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The separation of the estrogens by gas chromatog

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THE SEPARATION OF THE ESTROGENS BY GAS CHROMATOGRAPHY

by

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TABLES

FIGURES

TOPICAL OUTLINE

INTRODUCTION

Despite the elegant methods of analysis for estrogenic steroids based either on column partitioning (11, 25), countercurrent distribution (52), or paper chromatography (37), there still exists a need of a much more rapid and sensitive assay procedure. The advent of gas chromatography offered a possible solution to this problem.

A prime requisite of any gas chromatographic method is that the compounds be relatively volatile and thermally stable at the temperature of vaporization and separation. Accordingly the thermal stability of certain free and acetylated estrogens was studied. Once suitable derivatives had been found, the parameters of separation and detection were evaluated. Of prime concern were the quantitative determination of and detector sensitivities towards these substances. In order to have a quantitative procedure, it was necessary to determine optimal conditions for the preparation of the estrogen derivatives. It was concluded that gas chromatography can be applied to the separation and determination of estrogens in biological media.

I REVIEW OF THE LITERATURE

A. Estrogens

1. Definition of an estrogen

The term estrogen is best interpreted in an historical context. The first well defined term used by the British workers was "oestrin", who, after the isolation of estrone by Doisy, Veller and Thayer in 1929, narrowed this term to a single chemical entity. Previously, two other terms, "theelin" and "folliculin", were applied to that factor which induced estrus-like changes in the rat's vagina. Prior to 1929, the terms "oestrin", "theelin" and "folliculin" referred to a substance, which was then recognized only on the basis of its biological effect. The definition soon became obsolete because of the discovery of additional compounds which showed either a structural relationship to or a biological activity like that of estrone. Since 1929, however, many compounds, both natural and synthetic, have been classified as estrogens. Prior to the discovery of 2-methoxyestrone in urine in 1957, one could have defined an estrogen as any substance which could induce cornification of the vagina of the adult mouse. However, 2-methoxyestrone was found to have little estrogenic activity. At the present time the term estrogen may be applied to any compound which 1) will induce cornification of the vagina of the adult mouse, or 2) whose steroid structure includes a phenolic ring A.

2. Classification

a. Natural
b. Synthetic
c. Conjugates

A natural estrogen, is a compound isolated from natural sources (blood, urine, feces or tissue). The natural estrogens isolated from the human are shown in Table I. Synthetic estrogen refers to those compounds which can induce cornification of the vagina, but which have not been isolated from biological media. The term conjugate refers to the sulfate or the glucopyranosiduronate of any of the natural compounds.

3. Sources

In view of the fact that substances which have no steroid nucleus can have estrogenic activity (e.g. isoflavones) the observation that estrogenic activity is found in various plants, lower animals and bituminous substances has little significance - unless the biologically active material is identified (55). Estrone has been isolated from the palm kernal, and estriol has been obtained from the female willow flowers.

In the chordates, estradiol-17β and estrone have been isolated from the dogfish and estriol was identified tentatively in the gonads of the lungfish (140,39). In the invertebrates estradiol-17β was identified from the mollusks and the starfish. The physiological significance of these findings in the invertebrates is at the present time unknown.

TABLE I

ESTROGENS ISOLATED FROM URINE

Common Name	IUPAC Nomenclature	Abbreviation	Ref.
Estrone	3-Hydroxyestra-1:3:5(10)-trien-17-one.	E_1	45
Estradiol-17β	Estra-1:3:5(10)-triene-3:17β-diol.	E_2	123
Estriol	Estra-1:3:5(10)-triene-3:16α:17β-triol.	E_3	97
16-ketoestrone	3-Hydroxyestra-1:3:5(10)-triene-16:17-dione.	16-keto-E_1	121, 123
16-*epi*estriol	Estra-1:3:5(10)-triene-3:16β:17β-triol.	....	99
16-ketoestradiol-17β	3:17β-Dihydroxyestra-1:3:5(10)-trien-16-one.	16-keto-E_2-17β	87
16α-Hydroxyestrone	3:16α-Dihydroxyestra-1:3:5(10)-trien-17-one.	16α-OH-E_1	100
2-methoxyestrone	2-methoxy-3hydroxyestra-1:3:5(10)-trien-17-one.	....	59
2-methoxyestradiol	2-methoxy-estra-1:3:5(10)-triene-3:17β-diol	....	57
18-Hydroxyestrone	3:18-Dihydroxyestra-1:3:5(10)-trien-17-one.	18-OH-E_1	91
16β-hydroxyestrone	3:16β-Dihydroxyestra-1:3:5(10)-trien-17-one.	16β-OH-E_1	96
17-*epi*estriol	Estra-1:3:5(10)-triene-3:16α:17α-triol.	....	20

In the vertebrates, and particularly in the urines of members of the equine family, estrogens are more abundant than in any other species studied thus far. Table II compares the estrogen content of urines of equines and man.

TABLE II

ESTROGEN CONTENT OF URINES (54)

	µg./day
Stallion	1,700,000
Mare	2,000
Pregnant Mare	1,000,000
Sexually mature women	45 - 300
Pregnant women	15,000

Further, Girard isolated two unsaturated ring B estrogens, equilin and equilenin, from pregnancy urine of the mare.

In man, the main sources of estrogen production are the ovary and the placenta, and to a lesser extent the adrenal cortex and the testis(92).

The Ovary

It is believed that all ovarian epithelial cells may secrete estrogens, but that the follicular epithelium is probably the primary source. The theca interna, which is part of the follicle, shows a high degree of mitotic activity during maturation of the ovum. The corpus luteum after ovulation takes over the estrogen secretion. The nature of the estrogens secreted in the human ovary, has not yet been proven with certainty. The weight of evidence indicates that all of the three major estrogens (estrone, estradiol and estriol) are produced in the ovary (125, 92).

The Placenta

All of the three major estrogens have been isolated from human placental tissue. Studies showed that the placenta produces primarily estriol. There is good evidence to support the view that during pregnancy the placenta takes over from the corpus luteum the production of estrogens after the first trimester.

The Adrenal Cortex

The evidence that the human adrenal cortex produces the three major estrogens was obtained indirectly from a variety of pathological and surgical data. Small quantities of estrone, estradiol and estriol have been detected in the urine of normal men and postmenopausal and oophorectamized women. It is therefore, considered possible that the principal source for these compounds is the adrenal cortex.

The Testis

Pooled semen was shown to contain estrone, estradiol and estriol in the human. Little is known thus far of the histological source of the material.

B. Methods for the estimation of estrogens.

The methods which have been used for the estimation of estrogens may be classified as follows:

1. Biological estimation
2. Chemical analysis

1. Biological Methods

The biological assays are dependent on the action of the estrogens on the accessory genitalia and may, therefore, be classified further into those measuring:

a) Vaginal cornification
b) Uterine weight
c) Vaginal opening
d) Specific histological responses

a. Vaginal cornification

In 1917, it was observed by Stockard and Papanicolaou that a periodic cornification of the vaginal epithelium occurred in certain animals and that this could be easily recognized by microscopic examination of vaginal smears. In 1923, Allen and Doisy obtained from the follicular fluid of the sow's ovary a substance which could induce cornification of the mouse vagina. Little progress was made toward the isolation of the pure material until 1927, when Ascheim and Zondek discovered that estrogenic material was excreted in considerable quantity in the urine of pregnant women. With a reliable method of bioassay and a convenient source available, the work entered the final phase and culminated in 1929 with the isolation of the pure crystaline hormone, estrone, by Doisy and Butenandt.

Estrone when administered to an oophorectomized mouse will produce a change in the vagina in twenty-four hours. The epithelium undergoes changes of both the numbers and the forms of cells. Further, if no more estrone is injected, leucocytosis occurs and the epithelium desquamates. If, however, the administration is maintained at short intervals, leucocytosis does not occur and keratinization instead of desquamation is observed, these vaginal changes are not a general phenomena of all vertebrates (e.g.) the rabbit vagina becomes mucified instead of cornified.

The biological reaction described above is the basis for the assay based on the vaginal cornification of the oophorectomized mouse. The procedure was modified by Marrian and Parks (1929) who used aqueous solutions of estrogens injected subcutaneously into mice in four doses over thirty-six hours. Vaginal smears are taken once on the second day, three times on the third day and twice on the fourth day. A "positive" response is considered to be the presence of epithelial and cornified cells and the absence of leucocytes. Many modifications of this method have been described in the literature.

b. Uterine weight methods

Methods which are more precise and require less work are those proposed by Lauson (86) and by Evans (53). These methods utilize intact immature rats and mice. The aminals are injected with a portion of the extract twice daily for three days and are sacrificed seventy-two hours after the first administration, at which time the uteri are weighed.

Astwood observed that a rapid increase in uterine weight occurred during the first few (4-6) hours after injection of estrogens into immature rats. This increase was due mainly to accumulation of water. This observation was utilized by Astwood to develop a more rapid biological assay of estrogens (3).

A method utilizing oophorectomized immature rats as opposed to immature mice was described by Bulbring (26), who determined the estrogenic effect by weighing the uterus forty-eight hours after administration.

c. Vaginal opening

Methods measuring the vaginal opening in intact immature rats, mice and guinea pigs and the enlargement of the oviduct in the chich have been suggested by Hartman (64) and by Dorfman (46).

d. Specific histochemical responses

Among responses of the uterine epithelial cells of the rat and mouse to estrogen, two of the most specific are hypertrophy of the Golgi apparatus and depletion of basal phospholipid in uterine epithelium (48).

Other criteria are the appearance of glycogen and the mobilization of alkaline phosphatase.

TABLE III

BIOASSAY METHODS

Method	Estrogen	Lower Limit of Detection μg,	References
Hartman	Estradiol dipropionate	$4x10^{-4}$	64
Elfman	Estradiol benzoate	$2x10^{-5}$	48
Stadler	Estrone monobenzoate	$0.5x10^{-1}$	128
	Estradiol monobenzoate	$1\text{-}2x10^{-2}$	
Stadler	Estrone monobenzoate	0.05-0.1	128
	Estradiol monobenzoate	$5\text{-}6x10^{-4}$	
Lauson	Estrone	6-8	87
	Estradiol	0.2-0.4	
	Estriol	6-8	
Albrieux	Estrone	$2x10^{-2}$	2
Evans - Dao	Estrone Estradiol Estriol 16-epiestriol	0.02 0.005 0.03 0.06	53, 37
Dorfman	Stilbesterol	25	46
	Estradiol benzoate	50	
	Estradiol	100	
	Estrone	640	

TABLE III

BIOASSAY METHODS
(continued)

Method	Estrogen	Lower Limit of Detection µg.	References
Krichevsky	Estrone	$1x10^{-2}$	82
Bulbring	Estrone	0.2	26
Marrian + Parks	Estrone	0.4	101
Astwood	Estradiol	0.006	3

e. Evaluation of biological assays

Bioassay, when performed on purified fractions, has the advantage of high sensitivity, as indicated in Table III. Some procedures, however, are insensitive, particularly when the tissue chosen for assay is not a primary target organ for the hormone (46).

The main disadvantages are that, in general, the assay procedures are time consuming and expensive, and if there is no separation prior to assay, the analysis are unreliable. Not all procedures yield the same order of sensitivity to a given estrogen. These methods are influenced by solvents, route and spacing of administration of the hormone. Also, in a given biological extract "augmenting" or "potentiating" factors which enhance the effect of estrogens on the target organs have been observed (92). Those methods which involve introduction of the hormone in pellet form, show positive responses to a variety of compounds often found in biological extracts (83) including albumin, gelatin, cystine and cholesterol.

2. Chemical Methods

The chemical methods are dependant on two essential steps.

a.) Separation

b.) Detection

The methods of separation are further classified as:

1.) Reaction separation

2.) Chromatographic separation

The chromatographic methods can be distinguished on the basis of the following interacting phases:

Type	Mobile	Stationary
I	liquid	liquid
II	liquid	solid
III	gas	liquid

The (liquid-liquid) separations have acquired specific names depending on the materials and type of apparatus used to accomplish these fractionation, and they are classified as:

a. Extraction

b. Countercurrent distribution

c. Column chromatography

d. Paper partition chromatography

The physical separations of compounds are accomplished by operations which fall into two wide catagories:

1.) those depending on discontinuity of the mutual phase in any two boundary system, and

2.) chemical reactions which change the solubility properties of a compound either with respect to the given system or to some anticipated system.

Examples of discontinuity of a mutual phase are those of chromatography and its variants and examples of chemical reaction are the synthesis of derivatives. Application of all possible variations of the two wide catagories mentioned above has been made for the separations of estrogens. The forces involved above run the gamut from weak van der Waals attractions to the formation or breaking of a covalent bond. One generality of theoretical significance is that the free energy of the system will be lowered in an exothermic process. Here the decrease in ΔH, the heat of adsorption, in general, will be a function of some molecular parameter. By far the most common method has been that dependent on liquid-liquid interaction (31).

a. Separatory Funnel Extraction

The important variable in this type of separation is the distribution coefficient of the compounds to be fractionated between immiscible solvents. Mather (102) has studied these coefficients in a variety of binary systems and showed that the distribution of estrogens between benzene and aqueous 0.3 M Na_2CO_3 was sufficiently different to allow separation of estrone and estradiol from estriol. Mather's data for this system is shown below:

TABLE IV

SOLUBILITY OF ESTROGENS IN TWO PHASE SYSTEMS

Organic Phase	Aqueous Phase	Percent estrogen in organic phase Estrone	Estriol	Estradiol
Benzene	0.1 M HCl	99.7	14.	----
Benzene	0.3 M Na_2CO_3	99.6	2.5	95
Benzene	0.1 M NaOH	60.0	0.3	17
Benzene	1.0 M NaOH	20.0	---	5

b. Countercurrent distribution apparatus

Engel (52) studied the partition coefficients of the three major estrogens and developed the countercurrent distribution method for estrogen.

Engel in a careful and elegant study presented evidence both actual and theoretical for the separation of the estrogens by using a twenty-four transfer system with methanol - water in the upper phase and carbon tetrachloride in the lower phase, separation of estradiol: estriol 1:700 could be accomplished. Recoveries of the estrogens were of the order of 95 per cent.

The countercurrent distribution of the estrogens, based on their partition coefficients, not only accomplished separation, but the compounds are characterized by a reproducible physical constant, a feature which is most valuable in the identification of new metabolites.

c. Column chromatography

Here the equilibration occurs over a number of theoretical plates where one phase stays adsorbed on an inert support and a mobile phase acts as carrier.

Examples of this type of separation are found in Table VI.

The methods involving column chromatography can be used over a wide range of concentration.

Solid-liquid separations refer to adsorption chromatography using either column or paper techniques. Examples of these separations are given in Table VIII.

d. Paper partition chromatography

Paper partition chromatography has been applied to the separation of estrogens by a number of investigators because of its ability to resolve mixtures of small quantities of these hormones. Recoveries rarely approach the quantitative level since some material always remains at the origin or is distributed throughout the paper. Recoveries generally range from 80-90 per cent but rarely higher. A variety of solvent systems have been used including reversed phase and two dimensional systems. Examples of some of these systems are tabulated below:

TABLE V

PAPER PARTITION CHROMATOGRAPHY

Stationary Phase	Mobile Phase	Separates	References
Ethanol-water	Toluene-petroleum ether	Diazotized E_1, E_2, E_3	66
Propylene-Glycol	Toluene	E_1, E_2, E_3	78

TABLE V

PAPER PARTITION CHROMATOGRAPHY
(continued)

Stationary Phase	Mobile Phase	Separates	References
Propylene-Glycol	Benzene	E_1, E_2, E_3	16
Propylene-glycol and Ethylene-glycol	Benzene and Ethyl acetate	E_1, E_2, E_3	16
Ethylene-glycol	Ethylene-dichloride	E_1, E_2, E_3	16
Glycerol	Acetone methyl-ethyl ketone		16
Glycerol	Dichloro-methane Dichloro-ethane Chloroform Amyl alcohol	E_1, E_2, E_3	16
Capryl alcohol	water-$KHCO_3$-10% K_2CO_3		16
Methanol-water	Benzene	Epiestriol Estriol	37
Methanol-water	Benzene	E_1, E_2, E_3	107
Formamide	Chloroform	16αOH E_1 16βOH E_1 16 keto-E_2-17β	97
Formamide	Cyclohexane Dichloro-methane	E_1, E_2, E_3 and others	4
	o-dichloro-benzene	E_1, E_2, E_3 and others	4
Propylene-glycol	Methyl-cyclohexane	E_1, E_2, E_3 and others	4
n-heptane	Methanol	E_1, E_2, E_3	37

Legend

E_1, E_2, E_3, etc. same as Table I

TABLE [illegible]V

METHODS OF COLUMN SEPARATION OF ESTROGENS

Inert Support	Quantity	Column Diameter	Stationary Phase	Mobile Phase	Compound	Percent Recovery	Ref.
Alumina	30 grams	----	ethanol	benzene	$E_1E_2E_3$	80-100	129
Florisil	5.5 gms.	10 mm	ethanol	skelly-solve C	monoazotes diazotes	---	132
Celite	21 gms.	250 mm	NaOH	benzene	E_1, E_2 dehydro-equilenin	---	29
Celite	1.25 gms.	6 mm	NaOH	benzene	E_1,E_2	---	131
Celite	3 gms.	10.8 mm	NaOH $NaHCO_3$	benzene	E_1, E_2, E_3	82-100	15
Vulcanized Rubber	--------	8 mm	benzene	methanol water	E_1, E_2, E_3	88-100	109
Celite	--------	1 cm.	NaOH	benzene	E_1, E_2, E_3	80-90	10

Legend:

E_1, E_2, E_3, etc. See Table I

TABLE VII

REVERSE PHASE PAPER PARTITION CHROMATOGRAPHY

Inert Support	Coating	Stationary Phase	Mobile Phase	Separates	References
Paper	Silicones	Chloroform	Water-ethanol	E_1, E_2, E_3	84
100% cellulose acetate	------	n-decane	Water-methanol	E_1, E_2, E_3	30
Foil	------	n-tetra-decane	Water-methanol	E_1, E_2, E_3	30

TABLE VIII

ADSORPTION CHROMATOGRAPHY

Inert Support	Mobile Phase	Compounds	References
Paper with alumina	Benzene	E_1, E_2, E_3	27, 38
Alumina column	Benzene Petroleum Ether	E_1, E_2, E_3	23

Legend

E_1, E_2, E_3 See Table I

Chemical Separations

Estriol can be separated from estrone and estradiol by partitioning according to Mather. Estrone and estradiol can then be further separated by taking advantage of the reactivity of the ketone group of estrone. Estrone can form 1) a hydrazone or 2) a hydrazone with Girard's reagent which is water soluble. (102), (8), (6).

Methods of Detection and Estimation

Regarding methods of detection and estimation, two factors are of utmost importance. 1) specificity, and 2) sensitivity.

With respect to specificity, detection methods have been used ranging from iodine vapor (27) - which reacts with nearly all steroids - to the Zimmermann reaction which is specific for 17 ketones with no α substituents.

Methods B-3 to B-9 (Table IX) are dependent on functional groups present in the molecules and would seem to be the more desireable. However, because of their relative insensitivity or incomplete reaction, they are rarely used today. Rather, the nonspecific reactions of these compounds with strong acids (H_2SO_4 or H_3PO_4) have been carefully studied and the methods commonly used today are: 1) modifications of the Kober reaction or 2) fluorimetric techniques. Methods B-1, 2, 3 and C (Table IX) are closely related when the following scheme is considered

TABLE IX

METHODS OF ESTIMATION AND AMOUNTS REQUIRED

Method	Estrogen	Minimum amount detected	References
A.) Physicochemical			
1) Gravimetric (e.g.) 2,4-dinitrophenyl hydrazine	E_1	mg	8,6
2) Polarography	E_1, E_2, E_3 Girard derivatives	10 µg	67
3) Ultraviolet spectroscopy	E_1, E_2, E_3	15 µg	58
4) Infrared spectroscopy	E_1, E_2, E_3 Benzene sulfonate	mg	8
5) Radiochemical			87
B.) Colorimetric			
1) Kober	E_1, E_2, E_3	2 µg	24
2) David (specific for estriol)	E_3	10 mg	115
3) Bachman	E_3	10 mg	5

TABLE IX

METHODS OF ESTIMATION AND AMOUNTS REQUIRED
(continued)

Method	Estrogen	Minimum amount detected	References
4) Zimmermann	E_1	20 μg	6
5) 2, 4 dinitrophenyl-hydrazine	E_1	7 μg	134
6) Diazobenzene sulfonic acid	E_1, E_2, E_3	2 μg	120
7) Dibromoquinone chloroimide	Equilin + Equilenin	-----	6
8) p-nitrobenzene azo-dimethoxyaniline	Equilenin	5 μg	136
9) p-nitrobenzene azo dimethoxyaniline	E_1, E_2, E_3	3 μg	66
C.) <u>Fluorimetric</u>	E_1, E_2, E_3 and others	0.02 μg	4
	E_1, E_2, E_3	0.005 μg	
D) <u>Biochemical</u>			
1) Enzymes 17β dehydrogenase	E_1, E_2	5-10 μg	85
2) Isocitric dehydrogenase	E_1, E_2		135

....Estrogens + $H_2SO_4 + \Delta$ or H_3PO_4 → Yellow-green fluorescent pigment → Basis for fluorimetric method

Basic for colorimetric method. (Kober) .... → + $H_2O + H_2SO_4$ + Stabilizer

↓

Pink color

↓ time

Colorless

Due to the studies of Brown, Bates, Engel, Bauld and Axelrod (24, 10, 7, 51, 4) many of the variables of this reaction have become known, and using the Kober reaction the Edinburgh group was able to detect and isolate several new estrogens.

Workers in the fields of physiology, endocrinology, biochemistry and medicine have concerned themselves with studies involving the detection and analysis of the estrogens in biological media. In order to accomplish this they had to solve a variety of other problems relating to the hydrolysis, extraction and purification of the substance from biologic media. It is a well established fact that human urinary steroids are present as a water soluble conjugate of glucuronic or sulfuric acids (126, 116). Some may be excreted in the free form, or the free estrogens which are present may be augmented due to the hydrolysis of the conjugates in the urine. Little is known about the estrogenic hormones circulating in the blood. It is possible that estrogens circulate as the free, conjugated or protein bound steroids. For tissue, uncertainty exists. Before any of the analytical methods may be applied, a complex series of steps involving 1) Hydrolysis, 2) Extraction, 3) Isolation, 4) Purification and 5) Separation must be undertaken. In order to demonstrate the complexity of the application of many of these methods, Figures 1, 2 and 3, depict the schemes currently used by some investigators in determining estrogens in urine (25), tissue (107) and blood (122).

FIGURE 1

The Isolation and Estimation of Estrogens in Placental Tissue
(107)

Minced Tissue

extracted with 80% ethanol
extracted with 95% ethanol

Ethanol extract

filtered

Residue A
combine with B

ethanol
extract

butanol
extract

combined

concentrated, add water
ether extract

Ether
extract
Free estrogen
fraction
1

water phase, HCl reflux

discard water phase

Ether extract 2

Conjugated estrogen
fraction

Residue

extracted with butanol

Residue B
combine with A

5% NaOH, 24 hours

Neutral layer

3 Ether extraction

Protein bound
Estrogen fraction

Ether extract 1, 2, or 3

washed with 10% $NaHCO_3$

water discard

ether extract
1 CCl_4 to 20x ether
extract with N NaOH

ether discard

aqueous phase

pH adjusted

ether extraction

water phase discard

concentrate dried in benzene

water extraction

Estrone +
Estradiol

extract with ether

discard
water

Estriol

FIGURE 2

Urinary Assay of Estrogens
(25)

Urine

urine hydrolyzed with HCl

extracted with ether

extract washed successively with

1. saturated Na_2CO_3 soln., pH 10.5
2. NaOH
3. NaOH wash neutralized to pH 10 and shaken again with ether extracts
4. $NaHCO_3$
5. Water

Ether evaporated

Residue dissolved in ethanol
Benzene and petroleum ether equal parts added

water extract

Benzene/Petroleum Ether extract

extracted NaOH
NaOH extract (estrone + estradiol)
H_3BO_3 added
Methylated with dimethylsulfate,
NaOH and H_2O_2 added

extract with
petroleum ether

Petroleum ether extract washed
with water

chromatographed
on alumina

Estrone
methyl ether

Estradiol
methyl ether

water extracts (estriol)

H_3BO_3 + NaOH added
Methylated with dimethyl-
sulfate, NaOH + H_2O_2

extract
with Benzene

Benzene extract washed
with water

chromato-
graphed on
alumina

Estriol methyl ether

FIGURE 3

Plasma Estrogen Determination
(122)

Blood Plasma

pH 10 with N NaOH
extract with ether

Ether extract

Residue

washed with 1/10
volume water

water sol. extract

washed ether extraction
dried and redissolved in
light petroleum and
extracted with methanol

Light petroleum
residue

Methanolic extract

dried and redissolved
with methanol, applied
to paper chromatogram

Light petroleum:absolute
methanol, 16 hours at 37^{o}C

Estrogens eluted
Treated with Kober reagent
for measurement

While many of these methods of estrogen analysis have been widely employed by investigators in a variety of fields, the method most frequently used at present is that developed by Brown (25). However, there are still some improvements to be made in this method. In 1948, Marrian (92) concluded that the Kober reaction, together with the extraction procedure then available, was probably unreliable in urines containing less than 2 mg of estrogens per twenty-four hours and that accurate chemical determinations of urinary excretion of the female hormones were only possible during mid and late pregnancy. Since then (24, 25, 11), great improvements have been made so that according to Brown the mean recovery was between 80 - 90 per cent even at levels corresponding to 4 μg of added estrogen. It is probable that a further 10-20 per cent is lost during hydrolysis of the urine so that the overall recovery by this method of the order of 60-75 per cent. Today it is felt that the sensitivity of the Brown method is around 1.5 μg of each component per twenty-four hours. The figures below this level do not differ significantly from zero. It would seem that over a period of nine years through improvements in extraction and purification the method has undergone a 1000 fold increase in sensitivity. This procedure although exacting and time consuming has been used widely.

The method of Bauld (11), is similar in many respects to that of Brown, except that column partition chromatography of the free estrogens is carried out as opposed to adsorption chromatography of the ethers of the estrogens.

The limitations of these methods involve time consuming procedure and non-optimal acid hydrolysis, with a concomitant loss of 10-20 per cent of material. Neither method in its present form is capable of measuring 16-epiestriol, 16α hydroxyestrone or 2-methoxyestrone.

Although the fluorescence techniques are more sensitive than the Kober reaction, they have the disadvantage of being less specific. This lack of specificity arises from the fact that in relatively impure extracts substances other than estrogens are capable of producing fluorescence. Many methods have been proposed (73, 51, 106, 18, 19) but, for various reasons none have found wide clinical use although some of them have great research value (51). In 1956, Aitken and Preedy described an assay procedure for urinary estrogens involving gradient elution partition chromatography followed by fluorimetry, which later enabled them to measure plasma estrogen levels in normal pregnant women (1).

All methods discussed thus far have mainly concerned themselves with estrone, estradiol and estriol. However, Marrian and co-workers have discovered several other estrogens in the urine. By use of the Kober reaction as proposed by Bauld and Brown, Marrian (99) discovered a new "Kober Chromogen" in the crude estriol fraction. Later by countercurrent partition methods, they separated this substance completely from estriol and isolated a pure crystalline compound. This compound was then shown to be 16-epiestriol, a substance which many years before, had been obtained synthetically from estrone by Huffman. Watson and Marrian, later described a method for its determination(138).

A second Kober chromogen, 16α-hydroxyestrone was discovered and identified by Marrian (100). In late pregnancy urine quantities of 16α-hydroxyestrone ranging from 1-3 mg per twenty-four hours, have been found and it is therefore probable that, from the quantitative point of view, this steroid is as important as estrone. In urines of normal women during the menstrual cycle values ranging from 1-25 μg per twenty-four hours output have been obtained. Biologically (92) estriol and 16α-hydroxyestrone are equipotent when assayed by the vaginal smear test in oophorectomized mice. In this same fraction about 20 per cent of 16-ketoestradiol was found which at first was considered to be an artifact of base rearrangement from 16α-hydroxyestrone. Later, Levitz (87), isolated 16-ketoestradiol-17β as well as 16α-hydroxyestrone using a procedure which could not lead to such a rearrangement. It was, therefore, considered probable that 16β-hydroxyestrone also would exist. Marrian and Layne were indeed able to isolate this compound. The search was facilitated by having available a synthetic 16β-hydroxyestrone which was prepared from estrone, and the finding that 16α-hydroxyestrone, 16β-hydroxyestrone, and 16-ketoestradiol-17β could be easily separated by paper chromatography in a formamide-chloroform system. It is important to note that the three ring D α-ketolic estrogens are unstable to base and any future quantitative method attempting to estimate these compounds must take this fact into account. To date all of the clinical procedures use sodium hydroxide in their isolations. Furthermore, acid hydrolysis largely destroys these labile substances, suggesting the greater use of enzymatic hydrolysis. These ketols are stable in Girard reagent only at room temperature, but are destroyed at elevated temperatures. In base 16β-hydroxyestrone is readily rearranged to 16-ketoestradiol-17β; 16α-hydroxyestrone undergoes the same re-arrangement less rapidly; while 16-ketoestradiol-17β itself becomes auto-oxidized to marrianolic acid.(96).

The prescence of 16-ketoestrone is at the present time still under investigation. However, 2-methoxyestrone has been found by use of countercurrent methods and 18-hydroxyestrone has been detected by partition chromatography. Still another incompletely characterized estrogen which gives a Kober chromogen has been found and designated as KC-6B.

It is a tribute to the Edinburgh group that the methods which it developed has enabled them to detect new estrogens, while the discovery of these new compounds detracts from the usefulness of the Brown and Bauld procedures in giving a complete metabolic picture. Further, this newer work cast much doubt on the large volume of clinical data now at hand and the physiological and pathological conclusions derived therefrom. It would be instructive to compare the methods which have enabled these new compounds to be isolated and characterized. This is done in Table X.

TABLE X

METHODS OF SEPARATION FOR THE NEWER STEROIDS

Compound	Method of detection	Method of separation	Method of Identification	Year
16-epiestriol	Kober	countercurrent column	Comparison to synthetic standard	1955
16-ketoestradiol-17β	Kober specutlation	countercurrent column	Comparison to synthetic standard	1956
16α-hydroxyestrone	Speculation	Isotope dilution methods	Comparison to synthetic standard	1957
16β-hydroxyestrone	Speculation	Paper-chromatography	Comparison to synthetic standard	1958
2-methoxyestrone	Radioactivity compound less polar than estrone	countercurrent	Comparison to synthetic standard	1957
18-hydroxyestrone	Kober more polar than 16α-hydroxy-estrone and 16-ketoestriol	column	Comparison to synthetic standard	1957
KC-6B	Polarity Kober	more soluble in cold chloroform	not completed as yet	1957

A review of the methods of isolation of these new compounds shows that all possible methods of separation have been used for one compound or another ranging from the ease of cold chloroform solubility to the more refined techniques of countercurrent distribution. Consistently, when other methods have failed, countercurrent distribution has enabled investigators to isolate new components, bearing out the great usefulness of any method where a physical parameter related to the molecular structure is easily correlated with the ability to separate. A most fortunate coincidence was the fact that except for 2-methoxyestrone and 18-hydroxyestradiol pure synthetic compounds were already available.

Regarding these new compounds much work must be done in the following directions:

1) The determination of their physiological importance.

2) A further study of their chemistry.

3) A further study of their biochemistry.

4) New methods which will estimate all the important physiological estrogens.

The need for this new work is attested by Marrian (98) who states "It is emphasized that there is an urgent need for accurate and precise methods for the determination in urine for all the .... estrogens which are known.".

3. Contributions of Past Methods

A. Physiologic Levels of the Estrogens in Man

With the advent of the Brown method, the values of normal physiological levels of the estrogens in the human were reinvestigated, and it is generally agreed that the results obtained are the most reliable values published to date. Below is a table of the normal values taken from Loraine (92) and the work of Brown and co-workers (21,22,25). Brown followed the estrogenic excretion pattern in one subject through:

1. a complete menstrual cycle
2. a cycle in which pregnancy had occured
3. pregnancy itself
4. labor and puerperium
5. lactation, and
6. the period of re-establishment of normal menstruation.

The results from this study are shown in Table XI. From this report and others by Brown, some generalizations regarding excretory patterns may be made.

a. During the normal menstrual cycle, the ratio of estriol: estrone: estradiol is approximately 3:2:1. In other words, the sum of the estrone and estradiol excretions per twenty-four hours equals the quantity of estriol excreted.

TABLE XI

MEAN URINARY EXCRETION OF ESTROGENS IN NORMAL SUBJECTS*

(Micrograms/24 hours)

Subject	FEMALE							MALE	
State	Normal Menstrual Cycle			Pregnancy				Menopause	Normal
Substate	onset	peak	luteal	First Missed Cycle	Full Term	Ten Days After Puerperium	Lactation		
Estriol	6	27	22	40	30,000	37	5	3.3	3.5
Estradiol	2	9	7	10	750	0	0	0.6	1.5
Estrone	5	20	14	20	2,000	5	5	2.5	5.4
16-epiestriol					750				
16α-hydroxy-estrone					2,000				

* A composite table from the data compiled by Loraine (92) from the data of Brown (25, 21 and 22).

b. From fertilization to mid-pregnancy, the slope of the rise in excretion, is the same for the three estrogens. The ratio of estriol: estrone: estradiol is 40:4:1, is deduced from this fact.

c. At full term, this ratio changes slightly to 40:2.5:1. Deviation from these normal values and ratios have found wide use in correlating the following pathological conditions (92).

1. Disorders of menstruation
2. Ovarian tumors
3. Fibromyoma of the uterus
4. Genital carcinoma in women
5. Mammary carcinoma
6. Endometriosis
7. Abnormal pregnancy
8. Testicular tumors
9. Adrenocortical tumors and adrenocortical hyperplasia
10. Prostatic carcinoma
11. Liver diseases

B. Contribution of the Past Methods to the Elucidation of Estrogen Biosynthesis and Metabolism

Having available dependable methods of separation and detection investigators were then able to clarify the major pathway of the estrogens. In many of these investigations, the methods of separation most frequently used were those of countercurrent distribution (141, 142, 50), and paper partition chromatography (142, 141, 105). The detection of metabolites was accomplished by a variety of methods. The methods used were, colorimetry (105), ultraviolet spectroscopy(142), infrared spectroscopy (141) and radioassay (50). Using these methods, the major pathway of the estrogens was shown to be intimately related to the fate of the androgens (47).

In 1936, Fieser suggested that testosterone might be converted to the naturally occuring estrogens by demethylation of position 10 and the subsequent rearrangement of ring A (54). Meyer in 1955 (106) incubated 19-hydroxy-Δ^4-androstene-3.17-dione with human placental homogenate and isolated estrone. This conversion was also found to occur in the ovary and the adrenal. In vitro studies have shown that the three major estrogens can arise from testosterone on its incubation with a human ovary (142, 12). The finding of estriol in these studies (142) has been substaintiated by recent work (125), showing the presence of estriol in the ovarian fluid of normally menstruating women.

It was known that acetate and cholesterol were precursors of testosterone, and therefore it might be expected that they also would be involved in the synthesis of estrogens. Acetate on incubation with human embryonal testicular carcinoma preparations, was incorporated into both estradiol and estrone (141). Once estrone has been formed, its reduction to estradiol is easily accomplished by human placental 17β-dehydrogenases (85). Further, it was shown that estradiol on incubation with human liver slices yielded estriol (50).

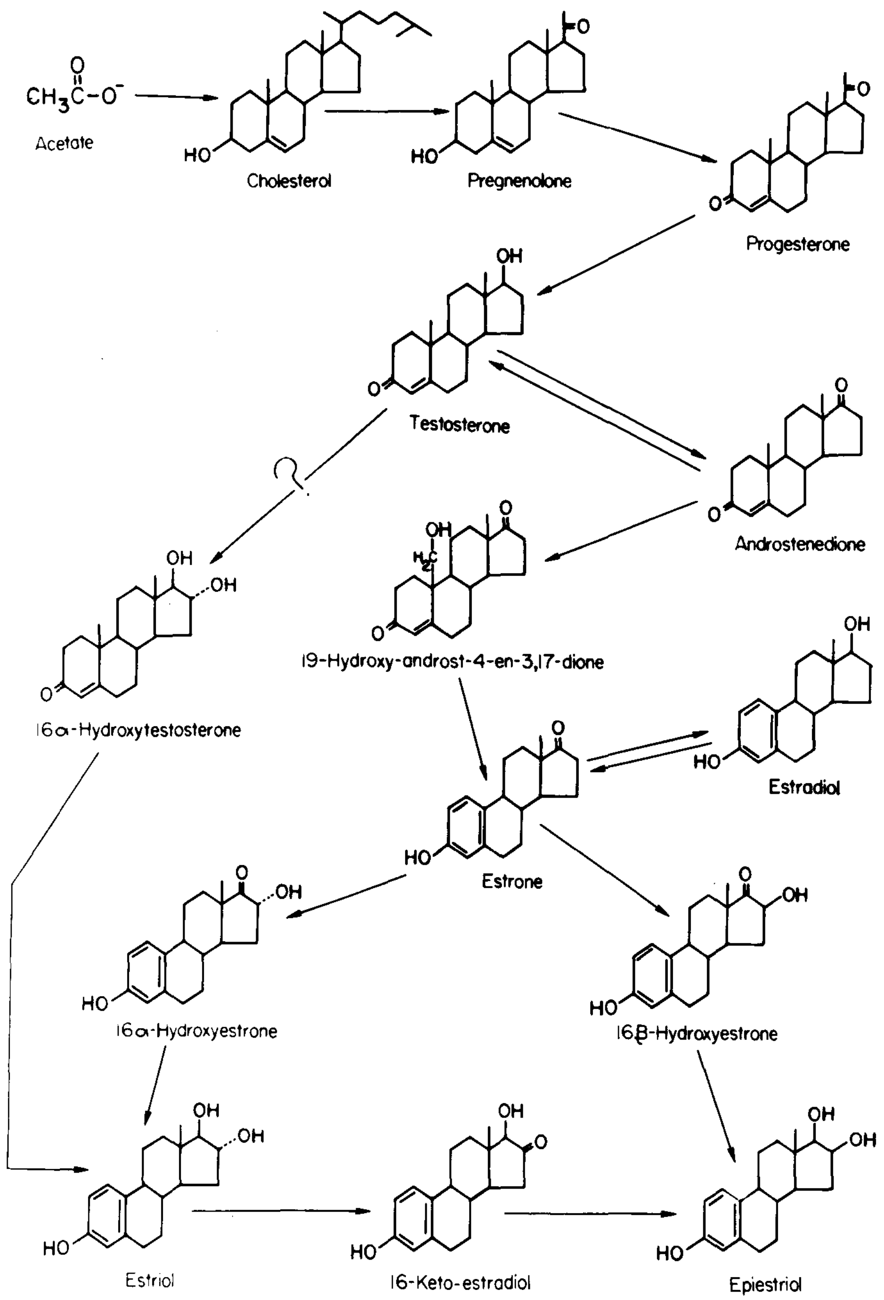
Estriol, however, can arise from a route which is independent of prior phenolic steroids. Ryan (118) on incubating 16α-hydroxytestosterone with human placenta isolated estriol. It is now generally not accepted that the major pathway to estriol is via estradiol. Rather, recent evidence indicates that estrone was converted to 16α-hydroxyestrone, which on incubation with placental tissue yielded estriol (118). Much of this information has been summarized by Marrian (96), who suggested that estrone on 16 hydroxylation gives two products, 16α and 16β-hydroxyestrone, where the latter has been found to be a product of the metabolism of estradiol. Estriol when administered to human subjects was also found to be metabolized to 16-ketoestradiol and 16-epiestriol, with the latter being visualized as a metabolic product of the former.

The metabolism of the estrogens as it appears to date, is summarized in Figure 4.

The pathways of estrogen-metabolism, delineated above, was primarily derived by tedious partition methods applied to urine extracts and tissue extracts. The discovery of new compounds within this scheme was largely based on two phenomena. Those substances having biological activity were, of course, discovered at the earliest. Later, the ability of estrogens to form a Kober chromogen was used to detect further compounds. It is presently recognized that only 30% of administered estrogen and probably of endogenous estrogen can be identified. The possibility then exists that other estrogen metabolites may occur in biological systems which have neither biological activity nor form a Kober chromogen, such as 2-methoxyestrone. The method described here recognizes molecular species not by virtue of a specific functional group or biological activity, but, by the very existance of the entire molecular structure and its' variations in either electronegativity or steric effects. It is, therefore, hoped that the application of gas chromatography to a further study of estrogen metabolism will yield the long sought answer regarding the total metabolic interconversions.

FIGURE 4

BIOSYNTHESIS OF THE ESTROGENS



C. Gas Chromatography

Gas chromatography is a physical method of molecular separation and is a type III interaction of mobile gas phase with either a liquid or a solid. If the interaction is gas-liquid the process is termed <u>gas liquid partition chromatography</u> (GLPC), and if the interaction is gas solid it is termed <u>gas adsorption chromatography</u> (GAC). In GLPC, the liquid phase is supported by an inert porous material with a large surface area. Depending on the nature of the liquid phase, one or more of the mechanisms listed below may be operating in the separation of closely related compounds.

1. Dipolar interactions
2. Steric interactions
3. Electrostatic attractions
4. Induced attractions
5. Weak complex formation.

Following elution from the column a suitable method of detection is used which is sensitive to either a physical, chemical or biological property of the substances being eluted.

The physical arrangement of a gas chromatographic system is represented in Figure 5. The essential components are:

1. a suitable pressure and flow regulating system for the inert gas carrier,
2. heating and heat control units,
3. a volatilizing surface (preheater),

4. a column consisting of:

    a. a porous surface

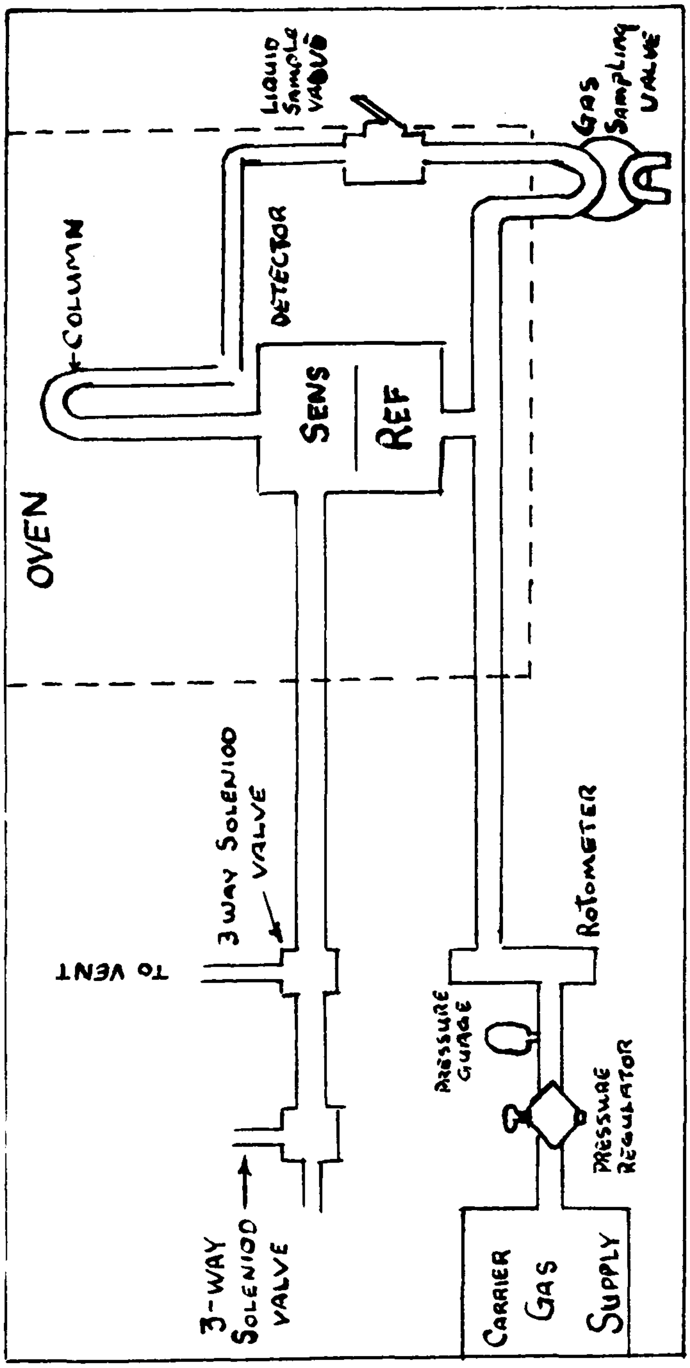
    b. a liquid phase

5. a detecting device

6. an insulated enclosure for items 4 and 5.

GAS CHROMATOGRAPH SCHEMATIC

FIGURE 5



1. Theory of Gas Chromatography and Interrelation of Variables.

For purposes of discussion, the theory is subdivided into:

1. Separation
2. Detection

Factors involved in separation (112,113).

The gas chromatographic column is visualized as consisting of a number of theoretical plates. If the various components of a mixture show varying degrees of interaction with this plate then separation will occur. Consider a component in the vapor phase which has no interaction with the plate. Then, the time at which it emerges from the column is t_o, and this period of time must be equal to the ratio of the volumn of the column to the flow rate:

$$t_o = \frac{\text{vol.}}{\text{vol./min}} = \text{min.}$$

Now consider a compound which shows interaction with the theoretical plate. Its time of emergence is t_a, and period of time is related to t_o by:

$$t_a = t_o + ut_o = t_a = t_o \quad (1 + u)$$

where u is equal to the ratio of molecules in the liquid phase to the number of molecules in the gas phase. The term u is the partition coefficient or liquid-gas equilibrium constant.

The ratio of the velocity of material in the gas phase to the velocity in the liquid phase is the R_f and it is:

$$R_f = p = \frac{t_o}{t_o + t_o u} = (1/1 + u)$$

Thus far only the interaction of one plate has been analyzed. The interaction with a large number of plates has been analyzed by electrical analogs and statistical models. In both cases, elution of a given component was found to be well represented by function of the general type: (62, 113)

$$y = k\ e^{-x^2 h}$$

In the case of the statistical model, the more detailed representation is:

$$y = (2\pi r)^{-1/2}\ e^{-tp^2 r/2}$$

Where y = number of molecules emerging from the column

r = number of theoretical plates

$p = R_f$

t = time difference from the maxima

The value of such a representation is that it enables the investigator to evaluate separation efficiency by calculating the number of theoretical plates.

Concept of the Theoretical Plate

It will be noted that the general equation above is a gaussian curve, symmetrical about zero time. It will further be noted that it is related in the detailed equation to the well known error function (113).

$$y = (2\pi)^{-1/2} \quad e^{-x^2/2}$$

It is further known that the second derivative has points of inflection at $\pm$ 1.

The integrated area under the curve can be approximated by integrating the power series for $e^{-x^2/2}$ (68).

That is to say, the area between +1 and -1 is equivalent to 68 per cent of the total material under the curve.

Reasoning by analogy from the detailed curve given previously the curve must have a minimum and 68 per cent of its area is eluted when:

$$x = 2t_o/p(r)^{1/2}$$

Since x is the time for 68 mole per cent area elution and is equal to $2t_m/(r)^{1/2}$ the number of theoretical plates r equals $4t^2/x^2$. Many variations of this formula exist in the literature (112). The one most commonly used approximates the number of plates by:

$$r = 16t^2/x^2$$

There is value in knowing the change of the number of plates with varying conditions, since information allows the investigator a rational basis for developing optimal conditions for separation. This point will be illustrated by a consideration of the Van Deemter relations(112).

Knowing the length of the column and the number of theoretical plates, the height equivalent to a theoretical plate (HETP) is easily evaluated.

It is apparant that the more efficient the column is the smaller is the HETP.

During the separation in a GLPC column, there are three mechanisms operating:

1. eddy diffusion - given as A
2. molecular diffusion - given as B
3. resistance to mass transfer - given as C.

Both the process of molecular diffusion and resistence to mass transfer are a function of carrier gas velocity (Z).

$$\text{then: HETP} = A + B/Z + CZ$$

Knowing that the HETP must be at a minimum for maximum efficiency the equation can be differentiated and solved for the minimum to show the best conditions. The results of the Van Deemter considerations are shown in the following Table XII.

TABLE XII

VAN DEEMTER PREDICTIONS

Change	Effect on HETP
Particle size of support and geometry	
1. increase	?
2. spherical	decrease
Carrier gas	
1. increase density	decrease
2. increase pressure	decrease
3. increase flow rate	minimum effect at optimum flow rate
Temperature	
1. increase	?
Liquid Phase	
1. Decrease of phase	decrease
2. Decrease viscosity	decrease

The variables effecting changes in the retention times.

Thus far only the variables effecting the separation have been considered, and it now remains to consider the variables which effect the retention time.

It will be recalled that for one theoretical plate and no interaction the retenion time is given by:

$$t_o = \frac{\text{volume}}{\text{flow rate}}$$

also,

volume = Area x length

and thus, tr_o, (the retention time with no column interaction).

$$Tr_o = \frac{\text{Area x length}}{\text{flow rate}}$$

if interaction occurs, then Tr_a (the retention time with column interaction)

$Tr_a = t_o\ (1+u)$ Note: Where u is an equilibrium constant and since it is usually much greater than 1 - this equation reduces to:

$$Tr_a = t_o u$$

Its dependence on temperature is given by

$u = B\ e{-q/RT}$ where -q is the heat of solution.

if

$$Tr_a = t_o u$$

$$\therefore \quad Tr_a = \frac{\text{Area x length}}{\text{flow rate}}\ B\ e^{-q/RT}$$

This expression states:

1. Retention time is inversely proportional to flow rate.

2. Retention time is directly proportional to column length.

3. Retention time is exponentially proportional to temperature.

4. Retention time is exponentially proportional to heat of solution

Summarizing then, the magnitude of separation is dependent on the magnitude of the differences of u, and the number of theoretical plates in the column.

2. Detection (112,113)

Compounds are detected on the basis of some chemical or physical property which is converted to a voltage, which after amplification is sufficient to drive the pen motor of a recorder. In this work only two types of detectors were used, the thermal conductivity and Sr^{90} ionization detectors.

The physical property, which is the basis for thermal conductivity detectors is the heat capacity (C_v). The energy of a colliding molecule on a surface is 3/2 RT.

But, the specific heat of any substance is equal to the quantity of heat required to raise the temperature of 1 gram of the material 1 degree.

$$C_v = E = Ek_1 - Ek_2 = 3/2R\ (T_2 - T_1)$$

$$\text{where } T_2 - T_1 = 1$$

$$C_v = 3/2R$$

The thermal conductivity (T_c) = C_v (γ), where γ is the viscosity of the gas mixture. As the gas or vapor emerges from the column it will remove a certain amount of heat from a heat source, depending on its heat capacity. If the heat source is coupled to a Wheatstone bridge where a change of temperature represents a change in resistance, then this change can be amplified to drive a recorder. The bridge is represented in Figure 6.

The sensing element, a thermistor, is a heat sensitive resistor, exhibiting a negative coefficient of resistance of about 4 per cent per degree.

Thus the higher the temperature rises the lower the resistance falls.

Refering to the diagram of the Wheatstone bridge, the two thermistors A and B are heated by the imposed voltage, where TA is entirely in an atmosphere of the eluting gas and TB is exposed to mixture of the eluting gas and the vapor. TB is now cooled at a rate different than A, the resistances do not match, and current flows in the bridge.

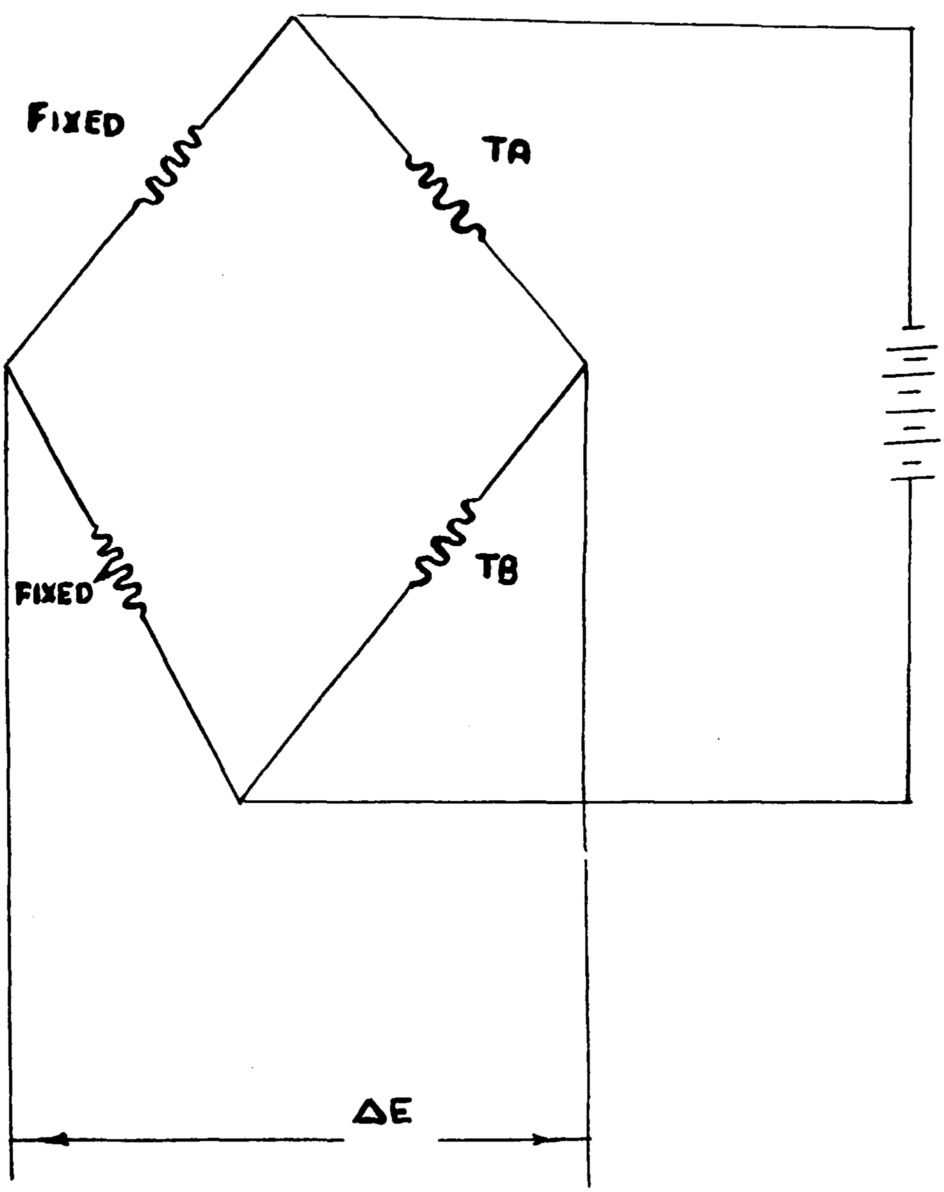
It is important to note that the sensitivity will be a function of the difference of the thermal conductivities. Thus for maximum sensitivity the gas choosen should be as different as possible from the organic moiety. Although hydrogen gas is the ideal choice, a consideration of safety has made helium the most commonly used gas in determining organic compounds. These detectors are sensitive to 10^{-10} moles of organic vapor up to about 225 C.

The ionization detectors are far more sensitive than the thermal conductivity detectors. The literature records sensitivities of the order of 10^{-15} moles (89).

This detector consists of a small metal ionization chanber with an internal volume from 3-10 mls. to which a high potential is applied. When argon is used as the carrier gas, the alpha or beta particle from radium D or Sr^{90} causes primary ionization (88) of the gas, and the electron which has been set free is accelerated in the applied potential to velocities sufficiently high to excite a large number of other argon molecules to their metastable state. As the organic vapor emerges from the column, the molecules collide with exited argon molecules. Since the ionization energies of such molecules are usually lower (although alkyl chlorides and others are higher) than the excitation potential of the metastable state (11.6 ev.), transfer of energy from argon to the organic vapor with subsequent ionization of the latter occurs. The secondary electrons produced in this process, are collected at the anode, giving rise to an ionization current which is proportional to the concentration of the vapor in the chamber. These secondary electrons also generate more metastable argon atoms, replacing those lost on collision with organic vapor.

FIGURE 6

THERMAL CONDUCTIVITY CELL



The sequence of events described, can be summarized as follows:

$Argon^{0} + \alpha$ or β ray $\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow Argon^{+} + e^{-}$ (primary)

$Argon^{0} + e^{-}$ + voltage $\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow Argon^{*}$(11.6ev) matastable

$Argon^{*}$ + organic material $\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow Argon^{+} + e^{-}$ (secondary)

e^{-} (secondary) = $Argon^{0}$ + voltage $\rightarrow\rightarrow$ $Argon^{*}$

e^{-} (secondary) $\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow\rightarrow$ Current in milliamps

At present, the limitation of this type of detector is due to the low ionization potential of metastable argon, and the temperature limit is about 450°C, using sapphire connections from the column to the cell. The temperature limitation is not serious since most compounds of interest will separate below this temperature. The limitation of the low ionization of argon (metastable) is more serious when the partial table below is considered (68).

TABLE XIII

IONIZATION POTENTIALS FOR VARIOUS COMPOUNDS

Compound	Ionization potential (EV)
Helium	24.6
Hydrogen	15.7
Nitrogen	15.6
Carbon Dioxide	14.4
Methane	14.4
Water	12.6
Oxygen	12.5
Acetylene	12.3
Cyclopropane	11.8
Ethylene	11.5
Argon (metastable)	11.5

Many other detector systems have been applied to gas chromatography. Below is a list of these detectors:

TABLE XIV

DETECTORS IN GAS CHROMATOGRAPHY

Detector System	Reference
Vapor density balance	75
Viscosity	76
Photometric titration	77
Surface potential	63
Dielectric constant	63
Heat of adsorption	139
Infrared absorption spectroscopy	103
Hydrogen flame ionization	95
Ionization	93
Radium D	
Krypton ^{85}	
Time-of-flight Mass Spectrometry	61
Electron Acceleration	119
Scintillation counting for radio-active compounds	80
Biologic	46

D. Physical Properties of the Estrogens and Feasibility of their Separation

Prior to undertaking the actual separation of the estrogens by gas chromatography, a number of calculations were performed to show feasibility of the method as applied to compounds of high molecular weight.

A review of the literature at the beginning of this investigation showed that except for one report on the identification of steroids by gas chromatography of the pyrolyzed side chain (32), there had been no attempt to separate the different steroids as such. There was, however, one report on the separation of the resin acids, which are polynuclear compounds related to the steroids. This separation was accomplished by separating the methyl esters of the acids (69). Since the start of the investigation, there have been three reports (133, 14, 130) on the separation of cholesterol and cholesterol derivatives, as well as some hormones. In one (133) of the papers, the following substances were reported to have been separated: androstane, allopregnane-3, 20 dione, pregnane-3, 20 dione, coprostane, cholestane, stigmastane, cholesterol, cholestan-3-one and stigmasterol. These separations were accomplished on a 7% silicone column at 260°C. It is important to note that a three minute difference was reported for the separation of coprostane and cholestant on an GE methyl silicone polymer SE-30. In still another report (14) short columns (3 ft.) were used at 280°C for the separation of cholesterol derivatives, and in this report infra-red data is offered as proof of compound stability. To date, However, there has been no report in the literature on the separation of the estrogens by gas chromatography nor has there been any attempt to develop a quantitative method for these compounds or other steroids which have been separated by gas chromatography.

In any separation by gas chromatography, there are two primary considerations. First, the compounds must be volatile and secondly, they must be stable at the temperature at which volatilization occurs.

From the data available at the time this investigation was begun, it appeared that the estrogens had a measurable sublimation vapor pressure, since both Butenandt and Girard had purified free estrogens by sublimation (28, 55). However, since these compounds have alcoholic and phenolic functional groups, it was reasoned that extra energy would be needed to overcome hydrogen-bonding forces which would require higher heats of vaporization than less polar derivatives. These energies can sometimes be of considerable magnitude as for example in comparing the heat of vaporization per gram of n-decane to that of methanol (34).

Compound	MW	Heat of Vaporization per gram	Heat of vaporization per mole
n-decane	142	60.2	8548
methanol	32	263.0	8416

Therefore, a search of the literature was made for the physical properties of the derivatives of the estrogens (60).

Compound	Boiling Point	Pressure mm of Hg.
estradiol divalerate	225^{o}	0.005
estradiol dicaproate	230^{o}	0.0005
estriol triacetate	210^{o}	0.03

From the data for estriol triacetate, it was possible to calculate its vapor pressure at various temperatures within the operating limits of commercial gas chromatographs, and knowing the sensitivities of the detectors, it was possible to determine if the amount volatilized would be sufficient to give a signal on the recorder. The method of approximation used was that of Haas and Newton (65).

TABLE XV

THE CALCULATED VAPOR PRESSURES OF THE ESTROGEN ACETATES FROM 230^{o} - 280^{o} C.

	VAPOR PRESSURE x 10^{-3} mm of Hg.		
Temperature	Estrone acetate	Estradiol diacetate/	Estriol triacetate
230^{o}	5.75	3.9	1.02
240^{o}	13.8	7.25	2.4
250^{o}	24.0	12.5	3.8
260^{o}	40.6	22.4	6.6
270^{o}	69.0	38.0	13.5
280^{o}	110.0	63.0	22.0

At 280^{o} C 10^{-1} to 10^{-2} mm of estrogen would be in the vapor state and assuming a detector cell with 5cc volume, there would be from 3×10^{-9} to 3×10^{-10} moles of these compounds in the detector. The Lovelock detector was reported to be sensitive to 10^{-15} moles of substance (89). Further, it was expected that after volatilization there would be few molecules in equilibrium with the liquid phase because of the fast flow rate of the eluting gas. This will result in an even higher concentration than the calculated value. This then suggested that the estrogen acetates were sufficiently volatile to be detected.

It would seem that since estriol triacetate was purified by distillation, that it might be stable at the temperatures necessary for gas chromatography. A review of the literature indicated that in the case of estradiol (72) dehydrogenation might be expected since it was reported that secondary alcohols easily pyrolyze to ketones. The model system of isopropyl alcohol had been studied by Parks and Kelley (110) showing a Δ F of-297 calories starting at 225^{o}C. Also, at the beginning of this investigation the most stable liquid phase available was silicone grease (33), which is relatively non-polar.

From these preliminary considerations and by calculation, the following was concluded:

1. The estrogen acetates were volatile.
2. The free estrogens were volatile, or at least had a sublimation vapor pressure.

3. The estrogen acetates were probably stable, since estriol triacetate had withstood distillation at reduced pressure.
4. Estradiol might show a tendency to dehydrogenate.
5. The steroid acetates could be measured by conventional gas chromatographic detectors.
6. The acetates might be more desirable, since the thermally most stable liquid phase available was non-polar.
7. Relative to the free estrogens the acetates might be more stable since the 17-hydroxyl group was no longer free to dehydrogenate.

On the basis of what was know about these compounds it would seem that the acetates were indeed the compounds of choice and some predictions concerning their separation could be made on the basis of what was known of their physical properties and the theory of gas chromatographic separation.

In a non-polar phase such as silicones, the non-polar vapors will have heats of solution, assuming it to be an ideal solution which can be substituted for heats of vaporization (114), since the interaction between vapor and liquid phase will be very similar to the interaction of the vapor molecules among themselves. In this case it is expected that the compounds will leave the column approximately in the order of their boiling points. Such a sequence is followed closely in practice (74). Polar vapors, however, will pass through the column more rapidly than non-polar vapors of the same boiling point, as they will have lost the strong dipole-dipole association energy which is present in their own liquid.

Thus, on a column of silicone at 100°C, ethyl acetate (bp. 77.2°C) is eluted between the peak of n-propyl alcohol (bp. 97.2°C) and isobutyl alcohol (bp. 108.1°C).

From this it can be appreciated that the acetates are more desirable than the alcohols when a greater degree of separation is desired using the same column length and equal conditions (90).

In the consideration of the factors which effect the retention time, the following equation was derived:

$$t_r = \frac{\text{length x area x a constant}}{\text{flow rate}} \quad e^{\frac{-Q}{RT}}$$

Where Q = heat of vaporization, and RT have their usual meanings, taking the log:

$$\ln t_r = \frac{-Q}{RT} + K$$

From this equation it appears that the log of the retention time will be proportional to the heat of vaporization of the compounds if ideal solution is also assumed. For the estrogen acetates the heat of vaporization can be estimated from the BP or by assuming that Trouton's constant holds for this series.

$$\frac{\text{Heat of vaporization}}{\text{Boiling point}} = \text{Trouton's constant}$$

In order to evaluate the boiling points of the acetates the data (60) for estriol triacetate was used to determine its boiling point at 760 mm, by the method of Haas and Newton (65).

$$T= \frac{(273+t)\ (2.88-\log p)}{\phi+(0.15)\ (2.88-\log p)}$$

Where $\underline{p}$ is the vapor pressure and

$\underline{\phi}$ is the entropy of vaporization

T=437, and BP=647^{o}C for estriol triacetate

Since no such data are available for estrone acetate or estradiol diacetate, the value of the boiling point for estriol triacetate was used to determine the boiling point numbers of a steroid nucleus to which molecular increments could be added in order to evaluate the boiling points of the estrone and estradiol acetates. The method used was that of Herbrandson and Nachod (17).

In this approximation the boiling point number (BPN) is given by:

$$\log BPN = 3\ (\log)\ (bp + 543) - (2.3620)$$

$$\log BPN = 2.1465$$

$$BPN = 138.2 \text{ for estriol triacetate}$$

The increments used were:

acetate = 10.65

ketone = 6.5

hydrogen= 1.0

BPN of a standard nucleus = 106.25

BPN of estrone monoacetate = 123.40

BPN of estradiol diacetate = 128.55

The boiling point is then given by:

$$BP = 230.14\ (BPN)^{1/3} - 543$$

Compound	Boiling Point
estriol triacetate	646°C
estradiol diacetate	617°C
estrone monoacetate	601°C

Since the determination of the boiling points was dependent on the addition of the molecular weights, as a first approximation the molecular weights can be substituted into the relation for the retention time:

$$\ln t_r = \frac{MWT}{RT} + K$$

From these relations it was concluded that separation is exponentially proportional to the molecular weight differences, and again it will be noted that the acetates have a great advantage over the free estrogens or the ethers in this respect. The greater the molecular weight difference the shorter a column may be used at a high flow rate. Since these compounds have a tendency to decompose a short column minimizes the heat exposure time and thereby minimizes decomposition. It could be concluded that the estrogen acetates could be detected and separated using short columns with high flow rates at 280°C.

With the derivations developed thus far and the data from the literature or calculation it was possible to predict the ratios of the retention times and to predict detector sensitivities to the acetates. In order to evaluate the relative retention times the calculated boiling points are considered as a linear function of the heat of vaporization, and the following simplification is made:

$$t_r = K\ e^z$$

where z = the ratio of the BP or MWT as scaled factors:

The predicted and experimentally determined retention time ratios are show in the following table:

TABLE XVI

COMPARISON OF PREDICTED AND DETERMINED RETENTION TIMES

Compound	Predicted from BP	Predicted from MWT	Found
Estrone acetate	1	1	1
Estradiol diacetate	1.4	1.5	1.46
Estriol triacetate	2.72	2.72	2.74

It would appear that the theory agrees well, with the experimental values. Once the retention time ratios had been determined, the assumption of detector linearity could then be used to determine the sensitivity of the detector for these compounds. The validity of the assumption is attested to by Lovelock (93) for the S_r^{90} detector and others have shown the linearity of the thermal conductivity detector (117). It will be recalled from the section on the theory of separation that Golay had shown that the eluted peak could be adequately represented by the general function:

$$P = K\ e^{-X^2 h}$$

where p is the only new term and it is a function of concentration.

At the maximum p=K. This is interpreted to mean that at the maximum, the signal on the recorder is a function of the rate at which the molecules pass through the detector. This rate of passage is a function of the retention time as previously discussed. Also, for an equiweight mixture, the number of molecules passing through the detector is a function of the molecular weight.

The completed form may be written as:

$$(1)\quad p = \frac{K}{MWT \times RT_r}\ e^{-X^2 RT}$$

for an equiweight mixture,

and

$$(2)\quad p = \frac{K}{RT_r}\ e^{-X^2 RT}$$

for an equimolar mixture.

These equations may now be used to predict both the separation and the sensitivities of the detector to the estrogen acetates.

The necessary information is given below:

Compound	MWT	Rel.MWT	Rel.BP
estrone acetate	312	1	1
estradiol diacetate	356	1.14	1.4
estriol triacetate	415	1.33	2.72

Using this data the following sensitivities may be calculated from the equations.

Compound	Predicted Sensitivity	Found experimental sensitivity	
		slope	actual
estrone acetate	1	1	1
estradiol diacetate	0.605	0.60	0.64
estriol triacetate	0.27	0.22	0.24

The theory is in good agreement with the experimental values. Utilizing all of the data derived thus far the theoretical curve (Figure 7) shown is derived from the calculations in Table XVII.

Comparison of theoretical values with experimental one (Figure 21) for an equiweight mixture, shows good correlation. One may also predict that estrone acetate and estradiol diacetate will show an unresolved tailing effect which was indeed noted in all the records at fast flow rates. One may anticipate that the theory, calculated from molecular weight alone, enables the investigator to predict most of the parameters for the detection and separation of a closely related series of steroids.

TABLE XVII

CALCULATED CHROMATOGRAM

Estrone Acetate	Estradiol Diacetate	Estriol Triacetate	Estrone Acetate	Estradiol Diacetate	Estriol Triacetate
X	X x 1.4	X x 2.72	p	p	p
0.0	0	0	51	31	14
0.2	0.28	0.54	49	29	12.5
0.4	0.56	1.10	43	25	9.1
0.6	0.83	1.63	35	18.5	5.3
0.8	1.10	2.10	26.5	12.2	2.5
1.0	1.40	2.72	18	7.5	0.83
1.4	1.96	----	6.6	1.8	----
1.73	----	----	2.5	----	----

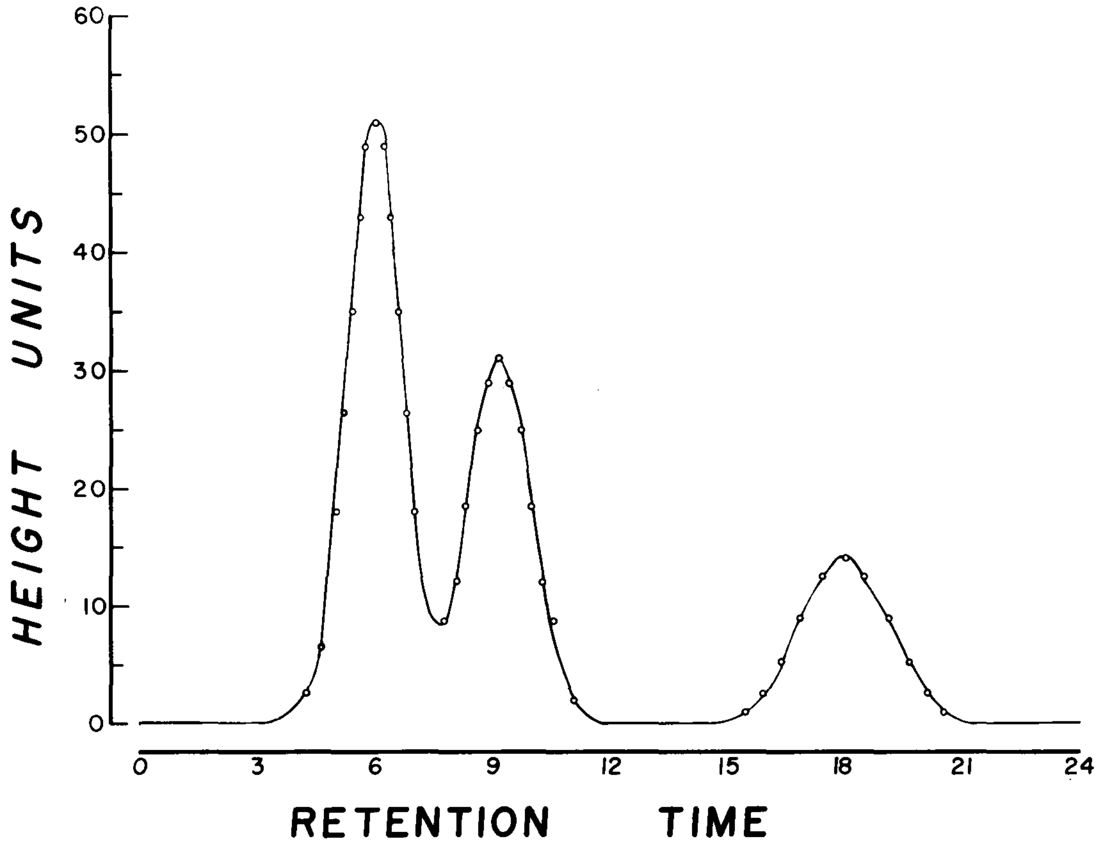
Legend

X = distance from maximum

p = peak height, (set to 51 in order to compare with Figure 21)

FIGURE 7

CALCULATED CHROMATOGRAM



II EXPERIMENTAL

Purification of Estradiol

A preliminary paper chromatogram of starting material-(Schering, A.G., Berlin) USP estradiol-17β having a melting point of 177°-178°C and an absorption maximum at 280 mμ of 2640-2690-showed it to be free of other phenols which could be separated in a propylene glycol-toluene (78). The infra-red spectrum of the melted material was identical to those on file at the spectroscopic division of this laboratory (127). The table below (XVIII) records the physical properties of the starting material.

TABLE XVIII

PHYSICAL PROPERTIES OF ESTRADIOL 17β

Property	Found	Literature Value	Reference
Melting Point	177°-178°	173°-179°	(42)
Rotation	+ 80	+ 76 - +83	(42)
UV max.	280 mμ	280 mμ	(45)
E max.	2640-2690	1760-2060	(45)
IR	identical	identical	(127)

This material was further purified by sublimation at 150°C and 0.02 mm. Hg, and showed an extinction coefficient at 280 mμ of 2900. Although this value was quite high compared to that reported in the literature, molecular changes were not suspected since at the temperature of sublimation no decomposition was found during a sealed vial reaction over a period of forty-eight hours.

A. Thermal Stability of Estradiol

1. Open System

Into tubes of approximately 8 inch length was added estradiol (10 mg.). The tubes were then evacuated to a pressure of 0.02 mm. of Hg and heated to a temperature of 100°C to 200°C. Under these conditions estradiol migrated upwards to a new location. Longer glass tubes (4-5') were heated with the steroid under a nitrogen atmosphere while at the outlet of the tube the material was trapped in absolute ethanol. This material was then analyzed by the Kober reaction.

In the pyrolysis experiments using the short tubes, it was found that estradiol sublimed at reduced pressure (0.02 mm of Hg) at temperatures of 100°-200°C without detectable decomposition. Using long columns with nitrogen and an ethanol trap it was seen that estradiol migrated into the trap. Again no visible decomposition was noted at 100° - 200° C, but at 300° - 400° C color changes were noted. At the lower temperatures and in the presence of oxygen decomposition proceeded readily.

Estradiol in the presence of air at 250°C gave a yellow compound and estrone produced a red compound which may be a polymeric quinone(55).

2. Closed System (Isotenoscope)

The thermal decomposition of estradiol was followed by measuring the changes in vapor pressure in an evacuated all glass monostat, called an isotenoscope(79,36). The basic vacuum system in shown in Figure 8 and the isotenoscope is shown i n Figure 9. Sublimation purified estradiol (= 400 mg) was introduced into the monometer at point A (Figure 9) and the apparatus was sealed at B. The isotenoscope was attached to the vacuum system as indicated in Figure 9 at position D. The pump was started and both sides of the monometer were brought to a reduced pressure simultaneously. The part of the monometer containing the isotenoscope was totally submerged in a lead-tin bath and heated to the melting point of estradiol and maintained at this temperature for fifteen minutes. This procedure insured that water and trapped air was removed prior to sealing the tube at point C. The temperature was allowed to return to room temperature and the side arm was sealed at C. The apparatus was brought to various temperature levels and after reaching equilibrium, the displacement of the needle E was corrected by leaking air through the vacuum system as shown in Figure 8. The changes in pressure to balance the indicator inside the isotenoscope was determined from the reading of the absolute manometer.

FIGURE 8

VACUUM SYSTEM

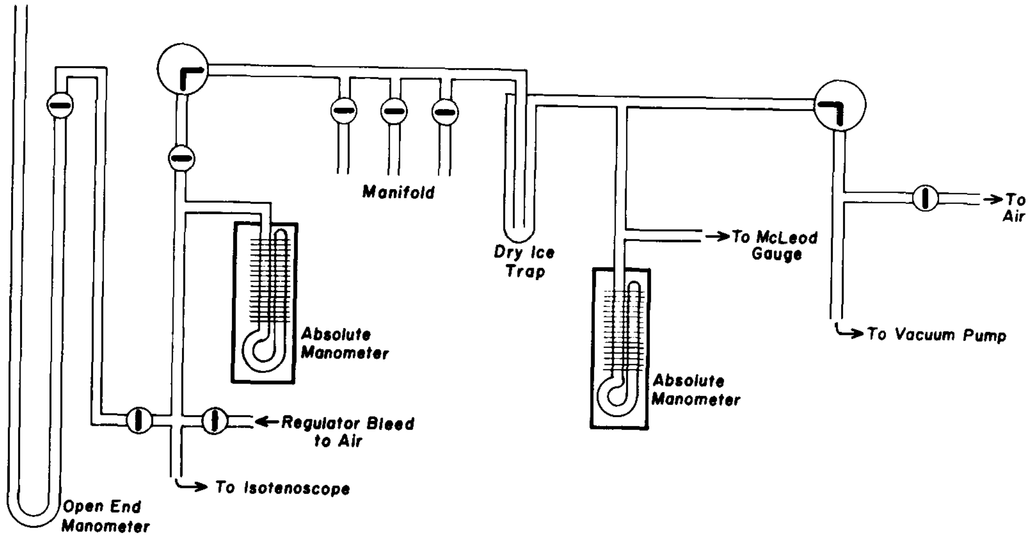
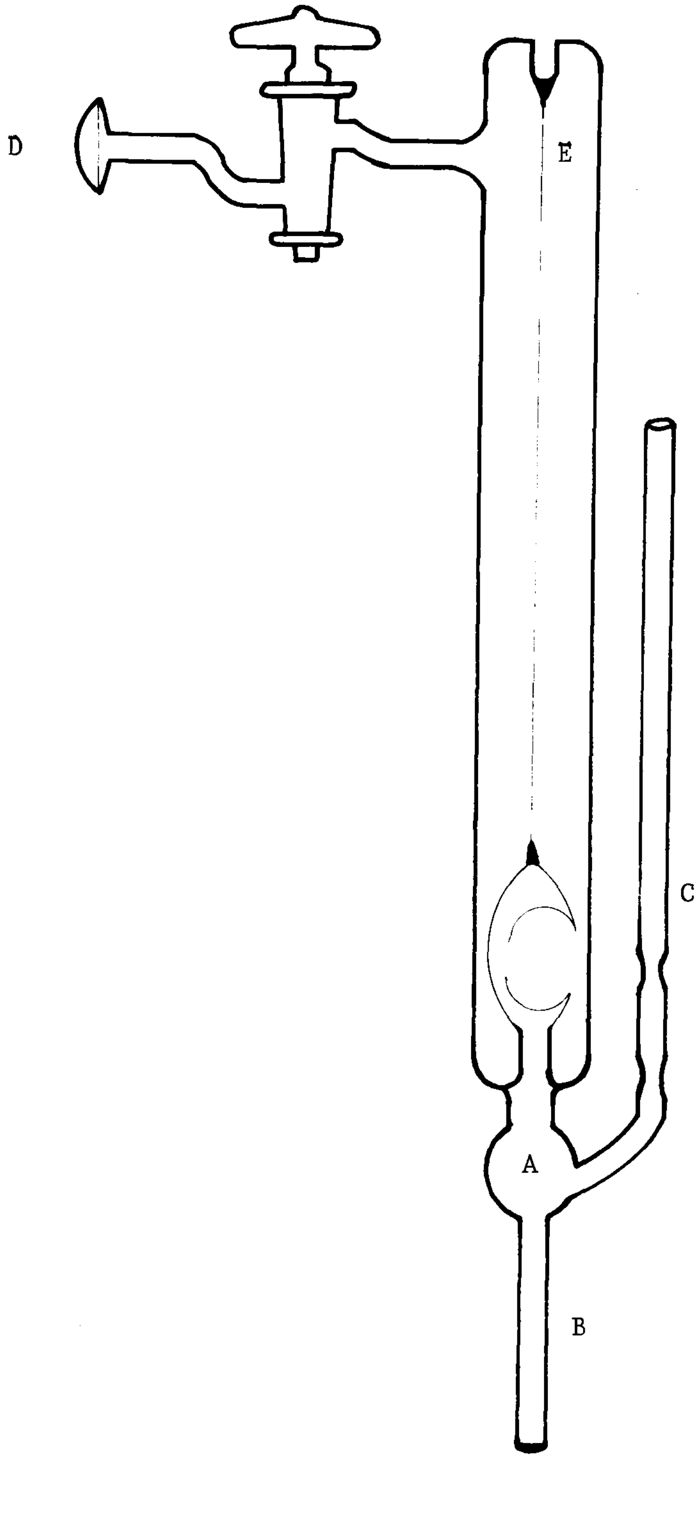


FIGURE 9

ISOTENOSCOPE (79) DESIGN OF I. B. JOHNS



Purified estradiol was introduced into the isotenoscope and heated at low pressure. No thermal decomposition was noticed at 230°C.

Conditions	Run 1	Run 2
Sample size	425 mg	390 mg
Volume of Vessel	16 cc	16 cc
Pressure	0.025 mm Hg	0.02 mm Hg

Data for the individual decompositions in given below:

TABLE XIX

DECOMPOSITION OF ESTRADIOL

Temperature° C	mm Hg	Minutes	Slope mm Hg / min.
275	0	0	0.40
	2	5	
	4	10	
287	2.5	10	0.432
	7.5	19	
	11.5	27	
	16.5	36	
	21.5	47	
	24.5	58	
317	5	10	0.488
	17	35	
	25	50	
	33	65	

TABLE XIX

DECOMPOSITION OF ESTRADIOL
(Continued)

Temperature° C	mm Hg	Minutes	Slope mm Hg / min.
335	0	20	0.93
	4	25	
	8.5	30	
	13	35	
	18	40	
	28	50	
	34	56.5	
	38	60	
347	4	4	0.96
	9.5	8	
	13.5	14	
	18.0	19.0	
	25.0	26.0	
375	32	2	16.0
	48	3	
	66	4	
	90	5.5	

TABLE XX

RETENTION TIMES OF GASES

Gas	Retention times (minutes)
Hydrogen	0.75
Methane	2.00
Nitrogen	1.50

The chromatogram was developed for ten minutes in order to determine any other gases. None were detected after nitrogen. Only hydrogen was found. The peak for hydrogen was negative due to its thermal conductivity difference relative to helium (Figure 11).

At the end of the experiment the residue was isolated and an infra-red spectrum showed a new absorption maximum at 5.8 μ which is known to be a carbonyl frequency.

B. Identification of Decomposition Products of Estradiol

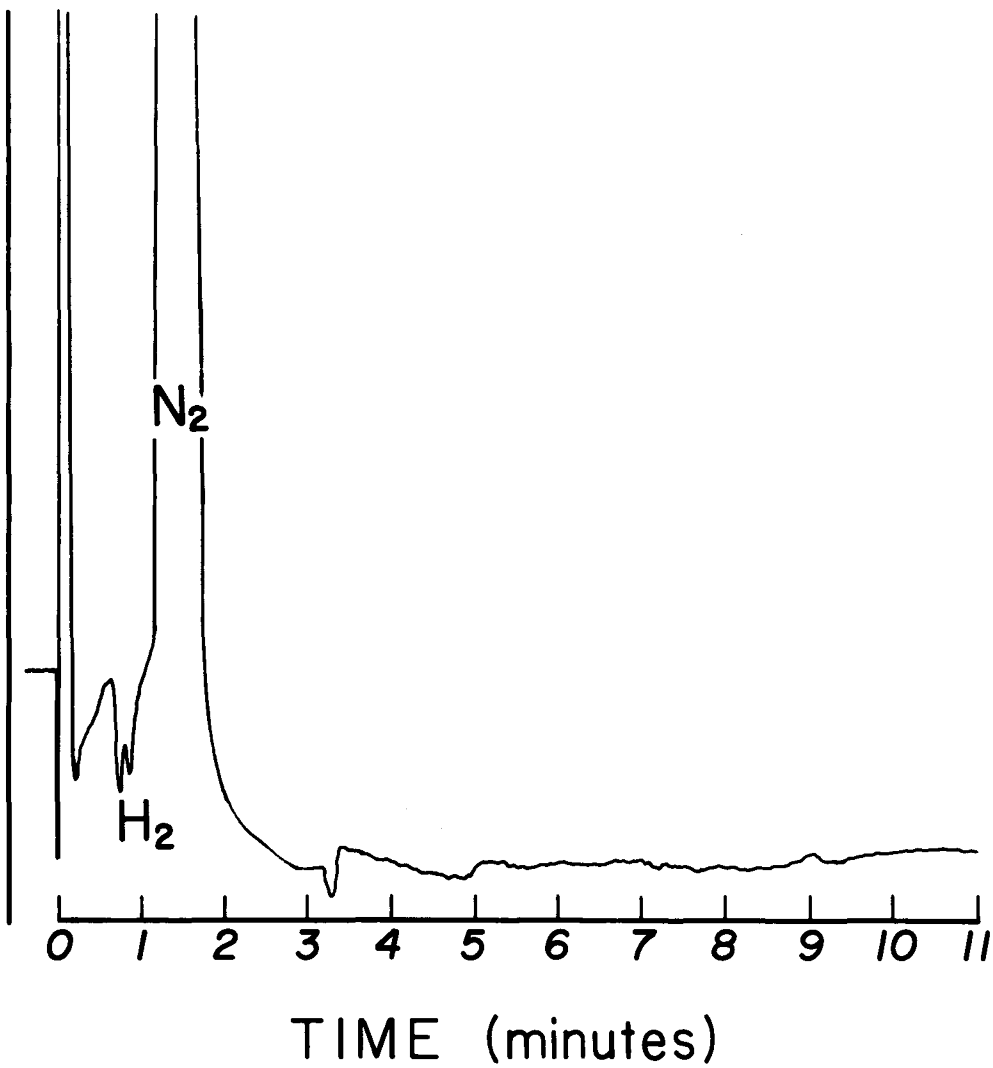
1. Gases

The gaseous material from the sealed tube reaction was transfered to an evacuated 200 cc gas flask which was allowed to come to atmospheric pressure. This gas mixture was then displaced by mercury into the gas inlet of the gas chromatograph. Using a silicagel column (Figure III) which had been calibrated for hydrogen, methane and nitrogen, the identity of the gas formed was determined by retention time and peak reversal.

The gaseous product was chromatographed using a properly conditioned 6 foot Silica column at 25^{o}C with a flow rate of 25 cc of helium per minute. The retention times for hydrogen, methane and nitrogen were determined (Table XX).

FIGURE 11

ESTROGEN BY GAS CHROMATOGRAPHY



2. Solid Residue

Solid reaction products were submitted to paper and column chromatography, and attempts to partially characterize the compounds by ultraviolet and infra-red absorption spectroscopy were made. The paper chromatographic system used throughout this work was that of Jellinck (78).

A plot of the slopes (Table XXI) against the reciprocal of the absolute temperature (Figure 30) suggests that up to 317°C only one reaction occurs. Assuming this to be C-17 dehydrogenation the expected product was estrone.

In order to acquire sufficient quantities of the decomposition products the following reaction was repeated several times.

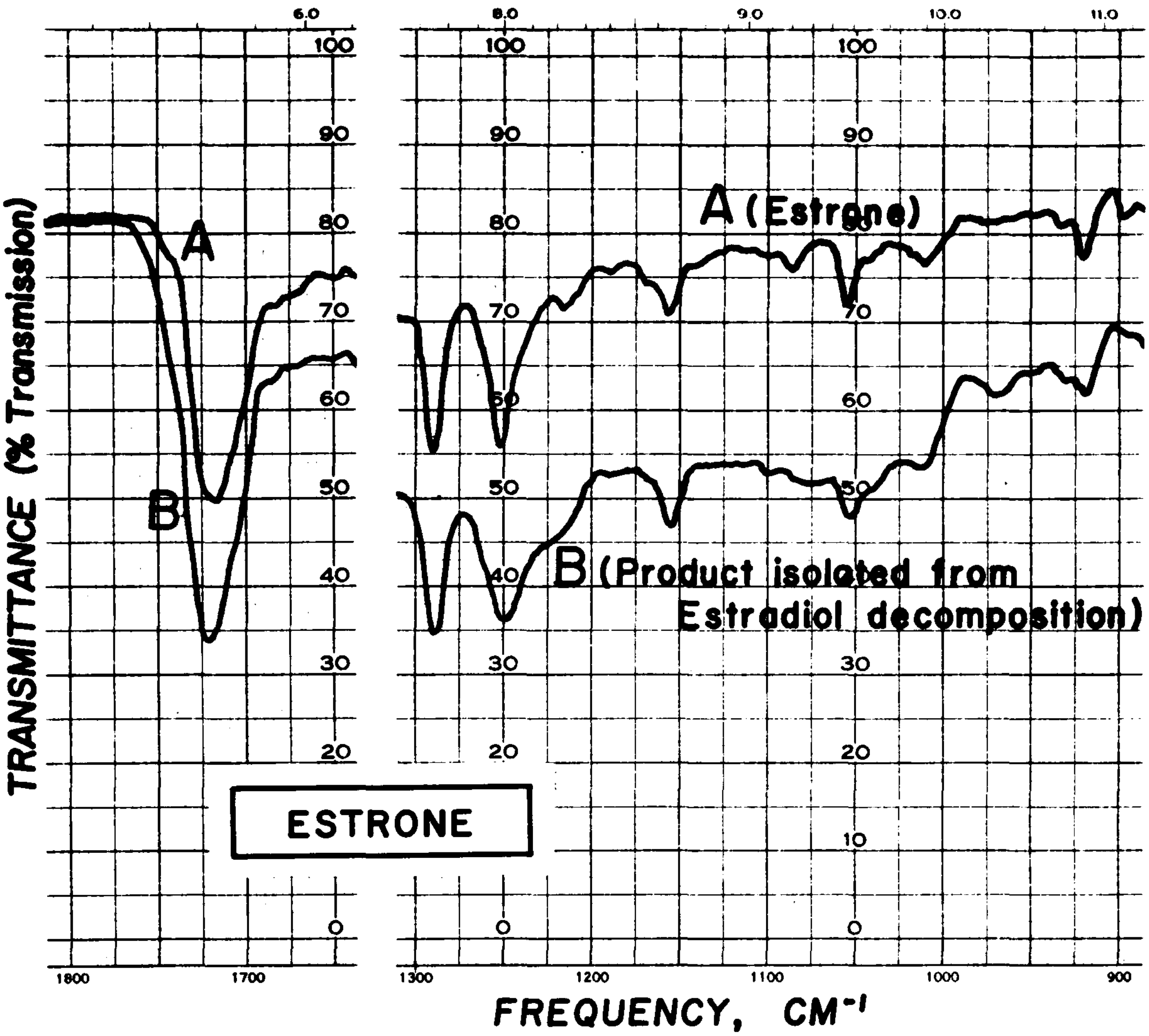
Vessels of the same volume as the monometer (16-18 cc) were prepared. Estradiol (50 mg) was introduced and after allowing for the evolution of trapped gases, the tube was sealed under the same conditions as before. The tubes were totally submerged in the lead-tin bath at various temperatures.

The contents of the tubes was dissolved in ethanol, reduced in volume and applied to a 16 cm paper strip. After development in a propylene glycol-toluene system a Zimmermann positive zone was found and eluted from the paper. This material was concentrated to about 0.5 cc of alcohol and water was added to precipitate out the steroid.

The mixture was centrifuged and the supernatant layer discarded. This procedure was repeated several times. The material finally obtained was dried and an infra-red spectrum was recorded. A comparison of the infra-red spectrum of this substance and that of authentic estrone is shown in Figure 12. Paper chromatograms showed that estrone traveled 17.8 cm in five hours and the sample showed a component at 18.4 cm in the same time. Addition of estrone to the sample chromatogram showed one single band at 18.0 cm. From this data it seemed plausible to conclude that estrone was primary product of decomposition at this temperature. This was further substantiated by infra-red analysis although the presence of a small quantity of equilin can not be ruled out. In order to determine the products formed at higher temperatures a series of closed tube pyrolyses were carried out at 400°C for varying times and the products analyzed by ultraviolet spectroscopy. These results are shown in Table XXII.

INFRARED SPECTRUM OF A DECOMPOSITION PRODUCT OF ESTRADIOL

FIGURE 12



ULTRA-VIOLET SPECTRUM OF A DECOMPOSITION PRODUCT OF ESTRADIOL

FIGURE 13

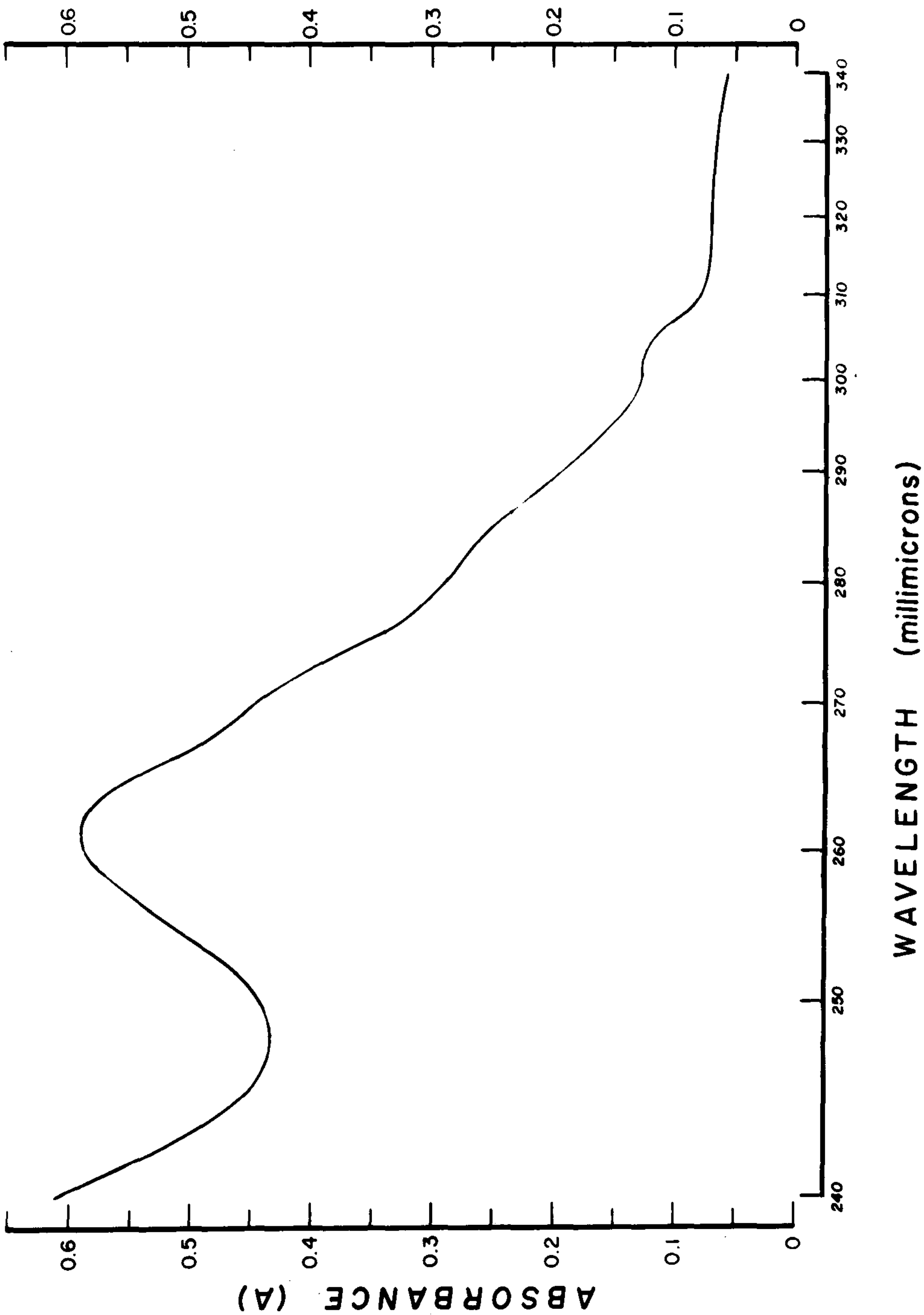


TABLE XXI

DATA FOR THE CONVERSION OF ESTRADIOL TO ESTRONE

K moles/min	Log K moles/min	k mm/min	Log k mm/min	T	TK	1/TK
1.87×10^{-7}	-6.723	0.40	-0.398	275	548	1.825×10^{-3}
1.98	-6.704	0.432	-0.365	287	560	1.785
2.13	-6.671	0.488	-0.312	317	590	1.695

TABLE XXII

PYROLYSIS AT 400°C

Volume (cc)	Estradiol (mgs)	Time (hours)	UVmax	IR Conclusion	Zimmermann Color	$FeCl_3$
17	50	4	261	carbonyl +	+	+
			a. 260	carbonyl +	+	+
16	50	5	b. 262	carbonyl -	-	+
			304	17-OH -	+	+
14	56	3	261	carbonyl +	+	+
16	73	1.5	280	carbonyl +	+	+

The compound showing maxima at 262 and 304 mμ was isolated and on chromatography on an alumina column it was eluted with 95 per cent ethanol, while on paper it was less polar than estrone (compound 32.8 cm, estrone 17.8 cm). The infra-red spectrum indicated the absence of the 17-hydroxy band at 1052 cm^{-1}. Its ultraviolet spectrum is shown in Figure B. The ultra-violet spectrum suggests this substance to be a 9 steroid on comparison to similar substances. (Table XXIII)

Pathway of Estradiol Decomposition

The decomposition of estradiol from 287°C to 375°C appears to involve first a conversion to estrone. Estrone is then dehydrogenated to form a 9-dehydro compound. Either estrone or 9-dehydro estrone is dehydrated to form a 9-dehydro 17-deoxy compound.

TABLE XXIII

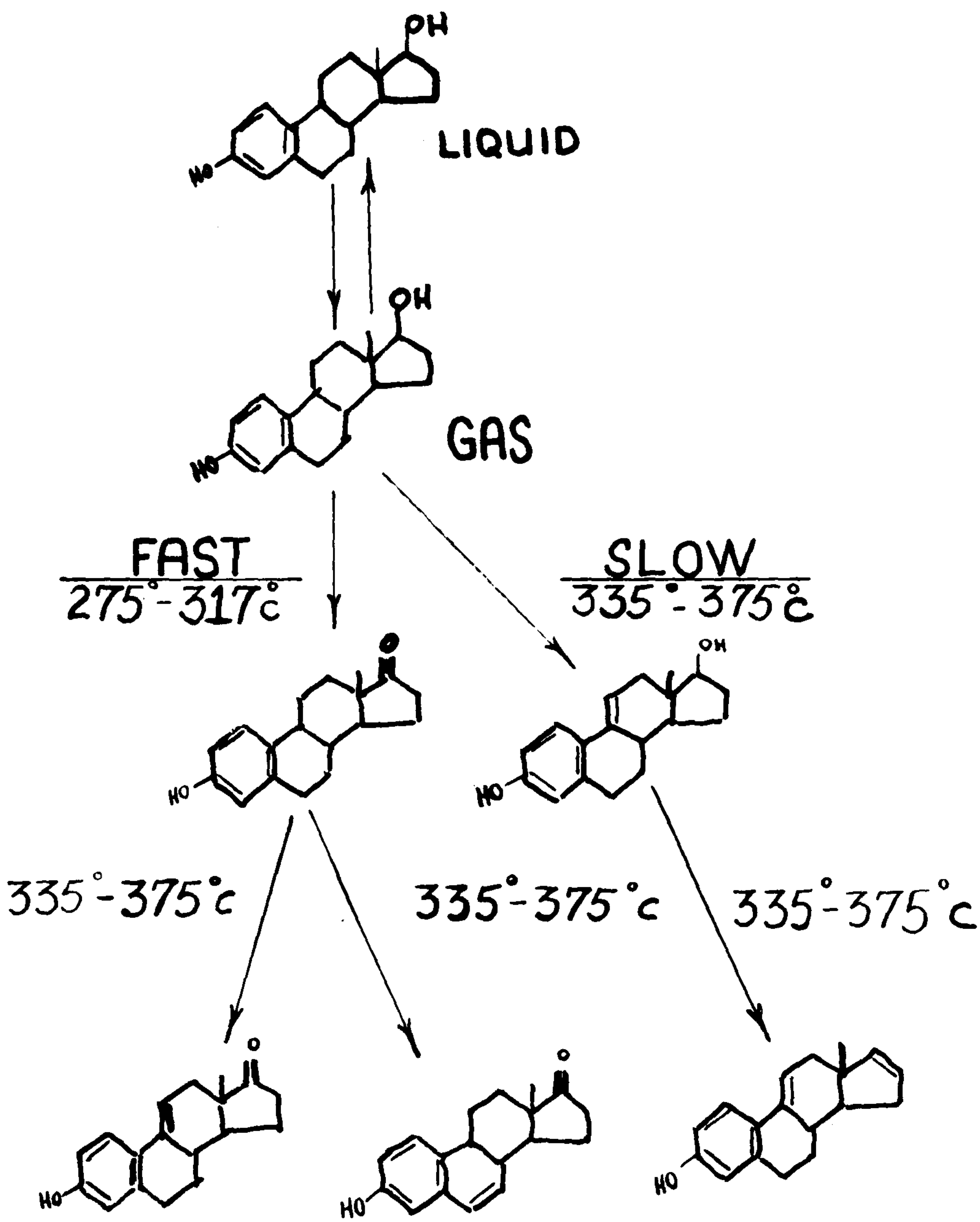
COMPARISON OF ULTRAVIOLET SPECTRA FOR Δ^6 AND Δ^9 COMPOUNDS

Compound	Maxima	Estinction Coefficient	Ratio	Reference
6	262,304	9,000 - 2,750	3.4	(55)
9	263.5 - 300	18,000 - 3,000	6.0	(9)
Isolated	262,303	--- ---	5.8	

The products of decomposition at 300°C were shown to be hydrogen and estrone. Also, the log of the rate of this reaction was linear with 1/TK from 275 to 317°C, so that it was concluded that only one reaction was proceeding in this temperature range. After 317°C linearity is lost indicating that perhaps mixed rates are present. Products of pyrolysis from decomposition at temperatures in excess of 317°C led to the isolation of compounds whose ultra-violet spectrum were consistent with unsaturation at position 9. In these same mixtures a compound was found that was devoid of an oxygen function at C-17 (Table XXII) leading to the conclusion that dehydration might have occured. These relationships are summarized in Figure 14.

FIGURE 14

SUMMARY OF PYROLYTIC DECOMPOSITION



Interpretation of Decomposition Rates

The rate of evolution of hydrogen corresponded to a zero order reaction. This means that the concentration of material which is activated is constant. In the heterogenous system studied it was assumed that the gas phase was kept constant at any given temperature. It is this factor which determines the concentration of activated molecules. There exists an example in the literature (35) illustrating this type of mechanism. In the study of the decomposition of N_2O_5 the vapor of the compound was in equilibrium with its crystalline form. Solid N_2O_5 does not decompose, but as a gas it decomposes slowly. When a molecule decomposes, its place is taken by another molecule from the solid portion and the concentration of the gaseous N_2O_5 is thereby maintained.

Another possible interpretation is that it is the liquid phase which underwent decomposition. If this were true, then the rate of pyrolysis is a linear function of the liquid concentration. In terms of gas chromatography, when 100 - 0.1 μg quantities were used the expected rate of decomposition at 250°C was of the order of 10^{-2}-10^{-4} μg per minute. A quantity which is negligible since it represented decomposition of only .01 % of the material per minute.

Degree of Decomposition

Assuming that one mole of hydrogen represents one mole of estradiol decomposed, one can calculate the amounts of estrone formed (Table XXIV).

TABLE XXIV

MICROGRAMS OF ESTRONE FORMED AT VARIOUS TEMPERATURE

Temperature C	mm/min H_2	Micrograms of Estrone/minute
275°	0.40	50.5
287°	0.432	56.6
317°	0.488	63.2

The Thermal Decomposition of Estrone

Estrone was pyrolyzed in the isotenoscope as described previously. Estrone (564 mg) was pyrolyzed at 305°C and 0.05 mm of Hg in a 16 cc isotenoscope. The results of this decomposition are given in Table XXV.

TABLE XXV

DECOMPOSITION OF ESTRONE AT 305°C

Time Minute	Pressure	mm/minute	Average Rate
10	7	0.7	
20	13	0.65	
30	19	0.635	0.65
43	27.5	0.630	
53	33.5	0.635	

At the termination of this run, air was admitted into the hot apparatus and a red compound was formed. This compound had a visible spectrum with a maximum (525 mμ) identical to the red compound formed by melting equilenin. Estrone when melted does not form such a red compound.

From this it was assumed that ring B dehydragenation probably had occured.

Assuming the mole for mole formation of hydrogen from estrone, decomposition occurs at a rate of 80 μg per minute.

C. Thermal Stability of Estrogen Acetates

The chromatographed estrogen acetates were trapped at the outlet of a gas chromatograph at an operating temperature of 280°C on a cold surface and dissolved in CS_2 for infra-red spectroscopic analysis. The same procedure was used for ultraviolet spectroscopic analysis except that absolute ethanol was used as solvent.

The ultraviolet spectra of the three acetates were determined after passing them over a 4.5 foot silicone grease column at 280°C with an injection block temperature of 340°C. All three estrogen acetates have absorption maxima at 275 and 268 mμ. Table XXVI shows the ratios of the maxima of the original acetate to that of the gas chromatographed compounds. All maxima and minima were discreet and when superimposed on the spectra of the original compounds were identical. The same procedure was followed for the preparation of the infra-red spectra of estrone, estradiol and estriol acetates, shown in figures 15, 16 and 17. A further criterion of stability is the absence of peaks in the chromatogram other than those expected. Figure 23, shows a run of the three acetates at 235°C and an injection temperature of 310°C. The compounds were in contact with the column for 1, 1.5 and 3 hours respectively. If decomposition should lead to compounds differing by as little as 2 hydrogen atoms, under these conditions a lack of symmetry in the peak would be expected. No such skewing was observed. In Figure 20, are the results obtained from chromatographing the three compounds at 280°C and again at 245°, in Figure 21, no new peaks are observed.

TABLE XXVI

COMPARISON OF ULTRAVIOLET SPECTRA OF ACETATES

Estriol triacetate		Estradiol diacetate		Estrone acetate	
Ratio 268/275 mμ		Ratio 268/275 mμ		Ratio 268/275 mμ	
Before Chromatography	After Chromatography	Before Chromatography	After Chromatography	Before Chromatography	After Chromatography
1.02	1.07	1.02	1.04	1.04	1.07

FIGURE 15

INFRARED SPECTRUM OF CHROMATOGRAPHED ESTRONE ACETATE

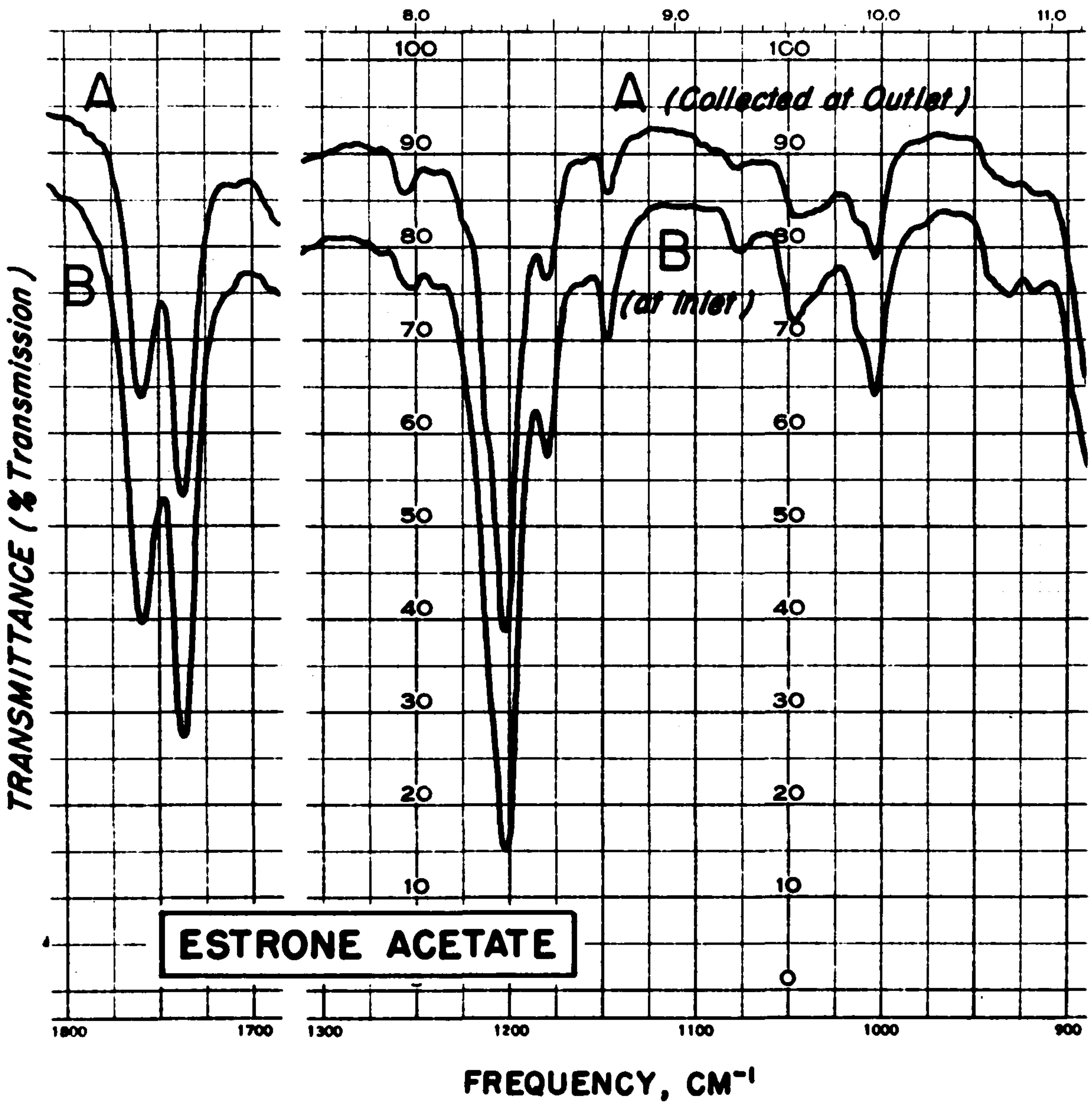


FIGURE 16

INFRARED SPECTRUM OF CHROMATOGRAPHED ESTRADIOL DIACETATE

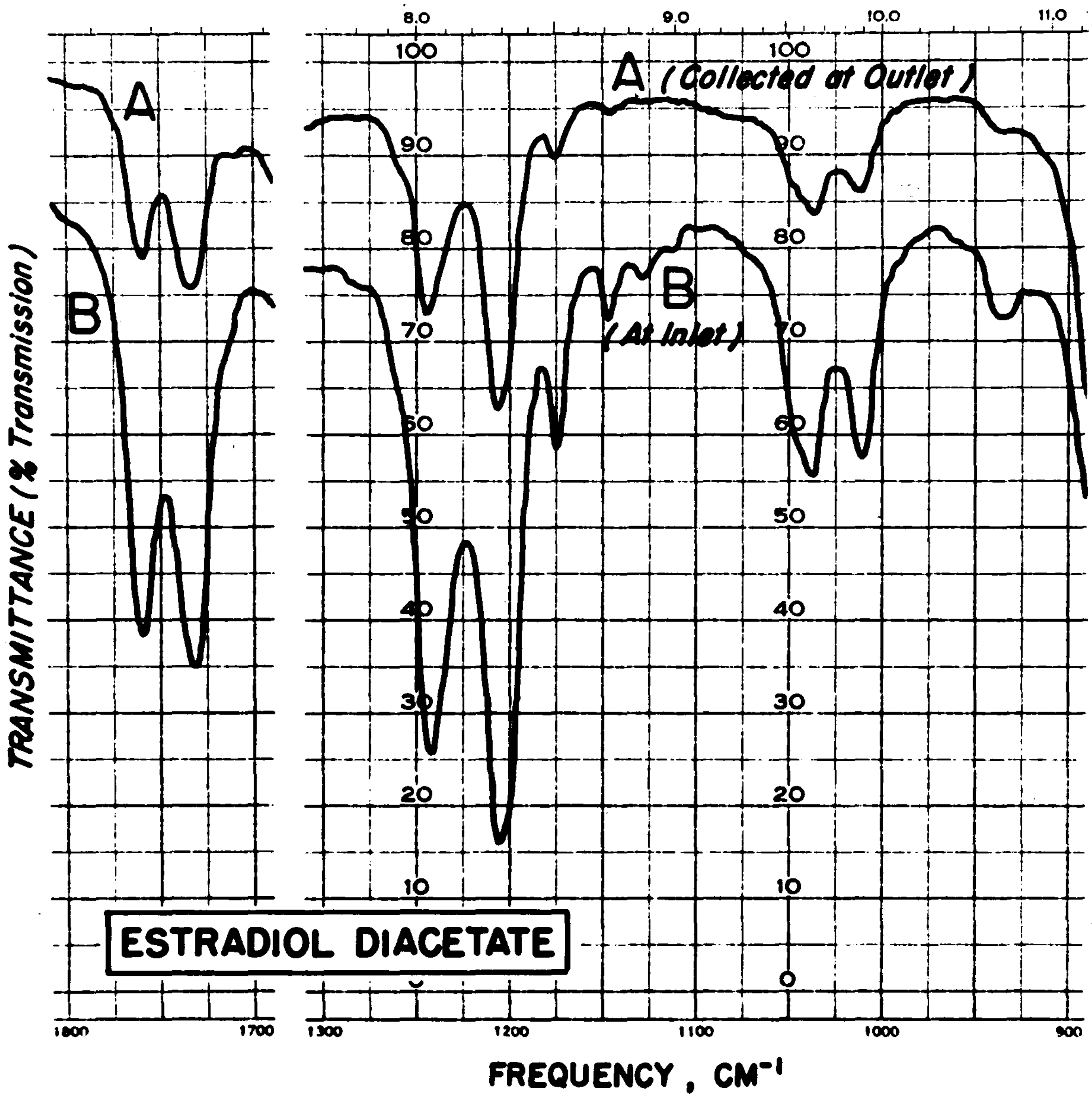
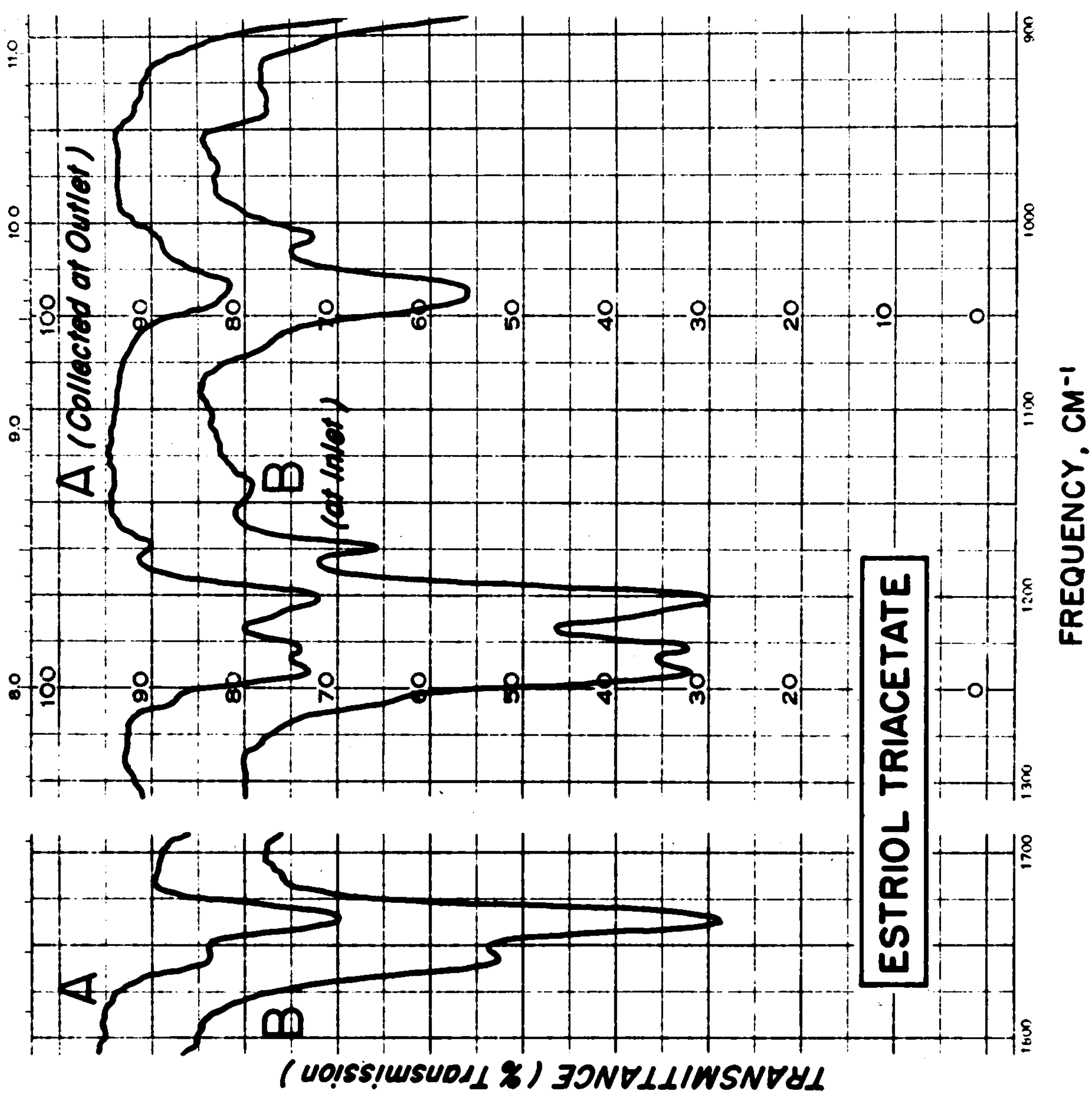


FIGURE 17

INFRARED SPECTRUM OF CHROMATOGRAPHED ESTRIOL TRIACETATE



Injection at 245^{o}C of 0.09 μg each of estrone, estradiol and estriol acetate yielded peaks for the first two compounds, but estriol triacetate was obscured because its expected peak height was of the order of the electromic noise level.

When free estradiol was chromatographed in a column at 280^{o}C and the vapor was trapped, the material on treatment with alkali and m-dinitrobenzene did not show formation of the Zimmermann color indicating the absence of any ketonic component. The peak was not symmetrical which may be ascribed to lack of interaction of this polar substance with the non-polar silicone coating on the column, rather than to decomposition.

The thermal stability of the acetates can also be modified by the solvent. With the large injection block at 300^{o}C (Aminco Gas Chromatograph) 300-400 μg of estradiol diacetate in 100 microliters of several different solvents were injected.

In methanol and ethanol there appeared a second peak which had a retention time consistent with that of estrone acetate. No such effect was noted when this steroid ester was dissolved in cyclohexane, acetone, pyridine, benezene and a mixture of pyridine and acetic anhydride prior to chromatography. Using a smaller injection block (Research Specialties Corporation), estradiol diacetate in 2 microliters of ethanol or methanol showed no evidence of deacetylation. The date relating to the decomposition due to primary alcohols and other solvents is shown in Table XVII.

The explanation for the behavior of estradiol diacetate with alcohols is probably derived from the general properties of esters which can undergo transesterification. Transesterification is a reaction which esters undergo when they are heated with alcohols or other esters. Double decomposition takes place and the alcohol residues are more or less completely exchanged (81). This reaction is accelerated by the presence of small amounts of acid or alkali, so that even a trace of these catalysts in a large block might easily account for this decomposition in the presence of alcohols.

TABLE XXVII

EFFECT OF SOLVENT ON THE DECOMPOSITION OF ESTRADIOL DIACETATE (Tr=5 minutes)

Solvent	MOLE PERCENT	
	Component with Tr=4 minutes	Component with Tr=5 minutes Estradiol diacetate
Acetone	0	100%
Ethanol	19%	81%
	16%	84%
Ethanol only	0	0
Methanol	25%	75%
Methanol only	0	0
Cyclohenane	0	100%
Pyridine	0	100%
Pyridine and Acetic anhydride	0	100%
Benzene	0	100%

D. SEPARATIONS AND VARIABLES

1. Methods

a. Column preparation

The column packing was prepared by dissolving 3 grams of Dow Corning Silicone grease in 50 ml of hot dicholoromethane. To this hot solution was added 7 grams of Chromsorb (40-60 mesh) and heating was continued until the solvent was evaporated and the granular residue was relatively dry and free flowing. The 1/4 inch copper tubing of selected length was plugged at one end with glass wool and the packing was slowly poured into the other end via a connecting funnel. Occasional gentle tapping was all that is required for proper filling. After filling the column, it was coiled and attached to the instrument. The packing was cured for 48 hours at 300°C with a slow flow of helium. After this initial curing, the column was primarily used for exploratory work. It takes nearly a month cure this type of column for work with an ionization detector. All of the work done in this investigation was carried out on one column which has been in continuous operation for over one year at temperatures from 245° to 300°C. Table XXVIII shows the retention times at various periods in the life of this column.

TABLE XXVIII

RETENTION TIMES OF THE ACETATES

Date	Pressure (PSI)	Flow (cc/min)	Temp. (C)	RETENTION TIMES OF THE ACETATES OF Estrone (min)	Estradiol (min)	Estriol (min)
June 17	25	400	235	11.6	16.9	31.8
July 25	25	400	235	11.3	16.5	31.5
Dec. 13	25	400	235	11.6	17.0	32.0

b. The preparation of derivatives.

1. Ethers

The estrogen (38 mg) was covered with a mixture of 8 ml of 90 per cent methanol containing 0.8 g of anhydrous K_2CO_3. The steroid was dissolved by heating the mixture at reflux after which 0.3 ml of dimethyl sulfate was added. When the initial reaction had subsided, another 0.3 ml of dimethyl sulfate was added. Refluxing was continued for a total of forty-five minutes from the first addition of dimethyl sulfate and then 4 ml of water was added and the resulting mixture distilled until turbid. The cooled mixture was then filtered, washed with water, and dried under vacuum. The residue was taken up in acetone, filtered and the acetone solution evaporated to dryness on the steam bath. Once the volume of acetone had decreased the product tended to crystallize spontaneously before all the acetone was removed. Both estradiol and estrone ethers were prepared in this manner. Yields of sixty-two per cent for estradiol methyl ether and sixty-eight per cent for estrone methyl ether were obtained (71).

Estriol acetate was prepared by dissolving 288 mg of the steroid in 10 ml of dry pyridine and adding two 5 ml portions of dry acetic anhydride. The reaction was allowed to proceed overnight while being shaken mechanically. The gas chromatograph showed this mixture to be 99 mole per cent of estriol triacetate with less than 1 per cent of an impurity which had a retention time similar to that of estradiol diacetate. After adding 25 ml of ice water the resulting precipitate was filtered, washed with water and dried under vacuum. This material was compared to the reference literature through infra-red spectroscopy and it melted at 125°-127°C. (Reported 128°C) Yields by this procedure were 90-95 per cent for the three acetates.

2. Column length

Columns of 1, 2 and 3 foot length were prepared and separation of 3-methoxyestrone was attempted on them. Separation from the solvent was observed with a one foot column at 300°C with a flow of 20-30 ml He/min. Separation of estrone acetate from estradiol diacetate required a three foot column at 280°C at a flow of 300-400 ml/min of helium.

3. Effect of temperature on the retention time

In Table XXIX are shown the retention times for the three estrogen acetates at three different temperatures. The determination at 280°C was carried out using a thermal conductivity cell and helium as a carrier. The other two readings were obtained on an ionization detector with argon as the carrier gas.

4. Effect of Pressure and Flow Rate on the Retention Time

The effect of increasing the pressure and flow rate result in the expected decrease in retention time. This data is shown in Table XXX.

TABLE XXIX

EFFECT OF TEMPERATURE ON THE RETENTION TIME

Temperature C	RETENTION TIME IN MINUTES estrone acetate	estradiol diacetate	estriol triacetate
235°	11.6	16.9	31.8
245°	8.3	12.0	22.5
280°	3.06	4.01	6.86

TABLE XXX

EFFECT OF PRESSURE AND FLOW RATE ON THE RETENTION TIME
AT CONSTANT TEMPERATURE (235°C)

Flow rate cc/min	Pressure PSI	RETENTION TIMES (MINUTES)		
		estrone acetate	estradiol diacetate	estriol triacetate
50	4	62	92	174
400	25	11.6	16.9	31.8
500	28	11.3	16.4	30.6
600	30	9.88	14.6	27.3

5. Comparison of the retention times and peak areas for different derivatives.

The retention times and peak areas for estrogen ethers and acetates were determined in order to evaluate their relative detector excitations. The area was determined by considering the peak as a triangle. The area can be calculated by multiplying the height times the base at 1/2 height. All determinations were carried out at 235°C at 25 psi using 6.25 µg of compound in 2 ml of solvent.

TABLE XXXI

Comparison of the Retention Times and Peak Areas For Different Derivatives

Compound	Retention Times in minutes	Area in mm^2
Estrone acetate	11.3	975
Estradiol diacetate	16.5	950
Estriol triacetate	31.5	764
Estrone methyl ether	7.80	595
Estradiol methyl ether	8.48	553

Although the numerical value for the retention time of the ethers show a difference in the table, the substances at these conditions emerge as one peak when injected together. Separation was achieved by changing column conditions to slower flow rates.

6. The Relation of the Log of the Retention Time to the Calculated Boiling Point and Molecular Weight.

It was predicted that the log of the retention times should be a linear function of the boiling points and molecular weight. This information is tabulated below and the data is also shown in Figures 18 and 19.

TABLE XXXII

RELATION OF RETENTION TIMES TO BOILING POINTS

Compound	Retention time minutes	Log of the Retention Time	Calculated Boiling Point C
Estrone acetate	3.06	0.486	601
Estradiol diacetate	4.01	0.613	617
Estriol triacetate	6.86	0.843	646

TABLE XXXIII

RELATION OF RETENTION TIMES TO MOLECULAR WEIGHTS

Compound	Retention Time in minutes	Log of the Retention Time	Molecular Weight
Estrone acetate	11.3	1.05	312
Estradiol diacetate	16.5	1.22	356
Estriol triacetate	31.5	1.497	415
Estrone methyl ether	7.80	0.892	284
Estradiol methyl ether	8.48	0.928	286

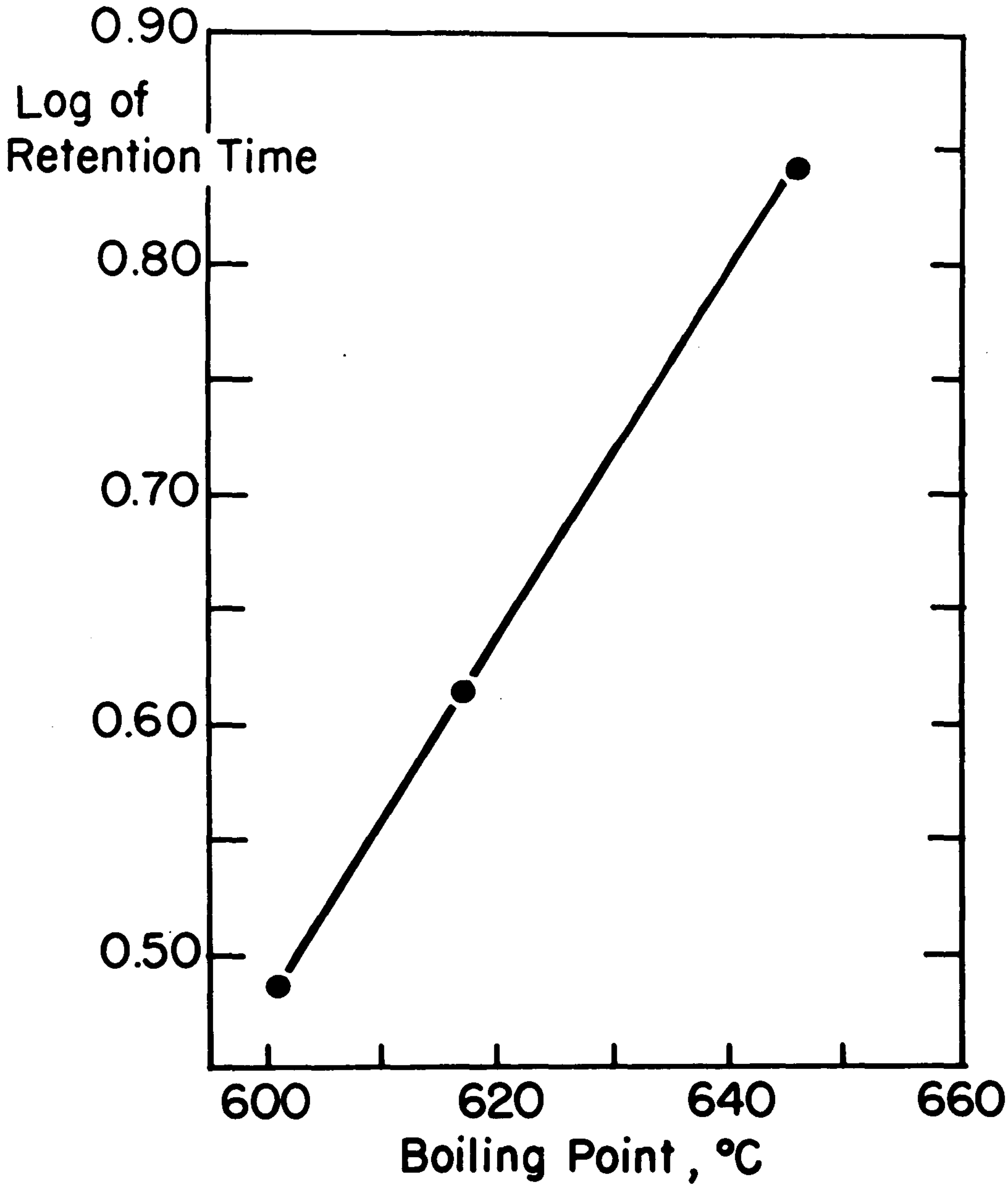
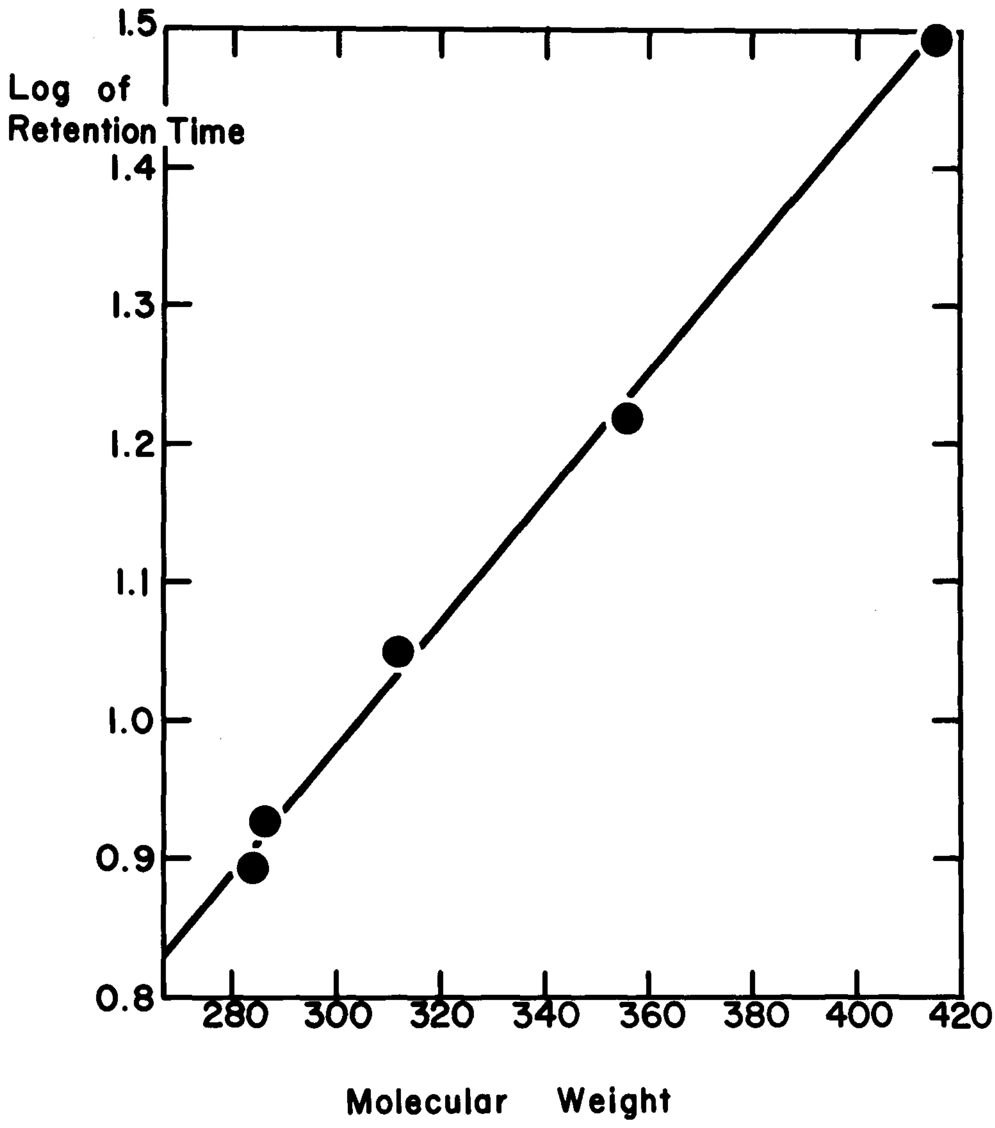


Fig. 18 RELATION OF THE BOILING POINT TO THE RETENTION TIME

RELATIONSHIP OF THE MOLECULAR WEIGHTS TO THE RETENTION TIMES

FIGURE 19



Both tables indicate that the observed results are in reasonable agreement with the theory and reinforced the decision to use the steroid acetates as opposed to the ethers. While it is true that lower temperatures can be used for the ethers, there would have been no net gain in the time for determination and separation. Figure 20 shows the separation of 200 μg of fully acetylated estrone, estradiol and estriol using a thermal conductivity detector. The flow rate shown in the diagram was uncorrected. Corrected flow rate was 375 ml of helium per minute.

Figures 21 and 22 show chromatograms for the three acetates using an ionization detector. Figure 21 shows the separations of 12.5 μg of each steroid in 2 ml of solvent and Figure 22 reproduces the results from a chromatogram of 1.56 μg of the acetates. Figure 23 illustrates the great resolution that can be attained at low flow rates and the peak symmetry is indicative of a low rate of decomposition with long retention times. This chromatogram shows the separation of 12.5 μg of the three acetates in 2 ml of solvent.

From a study of the various variables, Table XXXIV shows optimum conditions for separation.

TABLE XXXIV

OPTIMUM OPERATING CONDITIONS

Variable	Thermal Conductivity Detector	Ionization Detector
Concentration	100-400 μg / 2 ml	0.1 - 10 μg / ml
Column	30-70 silicone	30-70 silicone
Length	3 feet	3 feet
Solvent	acetone, pyridine, pyridine with acetic anhydride benzene.	same, also primary alcohols
Temperatures		
Column	280^{o}C	235 - 245^{o}C
Vaporizer	340^{o}C	340^{o}C
Flow	400 cc / min.	400 cc / min.
Gas	Helium	Argon
Injection size	50-100 μl	2-5 μl
Method of Injection	Syringe	Capillary pipette

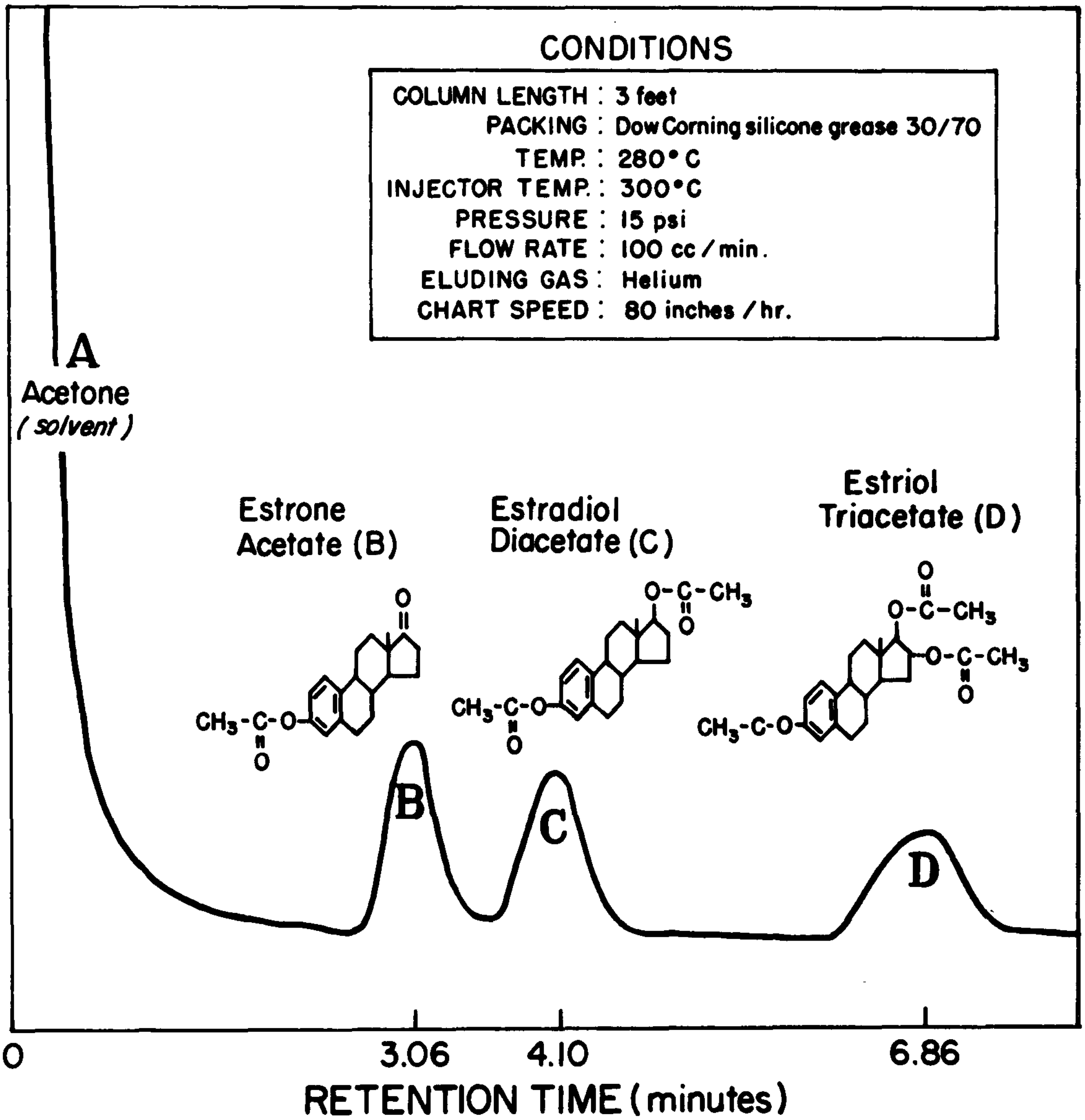


Fig. 20 SEPARATION OF THE ESTROGENS AS THE ACETATES

FIGURE 21

SEPARATION OF THE ESTROGEN ACETATES (12.5 ug)

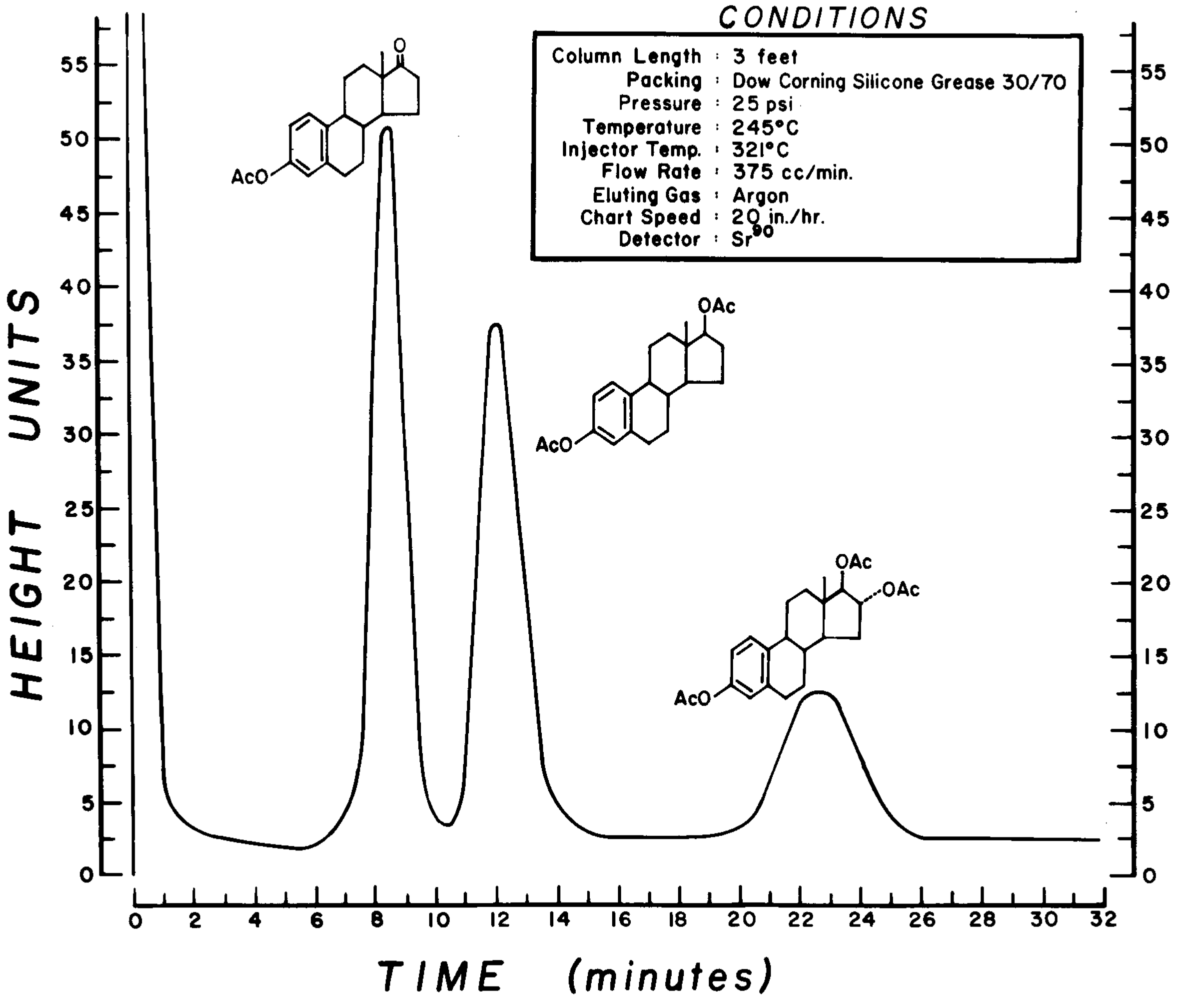


FIGURE 22

SEPARATION OF THE ESTROGEN ACETATES

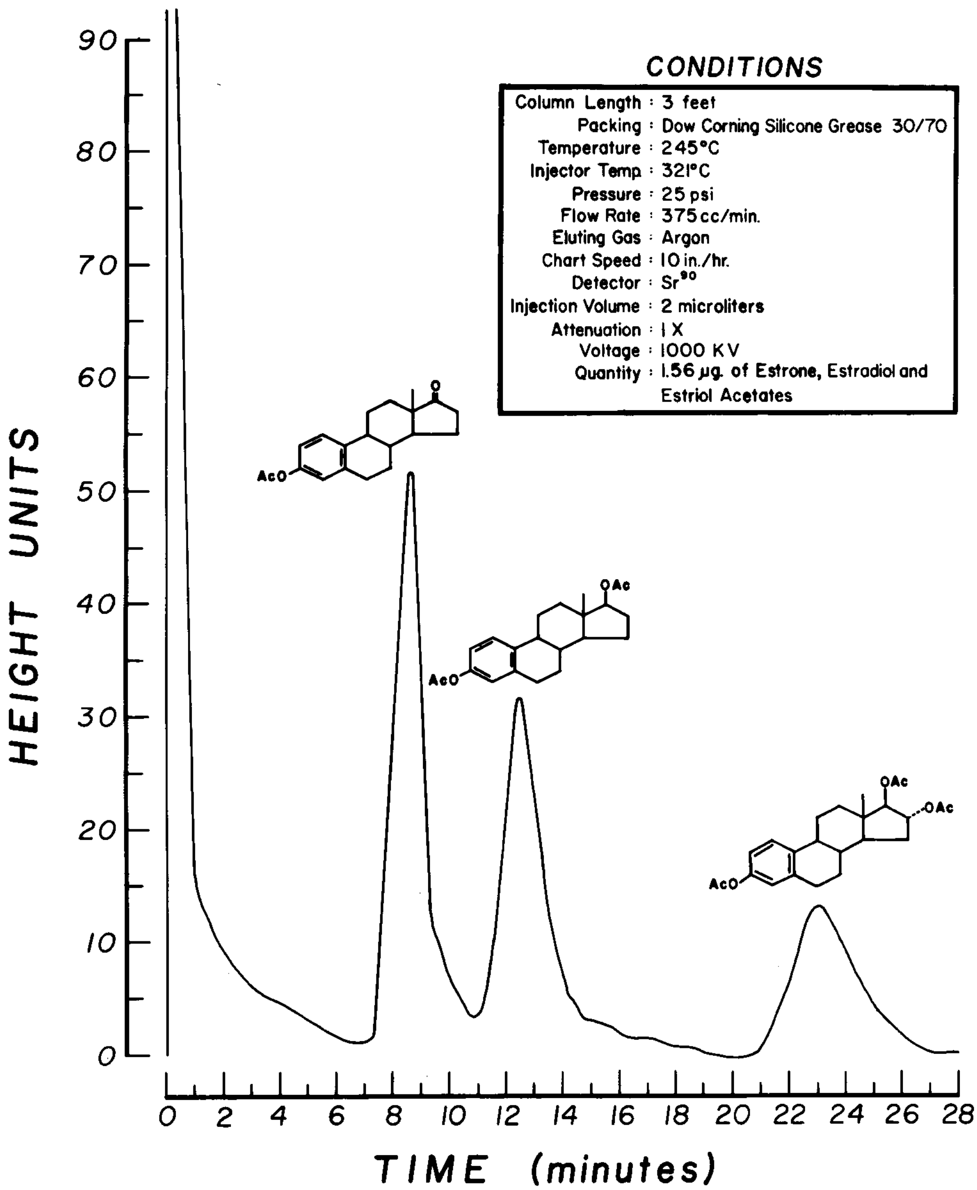
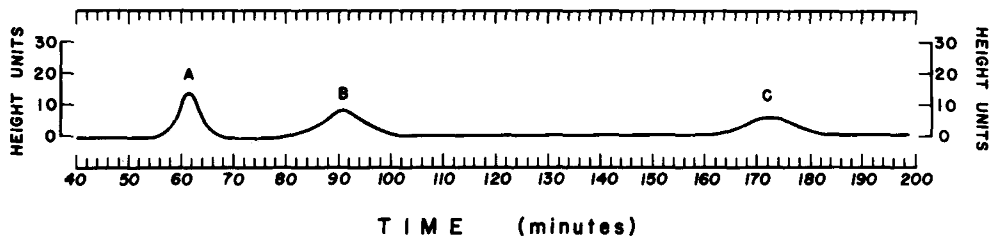


FIGURE 23

SEPARATION OF THE ESTROGEN ACETATES AT LOW FLOW RATES

A = Estrone Acetate B = Estradiol diacetate
C = Estriol triacetate



Conditions:

Column length:	3 feet
packing:	30/70 silicone grease
Temperature:	235°C
Injection temperature:	310°C
Pressure:	4 psi
Flow Rate:	50 cc./minute
Eluting Gas:	Argon
Chart speed:	10 inch/hour
Detector:	Sr^{90}

E. QUANTITATIVE RELATIONS

Having established optimum conditions for separation it now remained to demonstrate that both detectors show linear reproducible responses to changes in concentration. Accordingly, the objectives were as follows:

1. To determine the response of the thermal conductivity detector to a change in concentration of the estrogen acetates.
2. To determine the sensitivity (limits of detection) of the thermal conductivity detector.
3. To determine the response of the Sr^{90} detector to a change in concentration of the estrogen acetates.
4. To determine the sensitivity (limits of detection) of the Sr^{90} ionization detector.
5. To determine the reproducability of a series of determinations.
6. To correlate the response of the detector with the proposed theory.

Methods

1. Quantitative data from the chromatograms utilizing the thermal conductivity detector were obtained by measuring the area under the peak. This area was approximated by multiplying the height of the peak by

the width at 1/2 the height. In order to obtain adequate reproduction of individual measurements, short syringe needles had to be used. Also, injection had to be rapid, allowing for a short period (30-45 seconds) in the injection block. Each sample injection was followed by solvent of the vaporizing block with three successive acetone injections prior to the next determination. Following analysis of less than 400 μg no residual steroid was detectable during the washing procedure. It was also found that reproducibility was improved by injecting equal volumes and changing the concentrations.

2. The Research Specialties Company gas chromatograph, equipped with a Sr^{90} ionization detector was equipped with a capillary pipette introduction system. Since the detector was over 1000X more sensitive than the thermal conductivity detector, the sample size must be smaller. In this instrument, a capillary tube is joined to the column, then through a valve arrangement the gas flow is passed behind the capillary and the material is delivered into the column. Particular attention must be paid so that:

    1. Complete delivery of the material occurs.
    2. No material was allowed to accumulate on the outside of the pipette.
    3. No residue material be left in the pipette due to evaporation during injection.

3. No leaks occur in the gas splitting system which forces the solution from the capillary into the column.

After much trial and error the following procedure was adopted. A wide tube containing the material in a higher boiling solvent than acetone (benzene or toluene) was used. The capillary pipette was washed three times (the entire length including reservoir) with acetone and then air dried. Only the tip was submerged below the level of the solution and this was cleaned with tissue soaked in acetone, being careful not to touch the orifice.

With the ionization detector, it was found that the height was a sufficiently good index of concentration and areas were not in general determined, except for the theoretical confirmations.

B. <u>Results</u>

1. Response of the thermal conductivity detector to varying concentrations of estrone monoacetate and estradiol diacetate.

The following conditions were used:

Column Silicone:	30-70: 3 feet	Power 275 watts
PSI:	15	Amplifier:175 watts
Flow rate:	100cc/min.(uncorrected)	Control:256 max.
Column Temperature	275°C	Detector range: 300
Block:	340°C	Attenuation: 1
		Zero recorder 1
		Extra heat only for block

TABLE XXXV

ESTRONE ACETATE

Run #	µg/100 µl	mm Base	mm Height	mm^2 Area	mm^2 Average Area
1.	99.6	9.5	6.5	61.5	61.0
2.		9.0	6.5	58.5	
3.		12.0	5.5	66.0	
4.	149.4	9.0	10.0	90.0	95.0
5.		10.0	10.0	100.0	
6.	199.2	10.0	13.0	130.0	122.0
7.		10.0	12.0	120.0	
8.		10.0	11.5	115.0	
9.	258.8	10.0	17.0	170.0	177.0
10.		9.5	19.0	180.0	
11.		9.5	10.0	180.0	
12.	398.4	9.0	26.0	234.0	238.5
13.		10.0	24.0	240.0	
14.		10.0	24.0	240.0	
15.		10.0	24.0	240.0	

The linearity of detector response with respect to estrone acetate is demonstrated in Figure 24.

According to theory, the base of the triangle describing the area under the curve is a linear function of the retention time so that for any series of determinations it should be constant. In run #3, however, it is 12, where the other runs the value ranged from 9.5 - 10.0. It was suspected that a leak had developed in the septum.

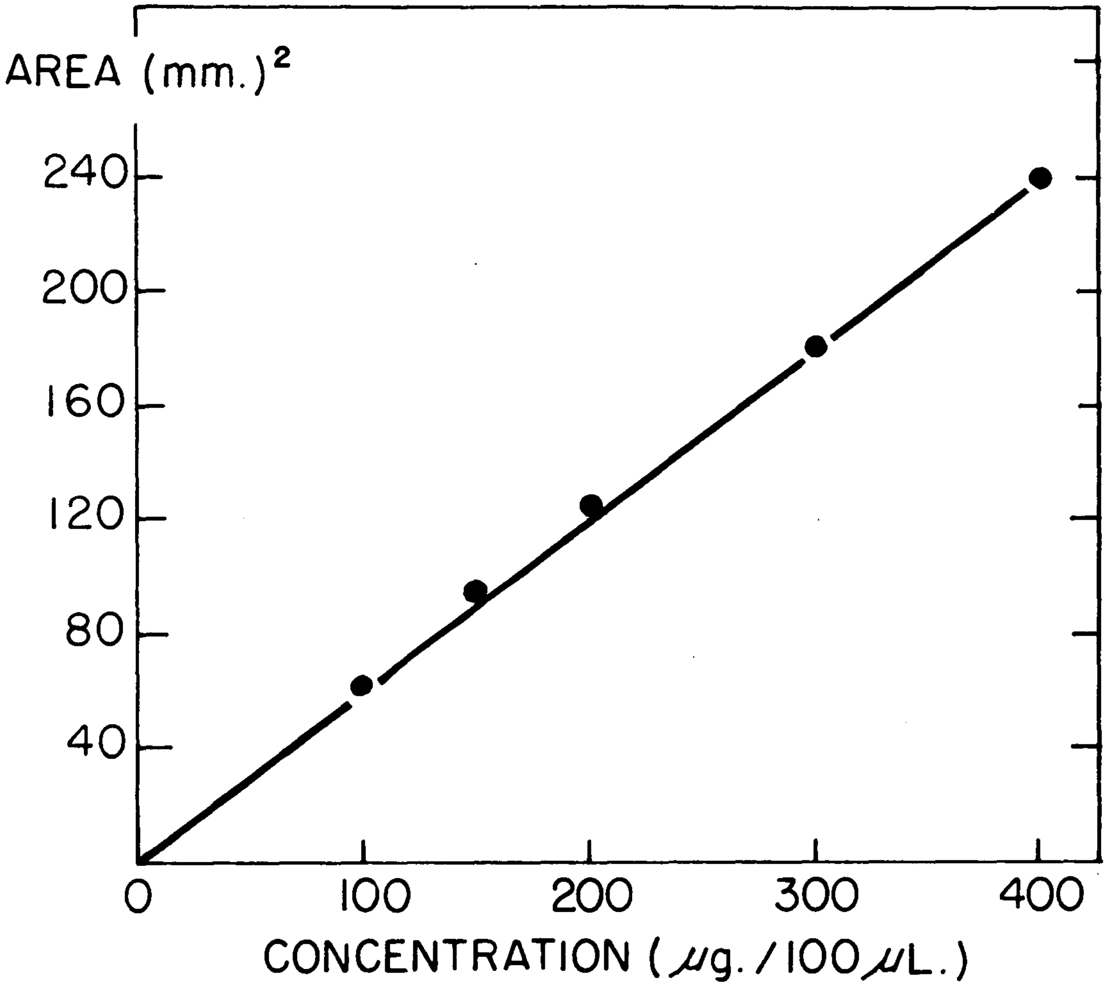


Fig. 24 RELATION OF THE CONCENTRATION TO THE AREA FOR ESTRONE ACETATE

TABLE XXXVI

ESTRADIOL DIACETATE

Run #	μg/100μl	mm Base	mm Height	mm^2 Area	mm^2 Average Area
1.	102.1	11.	6.0	66.	66.
2.		11.	6.0	66.	
3.		11.	6.0	66.	
4.	153.2	12.5	7.5	93.5	93.5
5.		12.5	7.5	93.5	
6.	204.2	11.	12.	132.	132.0
7.		11.	12.	132	
8.	306.3	11.5	17.	196.	193.0
9.		11.5	16.5	190.	
10.	408.8	11.5	23.5	270.	270.0
11.		11.0	24.5	270.	
12.		11.5	23.5	270.	

The linearity of the detector toward estradiol diacetate is demonstrated in Figure 25.

2. Response of the ionization detector to a change in concentration.

Solutions of varying concentration were prepared from a standard containing exactly 25.0 mg each of the three estrogen acetates. By serial dilution six different concentrations were prepared and 2 μl of each was introduced into the chromatograph

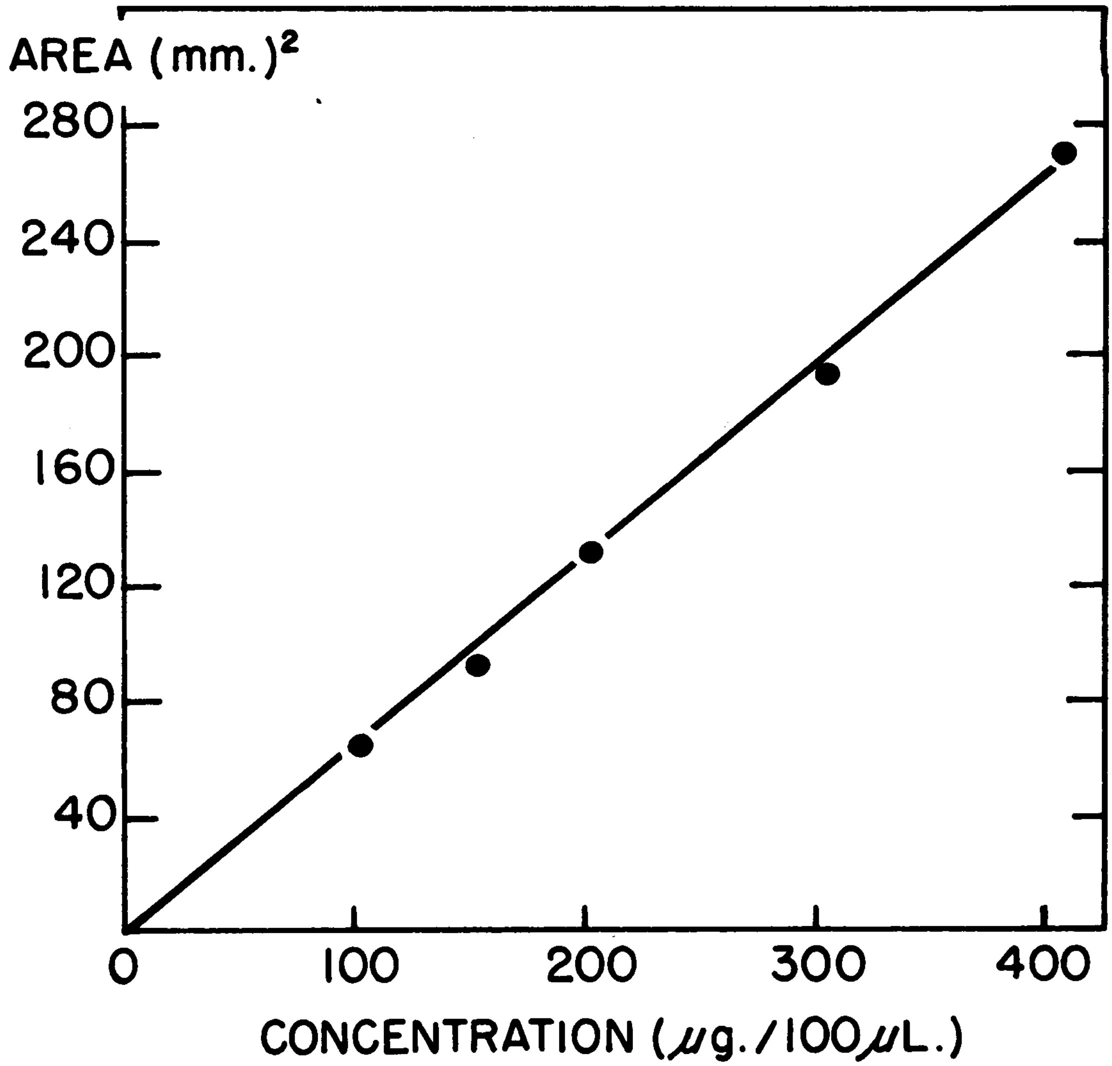


Fig 25 RELATION OF THE CONCENTRATION TO THE AREA FOR ESTRADIOL DIACETATE

with an ionization detector at optimum conditions reported previously. The data was then converted to a standard attenuation of 10X and evaluated. This information is recorded in Table XXXVII.

Conversion of all height units to a standard attenuation (10X), produced the results in Table XXXVIII.

The peak height for 0.0975 micrograms for estriol could not be determined since its displacement was of the same order of magnitude as the noise level of the instrument.

The linear response of the detector to a change in concentration is shown in Figure 26.

FIGURE 26

RELATION OF CONCENTRATION TO PEAK HEIGHTS

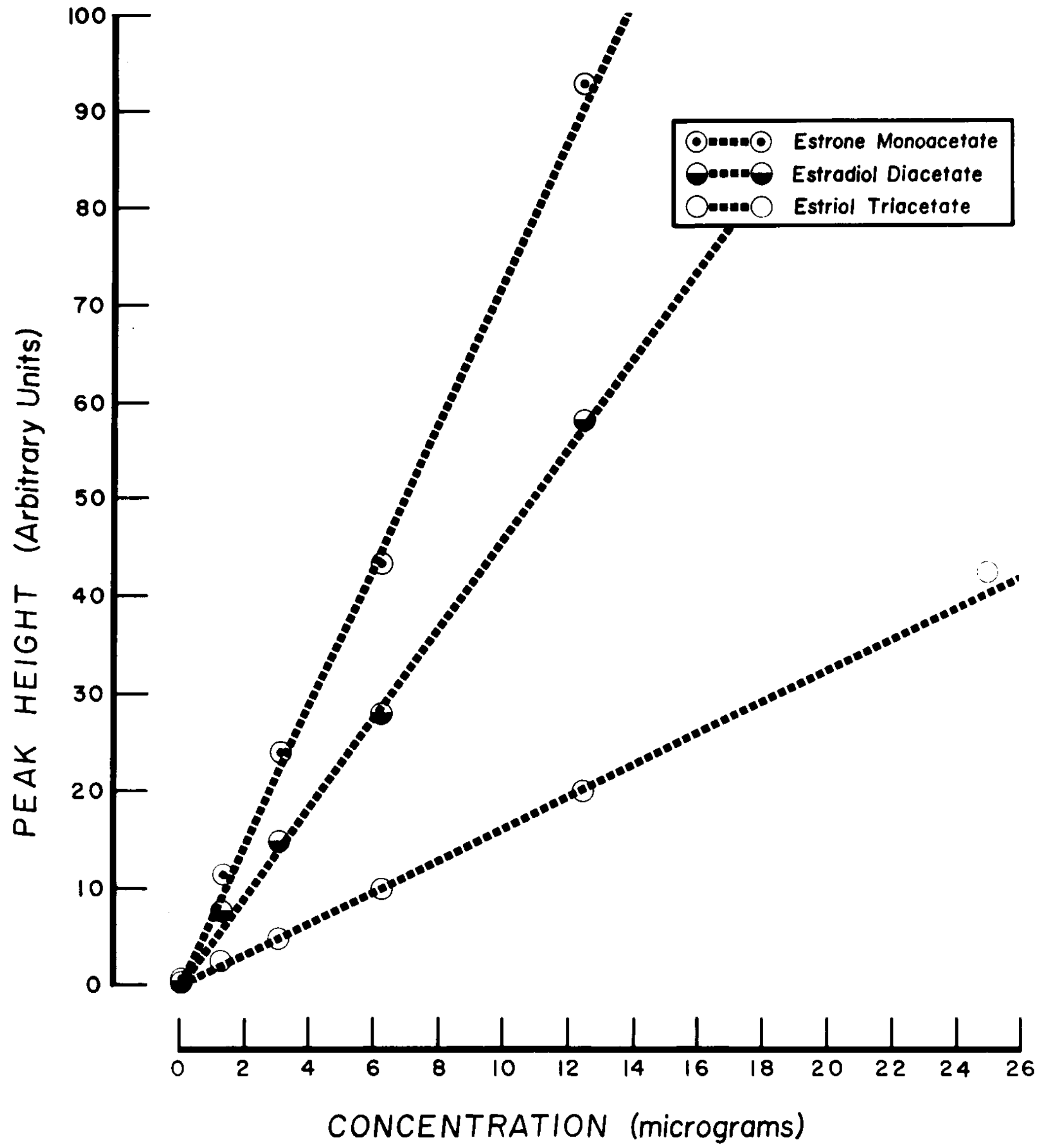


TABLE XXXVII

RESPONSE OF IONIZATION DETECTOR

Run #	μg /2 μl	Attenuation	Estrone Acetate Height units	Estradiol Diacetate Height units	Estriol Triacetate Height units
1.	25	50	40.5	25	8.5
2.	12.5	50	18.5	11.6	4.0
3.	6.25	10	43.3	27.8	10.0
4.	3.125	10	24.0	15.0	5.0
5.	1.56	5	27.0	15.0	5.0
6.	1.56	2	55.5	36.5	13.0
7.	0.0975	1	6.5	4.5	--

TABLE XXXVIII

(ATTENUATION AT 10X) - HEIGHT UNITS

μg /2 μl	Estrone Acetate	Estradiol Diacetate	Estriol Triacetate
25.0	202.5	125	42.5
12.5	92.5	58	20.0
6.25	43.3	27.8	10.0
3.125	24.0	15.0	5.0
1.56	11.5	7.5	2.5
0.0975	0.65	0.45	---

. Reproducibility

Studies of the reproducibility of single injections and mixtures were undertaken next. The major variables that effect reproducibility have been discussed previously. Below data is tabulated for six successive and equal injections.

The symbols are defined as follows:

x_i = individual values
$x_i-\bar{x}$ = deviation from the mean value
N = the number of trials.

N	X_i	$X_i-\bar{x}$	$(X_i-x)^2$
1.	27.	-0.166	0.0276
2.	28.	+0.833	0.693
3.	27.5	+0.334	0.112
4.	26.0	-0.833	0.693
5.	27.	-0.166	0.0276
6.	28.5	+1.334	1.7795
			3.3327

$\bar{x} = 27.166$ The standard deviation $= \left(\frac{(x_i-\bar{x})^2}{N-1}\right)^{1/2} = \pm 0.815$

The data from which the values were derived is shown in Figure 27. Reproducibility of both peak height and retention time of components of a mixture were also determined. This data is tabulated below.

Run #	Estrone acetate		Estriol Diacetate	
	Height	Retention Time (min.)	Height	Retention Time (min.)
1	151	8.24	95	12.00
2	149	8.24	95	12.00
3	150	8.24	94.5	12.00

The reproducibility of the retention times may also be evaluated from Figure 27.

An evaluation of detector sensitivity.

It was previously assumed that the detector sensitivity of the Sr^{90} detector was a function of the number of particles in the detector at any time t. Therefore, it was also assumed that the signal output (in millivolts) would be a function of the retention time. This determines the rate at which molecules pass through the detector relative to another compound. Also, this output in millivolts was expected to be a function of the number of molecules as was shown experimentally. Since these studies were carried out with equiweight mixtures, the signal would be expressed by:

$$\frac{1}{\text{Molecular weight} \ \times \ \text{Retention Time}}$$

From the slopes of the lines for concentration versus output and the retention times as well as the integrated areas, the theory can be compared with the experimental values. The necessary parameters are tabulated in Table XXXIX.

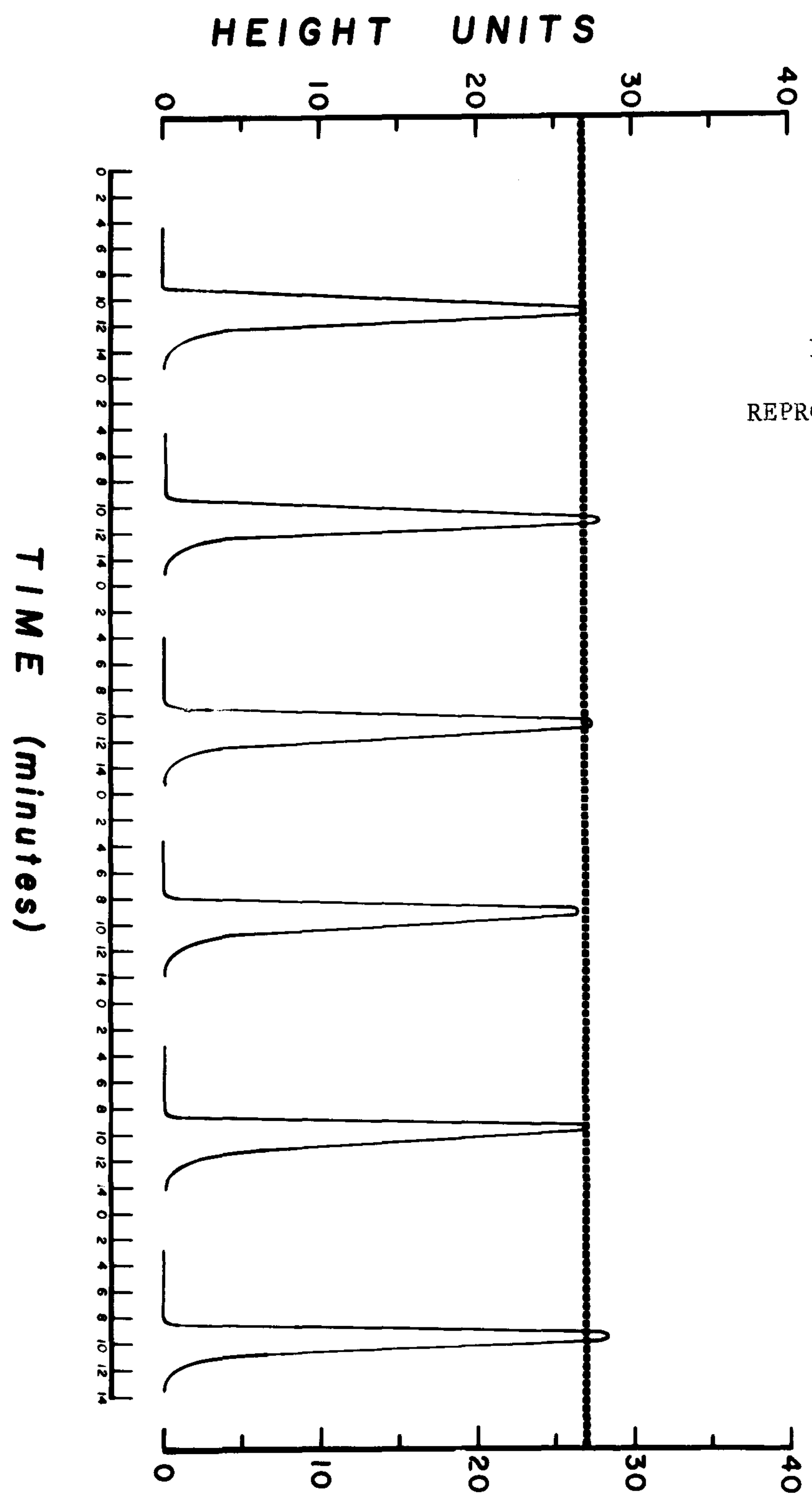


FIGURE 27

REPRODUCIBILITY

TABLE XXXIX

EVALUATED SENSITIVITIES

Compound as Acetates	Ratio of Slopes $\frac{E_x}{E_1}$	Predicted from theory $\frac{1}{\text{Ratio } T_r \times \text{Ratio MW}}$
Estrone	1	1.
Estradiol	0.60	0.605
Estriol	0.22	0.27

Summary

1. The thermal conductivity detector produced a linear response to a change of concentration. Due to the slower rate of elution it was slightly more sensitive to estradiol than estrone. The lower limit of detection was about 50 µg of steroid. The block could volatilize a maximum of about 400 µg, of substance. Linearity was determined between 100-400 µg.

2. The ionization detector showed a linear response to a change in concentration between 25 - 0.1 µg for all three compounds. The lower limit of detection is approximately 0.1 microgram for estrone and estradiol acetates, but, about 0.2 microgram for estriol.

3. Reproducibility was found to be of the order of ± 3 per cent. For six successive runs of estrone acetate the value obtained was 27.17 ± 0.75. Reproducibility of the retention time for mixtures was exact and identical with that for the individual compounds.

4. The sensitivity parameters were correlated and found to a function of the retention time and molecular weight for equiweight mixtures. The area also appeared to the function of these parameters. The sensitivity is inversely proportional to the product of the ratios of the retention time and molecular weight, but, the area is a function of the product of the retention time and molecular weight.

F. ACETYLATION STUDIES

The Mechanism of the Acetylation Reaction

Recent investigation of the mechanism of acetylation in the presence of tertiary amines suggests a reaction sequence involving the formation of the acetylpyridinium ion (13). In order to have the reactions proceed at a high velocity it seemed reasonable to work with high concentrations of acetic anhydride and pyridine to insure a high concentration of the reactive intermediate. Accordingly, the reactions were carried out without dilution in a pyridine-acetic anhydride solution.

The yields of derivatives from the acetylation reaction for the three major estrogens was determined by 1) gravimetry, 2) ultraviolet, 3) infrared absorption spectroscopy, and 4) by gas chromatography.

Acetylation of Estrone

1. Ultraviolet Spectral Analysis

This method of analysis is only useful in determining yields of estrone acetate. Acetylation of estrone can be followed by observing the disappearence of the absorption band at 280 mμ and the simultaneous appearance of the twin peaks at 268 mμ and 275 mμ. It is also useful in determining the extent of acetylation of the pheonolic hydroxyl group of estradiol, although it cannot give any information regarding the extent of acetylation of the c-17 hydroxyl group.

Estrone (137mg) was acetylated in pyridine (0.5ml) and acetic anhydride (0.5ml) for one hour at 40^{o}C.

Calibration:

Estrone Acetate (μg)	Absorbance Units
397.	0.93
198.5	0.49
132.5	0.32
99.0	0.23
79.5	0.18

Absorbance measurements for the reaction product gave the following results:

Run #	Absorbance Units
1	0.33
2	0.34
3	0.33
4	0.32
Average:	0.33

Using the above calibration this data was interpreted to represent a conversion of 87 per cent of estrone in one hour at the above conditions.

2. Gas Chromatographic Analysis

Samples of steroid were acetylated under varying conditions of time and temperature and the resulting products were injected into the gas chromatograph. Estrone and estradiol were injected directly after acetylation in pyridine and acetic anhydride. In the case of estriol, the pyridine and acetic anhydride were first evaporated by a stream of nitrogen at 40°-60°C. Estrone and estradiol were measured by direct comparison to a known standard and estriol was determined from a calibration curve.

Calibration of Standard:

Run	Estrone Acetate (μg/2μl)	Retention Time (Min.)	Peak Height (mm)
1	10	8.24	149
2	10	8.24	150
3	10	8.24	151

The acetylation reactions were run for varying times at constant temperatures with the following results:

Run #	Time Min.	Temp. °C	μg of Steroid/2 μl Expected	Found	Percent Recovery
1	30	60	5.78	5.05	87.5
2	40	60	5.78	5.12	89.0
3	50	60	5.78	5.28	91.5
4	60	60	5.78	5.12	89.0
5	70	60	5.78	5.22	90.5

Acetylation of 11.6 μg/2 μl of estrone for 48 hours at 25°C yielded 103 per cent of the corresponding acetate.

Acetylation of Estradiol

The acetylation of estradiol was thought to proceed in two steps. There occurs first an acetylation of the phenolic hydroxyl to the monoacetate followed by the esterification of the C-17 hydroxyl to yield the diacetate. The first step was studied by ultraviolet spectroscopy and the second by infrared spectroscopy. The overall conversion could then also be studied by gas chromatography.

With the assumption that the extinction coefficient of the monoacetate was similar to that of diacetate, a calibration curve was constructed for the diacetate of estradiol as shown in Table XL.

TABLE XL

CALIBRATION CURVE FOR ESTRADIOL DIACETATES

Estradiol Diacetate (grams/liter)	Absorbance 268 mμ	Absorbance 275 mμ		Max. 268 mμ	Max. 275 mμ
0.328	0.965	0.915		1100	1010
0.292	0.789	0.750		1000	965
0.220	0.580	0.560		995	960
0.146	0.360	0.350		925	900
0.073	0.200	0.195		1030	900
			Average	1010	963

Estradiol (14.85 mg) was acetylated in pyridine (6 ml) and acetic anhydride (4 ml) for one hour at 40°C. The average of four values obtained indicated a recovery of estradiol of 99 per cent of the theoretical. Comparison of the absorption coefficients at 268mμ and 275 mμ for estradiol diacetate and for the acetylation product gave the following data:

Wave length mμ	Absorbance of Estradiol Diacetate per mg	Absorbance of Reaction Product per mg
282	38.8	38.2
281	55.0	51.0
280	82.0	88.0
279	113.0	117.0
278	151.0	163.0
277	202.0	212.0
276	248.0	252.0

Because of the similarity of the values and the absence of visible distortion of the absorption peak at 280 mμ, it can be concluded that nearly all the estradiol had disappeared at the end of one hour. The nature of the product formed was determined by infrared spectroscopy.

Infrared Analysis - Changes in the 1760 and 1730 cm^{-1} vibration bands may be used to determine the extent of acetylation of both the phenolic and alcoholic hydroxyl groups. The 1730 cm^{-1} band is representative of the C-17-acetate, whereas the 1760 cm^{-1} band is characteristic for the phenolic acetate vibrations. All determinations by infrared spectrophotometry were carried out on a Perkin-Elmer Model 21 spectrophotmeter as follows: Gain 4.8; Speed 5: Suppression 2; Slit program 927; cell path length 0.50 mm for the sample and 0.517 mm for the CS_2 blank.

When pyridine and acetic anhydride were present in less than molar excess the only product formed was estradiol-3-acetated as determined from the carbonyl vibration bands. In the presence of an excess of these reagents a mixture of the mono and diacetates is formed in one hour. Quantitative determinations were performed by infrared spectroscopy to determine the ratio of mono acetate to diacetate.

Values for calibration of standards are shown below:

Estradiol Diacetate (μg)	Absorbance 1760 cm^{-1}	Absorbance 1730 cm^{-1}
1340	0.088	0.095
1070	0.070	0.075
805	0.054	0.058

Estradiol Mono-acetate μg	Absorbance 1760 cm^{-1}
3250	0.216
1625	0.116
812.5	0.060

Estradiol (1mg) was acetylated in pyridine (0.5 ml) and acetic anhydride (0.5 ml) for one hour at 60°C. The results are shown below:

Reaction #	Estradiol μg	Absorbance 1760 cm^{-1}	Absorbance 1730 cm^{-1}
1	1045	0.100	0.095
2	502	0.052	0.050
3	910	0.30	0.028

It will be noted that the ratio of the absorbance of the reaction product have reversed with respect to the standard. The added absorbance intensity at 1760 cm^{-1} is due to the presence of the monoacetate. Tabulated below are the percentage conversions at the end of one hour at 60°C.

Reaction #	Per cent Diacetate	Percent Monoacetate
1	92	8.1
2	92	8.2
3	92.5	7.5

From this data it appears that a reaction at the above conditions has the following composition at the end of one hour.

Compound	Percent of Mixture
Estradiol	0-1
Estradiol Monoacetate	7.5-8
Estradiol Diacetate	92-91

Allowing the reaction to proceed at 25°C overnight resulted in reversal of the peak intensities at 1730 and 1760 cm^{-1} with respect to those obtained after a one hour reaction. This information indicates that yield of the diacetate was 97.5 percent of the theoretical. These results are recorded in Figure 28, where A is the spectrum of authentic estradiol diacetate, B , the spectrum of the reaction product of estradiol at the end of one hour, at 60°C, C the spectrum of authentic estradiol-3 acetate and D the spectrum of the reaction product, of a reaction which was allowed to proceed overnight at 25°C.

Gas Chromatography

Standards:

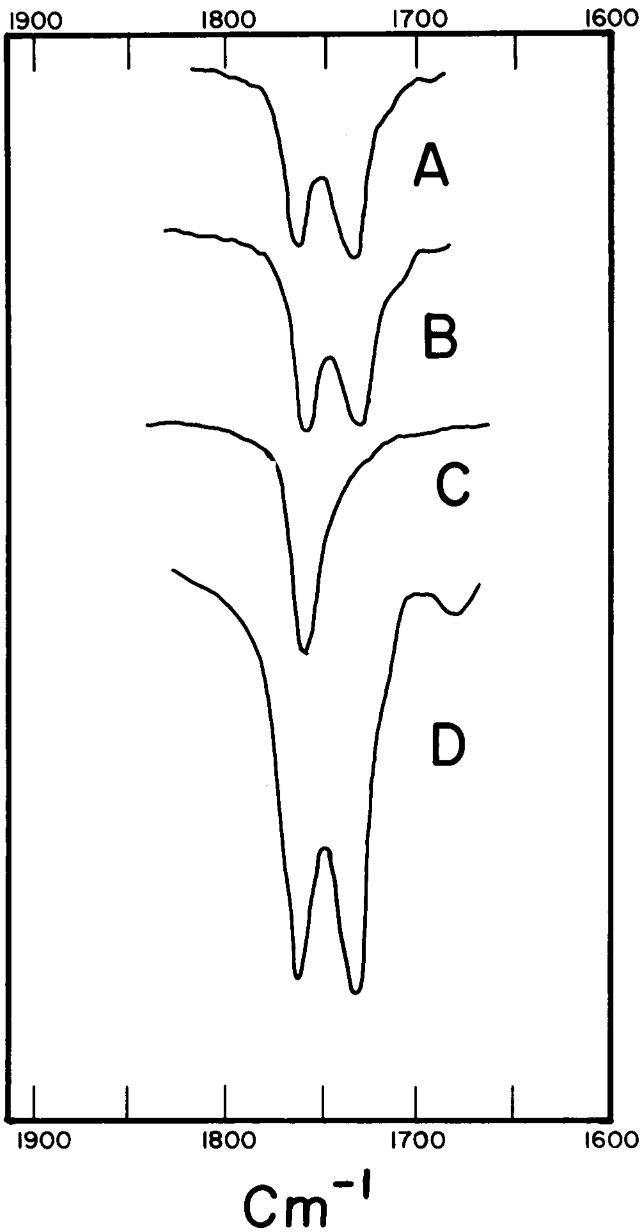
Run #	Estradiol diacetate µg/2 µl	Retention time min.	Peak Height mm
1	10	12.0	95
2	10	12.0	95
3	10	12.0	99.5

A study of the acetylation reactions gave the following results; with respect to different times of reaction.

Run #	Time Minutes	Temperature °C	Steroid Expected	ug /2 µl Found	Per cent Recovery
1	30	60	6.54	5.70	87
2	40	60	6.54	6.20	95
3	50	60	6.54	5.80	89
4	60	60	6.54	5.80	89
5	70	60	6.54	6.20	95

INFRARED SPECTRA OF ACETYLATION PRODUCTS OF ESTRADIOL

FIGURE 28



Acetylations were also studied at 25°C for forty-eight hours. The conversion in these studies were found to be 104 per cent of the theoretical.

Acetylation of Estriol

The acetylation of estradiol was studied both by gas chromatography and gravimetrically. As reported previously, the acetylation of 460 mg of estriol at 25°C for forty-eight hours produced 610 mg of its triacetate, representing a yield of ninety per cent of theoretical. Under the same conditions of time and temperature, but using gas chromatography as the method of analysis, the yield was found to be 111 per cent of theoretical. The conversion of estriol to estriol triacetate was found to be nearly complete after fourteen hours at 25°C. Approximately 1.3 per cent of a substance having a retention time consistant with that expected for estradiol diacetate was also found. This indicates an approximate conversion of ninety-eight per cent of estriol to its triacetate. A summary of the acetylation reactions are tabulated in Table XLI.

TABLE XLI

SUMMARY OF ACETYLATIONS

Compound	Time	Temp. C	µg of Steroid Added	Found	Method	Per cent Recovery
Estrone	48 hrs.	25	11.6	11.95	GLPC[1]	103.
Estrone	30 min.	60	5.78	5.05	GLPC	87.5
Estrone	40 min.	60	5.78	5.12	GLPC	89.0
Estrone	50 min.	60	5.78	5.28	GLPC	91.5
Estrone	60 min.	60	5.78	5.12	GLPC	89.0
Estrone	70 min.	60	5.78	5.22	GLPC	90.5
Estrone	60 min.	40	158.0	135.0	UV[2]	86.0
Estradiol	14 hrs.	25	11882.0	11600.0	IR[3]	97.5
Estradiol	48 hrs.	25	6.55	6.85	GLPC	104.0
Estradiol	30 min.	60	6.54	5.70	GLPC	87.0
Estradiol	40 min.	60	6.54	6.20	GLPC	95.0
Estradiol	50 min.	60	6.54	5.80	GLPC	89.0
Estradiol	60 min.	60	6.54	5.80	GLPC	89.0
Estradiol	70 min.	60	6.54	6.20	GLPC	95.0
Estradiol	60 min.	60	1370.0	1259.0	IR	91.5
Estradiol	60 min.	60	660.0	612.0	IR	93.0
Estradiol	60 min.	60	410.0	389.0	IR	92.5
Estriol	48 hrs.	25	6.48	7.20	GLPC	111.0
Estriol	14 hrs.	25	----	----	GLPC[4]	98.0
Estriol	14 hrs.	25	680 mg	610 mg	Grav.[5]	90.0

Methods:

1. Gas Liquid Partition Chromatography - all values in 2 µl
2. Ultraviolet absorption spectroscopy
3. Infrared absorption spectroscopy.
4. Only mole percents were taken
5. Gravimetric analysis

G. APPLICATION OF THE METHOD TO BIOLOGICAL MEDIA

In order to develop a reliable method to determine urinary estrogens several of the variables in the hydrolysis and extraction procedures were studied using gas chromatography. Among the variables studied were:

1. recovery of estrone and estradiol from a water solution and the effect of acid hydrolysis,
2. recovery of estrone and estriol from acid hydrolysed male urines under conditions of
    (a) high volumes, and
    (b) low volumes
3. the effect of sodium hydroxyde concentration on the recovery of estriol from acid hydrolysed male urines,
4. comparison of acid and enzyme hydrolysis, and
5. the estrone and estriol content of pregnancy urines.

The method for isolating the estrogens from urines involved treating a twenty-four hour urine collection with fifteen volume per cent of concentrated HCl and refluxing for one hour. This solution was extracted, after cooling, with first 400 cc of ether and later with two more portions of 200 ml of ether. The ether solution was reduced in volume and washed with Na_2CO_3 to remove strong acids.

The ether solution was then treated with 1N NaOH and extracted. The phenolic fraction was neutralized with dilute (6N) HCl and re-extracted with ether. The ether extract was then evaporated to dryness. The dried residue was dissolved in 2 ml. of pyridine and acetylated with 2 ml of acetic anhydride. The reaction was allowed to proceed for one hour at 65°C. The reaction mixture was evaporated to dryness and the residue dissolved in quantities ranging from 2 ml to 0.1 ml of acetone or toluene. A 2 microliter aliquot was introduced into the Sr^{90} chromatograph or 100 microliters injected into the chromatograph with the thermal conductivity cell. A urinary estrogen chromatogram is shown in Figure 29.

1. Recovery from Water

As shown in Table XLII, there was a twenty per cent loss of material which was not associated with acid hydrolysis in water. For various duplicate runs, the difference rarely exceeded five per cent which was the variation expected for the gas chromatographic reproducibility. These recoveries are comparable to those reported by Brown (25) where recovery of estrogens added to acid hydrolysed urines were of the order of eighty per cent, although this work used much lower steroid concentrations than as reported here.

TABLE XLII

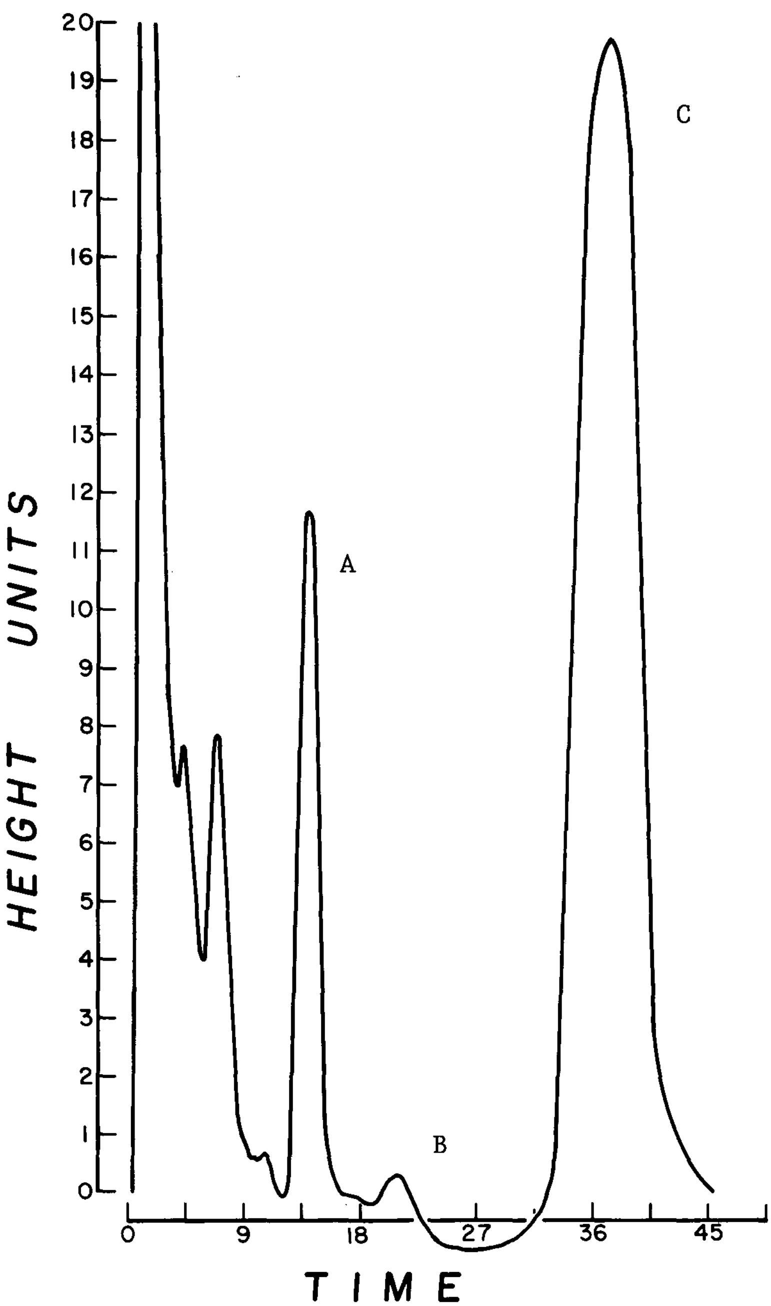
Recovery of Estrone and Estradiol from Water

Estrone					
Direct Extraction			Treated with HCl before Extraction[1]		
γ Added	γ Recovered	% Recovery	γ Added	γ Found	% Recovery
5020	4000	80	5220	4580	88
5020	3760	75	5220	4580	88
5310	5770	108	5260	4200	80
5310	4940	93	5260	4350	83
5310	4390	83	5260	4590	87
	Average	88 %		Average	85 %
Estradiol					
6120	5450	89	5940	4650	78
6120	5160	84	5940	4460	75
5980	7000	119	6020	5020	83
5980	5440	91	6020	4840	80
5980	5020	84	6020	5160	86
	Average	93 %		Average	80 %

1 The mixture was treated with 15 vol. % of HCl for one hour under reflux.

SEPARATION OF ESTROGEN FROM A PREGNANCY URINE

FIGURE 29



A - Estrone acetate
B - Estradiol diacetate
C - Estriol triacetate

2. Recovery of Estrone and Estriol from Acid Hydrolysed Male Urines

(a) High Volume

This data is presented in Table XLIII. In general it appears that estrone recoveries are lower than estriol by twenty per cent. This discrepency, at the moment, can not be explained. The recoveries of estriol appears to be nearly quantitative.

(b) Low Volume

In this study, 20-50 ml of acid hydrolysed male urines containing 1 mg of estrone and 3 mg of estriol was extracted and analyzed. The results are presented in Table XLIV. Again the recovery of estrone is lower by 20% relative to estriol recoveries.

In agreement with the results of Brown (25), the recoveries are independent of the volume as indicated by the overlap of the standard errors. They were for 5000 ml 95 ± 3.4 and for 20-50 ml 90 ± 3.1.

3. The Recovery of Estriol using 1N NaOH and 0.1 NaOH

No effect was observed on changing the concentration of the base, as indicated by the data presented in Table XLV. The recoveries with o.1 N NaOH were 99 per cent with a S.E. of 5, where as with 1N NaOH the recovery was 95 ± 3.9. Again the overlap of the standard error indicates no significant change.

TABLE XLIII

Recovery of Estrone and Estriol from Acid Hydrolysed

Male Urines using 1 N NaOH

Urine #	Run #	Estrone % Recovery		Estradiol % Recovery	
1	a	71	73	97	97
	b	75		97	
2	a	62		87	
3	a	98	100	108	110
	b	102		112	
4	a	77	77	91	93
	b	77		94	
5	a	60	57	76	72
	b	54		67	
6	a	--		73	
7	a	65		97	
8	a	84		106	
9	a	75		102	
10	a	77		101	
11	a	77		107	
		Average 75 ± 12		Average 95 ± 13	
		S.E. = 3.4		S.E. = 3.9	

TABLE XLIV

Low Volume Extractions

Urine #	Estrone % Recovered	Estriol % Recovered
1	78	86
2	61	95
3	70	86
4	71	83
5	77	100
Average	71 ± 7	90 ± 7
	S.E. = 3.1	S.E. = 3.1

TABLE XLV

Recovery of Estriol from Hydrolysed Male Urine using 0.1 N NaOH

Urine #	Run #	Estriol % Recovery	
1	a	107	
2	a	109	107
	b	105	
3	a	95	
4	a	85	
		Average 99 ± 10	
		S.E. = 5	

4. <u>Comparison of Enzyme and Acid Hydrolysis</u>

Several pregnancy urines were divided and one part of each was treated with an enzyme preparation and the other was heated with acid. In enzymatic hydrolysis, the urine is treated with an extract of *Helix pomatia* known to contain β-glucuronidase and sulfatases. The mixture was incubated at 37°C overnight at pH 5. The data presented in Table XLVI point to the unreliability of enzyme hydrolysis.

5. <u>The Estrone and Estradiol Content of Pregnancy Urines as Determined by Gas Chromatography</u>

Total urines (24 hour collections) were treated as described above with results consistent with published data (92). The reproducibility among several runs on the same sample is of the order of 2-5 per cent. This data is presented in Table XXXVI.

6. <u>Application to Enzyme Conversions</u>

The enzyme 17β-estradiol dehydrogenase, was available in this laboratory. It had been prepared by the method of Langer and Engel (85). Preliminary enzyme substrate reactions were run to determine enzyme activity in solutions of the following composition:

TABLE XLVI

Estrone and Estriol Content of Pregnancy Urines

Urine #	Run #	Acid Hydrolysis Estriol ug/24 hrs.	Acid Hydrolysis Estrone ug/24 hrs.	Enzyme Hydrolysis Estriol ug/24 hrs.
1.	1	7656	1354	9,624
	2	7925	1398	10,106
	3	7748	1398	10,489
	4	7656	1430	Avg. $\bar{X}$=10,073±354
	5	8100	1430	(± 3.54)
		$\bar{x}$=7817±172	$\bar{x}$=1213±24	
		S.E. = 77	S.E. = 11	
		(± 2.2%)	(± 2)	
				Enzyme Comparison + 30 %
2.	1	12,304	608	11,060
	2	12,556	580	12,403
	3	12,918	546	11,407
	4	11,714	642	12,250
	5	12,939	606	Avg. 11,810±526
		12,486 ± 453	516 ± 28	S.E. = 263
		S.E. = 202	S.E.=±12	(± 4.4)
		(± 3.6)	(±5.4)	
				Enzyme Comparison - 5.5 %

TABLE XLVI continued

Urine #	Run #	Acid Hydrolysis Estriol ug/24 hrs.	Acid Hydrolysis Estrone ug/24 hrs.	Enzyme Hydrolysis Estriol ug/24 hrs.
3.	1	4364		8154
	2	4594		7748
	3	4165		7951 ± 203
	4	4165		S.E. = 144
	5	4280		(±2.6%)
		Avg. = 4314 ± 159		
		S.E. = 71		Enzyme Comparison + 80 %
		(± 3.7%)		
4.	1	3167		943
	2	3167		943
	3	3261		971
	4	3170		926
		3191 ± 40		946 ± 16
		S.E. = 20		S.E. = 8
		(± 1.3%)		(±1.7%)

(1) 100-150 micrograms of estradiol in 0.1 cc of solvent

(2) 0.2 cc of the enzyme preparation

(3) 0.5 cc of DPN

(4) 0.3 cc of buffer pH 9 $NaHCO_3/Na_2CO_3$.

(5) 1.9 cc of distilled water

The total volume in the cuvette was 3 ml..

1. The reaction was followed by observing the increase in absorbance at 340 millimicrons.

The same reaction was carried out in tubes and the products formed analysed by gas chromatography. The ratio of estrone to estradiol from the ultraviolet data is compared with that obtained by gas chromatography.

Since the enzyme preparation was about 1 year old, it was necessary to determine its activity. From the extinction coefficient of DPNH it was found that the enzyme was active and converted 110 μg of estradiol to estrone in 1.5 hours at the conditions described previously. A comparison of the conversion of estradiol to estrone measured by ultra-violet spectroscopy and gas chromatography gave the following results:

Method	% Estrone	% Estradiol
Ultraviolet	69	31
Gas Chromatography	59	41

From these results it was concluded that gas chromatography could also be used as a method of following metabolic conversions of estrogens by enzymes or tissue preparations for *in vitro* investigations.

III DISCUSSION

The prime purpose of this work was the development of a rapid and sensitive procedure for the assay of estrogenic hormones. The method as developed and applied to pregnancy urines is quantitative and rapid. The major advantage of gas chromatography is simultaneous quantitation and separation.

The major problem to be faced before undertaking such a study was the question of thermal stability of the estrogens.

The system studied during decomposition was heterogeneous in so far as the molecules in the tube could at any time be in either a gas or a liquid phase. Therefore, decomposition could have occured in either the gas or liquid phase. If decomposition occurs in the liquid phase, then the reaction would be expected to be first order with respect to the concentration of estradiol. If the decomposition occured in the gas phase the reaction would be zero order in so far as the vapor pressure is constant at any given temperature. Since our determinations were done for periods of no longer than hours using large amounts of materials the chance that decomposition was occuring in the liquid phase as a first order reaction cannot be ruled out. Nevertheless, over the periods of time studied the reactions appeared to be zero order. This allows us to assume that decomposition occured in the vapor phase and that the liquid phase was more stable than the vapor phase. This reaction, assumed to be occuring in the vapor phase, was the dehydrogenation of a secondary alcohol to a ketone.

The rates were linear with the reciprocal of the absolute temperature. This does not help in distinguishing between a first order and zero order reaction since for a single reaction step either order would show the Clausius - Clapeyron relationship. One fact, however, which might indicate zero order reaction is that when the compounds were chromatographed and showed retention on a column, no decomposition was observed. This means that since the retention time was measurable the compound spent more time in the liquid phase of the column than in the gas phase thereby reducing the decomposition rate. From the theory of homogeneous gas reactions the rate of decomposition is determined by the vibration along a chemical bond (108). If the molecule, at any given plate, spends more time in the liquid phase then in the gas phase and if the liquid phase can absorb the extra vibrational energy, then the molecule is stabilized before moving to the next plate. On this basis then, it is possible to account for the fact that the decomposition rate in a column with a high surface area is minimal relative to the reaction rate in a closed tube. If the assumption is made that decomposition occurs only in the vapor phase then it would seem that in gas chromatography the more retentive the column packing material the less decomposition should be expected. The infra-red and ultra-violet spectra of the acetate as well as the symmetry of the gas chromatographic peaks confirm this expectation.

If the reaction had been homogeneous, in the gas phase, then a first order reaction should have been expected. It now remains to be shown that the assumption of deactivation is not in conflict with the accepted theories of gas phase reactions (108). In the tube, with estradiol in the gas phase the intermolecular collisions are continually producing molecules with higher than average energy and occassionally molecules with an energy above a certain critical value necessary for the activation that precedes decomposition. The Lindemann theory (108) assumes a certain time lag between activation and decomposition; the activated molecule does not immediately rupture, but moves around for a while in its activated state. Sometimes it may meet an energy-poor molecule and in the ensuing collision it may be depleted of enough energy to be deactivated. If there is a surface with a liquid phase the deactivation might also occur by collision with surface molecules. This situation may be represented as follows:

$$\text{estradiol (gas)} + \text{estradiol (gas)} \underset{K_{-2}}{\overset{K2}{\rightleftarrows}} \text{estradiol (g)} + \text{estradiol (activated) or estradiol (liquid)}$$

$$\text{Estradiol (activated)} \rightarrow\rightarrow\rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \rightarrow \text{estrone} + H_2$$

The process called <u>activation</u> consists essentially in transferring translational energy into energy stored in internal degrees of freedom, especially vibrational degrees of freedom. The fact that a molecule is moving rapidly (high translational kinetic energy) does not make it unstable. In order to cause reaction the energy must get into the chemical bonds where high-amplitude vibrations will lead to rupture, decomposition or rearrangements.

The transfer of energy from translational to vibrational can occur only in collisions with other molecules or with the wall of the vessel. The critical point of the Lindemann theory is that there is a time lag between the activation of the internal degrees of freedom and subsequent bond cleavage The reason is that a polyatomic molecule (like estradiol) can take up collisional energy into a number of its 3n-6 vibrational degrees of freedom and some time may elapse before this energy flows into the one bond that breaks.

The equations for the above proposed schema would indicate that:

$$\frac{d(\text{estradiol) activated}}{dt} = K_2 \text{ (estradiol (gas))}^2 - K_{-2}\text{(estradiol)}$$

$$d\,\frac{\text{(estrone)}}{dt} = K1 \text{ (estradiol activated)}$$

Because the value for (estradiol activated) is necessarily small, it may be set equal to zero and if the rate of deactivation of estradiol is much greater than its decomposition K_2 (E_2deactivation) K_1 the rate is

$$\frac{db}{dt} = \frac{K1\ K2}{K\text{-}2} \text{ (estradiol} = K_1 \text{ (}E_2\text{ gas)}$$

It now becomes evident that it is possible to obtain first order kinetics while preserving a collisional mechanism for activation. This situation will then exist whenever the activated molecule has a sufficiently long life-time for it to be deactivated by collision before it has a chance to break into fragments. Since in the present heterogenous system estradiol (gas) is constant, the kinetics observed would then be zero order due to the vapor pressure and

$$\frac{dB}{dt} = K_x \text{ when } K_x \text{ contains a}$$

term related to the vapor pressure of estradiol any any particular temperature.

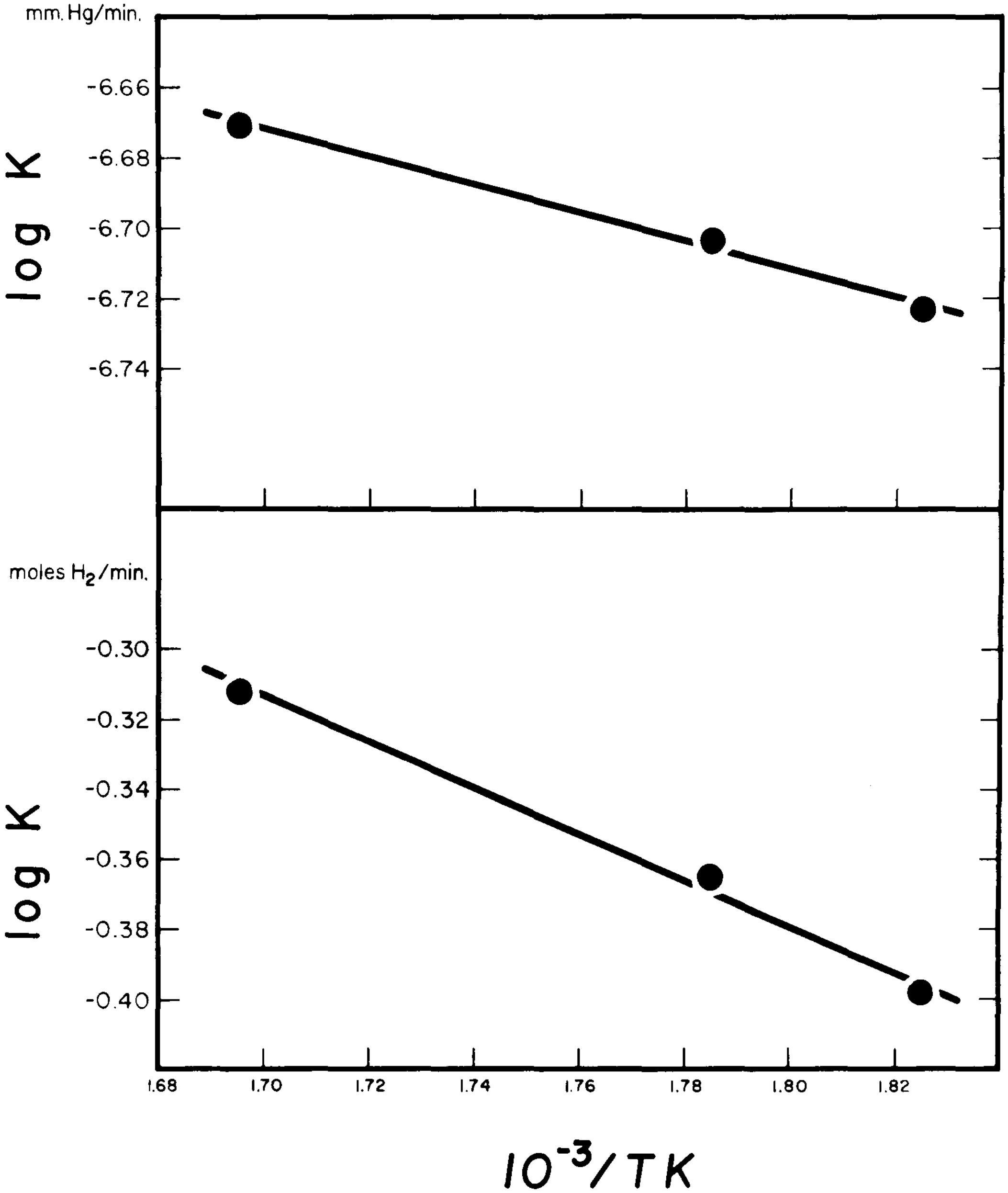
The plot of log K (log $\frac{dH_2}{dt}$ or log $\frac{d\,(\text{estrone})}{dt}$ vs. $\frac{1}{Tk}$ is usually linear for heterogenous reactions (108) and indeed between 275-300^{o}C - where it was suspected that only one dehydrogenation was proceeding - this was found to be true Figure 30. From the slope of the straight line an activation energy (E_a) is called the apparent activation energy since it is usually a composite quantity including not only the ture activation energy, but also the heat of adsorption of reactants and products.

Therefore, since the rate of deactivation is increased in a gas chromatographic system when the column coating is not catalytic but is deactivating, decomposition is not a particularly dominant factor. A small quantity of decomposition may have occurred but it could not be detected by any of the methods used in this investigation.

Theoretical considerations of volatility lead to the choice of non-polar derivatives. Also, practical consideration of the thermally stable liquid phase, further strengthened this choice. The final choice of the proper derivatives was based on several more factors. For one thing, the acetylation reaction with acetic anhydride in pyridine proceeded quickly and quantitatively (104). It produced mono-, di-, and tri-acetates thereby reducing polarity in polyhydroxy substances and allowed bettwe separation on the column. The great differences in molecular weight introduced by acetylation were particularly important in view of the fact that separation in gas chromatography is largely a function of molecular weight among a series of closely related substances(74).

APPARENT ACTIVATION ENERGY FOR THE DECOMPOSITION OF ESTRADIOL

Figure 30



As was pointed out previously (page 108) the retention times fit the following expression reasonably well (Figures 18, 19).

$$\ln T_r = \frac{MW}{RT} + K$$

Thus, it becomes apparent that greater separations can be obtained with greater differences in molecular weight. Since this relationship is logarithmic, the considerable difference in retention times between the methyl ethers and the three acetates dictated the choice of the acetates.

The acetates were shown to be stable at the conditions necessary for separation. The only decomposition observed occured when the steroid esters were introduced into the chromatograph from solutions of primary alcohols. This was interpreted as suggestive of transesterification. For this reason non-polar solvents should be used for introducing the esters into a gas chromatograph.

Experiments with columns of various lengths indicated that short columns of high silicone content were desirable. Estriol, the slowest member of the group, can be seen to emerge as a symmetrical peak anywhere from 7 to 180 minutes. The low flow rates may be used to determine the molecular weight of an unknown among a group of similar derivatives, as indicated by the equation presented above. It is expected that this parameter will facilitate identification of unknown compounds in mixtures isolated from biological sources. The method has been found to be reproducible both as to retention time and peak height in day to day usage. The sensitivity was found to be about 0.2 μg for the estradiol, estriol and estrone acetates. Increase in sensitivity may be attained by using conditions which decrease retention time since it was found that the sensitivity is primarily a function of the retention time (page 131). Another method of increasing sensitivity is to use derivatives which have short retention times. These statements apply principally to the Sr^{90} detector.

The acetylation reaction was studied and found to be sequential in nature. Careful evaluation of the esterification of estradiol has shown that with a reaction time of one hour at 60°C a yield of 92 per cent of the theoretical, can be obtained. The remaining 8 per cent was shown to be the 3-mono-acetate of estradiol. The presence of the mono-acetate impurity may be of importance since it may be confused with estrone acetate which has nearly the same retention time, except under conditions involving very long retention times. A yield of 98 per cent of the diacetate may be obtained by carrying out the reaction overnight.

Acetylation of estriol overnight also yields 98 per cent of the tri-acetate with about 2 per cent of a substance whose retention time is consistent with that expected for the diacetate of estriol.

The prime purpose in developing a new method of estrogen anaylsis was the necessity of having available a rapid as well as chemically specific determination. The fact that the gas chromatographic procedure allows rapid separation of the individual components concomitant with quantitative measurement easily achieves the two prime objectives.

Although no attempts were made at this time to develop a highly sensitive assay, since only crude urine extracts were utilized for the biological application of this method, the evidence presented in the earlier part of this thesis clearly suggests the feasibility of determining low concentrations of these hormones in biological media.

As has been pointed out earlier, the best prevailing method in terms of chemical specificity and high sensitivity is the procedure described by Brown (25). While no attempt can be made to compare the method described here with that of Brown as to low level analysis the chemical specificity, the recovery data and speed of analysis may indeed be compared.

Brown states that the sensitivity of the method seems to depend on the sensitivity of the color reaction and the accuracy of the color measurements and not on the extraction procedures. While gas chromatography is also independent of the extraction procedure, its' sensitivity is also independent of any other reaction other than the presence of the compound in the detector. Because of this independence of the extraction procedure, it is permissible to compare recovery studies with fairly large quantities of steroids to those carried out by Brown on relatively smaller samples. We were able to recover 75% of added estrone based on analyses of eleven samples, as compared to Brown's 82% recovery based on the analysis of ten samples. It appears then that our recoveries are some what lower but are consistantly so. In contrast, however, when Brown was able to recover only eighty-three per cent of estriol, our average estriol recovery is ninety-five per cent. The fact that estriol recovery is quantitative, while estrone recovery shows a consistant twenty-five per cent loss, might point to a selective adsorption which is not dissociated by ether extraction. This suggestion does not rule out other explanations.

Attempts to recover quantities of estrogens near the order of magnitudes described by Brown (60 μg/24 hrs.) were carried out by adding small quantities of estrone (20 μg) and estriol (60 μg) to acid hydrolysed male urines (20 ml and 50 ml). The results further verify Brown's statement that the mean recovery is independent of concentration since the new means determined at this concentration do not differ markedly from the previous data. See Table XLIII and XLIV.

No attempts were made to analyse urines containing smaller quantities (lower than 20 μg of estrone/ 20 ml). On the basis of the base line obtained from the total extraction of male twenty-four hour urines (base line reached in 5-7 minutes) one may predict that quantities of 10 μg and possibly less per twenty-four hours may be analysed by using small amounts of crude extracts.

Obviously further purification prior to gas chromatography all-owing attainment of base line in shorter time would further increase the sensitivity of the method. The choice of working on partially purified extracts must depend entirely on the nature of the project to which the method is applied.

By and large, it is safe to say that in terms of speed (the number of analysis which can be performed per unit time) the gas chromatographic procedure is by far superior to any presently available estrogen assay, in which fractionation of the individual components must be performed. The time gained by this method would allow the investigator considerable latitude in developing pre-purification methods, without severely holding up a given project.

In summary, the method reported here compares favorably with any previously reported estrogen assay. In comparison to biologic assay, gas chromatography has the advantages of rapid determination and chemical specificity. With respect to other chemical assays, the gas chromatographic procedure is at least as specific and far more rapid, although it has not yet been developed to its highest sensitivity, particularly since this latter factor is dependent on the further development of high sensitivity detectors.

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ABSTRACT

The prime purpose of this investigation was the development of a rapid and sensitive procedure for the assay of estrogenic hormones. The advent of gas chromatography offered a possible solution to this problem.

A pre-requisite of any gas chromatographic method is that the compounds to be chromatographed be volatile and thermally stable at the temperatures of vaporization and separation in the presence of the eluting gas. Accordingly, the thermal stability of certain free and acetylated estrogens was studied. The results of these studies indicated that decomposition was minimal when small quantities were subjected to gas chromatographic separation. Estrogen acetates were found to be stable up to 340°C by infrared spectroscopy, ultraviolet spectroscopy and by an evaluation of the gas chromatographic peak symmetry.

Once suitable derivatives had been found, the parameters of separation and detection were evaluated. The optimum conditions for separation were found to be: 1) a temperature of 240°-280°C, 2) column length of 3 feet, 3) a silicone grease liquid phase, 4) flow rates of 50-500 cc/min, and 5) non-polar solvents. The lower limits of detector sensitivity were found to be 0.2 μg for a Sr^{90} detector and 100 μg for the thermal conductivity cell detector. The ionization detector responded linearly in the range of 0.2 to 25 μg. The thermal conductivity cell detector responded linearly in the range of 100 to 400 μg.

In order to have a quantitative procedure, it was necessary to determine the optimal conditions for the preparation of the estrogen acetates. It was found by means of gas chromatography, infrared spectroscopy and ultraviolet spectroscopy that estrone was converted to its acetate in 86-90 per cent yield in 2×10^{-3} M solutions in one hour at 65°C. Estradiol, under the same conditions was found to be converted in 92 per cent yield to the diacetate. The remaining 8 per cent was found to be estradiol-3-acetate. By allowing this reaction to proceed overnight only two per cent of the monoacetate was found. Estriol, in reactions which were carried out under similar conditions, was found to be converted in 98 per cent yield to the triacetate.

In order to develop a urinary assay procedure, recovery studies from water and acid hydrolyzed urine, were carried out. The results indicated a 75-80 per cent recovery of estrone and estradiol and a 90-95 per cent recovery of estriol. The lowest concentration studied was the recovery of steroid from a solution containing 1 μg/ml. Studies at this lower concentration showed that the recoveries were independent of concentration.

The method described above has the advantage of chemical specificity, rapidity and reproducibility. As yet, it has not been developed to its highest sensitivity, however, coupled with prepurification procedures and with advances in instrumentation, it appears that the method will become useful for low level estrogen determination in biological media.

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