STUDIES OF OVARY INCUBATION AND STEROID CONSTITUENTS OF THE PLACENTA FROM A MARE TREATED WITH ACETATE-1-C^{14}

A Thesis

by

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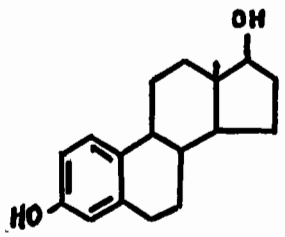
PART ONE

STUDIES OF OVARY INCUBATION

GENERAL INTRODUCTION

The ovary has been recognized as an endocrine organ for many years. This was established in many species, in classical fashion, by removing these organs and observing what occurred in the spayed animals (1). Early workers, Fellner (2) and many others, prepared placental extracts which would induce estrus in ovariectomized animals. A major advance was the 'vaginal smear' method for the bioassay of the estrogenic activity of extracts of tissue and urine devised by Allen and Doisy (3), before the chemical identity of the hormone, responsible for this activity was known. This technique aroused interest in estrogenic extracts and led to the isolation and identification of the estrogenic hormones from urine, ovarian follicidar fluid and placenta (4).

The isolation of the estrogens implemented the investigation of the metabolism of the individual estrogens. Conversions of these were shown in <u>in vivo</u> experiments: estradiol-β (I) given to male human subjects gave rise to estrone (II) and estriol (III) which were isolated from the urine in small yield (5, 6); when estrone was administered to rats, estradiol-β was recovered from the urine of these animals (7).



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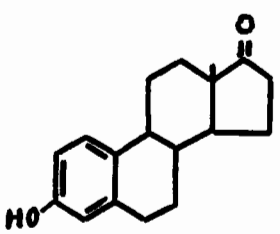

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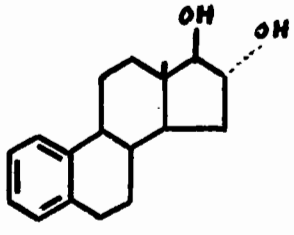

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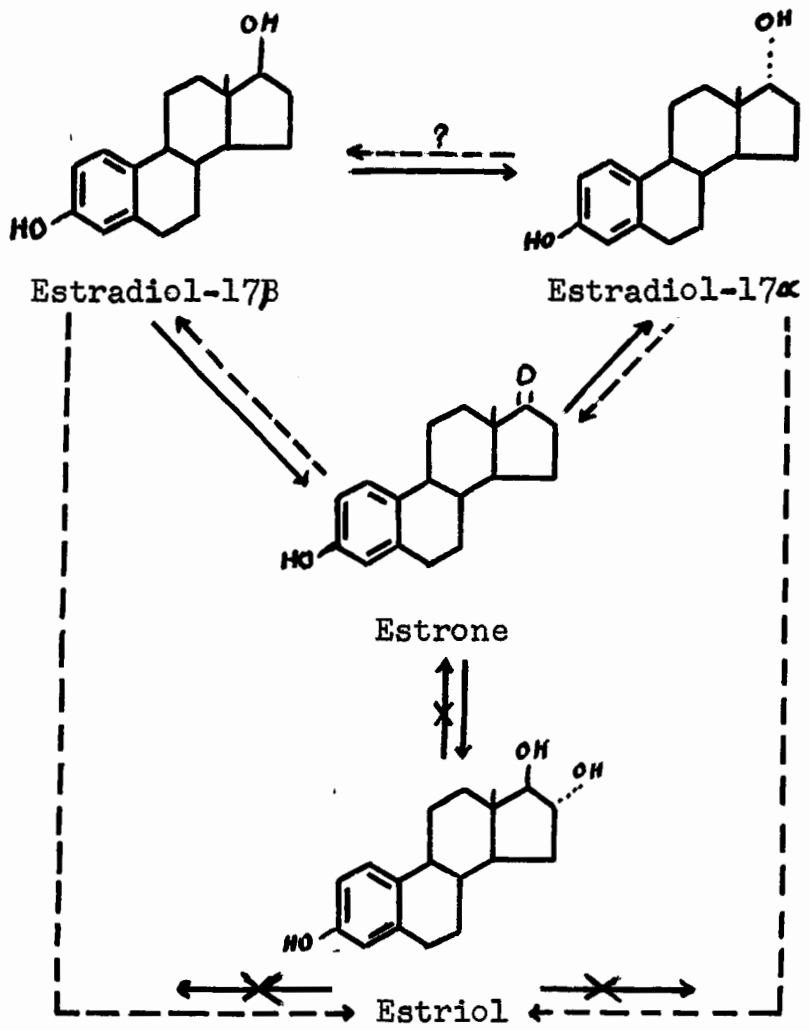


CHART A. INTERCONVERSION OF THE ESTROGENS

Solid arrow - established by isolation
Broken arrow - indicated by carbonate partition
Crossed arrow - contraindicated by either method

after Heard (8).

The information obtained with experiments of this type was summarized by Heard (8), whose chart is reproduced in this thesis, Chart A. In these experiments, the individual

estrogens were isolated from the urine and identified, or the urine was extracted, the extract was fractionated and colorimetric or bioassay methods of analysis were employed for the estimation of the estrogenic material present in the fraction. The results obtained by steroid isolation provided conclusive evidence for the conversions shown by solid lines in Chart A; those conversion results obtained by the partition procedure (broken lines) provided only indicative information. Interconversion of estrone and estradiol-β, activation of estrone and inactivation of estrone and estradiol-β by various tissues in in vivo experiments, have been shown by many workers, and are discussed in Part One of this thesis.

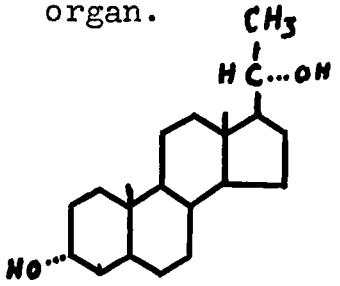
The interconversion of estrone and estradiol-β had not been shown by ovary slices. Also the production of estrogenic material by the incubation of ovarian tissue had not been reported. Experiments, whose aim was to provide answers for these problems, were performed as Part One of this thesis.

The endocrine functions of the placenta are well recognized to-day. After it was deduced that pregnanediol (IV), in the human, was the main excretory product of progesterone (V) (9),it was observed that the daily levels of excreted pregnanediol rose during pregnancy for several months after the corpus luteum had ceased to produce this hormone (4) and fell sharply after parturition (10). These facts indicated strongly that some organ, other than the corpus luteum, was producing progesterone and that this organ was probably the placenta. Supporting

evidence for this view was based on the discovery of pregnanediol in the urine of a woman ovariectomized during pregnancy (4) and on the high titers of urinary pregnanediol that continued after the expulsion of the fetus in abortion, when the placenta was retained (11). The production of gonadotrophin by the placenta has become widely accepted (12); this chorionic gonadotrophin has been detected in the urine of patients who have retained only a few chorionic villi after abortion (13) and has been extracted also from placental tissue by Bickenbach (14), who found that the extractable amounts of this hormone increased as pregnancy progressed. An accumulation of evidence suggests strongly that this organ also produces estrogens during pregnancy. Ovariectomy in various species, human, mares and monkeys, during pregnancy did not cause the level of excreted estrogens to fall to non-pregnancy values (15, 16, 4). A wealth of evidence of this kind, of which only a short account was given here, has attributed these three endocrine functions to placenta; for more detailed reviews of this field see Pincus (4) and Heard (8).

For many years the placenta was the main source of extracts containing'progestin' and 'estrin'; many extraction procedures were devised and improved to provide richer concentrations and better separations of these two hormonal principles (17, 18, 19, 20). These extracts were purified sufficiently for clinical purposes and the 'estrin' preparations were used in relieving ovarian

dysfunction (21). This estrogenic activity was explained by the discovery of estriol, estrone and estradiol-β in placental extracts (22, 23). The 'progestin' extracts were sufficiently potent to produce progestational activity in rabbits (24, 25). The isolation of progesterone from human placenta was not achieved until very recently. Samuels and his co-workers (26, 27) and Pearlman (28) have reported that placenta contained one mg. of progesterone per kg. wet weight of tissue. Pearlman (29) has also isolated allopregnanol-3(β)-one-20 (VI), pregnanediol-3(α), 20(α) (IV) and allopregnanediol-3(β), 20(α) (VII) from this organ.



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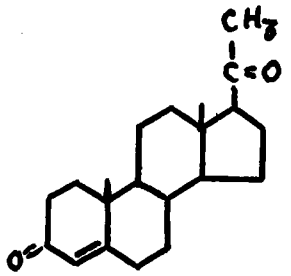

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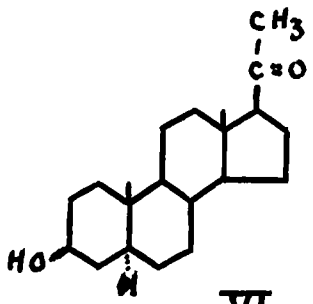

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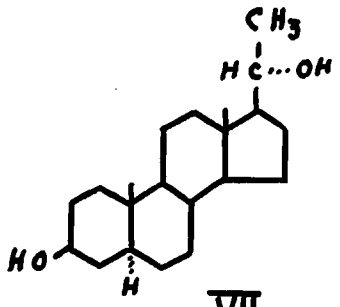

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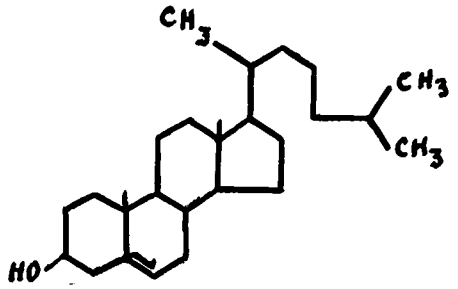


VIII

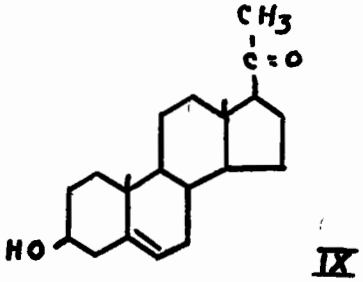
Placenta has been examined for the presence of other compounds. Both free and esterified cholesterol (VIII) have been reported to be present in this tissue which contained from 0.9 to 2.0% total cholesterol (30, 31, 32).

Cunningham and Kuhn have detected a low level of androgenic activity in extracts of placenta (33). Examination of this organ has been extended to determinations of xanthrophylls and carotenes present (34) and to the isolation of the pigment uteroverdin from canine placenta (35).

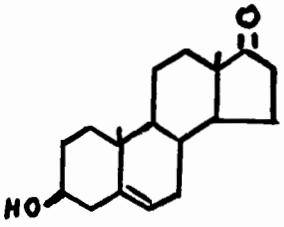
This wealth of placental constituents led us to believe that the examination of an equine placenta, removed from a mare previously injected with high levels of radioactive acetate, would be profitable. This investigation is described in Part Two.

A. INTRODUCTION

In vitro experiments have been performed with endocrine and other tissues resulting in the increased steroid content of the medium and/or of the tissue present in the incubation flask, especially when the appropriate hormone was added to the medium. Increased corticoid levels were found in the medium of such an incubation of quartered adrenals or adrenal slices and adrenocorticotrophic hormone (36). More of each of the three human estrogens were detected in placental tissue after incubation than was present before incubation (37). Similar experiments have shown the incorporation of acetate, labelled with C^{13}, C^{14} or D, into the cholesterol of many tissues: liver (38), mammary gland (39), spleen, gut and adrenal cortex (40, 41) and testes (42). More experiments of this kind established the incorporation of acetate into testosterone (VIII) by testis slices (42) and into corticoids by adrenal slices (43). Thus it seemed possible that similar experiments with surviving ovary slices might show the endogenous production of estrogens and the incorporation of acetate into the estrogen molecule. It seemed logical to begin these experiments by incubating ovary slices with non-



IX



X

radioactive acetate until benificient conditions for estrogen production were established.

Nissim (44) incubated beef ovary with various steroids, pregnenalone (IX), progesterone, dehydroisoandrosterone (X) and testosterone and noticed no increase in the estrogenic activity of the tissue or medium. The incorporation of acetate, labelled with C^{14}, into estrone and estradiol-β occurred when Werthessen et al. (45) perfused sow ovaries; large dilution with non-radioactive estrogens was carried out and the isolated estrogens each had a specific activity of 3 d./min/mg.

The fate of estrone, estradiol-β and estriol in in vitro experiments has been studied by many workers. Crépy examined the conjugation of these compounds with glucuronic acid during incubation with rat liver slices (46, 47). The inactivation of estrone and estradiol-β with liver and kidney preparations has been investigated (48, 49, 50, 51). In the case of estradiol-β this could be due, at least in part, to the conversion of estradiol-β to estrone (52), but Pearlman (53) has introduced evidence that estradiol-β is also transformed into unknown metabolites when incubated with rat liver slices. The interconversion of estrone and estradiol-β has been established by Ryan and Engel (54, 55), in incubations of these estrogens with many tissue preparations; the same authors introduced separation of estrogens with the Craig counter-current distribution apparatus (56). This interconversion can explain the activation of estrone observed

by other workers when this hormone was incubated with various tissues (48, 49, 50, 57, 58). This type of experiment had not been performed with ovarian tissue, and the problem of the fate of these two estrogens when incubated with ovary slices is examined in the experiments described in the following section of this thesis.

The detection and estimation of very small quantities of estrogens in biological extracts have been facilitated by the advent of paper chromatography. Bush (59) was able to separate the three human estrogens using a method that involved filter paper impregnated with alumina. Heftman (60) prepared diazo derivatives of these compounds and separated these by ascending chromatography; Nyc et al (61) proposed a system of chromatography that gave a separation of these estrogens but, as the R_F values fell within a narrow range did not promise reliable results. These methods were not considered to be satisfactory for our experiments, as recovery of free estrogenic material was desired; this initiated the investigation of many solvent systems, a search that resulted in the use of isooctane-benzene-water for chromatography with these compounds. While the following experiments were in progress, several additional chromatographic procedures were published; Boscott (62) separated the estrogens with a system which was made up of saturated aqueous sodium p-toluenesulphonate and tolune; Heusghem (63) has shown that a satisfactory separation of these compounds could be effected with this system, ammonium hydroxide and a mixture

of one part of chloroform, nine parts of benzene and one part of normal ammonium hydroxide. A three step method was devised by Axelrod (64); this method was adopted for the last seven experiments and described fully in the following section of this thesis.

Many colour reactions for the detection and estimation of estrogens have been reported. The reaction with phenosulfonic acid, which was described by Kober and which has survived through many modifications (65) is still widely used; several diazonium derivatives have proved useful (60, 66, 67, 68); such compounds as titanium sulfate, antimony pentachloride and zinc chloride gave useful colours with estrogenic material (69, 70, 61). More recently, ultraviolet spectroscopy and fluorimetry have been successfully adapted for assay of estrogens (71, 72, 73, 74).

Several of these reactions have been applied to the detection of spots of estrogenic material on paper strips; Nyc et al (61) used zinc chloride for this purpose; Axelrod (64) used 15% fuming sulfuric acid, fenic chloride, and antimony pentachloride. A publication by Boscott (66), in which he reported the use of diazotized sulfanilic acid and similar compounds to effect this detection, reached us shortly after a method of estrogen detection which involved spraying the strips with diazotized sulfanilic acid had been elaborated from the procedure of Schmulovitz and Wylie (68). This method, and also detection with 15% fuming sulfuric acid (64), proved valuable in the experiments to be described.

B. METHODS AND RESULTS

1. Paper Chromatography of Estrogens.

(a) Detection of Estrogens with Diazotized Sulfanilic Acid.

The following reagents were prepared by the method outlined by Schmulovitz and Wylie (68).

(i) 0.9 g. of sulfanilic acid in 100 ml. of H_2O, containing 9.0 ml. of concentrated hydrochloric acid.

(ii) 5.0% sodium nitrite in H_2O.

(iii) 1.1% sodium carbonate in H_2O.

1.5 ml. of (i) and 1.5 ml. of (ii) were mixed and cooled at 0°C for five minutes; 6.0 ml. of (ii) were added to the mixture and the solution cooled at 0°C for fifteen minutes. This solution was sprayed lightly on the strips; the strips were then sprayed with (iii). The spots of estrogenic material gave pink spots almost immediately.

(b) Experiments with Various Solvent Systems.

Strips, one cm. wide and 43 cm. long, were cut from Whatman No. 1 filter paper sheets, were boiled in methanol for thirty minutes and dried in air at room temperature.

When two phase systems were used, the aqueous and non-aqueous phases were shaken occasionally for one hour, to saturate each phase with the other. 20 µg. quantities of each estrogen were added to the strips at the starting lines and the strips were placed in position in the chromatography chamber. Portions of each phase were placed in separate containers on the floor of the chamber; the chamber was sealed with plasticene. After six hours the non-aqueous phase was

added to the trough, from which the strips were suspended; the chamber was resealed and the chromatograms were developed until the solvent front had travelled 25.5 cm. from the origin. The strips were removed, dried at room temperature in air and the R_F values of the individual estrogens were determined, by the sulfanilic acid colour test.

When single phase systems were used, the solvents were mixed and used immediately; the rest of the procedure was identical to that described in the preceding paragraph.

These experiments were carried out at room temperature which varied from 25 to 30°C.

The following estrogens were used throughout this work:
Estrone, laboratory sample, m.p. 257-259°C,
Estradiol-β,(1), laboratory sample, m.p. 176.5-177.5°C,
Estradiol-β,(2), Steroid Laboratories Ltd., m.p. 171-172°C,
Estriol, Parke-Davis, m.p. 264-267°C.
All melting points were determined on the Fisher-Johns apparatus.

Two Phase Systems: Single Organic Solvents and Water.

Benzene, toluene, carbon tetrachloride, cyclohexanol, n-butanol and isooctane were used, individually, to chromatograph the three estrogens. The R_F values, obtained with each of these solvents, are listed in Table 1, nos. 1-6.

Filter Paper Impregnated with Sodium Hydroxide.

The paper strips were drawn through aqueous sodium

hydroxide and dried at room temperature in air; the concentration of the solutions of alkali varied from 0.5 to 10% sodium hydroxide. Benzene-H_2O, and toluene-H_2O systems were used with these strips and typical results appear in Table 1; in experiments nos. 7-13, each estrogen was chromatographed individually, but in nos. 14 and 15, 20 μg. of each of the three estrogens were run together.

Single Phase Systems.

Methanol-H_2O mixtures, from 5%-50% methanol, gave poor results. Pyridine-H_2O systems gave poor results with one exception; 10% pyridine carried estriol from the starting-line and did not move estrone and estradiol-β. With a system composed of 25% acetone in water, estriol and estradiol-β travelled from the starting line and estrone did not. The R_F values obtained with these two systems are listed in Table 1, nos. 16-18.

Isooctane Benzene-Water Systems.

The chromatographic properties of mixtures of these solvents, in separating the three estrogens, were examined over a wide range of concentration, from 20% to 80% isooctane in benzene. More than sixty chromatograms were developed with these mixtures, and typical R_F values and separations obtained with them are shown in Table 1, nos. 19-30.

Four chromatograms, each containing the three estrogens, developed with 45% isooctane in benzene, gave satisfactory

Table 1

Chromatography of Estriol, Estradiol-B and Estrone with Various Solvent Systems

No.	System	Estriol,	R_F.	Estradiol-B,	R_F.	Estrone,	R_F.
1	Benzene-H_2O	0.0-4.3cm.	0.09	20.3-23.6cm.	0.86	22.8-24.1cm.	0.93
2	Toluene-H_2O	0.0-2.0cm.	0.04	19.5-23.6cm.	0.81	21.3-23.8cm.	0.89
3	Carbon Tetrachloride-H_2O	0.0-1.3cm.	0.03	17.0-21.1cm.	0.75	19.1-23.1cm.	0.83
4	n-Butanol-H_2O	22.3-23.9cm.	0.92	23.2-24.9cm.	0.95	22.3-23.9cm.	0.91
5	Cyclohexanol-H_2O	24.9-25.4cm.	0.99	24.4-25.5cm.	0.98	23.1-24.1cm.	0.93
6	Isooctane-H_2O	0.0cm.	0.00	0.0-2.5cm.	0.05	0.0-3.8cm.	0.07
7	Benzene-H_2O,Strips - 0.5% NaOH			22.3-23.6cm.	0.90	23.1-24.1cm.	0.93
8	Benzene-H_2O,Strips - 2.0% NaOH	0.0cm	0.00	19.9-23.6cm.	0.85	23.0-24.4cm.	0.93
9	Benzene-H_2O,Strips - 3.0% NaOH	0.0-4.2cm.	0.08	3.3-5.1cm.	0.16	3.8-5.1cm.	0.17
10	Toluene-H_2O,Strips - 1.0% NaOH	0.0-4.2cm.	0.08	22.1-24.1cm.	0.91		
11	*Toluene-H_2O,Strips - 1.0% NaOH	0.0cm.	0.00	19.1-21.6cm.		19.1-21.6cm.	
12	Toluene-H_2O,Strips - 2.0% NaOH	0.0-3.6cm.	0.07	21.1-23.4cm.	0.88	23.4-25.5cm.	0.96
13	Toluene-H_2O,Strips - 2.0% NaOH	0.0-5.1cm.	0.10	16.8-21.9cm.	0.76	23.4-25.5cm.	0.96
14	*Toluene-H_2O,Strips - 2.0% NaOH	0.0cm.	0.00	16.3-18.8cm.	0.69	22.9-25.5cm.	0.95
15	*Toluene-H_2O,Strips - 2.0% NaOH	0.0cm.	0.00	8.4-21.1cm.	0.58	22.9-25.5cm.	0.95

Table 1 (continued)

No.	System	Estriol,	R_F.	Estradiol-B,	R_F.	Estrone,	R_F.
16	10% Pyridine, 90% H_2O	7.0-17.3cm.	0.47	0.0 cm.	0.00	0.0cm.	0.00
17	25% Acetone, 75% H_2O	11.8-17.8cm.	0.57	5.1-14.7cm.	0.49	0.0cm.	0.00
18	25% Acetone, 75% H_2O	4.6-16.0cm.		4.6-16.0cm.		0.0cm.	0.00
19	20% Isooctane in Benzene-H_2O	0.0cm.	0.00	18.0-20.6cm.	0.76	21.9-24.7cm.	0.92
20	20% Isooctane in Benzene-H_2O	0.0cm.	0.00	18.5-21.1cm.	0.78	21.1-23.6cm.	0.88
21	*20% Isooctane in Benzene-H_2O	0.0cm.	0.00	17.8-20.8cm.	0.76	23.4-25.5cm.	0.96
22	*20% Isooctane in Benzene-H_2O	0.0cm.	0.00	17.8-20.6cm.	0.76	20.8-23.4cm.	0.86
23	40% Isooctane in Benzene-H_2O	0.0cm.	0.00	8.6-14.0cm.	0.45	17.3-21.3cm.	0.76
24	40% Isooctane in Benzene-H_2O	0.0cm.	0.00	8.6-14.5cm.	0.46	16.0-21.3cm.	0.74
25	*40% Isooctane in Benzene-H_2O	0.0cm.	0.00	9.4-15.0cm.	0.48	17.0-18.8cm.	0.72
26	*40% Isooctane in Benzene-H_2O	0.0cm.	0.00	10.4-16.3cm.	0.53	18.8-21.8cm.	0.80
27	50% Isooctane in Benzene-H_2O	0.0cm.	0.00	14.0-17.8cm.	0.62	18.0-21.6cm.	0.78
28	50% Isooctane in Benzene-H_2O	0.0cm.	0.00	11.3-18.3cm.	0.58	17.8-21.8cm.	0.78
29	*50% Isooctane in Benzene-H_2O	0.0cm.	0.00	10.4-15.5cm.	0.51	16.8-20.8cm.	0.74
30	*50% Isooctane in Benzene-H_2O	0.0cm.	0.00	8.1-14.5cm.	0.45	15.3-18.5cm.	0.66

*20 µg. of each estrogen were run together on the same chromatogram.

separations. Of ten similar chromatograms, when the moving phase was 50% isooctane in benzene, eight showed satisfactory separations. With 60% isooctane in benzene, streaking of estrogens on the strips occurred.

The addition of a small percentage of acetic acid to the system, 50% isooctane in benzene, decreased tailing of estrone and estradiol-β but increased the yellow background colour, when the strips were sprayed with diazotized sulfanilic acid and sodium carbonate solutions.

It was noted that at higher room temperatures, 30° C or higher, poor separations of estrone and estradiol-β were achieved with these systems.

(c) Axelrod's Method of Partition Chromatography.

This scheme (64) included three chromatographic systems, used in this order; methylcyclohexane-formamide, o-dichlorobenzene-formamide, and methylene chloride-formamide; with these the chromatographic technique was essentially the same as that described previously, with the following exceptions.

For each of the three systems, the strips were dipped in a mixture of one part of formamide and one part of absolute methanol, and blotted between sheets of filter paper before the samples were dried on the strips at the starting lines. With the first system, strips 3 cms. wide and 43 cms. long were used; with the remaining systems, strips 1 cm. wide and 43 cms. long were used. The developed chromatograms were dried in air at 40° C for 6 hours, and a narrow strip 1.5 mm. wide was cut from the centre of each chromatogram. Each narrow

strip was placed in fuming H_2SO_4 and, after one minute, examined in ordinary light, and, in the dark, under an ultraviolet light source. In ordinary light, estriol gave a pink, estradiol-β an orange-yellow and estrone an orange-tan colour; under ultraviolet light, estriol exhibited a pinkish-green, and estradiol-β and estrone, a greenish-yellow fluorescence. The corresponding areas on the remainder of each chromatogram were eluted with methanol, one ml. per square cm. of filter paper.

The chromatographic procedure could be summarized as follows: a sample which contained the three estrogens was chromatographed with methylcyclohexane-formamide for 24 hours; the location of the estrogens on the dry chromatogram was ascertained with fuming H_2SO_4 and the estrogens eluted with methanol; this extract was chromatographed with o-dichloro-benzene-formamide for 6 hours and the estrogens located and eluted as before; the eluate from the origin of this chromatogram was chromatographed with methylene chloride-formamide for 10 hours, and worked up as before.

The concentration of estrogenic material in the eluate was determined by ultraviolet absorption measurements at 280-281 mμ, using the methanol eluate from an equal area of bare paper strips as solvent blank.

Identification of the individual estrogens was achieved by dissolving each eluate in 3.0 ml. of concentrated H_2SO_4 and determining the ultraviolet absorption spectrum after two hours. The spectra of the four samples of estrogens

used in these experiments may be seen in Figures 1 and 2.

A model experiment was performed with this method. The same samples of estrogens were used as in the previous chromatographic experiments. The solvents needed for this method were redistilled. A stock extract of ovarian tissue was prepared as follows; 40 g. of beef ovary slices were digested and extracted as shown in Figure 5; the extract was dissolved in 10 ml. of chloroform; 0.5 ml. of this solution represented the digest extract of 2 g. of ovarian tissue.

The following samples were prepared for chromatography:

1. 0.5 ml. of stock extract,
2. 0.5 ml. of stock extract + 98 µg. of estriol + 98 µg. of estrone,
3. 0.5 ml. of stock extract + 98 µg. of estriol + 114 µg. of estradiol-β,
4. 0.5 ml. of stock extract + 98 µg. of estriol + 114 µg. of estradiol-β + 98 µg. of estrone,
5. 98 µg. of estrone + 98 µg. of estriol + 114 µg. of estradiol-β.

Each of these was evaporated to dryness under N_2, dissolved in methanol and chromatographed by the methylcyclohexane-formamide system. The following spots were found with fuming H_2SO_4:
chromatogram of Sample 1. origin - 1.7 cm.,
chromatogram of Sample 2. origin - 1.7 cm., 4.7 - 7.3 cm.,
chromatogram of Sample 3. origin - 2.5 cm.,
chromatogram of Sample 4. origin - 2.5 cm., 2.8 - 4.7 cm.,
chromatogram of Sample 5. 1.3 - 4.7 cm.

FIGURE 1

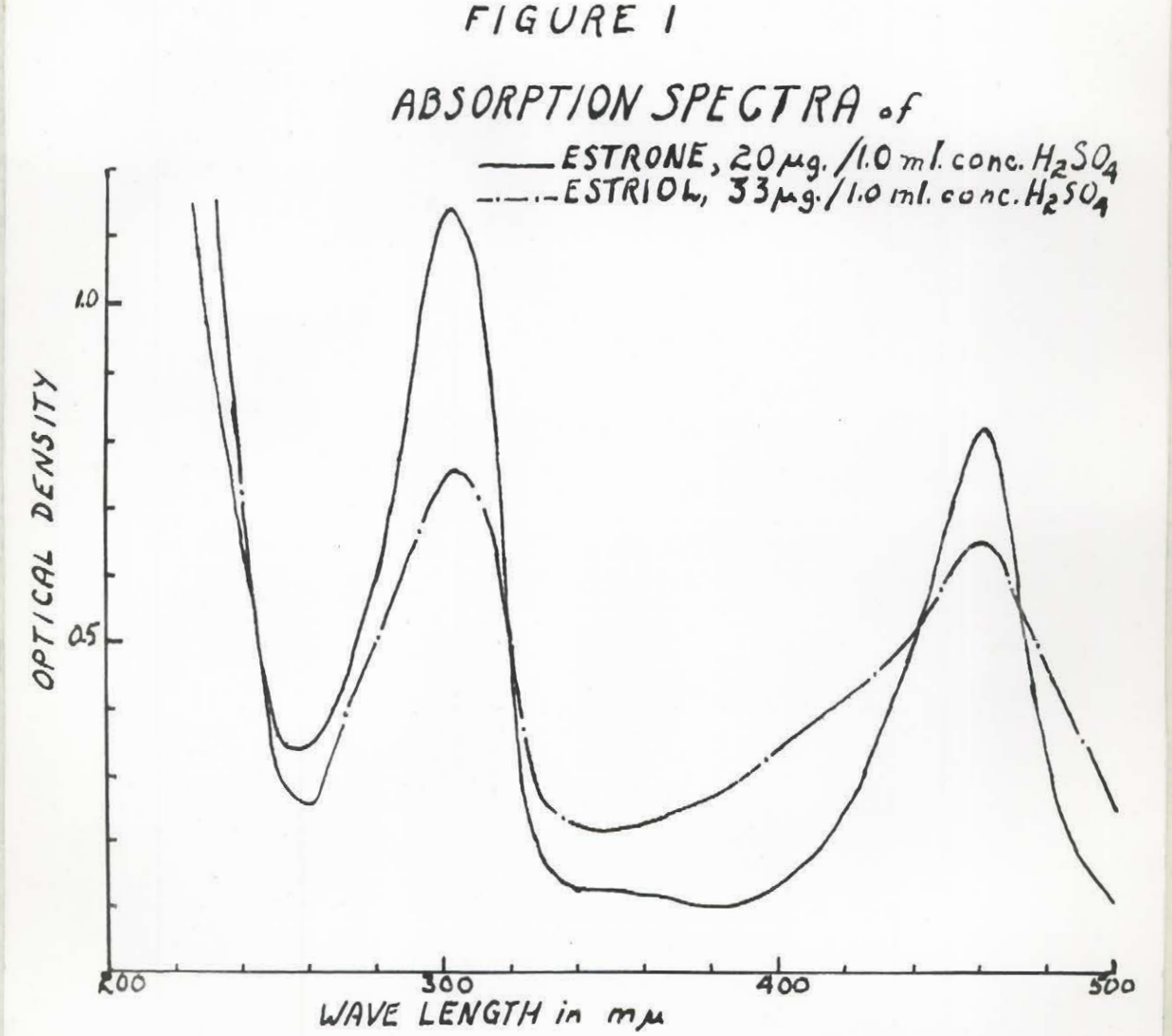
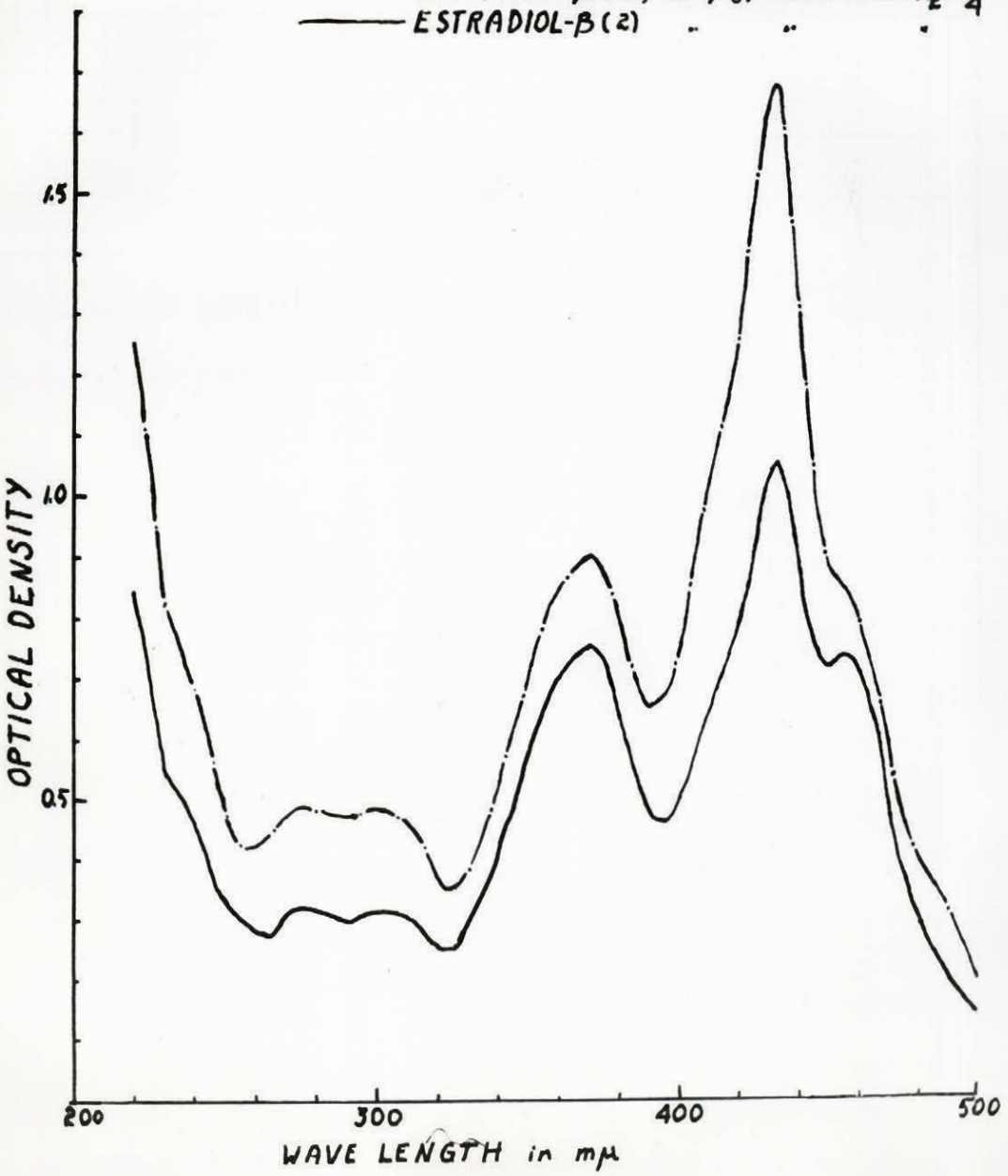

ABSORPTION SPECTRA of
ESTRONE, 20 µg. /1.0 ml. conc. H_2SO_4
ESTRIOL, 33 µg. /1.0 ml. conc. H_2SO_4
OPTICAL DENSITY
1.0
0.5
200
300
400
500
WAVE LENGTH in mµ

FIGURE 2

ABSORPTION SPECTRA of

—·—·— ESTRADIOL-β (1), 20 µg/1.0 ml. conc. H_2SO_4

——— ESTRADIOL-β (2) " " "



The spots were eluted separately with methanol; the first spots on each strip were called 1, 2 etc., and where there were second spots on the strips they were called 2A and 4A. No eluate weighed more than one mg., the maximum for a strip of one cm. width.

The eluates from the seven spots were chromatographed by the o-dichlorobenzene-formamide system; the following spots were found with fuming H_2SO_4:

1. origin,
2. origin, 21.6 - 24.1 cm.,
2A. 18.0 - 23.0 cm.,
3. origin, 10.2 - 13.3 cm.,
4. origin, 8.3 - 10.8 cm., 27.0 - 28.6 cm. (end of strip),
4A. 27.0 - 28.6 cm. (end of strip),
5. origin, 11.4 - 16.5 cm., 27.0 - 28.6 cm. (end of strip).

The eluates from the origins of strips 1, 2, 3, 4 and 5 were chromatographed with the third system, methylene chloride-formamide. The eluates from all the remaining spots were analyzed quantitatively for estrogen content; the estrogens were identified by the determination of the ultraviolet absorption spectra of the sulfuric acid chromogens obtained with these eluates.

The only eluate that contained enough estrone for quantitative measurement or identification was from strip 2A; the spectrum obtained with this eluate, in H_2SO_4, agreed exactly with that of authentic estrone; the second spot on strip 2, and the terminal spots on strips 4, 4A and 5,

probably contained traces of estrone. Estradiol-β was identified (Figure 3) and quantitatively determined in the eluates of the second spots of strips 3, 4 and 5.

The following spots were found on the chromatograms from the third system, with fuming H_2SO_4:

1. origin,
2. origin, 2.5-3.8 cm.,
3. origin, 1.8-2.5 cm.,
4. origin, 1.8-2.8 cm.,
5. origin, 1.6-3.8 cm.

The eluates from the second spots of strips 2, 3 and 5 were analyzed for estrogen content as described above and contained estriol.

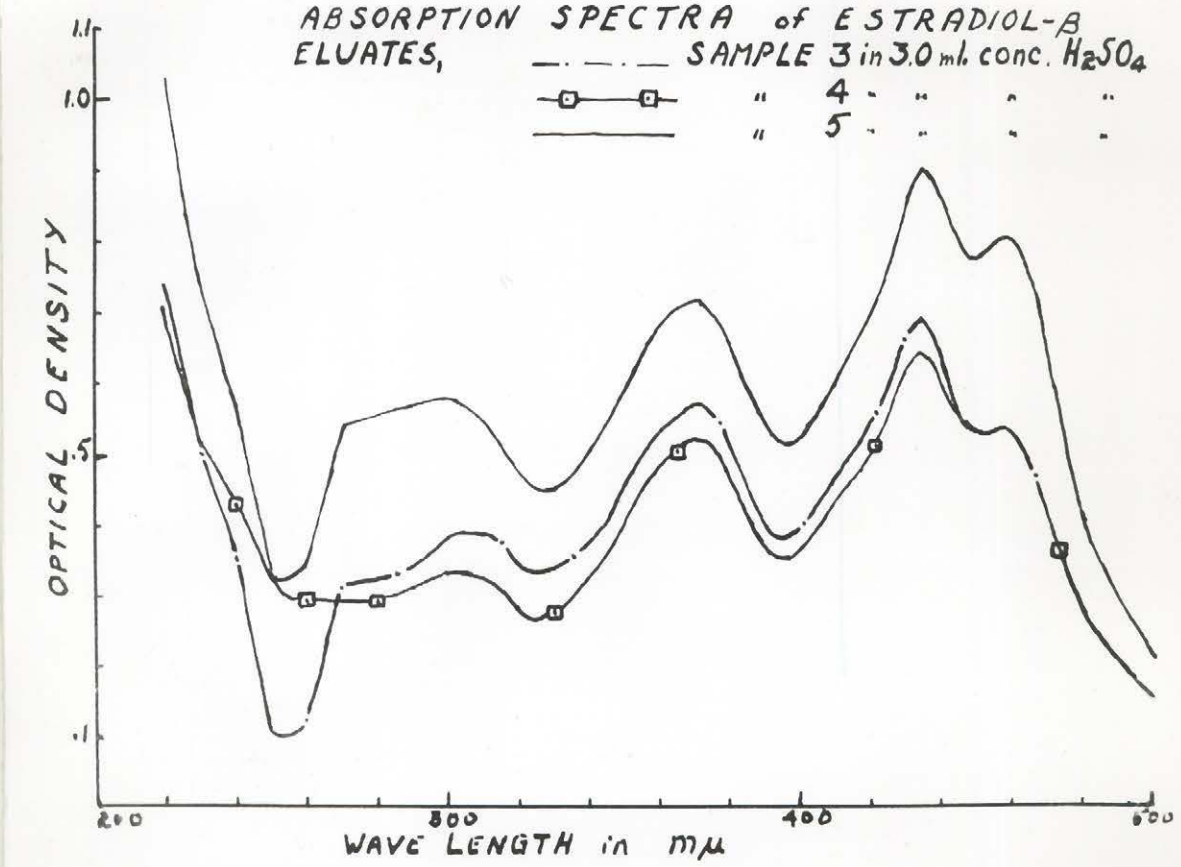
No estrogenic material was recovered from Sample 1. The recoveries of individual estrogens from the other samples are listed in Table 2.

TABLE 2

Recovery of Estrogens in Model Experiment

Sample No.	Estriol µg. Found	Estriol %Found	Estradiol-β µg. Found	Estradiol-β %Found	Estrone µg. Found	Estrone %Found
2	67	68			66	67
3	63	64	56	48		
4			43	37		
5	71	72	91	80		

FIGURE 3


ABSORPTION SPECTRA of ESTRADIOL-B
ELUATES, SAMPLE 3 in 3.0 ml. conc. H2SO4
" 4 " " " "
" 5 " " " "
OPTICAL DENSITY
1.1
1.0
.5
.1
200
300
400
600
WAVE LENGTH in mμ

2. Incubation of Rat Ovary Slices.

Young female rats of the Sprague-Dawley strain, weighing from 170-230 grams were anaesthetized with Nembutal and their ovaries were removed. The ovaries were placed in a Petrie dish that was chilled in ice, and which was lined with filter paper moistened with water. Fat and extraneous tissue were removed and each ovary was sliced into three or four thick slices. Eight ovaries were used for each experiment.

Krebs-Ringer bicarbonate (KRB) and Krebs-Ringer phosphate (KRP) buffer solutions were prepared according to Umbreit et al. (75). To each of these media enough sodium acetate was added to bring the final molarity of acetate in the medium to 0.008. In various incubations other substrates were included in the media, i.e.: 0.01 M glucose and 0.02 M succinate. In incubations nos. 5, 7 and 8, the saline solution used in preparing the media was replaced with a saline extract of pituitary powder; this extract contained 16.0 mg. of solids per ml. This powder was prepared in this laboratory by J. Purvis, by the lyophilization of bovine pituitary glands. 25 µg. of estriol and of estrone were added to the incubation flask in experiment no. 9, before the incubation, and in experiment no. 10 immediately after incubation.

The ovary slices were weighed into Warburg flasks, each of which contained 3.0 ml. of medium. The flasks were then gassed, with 5% CO_2 in O_2 when KRB was used and with O_2 when KRP was used, and incubated at 37°C for five hours.

The flask contents were filtered and the tissue was washed four times with 3.0 ml. of H_2O, which were added to the filtered media. In each case, the medium plus washings, and the tissue were worked up separately as shown in Figures 4 and 5.

Three fractions were obtained from each experiment: the extract of the neutral medium, N M; the extract of the acidified medium, A M; and the extract of the digested tissue, D. These were chromatographed with the 50% isooctane in benzene system, described previously; the strips were sprayed with the diazotized sulfanilic acid and sodium carbonate solutions and examined for the presence of estrogens.

Ten experiments were performed, as described in Table 3.

TABLE 3

Rat Ovary Incubations

INCUBATION NO.	TISSUE WEIGHT (mg.)	3.0 ml. of MEDIUM	ACETATE ADDED	GLUCOSE ADDED	OTHER SUBSTRATES
1	550	KRB	+		
2	510	"	+		
3	590	"	+	+	
4	430	"	+	+	0.02 M succinate
5	610	"	+		pituitary powder extract
6	560	KRP	+		
7	490	"	+		pituitary powder extract
8	480	"	+	+	pituitary powder extract
9	430	KRB	+		25 µg. estriol, 25 µg. estrone
10	420	"	+		25 µg. estriol, 25 µg. estrone

No spots were found on the chromatograms of the NM, AM and D fractions, from experiments nos. 1-8. Spots were found on corresponding chromatograms from experiments nos. 9 and 10; the results are shown in Table 4.

TABLE 4

Estrogens Found in Incubations Nos. 9 and 10

System, 50% Isooctane in Benzene-Water

SAMPLE	ESTRIOL	R_F	ESTRADIOL-B	R_F	ESTRONE	R_F
9 NM	0	0.00				
9 AM	0	0.00	10.2-12.7 cm.	0.45	16.5-18.5 cm.	0.74
9 D	0	0.00	12.5-14.0 cm.	0.52	14.7-16.5 cm.	0.62
10 NM	0	0.00			15.2-18.5 cm.	0.66
10 AM	0	0.00			16.0-19.1 cm.	0.69
10 D	0	0.00			18.3-19.9 cm.	0.75

On every strip spots at the origin were found, which indicated the presence of estriol. 9 NM showed the presence of estriol and no other spots. From 9 AM and 9 D three spots were found on each strip, indicating the presence of the three estrogens. 10 NM, 10 AM and 10 D each showed two spots that correspond to estriol and estrone.

FIGURE 4

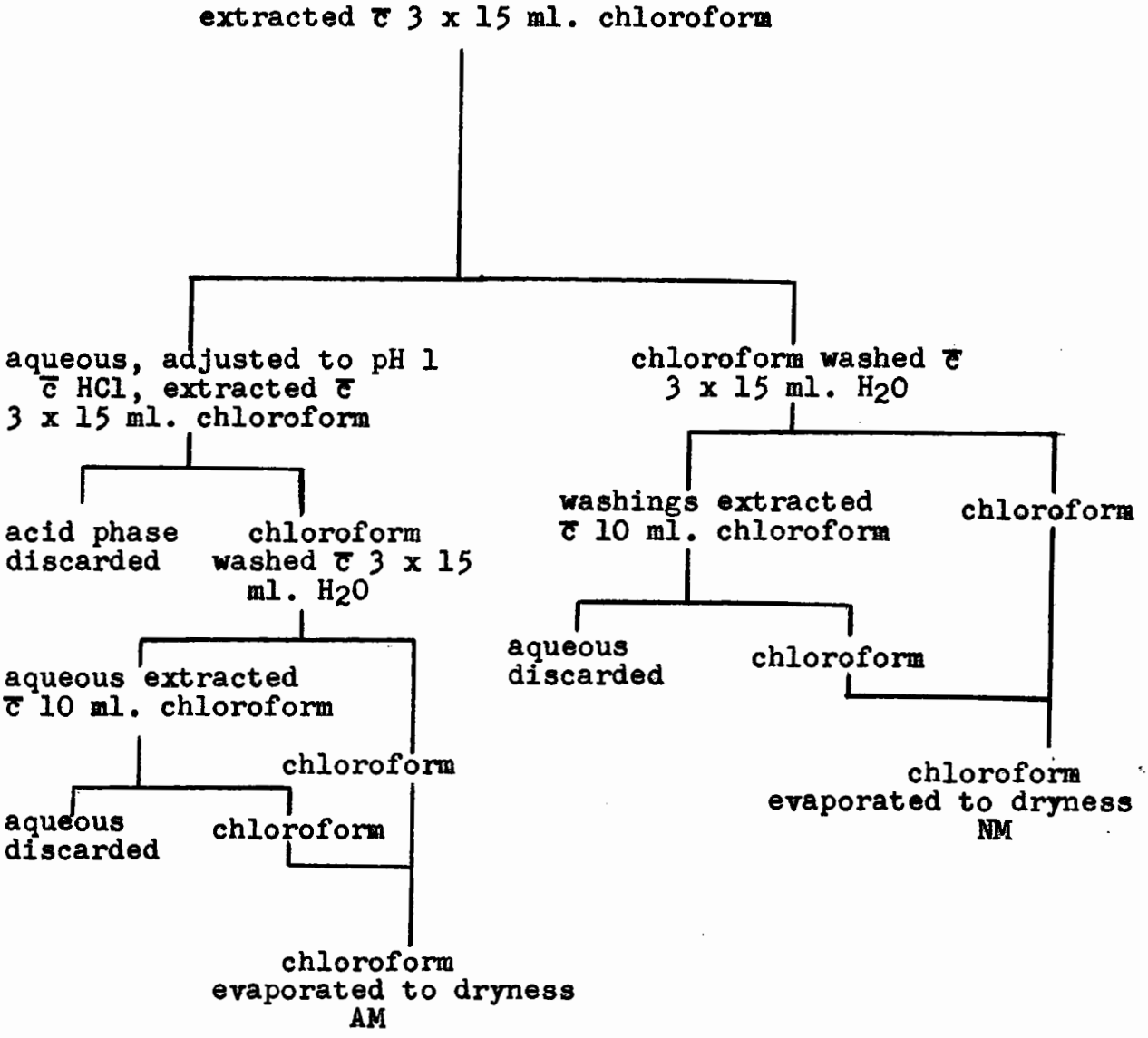
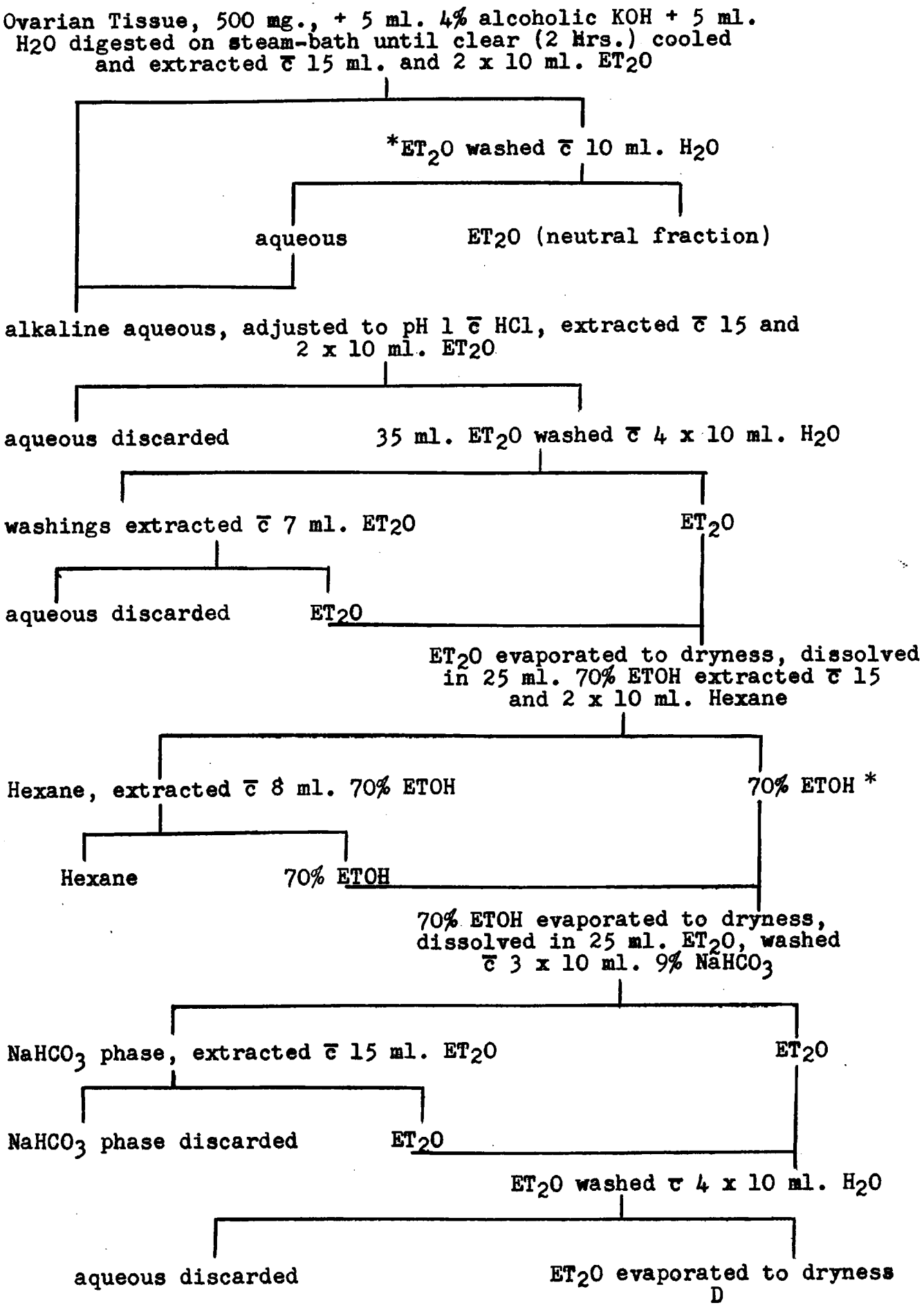

12 ml. Medium and Washings
extracted c̄ 3 x 15 ml. chloroform
aqueous, adjusted to pH 1 c̄ HCl, extracted c̄ 3 x 15 ml. chloroform
chloroform washed c̄ 3 x 15 ml. H2O
acid phase discarded
chloroform washed c̄ 3 x 15 ml. H2O
washings extracted c̄ 10 ml. chloroform
chloroform
aqueous discarded
chloroform
aqueous extracted c̄ 10 ml. chloroform
chloroform
aqueous discarded
chloroform
chloroform evaporated to dryness AM
chloroform evaporated to dryness NM

FIGURE 5



* ET_2O - diethyl ether; ETOH - ethanol

3. Incubation of Beef Ovary Slices.

The ovaries were removed from freshly killed cows at Canada Packers Ltd., and were carried to this laboratory in cold saline solution. Fat and extraneous tissue were dissected from the ovaries which were cut into thick slices with a Stadie microtome blade. These slices contained corpus luteum tissue.

5.0 g. of slices and 30 ml. of medium were placed in each 125 ml. Erlenmeyer flask, gassed as before and incubated 6 hours at 37°C. The incubation media used were identical to those of the rat ovary slice experiments. Two gonadotrophin extracts were obtained from Ayerst, McKenna and Harrison Ltd.;

P.M.S. - Pregnant Mares' Serum Gonadotrophin

A.P.L. - Chorionic Gonadotrophin

Each of these preparations contained 500 I.U. per ml.; one ml. quantities of these were added to the incubation media in various experiments, and to incubations nos. 17 and 18 at the end of the incubation.

L.A.P., lyophilized anterior pituitary powder was donated by Dr. Hans Selye; 60 mg. of this powder was added to the incubation medium in experiment no. 35.

The extraction of the neutral medium was omitted in this series of experiments and the extraction of the acidified medium was retained; in experiments nos. 19-35, the chloroform extraction of the medium (see Figure 4) was replaced by an ether extraction. Each sample of tissue was digested in 20 ml. of 4% potassium hydroxide in 95% ethanol,

TABLE 5

Beef Ovary Incubations

Incubation no.	Wet Wt. of Tissue	30 ml. of Medium	Acetate	Glucose	Other Substrates
11	5.0 g.	KRB			
12	5.0 g.	KRB	+		
13	5.0 g.	KRB	+		1 ml. P.M.S.
14	5.0 g.	KRB	+		1 ml. A.P.L.
15	5.0 g.	KRB	+		1 ml. P.M.S.+1 ml. A.P.L.
16	5.0 g.	KRB	+		1 ml. P.M.S.+1 ml. A.P.L.
17	5.0 g.	KRB	+		1 ml. P.M.S.+1 ml. A.P.L.
18	5.0 g.	KRB	+		1 ml. P.M.S.+1 ml. A.P.L.
19	5.0 g.	KRB		+	
20	5.0 g.	KRB	+	+	
21	5.0 g.	KRB	+	+	1 ml. P.M.S.
22	5.0 g.	KRB	+	+	1 ml. A.P.L.
23	5.0 g.	KRB	+	+	1 ml. P.M.S.+1 ml. A.P.L.
24	5.0 g.	KRP		+	
25	5.0 g.	KRP	+	+	
26	5.0 g.	KRP	+	+	1 ml. P.M.S.
27	5.0 g.	KRP	+	+	1 ml. A.P.L.
28	5.0 g.	KRP	+	+	1 ml. P.M.S.+1 ml. A.P.L.
29	5.0 g.	KRB		+	50 µg. estrone
30	5.0 g.	KRB		+	50 µg. estradiol-β
31	5.0 g.	KRP		+	50 µg. estrone
32	5.0 g.	KRP		+	50 µg. estradiol-β
33	5.0 g.	KRB			0.01M cyanide
34	5.0 g.	KRB			0.01M cyanide + 1 ml. P.M.S.
35	5.0 g.	KRB			0.01M cyanide + 60 mg. L.A.P.

for two or three hours on the steam-bath, until clear; 10 ml. of H_2O were added to this solution which was then extracted as shown in Figure 5. This modified scheme gave two fractions for chromatographic investigation, the medium extract, M, and the digest extract, D. Twenty-five incubations were performed; they are described in Table 5.

The M and D extracts from incubations nos. 11-32 were chromatographed with the 50% isooctane in benzene-water system, described previously; the strips were examined, using the colour reaction with diazotized sulfanilic acid. Very faint spots were found on chromatograms from experiments nos. 16, 19, 21, 23, 26 and 28. Definite spots, indicating the presence of both estrone and estradiol-β, were found on the chromatograms of the digest extracts of the incubations to which estrone or estradiol-β had been added, nos. 29-32. These results are shown in Table 6.

The D and M extracts from experiments nos. 33, 34 and 35 were chromatographed by Axelrod's systems (64), which have been described previously. Spots were detected with fuming H_2SO_4 on the chromatograms from the second step (the o-dichlorobenzene-formamide system); these spots were:-

33D, origin , 20.7-27.2 cm.,
34D, origin , 14.0-19.1 cm.,
35M, origin , 13.5-15.2 cm.

TABLE 6

Estrogens Detected in Incubations of Beef Ovaries

Fraction	Spot One	R_F	Spot Two	R_F
16M	9.7-10.7 cm.	0.40		
16D	11.4-18.5 cm.	0.59		
19D	8.4-15.5 cm.	0.47		
21D	7.4-15.5 cm.	0.45		
23D	15.0-18.5 cm.	0.66		
26D	16.7-18.3 cm.	0.69		
28D	17.0-18.3 cm.	0.69		
29M	15.5-16.5 cm.	0.63		
29D	15.1-17.5 cm.	0.64	18.8-20.2 cm.	0.78
30M	17.8-18.5 cm.	0.71		
30D	13.2-14.7 cm.	0.55	14.7-16.2 cm.	0.61
31M	16.0-17.3 cm.	0.65		
31D	12.9-15.5 cm.	0.56	16.0-17.3 cm.	0.66
32M	15.5-16.7 cm.	0.64		
32D	14.2-15.7 cm.	0.57	16.5-17.8 cm.	0.67

The eluates from the second spots of these chromatograms gave no ultraviolet peak at 281 mu; when the H_2SO_4 chromogens of these eluates were examined, the ultraviolet absorption spectra were dissimilar to those obtained with authentic estrone or estradiol-β.

The origins of the strips from the second chromatographic step were eluted and chromatographed with methylene chloride-formamide. Again spots were detected on the developed chromatograms, as follows:-

33D, origin, 2.3-5.1 cm.,
34D, origin, 2.3-4.6 cm.,
35D, origin, 3.8-6.3 cm..

The second spots on these chromatograms were eluted; examination of the ethanolic solution of these eluates at 281 mμ and of their H_2SO_4 chromogens, showed that no detectable amounts of estriol were present.

4. Incubation of Beef Ovary Slices with Radioactive Estrogens.

In this section of the work the C^{14} assay was carried out with a Nuclear Instrument and Chemical Corporation 64-scaler unit used in conjunction with a windowless Q-gas counter made by the same company. A standard plate which held a known amount of C^{14} was counted with all samples assayed and corrections for background radiation were made. Specific activities were expressed as disintegrations per minute per milligram (d./min./mg.), while the activities of unweighed fractions were expressed as disintegrations per minute (d./min.).

Most of the samples were counted according to the method of Yates (76), in which duplicate plates of a sample, and duplicate plates of the same sample plus a known number of disintegrations per minute, were counted and correction was made for the absorption of radiation by the material being assayed. Where 22 μg. of material, or less, were plated for counting, Yates' method was not necessary, and no corrections were made for self-absorption of radiation. In two determinations in experiments nos. 36 and 37, C^{14} was counted as $BaCO_3$, following the procedure of Calvin et al. (77).

The preparation of radioautographs of chromatograms followed the method outlined by Peron (78); the chromatograms were stapled to X-Ray film (Blue Brand, Double Coated, Eastman Kodak) and were stored in a light tight casette for 36 to 46 hours, depending on the radioactivity present on the strips. The radioactive spots appeared as dark areas

on the developed film.

The radioactive estrogens were prepared in this laboratory by Thompson (79), and were labelled in the 16 position with C^{14}. The following samples were used in these experiments:

Estrone, m.p. 240-251°C, 2,478,000 d./min./mg.,
Estradiol-β, m.p. 164.5-172.5°C, 2,305,000 d./min./mg.

These were analyzed chromatographically. Duplicate samples of estrone, each containing 74.5 µg. (185,000 d./min.) and two samples of estradiol-β, each containing 85.1 µg. (196,000 d./min.) were chromatographed with the o-dichlorobenzene-formamide system (64). The following spots were found by radioautography:

Estrone 1,	origin,		18.5-21.5 cm.,
Estrone 2,	origin,		15.2-22.3 cm.,
Estradiol-β 1,	origin,	2.5-9.4 cm.,	11.7-22.1 cm.,
Estradiol-β 2,	origin,	2.5-10.2 cm.,	13.5-21.8 cm.

The four strips had faint spots at the origins. The estrone chromatograms showed that there was no second constituent present in this sample of estrone. The spots from chromatograms of estradiol-β were eluted and the C^{14} content of the eluates was determined; the run-offs of these strips were pooled; the C^{14} determinations are shown in Table 7. The estrogen responsible for the centre spots was identified as estradiol-β, by the absorption spectrum of the H_2SO_4 chromagen. 30 µg. of authentic, non-radioactive estrone were added to each of the eluates of the third

TABLE 7

The Recovery of C^{14} from the Chromatograms of Radioactive Estradiol-β

Eluate	Chromatogram 1	Chromatogram 2
Origin	6,570 d./min.	6,430 d./min.
Estradiol-β (second spot)	146,500 d./min.	146,080 d./min.
Estrone (third spot)	2,400 d./min.	2,700 d./min.
Pooled Run-offs	520 d./min.	

spots; these mixtures were chromatographed by the same system and the strips were examined first by radioautography and then with fuming H_2SO_4; the following spots were found:

by radioautography, Strip 1, 16.5-22.4 cm., Strip 2, 15.0-22.4 cm.,
by fuming H_2SO_4, Strip 1, 17.0-21.6 cm., Strip 2, 17.0-20.2 cm.

Only one spot was found on each strip and it contained the C^{14}; thus the terminal spots on the original chromatograms of estradiol-β contained estrone, whose activity represented 1.65% of the total activity recovered from the strips. The estrone sample contained no estradiol-β.

Beef ovaries were obtained from Canada Packers Ltd., and were treated as described in the previous series of experiments. The medium used here was prepared as described previously. Four incubations were performed, and are described in Table 8.

Samples nos. 36 and 37 were gassed with O_2 and incubated in Warburg flasks for four hours at 37.5°C; the tissue continued to absorb O_2 during this period. In each flask the CO_2

TABLE 8

Incubations of Beef Ovary Slices

with Radioactive Estrogens

Incubation no.	Wet Weight of Tissue	Medium	Estrogens Added
36	1.00 g.	3.0 ml. KRP	146,000 d./min. in 105 µg. estrone
37	1.24 g.	3.0 ml. KRP	217,000 d./min. in 94 µg. estradiol-β
38	5.00 g.	30.0 ml. KRP	278,000 d./min. in 1,152 µg. estrone
39	5.00 g.	30.0 ml. KRP	412,000 d./min. in 980 ug. estradiol-β

produced was trapped in 0.25 ml. of 20% aqueous NaOH in the centre well, and 0.25 ml. of 2N HCl was tipped from the side-arm into the medium at the end of the incubation. The contents of each centre well were removed, and to each, 2.8 ml. of 0.2M Na_2CO_3 were added, followed by 1.0 ml. of M $BaCl_2$. Infinitely thick plates were prepared from the resulting precipitates according to Calvin et al. (77); each plate held approximately 20 mg. of $BaCO_3$, and, when counted, each was found to be radioactively inert. No radioactive CO_2 was produced in either incubation.

Samples nos. 38 and 39 were gassed with O_2 and incubated in 125 ml. Erlenmeyer flasks at 37.5°C for six hours.

The medium and tissue from incubations nos. 36-39 were worked up as outlined in the previous series of beef ovary incubations. C^{14} assays were carried out on all fractions and the d./min. estimations were listed in Table 9.

TABLE 9

C^{14} Content of the Fractions Obtained in the Extraction Scheme

(See Figures 4 and 5.)

Fraction	Experiment no. 36	Experiment no. 37	Experiment no. 38	Experiment no. 39
Medium extract, M	43,000 d./min.	67,000 d./min.	118,800 d./min.	130,500 d./min.
Medium aqueous residue	10,500 d./min.	5,700 d./min.	2,100 d./min.	6,600 d./min.
Digest neutral extract	53,000 d./min.	28,000 d./min.	19,000 d./min.	44,000 d./min.
Digest $CHCl_3$ extract	21,300 d./min.	24,900 d./min.	88,000 d./min.	145,800 d./min.
Digest aqueous residue	5,400 d./min.	4,800 d./min.	5,500 d./min.	9,600 d./min.
Hexane extract	250 d./min.	450 d./min.	750 d./min.	2,000 d./min.
$NaHCO_3$ extract	950 d./min.	3,600 d./min.	6,700 d./min.	11,400 d./min.
Water washes	500 d./min.	1,680 d./min.	190 d./min.	560 d./min.
Digest extract, D	11,800 d./min.	6,120 d./min.	67,000 d./min.	109,400 d./min.

The whole D and M extracts of incubations nos. 36 and 37, and aliquots of these from incubations nos. 38 and 39, 50% of each D extract, and 30% of each M extract, were chromatographed by the methylcyclohexane-formamide system (64). The chromatograms were examined by radioautography, and also, in some cases, with fuming H_2SO_4; the detected spots

are listed in Table 10; as detection with radioautography was much more sensitive than detection with fuming H_2SO_4, the latter method was discontinued except in the case of mixed chromatograms.

The elution of the spots (Table 10) was directed by the radioautographic results. These eluates, which were assayed for C^{14} content, were each dissolved in 3.0 ml. of methanol, of which 0.1 ml. was removed for counting; the d./min. values, and the recovery of C^{14}, are also shown in Table 10.

TABLE 10

Detection of Estrogenic Material after Chromatography with Methylcyclohexane-formamide, and Recovery of C^{14} in the Eluates.

Sample	Spots Detected by Radioautography	F. H_2SO_4	C^{14} Content of Eluate	% Recovery of C^{14}
36M	0.0-6.1 cm.	no spot found		
36D	0.0-3.0 cm.	0.0-2.3 cm.		
37M	0.0-6.9 cm.	0.0-2.5 cm.		
37D	0.0-3.0 cm.	0.0-1.5 cm.		
38M	0.0-6.4 cm.		31,050 d./min.	88.6
38D	0.0-6.4 cm.		27,170 d./min.	80.3
39M	0.0-5.1 cm.		27,450 d./min.	73.8
39D	0.0-4.4 cm.		44,160 d./min.	80.7

The eluates of 36M and 36D were chromatographed with o-dichlorobenzene-formamide; those of 37M and 37D were accidentally destroyed. Good separations were achieved with samples 36M and 36D. The spots were detected and eluted as

described above; 0.2 ml. of each eluate, in 3.0 ml. of methanol, were removed for C^{14} assay. The spots found and C^{14} content of the eluates are shown in Table 11. The paper from which the second and third spots of chromatogram 36M were eluted was extracted in a Soxhlet with methanol for three hours; the extracts were counted and contained no radioactivity.

TABLE 11

Spots Detected after Chromatography with o-Dichlorobenzene-Formamide, and C^{14} Content of Spots.

	Spots Detected 1	2	3
Sample 36M	origin	9.7-12.7 cm.	20.3-26.9 cm.
C^{14} content of eluates of spots from 36M		2,340 d./min.	20,430 d./min.
Sample 36D	origin	5.1-8.1 cm.	no spot
C^{14} content of eluates of spots from 36D		3,500 d./min.	

50 µg. of non-radioactive estrone and 50 µg. of estradiol-β were added to the eluates of the second spots; these were chromatographed with the same system. The strips were analyzed by both radioautography and fuming H_2SO_4, and the following spots found:

	by radioautography,	by fuming H_2SO_4,
36M	4.6-10.7 cm.,	4.0-10.3 cm., 21.1-25.4 cm.,
36D	4.6-10.3 cm.,	4.6-8.9 cm., 18.5-21.1 cm.

Only the first spots of these mixed chromatograms, representing estradiol-β, were radioactive; the second, non-

radioactive spots were estrone. Thus, the second spots, shown in Table 11, contained estradiol-β; the C^{14} content of these spots, corrected for sampling for C^{14} assay and for strip examination with fuming H_2SO_4, indicated the conversion of estrone to estradiol-β to this extent:

$$\frac{2,340 + 3,500 \text{ d./min.}}{146,000 \text{ d./min.}} \times \frac{3.0}{2.8} \times \frac{3.00}{2.85} \times 100 = 4.6\%$$

The eluates from the chromatograms of samples nos. 38 and 39, see Table 10, were chromatographed with o-dichlorobenzene-formamide; as a poor separation was achieved, the spots were eluted and rechromatographed with the same system. The eluates from the third spots (estrone) of the chromatograms of samples 38M and 38D were each mixed with 30 μg. of authentic estrone, and those of the second spots (estradiol-β) of the chromatograms of samples 39M and 39D were each mixed with 30 μg. of authentic estradiol-β; the mixtures were chromatographed with the same system. The chromatograms from this last step were examined by both methods described before. The following spots were found:

	by radioautography,	by fuming H_2SO_4,
38M	5.7-10.8 cm.,	5.1-9.5 cm.,
38D	4.4-9.6 cm.,	3.2-7.6 cm.,
39M	10.8-17.7 cm.,	10.2-16.5 cm.,
39D	10.8-17.7 cm.,	10.2-16.5 cm.

In each case only one spot was found and it contained the C^{14} present in the strip. The spots were eluted and the

FIGURE 7

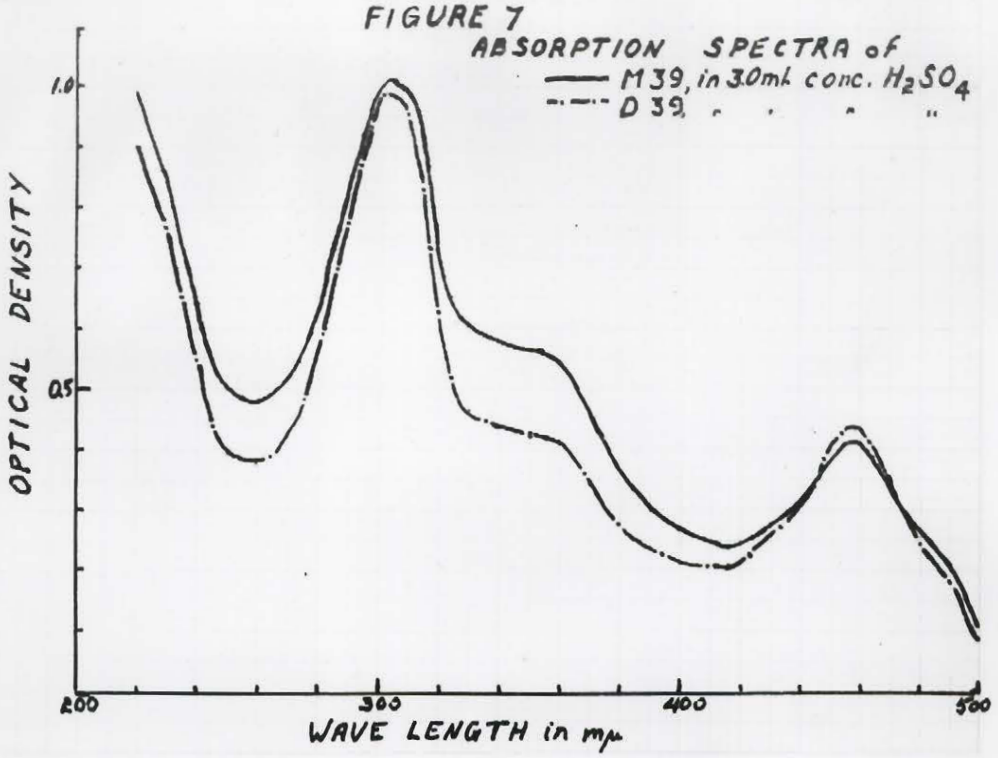
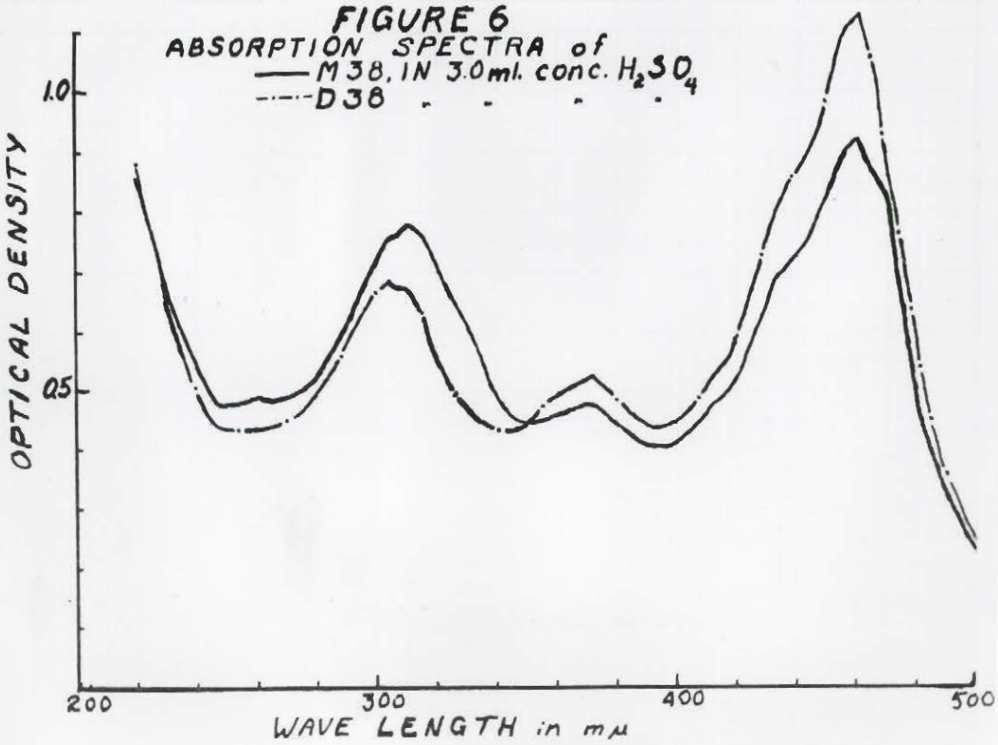


FIGURE 6



estrogenic content of each radioactive eluate was identified by the ultraviolet absorption spectrum of its H_2SO_4 chromogen (Figures 6 and 7). These spots from incubation no. 38 contained estradiol-β, and those from incubation no. 39 contained estrone. The eluates were assayed for C^{14} content after each chromatographic step; the results of the C^{14} assay of the eluates of the last chromatograms are given in Table 12; appropriate corrections for sampling for C^{14} assay and for strip examination with fuming H_2SO_4 and the corrected C^{14} contents of these last eluates are given in Table 12.

TABLE 12

Calculations Involved in Obtaining Corrected d./min. Content of Eluates of Radioactive Spots from the Last Chromatograms from Incubations nos. 38 and 39.

Sample	Observed d./min.	Correction Factors	Corrected d./min.
38M	1,165	30/29 x 3.0/2.8 x 3.0/2.8 x 1.0/0.85	1,541
38D	1,515	30/29 x 3.0/2.8 x 3.0/2.8 x 1.0/0.85	2,096
39M	975	30/29 x 3.0/2.8 x 3.0/2.5 x 1.0/0.85	1,468
39D	1,002	30/29 x 3.0/2.8 x 3.0/2.5 x 1.0/0.85	1,563

The four fractions contained:

38M 1,541 d./min. x 100/30 = 5,137 d./min. of estradiol-β

38D 2,096 d./min. x 100/50 = 4,192 d./min. of estradiol-β

39M 1,468 d./min. x 100/30 = 4,893 d./min. of estrone

39D 1,563 d./min. x 100/50 = 3,126 d./min. of estrone

The conversion of estrone to estradiol-β, in incubation no. 38

$$\frac{5,137 + 4,192 \text{ d./min.}}{278,000 \text{ d./min.}} \times 100 = 3.3\%$$

The conversion of estradiol-β to estrone, in incubation no. 39

$$\frac{4,893 + 3,126 \text{ d./min.}}{412,000 \text{ d./min.}} \times 100 = 1.95\% - 1.65\% = 0.30\%$$

C. DISCUSSION

The use of diazotized sulfanilic acid for the detection of estrogens on paper chromatograms was reported by Boscott (66). This method was developed independently in this laboratory by the author and was a direct application of the reagents,described by Schmulovitz and Wylie (68), to the chromatograms. This colour reaction was sensitive enough to detect 5 µg. of estrogen per two square centimeters of paper. Estriol gave the strongest, estradiol-β the next strongest and estrone the weakest pink colour. The colour faded; after twenty-four hours the spots were just discernible. Schmulovitz and Wylie used 10% aqueous sodium hydroxide to 'fix' these colours; this step proved to be unsatisfactory as this alkali solution moved the coloured spots.

Several systems (nos. 1, 2, 3, Table 1), each comprised of a single organic solvent and water, would separate estrone and estradiol-β from estriol but not from each other on paper chromatograms. The systems, benzene-water and toluene-water gave more promising results when used with paper strips that were pretreated with alkali. When the three estrogens were chromatographed with toluene-water, on paper that was previously treated with 2% aqueous sodium hydroxide, they separated (nos. 12 - 15, Table 1). The alkali on the strips made the colour reaction with diazotized sulfanilic acid quite unreliable (the spraying had to be very light); the spraying

with the solution of sodium carbonate was unnecessary in this case. For this reason the investigation of a separation of the estrogens using alkali treated paper was abandoned. It was possible that with other means of detection of the estrogens on the paper strips, the use of alkali treated paper could have provided a satisfactory method for the separation of these compounds.

Estrone and estradiol-β travelled from and estriol remained at the origin when these compounds were chromatographed with systems comprised of one of the more non-polar solvents and water (nos. 1-3, Table 1). When one of the more polar organic solvents was used as the non-aqueous phase, the three estrogens were carried almost to the solvent front (nos. 4-5, Table 1). With single phase systems, containing pyridine or acetone, estriol travelled from the origin at which estrone, or estrone and estradiol-β, remained (nos. 16 - 18, Table 1). The chromatographic effect of these last solvents was the reverse of that obtained with the non-polar systems such as benzene-water.

Typical results of chromatography with systems, whose non-aqueous phase varied from 20-60% isooctane in benzene, are given in Table 1, nos. 19 - 30. In all cases estriol remained at the origin; the best separation of estrone and estradiol-β was achieved with 40, 45 and 50% isooctane in benzene as the moving phase. These experiments were conducted at room temperature and it was noticed that better separations were obtained at 25°C. than those that occurred

at 30°C. As the content of isooctane was increased, the spots of estrogens on the developed chromatograms became longer until, at 60% isooctane:40% benzene, unreliable separations were observed. With 50% isooctane, some tailing of the estrogens was encountered, but a satisfactory separation of them was obtained. The R_F values of the three estrogens resulting from chromatography with this system were: estriol - 0.00, estradiol-β - 0.45-0.62, estrone - 0.66-0.78.

While the incubation experiments were in progress, Axelrod (64) published a new chromatographic method for the separation, quantitative estimation and identification of the three human estrogens. This method replaced chromatography with isooctane benzene for the last seven incubations. This three step procedure used: firstly, the methylcyclohexane-formamide system for the removal of impurities from the sample, secondly, the o-dichlorobenzene-formamide system which carried estrone and estradiol-β from the starting line and separated them, and thirdly, the methylene dichloride-formamide system which carried estriol a short distance from the starting line. This method was fully described previously. Axelrod's control experiments showed recoveries of 91-106% of estrone, 89-105% of estradiol-β, and 91-110% of estriol. A model experiment with his method (Table 2) gave much lower recoveries of these estrogens.

The ultraviolet absorption spectra of the sulfuric acid chromogens of estrone and estradiol, determined by Axelrod (64)

Bernstein et al(80) and by the author were in close agreement, and typical spectra of these may be seen in Figure 1. The analagous spectrum of estradiol-β that was reported by Axelrod did not conform with the data given by Bernstein et al. or with the spectra obtained in this laboratory; Axelrod found only two peaks, at 308 and at 455 mμ. Two samples of estradiol-β were used in these experiments; each showed an extra peak at 370 mμ. Aside from this previously unreported peak the spectra of these two samples agreed with the data given by Bernstein et al. The spectra of the chromogens of samples of estradiol-β (see Figure 3) recovered in the control experiment were distorted, but were so very different to that obtained from the chromogen of the estrone recovered in this experiment that they could be used for the identification of estradiol-β.

In in vitro studies of the incorporation of C^{14} labelled acetate into cholesterol and other steroids, the concentration of acetate in the medium varied from 0.002 M to 0.02 M, and positive results were obtained (81, 41, 40). Brady, in his experiments with testes slices (42), used 0.008 M acetate in Krebs-Ringer bicarbonate buffer; this concentration of acetate was adopted for the incubation of both rat ovary and beef ovary slices in this laboratory.

Szego and Samuels (82, 83) have shown that two-thirds of circulating estrogen were associated with protein in blood and were not extracted by diethyl ether while the remaining one-third could be extracted readily with this

solvent. These authors digested the precipitated blood protein with 40% aqueous sodium hydroxide and after one hour were able to extract the remaining two-thirds of the estrogen from the acidified digest. It was possible, that, if the surviving ovary slice produced any estrogenic material during its incubation, this material would be associated with the tissue and not free in the medium. Thus the ovary slices were separated from the medium at the end of each incubation, and digested on the steam-bath with 4% alcoholic potassium hydroxide; both the medium and alkaline alcoholic digest were extracted as shown in Figures 4 and 5. This treatment of the ovarian tissue was justified by the recovery of considerable estrogenic material from the digested tissue.

The chloroform extraction of the neutral medium did not remove all of the estrogenic material; more was obtained by a second extraction of the acidified medium (Figure 4 and Table 4). The first extraction was omitted from the extraction scheme used for the rest of the experiments, nos. 11 - 32.

In the extraction scheme for the tissue digests (Figure 5), the fatty acids were separated by the partition of the extracted material between 70% ethanol and hexane; as little radioactivity was found in each hexane phase of experiments 36 - 39, this separation introduced a negligible loss of estrogens (see Table 9). Bachman and Pettit (84) have shown that the extraction of a solution of the three human estrogens in ether with 9% aqueous sodium bicarbonate,

removed no estrogens from the ether phase but did remove more strongly acid compounds. The results of the incubation of C^{14} labelled estrogens showed that from 4.0 - 14.0% of radioactivity of the fractions, extracted in this manner, were found in the bicarbonate washings. It was probable that this step introduced a loss of estrogenic material.

No estrogenic material was found in the extracts of rat ovary incubations (nos. 1 - 8); these results were not influenced by the addition of glucose, succinic acid or a saline extract of pituitary powder to either Krebs-Ringer phosphate or Krebs-Ringer bicarbonate incubation media. Material that corresponded to estriol, estradiol-β and estrone, was found in the extracts of both the medium and digested tissue of incubation no. 9; the presence of estriol and estrone was shown in the extracts of both digest and medium of incubation no. 10 (see Table 4). From these results it seemed quite likely that estrone was converted to estradiol-β. Around 50% of the estrogens added to the incubation medium was recovered from the tissue, even when the estrogens were added at the end of the incubation, no. 10, and the separation of the tissue and medium was carried out a few minutes later. These facts indicated that estrogenic material became associated with the ovarian tissue very quickly.

A similar series of experiments was performed with beef ovary slices; P.M.S., a preparation that contained follicular stimulating hormone, A.P.L., which contained chorionic gonadotrophin, and glucose were added to both phosphate

and bicarbonate media before incubation. Very faint spots were found on a few chromatograms; these, by themselves, had little significance; but as they were derived from incubations in which P.M.S. had been added, it was possible that P.M.S. had a stimulating effect and that traces of estrogen had been produced in these experiments, nos. 19, 21, 23, 26 and 28, Tables 5 and 6.

It has been shown that estrone and estradiol were inactivated by kidney and liver tissue (50, 48, 51); this inactivation was prevented by the addition of cyanide to the incubation medium (49). Although liver and kidney were specified as the only tissues that caused this inactivation (50), the perfusion of ovaries and other organs resulted in a loss of estrone from the perfusate (85). It was considered possible that production of estrogens by ovary slices might have been masked by a simultaneous destruction of them by the same tissue. Three incubations (nos. 33, 34 and 35, Table 5) with cyanide, glucose, P.M.S. and anterior pituitary powder were performed. Spots were detected on the chromatograms of 33D, 34D and 35M, but confirmatory texts on the eluates of these spots gave negative results.

It is possible that the beef ovary slices produced traces of estrogens when P.M.S. was added to the incubations, and that not enough was found to allow positive detection of them by the chemical methods used in this work; a different answer might have been obtained if one of the bioassay methods of analysis had been employed or if larger amounts of

tissue had been used. Mitchell and Davis (37) found an increase in the estrogenic content of placenta by both chemical and bioassay methods of analysis; they also reported poor recovery of estrogens when they were incubated with placenta and on the basis of this finding employed large correction factors in their final answers. Thus the inconclusive and negative results that were obtained in these experiments do not preclude the possibility that estrogen production, in vitro, will eventually be demonstrated.

The interconversion of estrone and estradiol-β by ovary slices was clearly established in the previous section of this thesis. In incubations nos. 9, 29, 30, 31 and 32 this was shown quantitatively with rat and beef ovarian tissue. A quantitative estimate of the conversion of these hormones was obtained from incubations nos. 36 - 39 with radioactive estrogens. There was a loss of radioactive material in each chromatographic step, for example from 11.4 to 26.2% (see Table 10); also the recovery of estrone and estradiol-β in the model experiment with Axelrod's systems was much lower than the values cited in his paper (64). Due to these data it is believed that the actual conversions were much higher, perhaps by as much as 50%, than the values that were obtained, which were a conversion of 4.6% and 3.3% of estrone to estradiol-β, and a conversion of 0.3% of estradiol to estrone.

Ryan and Engel (54, 55) have shown that the in vitro interconversion of estrone and estradiol-β could be demonstrated with many other tissues, testes, kidney, placenta,

adrenals, breast and other tissues of human origin and with rat liver. Other workers have shown an activation of estrone when incubated with mammalian red blood cells, muscle, uterus, lung and other tissue (57, 58), with rabbit uterus (86), and with liver (49); this could be explained readily by assuming a conversion of at least part of the estrone to estradiol-β. The experiments of Heller (50, 48) indicated that this conversion, estrone to estradiol-β, occurred with liver and kidney slices if cyanide was present in the incubation medium, and with spleen and uterus slices without cyanide, as in each of these experiments the biological activity of the extracts was increased. Ledogar et al.(87) have stated that a dehydrogenase in liver, breast and testes changed estradiol-β to estrone, and that this enzyme was not present in ovary, uterus or adrenal tissue; the inactiviation of estradiol-β by liver (52, 48) could be partly due to the activity of this enzyme.

Ryan and Engel (54, 55), Pearlman et al. (53), Mitchell and Davis (37) are in agreement that estrogen recovery from in vitro experiments show loss of material, in some cases as great as 90%. Pearlman and De Meio (53) produced some evidence that estradiol-β was converted to unidentified metabolites, a view that is endorsed by Ryan and Engel (54) who have stated:

> "The 55% of the starting material unaccounted for represents conversion of estrone and/or estradiol to unknown metabolites".

This opinion could be used to explain the large d./min values

detected in the neutral fractions of the tissue digests of incubations nos. 36 - 39, and also the considerable amounts of radioactivity found in the 9% sodium bicarbonate extracts from these samples (see Table 9); however, another possible explanation is that part or all of the material containing C^{14} in these fractions is one or both of these estrogens.

D SUMMARY

1. The reaction of each of the three human estrogens with diazotized sulfanilic acid was applied successfully to the detection of estrogens on paper chromatograms.

2. Many chromatographic systems for separating estriol, estradiol-β and estrone were investigated and a satisfactory system was devised; it consisted of isooctane-benzene-water, 1:1:2.

3. The claims for quantitative recovery of the estrogens in his three step procedure, by Axelrod, were not substantiated by this author, who reports much lower recovery of the three human estrogens by the same method.

4. A high proportion of estrogenic material incubated with ovarian tissue was recovered from the tissue, which rapidly become associated with the estrogens.

5. Attempts to show the endogenous production of estrogens by ovary slices gave negative or inconclusive results.

6. Interconversion of estrone and estradiol-β by surviving beef ovary slices was established.

7. No estrogenic material was metabolized to CO_2 by ovary slices, and the possible conversion of this material to unknown metabolites was discussed.

PART TWO

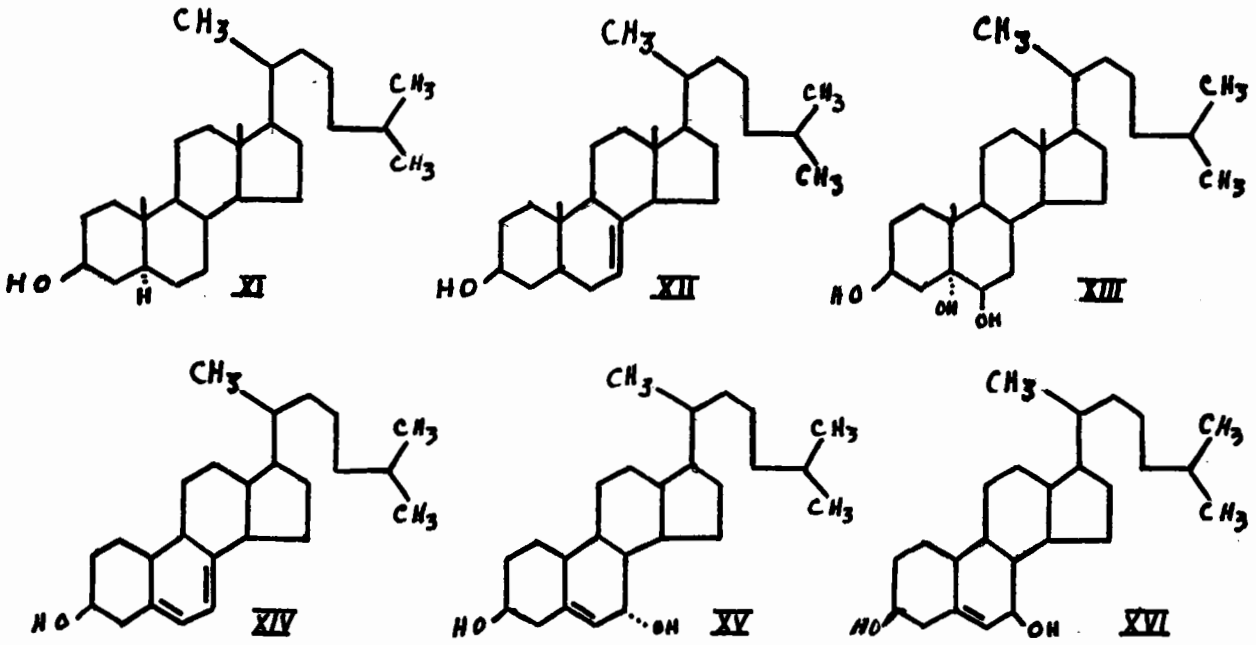
THE STEROID CONSTITUENTS OF THE PLACENTA FROM A MARE TREATED WITH ACETATE-1-C^{14}

A INTRODUCTION

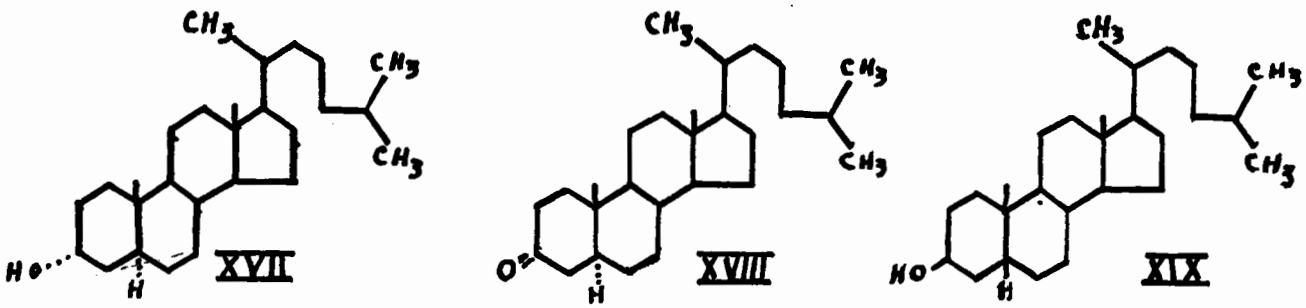
The isolation of progesterone from human placenta by two groups of workers (26, 27, 28) has explained the progestational activity of placental extracts. In the experiments of Salhanick et al. (27) the concentration of progesterone during the purification procedure was followed by the very sensitive bioassay technique devised by Hooker and Forbes (88, 89). Pearlman and Cerceo (28) used the ultra violet absorption peak at 240 mμ. to obtain an index of the progesterone content of the fractions they obtained in their investigation. Two C_{21} diols, pregnane-3(α), 20(α)-diol and allopregnane-3(β), 20(α)-diol, and allopregnanol-3(β)-20-one, compounds which could be metabolites of progesterone were also isolated from human placenta by Pearlman and Cerceo (29). The present investigation of the steroid contents of this equine placenta was undertaken with the aim of attempting to isolate progesterone and its metabolites from it, and to determine whether or not they had incorporated C^{14} from the labelled acetate administered to the pregnant mare.

Compounds, that could be companions of cholesterol, were encountered in the course of this investigation. In this group Fieser (90, 91) has included (β)-cholestanol (XI), lathosterol (XII) and cholestane-3(β), 5(α), 6(β)-triol (VIII). Mitchell (92) also included 7-dehydrocholesterol (XIV) in this group of sterols. Other compounds, whose occurrence in biological systems has been shown to be questionable, are

7(α)-hydroxycholesterol (XV) and 7(β)-hydroxycholesterol (XVI).



(β)-cholestanol was first known under the name dihydrocholesterol, and was found in gallstone cholesterol by Schoenheimer (93) and by Gardner et al. (94); this compound was also observed as a companion of cholesterol in bile, blood, brain, liver and other tissues by Schoenheimer et al. (95) and Fieser (90). The (α) isomer epicholestanol (XVII) and cholestane-3-one (XVIII) have never been found accompanying cholesterol from any natural source. (β)-cholestanol, like cholesterol, was shown to inhibit the haemolytic action

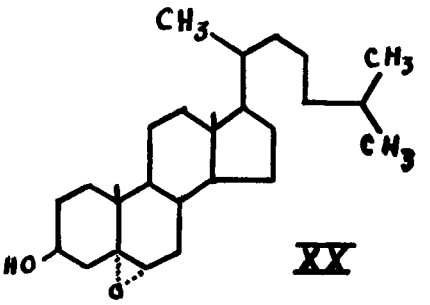


of digitonin (96); the (α) isomer did not. Also (β)-cholestanol was satisfactory as a growth factor for Trichomonas columbae, as was cholesterol while (α)-cholestanol and cholestane-3-one were not utilized by this organism (97). These experiments suggest biological specifity for the (β) isomer. Another saturated sterol encountered in biological investigations is coprosterol (XIX); Dam (98) has shown evidence that this sterol is formed from cholesterol by intestinal bacteria and explains the presence of coprosterol in feces on this basis.

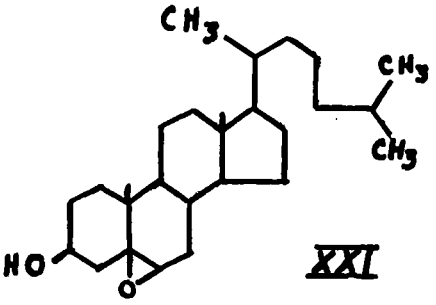
Cholestane-3(β), 5(α), 6(β)-triol was prepared for the first time by Pickard and Yates (99); these authors oxidized cholesteryl acetate with hydrogen peroxide and obtained a triol. The structure was assigned to this compound by Ellis et al. (100). Since its discovery this compound has been found in tissue by various workers: Haslewood (101) isolated this compound from ox liver extracts; Hardegger et al. (102) have shown its presence in an extract of arteriosclerotic aorta; Fieser et al. (91) observed that it was a companion of cholesterol from brain and gallstones; and Schwenk et al. (103) isolated it from commercial cholesterol and from a crude liver extract.

This triol has been prepared by the oxidation of cholesterol with alkaline permanganate by Windaus (104); he (105) also oxidized cholesterol with benzoyl peroxide and obtained cholesterol-(α)-oxide (XX) which was readily hydrated to give the triol. The oxidation of cholesterol and

cholesterol acetate with hydrogen peroxide and ferrous sulfate gave this triol (106). Both cholesterol-(α)-oxide and cholesterol-(β)-oxide (XXI) can be readily transformed to cholestanetriol by water at elevated temperatures (107, 108), by aluminum amalgam (109), or by heating in acetic acid (110).



XX



XXI

Blix and Lowenheilm (111) have summarized the work of Lifschütz, with his "oxycholesterol", as follows:

> "According to this author, cholesterol is transformed by the action of permanganate, or better, benzoyl peroxide in acetic acid solution, into a resinous product called oxycholesterol. This substance can be recognized and estimated with a colour reaction with glacial acetic and sulfuric acids, a reaction not given by cholesterol itself. Since oxycholesterol has not been obtained crystalline and has no definite melting point, the purity of the material is not yet assured. Lifschutz ascribes to it the formula $C_{26}H_{47}O_2$."

Blix et al. (111) and Moore and Willimot (112) found that the chromogenic material was produced when cholesterol was heated in air and when a suspension of cholesterol was aerated at 90°C. Shear and Kramer (113) observed that ultraviolet irradiation of cholesterol gave similar results.

The fact that these early workers were investigating mixtures, rather than pure compounds has impelled Bergström

and Wintersteiner (114) to make this statement:

> "Lifschütz apparently took it for granted that this oxycholesterol, claimed to be present as a preformed entity in the unsaponifiable matter of blood and tissues, and the chromogen he obtained from cholesterol by various, mostly oxidative, procedures in vitro, were represented by one and the same substance. This notion has now been dispelled not only by the isolation of two pure chromogens from natural sources, but also by the preparation in recent years of numerous other cholesterol derivatives of known constitution, which all give the Lifschutz reaction."

These two pure chromogens were 7(α) and 7(β)- hydroxycholesterol; the same authors (114, 115) report that cholestane-3(β), 5(α), 6(β)-triol does not give this reaction. These authors established that aeration of colloidal cholesterol produces both epimers of 7-hydroxycholesterol. This finding has cast doubt on the metabolic authenticity of these compounds when isolated from natural sources. 7(β)-hydroxycholesterol has been obtained from extracts of hog spleen by Prelog et al. (116), and from an extract of arteriosclerotic aorta by Hardegger et al. (102); Haslewood (101, 117) isolated this epimer from ox liver extracts and MacPhillamy (118) found it in an extract of swine liver. Wintersteiner and Ritzmann (119) were able to identify 7(α)-hydroxycholesterol as a constituent of an extract from pregnant mare serum. This compound was found also in the extract of the equine placenta examined in this thesis. As cholesterol is readily oxidized at position 7 (112, 113), the existence of 7(α)-hydroxycholesterol in biological systems is doubtful; this question

of whether or not this diol is an artifact accidentally produced during the fractionation scheme is discussed more fully in a later section of this thesis, in the light of new evidence obtained in this investigation.

B METHODS AND RESULTS

1. General Methods

The Separation of (α) and (β) Steroids by Digitonin

Butler and Marrian (120) precipitated (β) steroids with this reagent in enough 80% ethanol to give a final concentration of 1% digitonin; 4 to 5 g. of digitonin were used for each gram of steroid. The mixture was allowed to stand overnight; the precipitate was removed and washed once with 80% ethanol and twice with ether; the washings were added to the supernatant solution.

The dry precipitate was heated in enough pyridine to dissolve it, for 2 to 3 hours on the steam bath, and the freed digitonin was precipitated with 8 to 10 volumes of ether. This precipitate was washed as above, and the washings and supernatent contained the (β) steroids.

Brooks et al. (121) precipitated the excess digitonin and soluble digitonides from the original supernatant with a large volume of ether and treated the precipitate as before. This gave a second (β) fraction, from the soluble digitonides. The mother liquor from this precipitation contained the non (β) fraction.

Bergmann (122) treated digitonides differently; after heating with pyridine, the digitonide solution was evaporated nearly to dryness, by vacuum distillation at 40°C., and the solids extracted for several hours with ether in a Soxhlet

apparatus. The ether contained the steroid material.

The procedure outlined by Butler et al. (120) was used in the fractionation of the extract of this placenta. The steroid fraction from the soluble digitonides was obtained by the method of Brooks et al. (121) and in one instance the recovered digitonin was extracted with ether as outlined by Bergmann (122). After each lot of material was precipitated with digitonin, a few drops of a 1% solution of this reagent in 95% ethanol were added to the supernatant solution to make sure that the precipitation was complete.

When fractions were tested with digitonin, 70 - 100 µg. of material were dissolved in 0.2 ml. of 95% alcohol, and three drops of a 1% solution of digitonin were added. 70 µg. of cholesterol gave a precipitate immediately under these conditions.

The Separation of Ketonic and Non-Ketonic Steroids with Girard's Reagent 'T'

A modification of Girard's technique was described in a private communication sent to us by Dr. J. Carol (123). This modification was applied for our fractionation scheme.

13.5 g. of non-saponifiable material and 5 g. of trimethylaminoacetohydrazide were refluxed in 75 ml. of glacial acetic acid for 45 minutes. The solution was cooled rapidly and the pH of it was adjusted to 7 with ice-cold 6N NaOH. 100 ml. of ice-water were added and the resulting solution was extracted four times with 250 ml. of ether. This ether phase

contained the sterol fraction. The pH of the aqueous phase was adjusted to 1 with HCl and after 2 hours this solution was extracted four times with 150 ml. of ether; this last ether extraction was repeated 16 hours later, and each of these extractions of the acidified aqueous phase gave rise to a ketonic fraction.

Purification of Cholesterol by Bromination and Debromination

This purification scheme came to us from Dr. L. Fieser in a private communication (124). 5 ml. of a solution of 4.3 g. of bromine in 50 ml. of glacial acetic acid were added to 1.0 g. of cholesterol in 7.0 ml. of ether. The mixture was cooled in ice. The resulting paste was stirred for 10 minutes, and was centrifuged. The supernatant liquor was removed and the crystals were washed with a small volume of methanol. 15 ml. of ether, 5 ml. of glacial acetic acid and 0.2 g. of zinc dust were added to the precipitate and the mixture was well stirred. The dibromide dissolved in three minutes and then a white precipitate began to appear. After 10 minutes water was added dropwise until this precipitate disappeared. The solution was transferred to a separatory funnel and the ether phase was washed twice with small volumes of H_2O, and once with a small volume of dilute NaOH, and was evaporated to dryness. The dry residue after one or two crystallizations from methanol yielded pure cholesterol. When smaller amounts of cholesterol were used for purification, the quantities of solvents and reagents were adjusted proportionately.

Chromatography of Steroidal Material on Aluminum

Reichstein's method of chromatography (125) was used for the examination of the steroid fractions of both the ketonic and non-ketonic fractions.

30 g. of Al_2O_3 were recommended for use with 1 g. of material. The inside diameter (I.D.) of the column varied with the amount of Al_2O_3 used: for 4 g. of Al_2O_3, 10 mm. I.D.; for 8 g. of Al_2O_3, 16 mm. I.D.; and for 30 g. of Al_2O_3, 25 mm. I.D. were selected.

The Al_2O_3 column was prepared by introducing the Al_2O_3, as a fine slurry in the solvent chosen, into the column which was half-filled with the same solvent. The column was tapped gently until the Al_2O_3 had settled, and was washed by allowing more of the same solvent to percolate through it. The steroidal material was dissolved in this solvent, usually hexane, or hexane and benzene mixtures, and introduced to the column in which the level of solvent was allowed to drop until the meniscus was just above the top of the Al_2O_3. The next volume of solvent was added carefully, followed by enough ignited sea sand to form a protective layer over the surface of the Al_2O_3.

The column was developed by these solvents, in this sequence: hexane, benzene, ether and methanol. Chloroform was used between ether and methanol in some instances. The Al_2O_3 was acid-washed and reativated by heating at 180°C. for 16 hours before use. 20 ml. eluates were collected and evaporated to dryness.

Preparation of Benzoates and Acetates

The preparation of these derivatives was performed according to Klyne et al. (121, 126). The steroidal material was dissolved in pyridine, 1 ml. per 100 mg. of steroid. In preparing benzoates, 0.1 ml. of benzoyl chloride per each hydroxyl group on 100 mg. of steroid was added to the pyridine solution; for acetates, 0.2 ml. of acetyl chloride replaced the 0.1 ml. of benzoyl chloride. After standing 18 hours at room temperature, ice was added to the mixture which was allowed to stand for a further 2 or 3 hours.

The mixture was transferred to a separatory funnel, ether was added, and the ether phase was extracted once with aqueous N Na_2CO_3, once with N H_2SO_4 and three times with water. The ether phase was evaporated to dryness.

C^{14} Assay

The determination of the C^{14} content of samples was performed as described in Part 1 B.

Melting Point Determinations

These were obtained with the Köfler Hot Stage melting point apparatus.

The Liebermann-Burchard Reaction

This reaction occurs with all unsaturated sterols and is described by Sobotka (127).

Three drops of acetic anhydride and three drops of H_2SO_4

were added to 70 μg. of sterol in 0.3 ml. of chloroform. This quantity of cholesterol gave a positive test.

Ultraviolet Absorption Spectra of Steroids and of their Sulfuric Acid Chromogens

Ultraviolet absorption spectra were determined with a Beckman DU spectrophotometer.

The H_2SO_4 chromogens were prepared by the method of Bernstein et al (80); from 40 - 100 μg. of steroid were dissolved in 3.0 ml. of concentrated H_2SO_4. After 2 hours the samples were transferred to Beckman cells and the absorption spectra of these were determined.

The absorption spectra of unchanged sterols were determined in ethanol, methanol or propyl alcohol with the Beckman spectrophotometer.

The Reaction with Tetranitromethane

3 drops of tetranitromethane were added to 31 - 75 μg. of steroid in 0.2 ml. chloroform. A blank determination was made. 31 μg. of cholesterol gave a positive test.

Purification of Solvents

The method of Venning (128) was used in the preparation of peroxide-free diethyl ether.

95% ethanol was refluxed with zinc and sodium hydroxide and then distilled.

Glacial acetic acid was refluxed with potassium permanganate

and was then distilled.

Benzene was redistilled and stored over sodium wire.

Acetic anhydride was distilled from fused sodium acetate.

Hexane was redistilled, and the fraction that boiled from 67.0 - 68.5°C. was stored over sodium wire.

Anhydrous pyridine was obtained by refluxing with and distillation from barium oxide.

2. Preparation and Fractionation of the Placenta Extract

The Alkali Digestion and Extraction of Placenta from the Pregnant Mare treated with C^{14} Labelled Acetate

A mare in the ninth month of gestation was given acetate, labelled in the carboxyl group with C^{14}, by injection. A full description of the injection scheme and of the demise of the mare may be found in the thesis of R. Jacobs (129).

The placenta was removed from the mare shortly after death and was transported in ice to this laboratory. The placenta was minced and digested for 48 hours at room temperature in an equal volume of 5% aqueous NaOH. The volume of the digest was 17 litres. This digest was extracted as shown in Figure 8.

Two fractions were obtained from this placental digest, the non-saponifiable fraction (13.5 g. of semi-crystalline material) and the acidic fraction (a very dark oil). The aqueous phases were discarded.

The Fractionations of the Non-Saponifiable Material

This material was separated into ketonic and non-ketonic moieties with Girard's reagent 'T', as described previously. The sterol fraction and the first ketonic fraction were re-processed with this reagent. The separations are shown in more detail in Figure 9.

Sterols 1 and 2 were semi-crystalline, and an attempt

Figure 8

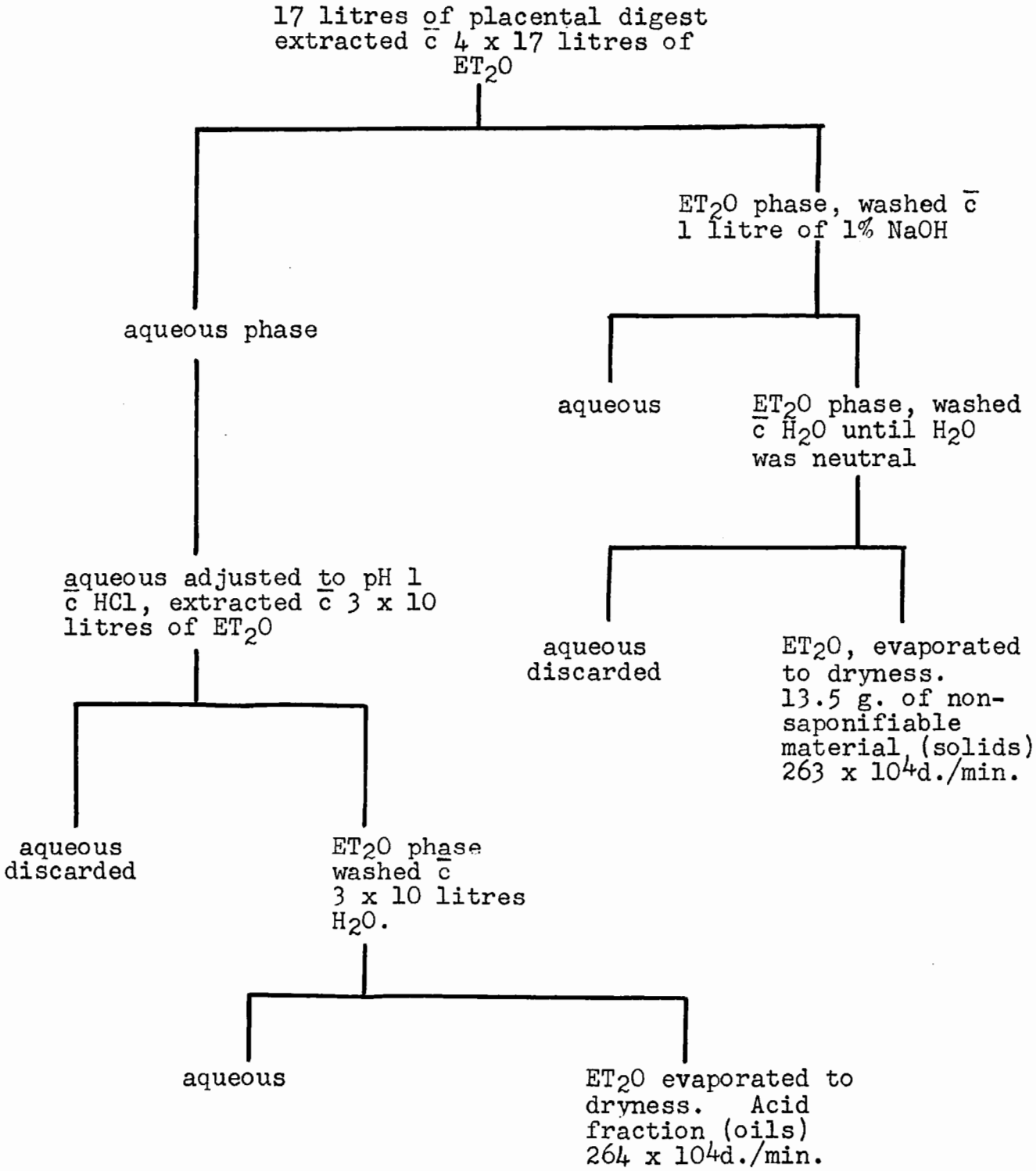
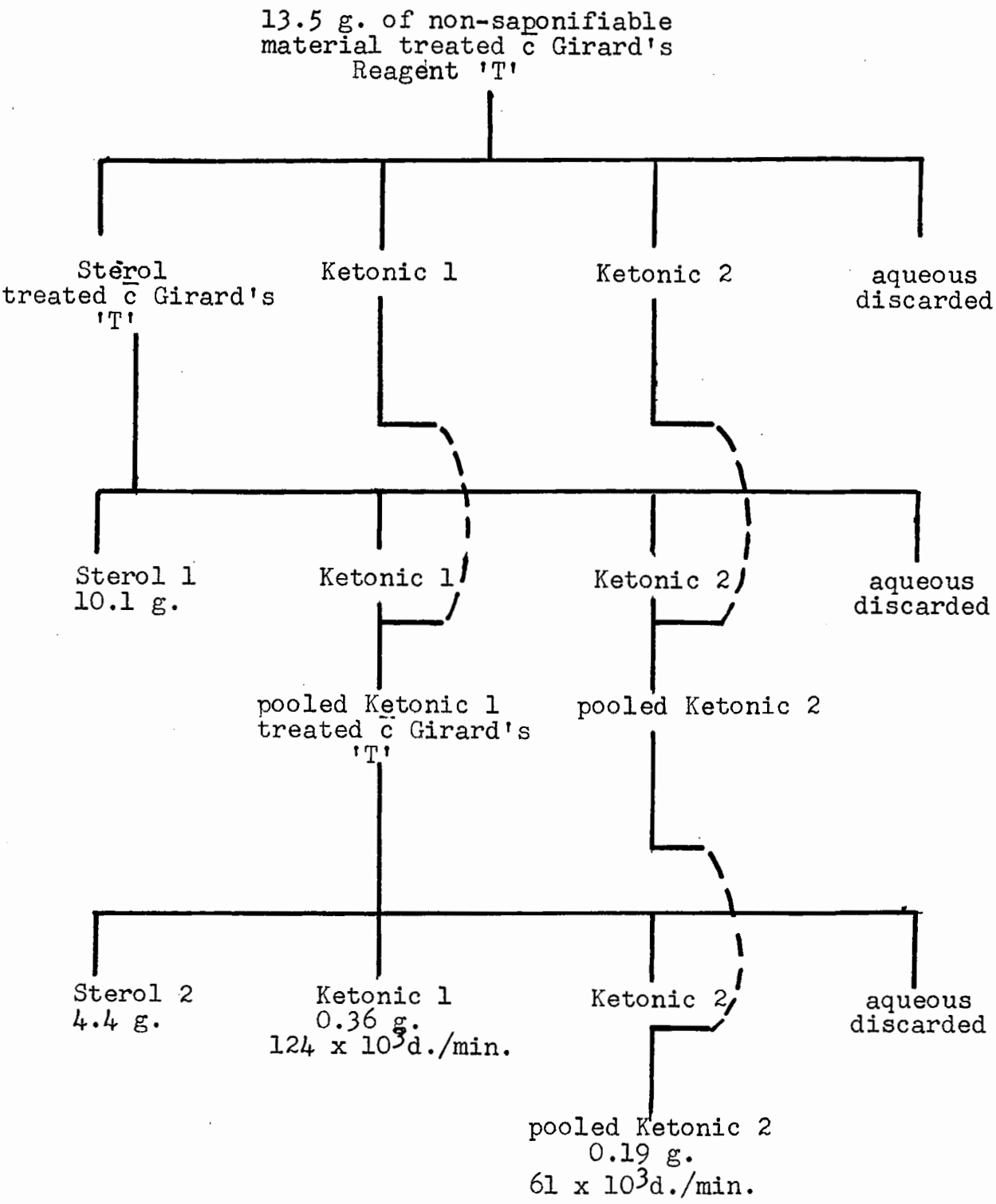

17 litres of placental digest extracted c 4 x 17 litres of ET_2O
ET_2O phase, washed c 1 litre of 1% NaOH
aqueous phase
aqueous
ET_2O phase, washed c H_2O until H_2O was neutral
aqueous adjusted to pH 1 c HCl, extracted c 3 x 10 litres of ET_2O
aqueous discarded
ET_2O, evaporated to dryness. 13.5 g. of non-saponifiable material (solids) 263 x 10^4d./min.
aqueous discarded
ET_2O phase washed c 3 x 10 litres H_2O.
aqueous
ET_2O evaporated to dryness. Acid fraction (oils) 264 x 10^4d./min.

Figure 9


13.5 g. of non-saponifiable material treated c Girard's Reagent 'T'
Sterol treated c Girard's 'T'
Ketonic 1
Ketonic 2
aqueous discarded
Sterol 1 10.1 g.
Ketonic 1
Ketonic 2
aqueous discarded
pooled Ketonic 1 treated c Girard's 'T'
pooled Ketonic 2
Sterol 2 4.4 g.
Ketonic 1 0.36 g. 124 x 10³d./min.
Ketonic 2
aqueous discarded
pooled Ketonic 2 0.19 g. 61 x 10³d./min.

was made to crystallize these from ethanol. A small yield of white material was obtained from each; these were pooled and called the M sample. This was crystallized from methanol and ethanol, and dried; 150 mg. of material were obtained whose melting point was 74.0-77.0°C.

Sample M was insoluble in cold methanol and ethanol, but was readily soluble in chloroform, hexane and ether. It gave positive tests in the Liebermann-Burchard and tetranitromethane reactions. It gave no precipitate with digitonin and the ultraviolet absorption spectrum of its sulfuric acid chromogen was very similar to that of cholesterol. The C^{14} content of this material was determined, and was 280 d./min./mg.

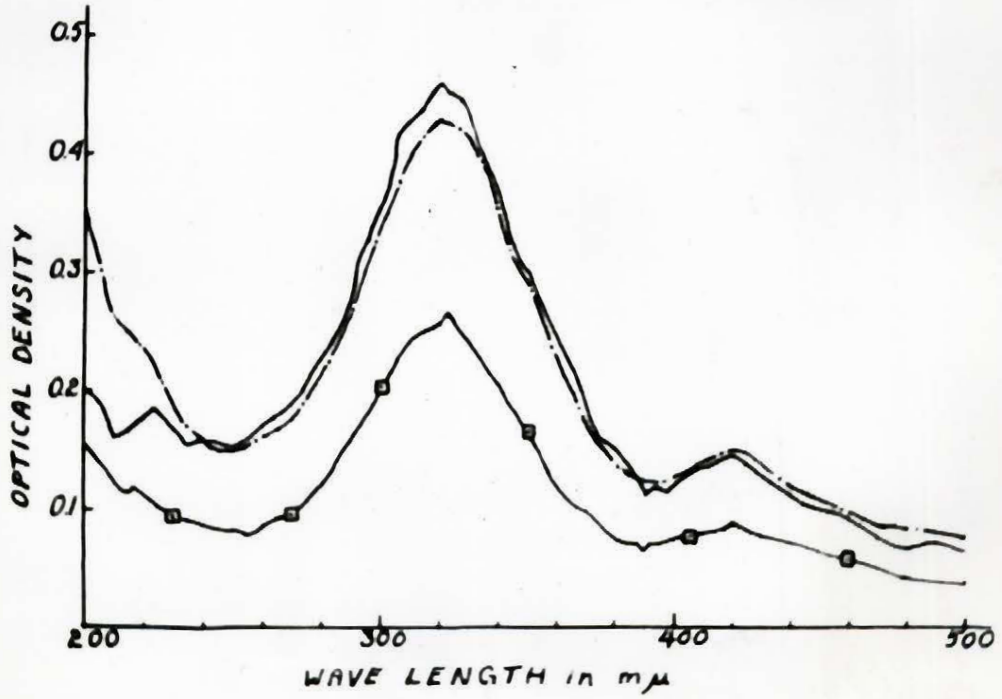
Sample M was chromatographed on Al_2O_3, and 54.2 mg. of material (labelled M1) were found in the hexane eluates; after crystallization from hot methanol, this compound melted at 68-73°C. The ultraviolet absorption spectrum of its H_2SO_4 chromogen was almost identical with that of cholesterol (see Figure 10). This material contained 250 d./min./mg. of radioactivity.

The remainder of sterols 1 and 2 were pooled and treated with digitonin as described previously. 4.30 g. of (β) material and 6.37 g. of non-(β) material were obtained; the former was labelled (β) 1.

1.02 g. of the non-(β) fraction were chromatographed on Al_2O_3. The first 40 ml. of the hexane eluates contained 164 mg. of solid material. After repeated crystallization from acetone 12.4 mg. of waxy crystals (labelled non-(β) 1)

FIGURE 10

ABSORPTION SPECTRA of
CHOLESTEROL, 18 µg./ml. conc. H_2SO_4
M1, 18 µg./ml. conc. H_2SO_4
NON-β 1, 27 µg./ml. conc. H_2SO_4



were obtained, which melted at 68.5 - 71.5°C., and which had this specific radioactivity, 304 d./min./mg. It was tested as was sample M, and gave the same results. The absorption spectrum of its sulfuric acid chromogen is also shown in Figure 10. This material was recombined with its mother liquors and brominated and debrominated as described under General Methods. 71.6 mg. of material were recovered from the insoluble bromide fraction and 20.2 from the soluble bromide fraction. The former fraction was recrystallized several times from acetone by Dr. H. Bolker, who obtained 6 mg. of material which melted at 67.0 - 68.0°C., and had this specific activity, 296 d./min./mg. Dr. Bolker determined the infrared absorption spectrum of this material, and found an absorption band at 1738 $cm.^{-1}$ The occurrence of this band in the spectrum established the presence of an ester linkage at position 3 of the steroid molecule.

The mother liquors from which this ester sample was obtained were pooled, and the 63 mg. obtained were saponified in 5 ml. of 2.4 N KOH in 95% ethanol for 8 hours on the steam bath. The digest was extracted with ether, and 42.4 mg. of non-saponifiable material were obtained. The pH of the aqueous phase was adjusted to 1 with HCl and this acidified digest was similarly extracted with ether. 25.4 mg. of acidic material were recovered. The non-saponifiable fraction was crystallized from methanol; crystals were obtained that melted at 137.5 - 141.5°C.; when mixed with a sample of authentic cholesterol, whose melting point was 142.0 - 145.5°C.,

this melting point was obtained, 141.0 - 145.5°C. The cholesterol from this non-saponifiable fraction had this specific activity, 223 d./min./mg.

Thus, the non-(β) fraction, obtained from the pooled sterols 1 and 2, contained esterified cholesterol. The residues from the remaining eluates of the chromatogram, carried out on 1.02 g. of this non-(β) fraction, were returned to this fraction which was then hydrolysed by 50 ml. of 2.4 N KOH in 95% ethanol, under N_2, on the steam bath. After 8 hours the digest was extracted as shown in Figure 11. This scheme provided two fractions, 3.94 g. of acidic, and 2.854 g. of non-saponifiable material. The latter fraction was separated into (β) and non-(β) fractions by digitonin. The (β) fraction was labelled (β)2, and weighed 0.545 g.; the specific activity of this material was 356 d./min./mg. A fraction containing material from the soluble digitonides was recovered, as described in General Methods, from the supernatant solution of the digitonide precipitation; this fraction weighed 0.36 g. and was labelled (β)3. The supernatant was evaporated to dryness and contained 0.985 g. of non-(β) material. The digitonin recovered from this separation was extracted with ether as described by Bergmann, and a further 0.75 g. of material were obtained, labelled (β)4.

Thus, four (β) sterol fractions and one non-(β) sterol fraction were separated; the separation scheme is summarized in Figure 12. Two ketonic fractions were obtained, K1 and K2.

Figure 11

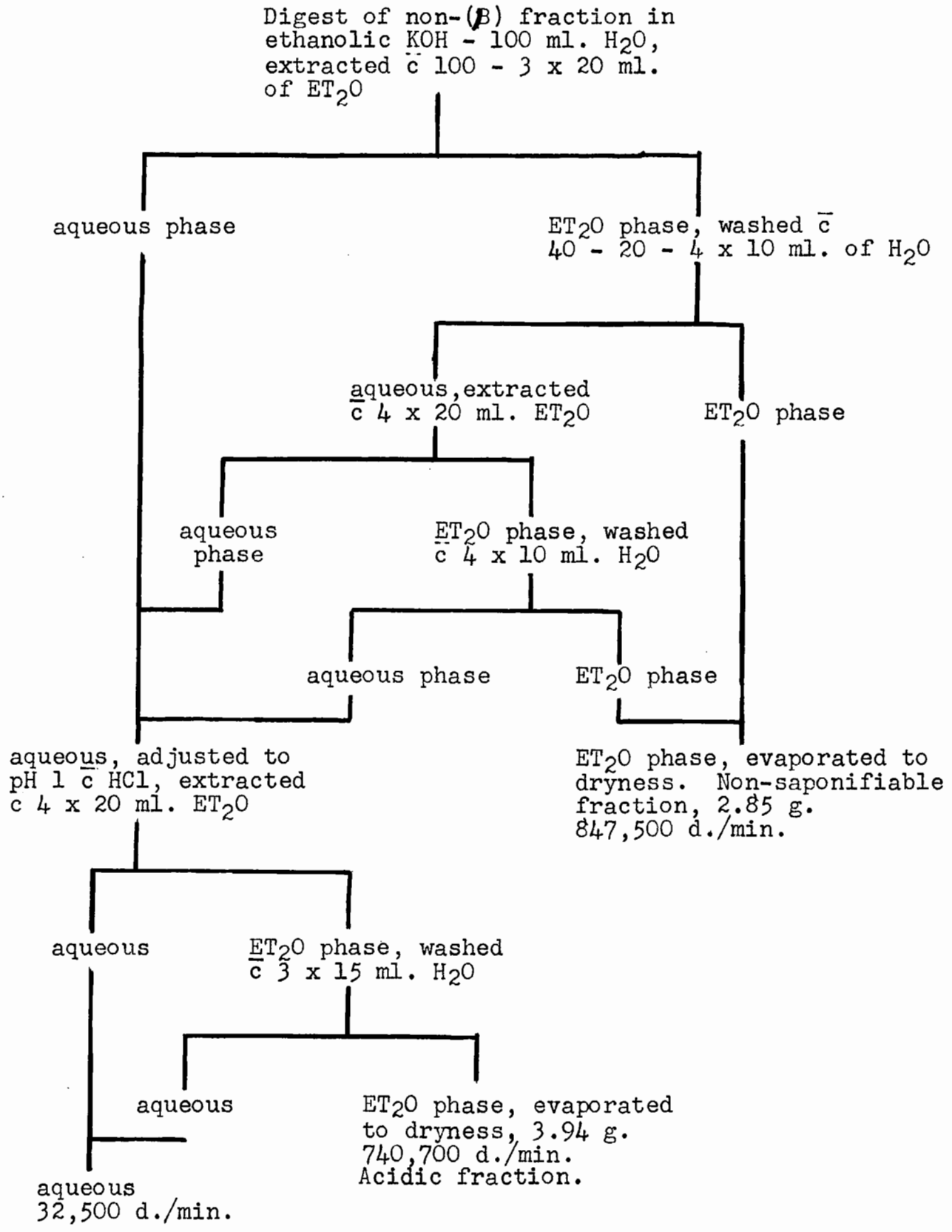
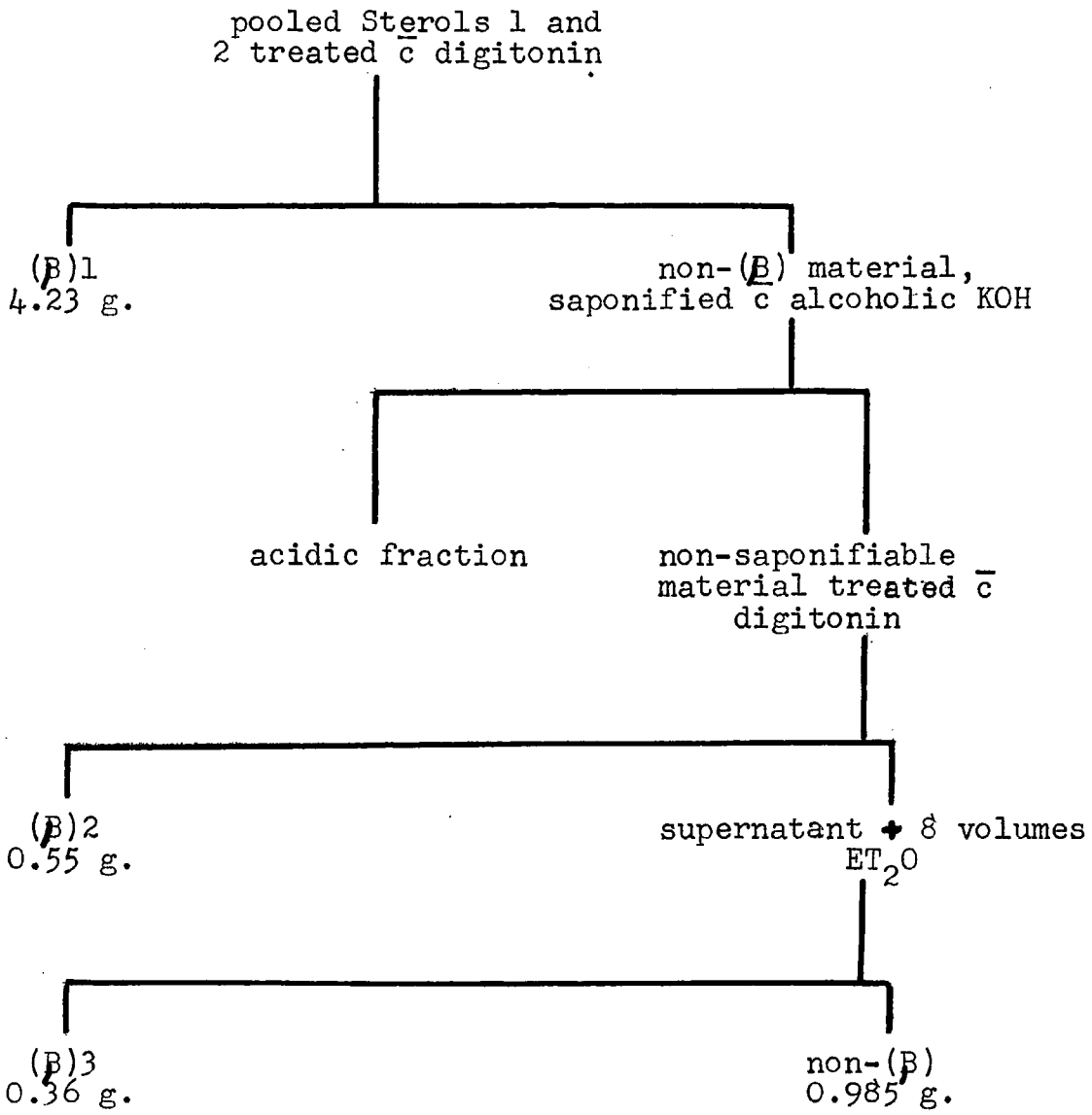

Digest of non-(B) fraction in ethanolic KOH - 100 ml. H_2O, extracted c 100 - 3 x 20 ml. of ET_2O
aqueous phase
ET_2O phase, washed c 40 - 20 - 4 x 10 ml. of H_2O
aqueous, extracted c 4 x 20 ml. ET_2O
ET_2O phase
aqueous phase
ET_2O phase, washed c 4 x 10 ml. H_2O
aqueous phase
ET_2O phase
aqueous, adjusted to pH 1 c HCl, extracted c 4 x 20 ml. ET_2O
ET_2O phase, evaporated to dryness. Non-saponifiable fraction, 2.85 g. 847,500 d./min.
aqueous
ET_2O phase, washed c 3 x 15 ml. H_2O
aqueous
ET_2O phase, evaporated to dryness, 3.94 g. 740,700 d./min. Acidic fraction.
aqueous 32,500 d./min.

Figure 12



Digitonin recovered from (β)2 and (β)3, extracted with ET_2O, gave (β)4, 0.75 g.

3. The Examination of the Sterol Fractions

1 g. of fraction (β)1 was purified by R. Jacobs of this laboratory; the radioactivity of the cholesterol in this fraction was determined before and after its purification through the dibromide. Jacobs' results (129) are reproduced in Table 13.

The remainder of (β)1 (3.23 g.), and (β)2 and (β)4 were chromatographed on Al_2O_3; chromatograms one, two and three (C1, C2, and C3) were performed as described under General Methods. In each case a large amount of crystalline material (later shown to be cholesterol) was found in the 5% methanol: 95% ether eluates, 2.87 g. from C1, 0.48 g. from C2 and 0.60 g. from C3. This material from C2, after crystallization from methanol, melted at 143.5 - 146°C.; with authentic cholesterol (Eastman Kodak, not purified through the dibromide, m.p. 142.0 - 145.5°C.) the mixed melting point was 143.0 - 146.0°C.

Purification of the Cholesterol from C1 and C2

The material from C1 was from the original (β) fraction (digitonin precipitable fraction) and that from C2 was from the esterified cholesterol in the original non-(β) fraction. These fractions were assayed for C^{14} content before and after bromination and debromination (performed as described under General Methods); the specific activities and melting points are listed in Table 13. The specific activity of the cholesterol obtained from the esters (β)2 was considerably

TABLE 13

The Radioactivity of Cholesterol from C1 and C2, before and after, and Melting Points after, Purification through the Dibromide

Sample	d./min./mg. before purification	d./min./mg. after purification	m.p. after purification
* (β)1	532	676	149.5-151.0°C.
Cholesterol (C1)	581	556	148.7-150.0°C.
Cholesterol (C2)	345	395	147.5-149.5°C.

* from Jacobs (129)

lower than that of (β)1.

Second fractions of solids were obtained from the 25% methanol: 75% ether eluate of these chromatograms: 34 mg. from C1, 20 mg. from C2 and 24 mg. from C3. The melting points of these ranged from 180 - 184°C. to 218 - 229°C., and did not improve on recrystallization. They were labelled X samples.

Different fractions of the first three chromatograms were pooled, benzoylated as described under General Methods and chromatographed on Al_2O_3. C4 was the pooled glacial acetic acid strippings and material that was eluted after the X samples of C1, C2 and C3; C5 was the pooled fractions of oils that came after cholesterol and before the X samples of C1, C2 and C3; C6 was the pooled oils from the eluates that preceded those that contained cholesterol from C1, C2 and C3.

Authentic cholesterol benzoate was prepared, as described previously; this compound melted at 144.0 - 145.5°C. and

cleared at 178°C.

Very small amounts of crystalline material were recovered from the hexane and 50% hexane : 50% benzene eluates of C4 and C5. Melting points, and mixed melting points with authentic cholesterol benzoate identified these samples as cholesterol benzoate.

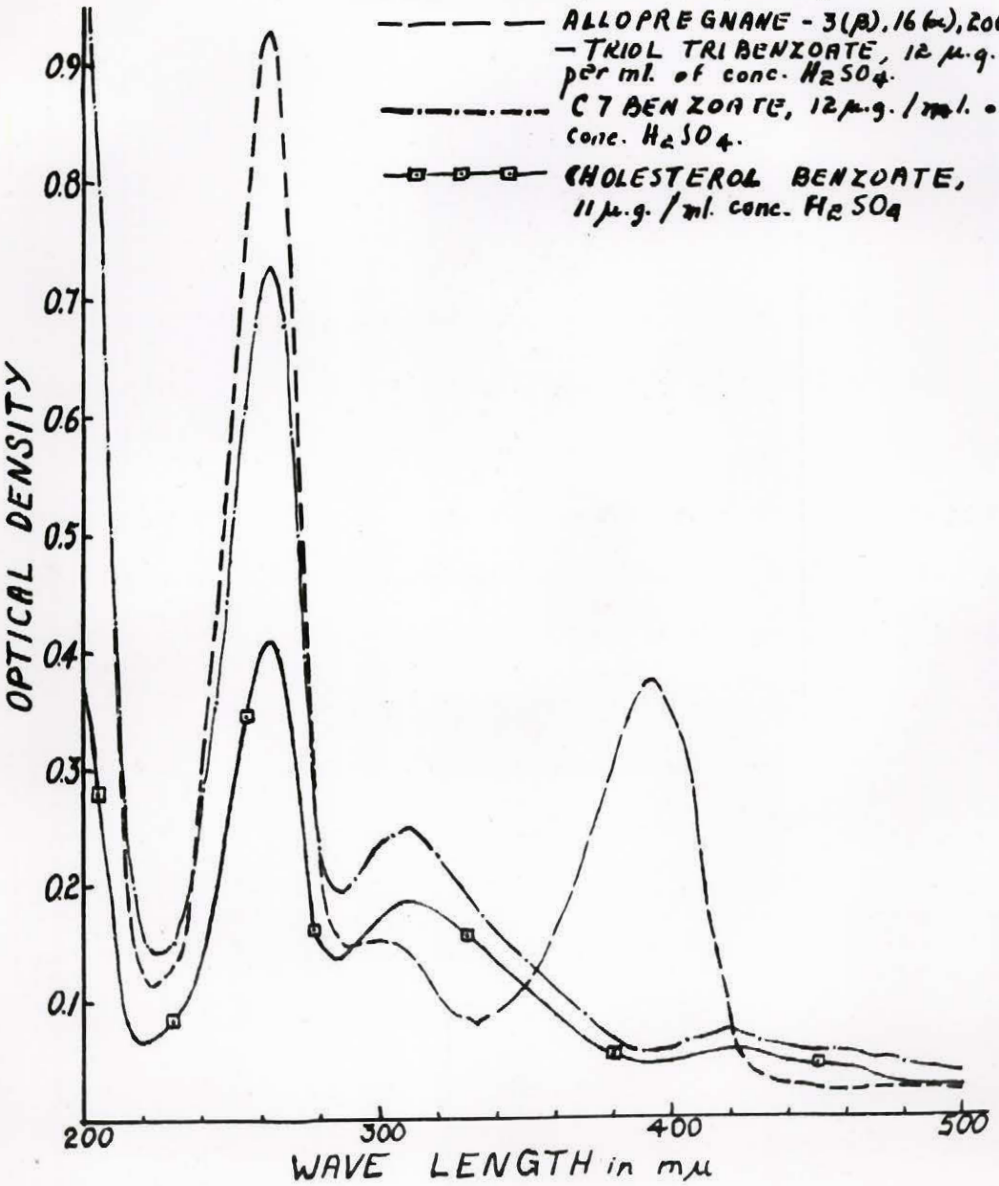
Isolation of an Unknown Sterol Benzoate

The X samples from C1, C2 and C3 were benzoylated and chromatographed on Al_2O_3, as C7. 16.2 mg. of solids were obtained from the 50% hexane : 50% benzene eluates. After repeated crystallization from chloroform-methanol, 4.1 mg., that melted at 213.5 - 217.0°C., were obtained.

Authentic allopregnane-3(β), 16(α), 20(β)-triol tribenzoate, melting point 217.0 - 218.2°C., was prepared, from a laboratory sample of the triol; authentic allopregnane-3(β), 20(β) diol dibenzoate, melting point 229.5 - 234.0°C. was provided by Dr. V.J. O'Donnell. The mixed melting points of the unknown benzoate with these authentic samples were depressed. With the triol tribenzoate the mixed melting point was 193.0 - 211.0°C., and with the diol dibenzoate, 197.2 - 221.5°C.

The absorption spectra of the H_2SO_4 chromogens of this unknown benzoate (labelled C7 benzoate), of allopregnane-3(β), 16(α), 20(β)-triol tribenzoate and of cholesterol benzoate are shown in Figure 13. The specific activity of the unknown benzoate was determined, 662 d./min./mg.

FIGURE 13
ABSORPTION SPECTRA of



All the other fractions from C7 (oils) were pooled and rechromatographed with Al_2O_3, as C8. The 15% benzene : 85% hexane eluates contained solids which, after recrystallization from chloroform-methanol, yielded approximately 1.5 mg. of crystals which melted at 206.0 - 213.0°C. This material was presumed to be more of the unknown benzoate, obtained from C7.

(β)3, the material recovered from the soluble digitonide fraction, was chromatographed on Al_2O_3, C9. 67 mg. of white solids (C9-1) were found in the 20% ether : 80% benzene eluates. This fraction, after crystallization from methanol, gave 17 mg. of crystals with a melting point of 146.5 - 148.5°C. The mixed melting point of this material with authentic cholesterol was 145.0 - 149.0°C. Thus (β)3 also contained cholesterol.

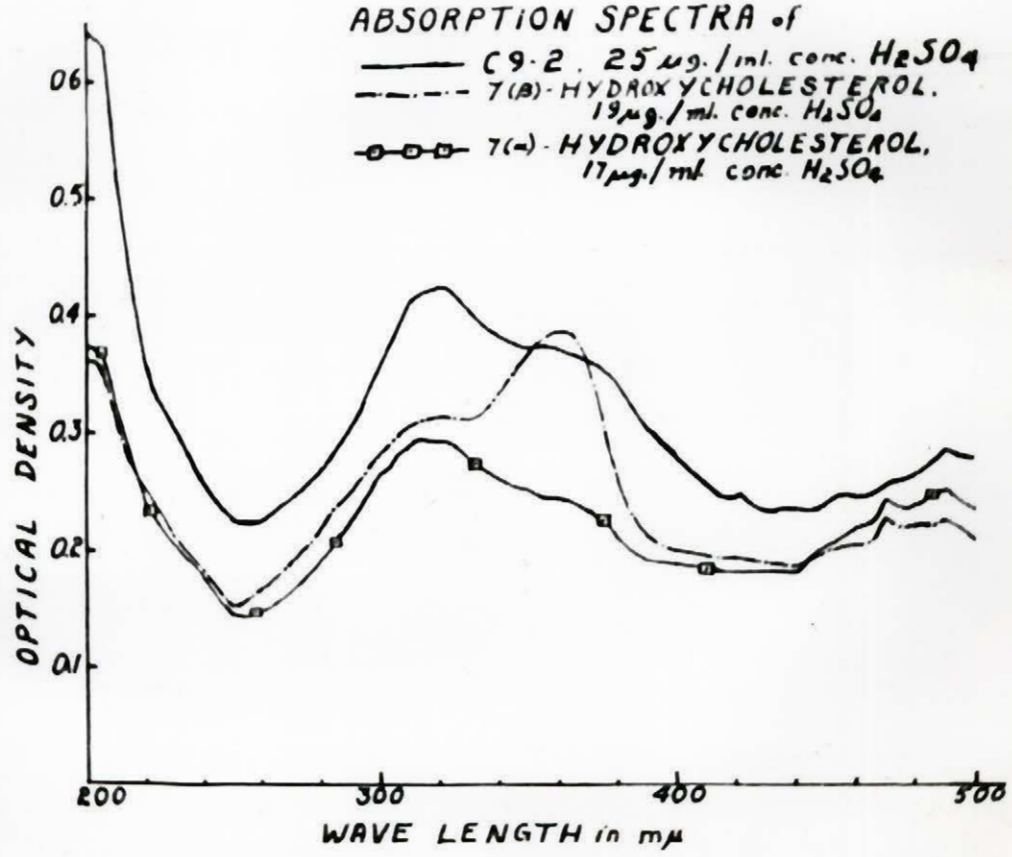
The Isolation of 7(α)-Hydroxycholesterol

A second fraction, C9-2, 43 mg. of crystalline material was eluted from C9 with 15 to 40% chloroform in ether. This fraction was crystallized from methanol, then from acetone, giving 7.2 mg. that melted at 177.0 - 189.5°C. A small second crop was recovered from the mother liquors, and, after recrystallization from acetone, gave 4.0 mg. that melted at 165.0 - 181.0°C.

Authentic samples of 7(α)-hydroxycholesterol and 7(β)-hydroxycholesterol were given to us by Dr. O. Wintersteiner. These two compounds and the second crop of crystals from C9-2 were examined by several tests and the results appear in Table 14; melting point determinations, done with the first

FIGURE 14

ABSORPTION SPECTRA of



crop from C9-2, also are given in Table 14. The absorption spectra of the H_2SO_4 chromogens of the two isomers and of C9-2 were obtained (see Figure 14). These results suggested strongly that C9-2 was 7(α)-hydroxycholesterol. The specific activity of C9-2 was 340 d./min./mg.

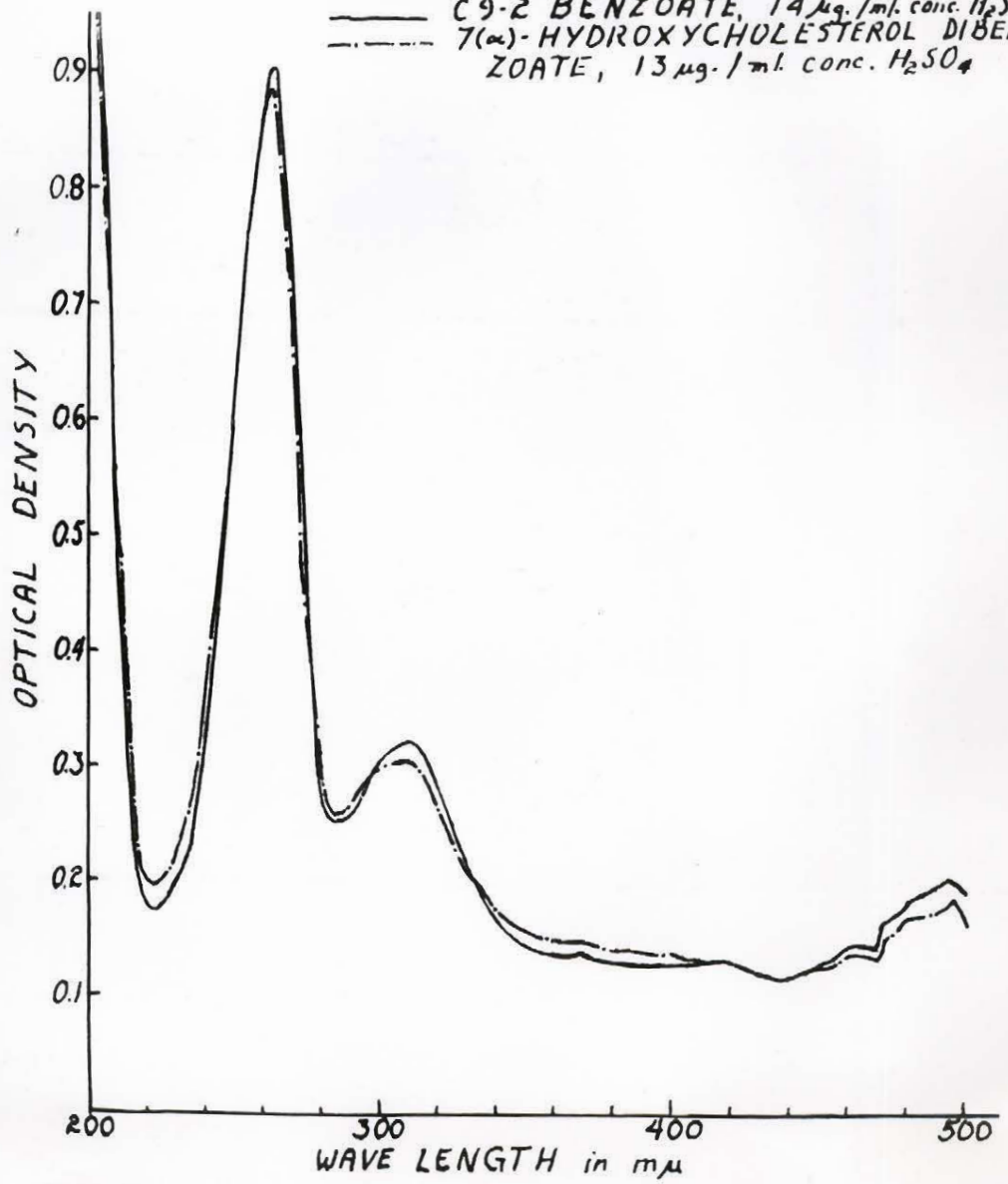
TABLE 14

Comparison of C9-2 and 7(α)-Hydroxycholesterol and 7(β)-Hydroxycholesterol

	C9-2	7(α)-Hydroxy-cholesterol	7(β)-Hydroxy-cholesterol
m.p.	179.0-190.0°C.	166.5-179.0°C.	146.0-160.0°C.
Mixed m.p. of C9-2 & authentic samples		162.5-178.5°C.	146.5-152.5°C.
Digitonin	-ve	-ve	+ve
Liebermann-Burchard	+ve	+ve	+ve
H_2SO_4	greenish-pink	greenish-pink	greenish-pink
Tetranitro-methane	+ve	+ve	+ve

The first crop of C9-2 and 7.0 mg. of the authentic 7(α)-hydroxycholesterol were each benzoylated; the dibenzoates of each were recrystallized from chloroform-methanol. The melting point of C9-2 dibenzoate was 147.5 - 152.0°C., that of the 7(α)-hydroxycholesterol dibenzoate was 148.5 - 151.5°C., and the mixed melting point of these was 148.0 - 151.7°C. The

FIGURE 15
ABSORPTION SPECTRA of
——— C9-2 BENZOATE, 14 µg./ml. conc. H_2SO_4
—·—·— 7(α)-HYDROXYCHOLESTEROL DIBENZOATE, 13 µg./ml. conc. H_2SO_4



absorption spectra of the H_2SO_4 chromogens of these two dibenzoates were in close agreement, and are shown in Figure 15. The specific activity of the C9-2 dibenzoate was 181 d./min./mg.; this corresponded to 275 d./min./mg. for the C^{14} content of the free diol.

Thus C9-2 was identified as 7(α)-hydroxycholesterol.

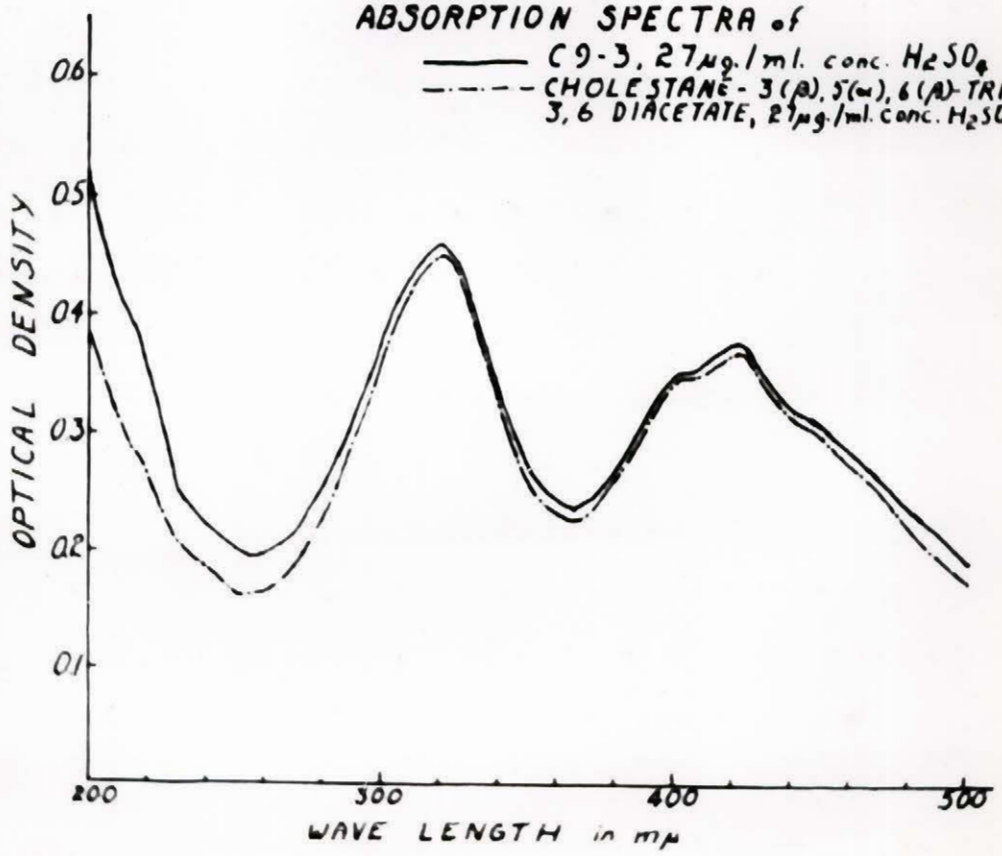
Isolation of Cholestane-3(β), 5(α), 6(β)-triol as the Diacetate

A third fraction, obtained from Chromatogram 9, C9-3, consisting of 82 mg. of solids was eluted with 5% methanol : 95% chloroform, which melted at 155.0 - 195.0°C. Recrystallization from acetone gave material that melted from 180.0 - 219.0°C.; this material could not be purified further by recrystallization. The mother liquors from these recrystallizations were pooled, giving 60 mg., of which 20 mg. were acetylated, as outlined under General Methods. Two crystallizations from methanol gave 3.1 mg. of acetate that melted at 164.0 - 167.5°C.

An authentic specimen of cholestane-3(β), 5(α), 6(β)-triol, 3, 6 diacetate was obtained from Dr. L. Fieser; this sample melted at 166.5 - 170.0°C. The mixed melting point of this authentic sample with the unknown acetate was 163.5 - 169.0°C. The absorption spectra of the H_2SO_4 chromogens of these compounds were in very close agreement (see Figure 16). Thus it was concluded that the unknown compound, C9-3, was identical with the authentic sample of cholestanetriol supplied by Dr. Fieser.

The specific activity of the cholestanetriol diacetate

FIGURE 16
ABSORPTION SPECTRA of
—— C9-3, 27µg./ml. conc. H_2SO_4
-·-·-- CHOLESTANE - 3(β), 5(α), 6(β)-TRIOL 3, 6 DIACETATE, 27µg./ml. conc. H_2SO_4



from C9-3 was 319 d./min./mg.; this represented a specific activity of 389 d./min./mg. for the free cholestane-3(β), 5(α), 6(β)-triol.

Isolation of a Sterol from the Non-(β) Fraction

The non-(β) material was chromatographed on Al_2O_3, C10. Most of the eluates from this chromatogram contained oils that could not be induced to crystallize. 35 mg. (C10-1) of oily material were eluted with 40% ether : 60% benzene; after several crystallizations from benzene-methanol, and acetone, 3.7 mg. were obtained that melted at 171.5 - 181.0°C. This material gave only a very faint colour with H_2SO_4. The Liebermann-Burchard, tetranitromethane and digitonin tests gave negative results. The specific activity of this material was 820 d./min./mg., and the absorption spectrum of the H_2SO_4 chromogen was very similar to that given by cholesterol (see Figure 17). This material was recrystallized from acetone, giving 3.1 mg., melting point 178.0 - 185.5°C. A last crystallization from methanol resulted in a small yield of crystals, circa 2.0 mg., which melted at 180.0 - 184.7°C.

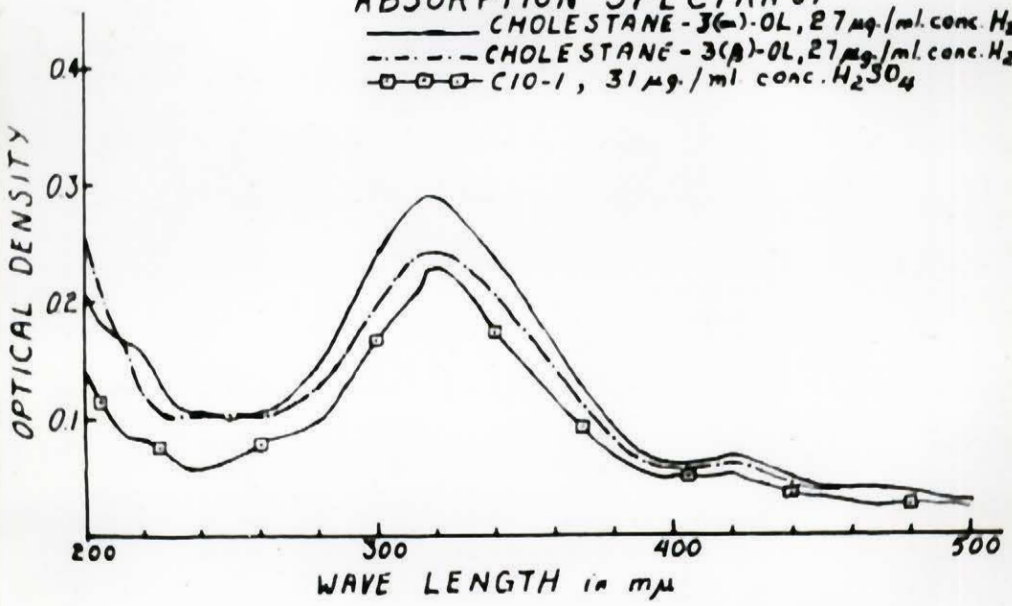
It was suspected that this material might be cholestane-3(α)-ol; an authentic sample of this compound was prepared by the reduction of cholestanone with sodium borohydride ($NaBH_4$), following the method of Belleau et al. (130). 1 g. of cholestanone (m.p. 129.5 - 130.5°C.) was dissolved in 150 ml. of 95% ethanol; to this solution was added a solution of 2 g. of $NaBH_4$ in 50 ml. of 70% ethanol. After 2 hours at 5°C.,

200 ml. of H_2O were added to the mixture which was extracted four times with 150 ml. of ether. The ether phase was evaporated to dryness and the residue treated with digitonin (see General Methods). 857 mg. of (β) material were obtained. The (α) fraction was very oily and was chromatographed on Al_2O_3. 128 mg. of (α) material were recovered from the 50% benzene : 50% hexane eluates. This fraction was recrystallized from 95% ethanol, giving crystals that melted at 181.5 - 185.0°C. This material was given a second treatment with digitonin. A small (β) fraction was removed, and the (α) fraction was crystallized several times from 95% ethanol, giving cholestane-3(α)-ol which melted at 185.5 - 187.0°C.

The mixed melting point of this authentic compound with the material from C10-1 was determined twice, 181.6 - 186.7°C. and 178.5 - 188.5°C. The absorption spectra of the H_2SO_4 chromogens of both isomers (α and β) of cholestane-3-ol were determined and appear in Figure 17.

The acetate of cholestane-3(α)-ol was prepared and, after repeated crystallization from methanol, material was obtained that melted at 95.5 - 97.0°C. The material from C10-1 was acetylated, and a very small yield of crystalline material, less than 1 mg., which melted at 82.3 - 91.0°C., was obtained after crystallization from acetone and 80% methanol. Insufficient material compelled the breaking off of this investigation at this point.

FIGURE 17
ABSORPTION SPECTRA of
——— CHOLESTANE - 3(-)-OL, 27 µg./ml. conc. H_2SO_4
-·-·-·- CHOLESTANE - 3(β)-OL, 27 µg./ml. conc. H_2SO_4
-o-o-o- C10-1, 31 µg./ml conc. H_2SO_4



Elution of C10 with 2% methanol : 98% chloroform provided a second fraction, C10-2, 182 mg. of oily solids. Repeated crystallization from acetone and from methanol gave white material that melted at 74.5 - 77.5°C. The Liebermann-Burchard, tetranitromethane and H_2SO_4 tests all gave negative results. The specific activity of this material was 656 d./min./mg.

4. Examination of the Ketonic Fractions

These two ketonic fractions, K1 and K2, were separated by digitonin into (β)1, material from the insoluble digitonides; (β)2, material from the soluble digitonides and non-(β) material, as described under General Methods. Part of K1 would not dissolve in 80% ethanol, prior to treatment with digitonin, and was removed as a separate fraction. These fractions were weighed and assayed for C^{14} content. The d./min. estimations and the weights of these fractions are given in Figures 18 and 19.

The ultraviolet absorption spectra of K1 and K2, before fractionation with digitonin, and of the fractions obtained from them were determined from 220 - 260 mμ. From the optical density of the peak at 240 mμ., the concentration of α,β unsaturated ketonic material in each fraction was calculated ($\epsilon^{alc.}_{max.}$ 240 mμ., for progesterone, ϵ = 16,200) as described by Pearlman et al. (28, 29); these data also appear in Figures 18 and 19.

K1(β)1, K1(β)2, K2(β)1, K2(β)2 and K1(ETOH insol.) were shown to contain little or no α,β unsaturated material; this material was recovered in K1 non-(β) and K2 non-(β).

K1 non-(β) was chromatographed on Al_2O_3, and the fractions eluted with 50% benzene : 50% hexane to 70% benzene : 30% ether contained 9.5 mg. of α,β unsaturated ketonic material. These eluates were pooled and to them 20 mg. of authentic progesterone (m.p. 128.0 - 129.5°C.) were added and the mixture was re-chromatographed on Al_2O_3. 19.7 mg. of progesterone came off

Figure 18

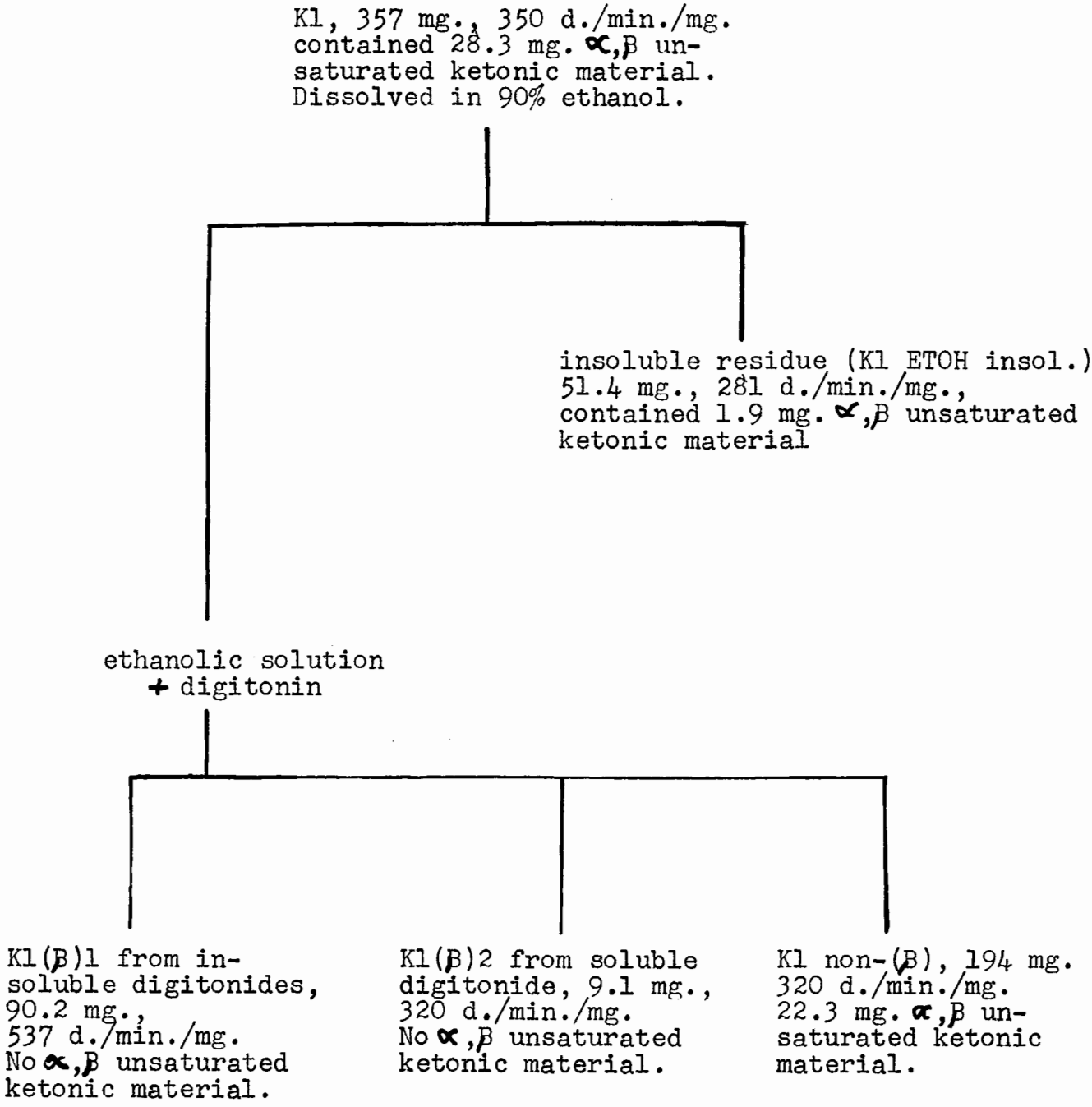
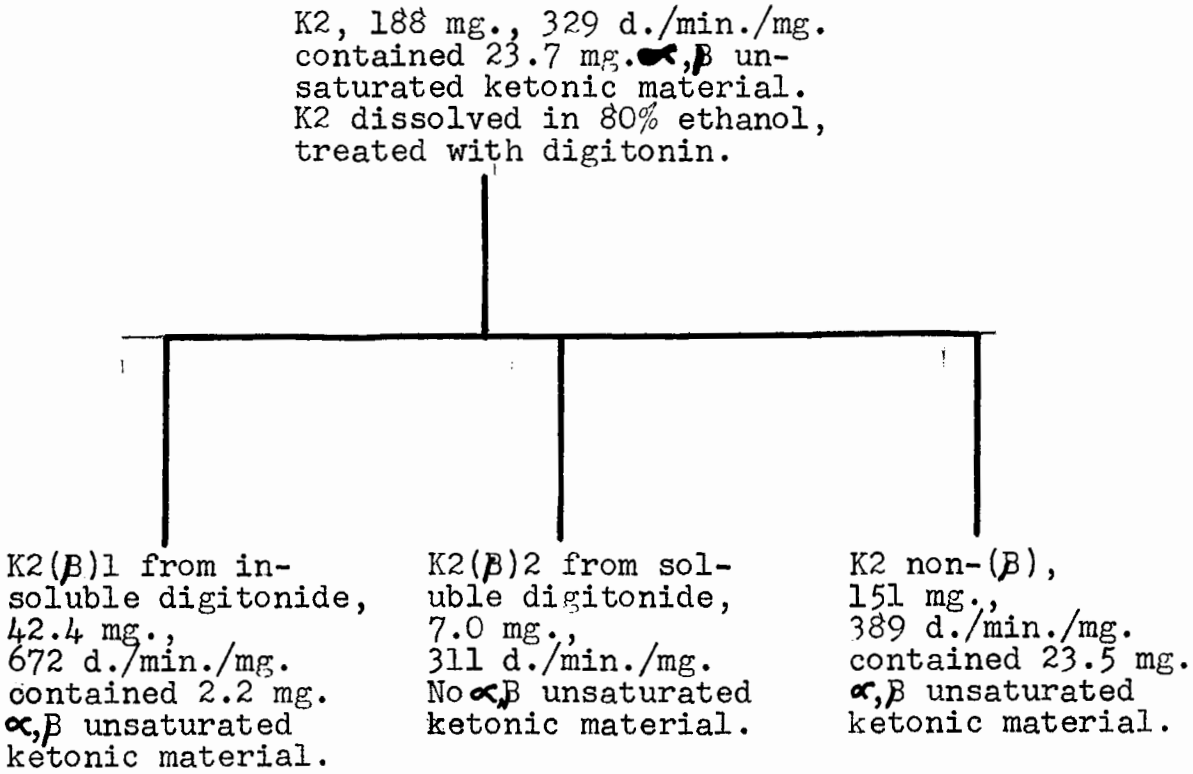

Kl, 357 mg., 350 d./min./mg.
contained 28.3 mg. α,β unsaturated ketonic material.
Dissolved in 90% ethanol.
insoluble residue (Kl ETOH insol.)
51.4 mg., 281 d./min./mg.,
contained 1.9 mg. α,β unsaturated ketonic material
ethanolic solution
+ digitonin
Kl(β)1 from insoluble digitonides,
90.2 mg.,
537 d./min./mg.
No α,β unsaturated ketonic material.
Kl(β)2 from soluble digitonide, 9.1 mg.,
320 d./min./mg.
No α,β unsaturated ketonic material.
Kl non-(β), 194 mg.
320 d./min./mg.
22.3 mg. α,β unsaturated ketonic material.

Figure 19


K2, 188 mg., 329 d./min./mg. contained 23.7 mg. α,β unsaturated ketonic material. K2 dissolved in 80% ethanol, treated with digitonin.
K2(β)1 from insoluble digitonide, 42.4 mg., 672 d./min./mg. contained 2.2 mg. α,β unsaturated ketonic material.
K2(β)2 from soluble digitonide, 7.0 mg., 311 d./min./mg. No α,β unsaturated ketonic material.
K2 non-(β), 151 mg., 389 d./min./mg. contained 23.5 mg. α,β unsaturated ketonic material.

the column in 50% benzene : 50% hexane, and after crystallization from ether, melted at 129 - 129.5°C. This material was assayed for C^{14} content and no significant radioactivity was detected.

K2 non-(β) was also chromatographed on Al_2O_3; 12.1 mg. of α,β unsaturated ketonic material were recovered in the 50% benzene : 50% hexane eluates. The first of these eluates weighed 16.2 mg. and contained 7.0 mg. of α,β unsaturated ketonic material; the following eluates held less. This first eluate could not be induced to crystallize; it and the other eluates containing α,β unsaturated ketonic material were pooled and to them 15 mg. of authentic progesterone were added. This mixture was chromatographed on Al_2O_3, and elution with 50% benzene : 50% hexane and with benzene removed 12.1 mg. of progesterone from the column. The progesterone was recrystallized from ether : hexane, and melted at 126.0 - 128.0°C. This specific activity of this material was very low, 3.8 d./min./mg.

C DISCUSSION

The digestion of this equine placenta in 5% aqueous sodium hydroxide was adopted from the methods used by Pearlman et al. (28), Noall et al. (26) and Salhanick et al. (27). In a previous attempt to isolate progesterone from human placenta, Pearlman and Cerceo (29) extracted the tissue with acetone, and no progesterone was found in this extract. When the same authors digested the tissue with alkali (28), partitioned the dried ethereal extract between aqueous methanol and petroleum ether and fractionated the methanol phase by counter-current distribution, they found approximately one mg. of progesterone per Kg. of tissue. Noall et al. (26) also used this alkali digestion as a first step in their procedure which led to the isolation of progesterone from placenta; they partitioned their dried ethereal extract with Girard's Reagent 'T' and chromatography. These results directed us to use the alkaline digestion for this equine placenta.

Persistent emulsions were encountered in separating the ketonic and non-ketonic fractions and necessitated repeating this technique (123) on both sterol and ketonic moieties. The recovery of (β) sterol, as outlined by Bergmann (122), ensured a more complete removal of (β) material from the residual digitonin and provided a third sterol sample which precipitated with digitonin, the (β)4 fraction.

The presence of cholesterol esters in the M sample, and in the original non-(β) fraction, the sterol material that did

not precipitate with digitonin, was surprising. It was expected that all esterified material in the placenta would have been hydrolyzed during the alkali digestion. The identity of esterified cholesterol was indicated by the melting points of these samples, 74.0 - 77.0°C. and 67.0 - 68.0°C. (for example, both the stearate and palmitate of cholesterol melt at 78°C. (131)). The absorption spectra of the H_2SO_4 chromogens of these samples of esterified cholesterol were very similar to that of cholesterol (see Figure 10). Conclusive evidence for the ester linkage at position 3 of these samples was provided by the infra-red spectroscopic data obtained by Dr. H. Bolker, in which an absorption band at 1738 $cm.^{-1}$ appeared. The presence of esterified cholesterol was corroborated by the recovery of cholesterol, with a specific activity of 223 d./min./mg., after a small portion of this material was saponified with 5% alcoholic potassium hydroxide.

Because of the presence of esterified cholesterol, the original non-(β) fraction was hydrolyzed in ethanolic alkali. This hydrolysis was performed in an atmosphere of nitrogen, to avoid oxidation of the sterol material. The specific activity of the cholesterol from this fraction was 395 d./min./mg., much lower than that of the cholesterol (556 d./min./mg.) obtained from (β)1, the original (β) fraction. Thus, the specific activity of the cholesterol in those esters that resisted the original hydrolysis by 5% aqueous sodium hydroxide was lower than that of the cholesterol from the free cholesterol and the more easily saponified cholesteryl esters of the placenta.

A probable explanation for the discrepancy in specific activity of the cholesterol obtained from the sample of purified ester, 223 d./min./mg., and of that from the whole fraction, is that the cholesterol of different esters varied in its C^{14} content. Rosenfeld et al. (132) obtained similar results with plasma cholesterol when C^{14} labelled acetate was given to two patients; the free cholesterol contained approximately three times more C^{14} than the esterified cholesterol, when the specific activity of each was determined from blood taken from these patients six hours after the dose of acetate was administered. This observation of the slower rate of incorporation of C^{14} into esterified cholesterol is supported by the work of Hagerman et al. (133, 134), who showed that, in the incubation of canine blood, no interchange of cholesterol occurred between free and esterified cholesterol. Thus we may conclude that the metabolism, or, at least, the turnover of free and esterified cholesterol differed in this placenta.

Brooks et al. (121) reported that sterol separation by chromatography was more complete when the sterols were previously benzoylated; these authors got improved separations of C_{21} sterols by this method. As the X samples resisted purification by recrystallization, and, as C_{21} sterols would not be unexpected since their discovery in human placenta (29), the X samples were pooled and benzoylated before chromatography on alumina. Other fractions of C1, C2 and C3 were treated similarly.

The unknown benzoate recovered from C7 was probably a diol.

In the absorption spectra of the H_2SO_4 chromogens of benzoylated sterols, the peaks at 264 mμ. were contributed by the benzoyl part of the molecule (see Figures 13 and 15). This peak increased as the degree of benzoylation increased. The data obtained with the unknown benzoate (see Figure 13), suggested that this compound was a diol; also the absorption spectrum was almost identical with that found for the H_2SO_4 chromogen of authentic allopregnane-3(β), 20(β)-diol dibenzoate by O'Donnell (135). It must be remembered that the mixed melting point of the unknown benzoate and this dibenzoate was depressed. The small yield of material limited the investigation of the identity of this compound.

7(α) hydroxycholesterol was found in (β)3, the sterols from the soluble digitonide fraction. The melting point of the free sterol was of little use in this identification; mixed melting with authentic samples of both epimers of 7-hydroxycholesterol, donated by Dr. O.Wintersteiner, gave inconclusive results (see Table 14). Wintersteiner, in a private communication (136), referring to these epimers, stated:

> "We never regarded the melting point as a good criterion of purity."

This statement is explained further by Wintersteiner and Ruigh (137), who found that the 7(α) epimer, which melted at 186.0°C., contained one mole of methanol of crystallization, and the dry form of this epimer melted at 154.0 - 157.0°C; they obtained mixed crystals of the two forms which gave intermediate

melting points and concluded that the melting point was inferior to the rotation determination, as a criterion of purity. On this basis, we also did not regard the melting point of the free diol as a datum of prime significance. Two tests associated the isolated diol with the authentic sample of 7(α)-hydroxycholesterol and not with the 7(β) epimer; neither precipitated with digitonin while the (β) epimer did precipitate, and the absorption spectra of their H_2SO_4 chromogens were in close agreement and quite different to that obtained with the 7 (β) epimer (see Figure 14).

The identity of the isolated compound was finally established by comparing the dibenzoate prepared from it with the dibenzoate of the authentic sample of 7(α)-hydroxycholesterol. The benzoates were in close agreement, and we concluded that the isolated material was 7(α)-hydroxycholesterol with a specific activity of 305 d./min./mg. (the average of C^{14} assays performed on the free diol and on its dibenzoate).

Cholestane-3(β), 5(α), 6(β)-triol was also isolated from the (β)3 fraction. Purification and identification of the free triol was not achieved, and it was identified as the 3,6 diacetate, a compound which can be characterized much more easily. Comparison of this compound with an authentic sample from Dr. L. Fieser, established its identity; the specific activity of the isolated material was 389 d./min./mg. for the free triol.

The 7-hydroxy sterols have been found in extracts of various tissues (101, 102, 116, 117, 118). Whether or not

these compounds arise from the oxidation of cholesterol during extraction and fractionation is still an unresolved problem. Wintersteiner and Ritzman (119), in their isolation of 7(α)-hydroxycholesterol from pregnant mares' serum, performed all operations involving heat and prolonged standing in an atmosphere of nitrogen and took all possible precautions to prevent the oxidation of cholesterol; they came to this conclusion:

> "On purely chemical grounds, the possibility that 7(α)-hydroxycholesterol may have been formed by such accidental oxidation seems remote."

Wintersteiner and Bergstrom (114, 115) examined this problem further and, after aerating cholesterol, in colloidal suspension, found evidence for the production of 7(α) and 7(β)-hydroxycholesterol by autoxidation of cholesterol. Only the latter compound was isolated; the former had been changed to another diol (not identified) during the separation of the ketonic and non-ketonic material. These authors concluded that position 7 in the cholesterol is readily oxidized by molecular oxygen, but did not rule out the possibility that these compounds might be made in the animal body:

> "There is no reason then, why this type of autoxidation should not occur in vivo, as its prerequisites, held in colloidal solution, and high oxygen pressure, certainly obtain in biological systems."

The production of both epimers of 7-hydroxycholesterol during the purification of cholesterol through the dibromide has been shown recently (138); the two epimers were identified

by paper chromatography. Chury (139) incubated cholesterol with spleen, and isolated 7-hydroxycholesterol in the incubation mixture and also in his control experiment; he believed that this compound arose from autoxidation of cholesterol and not from any action of the tissue. This experimental evidence supports the view that oxidation at position 7 of the cholesterol molecule can occur during the isolation procedure.

The 7-hydroxycholesterols have been formed from cholesterol by several methods. The reduction of the acetate of 7-ketocholesterol, prepared from cholesteryl acetate with CrO_3, by aluminum tri-isopropoxide gives both 7(α) and 7(β)-hydroxycholesterol (140). Wintersteiner and Ruigh (137) obtained a 10% yield of 7(α)-hydroxycholesterol from the oxidation of cholesterol hydrogen phthalate with $KMnO_4$; this reaction was previously examined by Barr et al. (141) with similar results. An interesting series of experiments was performed by Keller et al. (142) and Clemo et al. (106). These workers examined the action of X-rays and also hydroxyl radicals on cholesterol in acetic acid, and in each case recovered cholestane-3(β), 5(α), 6(β)-triol and 3(β)-hydroxycholestene-5-7-one; this latter compound is easily reduced to a mixture of the epimeric 7-hydroxycholesterols (143, 144).

The most probable sites of oxidation in the digestion and extraction schemes used in the experiments by the author of this thesis were the alkaline digestion of the placenta, in air, and the ether extraction of this alkaline digest. Ether was also used in the next step, the separation of ketonic and non-ketonic

material; Wintersteiner (115) found that this technique transformed the 7(α)-hydroxycholesterol to another sterol which was not identified, while the 7(β)-hydroxycholesterol was unchanged. The specific activity of the 7(α)-hydroxycholesterol, 305 d./min./mg., isolated from the equine placental extract was much lower than that of the cholesterol, 556 d./min./mg., that came from the original (β) fraction, made up of the free cholesterol and that from easily saponified esters. This evidence and the alteration of this compound during treatment with Girard's Reagent 'T' (123), described by Wintersteiner (115), have led us to conclude that the 7(α)-hydroxycholesterol did not arise, as an artifact from the original (β) fraction.

However, it is possible that this compound arose from, either the oxidation of esterified cholesterol during the treatment mentioned above, or during the saponification of the of the original non-(β) fraction. The specific activity of the cholesterol from this fraction, from the esters which withstood the original saponification with 5% aqueous sodium hydroxide, was 395 d./min./mg., closer to the value obtained for the 7(α)-hydroxycholesterol. The saponification of the non-(β) fraction was carried out in 95% ethanol, in which the cholesterol was in true, not in colloidal, solution, in an atmosphere of nitrogen which minimized the possibility of autoxidation of cholesterol at this stage. Thus we have arrived at this view, that the 7(α)-hydroxycholesterol was present in the placental tissue, or arose from the oxidation of esterified

cholesterol during the original saponification with 5% sodium hydroxide.

It is possible that the cholestane-3(β), 5(α), 6(β)-triol isolated from this placental extract was an artifact also. Fieser et al. (91) have isolated this compound from brain in such a manner that they have reported:

> "We feel that the triol, isolated from sterol extracted from brain by acetone at room temperature and without saponification, can hardly be an artifact and hence that the triol is a true companion of cholesterol in brain tissue."

Windaus (104) prepared this triol by oxidizing cholesterol with alkaline permanganate; he obtained only 4 - 5% of the triol. The same author (105) found that the oxidation of cholesterol with benzoyl hydroperoxide gave cholesterol (α)-oxide which readily gave this triol when heated with water. Stavely (145) has observed that heating cholesterol (α)-oxide with acetic acid gave this triol. When esterified cholesterol was oxidized with alkaline permanganate by Barr et al. (141), no cholestanetriol was obtained; he isolated only 7(α)-hydroxycholesterol. This triol was found in the reaction mixture after cholesterol was treated with hydrogen peroxide and ferrous sulfate (106); this reaction gave similar results with esterified cholesterol. Keller et al. (142) have shown also that similar results were obtained when free or esterified cholesterol in acetic acid was exposed to X-ray irradiation. Thus we can assume that both free and esterified cholesterol are oxidized to the triol by hydroxyl radicals and by X-ray

irradiation. The available evidence suggests that free cholesterol only is oxidized to the triol with alkaline permanganate; esterified cholesterol, treated similarly is oxidized to 7(α)-hydroxycholesterol but not to cholestanetriol (137). No cholestanetriol was found after the autoxidation of colloidal cholesterol with molecular oxygen (114, 115).

The cholestane-3(β), 5(α), 6(β)-triol isolated in this thesis had a specific activity of 390 d./min./mg.; this value is very close to that (395 d./min./mg.) obtained from the cholesterol from the original non-(β) fraction, which contained esterified cholesterol, and it could not have arisen from the cholesterol from the original (β) fraction, which had a specific activity of 556 d./min./mg. As the saponification of the esterified cholesterol (original non-(β) fraction) was performed in an atmosphere of nitrogen, it is unlikely that the triol was produced from cholesterol in the course of this operation. It is also unlikely that this triol arose from the oxidation of esterified cholesterol during the original tissue digestion and extraction; the evidence in the preceding paragraph indicates that free cholesterol is oxidized by peroxide to give this triol as readily as esterified cholesterol, and by alkaline permanganate more readily than the ester. If oxidation to the triol had occurred during these operations, both free and esterified cholesterol would have been oxidized, and the specific activity of the cholestanetriol would have been higher than the value observed. We have arrived, therefore, at this opinion, that the cholestanetriol was a true companion of cholesterol and did

not arise as an artifact from the accidental oxidation of cholesterol.

It is well established that (β)-cholestanol is a companion of cholesterol and may be found in many tissues (91). As epi-cholestanol has not been found in any extract of biological origin, it is surprising that evidence for the presence of the (α) epimer of cholestanol should have been encountered in this investigation.

The catalytic reduction of cholestanone with platinum and hydrogen in acidic solution has been used to prepare (α)-cholestanol; if this reduction is performed in neutral solution the (β) isomer is produced (146). Alkoxides also have been used to prepare these epimers, and the (β) isomer predominated in the products of these reactions (147). More recently, hydrides such as $LiAlH_4$ and $NaBH_4$ have found application for the reduction of ketonic steroids. Shoppee et al. (148) and Nace et al. (149) reduced cholestanone with $LiAlH_4$ and observed that the (β) isomer formed 90% of the reaction products which contained only a very low percentage of (α)-cholestanol. Etiocholane-3, 17-dione was reduced with $NaBH_4$ by Elisberg et al. (150), giving 70% of 3(α)-hydroxyetiocholane-17-one and 15% of the (β) isomer. Belleau and Gallagher (130) obtained 70 - 85% of cholesterol from the reduction of Δ^4-cholestene-3-one with this reagent. The reduction of cholestane-3-one with $NaBH_4$, performed here, gave results very similar to those obtained in the reduction of this ketone with $LiAlH_4$; 12% of (α)-cholestanol and 86% of (β)-cholestanol were found in the reaction products of this reduction

with $NaBH_4$.

No conclusive evidence for the presence of progesterone was observed in this placental extract. The absorption peak at 240 mμ. is not specific for progesterone, but is characteristic for any α,β unsaturated ketone. Pearlman et al. (29), in their investigation that failed to show the presence of progesterone in human placenta, also used the absorption at this wave length to show the presence of α,β unsaturated ketonic material in their placental extracts, but isolated no progesterone from the ketonic fraction. At least part of the absorption at 240 mμ., observed by these authors, was due to the presence of another unidentified ketone. In our investigation of this equine placenta, the presence of α,β unsaturated ketonic material was observed, but no progesterone was isolated.

In the separation of ketonic and non-ketonic compounds with Girard's Reagent 'T', Beall (151), following Reichstein's procedure, found that progesterone was easily freed from the water-soluble complex by hydrolysis with HCl at pH 3. He extracted this material after one hour and after two hours, and showed, by bioassay analysis, that all of the progestational material was contained in the first extract. In the adaptation of Girard's method used in this thesis (123), the water-soluble complexes were hydrolysed by HCl at pH 1, and two extracts, K1 and K2, obtained after two and sixteen hours, were examined for progesterone content. On the basis of Beall's findings (151), any progesterone present in this placental extract would have been carried into K1 and K2.

As no fractions, obtained by chromatographing the non-(β) fractions of K1 and K2, could be induced to crystallize, carrier progesterone was added to each of these. The progesterone recovered from K1 contained no C^{14}, and that from K2 contained only a trace of C^{14}. We are thus led to this conclusion, that, either no progesterone was present in this ketonic extract, or that any progesterone present in it contained no or very little C^{14}.

D SUMMARY

1. The specific activity of cholesterol from those esters that resisted saponification by 5% sodium hydroxide was much lower than that of cholesterol from the free cholesterol and those esters that were saponified by 5% sodium hydroxide.

2. An unknown sterol dibenzoate was isolated.

3. 7(α)-hydroxycholesterol and cholestane-3(β), 5(α), 6(β)-triol were isolated from the sterols obtained from the soluble digitonides; the possibility that these might have been artifacts is discussed.

4. Evidence for the presence of cholestane-3(α)-ol was encountered; this material had a specific activity much higher than that of cholesterol.

5. The reduction of cholestane-3-one with sodium $NaBH_4$ was performed for the first time. The proportion of the (α) and (β) isomers of cholestanol in the reaction products was similar to that obtained from the reduction of this ketone with $LiAlH_4$.

6. No evidence for the presence of radioactive progesterone in this placental extract was observed.

CLAIMS TO ORIGINAL RESEARCH

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

1. A method was developed for the detection of estrogenic material on paper chromatograms by diazotized sulfanilic acid.

2. The interconversion of estrone and estradiol-17(β) was shown to occur with ovarian tissue in <u>in vitro</u> experiments, although the endogenous production of estrogens under similar conditions could not be shown.

3. The specific activity of the cholesterol from those esters that resisted saponification with 5% NaOH was much lower than that of the cholesterol obtained from the free cholesterol and the saponifiable esters. These fractions were obtained from the placenta of a mare treated with acetate-1-C^{14}.

4. 7(α)-hydroxycholesterol and cholestane-3(β), 5(α), 6(β)-triol were isolated from this placental extract. These compounds were either biosynthesized from acetate-1-C^{14} in the pregnant mare or were formed by oxidation from esterified cholesterol in the placental extract.

5. Cholestane-3(α)-ol was prepared from cholestane-3-one with $NaBH_4$ and evidence for the presence of this sterol in the placental extract was encountered.

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