified by distillation in an all glass apparatus. This reagent was stored in the dark.

Pyridine: Pyridine was distilled from solid KOH and stored over BaO.

Figure 6:

Absorption in Sulphuric Acid



Buffer Solution: Acetate buffer was prepared by dissolving 220 g. of $CH_3COONa.3H_2O$ in 600 ml. of water containing 20 ml. of glacial acetic acid. The solution was diluted to 1 litre in a volumetric flask.

B.Q.C. Reagent: A 0.5% solution of reagent grade dibromoquinonechloroimide (B.Q.C.) in ethanol was prepared just prior to use. A fresh solution of B.Q.C. had to be made for each determination because it is light sensitive and deteriorated progressively in light. The solid reagent was stored in a vacuum desiccator in the dark.

Kober-Haenni Reagent: To a solution of 1.054 g. of $FeSO_4.(NH_4)_2SO_4.6H_2O$ (Mohr's Salt) in 20 ml. of H_2O was added 1 ml. of 30% H_2O_2. The solution was heated and mixed till effervescence ceased and then diluted to exactly 50 ml. Three volumes of this solution (6 ml.) was diluted in a 200 ml. volumetric flask with concentrated H_2SO_4.

U.S.P. phenol was redistilled, and tared in a glass-stoppered flask. The phenol was solidified by placing the tared flask in an ice bath, and to ensure complete crystallization, the top crust was broken with a glass rod. The tared flask was weighed, and to the phenol was added 1.13 times its weight of iron-sulfuric acid solution. The flask was stoppered and allowed to stand without cooling but with occasional shaking until the phenol liquefied. The mixture was then shaken vigorously and allowed to stand in the dark

for 24 hours. To this mixture was added 23.5% of its weight of a solution of 100 volumes of H_2SO_4 in 110 volumes of H_2O and the resulting viscous solution was shaken to obtain complete homogeneity. This stock solution was stored in a glass-stoppered bottle in the dark. Before removing an aliquot, the stock solution was shaken vigorously.

For Kober determinations, 10 ml. of the stock solution was diluted to 100 ml. with 24 N H_2SO_4 just prior to use and the mixture was shaken vigorously to obtain complete homogeneity.

In all these colorimetric determinations, the optical density was determined with the use of an Evelyn photometer equipped with the appropriate filters.

Standard solution: Approximately 37.5 mg. of pure estrone, equilin and equilenin were accurately weighed on a Gram-atic balance and diluted with 95% ethanol to 500 ml. in volumetric flasks. The concentrations of estrone, equilin and equilenin were 75.12, 74.82, and 74.98 µg./ml. respectively. The standard solutions were stored in dark, plastic covered bottles.

Sample solution: The sample solution used was prepared as described above. It contained 4.62 µg./ml. of estrone, 41.00 µg./ml. of equilin and 19.34 µg./ml. of equilenin.

B.Q.C. Reaction after Esterification.

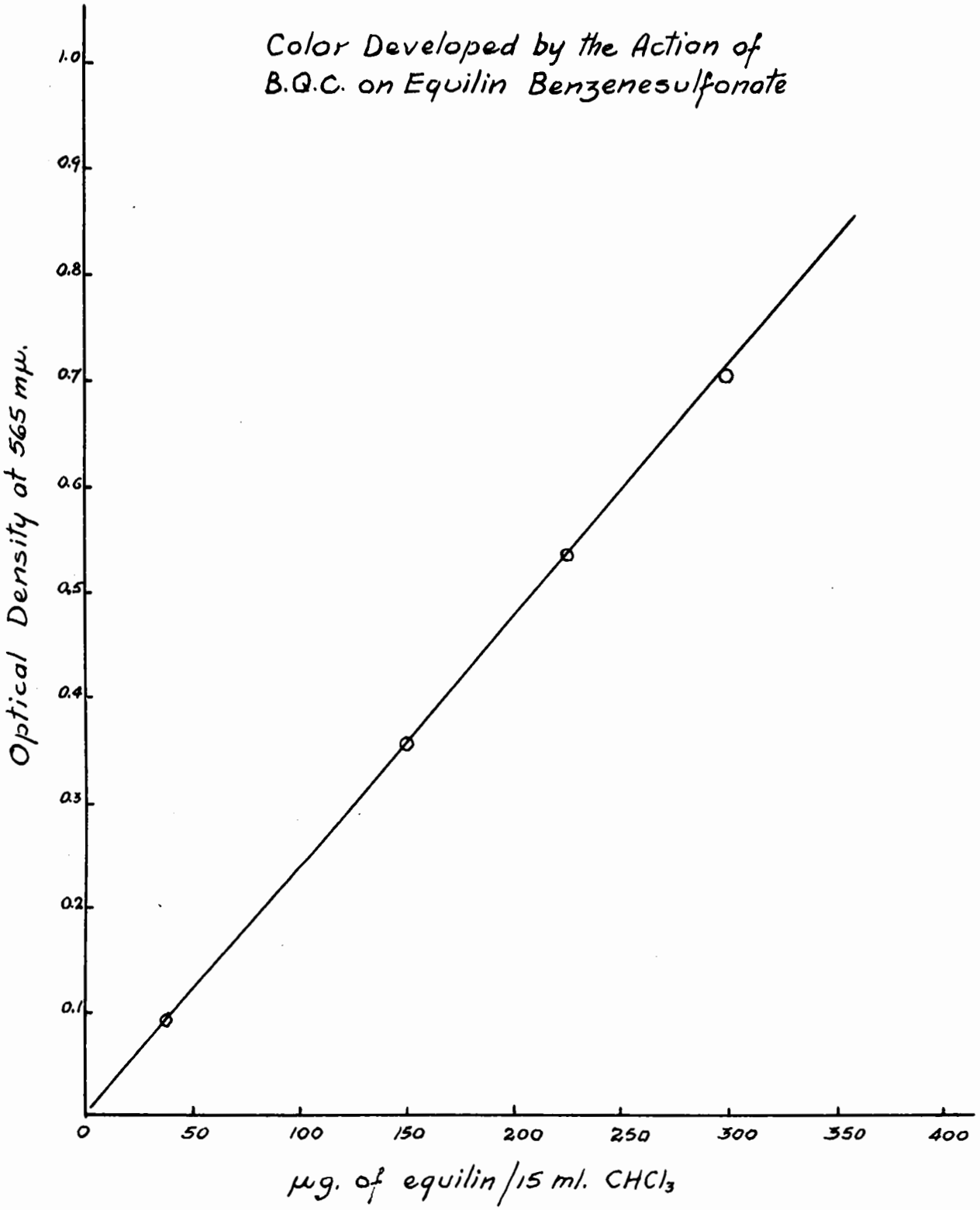
Two 5 ml. aliquots of the sample solution, contained in 50 ml. Erlenmeyer flasks, were carefully evaporated just to dryness at 60°C under a stream of nitrogen. A similar treatment was given to a 5 ml. alcohol blank and a series of alcoholic solutions containing respectively, 37.41 µg., 74.84 µg., and 149.64 µg. of equilin, 99.92 µg. of equilenin and 751.20 µg. of estrone. The flasks were immediately placed in a vacuum desiccator and dried for 1 hour. Two ml. of dry pyridine and 0.2 ml. of benzenesulfonylchloride were pipetted into each flask and esterification was allowed to proceed for 12 hours at room temperature. Fifteen ml. of H_2O was added to each flask with shaking. The contents of the flasks were then washed into a 125 ml. separatory funnel with the aid of 2 x 10 ml. portions of H_2O and 20 ml. of $CHCl_3$. The separatory funnels were shaken vigorously for exactly one minute and the chloroform layer was separated into a 50 ml. glass-stoppered Erlenmeyer. The aqueous phase was again extracted with 20 ml. of $CHCl_3$ and the combined chloroform extracts were taken to dryness under N_2 at 60°C. To each flask was added 2 ml. of ethanol and the residues were dissolved by gently warming the stoppered flasks on the steam bath. The flasks were cooled and to each was added 4.0 ml. of buffer solution and 2.0 ml. of 0.5% B.Q.C. reagent. Color development proceeded at room temperature for 4 hours.

The contents of the flasks were then rinsed into separatory funnels with exactly 15 ml. of $CHCl_3$ and mixed carefully. To each separatory funnel was added 20 ml. of 10% NaOH and the mixture was immediately shaken vigorously for exactly one minute. The $CHCl_3$ layers were allowed to separate and then filtered into calibrated colorimeter tubes through filter paper containing anhydrous Na_2SO_4. The optical density of the sample solutions and of the standard solutions were determined relative to the blank using a 565 mμ filter. In a separate experiment, conducted as described above, a standard curve for esterified equilin was constructed using 37.41 μg., 223.86 μg., and 299.28 μg. of equilin. (Fig. 7, p. 72).

The B.Q.C. Reaction.

Two 5 ml. aliquots of the sample solution were pipetted into test tubes (2.5 x 12 cm.) fitted with standard tapered joints (24/40). A 5 ml. alcohol blank was prepared in the same manner as well as a set of alcoholic solutions containing respectively 37.49 μg., 74.98 μg. and 149.96 μg. of equilenin, 50 μg. of equilin and 375.6 μg. of estrone. The final volume in each tube was adjusted to 5 ml. with 95% ethyl alcohol. To each tube was added 5.0 ml. of buffer solution and 1.0 ml. of 0.5% B.Q.C. reagent. The tubes were incubated for 5 minutes in a bath thermostatically controlled at 70°C and were then immediately cooled in ice water.

Figure 7.

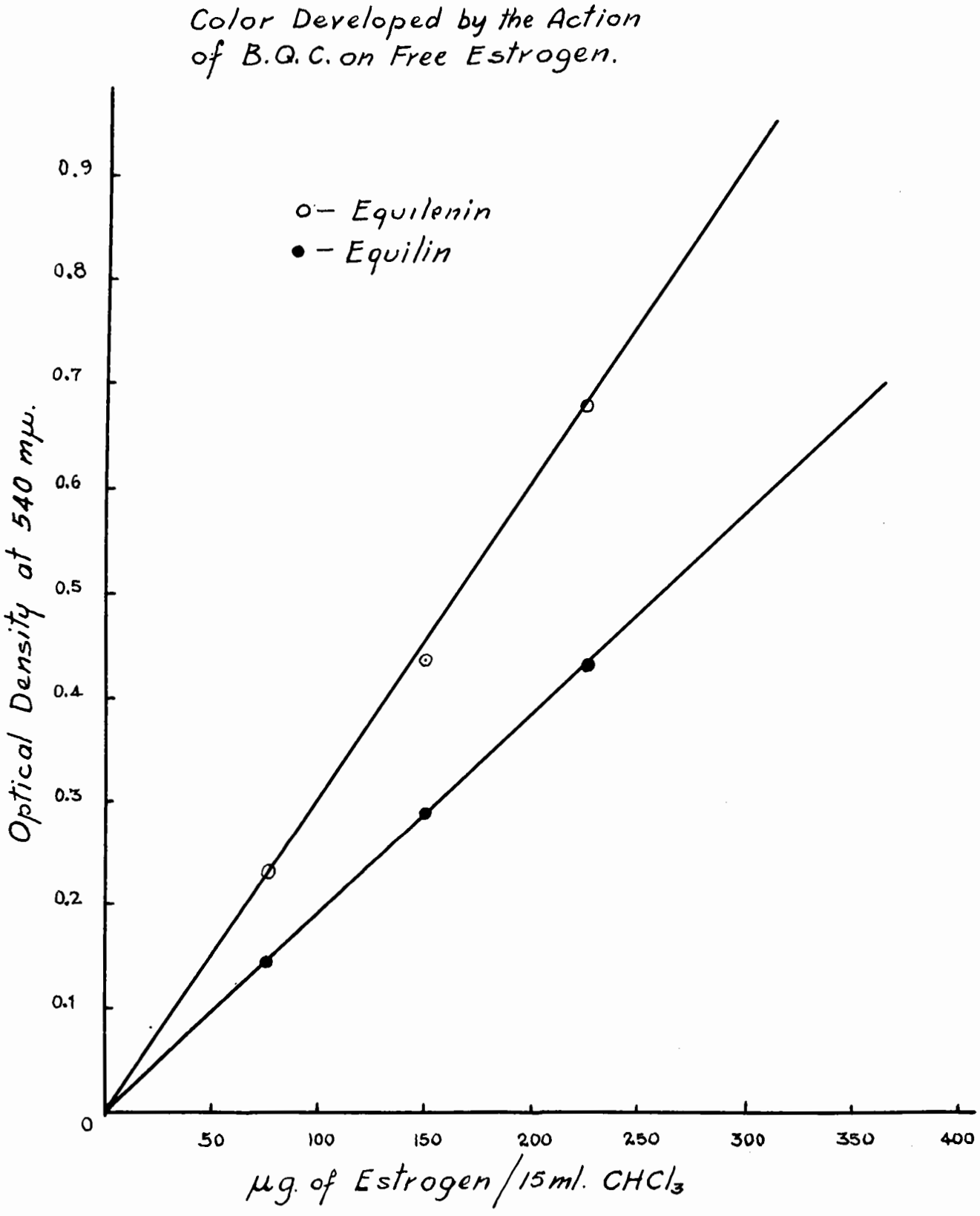

Color Developed by the Action of
B.Q.C. on Equilin Benzenesulfonate
Optical Density at 565 mμ.
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
150
200
250
300
350
400
μg. of equilin/15 ml. CHCl3

The contents of each tube were rinsed into a separatory funnel with the aid of exactly 15 ml. of $CHCl_3$ (the chloroform was pipetted with the aid of suction) and mixed by careful shaking. To each separatory funnel was added 20 ml. of 10% NaOH. After vigorous shaking for one minute the chloroform layer was allowed to separate. The clear chloroform solution was filtered through filter paper containing anhydrous Na_2SO_4 into calibrated colorimeter tubes. Optical densities of the sample solutions and the standard solution were read relative to the blank in the Evelyn Photometer using a 540 mμ. filter. In a separate experiment a standard curve of equilin and equilenin was constructed (Fig. 8, p. 74) by subjecting a series of alcoholic standards to the process described above.

Kober Reaction.

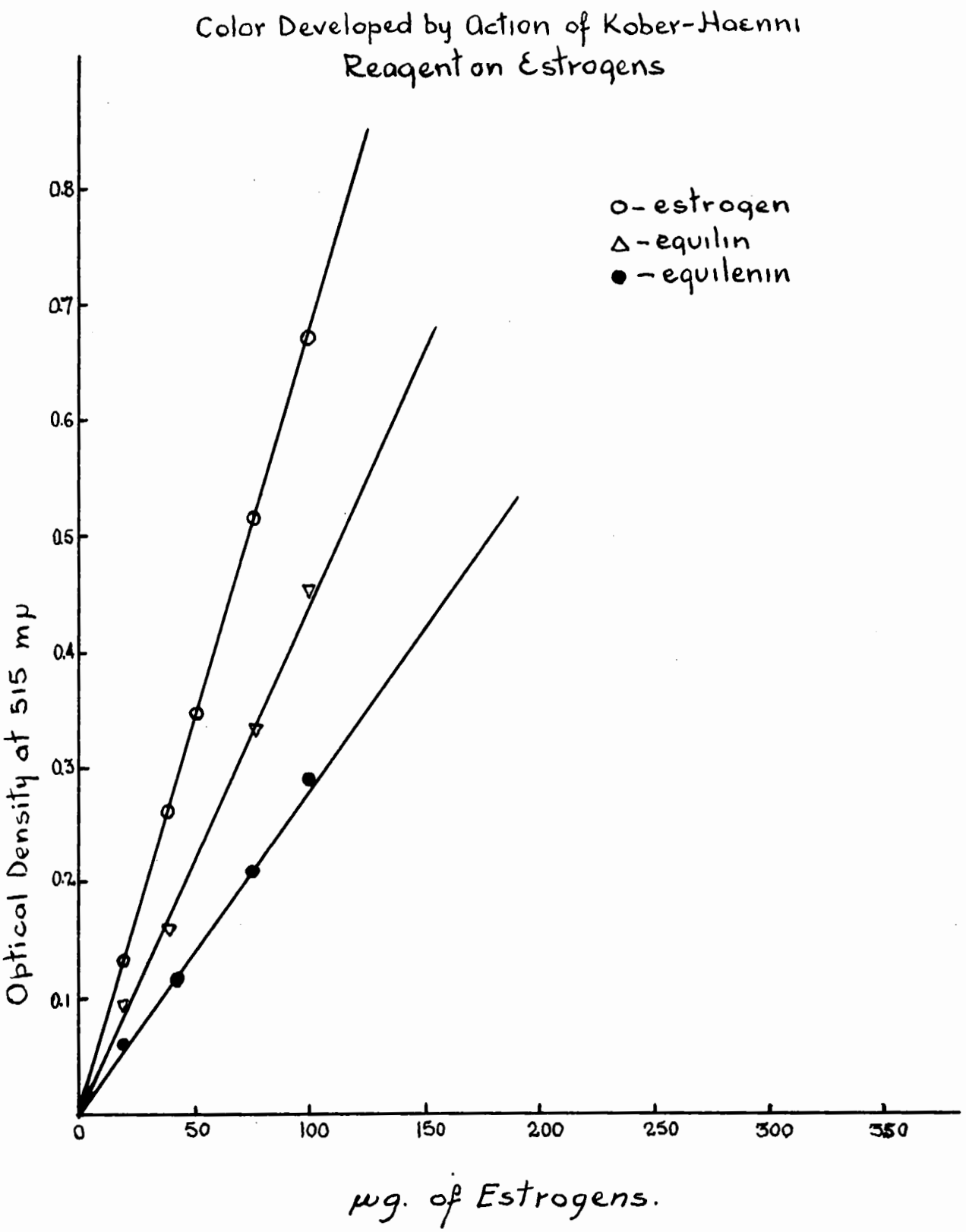
Ten ml. of Kober-Haenni reagent was slowly added to duplicate 1 ml. aliquots of the sample solution in standard tapered tubes. The tubes were vigorously shaken during the addition of the reagent and for a few minutes after. Similar treatment was applied to a 1 ml. alcohol blank and alcoholic solutions containing 18.78 μg., 37.58 μg. and 75.12 μg. of estrone respectively, 74.82 μg. of equilin and 75.12 μg. of equilenin. The loosely stoppered tubes were incubated at 75-80°C for 2 hours in a thermostatically controlled bath.

Figure 8.


Color Developed by the Action of B.Q.C. on Free Estrogen.
o – Equilenin
● – Equilin
Optical Density at 540 mμ.
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
50
100
150
200
250
300
350
400
μg. of Estrogen / 15 ml. CHCl3

Then the tubes were cooled in an ice bath, mixed by shaking and allowed to stand at room temperature for 15 minutes. The contents of each tube were poured into a calibrated colorimeter tube. The optical densities of the sample solutions and standard solutions were determined, relative to the blank, in the Evelyn Photometer using a 515 mμ. filter. Using the procedure described above, standard curves of estrone, equilin and equilenin were constructed with the appropriate standard solutions.(Fig. 9, p. 76).

Figure 9.


Color Developed by Action of Kober-Haenni Reagent on Estrogens
o - estrogen
Δ - equilin
● - equilenin
Optical Density at 515 mμ
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
150
200
250
300
350
μg. of Estrogens.

Calculations.

A. The equilin content of the sample solution was computed by comparison of the color developed by the action of the B.Q.C. reagent on the esterified sample with the colors developed by the equilin benzenesulfonate standards. It was found that "authentic" estrone (isolated from mare's urine) gave a color with the B.Q.C. reagent due to contamination with equilin. Highly purified estrone (4 recrystallizations from absolute ethanol) gave no color with the B.Q.C. reagent.

B. Knowing the equilin content of the sample solution, one can subtract it from the B.Q.C. color obtained without esterification and thus determine the equilenin content of the sample by comparison with the proper standards.

C. The Kober reaction is given by equilin, equilenin and estrone. Knowing the equilin and equilenin content of the sample, these volumes can be subtracted from the color developed by the Kober reagent on the sample solution and the estrone content can be computed by comparison with the estrone standard curve.

Recoveries of the Estrogens in the B.Q.C. Reaction.

When the B.Q.C. method was applied to a sample solution of the authentic estrogens, the following recoveries were obtained on duplicate determinations.

Recovery of Added Estrogens

	Sample Solution	Estrogen Recovered	Average Recovery
	µg./ml.	µg./ml.	µg./ml.
Estrone	41.62	32.5; 34.7	33.6
Equilin	41.00	45.0; 43.2	44.1
Equilenin	19.34	18.2; 19.1	18.6

B.Q.C. Analysis of the Mother Liquors from the Ketonic Strong Phenols.

The mother liquors of the ketonic strong phenols were diluted to a definite volume containing a final concentration of 81.2 µg./ml. Aliquots of this solution were used to determine the titer of equilin, equilenin and estrone as described above. All the determinations were done in duplicate. The results obtained from the B.Q.C. analysis indicated the presence of 15.0 mg. of equilin, 11.25 mg. of equilenin and 37.5 mg. of estrone in the mother liquors of the ketonic strong phenols.

Chromatographic Separation of Estrone, Equilin and Equilenin.

The chromatography procedure described here is a modification of the method originally described by Bane, Carol and Haenni (111). A glass tube, 24 mm. in diameter, with a stopcock sealed at the end, served as the chromatography column. The constricted end of the tube was packed with fine glass wool and the tube was then filled one quarter full with benzene. A pad of glass wool was gently compressed into the bottom of the tube to a height of 1 cm. To 1 g. of Celite (No. 545, Johns Manville) contained in a small beaker was added sufficient benzene to cover the adsorbent and 0.5 ml. of water was pipetted uniformly onto the benzene. The mixture was then stirred vigorously until the Celite was uniformly wet. After allowing the benzene to drain slowly from the chromatography tube, the Celite was transferred to it in small portions with the aid of a spatula. A flocculent suspension was formed in the tube by slowly working a packing rod (a cork stopper fitted with a wooden handle,) up and down as a piston through the Celite-benzene-water mixture. The Celite was then gently compressed with the rod to form a sharply defined level surface approximately 1 cm. high. To 20 g. of Celite contained in a large mortar was added 100 ml. of benzene, and exactly 20 ml. of 0.4 N NaOH was uniformly pipetted on the surface. The mixture was vigorously stirred until the Celite was uniformly wet and then was transferred in small portions to the glass tube,

suspending each portion and gently packing as described above. During this operation the benzene in the tube was slowly allowed to drain out. When all the Celite had been packed into the tube, the top surface was leveled and covered with a wad of glass wool. The final height of the Celite column was 18 cm. During chromatography the Celite was always covered with benzene.

For adsorption on Celite the estrogens were dissolved in a minimum volume of benzene and the solution was slowly added to the column when the level of the benzene in the column was almost at the same level as the Celite. The flask containing the estrogens was rinsed with several portions of benzene and these benzene washes were added to the column. A total of forty 10 ml. fractions were collected and the estrogens eluted were assayed as follows.

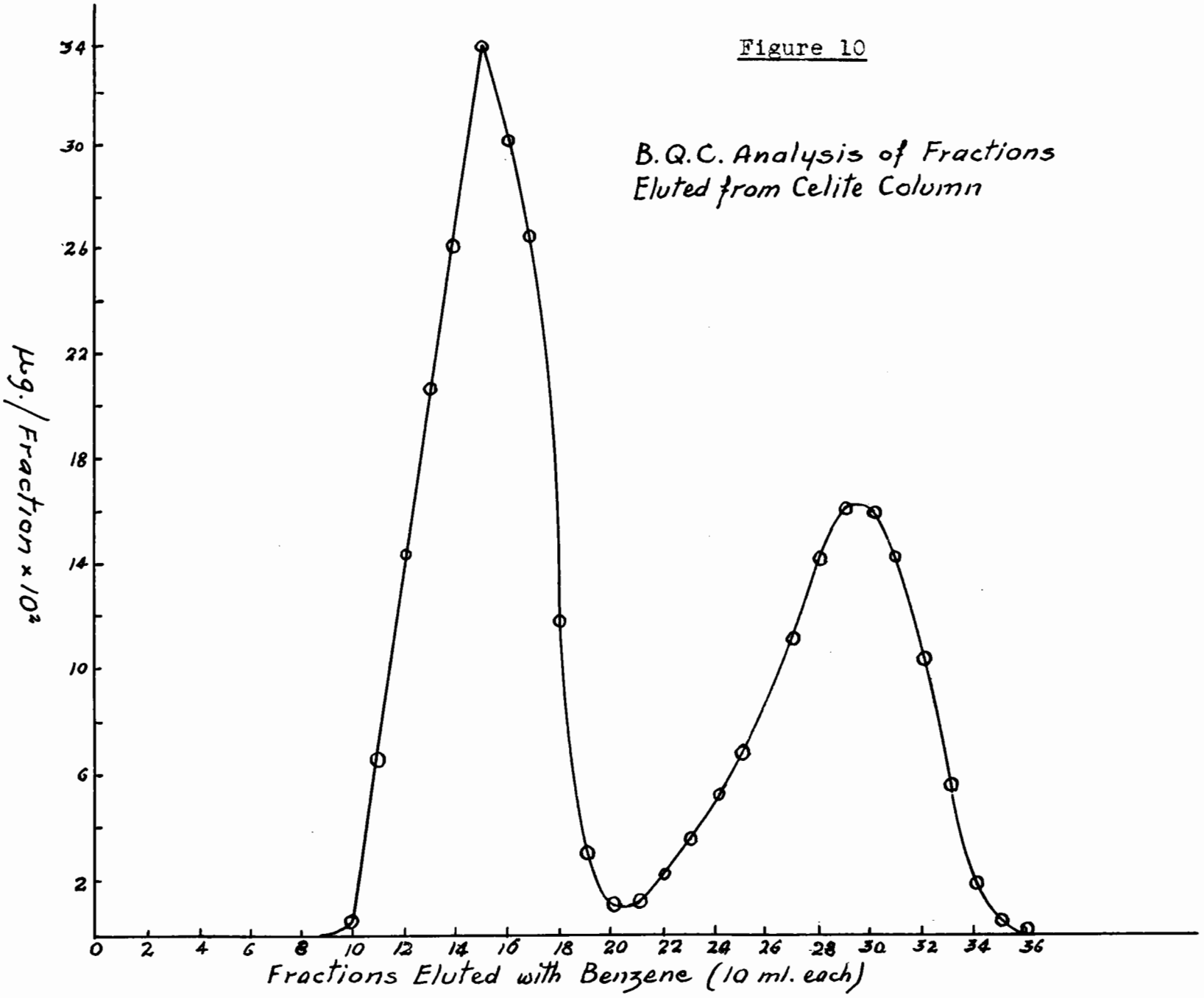
A 1 ml. aliquot was removed from each fraction; the benzene was evaporated at 60°C, under a stream of nitrogen and the unesterified B.Q.C. reaction was done on each aliquot using appropriate standard estrogen solutions as described above. This afforded a means of determining approximately how much, and where equilin and equilenin were eluted.

In an number of trial experiments with mixtures of the authentic estrogens, it was found that the best resolution on Celite was obtained when 10 ml. portions of benzene were

collected. In a typical trial experiment a mixture of 152.6 mg. of estrone, 15.6 mg. of equilin and 11.94 mg. of equilenin was adsorbed on the Celite column from a benzene solution. Ten ml. fractions were collected and 1 ml. aliquots of these were analysed by the B.Q.C. method as described above. The results obtained from the analysis of the fractions eluted were graphed as shown in Fig. 10, p. 81. Since fraction 9 did not give a positive B.Q.C. reaction, fractions 1 to 9 were combined. Fractions 21 to 35 were combined because from the graph it appeared that one compound was eluted in these fractions. These latter fractions exhibited an absorption maximum at 340 mμ., which is characteristic of equilenin. From past experience with this chromatography method it was known that fractions 10 to 20 contained equilin in admixture with estrone. The results obtained on analysis of the fractions eluted are tabulated below.

Fractions Combined	Weight in mg.	B.Q.C. Assay mg.
1-9	56.8	
10-20	92.0	
21-35	13.2	11.87

Figure 10


B.Q.C. Analysis of Fractions
Eluted from Celite Column
μg./Fraction x 10²
34
30
26
22
18
14
10
6
2
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Fractions Eluted with Benzene (10 ml. each)

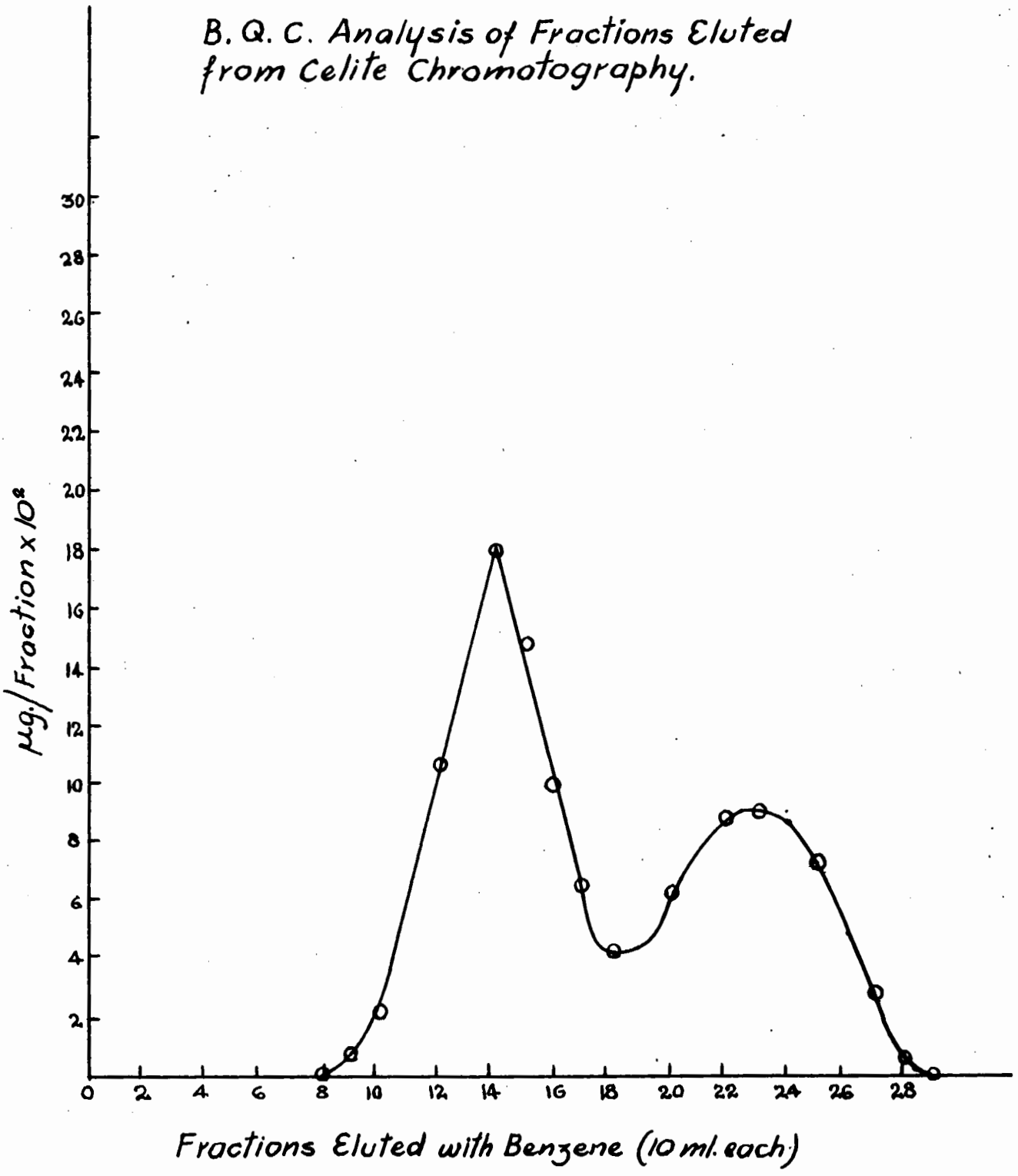
Fractions 1 to 9, when combined, crystallized from 95% ethyl alcohol yielding 42 mg. of small colorless needles. These crystals were judged to be pure estrone from their m.p., admixture m.p. with authentic estrone and from the negative reaction obtained with the B.Q.C. reagent.

Fractions 21-35 crystallized from 95% ethyl alcohol yielding 8.34 mg. of light yellow crystals. This material was identified as equilenin from its characteristic ultraviolet absorption maximum at 340 mμ., its m.p. and admixture m.p. with authentic equilenin.

Fractions 10-20 gave a positive B.Q.C. reaction and weighed 92.0 mg. Since only 15.6 mg. of equilin were originally adsorbed on the Celite, the balance (76.4 mg.) must therefore have been estrone. Therefore these fractions were rechromatographed on Celite under the identical conditions described above. The fractions eluted were analysed for their B.Q.C. color and the results are shown in Fig. 11, p.83. Fractions 1 to 8, 9 to 18, and 19 to 28 were combined and weighed. The results obtained on analysis of these fractions are tabulated below.

Fractions combined	Weight in mg.	B.Q.C. assay in mg.
1-8	56.0	
9-18	22.18	8.76
19-28	6.45	6.02

Figure 11.


B. Q. C. Analysis of Fractions Eluted from Celite Chromotography.
μg./Fraction x 10²
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Fractions Eluted with Benzene (10 ml. each)

Fractions 1 to 8 were crystallized from 95% ethyl alcohol yielding an additional 40 mg. of pure estrone. On crystallization from ethyl acetate, fractions 19-28 yielded 3.15 mg. of colorless needles which were identified as pure equilin from its m.p. and admixture m.p. with authentic equilin and from the B.Q.C. reaction on the p-toluenesulfonate. Fractions 9-18 were crystallized from 95% ethyl alcohol but the m.p. obtained was 221-226°C, and it could not be elevated by recrystallization. Thus a large portion of the equilin could not be separated from the estrone but for our purpose a quantitative separation was not essential.

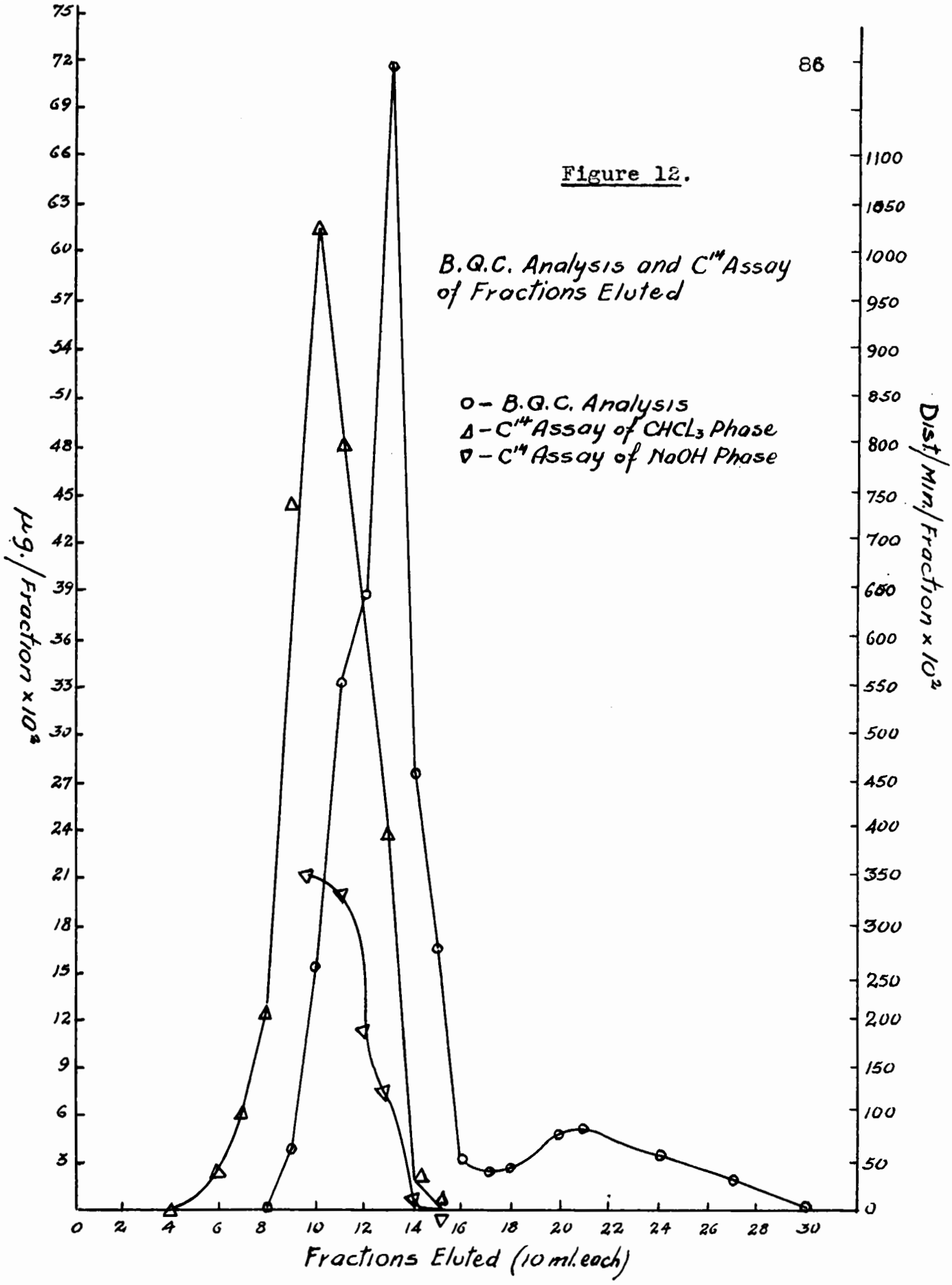
The mother liquors from the ketonic strong phenols weighed 203 mg. and contained 806,500 d./min. It had been previously determined that this fraction contained 37.5 mg. of estrone, 15.0 mg. of equilin and 11.25 mg. of equilenin. These mother liquors were dissolved in 25 ml. of benzene and the solution was adsorbed on 21 g. of Celite impregnated with 20 ml. of 0.4N NaOH. The estrogens were eluted with 10 ml. portions of benzene and from each fraction a 1 ml. aliquot was removed for B.Q.C. assay. Along with the B.Q.C. assay, each fraction was analysed in the following manner.

a) After determining the optical density of the chloroform solutions (15 ml.) containing the B.Q.C. colored complexes, a 1 ml. aliquot was removed for C^{14} assay. These aliquots were plated as infinitely thin plates.

b) The NaOH washings of the chloroform solutions containing the B.Q.C. complexes (c.f. page 73) were acidified to pH 1 with 2.5 N HCl and were extracted three times with 30 ml. portions of peroxide free ether. The combined ether extracts were washed with 3 portions of 25 ml. 1 M Na_2CO_3, in order to remove the acetic acid present (used as a buffer in the B.Q.C. reaction), and the ether was evaporated on the water bath. The residues thus obtained were dissolved in ethyl alcohol and made up to volume in a 10 ml. volumetric flask. One ml. aliquots of these solutions were plated for C^{14} assay. The results obtained from the analysis of each fraction eluted, with the methods described above, are graphically presented in Fig. 12, p. 86. From this graph it is evident that fractions 15 to 29 only contained small amounts of radioactive material, since both the chloroform solutions and the NaOH washes obtained in the B.Q.C. analysis of these fractions did not contain any C^{14}.

Fractions 1 to 8 did not give the B.Q.C. reaction and therefore must have contained pure estrone. As a result these fractions were combined. Fractions 9 to 13 must have contained estrone plus equilin because both the NaOH washings and the chloroformic solutions of these fractions contained appreciable quantities of radioactivity. Since fractions 14 to 16 did not contain appreciable quantities of C^{14} but still gave the B.Q.C. reaction, they must have contained pure equilin, uncontaminated

Figure 12.


B.Q.C. Analysis and C14 Assay of Fractions Eluted
o – B.Q.C. Analysis
Δ – C14 Assay of CHCl3 Phase
▽ – C14 Assay of NaOH Phase
μg./Fraction x 10²
Dist./Min./Fraction x 10²
Fractions Eluted (10 ml. each)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
75 72 69 66 63 60 57 54 51 48 45 42 39 36 33 30 27 24 21 18 15 12 9 6 3
1100 1050 1000 950 900 850 800 750 700 650 600 550 500 450 400 350 300 250 200 150 100 50 0

with estrone. From the shape of the B.Q.C. curve in Fig. 12, it is evident that fractions 17 to 24 contained a single compound which from our previous experience with this method of chromatography was judged to be equilenin. The results obtained from the chromatography on Celite of the mother liquors from the ketonic strong phenols are tabulated below.

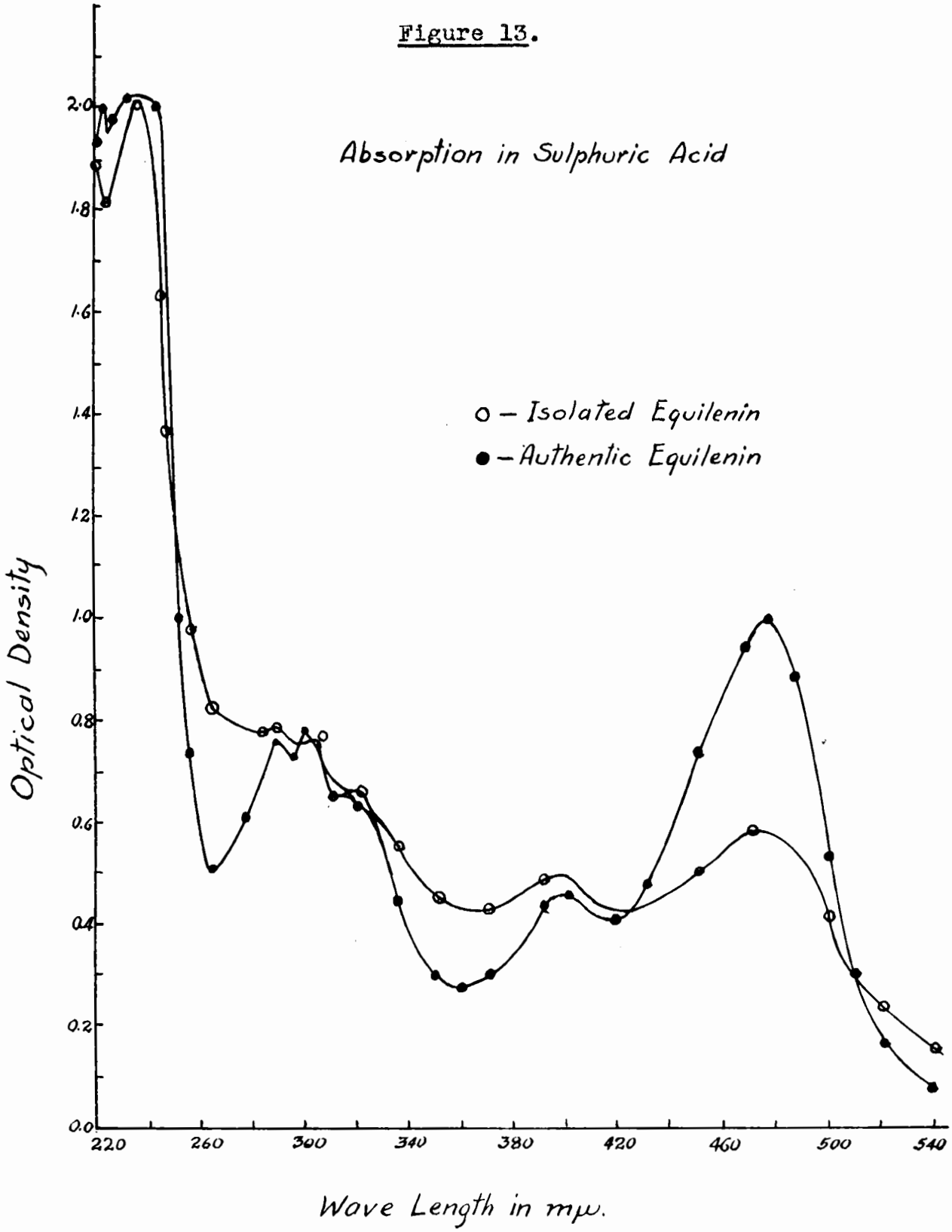
Fractions Combined	Weight of Fractions mg.	B.Q.C. assay mg.	Total radioactivity d./min.	Specific activity d./min./mg.
1-8	50.25		401,600	37,620
9-13	42.96	9.07 (as equilin)	395,000	
14-16	9.72	4.66 (as equilin)	1,827	392
17-24	11.52	3.92 (as equilenin)	1,619	413

On crystallization from ethyl acetate and 95% ethyl alcohol, fractions 1-8 yielded 8 mg. of estrone (m.p. 256-258°C). On admixture with authentic estrone, there was no depression in m.p. The estrone had a specific activity of 37,620 d./min./mg. Fractions 9-13 were set aside for rechromatography and fractions 14-16 were saved for combination with more equilin expected from the rechromatography of fractions 9 to 13. It is worthy to note that the equilin and equilenin eluates (fractions 14-16 and fractions 17-24) contained very small amounts of radioactivity. Based on

the B.Q.C. assay of these fractions the specific activities of equilin and equilenin were 392 and 413 d./min./mg. respectively, which is about 1% of the specific activity of the isolated estrone.

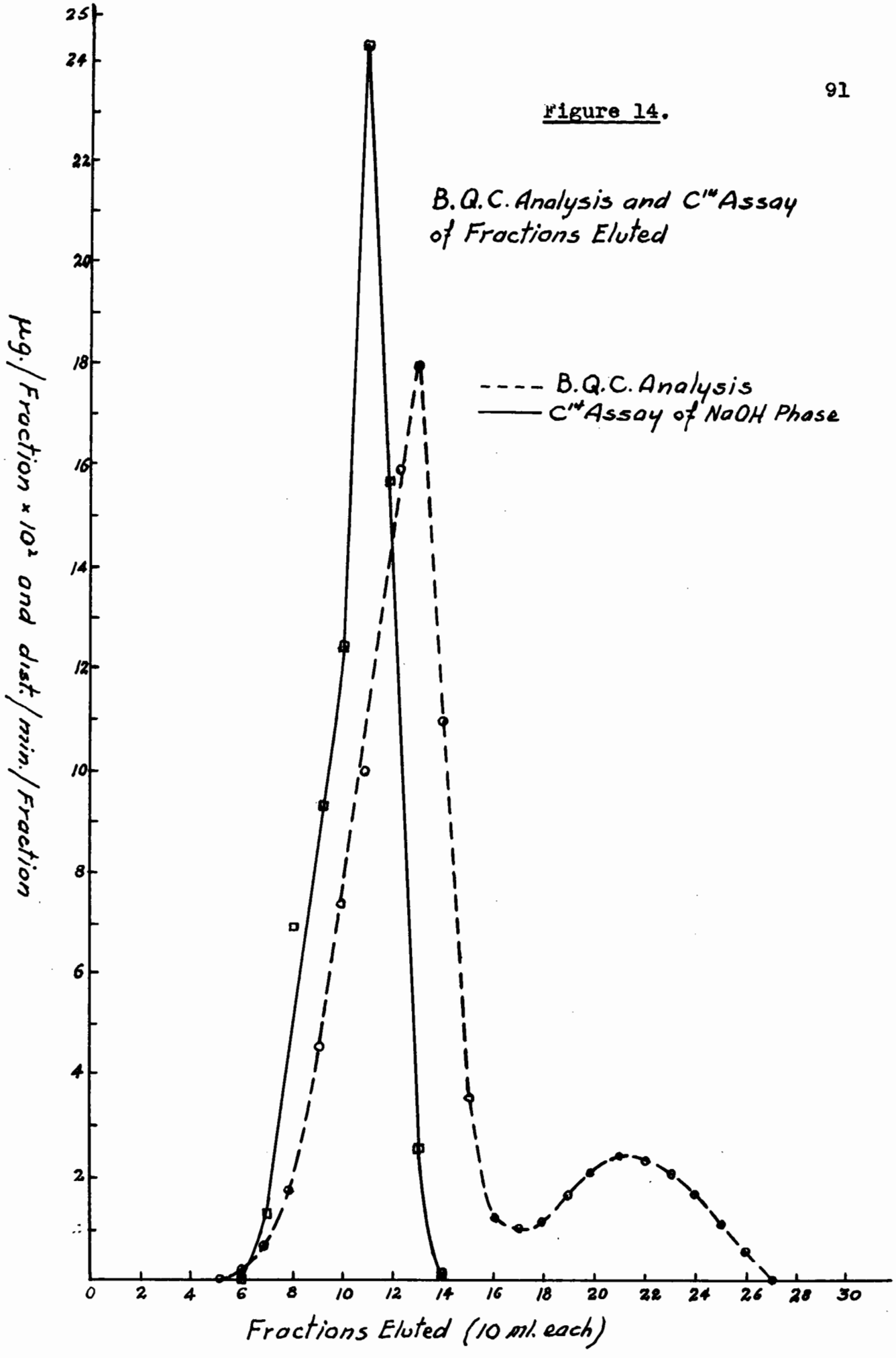
Fractions 17-24 could not be induced to crystallize from either ethyl acetate, 95% ethyl alcohol or benzene because of the relatively large amounts of impurities present. However, the equilenin present in these eluates was identified by its ultraviolet absorption spectrum in sulphuric acid, Fig. 13, p. 89. The spectrum of the isolated equilenin corresponded closely to the spectrum given by authentic equilenin except that some of the bands were not as well defined as in the spectrum of the latter. This is probably due to the impurities present in the equilenin eluates. In ultraviolet light the equilenin eluates exhibited an absorption maximum at 342 mμ., a region where no other estrogen absorbs light. Further confirmatory data which characterized the equilenin was obtained from paper chromatography, but this will be treated in a separate section.

Figure 13.


Absorption in Sulphuric Acid
o – Isolated Equilenin
● – Authentic Equilenin
Optical Density
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
220
260
300
340
380
420
460
500
540
Wave Length in mμ.

Fractions 9 to 13 (42.96 mg., 395,000 d./min.) were rechromatographed on 21 g. of Celite, impregnated with 20 ml. of 0.4N NaOH in the manner previously described. Each fraction eluted was analysed with the B.Q.C. reagent and the NaOH washes of the chloroform solution, containing the B.Q.C. complexes, were also treated as described above. In this instance, the chloroform solutions containing the B.Q.C. complexes were not assayed for their C^{14} content. The results obtained from this chromatogram are shown graphically in Fig. 14, p. 91.

Figure 14.


B.Q.C. Analysis and C14 Assay of Fractions Eluted
---- B.Q.C. Analysis
—— C14 Assay of NaOH Phase
μg./Fraction × 10² and dist./min./Fraction
Fractions Eluted (10 ml. each)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

From Fig. 14, it is seen that fractions 1 to 6 did not give a positive B.Q.C. reaction nor did they contain appreciable quantities of C^{14}. Therefore these fractions were not further examined. Since the NaOH washings from fraction 14 did not contain any C^{14}, it was deduced that estrone ceased to be eluted in this fraction. This defined the zone of overlapping between estrone and equilin in fractions 7 to 13. In the trial experiments with this Celite column, it was found that equilin was eluted in the region corresponding to fractions 14 to 29. Thus these fractions were combined and they will be called the equilin eluates. Fractions 7 to 13 gave a positive B.Q.C. reaction and the NaOH washes of the corresponding chloroform solution containing the B.Q.C. complexes were radioactive. From these facts, it was deduced that these fractions contained a mixture of equilin and estrone. The results obtained from this chromatogram are tabulated below.

Fractions combined	Weight of Fractions mg.	B.Q.C. assay mg.	Total radioactivity d./mg.	Specific activity d./min./mg.
1-6	3.78		8,400	
7-13	29.38	5.89 (as equilin)	366,200	
14-29	10.75	3.23 (as equilin)	1,800	557

When all the fractions had been eluted, the Celite column was washed with methanol. The methanol washes were concentrated to a small volume and on assay for C^{14}, and were found to contain 11,200 d./min. This result indicated that benzene eluted almost all of the C^{14} containing material on the Celite column.

Fractions 7 to 13, on crystallization from 95% ethyl alcohol, yielded 6.36 mg. of yellow needles (m.p. 248-252°C). Recrystallization from methanol gave 4.73 mg. of colorless needles, (m.p. 256-258°C), which did not show a depression on admixture with authentic estrone. The mother liquors from these fractions were chromatographed on paper in the hope or separating equilin from the estrone; this work will be reported in a separate section.

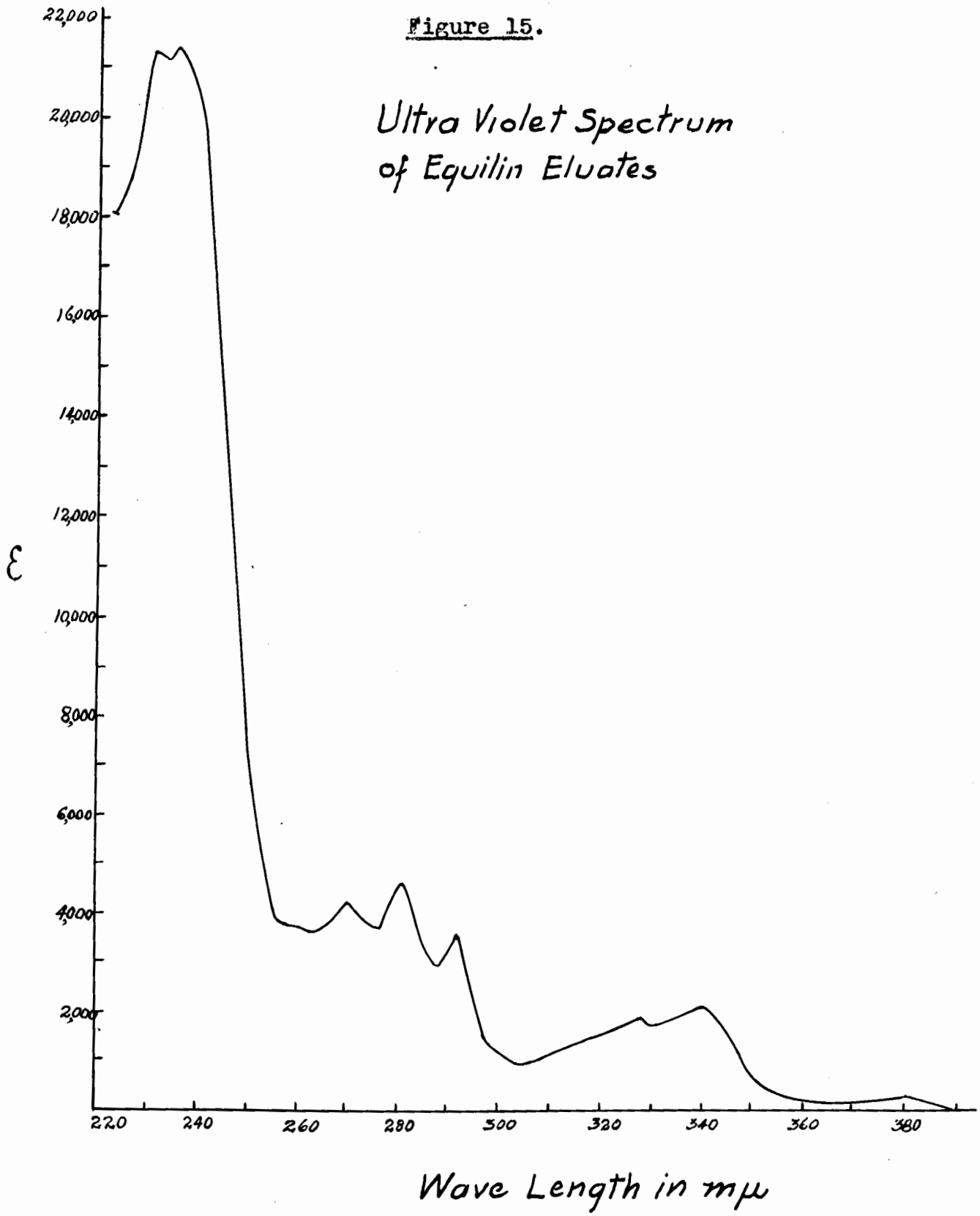
The Attempted Isolation of Equilin.

Fractions 14 to 29 were combined with the equilin eluates (fractions 14 to 16) from the first Celite chromatogram. The combined equilin eluates (20.47 mg.) could not be induced to crystallize from ethyl acetate, 95% ethyl alcohol or 80% aqueous methanol. As a result, the equilin eluates were sublimed under high vacuum in order to separate the equilin. These eluates were transferred to a sublimation tube (24 x 1 cm.) with acetone and the solvent was evaporated under a stream of nitrogen. The resulting oil was dried under the high vaduum

(ca 0.08 mm. Hg) and heating commenced. During the first two hours the temperature was elevated to 100°C and a small amount of oily material distilled. The main fraction distilled over at 130-135°C and crystallized in the sublimation tube. This temperature was maintained for 3 hours in order to get a complete removal of the equilin. The main fractions, distilling at 130-135°C (6.42 mg.), were dissolved in 95% ethyl alcohol and some oily material precipitated. The supernatant solution was removed and reduced to dryness, yielding a yellow oil. This oil, on crystallization from ethyl acetate, yielded 2.28 mg. of brown crystals, m.p. 238-242°C. When these crystals were mixed with authentic equilenin (m.p. 255-256°C) the m.p. obtained was 242-246°C. The m.p. and mixed m.p. of this semi-pure material indicated that it was equilenin. Because of the small amount of crystals obtained (2.28 mg.), purification by recrystallizing was precluded. As a result, an attempt was made to identify this crystalline material by its ultraviolet absorption spectrum, its ultraviolet absorption spectrum in sulphuric acid and by paper chromatography. For these determinations 0.636 mg. of the crystals was weighed on the microbalance and was dissolved in 10 ml. of 95% ethanol in a volumetric flask.

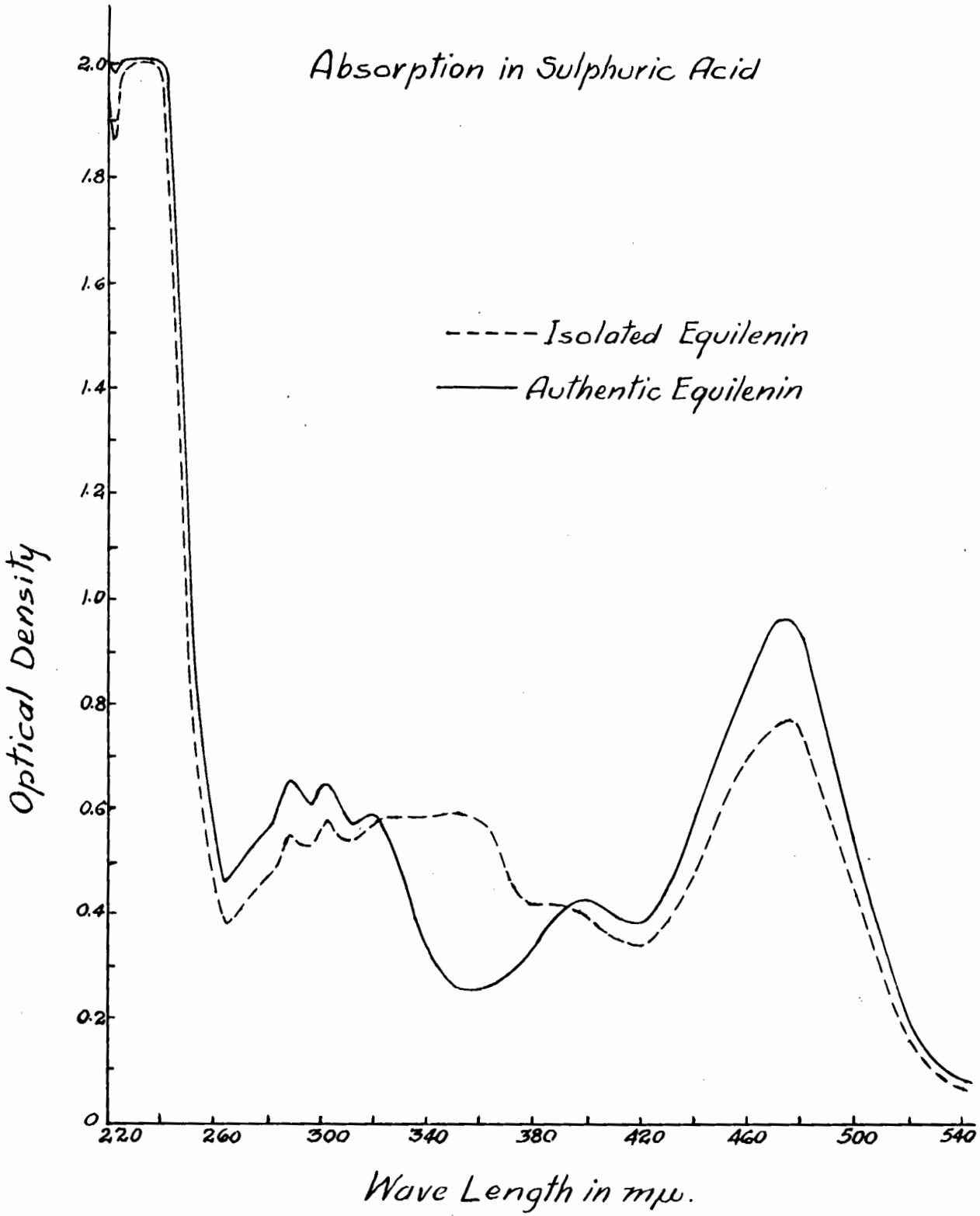
The ultraviolet spectrum of this material (Fig. 15, p.95, was identical with the published spectrum of equilenin (36,102).

Figure 15.


Ultra Violet Spectrum
of Equilin Eluates
22,000
20,000
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
ε
220
240
260
280
300
320
340
360
380
Wave Length in mμ

From the absorption maximum at 340 mμ., it was calculated that the material was 83% pure. When these crystals were dissolved in sulphuric acid, the ultraviolet spectrum obtained (Fig. 16, p. 97) was identical with the spectrum given by equilenin except in the region 320-380 mμ. In this region, equilin exhibits its maximum absorption in sulphuric acid; therefore this equilenin is probably contaminated with a small amount of equilin. The above data definitely shows that the equilin eluates contained equilenin. This equilenin, when counted as an infinitely thin plate, had a specific activity of 380 d./min./mg.

Figure 16.


Absorption in Sulphuric Acid
----- Isolated Equilenin
—— Authentic Equilenin
Optical Density
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
220
260
300
340
380
420
460
500
540
Wave Length in mμ.

Paper Chromatography of Estrone, Equilin and Equilenin.

The methods of chromatography used here were essentially those described by Burton, Zaffaroni, and Kentmann (105). A detailed description of the methods used will be presented in Part II of this thesis. Here, it is sufficient to describe the systems of chromatography used and the results obtained with them.

When we first used paper chromatography, the estrogen spots were located by spraying the paper strips with diazotized sulfanilic acid. This reagent was prepared as follows.

Solution A: This consisted of a solution containing 0.9 g. of sulfanilic acid in 100 ml. of 9% hydrochloric acid (by volume).

Solution B: This consisted of an aqueous solution of 5% sodium nitrite.

The sulfanilic acid was diazotized by mixing 3.0 ml. of A with 3.0 ml. of B and cooling the mixture in an ice bath for 5 min. Then 12 ml. of solution B was added to the mixture and the resulting solution was cooled in the ice bath for an additional 15 min. The paper strips were immediately sprayed, first with the diazotized sulfanilic acid and then with a solution of 1.1% Na_2CO_3. The sensitivity of this reagent for the estrogens was 15 $\mu g./cm^2$ of paper.

Equilenin gave an intense pink color with diazotized sulfanilic acid, while the pink colors obtained with equilin and estrone were of a lesser intensity. More recently, we have been using 15% fuming sulphuric acid to locate the estrogens on the paper strips by the method described by Axelrod (106). Estrone, equilin and equilenin gave rose-tan colors when the paper strips containing them were immersed in fuming sulphuric acid. These three estrogens also gave a green-yellow fluorescence when the paper strips immersed in sulphuric acid were illuminated with an ultraviolet lamp in the dark. The chromatography systems used in this investigation will now be discussed individually.

a) The first system used in attempting to separate estrone, equilin and equilenin was toluene-propylene glycol. This system was found to be effective in separating adrenocortical steroids (105). One cm. paper strips (Whatman No. I) were used with the descending chromatography method of Burton et al. (105). It was found that in 24 hours of chromatography, the mobilities of estrone, equilin and equilenin were 14.0, 10.8 and 10.0 respectively. When different combinations of the three estrogens were chromatographed together, estrone and equilenin separated, but estrone and equilin, as well as equilin and equilenin, travelled together. This method was therefore abandoned. Likewise, the benzene-propylene glycol system yielded similar results.

b) Because equilin, estrone and equilenin can be separated on Celite impregnated with 0.4N NaOH, it was logical to try to separate these estrogens on paper strips impregnated with NaOH. Therefore 1 cm. paper strips (Whatman No. I) were immersed in varying concentrations of NaOH (0.4N to 2N), previously saturated with benzene. Twenty five µg. each of estrone, equilin and equilenin were applied to the paper strips and benzene, saturated with the alkali solution, used, was employed as a mobile phase. The paper strips were then dried at 80°C for 4 hours and sprayed with diazotized sulfanilic acid. It was found that this system gave no separation of the estrogens and furthermore the paper strips had a tendency to fall apart in handling. This system was therefore abandoned.

c) The next system that was tried was that of Boscott (104). In this system, Whatman No. 3 paper was impregnated with a saturated solution of sodium p-toluenesulphonate, and the mobile phase was toluene equilibrated with the latter. The R_f values found for estrone, equilin and equilenin, using this method, were 0.55, 0.25, and 0.10 respectively. Diazotized sulfanilic acid was used to locate the estrogens.

Using this system, it was found that the material in the equilenin eluates (fractions 17-24, p.88) exhibited an R_f value of 0.14. Because it was impossible to elute the estrogens from these salt impregnated paper strips, an

aliquot of the equilenin eluates was mixed with authentic equilenin and the mixture was chromatographed. It was found that the two travelled together and had an R_f value of 0.12. This fact, coupled with the data previously presented, leaves no doubt that the equilenin eluates did contain authentic equilenin.

Using the technique described above, it was also found that the crystals obtained from the equilin eluates exhibited the same R_f value as equilenin and travelled with authentic equilenin on admixture chromatography. This is further confirmation that the equilin eluates contained equilenin. Since it is impossible to elute the estrogens from the treated filter papers in the above system, attempts were made to find a system which would separate equilin, equilenin and estrone using untreated filter paper. Such a system has recently been devised by Axelrod (106) for the separation of estrone, estradiol-17β and estriol.

d) Axelrod found that the system o-dichlorobenzene-formamide gives a good separation of estrone, estradiol-17β and estriol. When this system was applied for the separation of estrone, equilin and equilenin, the R_f values found in 8 hours of chromatography were 7.4, 5.5 and 4.0, respectively. With this system it was possible to elute the estrogens and to do mixed chromatograms. Here 15% fuming sulphuric was

used to detect the estrogens on the paper strips. In confirmation of the earlier findings, it was found that the crystalline material in the equilin eluates exhibited an R_f value of 3.8 in this system and travelled with equilenin in mixed chromatography. Likewise, the material in the equilenin eluates behaved like equilenin on chromatography and on mixed chromatography with authentic equilenin. An attempt was made to separate estrone and equilin from the mother liquors of fractions 7 to 13, p. 92. These mother liquors contained estrone and equilin from the zone where the two overlapped on the second Celite chromatogram. It was found that o-dichlorobenzene-formamide does not effect a total separation of estrone and equilin due to streaking of the two steroids on the paper. However, it was evident that there were two compounds in the mother liquors of fractions 7 to 13. The search for a better method of separating estrone, equilin and equilenin on untreated paper strips is being continued in this laboratory.

In conclusion, it should be stated that the weak phenols and the acid, obtained on extraction of the urine collected in this investigation, did not exhibit any equilin or equilenin when reacted with the B.Q.C. reagent. The neutral fraction obtained from urine of the pregnant mare injected with estrone-16-C^{14} is being investigated by Mr. V.J.O'Donnell.

B. The Anabolism of Estrone, Equilin and Equilenin from Acetate.

1. Introduction and Discussion.

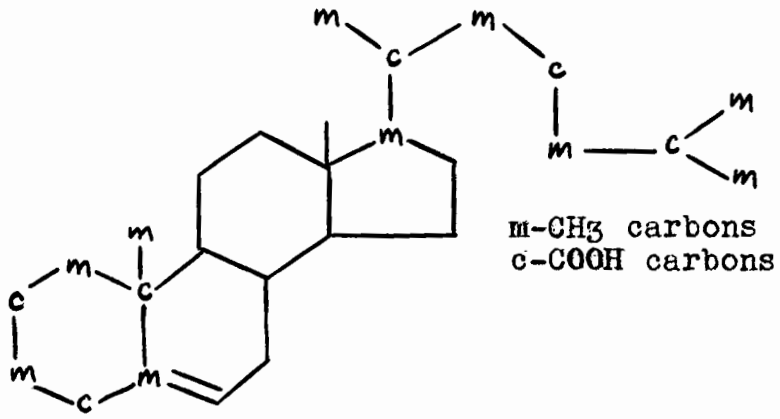
In section A of this thesis, it was shown that estrone is not converted to equilenin and probably not to equilin. It was also found in this laboratory that while cholesterol administered to the pregnant mare does not give rise to urinary estrone (112), acetate does (113). Recently Werthessen et al. (114) have perfused sow ovaries with C^{14}-carboxyl labeled acetate and were able to isolate estrone and estradiol-17β after large isotopic dilutions. The specific activities of the isolated estrogens was 3 d./min./mg. Since all the available evidence indicated that acetate was a precursor of estrone, it was logical to design experiments to determine whether it is also converted to equilin and equilenin by the pregnant mare. However, before entering upon a discussion of the experimental methods used in this investigation and the results obtained, a brief outline will be presented on the central position occupied by acetate in the biogenesis of sterols and steroid hormones. The investigations which will be described below were all performed with the use of deuterium-, carbon-15-, and carbon-14-, labeled compounds respectively.

In 1937, Sonderhoff and Thomas (115) demonstrated that resting yeast tends to concentrate deuterioacetic acid in the unsaponifiable fraction of the cells. This fraction consists principally of ergosterol. This observation was the first evidence to suggest that acetate can be converted to sterols by the plant organism. Some years later Bloch and Rittenberg (116) demonstrated that acetic acid is a precursor of cholesterol in animals. They fed sodium deuterioacetate to adult mice and growing rats and found that the fecal sterols and the body cholesterol of the sacrificed animals contained more than three times as much deuterium as the body fluids. These results demonstrated beyond any doubt that cholesterol is formed from acetate in the animal body.

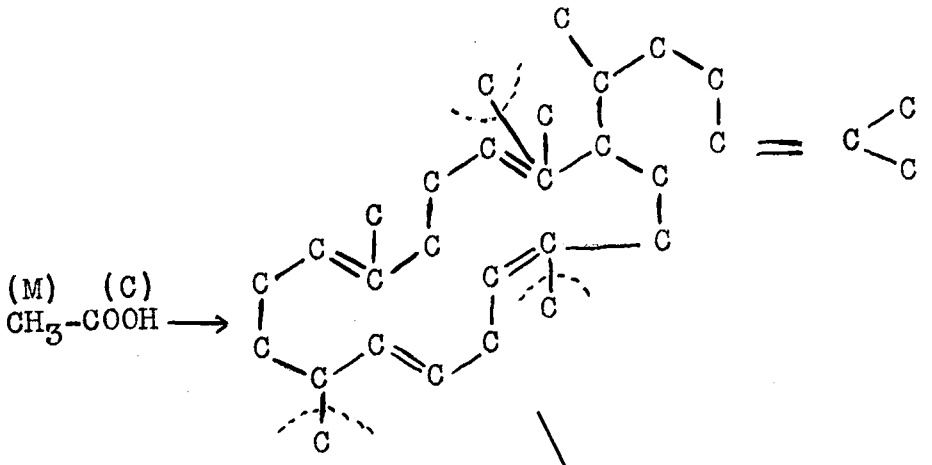
It was early recognized that the liver is an important site in the conversion of acetate to cholesterol (117) and in recent years it has been shown that adrenal cortex slices are also capable of performing this conversion (118). The use of the liver slice technique has made it practicable to study the conversion of a large number of simple organic molecules to cholesterol. These studies have been recently reviewed by Bloch (119), who pointed out that only the compounds which bear a close metabolic relation to acetic acid are efficient precursors of cholesterol and that intermediates of carbohydrate metabolism or compounds which are convertible to carbohydrate are relatively poor sources. Isovaleric acid has been

found to be 5.5 times as efficient as acetate (119). Some evidence has been presented which suggests that acetoacetic acid can be converted directly to cholesterol by rat liver slices (120,121).

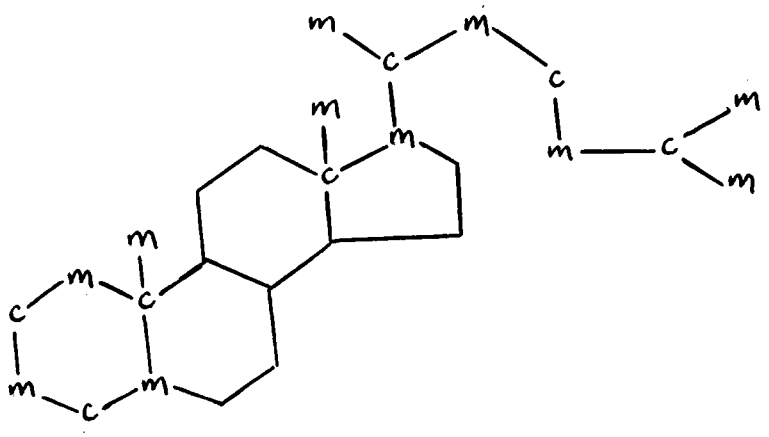
In consideration of the results of the above investigations, Bloch and his associates (119,122,123) and Hunter, Popjak and Cornforth (124) have degraded biologically synthesized cholesterol in order to ascertain the origin of the individual carbon atoms of the molecule. These investigators incubated liver slices with C^{14} -methyl labeled and C^{14} -carboxyl labeled acetic acid, respectively, and isolated the cholesterol thus formed. With the use either of known chemical reactions or reactions specifically designed for this study, the A, B and D rings of cholesterol as well as the isooctyl side chain were degraded, the individual carbon atoms contained therein were identified and their C^{14} content determined. Bloch and his associates concentrated their efforts on the degradation of the isooctyl side chain and ring D of cholesterol while the British group degraded ring A and B of the molecule. The results obtained from their combined efforts are summarized in the diagram below.



The degradation of cholesterol demonstrated that acetic acid can furnish all the carbon atoms required in the biosynthesis of this sterol. This has also been proven in the biosynthesis of ergosterol (125). However, the nature of intermediates of larger molecular weight in the biosynthesis of cholesterol has, until recently, been a topic of speculation. In 1926, Heilbron et al. (126), after elucidating the structure of the terpenoid hydrocarbon squalene (XII), suggested that this compound might be an intermediate in the biosynthesis of steroids. This year, Langdon and Bloch found that the rat can synthesize squalene (XII) from acetate (127), and that squalene is a precursor of cholesterol (XIII,128). Both carbon atoms of acetic acid were utilized in the synthesis of squalene and the liver was found to be the site of the synthesis. The metabolic reactions (CH_3-COOH $\longrightarrow$ XII $\longrightarrow$ XIII) involved in this synthesis are shown below. (page 107).

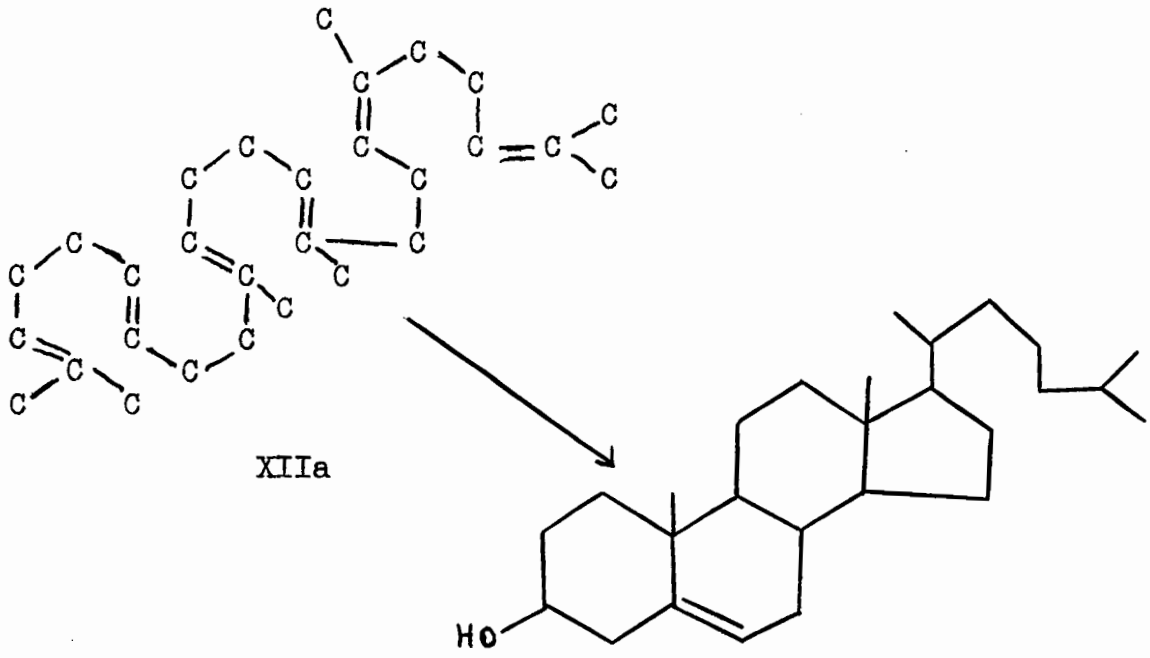


XII



XIII

Langdon and Bloch (128) suggested that the direct cyclization of squalene to form cholesterol, originally proposed by Robinson (129), coincides well with the experimentally determined distribution of the acetate carbons in cholesterol. In the cyclization of squalene, the branched methyl groups that are attached to carbon atoms corresponding to positions 4, 13 and 14 of cholesterol would have to be eliminated. However, more recently Woodward and Bloch (130) and Dauben et al. (131) have presented evidence which suggests that squalene does not undergo direct cyclization in the conversion to cholesterol. These authors proposed the alternative mechanism (XIIa ⟶ XIII) shown below.



XIIa

XIII

Woodward and Bloch and Dauben et al. (129), proposed that each isoprene unit of squalene is composed of 3 methyl and 2 carboxyl carbons of acetate, arranged as shown.

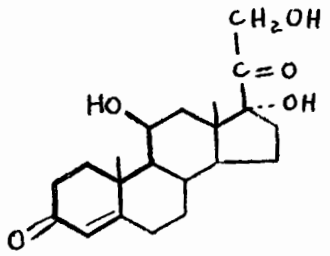
The results obtained from the degradation studies of biosynthesized cholesterol indicated that biosynthesis might proceed by condensation of isoprene units labeled as above (123).

Very recently Langdon and Bloch (132,133) found that there is almost a complete suppression in the conversion of acetate to cholesterol in liver slices of rats fed squalene (XII). They also showed that the incorporation of C^{14}- acetate into liver sterols in vitro is reduced after feeding of cholesterol, Δ^7-cholesterol and 7-dehydrocholesterol. These results indicated that Δ^7-cholesterol and possibly 7-dehydrocholesterol are intermediates in the conversion of squalene to cholesterol.

In the last four years two groups of investigators have done a great deal of work to elucidate the biogenesis of the steroids elaborated by the adrenal gland. At the Worcester Foundation, Dr. G.Pincus and his associates have perfused beef adrenal glands with both C^{14}-acetate and C^{14}-labeled cholesterol respectively. It was found that both acetate and cholesterol can be transformed by the isolated adrenal gland into adrenocortical steroids (134). A second group of investigators at

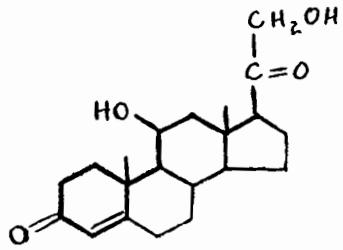
the Upjohn Company, under the leadership of Dr. W.J.Haines, have incubated hog adrenal cortex slices with C^{14}-acetate, and they too found that acetate is a precursor of adrenocortical steroids (135).

After perfusion of C^{14}-carboxyl labeled acetate through beef adrenal glands (134) the perfusate contained radioactive 17-hydroxycorticosterone (XIV) and corticosterone (XV). Incubation of hog adrenal cortex slices with C^{14}-carboxyl labeled acetate led to the identification of radioactive (XIV), (XV), 11-dehydro-17-hydroxycorticosterone (XVI) and small amounts of 11-desoxy-17-hydroxycorticosterone (XVII) in the neutral extracts of the incubation medium and the slices (135,136). These <u>in vitro</u> investigations clearly demonstrate that acetate is a precursor of the adrenocortical hormones. Acetate was also shown to be a precursor of the adrenocortical hormones <u>in vivo</u>. In this laboratory the ethanol soluble extract of the adrenal glands of a pregnant mare injected with C^{14}-carboxyl labeled acetate was found to contain radioactive (XIV), (XVII), (XV) and an unidentified substance called compound X (137).



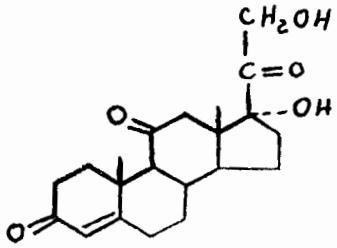
XIV

Kendall's F



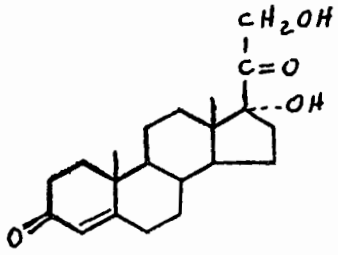
XV

Kendall's B



XVI

Kendall's E



XVII

Reichstein's S

An issue which has been much debated in recent years is whether cholesterol is an obligatory intermediate in the biosynthesis of steroids from acetate. Pincus and his associates (138) have some evidence which suggests that in the conversion of acetate to compounds F and B by the perfused adrenal gland, cholesterol is not an obligatory intermediate. These investigators found that when C^{14}-labeled acetate was perfused through the adrenal gland, the specific activities of the isolated compounds F and B (XIV and XV) was 6.4 times that of isolated adrenal-free cholesterol. When C^{14}-labeled cholesterol was

perfused through the adrenal glands, the specific activities of compounds F and B was 0.4 times that of adrenal-free cholesterol. Thus, these authors reason that if acetate were to go to the adrenocorticoids through cholesterol as an obligatory intermediate, then the specific activity of compounds F and B should not be greater than that of the cholesterol in the adrenal gland. Therefore it seems very likely that acetate is a precursor of compounds F and B in the perfused hog adrenal gland, without necessarily involving cholesterol.

In this laboratory the pregnant mare injected with C^{14}-carboxyl labeled acetate was sacrificed on day 10. On this day the isolated urinary estrone had a specific activity 10 times greater than that of blood cholesterol or maternal liver cholesterol (113,139). Thus acetate is utilized to a greater extent in the synthesis of estrone than in the synthesis of maternal blood cholesterol or maternal liver cholesterol. Furthermore, since administered cholesterol does not give rise to estrone in the pregnant mare (112), it is very likely that it is not an obligatory intermediate in the biosynthesis of estrone from acetate in this species.

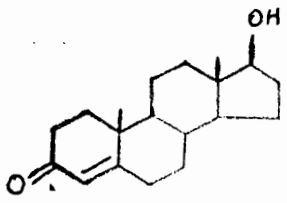
In collaboration with this laboratory, Dr. K.Savard, at the Worcester Foundation succeeded in isolating radioactive 5α-pregnane-3β-ol-20-one from the neutral fraction of the urine collected on days 4 and 5 from the pregnant mare injected with C^{14}-carboxyl labeled acetate. This finding is of great

interest since 5α-pregnane-3β-ol-20-one is generally regarded as a saturated excretory product of progesterone in the pregnant mare (46). Likewise, the administration of deuteriocholesterol to a woman in the eighth month of pregnancy led to the excretion in the urine of deuterium rich pregnane-3α,20α-diol (140), which is considered to be a urinary metabolite of progesterone in man.

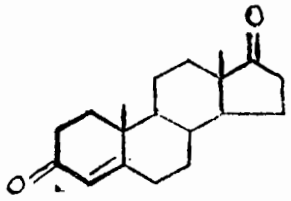
In recent years, it has been found that acetate is also a precursor of testosterone and its saturated excretory products. Brady (141) incubated hog, rabbit and human testicular slices with C^{14}-carboxyl labeled acetate and isolated radioactive testosterone (XVIII) and cholesterol from the tissues and incubation medium. Human chorionic gonadotropin, when added to the incubation medium, markedly stimulated the conversion of acetate to testosterone, but had little effect upon the rate of cholesterol synthesis. Also, the specific activity of the testosterone (by isotope dilution) approached that of the isolated cholesterol, in spite of the great dilution of testosterone caused by the carrier. These findings strongly suggest that cholesterol may not be an intermediate in the biosynthesis of testosterone.

Savard, Dorfman and Poutasse (142) perfused human testes with C^{14}-carboxyl labeled acetate and isolated from the perfusate radioactive testosterone (XVIII) and radioactive androst-4-en-3,17-dione (XIX). In a second investigation

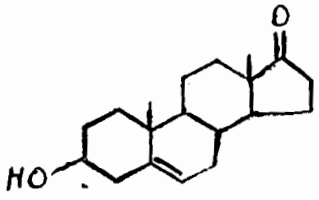
Dorfman et al. (143) administered C^{14}-carboxyl labeled acetate to a male patient suffering from an adrenal carcinoma and they isolated the following radioactive urinary metabolites; dehydroisoandrosterone (XX), androsterone (XXI), etiocholane -3 -ol-17-one (XXII) and androst-5-ene-3 ,17 -diol (XXIII). The androgens derived from acetate are listed below.



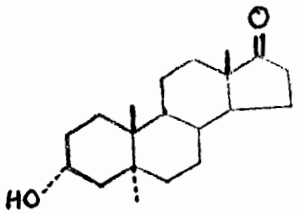
XVIII



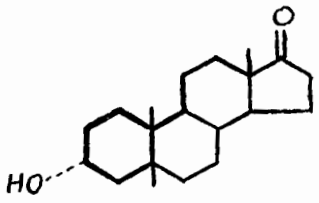
XIX



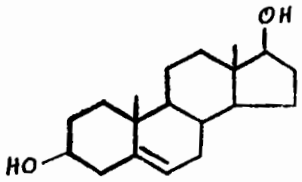
XX



XXI



XXII



XXIII

Thus it is seen that acetate is a precursor of a) cholesterol and ergosterol, b) estrone and estradiol-17β, c) adrenocorticoids, d) testosterone and its saturated excretory products and e) the saturated excretory products of progesterone. It is also generally agreed that cholesterol is not an obligatory intermediate in the conversion of acetate to many of these steroids.

From the above discussion it is evident that acetate occupies a central position in the biogenesis of the sterols and steroid hormones. Because acetate gives rise to estrone in the pregnant mare and since estrone is not converted to equilin or equilenin, it was of great interest to determine whether acetate is a precursor of equilin and equilenin. We shall now discuss the experimental condition employed and the results obtained, in this investigation.

The administration of the labeled acetate and the hydrolysis and extraction of the urine, to be described below, were done by Mr. R.Jacobs and Dr. B.Brooksbank. A hybrid mare in her ninth month of gestation was injected, over a period of four days, with 53.62 millicurries of C^{14}-carboxyl labeled sodium acetate. The urine was collected daily for 10 days and then the animal was sacrificed and the tissues of the colt and the mare were removed for future examination. Each 24 hour output of urine was hydrolysed and partitioned separately.

The urine collected on each day was hydrolysed and partitioned into acids, strong phenols, weak phenols and neutrals as described in section A of this thesis. Estrone was isolated from the weak phenolic fraction obtained from each day, by the methods previously described. The isolated estrone was obtained in a pure state by chromatography on Celite impregnated with 0.4 N NaOH as previously described. Estrone isolated on day 5 had the highest specific activity (1664 d./min./mg.). This finding indicated that 24 hours after the administration of labeled acetate ceased, the conversion of acetate to estrone was maximal.

In order to isolate estrone, equilin and equilenin from the strong phenols, it was necessary to determine the relative concentration of the three estrogens in this fraction. Therefore the strong phenols obtained from the urine collected on

day 4 was partitioned and analysed for estrone, equilin and equilenin by the B.Q.C. method previously described. The strong phenols from day 4 were chosen because they contained a larger amount of radioactivity than the corresponding fractions obtained on other experimental days.

The strong phenols (day 4) were first subjected to high vacuum distillation in order to remove the volatile light oils. In this purification process there was a 39% diminution in weight and only about a 10% decrease in radioactivity. The light oils were counted before they had evaporated and they were found to be radioactive. These light oils when plated from an absolute methanol solution and allowed to evaporate completely, did not exhibit any radioactivity. From the distillate obtained above it was possible to isolate benzoic acid, which on assay was found to be devoid of radioactivity. This finding confirmed the earlier observation of O'Donnell (144), who isolated non-radioactive benzoic acid from the combined acid fractions obtained from the urine excreted by the mare used in this investigation. The biological synthesis of a benzene ring remains to be demonstrated.

The residual strong phenols (day 4) from the high vacuum distillation, were separated into a ketonic and non-ketonic fractions by reaction with Girard's reagent T. As was previously observed, tarry oils formed at the interface between

the ether solution containing the non-ketones and the aqueous solution containing the Girard complex of the ketones. Because these tarry oils contained about 50% of the radioactivity originally present in the residual strong phenols (day 4), they were fractionated into ketones and non-ketones with Girard's reagent T. In this fractionation there was a sizable loss in radioactivity as well as in weight. These losses have been repeatedly encountered in the Girard reaction on the tarry oils.

B.Q.C. analysis of the ketonic fractions obtained from the Girard separation of the strong phenols (day 4) revealed that in one day this pregnant mare excreted 50 mg. of equilin plus equilenin and 90 mg. of estrone. These values are in sharp contrast to the quantities of these three estrogens excreted during 3 days by the pregnant mare injected with estrone-16-C^{14}; i.e. 26.25 mg. of equilin plus equilenin and 37.5 mg. of estrone. Doisy (33) observed many years ago, that the total amount of estrogens (bio-assay values) excreted by the pregnant mare varied greatly with individual animals.

In order to facilitate the separation of estrone, equilin and equilenin, the strong phenols from days 1 to 10 were combined (day 8 was lost in extraction) and separated into ketones and non-ketones. It was presumed that the combined ketonic strong phenols would then contain sufficient quantities of the three estrogens to allow facile separation of estrone, equilin and equilenin.

Following high vacuum distillation of the volatile light oils, the combined strong phenols were separated into ketones and non-ketones by reaction with Girard's reagent T. Here also tarry oils were encountered. The non-ketones and the tarry oils were subjected to a second Girard separation and here pure estrone was isolated from the ketonic fraction of the tarry oils.

The combined ketonic strong phenols (including day 4) weighed 3.41 g. Since the maximum quantity of ketonic strong phenols that can be readily separated on the Celite chromatogram is approximately 200 mg., a method had to be devised to purify the ketonic strong phenols in order to adsorb them on the Celite column. Stimmel (145) had earlier found that estrone, estradiol-17β and estriol, present in human pregnancy urine, can be separated by chromatography on activated alumina. Estrone, estradiol-17β and estriol were separated by eluting the alumina column with 2% methanol-benzene, 5% methanol-benzene and 30% methanol-benzene respectively. The above author found that most of the impurities normally present in the urine remained at the top of the alumina column. Therefore in a trial experiment, 1/10 of the combined ketonic strong phenols (0.341 g.) was adsorbed on a column of activated alumina and the estrogens were eluted as described above. It was found that 10% methanol-benzene eluted almost all of the estrogens on the column as well as brown impurities. Neither

benzene nor 5% methanol-benzene was capable of eluting the estrogens on the alumina column. Therefore this method of chromatography was abandoned.

It was then decided to adsorb small portions of the combined ketonic strong phenols on Celite impregnated with 0.4 N NaOH and to pool the eluted fractions containing the three estrogens until sufficient quantities of material had accumulated to allow facile isolation. In the first experiment 0.545 g. of the ketonic strong phenols was adsorbed on 31 g. of Celite impregnated with 30 ml. of 0.4N NaOH and the estrogens were eluted with 20 ml. portions of benzene. On examination of the fraction eluted it was found that estrone, equilin and equilenin had separated in pure form and in quantities sufficient for identification. The three estrogens were identified by their a) melting points, b) mixed melting points with authentic samples of the corresponding estrogen, c) ultraviolet spectra in 95% ethanol, d) ultraviolet spectra in sulphuric acid. When assayed for C^{14} as infinitely thin plates, the specific activity of estrone, equilin and equilenin was 641, 328 and 304 d./min./mg. respectively.

Estrone contained approximately twice the radioactivity of equilin or equilenin. This finding indicated that twice as much carboxyl carbon of acetate was incorporated into estrone as into equilin or equilenin. It may well be that acetate is not incorporated into the naphthalene ring of

equilenin or ring B of equilin. A conclusive answer to this problem can only be obtained by degrading the three estrogens in a manner that would leave ring A of estrone intact and leave ring A and B of equilin and equilenin intact. Carbon-14 assay of these moieties, left after degradation, would then tell us whether the benzene ring of estrone or the naphthalene ring of equilenin contained any C^{14}.

If one can draw a parallel between the metabolic changes taking place in a pregnant rabbit and the metabolic changes occurring in a pregnant mare, then some conclusions can be drawn of the possible sites of formation of the estrogens. Popjak and Beeckmans (146) found that in the pregnant rabbit, all foetal cholesterol is synthesized within the body of the foetus and that the foetal placenta synthesizes cholesterol and also absorbs it from the maternal circulation. In the case of the pregnant mare injected with C^{14}-carboxyl labeled acetate, the following facts have thus far been revealed concerning the origin of cholesterol. At death (day 10) it was found that, a) the specific activity of the isolated urinary estrone was greater by a factor of ten than the maternal blood cholesterol, b) foetal liver cholesterol had a higher specific activity than maternal liver cholesterol and c) cholesterol from the foetal placenta had the highest specific activity. These findings are in agreement with the results obtained by

Popjak and Beeckmans (146) for the pregnant rabbit and indicate that estrone, equilin and equilenin may be synthesized from acetate by the tissues of the foetus or by the foetal placenta.

In the case of the pregnant human female, estrone, estradiol-17β and estriol have been isolated from placental tissue (147,148,149). Recently Mitchell (150) was able to identify estrone, estradiol-17β and estriol by the application of paper chromatography to separate the estrogens extracted from 100 g. of human placenta. That the placenta of the pregnant mare is capable of synthesizing estrogens was demonstrated many years ago by Hart and Cole (151). These investigators found that bilateral removal of the ovaries in a pregnant mare produced an initial drop in the estrogen level in the urine, followed by a rise to the normal level, which was maintained for the remainder of the pregnancy. These findings indicate that the placenta synthesizes estrogens during pregnancy.

2. Experimental.

The processing of the urine of the mare injected with sodium acetate-1-C^{14} was done in this laboratory by Mr. R. Jacobs and Dr. B.Brooksbank. This author examined the strong phenolic fractions. Since the methods used in this investigation are identical to those described in section A of this thesis, they will not be described here in detail.

Injection of Labeled Acetate and Collection of Urine.

One hybrid mare in the ninth month of gestation was injected intrajugularly with 619 mg. of sodium acetate containing 53.62 millicurries of $CH_3.C^{14}OONa$. The injections were made thrice daily through days 1 to 4. Urine was collected for 10 days and full 24 hour outputs of urine were processed each day. The urine exhibited a Kober value of ca. 220 mg. per diem. throughout. On day ten, the animal was killed (chloral) and various tissues of the mare and colt were taken for chemical examination; sections of all tissues were taken for histological and radioautographic study.

Fractionation of the Urine.

Each 24 hour output of urine from days 1 to 10 was processed separately. The urine was acid hydrolysed with an equal volume of toluene and then the organic phase was fractionated into acids, strong phenols, weak phenols and neutrals,

as described in section A of this thesis. The maximum urinary excretion of C^{14} occurred on day 4. However, estrone isolated from the weak phenols of the day 5 urine had a higher specific activity (1164 d./min./mg.) than that of day 4 (1117 d./min./mg.)

Vacuum Distillation of the Strong Phenols (Day 4).

The strong phenols from the urine collected on day 4 ($S.P._4$) weighed 5.67 g. and contained 1.35×10^6 d./min. The volatile light oils were distilled from this fraction under high vacuum as described in section A of this thesis. In this instance, the light oils started to distill at 65°C and they were collected in a tube surrounded by an acetone-dry ice mixture in a Thermos container. The material distilling above 65°C crystallized in the receiving tube. Then the temperature was elevated to 100°C. Distillation was allowed to continue at this temperature for four hours. The results obtained from this distillation are tabulated below.

Fraction No.	Nature	Temp. °C	Weight g.	C^{14} Assay d./min.
1	light oils	65-70	2.125	
2	crystalline	75-100	0.119	
3	oils (non-volatile)		3.412	1,237,000

From the above data it was deduced that 10% of the radioactivity originally present in the strong phenols of day 4 distilled with the volatile light oils (Fractions 1 and 2). Fraction 2, recrystallized from water, yielded 32 mg. of colorless crystals (m.p. 121-122°C). These crystals did not show a depression in m.p. on admixture with pure benzoic acid. The isolated benzoic acid did not contain any radioactivity.

The light oils, which were removed from the strong phenols by high vacuum distillation, contained appreciable amounts of volatile radioactivity. This was demonstrated as follows. The light oils were diluted 1 part in 25,000 with methanol and were plated as infinitely thin plates. When the plates were allowed to evaporate completely to dryness they did not contain detectable amounts of C^{14}. However, when these light oils were diluted 1 part in 250 and 1 ml. aliquots were plated and dried to the point where only the methanol had evaporated, there was a detectable amount of radioactivity in them. It was found that the radioactivity on these plates decreased with time as is shown below.

Time in min.	Radioactivity d./min./plate	
	Plate 1.	Plate 2.
10	124	37
20	115	22
30	108	9
40	94	0

Plate 1 was counted first and plate 2 was counted 45 minutes later in each instance. The background count was subtracted from the observed count on each plate.

Girard Fractionation of the Strong Phenols (Day 4).

The non-volatile strong phenols (3.412g.) were dissolved in 50 ml. of absolute ethanol and to this solution was added 4.89 g. of Girard's reagent T plus 5 ml. of glacial acetic acid. The mixture was refluxed for 1 hour and then the ketones were separated from the non-ketones as described in section A of this thesis. In this separation tarry oils were encountered at the interface between the aqueous phase containing the Girard complex of the ketones and the ether solution containing the non-ketones. These tarry oils were dissolved in 95% ethanol and taken to dryness under reduced pressure. This fraction was submitted to a second treatment with Girard's reagent T. The ketones, non-ketones and tarry oils were assayed for C^{14} by the method of Yates (29) and the results are tabulated below.

Fraction	Weight g.	Radioactivity d./min.
Strong Phenols	3.412	1,210,000
Ketones	0.238	117,500
Non-ketones	1.091	242,500
Tarry oils	1.998	525,000

Girard Reaction on the Tarry Oils.

The tarry oils (1.998 g.) were dissolved in 40 ml. of absolute ethanol and to this solution was added 1.98 g. of Girard's reagent T plus 4 ml. of glacial acetic acid. This mixture was refluxed for 1 hour and then the ketones were separated from the non-ketones as described in section A of this thesis. After diluting the ketones and non-ketones thus obtained to a definite volume with absolute methanol, aliquots were assayed for C^{14} by the method of Yates (29). The results obtained from the Girard fractionation are tabulated below.

Fraction	Weight g.	Radioactivity d./min.
Strong Phenols	3.412	1,210,000
Ketones	0.238	117,500
Non-Ketones	1.091	242,500
Tarry Oils	1.998	525,000
Ketones	0.289	185,000
Non-Ketones	0.762	285,000
Total Ketones	0.527	302,500
Total Non-Ketones	1.852	527,500

B.Q.C. Analysis of the Ketonic Strong Phenols (Day 4).

The esterified B.Q.C. reaction, the unesterified B.Q.C. reaction and the Kober reaction on the ketonic fractions from the strong phenols (day 4) and the tarry oils were done as described in section A of this thesis. The results obtained are tabulated below.

	Equilin mg.	Equilenin mg.	Estrone mg.
Ketones from the first Girard reaction	6.18	3.83	17.50
Ketones from the tarry oils	24.70	15.30	72.50
Total	30.88	19.13	90.00

High Vacuum Distillation of the Combined Strong Phenols.

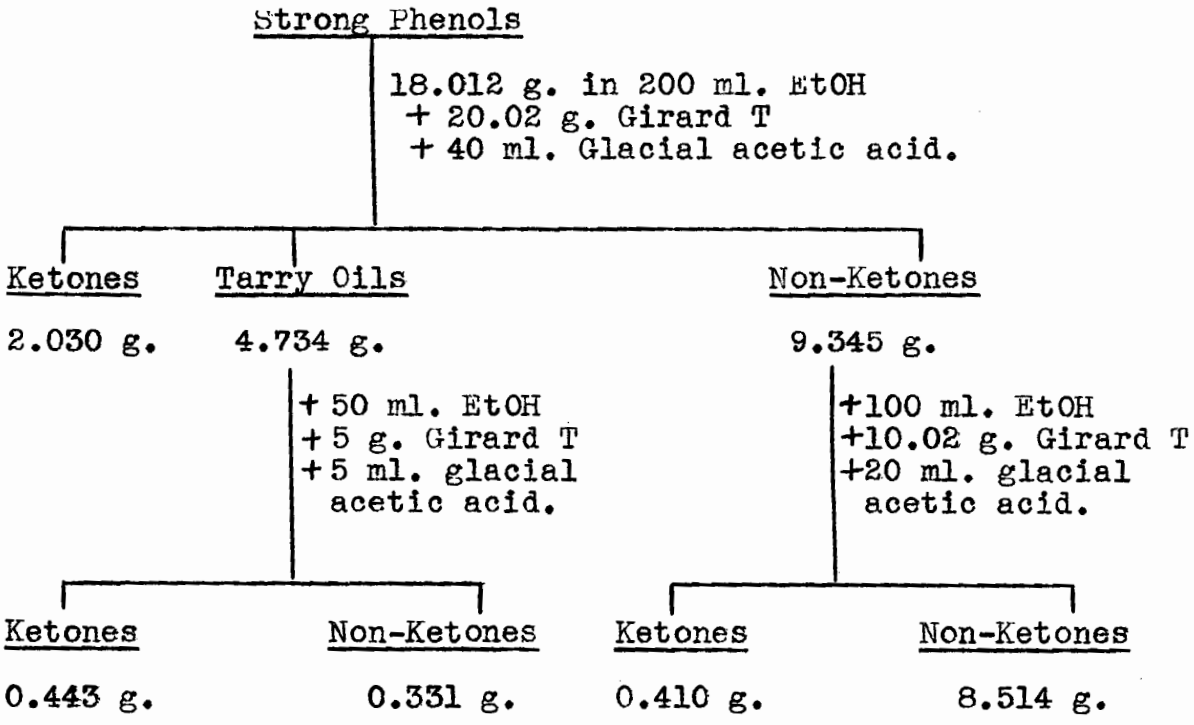
The combined strong phenols (8 days; day 8 was lost in extraction) weighed 46.027 g. and contained 1,989,620 d./min. when assayed by the method of Yates (29). In order to exclude the volatile light oils from the C^{14} assay of the above fraction, the plates were air dried for 4 hours prior to counting them.

The light oils were distilled under high vacuum as previously described. The apparatus used consisted of a 250 cc. round bottom flask sealed at the neck to a piece of tubing

(3 cm. diameter) which in turn was connected by standard tapered joints to a large test tube immersed in acetone-dry ice. The round bottom flask containing the combined strong phenols was heated in an air oven as previously described. At 49°C distillation commenced (mainly benzoic acid) and the process was continued until the temperature reached 115° C (a total of 10 hours). At the end of this period of time, distillation ceased. The distillate weighed 28.0 g. and no attempt was made to determine its C^{14} content. The residual strong phenols weighed 18.012 g. and were found to contain 2,025,600 d./min. when assayed by the method of Yates (29).

Girard Separation of the Combined Strong Phenols.

The combined non-volatile strong phenols (18.012 g.) were dissolved in 200 ml. of absolute ethanol and to this solution was added 20.02 g. of Girard's reagent T plus 40 ml. of glacial acetic acid. The mixture was refluxed for 1 hour and was then separated into ketones and non-ketones as previously described. The yield of ketones, non-ketones and tarry oils was 2.030 g., 10.322 g., and 4.734 g., respectively. In order to obtain a better separation of ketones from non-ketones, the tarry oils and the non-ketones were again reacted with Girard's reagent T. The Girard fractionation of the combined strong phenols is outlined schematically below.



Total Ketones - 2.883 g.

Total Non-Ketones - 8.845 g.

The fractions resulting from the Girard reaction on the combined strong phenols were diluted with absolute methanol and aliquots were assayed for C^{14} by the method of Yates (29). In the table below is summarized the results obtained from the analysis of the above fractions.

Fraction	Weight mg.	Radioactivity d./min.
Strong Phenols	18.012	2,025,600
Ketones	2.030	456,250
Non-Ketones	9.345	837,500
Ketones	0.410	36,000
Non-Ketones	8.514	776,000
Tarry Oils	4.734	492,000
Ketones	0.443	132,500
Non-Ketones	0.331	40,000
Total Ketones	2.883	624,750
Total Non-Ketones	8.845	816,000

In the Girard reaction on the tarry oils, 0.761 g. of material did not dissolve in absolute ethanol and was recovered at the end of the reaction. The low yield (in weight and radioactivity) obtained from this reaction cannot at present be explained. However, the ketonic fraction from

these tarry oils were crystalline when taken to dryness and recrystallizations from ethyl acetate and absolute methanol yielded 32 mg. of colorless needles, m.p. 255-256°C. The m.p. of these crystals was not depressed on admixture with authentic estrone, m.p. 258-260°C. When dissolved in sulphuric acid the ultraviolet spectrum given by this material was identical with the spectrum exhibited by authentic estrone (Fig. 17, page 133.). From the evidence presented above, it was concluded that these crystals were pure estrone. The mother liquors from the crystallizations of the estrone from the ketonic fraction of the tarry oils were added to the ketonic strong phenolic fraction.

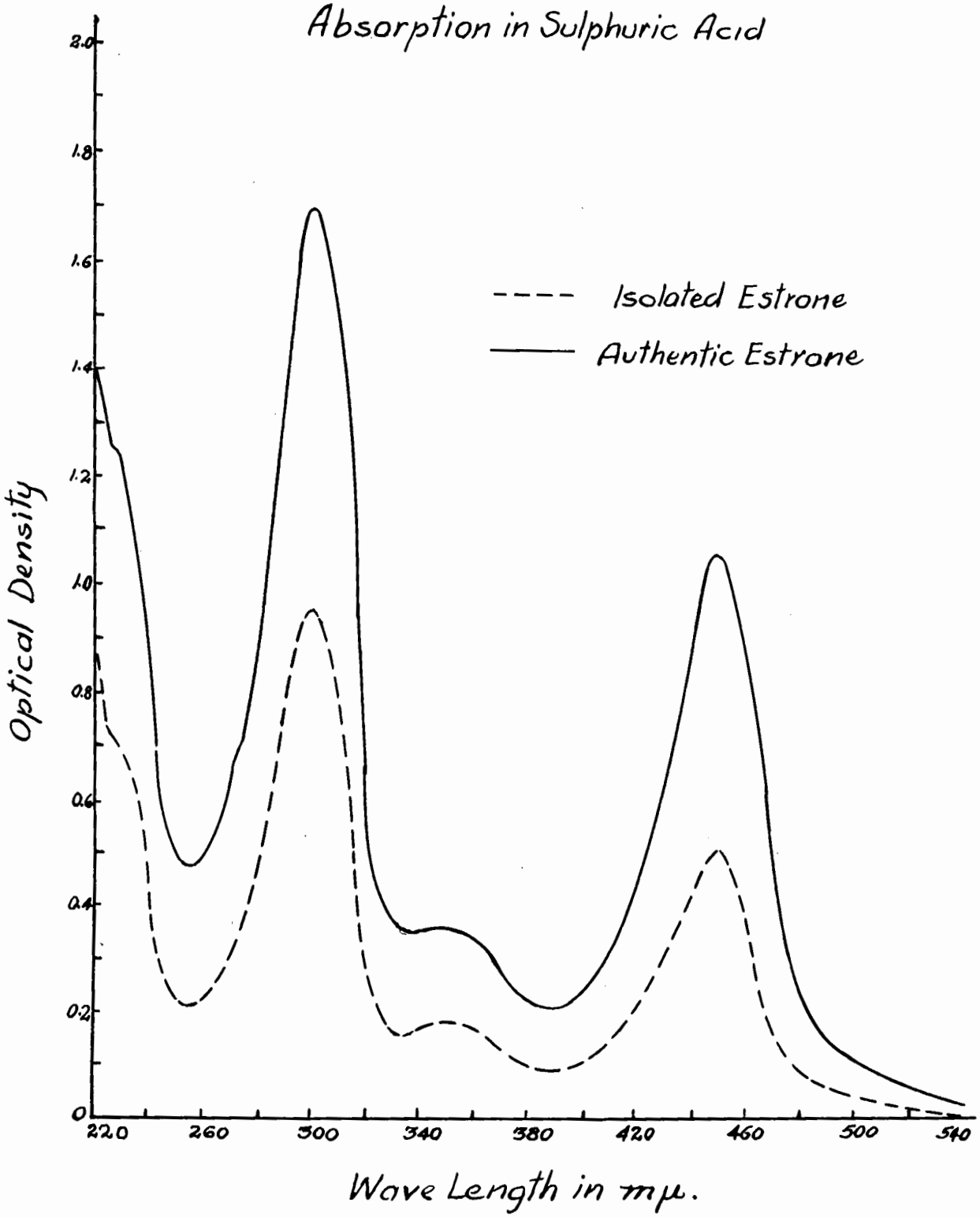
When pooled, the ketonic fractions of the combined strong phenols and the strong phenols (day 4) weighed 3.410 g. An aliquot of this ketonic fraction was removed for chromatography on alumina.

Alumina Chromatography of the Ketonic Strong Phenols.

A portion (0.3326 g.) of the combined ketonic strong phenols was dissolved in 15 ml. of benzene with gentle heating and the solution was adsorbed on 4 g. of activated alumina# contained in a glass tube (9 cm. x 1.2 cm.). The estrogens were eluted with 10 ml. portions of anhydrous benzene (fractions 1 to 11).

The alumina was washed first with 6 N HCl and then exhaustively with distilled water and activated by heating at 200°C for 24 hours.

Figure 17.


Absorption in Sulphuric Acid
Isolated Estrone
Authentic Estrone
Optical Density
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
220
260
300
340
380
420
460
500
540
Wave Length in mμ.

5% methanol-benzene (fractions 12 to 21) and 10% methanol-benzene (fractions 22 to 36). Nothing was eluted with either benzene or 5% methanol-benzene; most of the material was eluted in fractions 22 to 24 with 10% methanol-benzene. There was no separation of estrone, equilin and equilenin in the eluates and as a result this method of chromatography was abandoned.

Chromatography of the Ketonic Strong Phenols on Celite.

A portion (0.5453 g.) of the combined ketonic strong phenols was adsorbed from a benzene solution on 31 g. of Celite previously impregnated with 30 ml. of 0.4 N NaOH solution in the manner previously described. The estrogens were eluted with 20 ml. portions of benzene.

Fraction No.	Eluant	Eluates			
		Weight mg.	B.Q.C. reaction	Absorption at 340 mμ.	m.p. °C.
1 - 3	Benzene	26.24	-	-	
4 - 7	"	100.08	-	-	257-258
8 - 15	"	37.88	+	-	238-241
16-- 17	"	2.52	-	-	
18 - 28	"	22.16	+	+	258-260
29 - 33	"	2.54	-	-	

From each fraction eluted, an aliquot containing about 100 µg. of material was taken to dryness and the B.Q.C. reaction was done on it as previously described. Similarly, an aliquot containing about 60 µg. was removed from each fraction and taken to dryness. The residues were dissolved in 3 ml. of 95% ethanol and the solutions were analysed for their absorption maximum in the ultraviolet at 340 mµ.

Fractions 4 to 7 did not give the B.Q.C. reaction and did not exhibit an absorption maximum at 340 mµ. Therefore these fractions did not contain equilin nor equilenin. After two recrystallizations from absolute methanol, these fractions yielded 54.2 mg. of colorless crystals (m.p. 257-258°C) whose m.p. was not depressed on admixture with authentic estrone (m.p. 258-260°C).

Fractions 8 to 15 gave a positive B.Q.C. reaction but they did not exhibit an absorption maximum at 340 mµ. Therefore these fractions must have contained equilin but no equilenin. After two recrystallizations from ethyl acetate, these fractions yielded 14.5 mg. of small colorless needles (m.p. 235-238°C) whose m.p. was not depressed on admixture with authentic equilin.

Fractions 18 to 28 gave a positive B.Q.C. reaction and exhibited an absorption maximum at 340 mµ. when tested in ultraviolet light. Therefore these fractions must have contained equilenin. When taken to dryness these fractions turned purplish-red. These fractions were induced to

crystallize from ethyl acetate, and on recrystallization from absolute methanol yielded 11.20 mg. of light red needles (m.p. 258-260°C). On admixture with authentic equilenin (m.p. 255-256°C), these needles did not show a depression in m.p. However, on admixture with authentic equilin there was an 18° depression in m.p.

Ultraviolet Analysis of Estrone, Equilin and Equilenin.

Approximately 60 µg. of estrone, equilin and equilenin in 3 ml. of 95% ethanol was used to determine their ultraviolet absorption values at the wave lengths indicated in the table below.

Estrogen	log ε found	log ε given (53)
Estrone	3.39 (281 mµ.)	3.40 (280 mµ.)
Equilin	3.40 (282 mµ.)	3.40 (280 mµ.)
Equilenin	3.41 (340 mµ.) 3.72 (279-280 mµ.)	3.40 (340 mµ.) 3.70 (280 mµ.)

Ultraviolet spectra of estrone, equilin and equilenin in sulphuric acid (80-90 µg. in 3 ml. of conc. H_2SO_4) were determined alongside of samples of the corresponding authentic estrogens. The results obtained are shown graphically in Fig. 18, p. 137, Fig. 19, p. 138, and Fig. 20, p. 139. It is seen from Fig. 18, p. 137, that the two curves do not

Figure 18

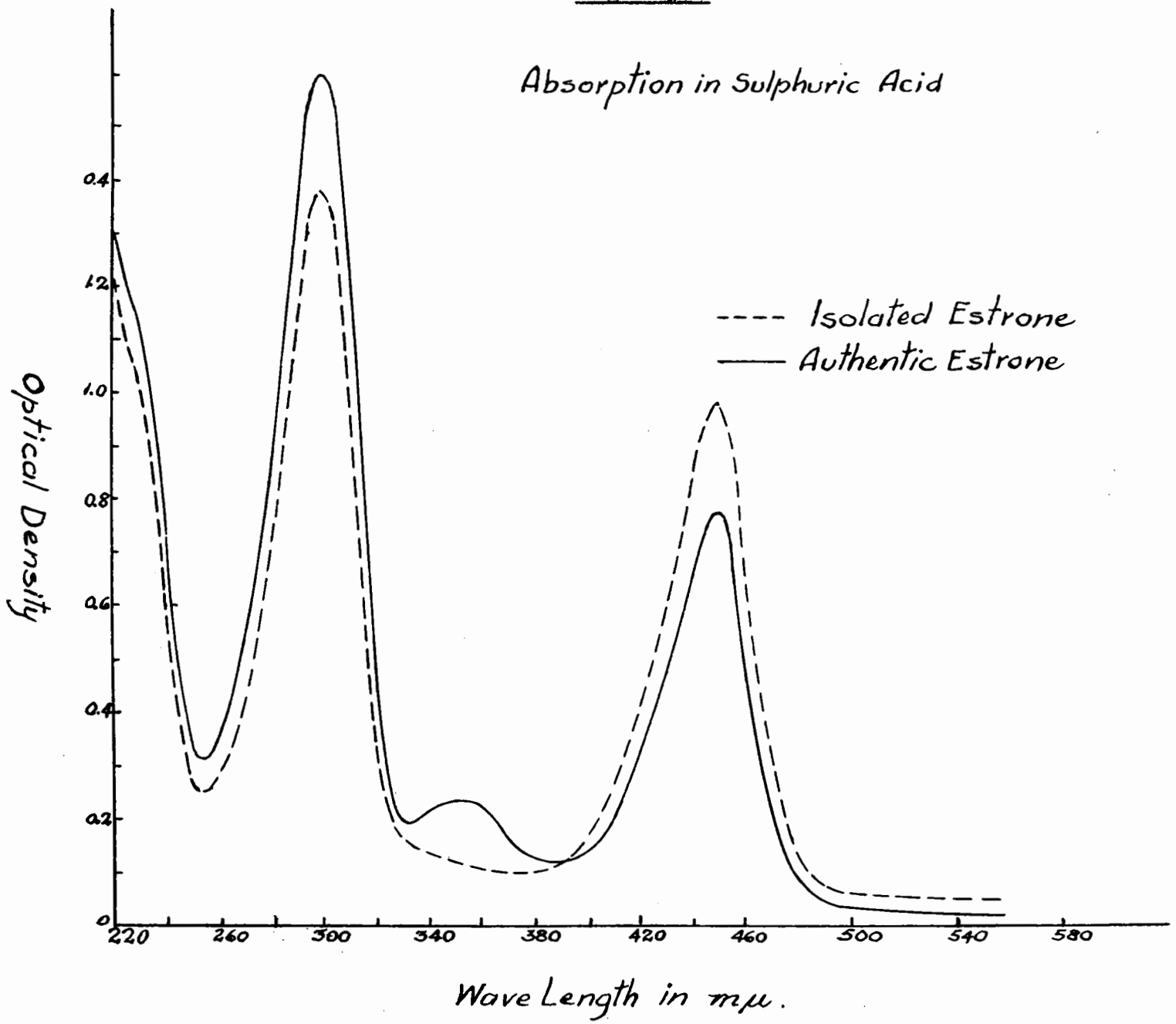

Absorption in Sulphuric Acid
---- Isolated Estrone
—— Authentic Estrone
Optical Density
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
220
260
300
340
380
420
460
500
540
580
Wave Length in mμ.

Figure 19.

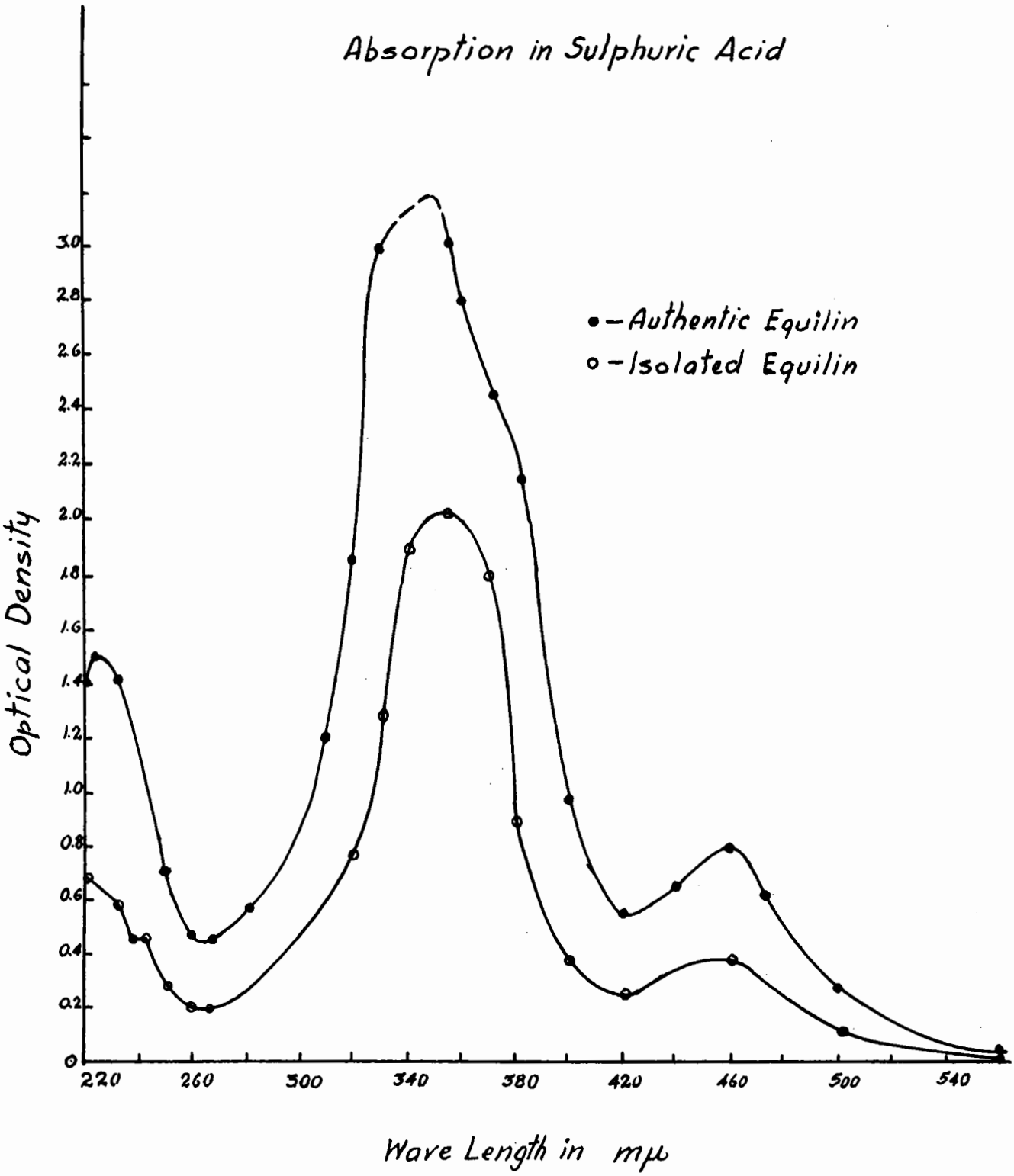
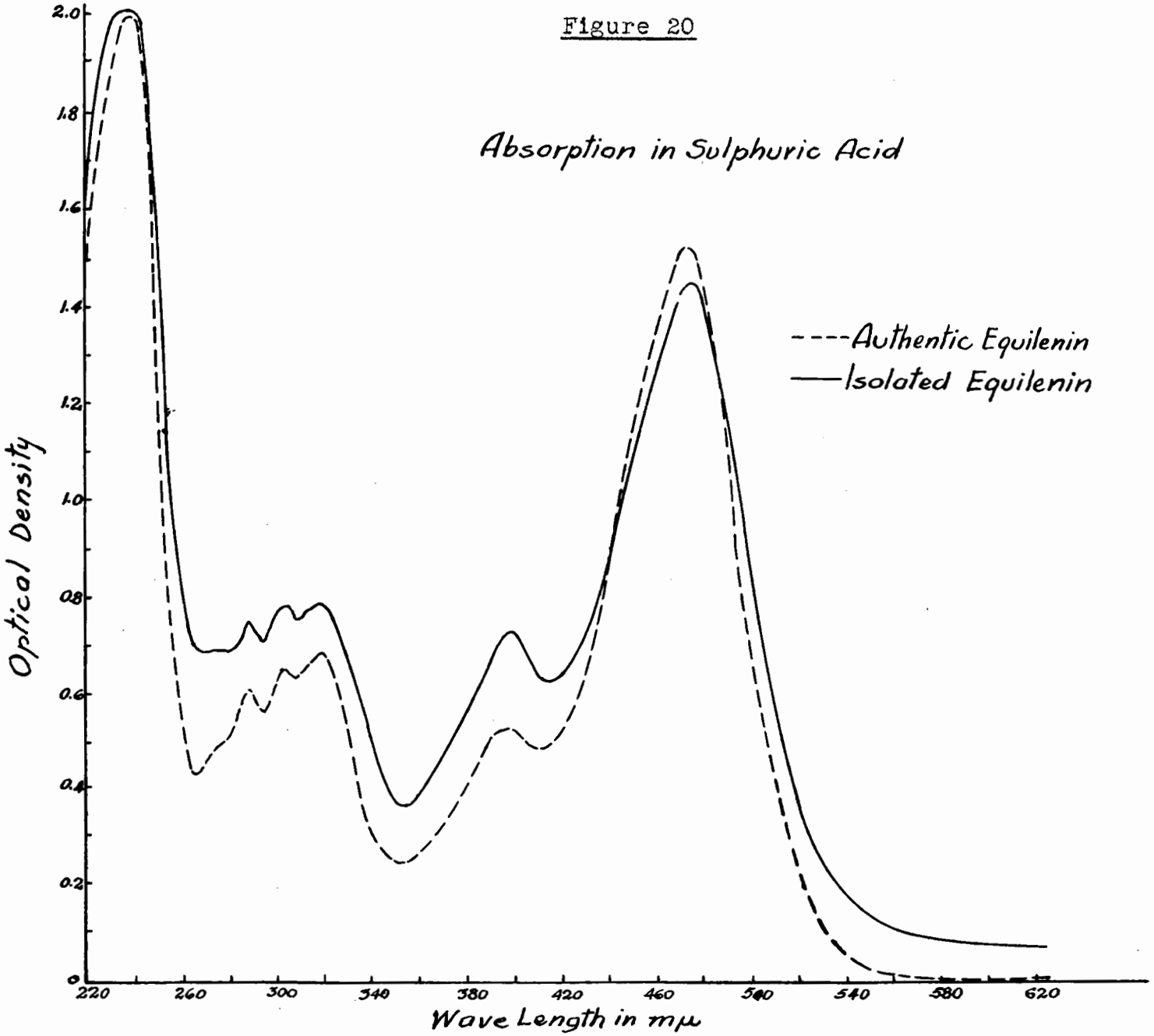

Absorption in Sulphuric Acid
•—Authentic Equilin
o—Isolated Equilin
Optical Density
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
220
260
300
340
380
420
460
500
540
Wave Length in mμ

Figure 20


Absorption in Sulphuric Acid
----Authentic Equilenin
——Isolated Equilenin
Optical Density
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
220
260
300
340
380
420
460
500
540
580
620
Wave Length in mμ

correspond closely in the region 340-380 mμ. In all the ultraviolet spectra of estrone in sulphuric acid thus far examined, it was found that authentic estrone (isolated from mare's pregnancy urine) has a small peak at 350 mμ. while estrone purified by Celite chromatography does not exhibit a peak in this region. From this it was deduced that the "authentic" estrone must be contaminated with a small amount of equilin since the latter exhibits its maximum absorption in sulphuric acid in this region.

C^{14} Assay of Estrone, Equilin and Equilenin.

The three isolated estrogens were plated as infinitely thin plates (40-70 μg./cm^2) from methanolic solutions and were thus assayed for their C^{14} content.

Estrogen	Specific Activity d./min./mg.
Estrone	641
Equilin	328
Equilenin	304

PART II

URINARY ESTROGENS IN THE NORMAL MENSTRUAL CYCLE.

1. Introduction and Discussion.

Because human pregnancy urine contains large quantities of estrogens (9) and because the state of pregnancy is of such great clinical interest, a large number of investigations have been reported on this source of estrogenic activity. Following the discovery of Zondek (9), that human pregnancy urine contains large quantities of estrogens, estrone (10,11), estradiol-17β(151), and estriol (149) were isolated from this urine. Furthermore, most of the methods of hydrolysis, extraction and quantitative determination of urinary estrogens have been developed with human pregnancy urine. As a result, a great deal of information has been gathered concerning the excretion of estrogens in this urine. Doisy et al. (58), by comparing the data published by Smith et al. (152) and Bachman and Pettit (88), estimated that in the last stages of human pregnancy about 30 mg. of total estrogens are excreted daily. This high level of urinary excretion has made it possible for investigators to devise suitable methods for the separation and quantitative determination of the urinary estrogens.

In sharp contrast to the amount of information that has been gained concerning the estrogens in human pregnancy urine,

very little is known about the excretion of these hormones in the normal menstrual cycle. This impasse is mainly due to the low level of estrogens excreted by the normal female. Doisy et al.(58), after evaluating the data published, estimated that about 1 mg. of total estrogens (bioassay value) is excreted during a normal menstrual cycle. It is generally agreed that the maximum daily excretion of estrogens during a normal menstrual cycle is about 100 µg., determined by bioassay (153).

Until very recently the only reliable method for the determination of such small quantities of urinary estrogens was the bioassay technique (153). In reviewing bioassay methods for urinary estrogens, Emmens (154) pointed out that the typical vaginal smear test can give an estimate of the potency of an extract within 80- 125% (P = 0.95) using about 50 animals per substance under test and 50 animals on the standard preparation. Therefore the values reported for the urinary estrogens excreted by the normal female, using bioassay methods must be regarded as questionable. It is also difficult to assess satisfactorily the results of investigations on the hormone content of normal female urine because the methods of extraction and bioassay are by no means standardized, thus making it difficult to compare results obtained by different investigators. At this point, it will be helpful to review

briefly some of the findings concerning the estrogens excreted in the normal menstrual cycle.

The cyclical sexual phenomena occurring during the normal menstrual cycle has recently been reviewed by Robson (155). It is sufficient to state here that it has been convenient to divide the normal menstrual cycle into two phases, the follicular phase (preovulatory phase) and the luteal phase (postovulatory phase). The average length of the normal menstrual cycle is generally considered to be 28 days, but cycles of 21 and 36 days are not uncommon (155). Ovulation usually occurs at the mid-menstrual period, but in a few cases anovulatory menstrual cycles have been described (156).

The investigations done on human urine prior to the discovery that the estrogens present therein occur as water soluble conjugates, which are only rendered ether soluble on acid hydrolysis (71), cannot be considered very reliable. However, in the light of our present knowledge these earlier investigations seem to be qualitatively correct and for this reason they will briefly be reviewed here.

In 1926, Loewe and Longe (157) were the first to find that normal female urine contained estrogenic activity. Siebke (158), in 1930, found that there was a peak in the excretion of estrogenic activity on the ninth day before menstruation. Later, Gustavson and Green (159) found that there

was a rapid rise in the output of estrogens about the time of ovulation and a second rise between the 14th and 21st days of the cycle followed by a gradual fall. That there were two peaks in the excretion of estrogens in the normal menstrual cycle was confirmed by a number of investigators (160,161,162). The investigations cited above were done without hydrolysing the urinary estrogen conjugates and must therefore be considered only as qualitative. It was not until Cohen and Marrian (71) showed the importance of hydrolysing human urine, in order to extract the estrogens present, that reliable data appeared on the excretion of estrogens in the normal menstrual cycle.

In 1936, Gustavson et al. (163) repeated their investigations but this time they acid-hydrolysed the urine prior to extraction and bioassay of the estrogens present. These authors again found two peaks in the excretion of estrogens in the normal menstrual cycle and they claimed that 1.3 mg. of total estrogens, equivalent to estrone, was excreted throughout the cycle. About the same time Smith and Smith (164) published their findings of the analysis of the urines of a large number of females having normal menstrual cycles. The Smiths observed a rise in the urinary excretion of estrogens on the 12th day before menstruation. In a few women, the Smiths did not observe a rise in urinary estrogens in the middle of the

menstrual cycle, but in these cases there was a rise just before bleeding commenced. These women, the Smiths assumed, had anovulatory cycles.

In 1938, Smith, Smith and Pincus (152) determined the urinary estrogens excreted in a normal menstrual cycle by their own methods of hydrolysis and extraction and by the methods described by Cohen and Marrian (71). Smith et al. (165) hydrolysed the urine by refluxing with 15 volume per cent concentrated HCl for 10 minutes and then extracted the liberated estrogens with benzene. Cohen and Marrian (71) hydrolysed the urine by first adjusting it to pH 1.0 with concentrated HCl, adding an additional 3.3 ml. of 12 N HCl per 100 ml. of urine and then autoclaving the mixture at 120°C for 2 hours. As a further refinement, Smith et al. (152) separated estrone from estriol by the procedure of Cohen and Marrian (71) and compared the values obtained by their bioassay method with the colorimetric method for urinary estrogens published by the latter (71).

The results obtained by Smith et al. (152), using their own methods for the determination of the urinary estrogens in a normal menstrual cycle, compared very well with the results obtained using the Cohen and Marrian (71) methods. These authors found that throughout the menstrual cycle, estrone was excreted in larger amounts than estriol but that

the excretion of estriol was greater in the luteal phase than during menstruation or the period of follicle ripening (follicular phase). Smith et al. (152) also found two peaks in the excretion of urinary estrogens during the menstrual cycle. Maximum excretion of the estrogens was observed on the 14th to 16th days before menstruation. These authors found that 1.67 mg. of estrogens (expressed as estrone) was excreted throughout the normal menstrual cycle. Of the estrogens excreted, 0.99 mg. was found in the estrone fraction and 0.46 mg. was in the estriol fraction. At the time that these investigations were done, it was not known that estradiol-17β is a normal constituent of human non-pregnancy urine. Because estradiol-17β is ten times more potent biologically than estrone (154), the values found for urinary estrone and estriol in the normal menstrual cycle by Smith et al. (152) cannot be considered as accurate.

In another investigation of the estrogens excreted during the normal menstrual cycle, Smith and Smith (166) found evidence which suggested that estradiol-17β (called "x" estrogen at the time) was extracted in the estrone fraction in the Cohen and Marrian fractionation procedure (71). This finding explained the elevated biological activity which was observed in the estrone fraction as compared with the values obtained for this fraction with the colorimetric procedure of Cohen and Marrian (71).

Based on their observations of the excretion of urinary estrogens in the normal menstrual cycle, and from the results published by other investigators, Smith and Smith (166) proposed a theory to explain the variation in excretion of the urinary estrogens in the normal female. Pincus and Zahl (167) determined the urinary excretion of estrone and estriol under various experimental conditions following the administration of known amounts of either estrone, estradiol-17β or estriol to rabbits. They found that, a) estradiol-17β is converted into estrone by rabbits with intact ovaries and that this conversion is reversible; b) estrone is converted into estriol when the uterus is present and under ovarian control, this conversion being irreversible and greatly facilitated by luteal secretion and c) that the luteal hormone partially protects these three estrogens against destruction, thus permitting both utilization and excretion, there probably being no renal threshold. About this time, Venning and Browne (168) found that the urinary excretion of pregnandiol, a urinary metabolite of progesterone, is very high during the luteal phase of the menstrual cycle and falls to a low value 1 to 3 days before menstruation.

From the data cited above and from their own observations of the increased urinary excretion of estriol during the luteal phase of the menstrual cycle, Smith and Smith (166) proposed

the following theory concerning the metabolism of the estrogens during the normal menstrual cycle.

1. Estradiol-17β is convertible to estrone and the conversion is reversible.

2. Progesterone, acting through the uterus, brings about the conversion of estrone to estriol.

3. Progesterone partially protects the estrogens against destruction, thereby allowing greater utilization and excretion.

4. A deficiency of progesterone, therefore results in a) reduced conversion to estriol, b) reduced conversion of estradiol to estrone and c) a greater destruction of all estrogens.

This theory of the Smiths is based to a large extent on the levels of the estrogens excreted during the normal menstrual cycle. Since the methods used in the hydrolysis and extraction of the urine are the limiting factors which determine the accuracy with which the urinary estrogens can be measured, we will now proceed to discuss these methods.

A large number of methods for the hydrolysis and colorimetric estimation of urinary estrogens have been published in the last 15 years. These methods have recently been extensively reviewed by Van Bruggen (169), Marrian and Bauld (170) and Marrian (153). A review of these topics here,

would therefore be superfluous. However, a few of the more important methods pertaining to this investigation will be discussed.

It is generally agreed that the Smith and Smith (165) method of refluxing the urine with 15 volumes per cent HCl for 10 minutes does not effect a complete hydrolysis of the urinary estrogens. Marrian and Bauld (170) suggested that the time of refluxing should be extended to 60 minutes. These authors also recommended that during hydrolysis, the urine should not be in contact with the air, since it has been shown that under these conditions the oxygen in air destroys the estrogens (26).

In recent years, it has been found that enzymatic hydrolysis of the urine is a very effective method of splitting the estrogen conjugates and that this method yields very pure estrogen extracts. Buehler et al. (171) have found that glucuronidase prepared from E. coli can effectively hydrolyse the conjugated estrogens in the urine of pregnant women. These authors reported yields of total estrogen (determined biologically and colorimetrically) by enzymatic hydrolysis which in nearly every case were somewhat higher than those obtained by acid hydrolysis. Recently Beer et al. (172) administered estrone-16-C^{14} and 17β-estradiol-16-C^{14} to a human female, and the urinary estrogens were hydrolysed

both with acid and calf-spleen-β-glucuronidase. These authors found that acid hydrolysis yielded larger amounts of radioactive material extractible with ether but that fractionation of the urine after hydrolysis by these two procedures yielded fractions containing different amounts of C^{14}. Thus it is possible that acid hydrolysis may alter the estrogens.

Butenandt and Hofstetter (173), did not actually isolate estrone sulphate from human pregnancy urine, but they presented evidence which strongly suggested that estrone is excreted by the human as the sulphuric acid ester. Crepy (174) incubated estrone, estradiol-17β and estriol with liver slices and concluded that estradiol-17β and estriol were conjugated for the most part with glucuronic acid, whereas estrone seemed to be conjugated with another substance resembling sulphuric acid. These observations prompted Cohen and Bates (175) to hydrolyse human pregnancy urine enzymatically, with a sulphatase preparation from aspergillus oryzae. These authors found that sulphatase was 20% more efficient in splitting the estrogen conjugates than was acid hydrolysis.

Recently, Stimmel (176), using chromatography on alumina to purify the estrogens, and a modified Kober colorimetric method for their determination, measured the amounts of free, β-glucuronidase, phenolsulphatase and hydrochloric acid

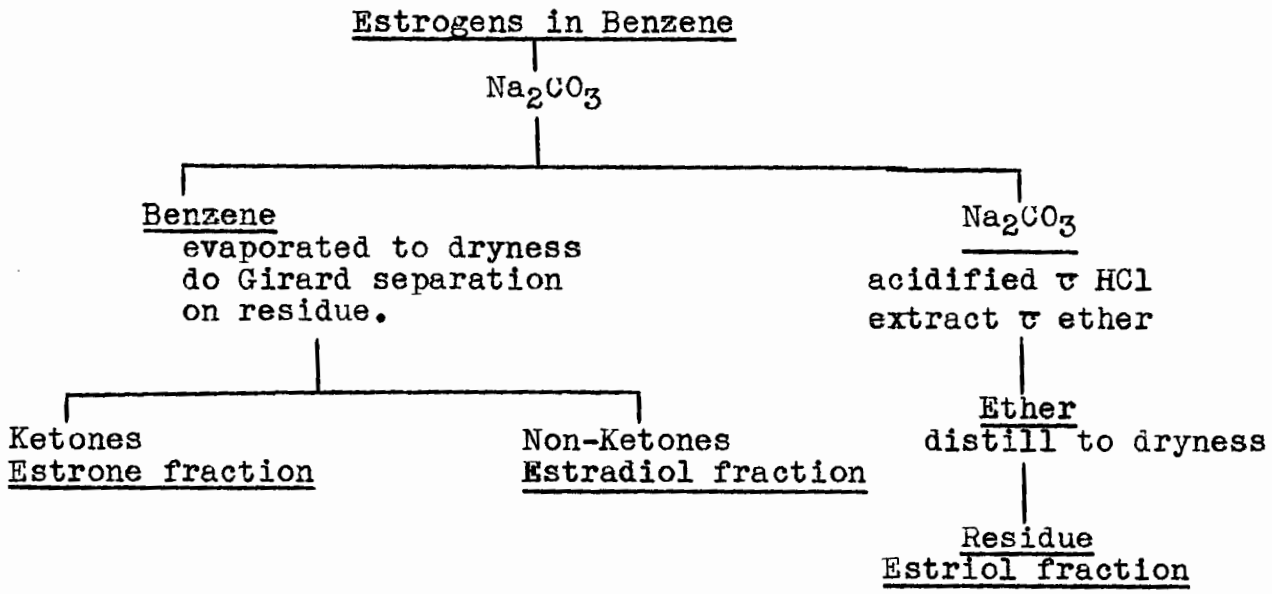
hydrolysable estrogens in human pregnancy urine. The results obtained using 24 hour samples of urine were as follows; a) only traces of free estrogens occur in the urine, b) the major portion of the conjugated estrogens (estrone, estradiol -17β and estriol) were β -glucuronidase hydrolysable, a small portion however, could only be hydrolysed with hydrochloric acid and c) phenolsulphatase only hydrolysed 20% of the total conjugated estrogens. From the data cited above it is evident that there are estrogen conjugates in the urine other than glucuronides or sulphuric acid esters, which can only be hydrolysed with hydrochloric acid. Although direct evidence of the nature of the estrogens conjugates in human non-pregnancy urine is lacking, it has been generally assumed that the estrogens are conjugated in this urine in the same way as in human pregnancy urine.

When it was established that aside from estrone and estriol, estradiol-17β is also present in human pregnancy urine, a number of investigators set out to develop solvent partition methods for the separation of these three urinary estrogens. The first advance in this direction was made by Mather (87) who investigated the distribution of estrone, estradiol-17β and estriol among imiscible solvent pairs and found that washing a benzene solution containing the three estrogens with 0.3 M Na_2CO_3 quantitatively removes estriol from estrone and estradiol-17β.

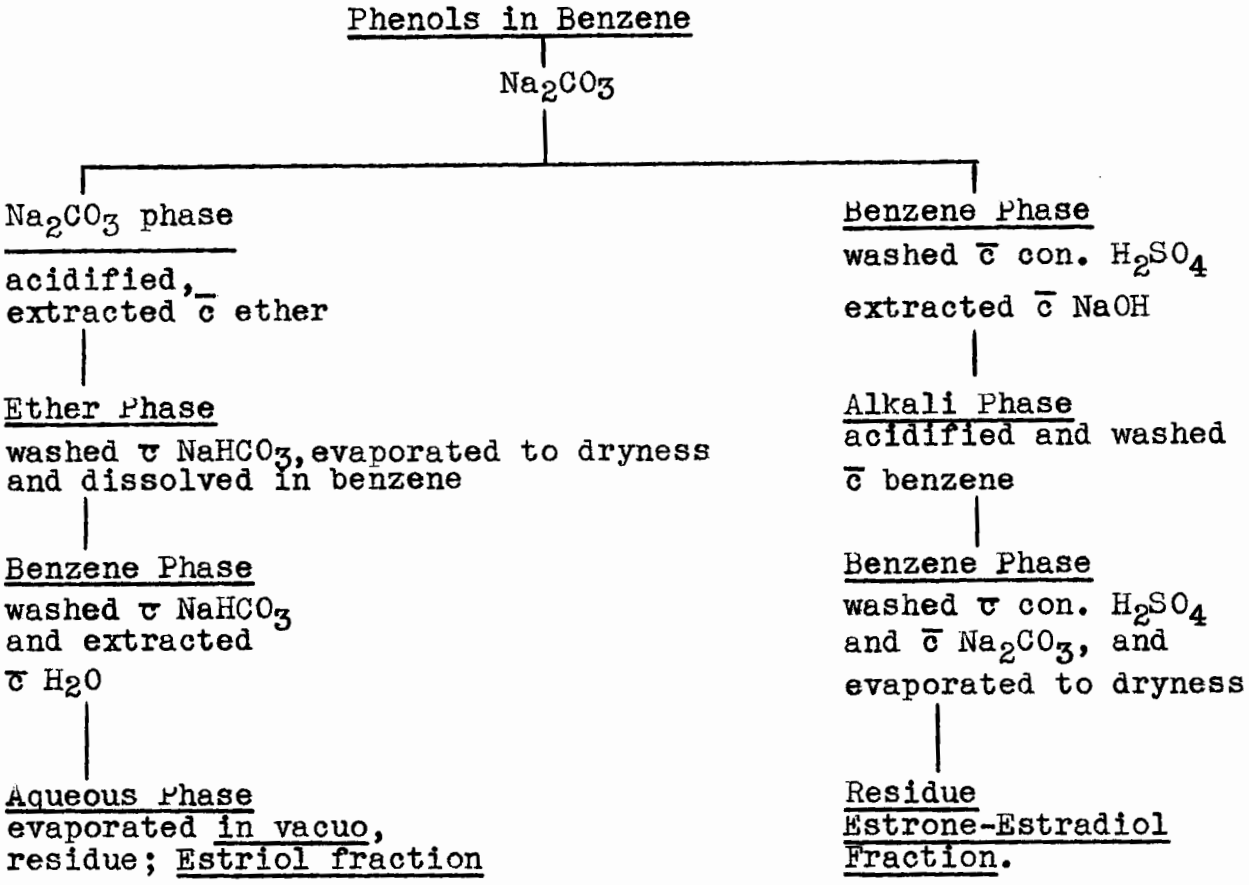
At the same time, Bachman and Pettit (88) published some data on the extraction and partition of estrogens added to human pregnancy urine, and the partition of crystalline estrogens added to ether and benzene solutions. These authors found that washing an ether solution containing the three estrogens with 9% $NaHCO_3$ does not remove any of the estrogens, whereas 9% Na_2CO_3 removes about 20% of the estriol present. Consequently, these authors used a 9% $NaHCO_3$ wash to remove organic acids from the ether extract of acid hydrolysed urine. Bachman and Pettit (88) also found that 0.3 M Na_2CO_3 will separate estriol from estrone and estradiol-17β when a benzene solution of the three estrogens is washed with this weak base. These authors showed that washing the estrone-estradiol fraction contained in benzene with concentrated sulphuric acid removes a lot of the pigments present in this fraction, without removing the estrogens.

In 1945, Pincus (177) published a procedure for the extraction and partition of estrogens which was essentially a modification of the procedure earlier published by Mather (87). However, Pincus (177) separated estrone from estradiol -17β with the use of a micro-Girard method. The fractionation procedures of Bachman and Pettit (88) and Pincus (177) are shown schematically below.

Pincus Modification of the Mather Partition.



Bachman and Pettit Partition



It is important to note that Pincus (177) employed a saturated solution of Na_2CO_3 to wash the initial ether extract of the hydrolysed urine, a procedure which, in view of Bachman and Pettit's work, results in a significant loss of estriol early in its handling.

Recently, Friedgood and Garst (178) have published the results of an extensive investigation into the application of ultraviolet spectroscopy to the determination of urinary

estrogens, but unfortunately they have limited their study to the fractionation of the crystalline hormones. These authors pointed out the inadequacies of the methods of extraction and assay of the estrogens excreted in human pregnancy urine, which had been previously published by Cohen and Marrian (71), Smith et al. (165) and Pincus et al. (179). In the light of the findings of Mather (87), Bachman and Pettit (88) and Pincus (177), it is evident that the Cohen and Marrian procedure (section A of this thesis) for the fractionation of urinary estrogens (71), which was adapted by Smith and Smith, does not give an accurate account of the estrogen titer in human urine. As a result, the estrogen content of human pregnancy and non-pregnancy urine has been reinvestigated ab initio in recent years, employing improved methods of hydrolysis, extraction and assay of the urinary hormones. In reviewing these methods, particular attention will be given to the estrogen content of human non-pregnancy urine.

In 1943, Jayle, Crepy and Judas (180) studied the estrogens excreted in the urine during the normal menstrual cycle of eight women, using their modified Kober method for the estimation of the estrogens. The curves for the excretion of the estrogens, obtained by these authors, were generally the same for the eight cycles studied. There was, a) a moderate initial

rise in estrogen excretion before menstruation, followed by a second peak at ovulation having a maximum of between 250 and 450 µg. of estrogens per litre of urine, and b) this was followed by a depression in estrogen excretion and the luteal phase was marked by a flattened peak which fell to about 100 µg. of estrogen per litre a few days before menstruation. These results are only qualitative since the colorimetric method used by these investigators is accurate for the determination of urines containing about 1000 µg. of estrogen per litre.

Stimmel (181) has recently devised a color correction equation for the Kober reagent in order to make allowance for the high readings given by the reagent, which are mainly due to non-specific absorbing impurities. He applied this method, as well as his alumina chromatography procedure (145), to the determination of the estrogens excreted during a normal menstrual cycle. However, Stimmel found that he could only measure the estrogens excreted during the mid-menstrual rise in the cycle, for which he obtained 23 µg. of estrone, 20 µg. of estradiol and 18 µg. of estriol per 48 hour samples of urine. During the rest of the menstrual cycle, the amounts of estrogens excreted were below the sensitivity of his method of assay.

In recent years, a number of refinements have been introduced into the measurement of urinary estrogens. In 1947, Bates and Cohen (182), Jailer (183) and Finkelstein, Hestrin

and Koch (184) simultaneously described methods for the quantitative estimation of estrogens using fluorimetry. The chemical basis of the reaction is similar to, if not identical with that of the Kober reaction. With this method of assay only 0.1-5 µg. of estrogens are required.

Jailer (185) applied the fluorometric method for the daily determination of estrone and estradiol excreted in the urine of 3 women with normal menstrual cycles. He used the Bachman and Pettit method (88) for the fractionation of the urine. The curves obtained for the urinary excretion of the estrogens during the menstrual cycle had the same shape as those published earlier by Gustavson et al. (163) who analysed the urinary estrogens by bioassay. There was a peak in the urinary excretion of the estrogens immediately preceding ovulation as judged by the abrupt rise in body temperature. Jailer (185) stated that he could not measure estriol by this method because of the interference from urinary chromogens, which did not separate from the estriol fraction by differential solubility.

Salter et al. (186) devised a fractionation procedure for the extraction of "estroids" from non-pregnancy female urine, which was a modification of the methods described by Bachman and Pettit (88) and Pincus (177). The "estroids" consisted mainly of estrone and estradiol and some estriol, and were expressed as estradiol equivalents. For the estimation of the

urinary estrogens these authors used a modified Kober reaction which they claimed to be capable of removing the brown interfering pigments usually encountered in this reaction. These investigators analysed the estrogens excreted in a normal menstrual cycle, but their results are not reliable since their methods were only capable of recovering 50% of the estrogens added to the urine.

Engel et al. (187) have recently developed a countercurrent distribution method for the separation of the estrogens. These investigators have applied the above method for the fractionation of the urine collected during the preovulatory and postovulatory phases of a normal menstrual cycle. They found 15 µg. of estrone in the first 14 days of the cycle and 12 µg. of estrone plus 34 µg. of estriol in the last 12 days of the cycle. No estriol was found in the preovulatory phase, and estradiol-17β was not detected throughout the cycle.

In 1952, Smith and Smith (188) published some results of recent investigations of the estrogens excreted during the normal menstrual cycle. The Smiths hydrolysed the urine by refluxing it for 10 minutes with 15 volumes per cent HCl and they extracted estriol out of benzene with 0.3 M Na_2CO_3, and estrone plus estradiol-17β out of ether with 0.1 N NaOH. Estrone was separated from estradiol by the use of Girard's reagent T. In this fractionation procedure, the Smiths found

that 10% of the estradiol entered the estriol fraction and they therefore corrected for it in the bioassay of the estriol fraction. The results obtained by the Smiths are tabulated below.

Fraction	Estrogens found µg./24 hours	Type of urine
Estradiol	5.6	Luteal phase
Estrone	50.0	
Estriol	95.0	
Estradiol	1.1	Follicular phase
Estrone	5.0	
Estriol	4.0	

From the discussion presented above, it is evident that a reliable method for the determination of the low estrogen titer present in normal female urine has not yet been evolved. Because of this situation, we endeavoured to develop a procedure employing paper chromatography for the determination of the estrogen content of normal female urine. The methods used in this investigation and the preliminary results obtained will now be discussed.

The theory of Smith and Smith (166), concerning the metabolism of estrogens in the human female, is based largely

on the relative amounts of estrone, estradiol-17β and estriol excreted in the urine. It was therefore assumed that an accurate determination of the three estrogens in the luteal and follicular phases of the normal menstrual cycle, with the use of paper chromatography, would either strengthen this theory or disprove it.

The urine analysed in this investigation was donated by a young woman, 20 years of age, who had a normal menstrual history since puberty at the age of 13. This young woman had a regular menstrual cycle lasting 28 to 31 days. The time of ovulation was determined by observing the basal body temperatures taken rectally on awakening each morning. Buxton and Atkinson (189) have used this method extensively and they found that there is a 0.6-1.0^{o}F rise in basal body temperature at ovulation. The basal body temperature curves obtained throughout the menstrual cycle were similar in shape to the curves published in the literature (189). The curve obtained during the second menstrual cycle analysed in this investigation, is shown in Figure 21, p. 161.

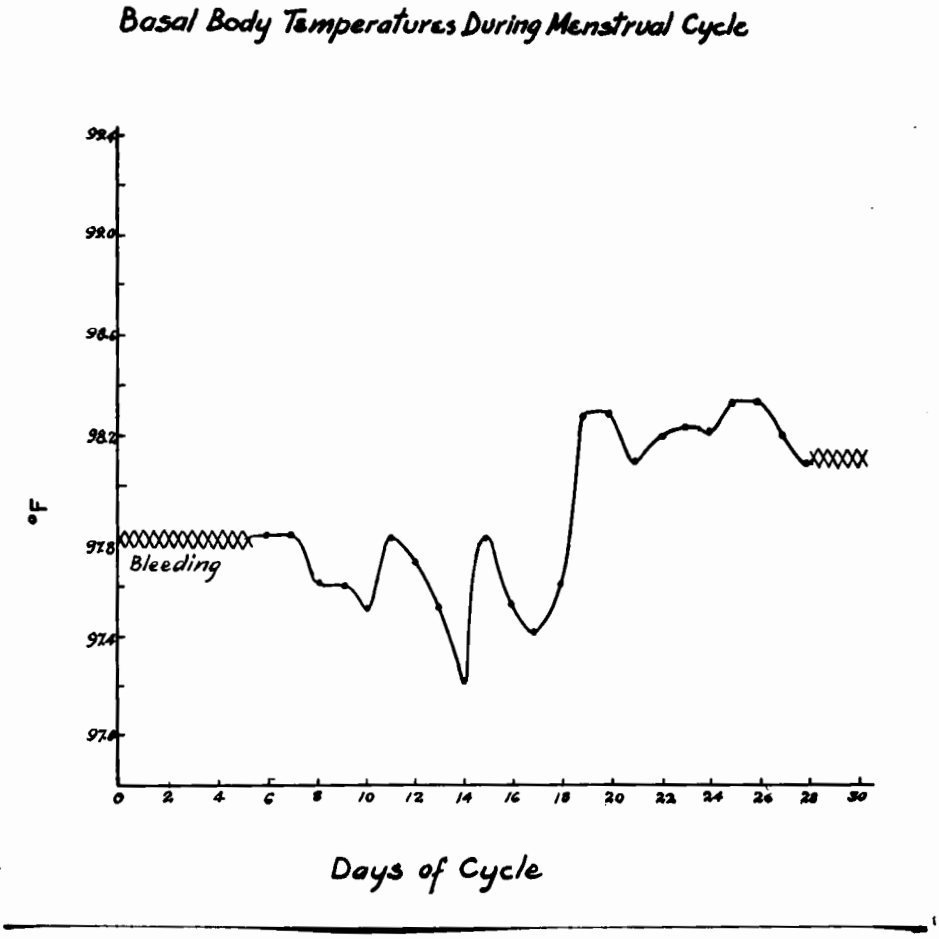

Basal Body Temperatures During Menstrual Cycle
°F
98.4
98.0
98.6
98.2
97.8
97.4
97.0
Bleeding
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Days of Cycle

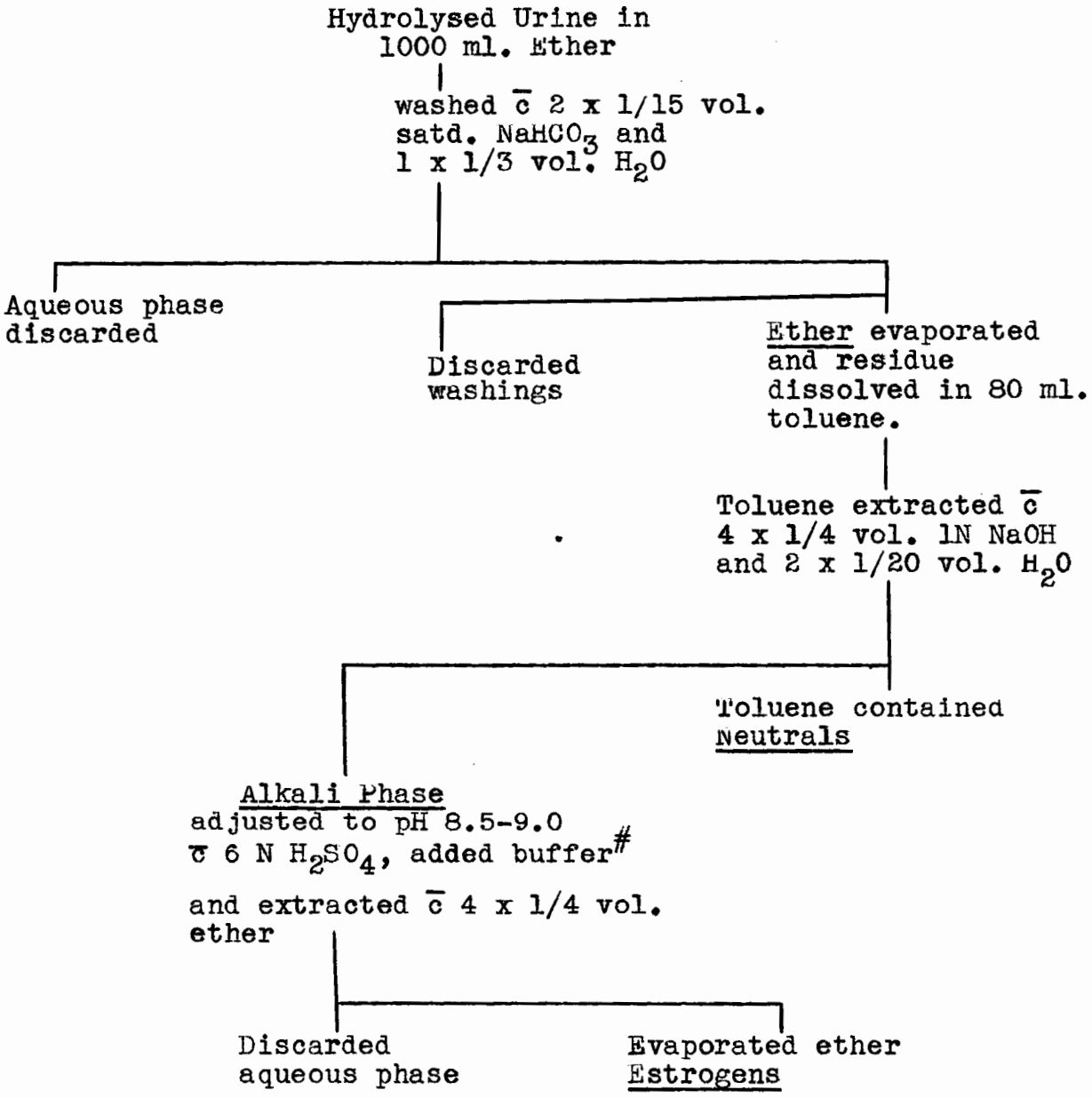
Figure 21.

In figure 21, it is seen that there is a rise in body temperature on day 15, which indicated that this day was the day of ovulation. The urine collected on days 5 to 13 and days 17 to 28 were designated as the preovulatory and post-ovulatory urines respectively. Two menstrual cycles were examined in this investigation.

The urine collected during the two menstrual cycles was hydrolysed by two methods. In the first menstrual cycle examined, the urine was hydrolysed by refluxing it with 15 volumes % HCl for 10 minutes. This procedure has been extensively used by the Smiths (188), but Marrian and Bauld (170) and a number of other investigators claim that the period of reflux is too short to effect a complete hydrolysis of the estrogen conjugates. In the second menstrual cycle examined, the urine was hydrolysed by refluxing it with 15 volumes % HCl for 60 minutes. This second method of hydrolysis has been shown to be superior to the 10 minute hydrolysis (170). The two methods of hydrolysis were used in order to determine for ourselves their relative merits.

The acid hydrolysed urine corresponding to the pre-and postovulatory phases of the menstrual cycle was fractionated by the method described by Engel et al. (187). This procedure has been found to give a 90% recovery of estrone, estradiol-17β and estriol added to normal female urine.

The fractionation scheme as adapted for this investigation is outlined below.



The buffer solution was prepared by mixing 20 volumes of saturated $KHCO_3$ with 1 volume saturated K_2CO_3 and 2 ml. of buffer per 100 ml. of solution was used.

Engel et al. (187) found that a complete recovery of estrone, estradiol-17β and estriol is obtained by ether extraction of the alkaline extracts containing these estrogens, after adjusting the pH of the solution to 9 $\pm$ 0.5. These authors also found that extracting the estrogens at pH 9 removed a lot of colored pigments which interfere with the subsequent determination of the estrogens.

When this investigation was started a few methods were available for the separation of estrone, estradiol-17β and estriol by paper chromatography (103,104,190,191). However, all these methods utilized paper strips which had been treated in various ways in order to facilitate the separation of the estrogens. The difficulties encountered in the use of pretreated paper strips were discussed in section A of this thesis. Dr. K.Savard, of the Worcester Foundation (192), suggested that the chromatography system, o-dichlorobenzene-formamide-methanol (1:1) was capable of separating the natural estrogens. In trial experiments with this method of chromatography using pure estrone, estradiol-17β and estriol, the R_f values obtained in 3.75 hours were 17.7, 5.0 and 0.0 respectively. The procedures of chromatography used were essentially those described by Burton et al. (105).

While this investigation was in progress, three methods were published for the separation of microgram quantities of

the natural estrogens. Bitman and Sykes (193) separated estrone, estradiol-17β and estriol by chromatography of the estrogens on Celite previously impregnated with 2.3 N NaOH. This method is capable of separating 2-10 µg. amounts of the three estrogens. Heusghem (194) found that paper strips saturated with ammonium hydroxide as the stationary phase and a mixture of 1 part of chloroform, 9 parts of benzene and 1 part of N ammonium hydroxide as the mobile phase, can readily separate the three natural estrogens. Axelrod (106) published a paper chromatography method for the separation of the natural estrogens using the system o-dichlorobenzene-formamide-methanol (1:1). With this method, Axelrod was able to demonstrate the presence of estrone, estradiol-17β and estriol in a 48 hour sample of normal female urine.

In a preliminary experiment using normal female urine, the methods described by Axelrod (106) gave very promising results, and they will therefore be dealt with here in some detail. Axelrod used formamide-methanol (1:1) as a stationary phase in all his experiments, but he devised different mobile phases for a variety of uses. With methylcyclohexane as a mobile phase, the estrogens remain at the origin, but the impurities extracted with the estrogens travel away from the origin. Methylene chloride when used as a mobile phase can separate estriol from the polar impurities at the origin in 10 hours of chromatography.

To identify the estrogens on the paper strips, Axelrod (106) devised a large number of spot tests which yield characteristic colors and distinct ultraviolet fluorescence.

For the quantitative determination of the estrogens, Axelrod (106) eluted them from the paper strips with absolute methanol and analysed their ultraviolet absorption at 280-282 mμ. As a blank in these determinations, a methanol extract of a paper strip equal in area to the estrogen spot eluted was used. Using this method, quantitative recoveries of the estrogens were reported. As an aid in the identification of the estrogens, Axelrod found that in sulphuric acid, estrone, estradiol-17β and estriol gave characteristic ultraviolet absorption spectra.

In this investigation, the procedure of Axelrod (106), described above, was used throughout. The urine collected daily from a normal female, was hydrolysed by refluxing it for either 10 minutes or 60 minutes with 15 volumes % HCl under a cover of toluene. The toluene served to protect the estrogens from destruction by the oxygen of the air. Cohen and Bates (175) found that when using a cover of toluene during acid hydrolysis of the urine larger amounts of extractible estrogens were obtained. The acid hydrolysed urine was then extracted thrice with 1/2 volumes of toluene and the toluene extracts were reduced to dryness. This procedure was repeated with all

the urine collected and the pooled extracts from the pre and postovulatory phases were fractionated by the procedure of Engel et al. (187) which was previously described.

After processing the urine collected during two menstrual cycles, four urinary fractions were obtained; two preovulatory phase fractions and two postovulatory phase fractions. The first pre-and postovulatory fractions were hydrolysed for 10 minutes and the second pre-and postovulatory fractions were hydrolysed for 60 minutes. The procedure used to separate the estrogens contained in these fractions is as follows. A portion of each fraction (ca. 50 mg.) was first chromatographed in the system methylcyclohexane-formamide-methanol (1:1) using a wide paper strip (13-15 cm.). The estrogens were then eluted from the origin of this paper strip and were chromatographed with the system o-dichlorobenzene-formamide-methanol (1:1), using a paper strip 3-5 cm. wide. In this latter system a number of distinct areas were usually obtained and these were eluted and rechromatographed in the system o-dichlorobenzene-formamide-methanol (1:1).

The areas thus obtained were now eluted with absolute methanol along with an equal area of a paper strip run as a blank. The methanol eluates were tested in the ultraviolet at 280-281 mμ. using the methanol eluates of the blank paper strips as a solvent blank. After determining the concentration

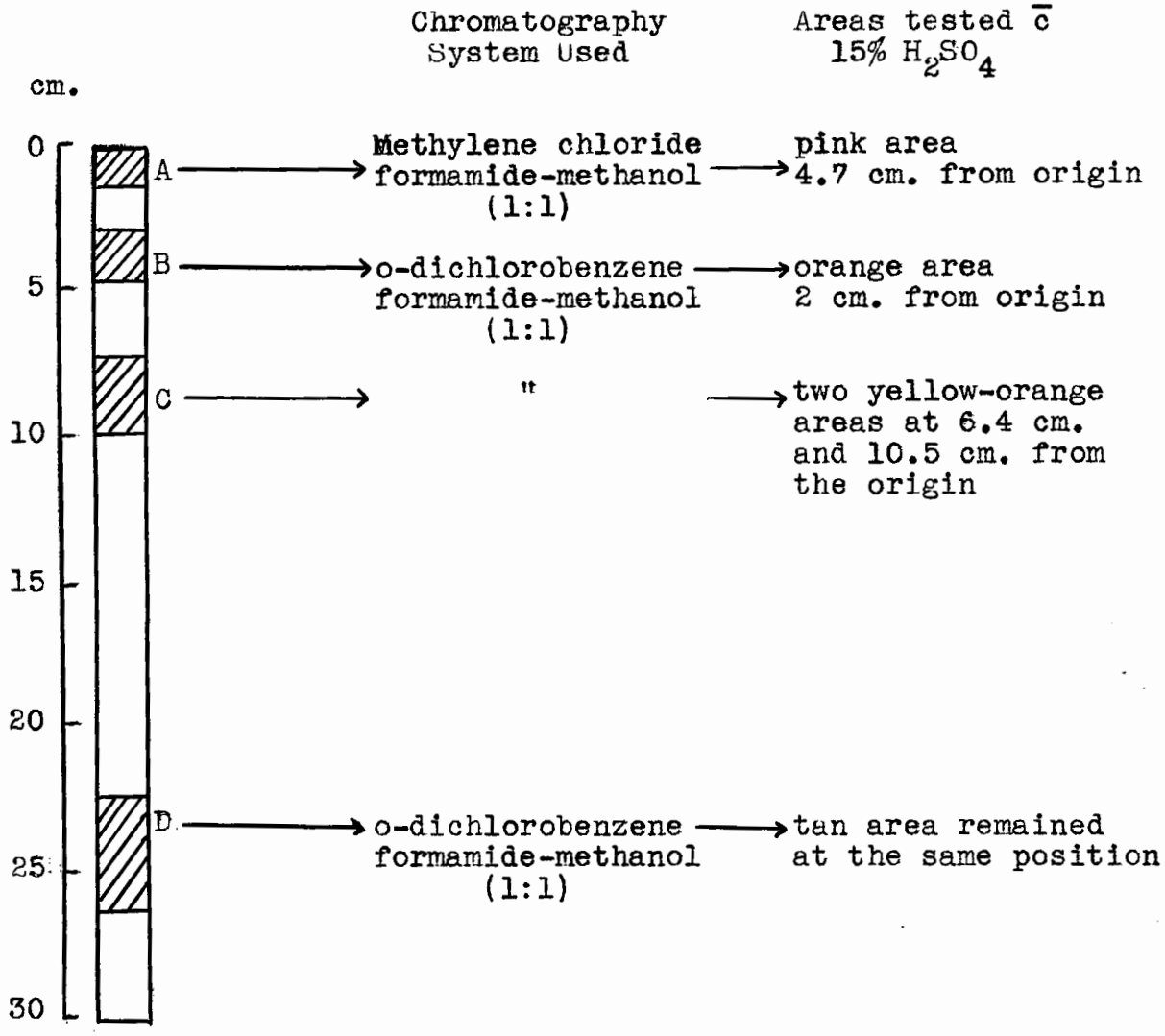
of the estrogen, an aliquot (80-90 µg.) was taken to dryness and the residue was used to find its ultraviolet spectrum in sulphuric acid, by the method of Zaffaroni (91). Since estriol stays at the origin in the two chromatography systems described above, it was chromatographed separately in the system methylene chloride-formamide-methanol (1:1) for 12 hours in order to separate it from the polar impurities at the origin. The estriol was then eluted and analysed by the methods described above.

In order to locate the estrogens on the paper strips, a 2 mm. portion was cut out and immersed in 15% fuming sulphuric acid. The estriol area gave a pink color in this test, while estradiol-17β turned orange-yellow and estrone gave an orange-tan color. This method had a sensitivity of 5 µg. per cm.^{2} for the three estrogens. After the colors produced by the estrogens in sulphuric acid were observed, the strips were examined for their fluorescence using an ultraviolet light source. Estriol gave a pink-green fluorescence in sulphuric acid, while estradiol-17β and estrone gave a greenish-yellow fluorescence. The fluorescence reaction had a sensitivity of about 1 µg. per cm.^{2} for estrone, and estradiol-17β, and 5 µg. per cm.^{2} for estriol. In every chromatogram run, the strips were analysed by this sulphuric acid method.

The estrogen extract of the first preovulatory phase weighed 275 mg. This extract contained the estrogens extracted from the urine collected on the first 10 days following menstruation. A 27.5 mg. portion of this extract was chromatographed on 3 cm. wide paper strips as outlined above, and the areas thus obtained were eluted and analysed by the methods previously described. It was found that the impurities present in the extracts interfered with all the methods used to analyse the estrogens. These results indicated that these extracts would have to be purified prior to separations of the estrogens by paper chromatography.

In this laboratory, it was found that crude urinary estrogen extracts can be purified by chromatography on Celite-magnesium silicate (1:1). Therefore, 193 mg. of the first preovulatory fraction (corresponding to 7 days of the cycle) was adsorbed from a benzene solution on a column of 4 g. of Celite-magnesium silicate (1:1), and the estrogens were eluted with, a) 150 ml. of benzene, b) 300 ml. of benzene, c) 200 ml. of 10% ether-benzene and d) 200 ml. of 25% ether-benzene. Fraction a) removed a lot of oily material but in fractions b) and c) red pigments were eluted which usually travel with the estrogens. Fraction d) removed a lot of brown colored material from the top of the column. After the column was eluted as described above, a large quantity of dark brown pigments remained at the top of the column.

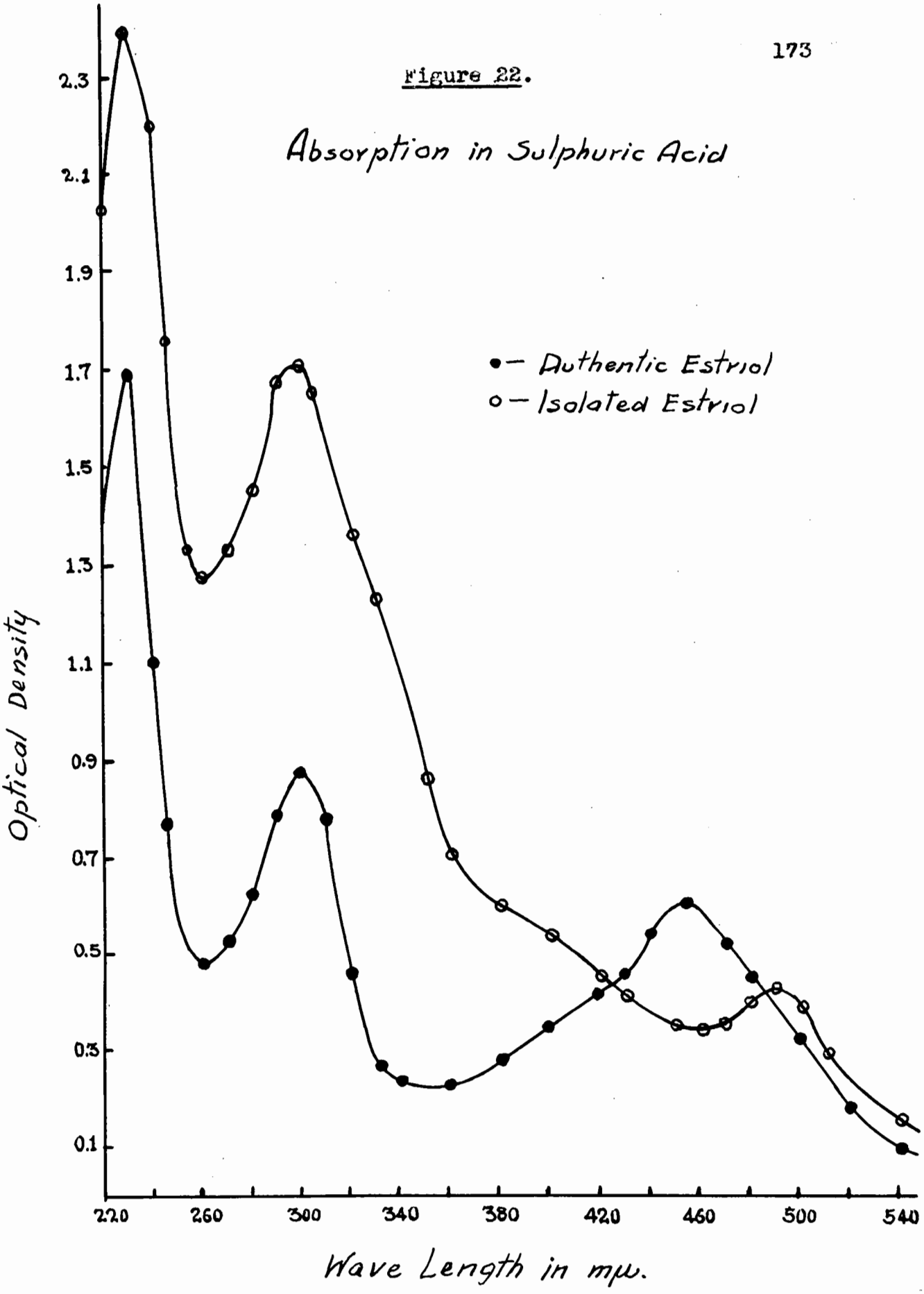
Fractions b) and c) when combined weighed 25.27 mg. and they were chromatographed on a paper strip 10 cm. wide, for 24 hours, in the system methylcyclohexane-formamide-methanol (1:1). At the end of this chromatogram, the material eluted from the origin weighed 5.76 mg. which indicated that methylcyclohexane removed a lot of impurities from the paper strip. This purified fraction was now chromatographed on a paper strip 3 cm. wide, for 6 hours, with the system o-dichlorobenzene-formamide-methanol (1:1). On testing a narrow portion of this paper strip in 15% fuming sulphuric acid, 4 distinct areas were noted as shown diagramatically below. These 4 areas were eluted with absolute methanol and chromatographed in the systems indicated below.



Area A was eluted from the paper strip with absolute methanol, after chromatography in methylene chloride-formamide-methanol (1:1) for 12 hours. From the extinction coefficient of this methanol solution in the ultraviolet at 280 mμ., it was calculated that there was originally 158 μg. of estriol present in fractions b) and c) from the Celite-magnesium silicate column. From this result it was calculated that the

amount of estriol excreted in the first preovulatory phase was 22.5 µg./24 hours. The ultraviolet spectrum in sulphuric acid of this estriol corresponded closely to the spectrum given by authentic estriol (fig. 22, p. 173). In Fig. 22, it is seen that in the case of the isolated estriol, the absorption peak at 454 mu. was shifted to 490 mµ., indicating that the estriol was not entirely pure.

Figure 22.


Absorption in Sulphuric Acid
● — Authentic Estriol
○ — Isolated Estriol
Optical Density
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
220
260
300
340
380
420
460
500
540
Wave Length in mμ.

When areas B, C, and D were rechromatographed, and the spots obtained were eluted with absolute methanol, and examined spectroscopically as described above, there was no indication of the presence of any estrogens. The material eluted in fraction d) from the Celite-magnesium silicate column (25% ether-benzene) was processed in the same manner as described for fractions b) and c). Here, however, no estrogens could be detected by paper chromatography. This result indicated that all estrogens were eluted from the Celite-magnesium silicate column with benzene and 10% ether-benzene. Since estriol was the only estrogen that could be readily identified in the urinary extract of the first preovulatory phase, it was decided to limit this investigation to isolation of this hormone from the remaining extracts to be processed.

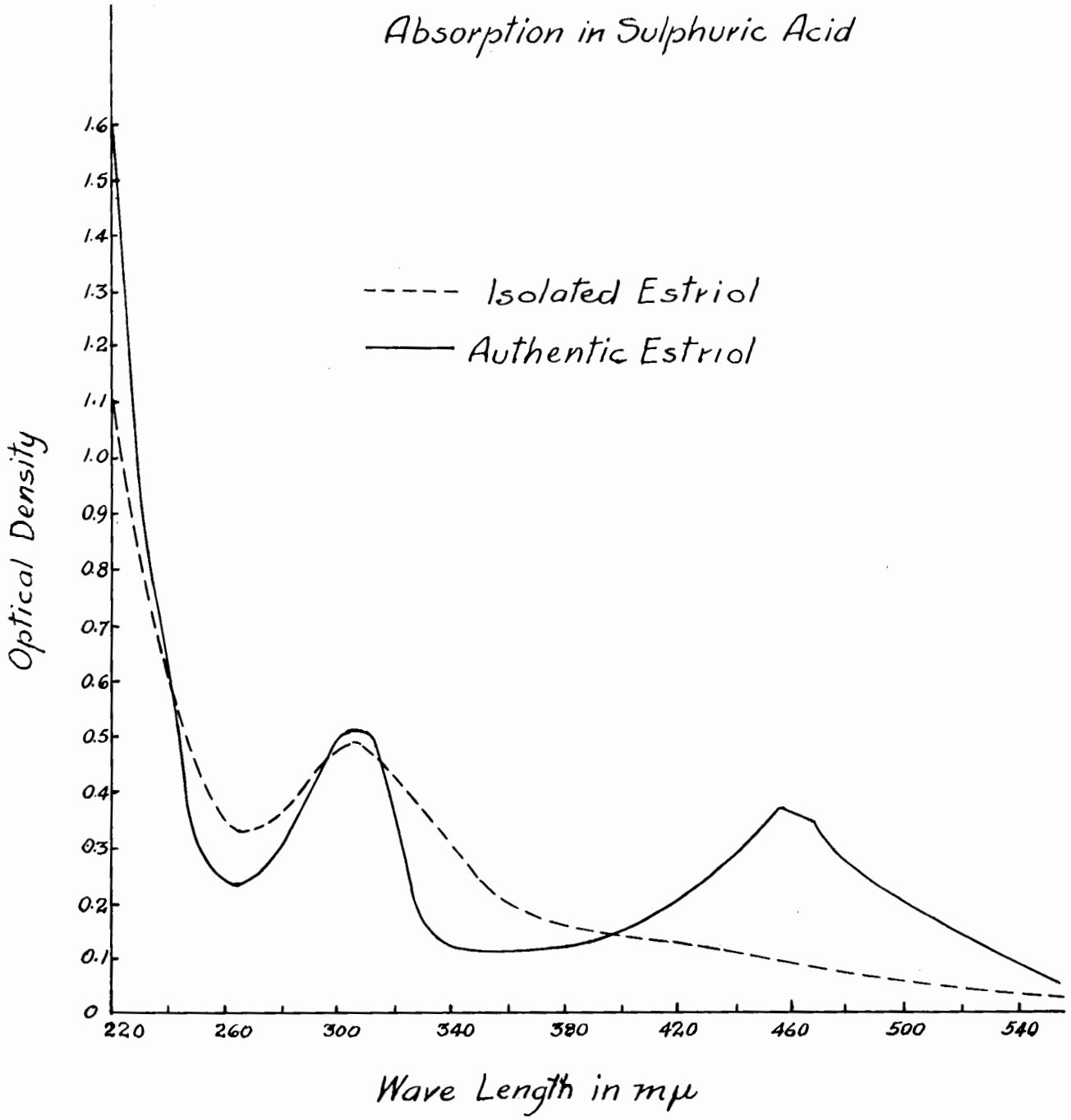
The estrogen extracts from the first postovulatory phase, the second preovulatory phase and the second postovulatory phase were chromatographed on Celite-magnesium silicate (1:1). In these chromatograms, all the eluates were pooled and reduced to dryness. From the purified extracts thus obtained, 40 to 50 µg. of material were removed for paper chromatography. The methods of chromatography were identical to those used in separating estriol from the first preovulatory phase.

In every instance, the amount of estriol isolated was determined from the extinction coefficient in the ultraviolet

at 281 mμ., and then a portion of the estriol was used to obtain its ultraviolet spectrum in sulphuric acid. The ultraviolet spectrum of the isolated estriol, taken in absolute methanol, was identical to the curve published by Friedgood and Garst (178). However, the ultraviolet spectrum of the isolated estriol in sulphuric acid did not correspond closely to the spectrum given by authentic estriol in the region 400-520 mμ. This is demonstrated in Fig. 23, p. 176, which represents the absorption in sulphuric acid of estriol isolated from the second postovulatory fraction.

Similar absorption spectra were given by the estriol isolated from the 2nd preovulatory fraction and the 1st postovulatory fraction.

Figure 23.


Absorption in Sulphuric Acid
----- Isolated Estriol
—— Authentic Estriol
Optical Density
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220
260
300
340
380
420
460
500
540
Wave Length in mμ

The non-correspondence of the two curves is probably due to some polar impurities in the urine, which travel with estriol on the paper strips. It is important to note that the brown color interfering with the Kober reaction on urinary estrogens shows considerable absorption in the region 420-520 mμ. (153). Also, Jailer (185) encountered great difficulty in determining urinary estriol when he attempted to measure its fluorimetry in 60 to 70% sulphuric acid.

Rechromatography of the isolated estriol in the system methylene chloride-formamide-methanol (1:1) did not remove the material interfering with the absorption of estriol in sulphuric acid. It is therefore evident that a superior method will have to be developed for the purification of the estrogen extracts before separating the estrogens by paper chromatography. It may well be that passage of the crude estrogen extracts through amberlite IR4B, as described by Bauld (195), will separate the interfering pigments from the estrogens.

The amounts of estriol present in the urine collected during two menstrual cycles are shown below (page 178.)

Nature of Urine	Method of Hydrolysis	Estriol found µg./24 hours.
1st preovulatory	10 min. reflux with 15 vol. % HCl	22
1st postovulatory		127
2nd preovulatory	60 min. reflux with 15 vol. % HCl	195
2nd postovulatory		92

From the results shown above, it can be seen that in the first menstrual cycle examined, more estriol was excreted in the luteal phase than in the follicular phase. In the second menstrual cycle examined, the reverse situation was found. Generally speaking it can be said that 60 minutes hydrolysis with 15 vol. % HCl yielded larger amounts of urinary estriol than the 10 minute hydrolysis. The low value for estriol found in the 2nd postovulatory urine may be due to the fact that this fraction had to be chromatographed twice on Celite-magnesium silicate (1:1) in order to purify it.

Axelrod (106) was able to demonstrate the presence of estrone, estradiol-17β and estriol in a 48 hour sample of normal female urine. However, he hydrolysed the urine by acidifying it to pH 1 with HCl, and he then separated the

free estrogens by continuous ether extraction. This mild treatment of the urine did not hydrolyse all the estrogen conjugates, but it yielded an extract which was relatively free of impurities. From such an extract, Axelrod was successful in isolating the three natural estrogens.

The results obtained in this investigation indicate that there is more estriol excreted in normal female urine than had been previously found by other investigators. However, these results do not allow an evaluation of the theory of Smith and Smith (166) concerning the metabolism of the estrogens in the normal female.

2. Experimental.

The urine used in this investigation was collected from a 20 year old woman throughout two menstrual cycles. This young woman had a normal menstrual history since puberty at the age of 13, and the length of her cycles was 28 to 31 days.

Collection and Hydrolysis of the Urine.

The urine was collected in glass bottles containing about 200 ml. of toluene. When it was not possible to hydrolyse the urine within 24 hours after collection, the urine was stored in the cold with a cover of toluene. Each 24 hour specimen of urine was covered with an equal volume of toluene and the mixture was heated until it refluxed. Then 15 vol. % conc. HCl acid was added to the mixture through the condenser, thus making it possible to time accurately the period of hydrolysis. The urine collected during the first menstrual cycle was hydrolysed for 10 minutes, and the urine from the second menstrual cycle was hydrolysed for 60 minutes.

The hydrolysed urine was immediately cooled and the toluene layer was separated. Then the hydrolysed urine was extracted three times with half volumes of toluene. Emulsions were encountered during the toluene extraction and these were broken by filtration. The total toluene extract was reduced to a small volume by distillation of the toluene on the steam bath

under reduced pressure. This toluene extract, corresponding to a 24 hour specimen of urine, was stored in the cold.

Basal Body Temperatures.

During the two menstrual cycles in which the urine was examined, the day of ovulation was determined by observing the basal body temperatures throughout the cycle. Rectal temperatures were taken on awakening each morning, and the temperatures obtained were plotted as shown in the previous section. In the first menstrual cycle there was a rise in body temperature on the 16th day, and the cycle was 28 days. Therefore the toluene extracts of the acid hydrolysed urine from day 5 to 14 were combined and were designated as the 1st preovulatory fraction, while the toluene extracts from the last 10 days were designated as the 1st postovulatory fraction. Menstrual bleeding occurred during the first 5 days of the cycle.

The duration of the second menstrual cycle was 28 days, and the rise in body temperature occurred on the 15th day. The toluene extracts from the first 10 days following menstruation were combined and designated as the 2nd preovulatory fraction; the toluene extracts from the last 10 days of the cycle were combined and designated as the 2nd postovulatory fraction.

Extraction of the Urine.

The toluene extracts of the four fractions described above were reduced to dryness in vacuo on the steam bath and the residues were dissolved in 1000 ml. of peroxide free ether. Then the ether solutions were extracted by the method of Engel et al. (187), which was described in detail in the previous section. This extraction procedure yielded the estrogen extracts which were dried in vacuo and weighed.

Fraction	Weight mg.
1st Preovulatory	275
1st Postovulatory	361
2nd Preovulatory	283
2nd Postovulatory	902

Paper Chromatography.

The methods of chromatography used were essentially those described by Burton, Zaffaroni and Keutman (105) and Axelrod (106), except for some minor innovations.

Chromatographic Apparatus.

Three chromatography chambers were used. Each of these consisted of a Mallinkrodt 5 lb. ether can, the top of which was removed; a shelf was welded 1/3 of the distance across

the diameter. The chambers were covered with glass plates, 15 inches square, and made air tight by sealing the outside edge of the chamber to the glass plate with plasticine. Porcelain-enameled sterilizing trays, 8 x 2 1/2 x 1 3/8 inches, were used as reservoir troughs. Rectangular glass plates 7 x 3 3/8 inches, ground along one long edge, were used to support the paper strips in the troughs. A second glass plate 7 x 3 3/8 inches, was used as cover plates for the paper strips.

Solvents.

The solvents used were all distilled in a glass apparatus; these were: formamide, o-dichlorobenzene, methylene chloride, methyloyclohexane, and absolute methanol.

Filter Paper.

Whatman No. 1 paper, supplied in sheets $18\frac{1}{4}$ x $22\frac{1}{2}$ inches, was used. The filter paper was washed with water and 95% redistilled ethanol respectively, and then air dried. For chromatography, the filter paper was cut into strips 42 cm. long and 1 cm. wide with a Stadie blade. The starting line was ruled 12 cm. from the upper edge in each case. The filter paper used to blot the paper strips was also washed and dried as described above.

Color Reactions.

To locate the estrogens on the paper strips, 15% fuming sulphuric acid was used throughout. The strips, immersed in sulphuric acid, were observed for fluorescence using an ultraviolet lamp, model SL 3600, obtained from the Ultra-Violet Products, Inc., South Pasadena, Calif.

After each chromatogram was dried at 50°C for 12 hours, narrow strips, 2 cm. in width, were cut from the middle of the chromatogram and treated with sulphuric acid in the following manner: the fuming sulphuric acid was pipetted along the length of a glass plate and the strip was immersed in the acid with the aid of a glass rod. The colors developed in 1 minute were noted, and then the strip was observed in the dark under the ultraviolet lamp for fluorescence. The colors and fluorescence given by pure estrogens are tabulated below.

	Color	Fluorescence
Estrone	Orange-tan	Yellow
Estradiol-17β	Orange-yellow	Green
Estriol	Rose-tan	Pink-green

The sensitivity of this color reaction for the three estrogens was 5 μg./cm.^{2}; the sensitivity of the fluorescence reaction was 1 μg./cm.^{2} for estrone and estradiol-17β , and 5 μg./cm^2 for estriol.

Chromatography Systems.

The chromatography systems used in this investigation were those described by Axelrod (106). o-Dichlorobenzene-formamide-methanol (1:1) separated the estrogens in 6 hours of chromatography. The system methylene chloride-formamide-methanol (1:1) separated the estriol from highly polar pigments present at the starting line (in 10 hours) and thereby purified the compound. Chromatography with the system methylcyclohexane-formamide-methanol (1:1) for 24 hours removed a large quantity of the interfering pigments present in the urine and did not move the estrogens from the starting line.

Methods.

The chromatography chambers were lined with filter paper, and the papers were thoroughly wetted with either methylcyclohexane, o-dichlorobenzene or methylene chloride saturated with formamide respectively. Sufficient excess solvent was placed in the bottom of the chambers to keep the lining paper wet, thus ensuring saturation of the atmosphere with respect to the volatile solvent. For all solvent systems used, the filter paper strips of appropriate size were first dipped into a solution of 1 part of formamide and 1 part of absolute methanol (by volume) and then blotted between filter papers.

In trial experiments with pure estrogens, standard solutions

were used which contained 52 µg. of estrone/ml., 78 µg. of estradiol-17β/ml., and 68 µg. of estriol/ml. respectively. A 0.5 ml. aliquot of the standard solution was carefully reduced to dryness in a 3 ml. centrifuge tube at 60°C, with the aid of nitrogen. To each residue was added two drops of absolute methanol, and the solution thus obtained was applied to the paper strips. Mixtures of the three estrogens were prepared in the same way.

When the urinary extracts were chromatographed, known aliquots were reduced to dryness in a 3 ml. centrifuge tube and dissolved in a minimum volume of absolute methanol.

The steroid solutions were applied with a capillary tube to the starting line of the paper strips and an additional drop of methanol was added to the centrifuge tube to ensure a complete transfer of the estrogens. A stream of nitrogen was used intermittently to evaporate the methanol quickly and thus limit the diameter of the area of application to 0.5 cm. or less. After the steroid solution was applied to the paper strip, the strip was suspended from the glass plate in the solvent trough and held in place with a small evaporating glass. A maximum of 8 paper strips were chromatographed at one time. When all the paper strips were suspended in the chromatography chamber, the uppermost edge of each strip was carefully aligned with the non-ground long edge of the glass plate, and a second glass plate was placed on top of the strips.

The chromatography chamber was then sealed with plasticine, and the paper strips were allowed to equilibrate with the mobile phase at the bottom of the chamber for at least 1 hour. Then 300 ml. of the mobile phase was added to the trough through a ground hole in the glass cover.

The period of development of the estrogens varied with the mobile phase used. For methylcyclohexane, it was 24 hours; for o-dichlorobenzene, it was 6 hours; and for methylene chloride, it was 10 hours. At the end of the period of development, the paper strips were removed from the chromatography chamber and dried at 50°C for 12 hours. The dry strips were then treated with fuming sulphuric acid to detect the positions of the estrogens.

The procedure described above was used with all the solvent systems in this investigation. After chromatography in the system o-dichlorobenzene-formamide-methanol (1:1), the undeveloped sections of the chromatograms containing the estrogens were eluted with absolute methanol. The methanol solutions were reduced to dryness and the residues were rechromatographed to purify the compounds. The estrogens were again eluted with absolute methanol and their absorption maxima were determined at 280 to 282 mμ., using a Beckman ultraviolet spectrophotometer. As a solvent blank in these determinations, a methanol extract was prepared of a control strip similar in area to each of the

estrogen areas on the paper strips. This procedure compensated for the background material from the paper chromatograms containing the estrogens. From the extinction coefficient thus determined, it was possible to calculate the quantity of estrogen eluted from the paper strips. An aliquot of the estrogen containing 80-90 µg. was removed and brought to dryness at 60°C, with the aid of a stream of nitrogen. The residue was dissolved in 3 ml. of conc. H_2SO_4 and the solution was allowed to stand at room temperature for 2 hours. Then the absorption spectrum was determined at wave-lengths from 220 to 600 mµ.

After chromatography of the estrogens in the system o-dichlorobenzene-formamide-methanol (1:1), the area corresponding to estriol was eluted with absolute methanol; the methanol solution was reduced to dryness and the residue was chromatographed in the system methylene chloride-formamide-methanol (1:1). In this system, estriol separated from the highly polar pigments present at the origin, and it was eluted with absolute methanol. This methanol solution was then used to determine the quantity of estriol present, and for the determination of the ultraviolet spectrum of estriol in sulphuric acid.

In the final calculation of the quantities of the estrogens present in the urinary extracts, account was taken of the losses due to the strips cut from the chromatograms for identification purposes.

Paper Chromatography of the Pure Estrogens.

Using 1 cm. wide paper strips, 58 µg. of estrone, 39 µg. of estradiol-17β and 54 µg. of estriol were chromatographed in the system o-dichlorobenzene-formamide-methanol (1:1) for 6 hours. In this period of time estriol remained at the origin and estrone and estradiol-17β traveled 6 cm. and 22 cm. respectively. The estrogens were detected on the paper strips by their color and fluorescence in fuming sulphuric acid.

Attempt to Separate the Estrogens of the 1st Preovulatory Fraction.

The estrogen extract of the first preovulatory phase weighed 275 mg. and corresponded to the urine collected during 10 days of the cycle. A 5.5 mg. portion of this extract was chromatographed on a 3 cm. wide paper strip in the system methylcyclohexane-formamide-methanol (1:1) for 24 hours. The material left at the origin after chromatography was eluted with absolute methanol and chromatographed in the system o-dichlorobenzene-formamide-methanol (1:1), for 6 hours, using a 3 cm. wide paper strip. In this chromatogram, three distinct spots were observed with the fuming sulphuric acid color reaction. The three spots were at 6.1 cm., 14.75 cm., and 27 cm., on the paper strip. These spots contained a lot of colored material and they were therefore eluted and chromatographed separately on 1 cm. wide paper strips for 6 hours in the system

o-dichlorobenzene-formamide-methanol (1:1). The first spot now remained at the origin, the second spot now traveled 8.8 cm. and the third traveled 24.5 cm. These three spots were again eluted from the paper strips and the spot at the origin was chromatographed on a 1 cm. wide strip in the system methylene chloride-formamide-methanol (1:1) for 10 hours. In this latter chromatogram some colored material traveled 5 cm. and this material was eluted with absolute methanol. The three methanol solutions thus obtained were examined in the ultraviolet at 280-282 mμ., but they did not show an absorption peak in this region of the spectrum. These results indicated that the estrogen extract would have to be purified prior to paper strip chromatography.

Chromatography on Celite-Magnesium Silicate.

A mixture of 2 g. of Celite (Johns Manville No. 545) and 2 g. of magnesium silicate was added to 100 ml. of benzene and the slurry thus obtained was poured into a glass column, 7.5 in. x 2 in. The Celite-magnesium silicate was allowed to settle in benzene for two hours and the top of the absorbent was then covered with a piece of filter paper. Then 193 mg. of the first preovulatory fraction, dissolved in 5 ml. of benzene, was adsorbed on the column. This portion of the extract represented the urine collected during 7 days of the menstrual cycle. The estrogens were eluted as follows.

Fraction	Eluant	Eluate	Weight mg.
a	150 ml. benzene	yellow oils	14.00
b	300 ml. benzene	red oils	13.17
c	200 ml. 10% ether-benzene	red oils	12.18
d	200 ml. 25% ether-benzene	dark brown oils	42.5

Fractions b and c when combined weighed 25.27 mg. and these fractions were chromatographed on paper.

Paper Chromatography of the 1st Preovulatory Fraction.

Fractions b and c from the Celite-magnesium silicate column (25.27 mg.) were chromatographed for 24 hours on a 10 cm.-wide paper strip in the system methylcyclohexane-formamide-methanol (1:1). At the end of the period of chromatography the paper strip was dried and a narrow strip (2mm.) was tested in fuming sulphuric acid. It was found that the estrogens remained at the origin in the above chromatogram. The estrogens were eluted from the origin with absolute methanol and the methanol extract was reduced to dryness at 60°C with the aid of nitrogen. The residue (5.76 mg.) was chromatographed on a 3 cm.-wide paper strip for 6 hours in the system o-dichlorobenzene-formamide-methanol (1:1). At the end of the chromatography period the strip was dried and a 2 mm. portion was cut out and immersed

in fuming sulphuric acid. Four distinct spots were located on the paper strip. These will be called spots A, B, C, and D.

Spot A was located at the origin and it was pink in color; spot B was located 3.5 cm. from the origin and it was cherry red in color. Spot C was located 8.5 cm. from the origin and it was orange-yellow in color and spot D was 24 cm. from the origin and it was a tan color. Spot A was eluted with absolute methanol and chromatographed on a 3 cm.-wide paper strip for 10 hours in the system methylene chloride-formamide-methanol (1:1). It was found that this spot traveled 4.7 cm. in methylene chloride.

Spots B, C and D were rechromatographed on 3 cm.-wide paper strips for 6 hours in o-dichlorobenzene-formamide-methanol (1:1) and they gave the following results. Spot B gave a yellow spot in sulphuric acid, 2 cm. from the origin. Spot C yielded two areas, 6.4 cm. and 10.5 cm. from the origin and spot D remained in the same position.

All the areas resulting from the chromatography of spots B, C and D were eluted with absolute methanol and they were tested in the ultraviolet for absorption at 280-282 mμ. It was found that none of these spots exhibited an absorption maximum in this region.

However, spot A when eluted did give an absorption maximum

at 281 mμ. From the extinction coefficient at 281 mμ. it was calculated that the material eluted from spot A consisted of 122 μg. of estriol. This estriol was dissolved in 3 ml. of conc. H_2SO_4 and set aside for 2 hours at room temperature. Then the ultraviolet spectrum of the solution was determined between 220 and 660 mμ. The spectrum obtained was similar to the spectrum given by authentic estriol. From the above results it was calculated that during the 1st preovulatory phase, the excretion of estriol was 22 μg./24 hours, on the average.

Isolation of Estriol from the 1st Postovulatory Fraction.

The estrogen extract of the 1st postovulatory urine weighed 361 mg. This extract was chromatographed on 4 g. of Celite-magnesium silicate as previously described. The estrogens were eluted with a) 200 ml. of benzene, b) 200 ml. of 10% ether-benzene and c) 400 ml. of 25% ether-benzene. The eluates were combined and they weighed 104 mg. A 52 mg. portion of these eluates was chromatographed on a 15 cm.-wide paper strip for 24 hours in the system methylcyclohexane-formamide-methanol (1:1). At the end of this chromatography 23.7 mg. of material was eluted from the origin and chromatographed on a 10 cm.-wide paper strip for 6 hours in the system o-dichlorobenzene-formamide-methanol (1:1). In this instance and in the other fractions to be described, only the material present at the origin, which corresponded to the estriol region, was further chromatographed. The

material eluted from the origin was chromatographed on a 3 cm.-wide paper strip for 10 hours in the system methylene chloride-formamide-methanol (1:1). In this chromatogram the material present at the origin traveled 5 cm. down the strip. This material was eluted with absolute methanol and the methanol solution was tested in the ultraviolet for absorption at 280-282 mμ. It was found that this material exhibited a strong absorption maximum at 280 mμ., and from the extinction coefficient at this wave length, it was calculated that the methanol solution contained 633 μg. of estriol. An 80 μg. portion of the estriol was used to determine its ultraviolet spectrum in sulphuric acid. From the above data it was calculated that during the 1st postovulatory phase the average daily excretion of estriol amounted to 127 μg.

Isolation of Estriol from the 2nd Preovulatory Fraction.

The estrogen extract of the urine collected for 10 days in the second preovulatory phase weighed 283 mg. Of this extract 226 mg. (corresponding to 8 days) was chromatographed on celite-magnesium silicate as previously described. The total amount of material eluted from this column weighed 121 mg. A 71.86 mg. portion was removed and chromatographed on a 15 cm.-wide paper strip for 24 hours in methylcyclohexane-formamide-methanol (1:1). From this chromatogram 28.3 mg. of material was eluted from the origin. This material was chromatographed in the o-dichloro-

benzene and methylene chloride systems as described above.

As was previously described, the estriol isolated was quantitatively determined from its extinction coefficient at 281 mμ. and its identity was confirmed from its ultraviolet absorption spectrum in sulphuric acid. From the data obtained it was calculated that during the 2nd preovulatory phase the average daily excretion of estriol was 195 μg.

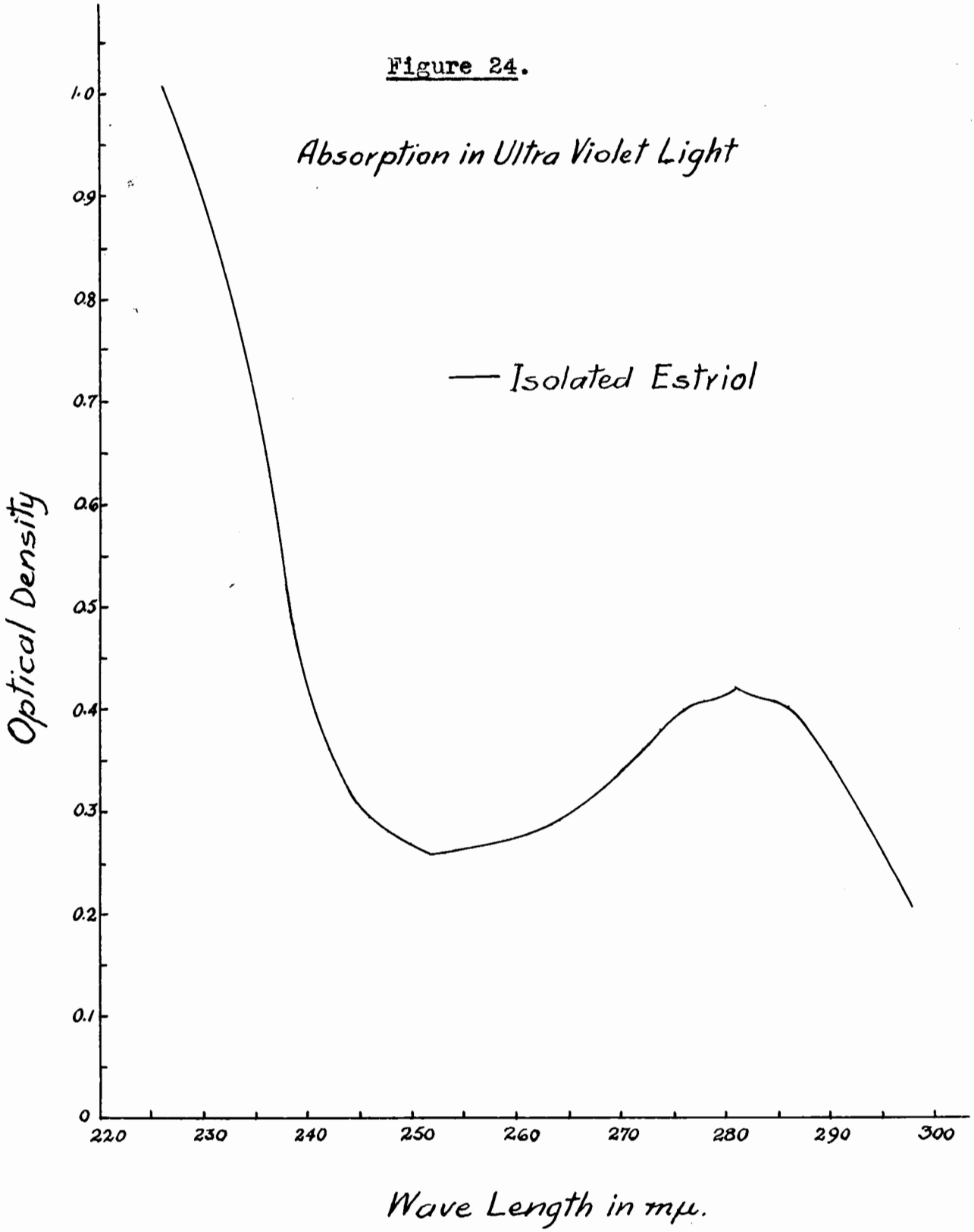
Isolation of Estriol from the 2nd Postovulatory Fraction.

The estrogen extract of the urine collected for 10 days during the 2nd postovulatory phase weighed 902 mg. This extract was chromatographed on 4 g. of Celite-magnesium silicate (1:1), as previously described. It was found that this column was too short to allow a purification of the estrogens and as a result the material adsorbed on the column was stripped with 50% ether-benzene. This process yielded 734 mg. of oily material. This partially purified material was adsorbed on 8 g. of Celite-magnesium silicate (1:1) and the estrogens were chromatographed as described above. The eluates from this chromatogram yielded 423 mg. of oils.

From these eluates 111 mg. was chromatographed on a 15 cm.-wide paper strip, for 24 hours, in the system methylcyclohexane-formamide-methanol (1:1). The material eluted from the origin of this chromatogram (4.15 mg.) was chromatographed in the

systems o-dichlorobenzene and methylene chloride as described above. Finally the estriol separated from the origin in the system methylene chloride-formamide-methanol (1:1) was eluted from the paper strip with absolute methanol. This estriol gave a characteristic ultraviolet absorption spectrum in absolute methanol, figure 24, p. 196a. From the extinction coefficient at 281 mμ., it was calculated that the portion of the total fraction used in this separation contained 143 μg. of estriol. The ultraviolet absorption spectrum in sulphuric acid given by this estriol was similar in shape to the spectrum given by authentic estriol. From the above data it was calculated that the average daily excretion of estriol during the second postovulatory phase was 92 μg.

Figure 24.


Absorption in Ultra Violet Light
— Isolated Estriol
Optical Density
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220
230
240
250
260
270
280
290
300
Wave Length in mμ.

SUMMARY

In Part I, Section A, a critical survey of available methods for the hydrolysis, extraction, separation and identification of the estrogens present in mare's pregnancy urine was presented. Estrone-16-C^{14} was administered to a pregnant mare and the distribution of C^{14} in the blood, saliva, urine and feces was investigated. Of the injected radioactivity, 39.64% was excreted in the urine, a detectable amount was found in the saliva, and an alcoholic extract of the plasma proteins contained some C^{14}. The feces did not contain any C^{14}, which leads us to believe that there is a marked species difference in the metabolism of estrone.

In the course of this study a modified partition chromatography procedure for the separation of estrone, equilin and equilenin was evolved. With this method, estrone, equilin and equilenin were separated from the ketonic strong phenolic fraction of the urine. The isolated equilenin, and probably equilin contained 1% of the radioactivity present in the isolated estrone. It was shown that the radioactivity present in the ring B unsaturated estrogens can be readily accounted for by contamination with estrone. This was considered as evidence that estrone is not converted to equilin and equilenin.

In Part I, Section B, a survey of the central position occupied by acetate in the biosynthesis of sterols and a large variety of steroid hormones was presented. Estrone, equilin and equilenin were isolated from the urine of a pregnant mare injected with C^{14} carboxyl labeled sodium acetate. The isolated equilin and equilenin contained approximately half the radioactivity present in the isolated estrone. In addition it was found that the volatile light oils which are extracted with the estrogens contained C^{14}.

The second and last part dealt with the determination of the urinary estrogens excreted in the pre- and postovulatory phases of a normal human subject. With the use of paper chromatography it was only possible to isolate estriol from the estrogen extracts of the urine. The preliminary results obtained indicate that there are larger quantities of estriol in normal female urine than those previously found by other investigators.

CLAIMS TO ORIGINAL RESEARCH

The following contributions presented in this thesis are claimed by the writer to be of an original nature.

1. After the injection of estrone-16-C^{14} into a pregnant mare, 39.64% of the radioactivity was excreted in the urine. No radioactivity was found in the feces and only traces were found in the saliva and plasma.
2. A modified partition chromatography method for the separation of urinary estrone, equilin and equilenin was described.
3. The failure of estrone to be converted to equilin and equilenin by the pregnant mare was demonstrated.
4. C^{14} carboxyl labeled acetate was found to be incorporated into estrone, equilin and equilenin by the pregnant mare. Estrone contained twice the radioactivity present in equilin and equilenin.
5. Estriol was isolated by paper chromatography from the pre- and postovulatory urine of a normal human.

BIBLIOGRAPHY

1. Selye, H., Textbook of Endocrinology, Acta Endocrinologica Inc., Montreal, 1951.

2. Knauer, E., Arch. Gynakol., 60, 322 (1900).

3. Halbon, J., Monatschr. f. Geburtsh. u. Gynak., 12, 496 (1900).

4. Marshall, F.H.A., and Jolly, W.A., Phil. Trans. Roy. Soc., 198, 99 (1905).

5. Adler, L., Arch. f. Gynak., 95, 349 (1912).

6. Iscovesco, H., Compt. rend. soc. biol., 73, 104 (1912).

7. Fellner, O.O., Arch. Gynak., 100, 641 (1913).

8. Allen, E., and Doisy, E.A., J. Am. Med. Assoc., 81, 819 (1925).

9. Ascheim, S., and Zondek, B., Klin. Wochschr., 6, 1322 (1927).

10. Doisy, E.A., Veler, C.D., and Thayer, S.A., Am. J. Physiol., 90, 329 (1929).

11. Butenandt, A., Naturwissenschaften., 17, 878 (1929).

12. Dingemanse, E., de Jongh, S.E., Kober, S., and Laqueur, E., Deut. med. Wochschr., 56, 301 (1930).

13. d'Amour, F.E., and Gustavson, R.G., J. Pharmacol. Exptl. Therap., 40, 485 (1930).

14. Butenandt, A., Nature, 130, 238 (1932).

15. Marrian, G.F., and Haslewood, G.A.D., J. Soc. Chem. Ind., 51, 2777 (1932).

16. Marrian, G.F., J. Soc. Chem. Ind., 49, 515 (1930).

17. Butenandt, A., and Hildebrandt, F., Z. f. physiol. Chem., 199, 243 (1931).

18. Doisy, E.A., J. Biol. Chem., 86, 499 (1930).

19. Zondek, B., Klin. Wochschr., 9, 2285 (1930).

20. de Jongh, S.E., Kober, S., and Laqueur, E., Biochem. Z., 240, 247 (1931).

21. Girard, A., Compt. rend. acad. sci., 195, 981 (1932).

22. Cook, J.W., Dodds, E.C., and Hewett, C.L., Nature, 131, 56 (1933).

23. Pincus, G., and Pearlman, W.H., Vitamins and Hormones, 1, 326 (1943).

24. Doisy, E.A., Thayer, S.A., and Van Bruggen, J.T., Federation Proc., 1, 202 (1942).

25. Heard, R.D.H., Recent Progress in Hormone Research, 4, 25 (1949).

26. Heard, R.D.H., and Saffran, J.C., Recent Progress in Hormone Research, 4, 43 (1949).

27. Ruben, S., and Kamen, M.D., Phys. Rev., 58, 149 (1940).

28. Jamieson, R., Ph.D. Thesis, McGill University, 1947.

29. Yates, C., Ph.D. Thesis, McGill University, 1951.

30. Zeigler, P., Ph.D. Thesis, McGill University, 1951.

31. Thompson, L., Ph.D. Thesis, McGill University, 1951.

32. Zondek, B., Nature, 133, 209 (1934).

33. Doisy, E.A., Sex and Internal Secretions, Williams and Williams Co., Baltimore 1939, p. 851.

34. a) Girard, A., Sandulesco, G., Fridenson, A., And Rutgers J.J., Compt. rend. acad. sci., 194, 909 (1932).

b) Girard, A., Sandulesco, G., Fridenson, A., and Rutgers, J.J., Compt. rend. acad. sci., 195, 981 (1932).

c) Sandulesco, G., Tohing, W.W., and Girard, A., Compt. rend. acad. sci., 196, 137 (1933).

35. Cohen, A., Cook, J.W., and Hewett, C.L., J. Chem. Soc., 445 (1935).

36. Hirschman, H., and Wintersteiner, O., J. Bio. Chem., 122, 303 (1938).

37. Prelog, V., and Fuhrrer, J., Helv. Chim. Acta., 28, 583 (1945).

38. Wintersteiner, O., Schwenk, E., and Whitman, B., Proc. Soc. Exptl. Biol. Med., 32, 1087 (1935).

39. Heard, R.D.H., and Hoffman, M.M., J. Biol. Chem., 135, 801 (1940).

40. Hirschman, H., and Wintersteiner, O., J. Biol. Chem., 126, 737 (1938).

41. Marker, R.E., Kamm, O., Crooks, H.M. Jr., Oakwood, T.S., Lawson, E.J., and Wittle, E.L., J. Am. Chem. Soc., 59, 2297 (1937).

42. Marker, R.E., and Rohrmann, E., J. Am. Chem. Soc., 61, 2537 (1939).

43. Bauld, W.S., and Heard, R.D.H., Unpublished observations, 1940.

44. Brooks, R.V., Klyne, W., and Miller, E., Biochem. J., 54, 694 (1952).

45. Heard, R.D.H., and McKay, A.F., J. Biol. Chem., 131, 371 (1939).

46. Marker, R.E., Lawson, E.J., Wittle, E.L., and Crooks, H.M. Jr., J. Am. Chem. Soc., 60, 1559 (1938).

47. Oppenauer, R., Z. physiol. Chem., 270, 97 (1941).

48. Marker, R.E., and Rohrmann, E., J. Am. Chem. Soc., 60, 1565 (1938).

49. Klyne, W., and Marrian, G.F., Biochem. J., 39, Proc. xlv (1945).

50. Haslewood, G.A.D., Marrian, G.F., and Smith, E.R., Biochem. J., 28, 1316 (1934).

51. Heard, R.D.H., J. Am. Chem. Soc., 60, 493 (1938).

52. Jacobs, J.D., and Laqueur, E., Rec. trav. chim., 58, 77 (1939).

53. Fieser, L.F. and Fieser, M., Natural Products Related to Phenanthrene, American Chemical Society Monograph Series, New York, 3rd Ed., 1949.

54. Marker, R.E., and Rohrman, E., J. Am. Chem. Soc., 60, 2719 (1928).

55. Klyne, W., and Shoppee, C.W., Chemistry and Industry, p. 470, 1952.

56. Browne, J.S.L., cited by Collip, J.B., Proc. California Acad. Med., 1, 38 (1931).

57. Marrian, G.F., J. Soc. Chem. Ind. (London), 49, 515 (1930).

58. Doisy, E.A., Thayer, S.A., Levin, L., and Curtis, J.M., Proc. Soc. Exp. Biol. Med., 28, 88 (1930).

59. Shoppee, W.C., Chemistry and Industry, p. 810, 1950.

60. Schwenk, E., and Hildebrandt, F., Naturwissenschafter, 20, 658 (1932).

61. Banes, D., Carol, J., and Haenni, E.O., J. Biol. Chem., 187, 557 (1950).

62. Heard, R.D.H., Unpublished results.

63. Huffman, M.N., and Lott, M.H., J. Am. Chem. Soc., 73, 878 (1951).

64. Inhoffen, H.H., Longemann, W., Hohlweg, W., and Serini, A., Ber., 171, 1024 (1938).

65. Doisy, E.A., Thayer, S.A., and van Bruggen, J.T., Federation Proc., 1, 202 (1942).

66. Dickens, F., Biological Oxidation of Aromatic Rings, Cambridge University Press, 1950, p. 73.

67. Cole, H.H., and Saunders, F.J., Endocrinology, 19, 199 (1935).

68. Cole, H.H., and Hart, G.H., J. Am. Vet. Med. Assoc., 101, 124 (1942).

69. Beall, D., and Edson, M., Biochem. J., 30, 577 (1936).

70. Kober, S., Biochem. Z., 293, 209 (1931).

71. Cohen, S.L., and Marrian, G.F., Biochem. J., 28, 1603 (1934).

72. Beall, D., and Marrian, G.F., J. Soc. Chem. Ind., 51, 309T (1934).

73. Curtis, J.M., MacCorquodale, D.T., Thayer, S.A., and Doisy, E.A., J. Biol. Chem., 107, 191 (1934).

74. Edson, M., and Heard, R.D.H., J. Biol. Chem., 130, 579 (1939).

75. Cohen, S.L., and Marrian, G.F., Biochem. J., 30, 57 (1936).

76. Schachter, B., and Marrian, G.F., J. Biol. Chem., 126, 663 (1938).

77. Klyne, W., Schachter, B., and Marrian, G.F., Biochem. J., 43, 231 (1948).

78. Klyne, W., ibid., 43, 611 (1948).

79. Paterson, J.Y.F., and Klyne, W., ibid., 43, 614 (1948).

80. Cohen, H., and Bates, R.W., Endocrinology, 44, 317 (1949).

81. Haussler, E.P., Festschrift E.C. Barrell, p. 327, 1936 (Basel).

82. Rakoff, A.E., Paschkis, K.E., and Cantarow, A., Am. J. Obstet. Gynecol., 46, 856 (1943).

83. Szego, C.M., and Roberts, S., Proc. Soc. Exp. Biol. Med., 61, 161 (1946).

84. Calvin, M., Heidelberger, C., Reid, J.C., Talbert, B.M., and Yankwich, P.F., Carbon-14, John Wiley and Sons, Inc., New York, 1949.

85. Kemp, T., and Pederson-Bjergaard, K., Endokrinologie, 13, 156 (1933).

86. Budy, A.M., Federation Proc., 12, 306 (1953).

87. Mather, A., J. Biol Chem., 144, 617 (1942).

88. Bachman, C., and Pettit, D.S., ibid., 138, 689 (1941).

89. Girard, A., and Sandulesco, G., Helv. Chim. Acta., 19, 1095 (1936).

90. Doisy, E.A. Jr., Huffman, M.N., Thayer, S.A., and Doisy, E.A., J. Biol. Chem., 138, 283 (1941).

91. Zaffaroni, A., J. Am. Chem. Soc., 72, 3828 (1950).

92. Zaffaroni, A., Recent Progress in Hormone Research, Vol. 8, p. 51. Academic Press Inc., New York, 1953.

93. Marx, W., and Sobotka, H., J. Biol. Chem., 124, 693 (1938).

94. Umberger, E.J., and Curtis, J.M., ibid., 178, 275 (1949).

95. Banes, D., J. Am. Pharm. Assoc., 39, 37 (1950).

96. Grant, G.A., and Beall, D., Recent Progress in Hormone Research, vol. 5, p. 307. Academic Press, Inc., New York, 1950.

97. Duschinsky, R., and Lederer, E., Bull. soc. chim. biol., 17, 1534 (1935).

98. Veitch, F.P., and Milone, H.S., J. Biol. Chem., 157, 417 (1945).

99. Banes, D., Carol, J., and Haenni, E.O., Private communication.

100. Banes, D., Carol, J., and Haenni, E.O., J. Biol. Chem., 187, 557 (1950).

101. Herrmann, E., Monatschr. f. Geburtsch. u. Gynak., 41, 1 (1915).

102. Dirscherl, W., and Hanusch, F., Z. physiol Chem., 233, 13 (1935).

103. Heftman, E., Science, 111, 571 (1950), and J. Am. Chem. Soc., 73, 851 (1951).

104. Boscott, R.J., Chemistry and Industry, p. 427, 1952.

105. Burton, R.B., Zaffaroni, A., and Keutmann, E.H., J. Biol. Chem., 188, 763 (1951).

106. Axelrod, L.R., J. Biol. Chem., 201, 59 (1953).

107. Tracerlog, No. 1, January, 1947.

108. Tracerlog, No. 3, March, 1947.

109. Venning, E.H., Methods in Medical Research, 2, 276 (1950). The Year Book Publishers, Inc., Chicago.

110. Manske, R.H.F., J. Am. Chem. Soc., 53, 1106 (1931).

111. Dr. J. Carol, Private communication.

112. Heard, R.D.H., and O'Donnell, V.J., Endocrinology (in press).

113. Jacobs, R., Unpublished results.

114. Werthessen, N.T., Schwenk, E., and Baker, C., Science, 117, 380 (1953).

115. Sonderhoff, R., and Thomas, H., Ann., 530, 195 (1937).

116. Bloch, K., and Rittenberg, D., J. Biol. Chem., 143, 297 (1942). ibid., 145, 625 (1942).

117. Bloch, K., Borek, E., and Rittenberg, D., J. Biol. Chem., 162, 441 (1946).

118. Srere, P.A., Chaikoff, I.L., and Dauben, W.G., ibid., 176, 829 (1948).

119. Bloch, K., Recent Progress in Hormone Research, 6, 11 (1951).

120. Curran, G., J. Biol. Chem., 191, 775 (1951).

121. Brady, R.D., and Gurin, S., ibid., 189, 371 (1951).

122. Little, H.N., and Bloch, K., ibid., 183, 33 (1950).

123. Wuersch, J., Huang, R.L., and Bloch, K., ibid., 195, 439 (1952).

124. Hunter, G.D., Popjak, G., and Cornforth, J.W., Biochem. J., 54, 590 (1953).

125. Ottke, R.C., Tatum, E.L., Zabin, I., and Bloch, K., J. Biol. Chem., 189, 429 (1951).

126. Heilbron, I.M., Kamm, E.D., and Owens, W.M., J. Chem. Soc., 1630 (1926).

127. Langdon, R.G., and Bloch, K., J. Biol. Chem., 200, 129 (1953).

128. Langdon, R.G., and Bloch, K., ibid., 200, 135 (1953).

129. Robinson, R., J. Soc. Chem. Ind., 53, 1062 (1934).

130. Woodward, R.B., and Bloch, K., J. Amer. Chem. Soc., 75, 2023 (1953).

131. Dauben, W.G., Abraham, S., Hotta, S., and Chaikoff, I.L., ibid., 75, 3038 (1953).

132. Langdon, R.G., and Bloch, K., Federation Proc., 12, 235 (1953).

133. Langdon, R.G., and Bloch, K., J. Biol. Chem., 202, 77 (1953).

134. Zaffaroni, A., Hechter, O., and Pincus, G., J. Am. Chem. Soc., 73, 1390 (1951).

135. Haines, W.J., Nielson, E.D., Drake, N.A., and Woods, O.R., Arch. Biochem. Biophys., 32, 218 (1951).

136. Haines, W.J., Recent Progress in Hormone Research, 7, 255 (1952).

137. Peron, F., Ph.D. Thesis, McGill University, 1953.

138. Pincus, G., and Hechter, O., Recent Progress in Hormone Research, 7, 300 (1952).

139. Schwenk, E., and Savard, K., Unpublished results.

140. Bloch, K., J. Biol. Chem., 157, 661 (1945).

141. Brady, R.O., J. Biol. Chem., 193, 145 (1951).

142. Savard, K., Dorfman, R.I., and Poutasse, E., J. Clin. Endocrinol. and Metab., 12, 935 (1952).

143. Dorfman, R.I., Ungar, F., and Savard, K., Proc. 2nd. International Congress of Biochemistry, p. 126, 1952.

144. O'Donnell, V.J., Unpublished results.

145. Stimmel, B.F., J. Biol. Chem., 162, 99 (1946).

146. Popjak, G., and Beeckmans, M.L., Biochem. J. 46, 547 (1950).

147. Westerfeld, W.W., MacCorquodale, D.W., Thayer, S.A., and Doisy, E.A., J. Biol. Chem., 126, 195 (1938).

148. Huffman, M.N., Thayer, S.A., and Doisy, E.A., ibid., 133, 567 (1940).

149. Cohen, S.L., and Marrian, G.F., Chemistry and Industry, 54, 1025 (1935).

150. Mitchell, F.L., Nature, 170, 621 (1952).

151. Smith, G.V., Smith, O.W., Huffman, M.N., MacCorquodale, D.W., Thayer, S.A., and Doisy, E.A., J. Biol. Chem., 130, 431 (1939).

152. Smith, G.V., Smith, O.W., and Pincus, G., Am. J. Physiol., 121, 98 (1938).

153. Marrian, G.F., Proc. Soc. Endocrinol., June 28, 1947.

154. Emmens, C.W., Hormone Assay, Academic Press Inc., New York, 1950.

155. Robson, J.M., Recent Advances in Sex and Reproductive Physiology, 3rd. Edition, The Blakiston Co., Toronto, 1947.

156. Buxton, C.L., The Short Luteal Phase in Menstruation and its Disorders, Charles C. Thomas, 1947, Springfield, Ill.

157. Loewe, S., and Lange, F., Klin. Wochschr., 5, 1038 (1926).

158. Siebke, H., Zbl. f. Gynak., 54, 1601 (1930).

159. Gustavson, R.G., and Green, D.F., J. Biol. Chem., 105, Proc., 34 (1934).

160. Frank, R.T., Goldberger, M.A., and Spielman, F., J. Am. Med. Assoc., 103, 393 (1934).

161. Yerby, L.D., Proc. Soc. Exp. Biol. and Med., 36, 496 (1937).

162. Spurrell, W.R., and Ucko, H., Guy's Hospital Report, 88, 230 (1938).

163. Gustavson, R.G., Wood, T., and Hays, E., J. Biol. Chem., 114, Proc. 46 (1936).

164. Smith, G.V., and Smith, O.W., New Eng. J. Med., 215, 908 (1936).

165. Smith, O.W., Smith, G.V., and Schiller, S., Endocrinology, 25, 509 (1939).

166. Smith, G.V., and Smith, O.W., Am. J. Obstet. Gynecol., 36, 769 (1938).

167. Pincus, G., and Zahl, P.A., J. Gen. Physiol., 20, 879 (1937).

168. Venning, E.H., and Browne, J.S.L., Endocrinology, 21, 711 (1937).

169. Van Bruggen, J.T., J. Lab. Clin. Med., 33, 207 (1948).

170. Marrian, G.F., and Bauld, W.S., Acta endocrinologica, 7, 240 (1951).

171. Buehler, H.J., Katzman, P.A., Doisy, P.P. and Doisy, E.A., Proc. Soc. Exp. Biol. Med., 72, 297 (1949).

172. Beer, C.T., Dobriner, K., Eidinoff, M.L., and Gallagher, T.F., Federation Proc., 12, 176 (1953).

173. Butenandt, A., and Hofstetter, H., Z. f. physiol. Chem., 259, 222 (1939).

174. Crepy, O., Compt. rend. acad. sci., 223, 646 (1946).

175. Cohen, H., and Bates, R.W., Endocrinology, 45, 86 (1949).

176. Stimmel, B.F., J. Clin. Endocrinol. Metab., 12, 950 (1952).

177. Pincus, G., J. Clin. Endocrinol., 5, 291 (1945).

178. Friedgood, H.B., and Garst, J.B., Recent Progress in Hormone Research, 2, 31 (1948).

179. Pincus, G., Wheeler, G., Young, G., and Zahl, P.A., J. Biol. Chem., 116, 253 (1936).

180. Jayle, M.F., Crepy, O., and Judas, O., Bull. soc. chim. biol., 25, 301 (1943).

181. Stimmel, B.F., J. Biol. Chem., 178, 217 (1949).

182. Bates, R.W., and Cohen, H., Federation Proc., 6, 236 (1947).

183. Jailer, J., Endocrinology, 41, 198 (1947).

184. Finkelstein, M., Hestrin, S., and Koch, W., Proc. Soc. Exp. Biol., N.Y., 64, 64 (1947).

185. Jailer, J.W., J. Clin. Endocrinol., 8, 564 (1948).

186. Salter, W.T., Humm, F.D., and Oesterling, M.J., ibid., 8, 295 (1948).

187. Engel, L.L., Slaunwhite, W.R. Jr., Carter, P. and Nathanson, I.T., J. Biol. Chem., 185, 255 (1950).

188. Smith, O.W., and Smith, G.V., Recent Progress in Hormone Research, 7, 209 (1952).

189. Buxton, C.L., and Atkinson, W.B., J. Clin. Endocrinol., 8, 544 (1948).

190. Bush, I.E., Nature, 166, 445 (1950).

191. Nyc, J.F., Garst, J.B., Friedgood, H.B., and Marion, D.M., Arch. Biochem., 29, 219 (1950).

192. Savard, K., Private communication.

193. Bitman, J., and Sykes, J.F., Science, 117, 356 (1953).

194. Heusghem, C., Nature, 171, 42 (1953).

195. Bauld, W.S., Colloquia on Endocrinology, 2, 72 (1952).