ON THE PREPARATION OF ESTRONE-16-C^{14} AND THE STUDY OF ITS METABOLISM

A Thesis

by

Lloyd M. Thompson

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GENERAL INTRODUCTION

In 1916 Lanthrop and Loeb (1) demonstrated a diminished incidence of spontaneous mammary cancer in female mice of known high incidence strains as a sequel to castration. This observation clearly implicated the ovarian hormone secretions (2), acting in conjunction with hereditary factors, in the transformation of normal to cancerous tissue in the mammary gland.

In his summary of studies on the use of steroid hormones in experimental carcinogenesis, Gardner (3) has stressed the increased incidence of neoplastic lesions on prolonged administration of estrogenic hormones. These lesions are produced in a variety of organs including the uterus, pituitary and testes. The decreased incidence, but not disappearance of mammary and cervical tumors following the simultaneous administration of estrogen and androgen serves to point out the unknown hormonal actions involved. Noble and Collip (4) have also shown regression of estrogen-induced mammary tumors in rats with simultaneously administered progesterone.

The answers to the vital questions of how and where the various steroid hormones exert these damaging and protective functions in the animal body must await a clearer understanding of the intermediary metabolism of these compounds.

The importance of clearly labelled steroids in the elucidation of metabolic pathways is at once apparent.

The concept of labelling or tagging a molecule with a marked atom or atoms is far from new and indeed has been employed with some success for a considerable period of time. Early studies of fat metabolism employed foreign chemical groups incorporated into fatty acids. In the last decade more reliable studies have been accomplished by the use of fatty acids whose labels are more acceptable from the physiological point of view. These more elegant methods use as labels both the heavy and radioactive isotopes of the component atoms of the molecule concerned.

As in the fatty acid series, so in the estrogenic group of compounds, labels have been used which are foreign to the hormone molecule. Examples of these types of labelled compounds are those which have been prepared by reactions between radioactive bromine and iodine and certain of the synthetic and natural estrogens. The application of triphenylethylene by both Gardner (5) and Bonser (6) as an estrogen to increase tumor incidence in mice has been followed by distribution studies of radioactive bromine-labelled triphenylethylene (7). In the natural series investigations of equilin, brominated with radioactive bromine (8), and β-estradiol,

iodinated with radioactive iodine (9), have been described. While these studies are of considerable interest it would be rash to assume that the intermediary metabolic behaviour of these non-physiological compounds bears any real relationship to that of the parent hormone itself. These derivatives are generally inert in a biological sense and have been so altered that their usefulness as a guide to hormonal metabolism is in grave doubt. The use of labelled estrogens which are more acceptable to the organism is thus very desirable.

The in vivo synthesis of sterols has been studied by Sonderhoff and Thomas (10) and Bloch and Rittenberg (11) using deuterium as a tracer element, replacing some of the hydrogen in the steroid molecule. The latter workers have extended their studies by the use of carbon 13 as well as deuterium (12). In the in vivo breakdown of cholesterol, deuteriocholesterol has been demonstrated to give rise to deuterium-rich cholic acid (13) and pregnanediol (14).

The discovery of carbon 14 by Ruben and Kamen (15) has heralded a further impetus in the tagging of carbon-containing molecules. In contrast to the heavy isotopes of carbon and hydrogen, this radioactive isotope is capable of detection in very high dilution, and hence offers excellent applicability to the metabolic studies of various steroid

hormones possessing physiological levels estimated in minute quantities. Indeed, preliminary investigations on the metabolism of radioactive progesterone, labelled with carbon 14 in the terminal carbon atom of the side chain, have demonstrated the in vivo breakdown of this hormone, with the resulting liberation of radioactive carbon dioxide in the expired air (16, 17).

The availability of carbon 14 now permits the possible tagging of steroids with sufficient concentrations of activity for use in metabolic studies. The work presented in this thesis describes the preparation of radioactive estrone tagged at position 16 with carbon 14 and the study of certain aspects of its metabolism.

PART I

A. THE PREPARATION OF ESTRONE-16-C^{14}

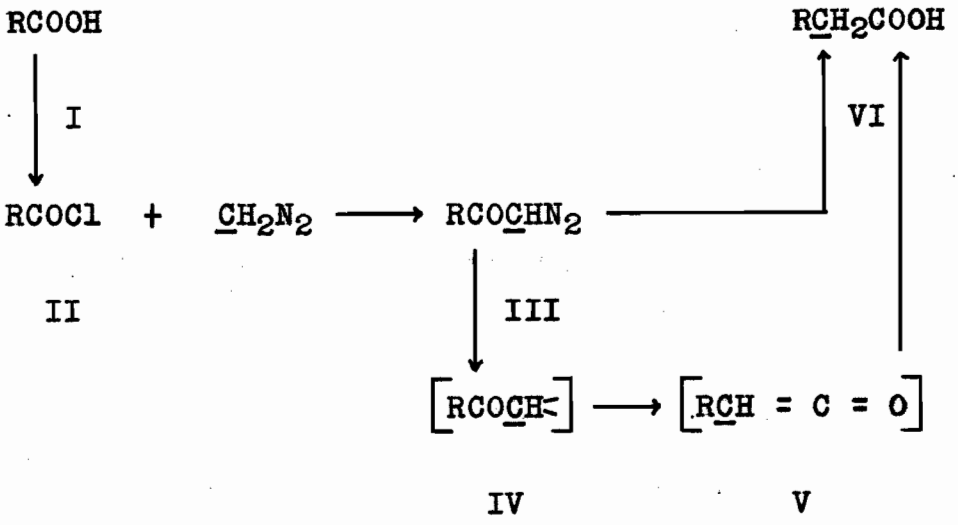
1. Introduction

Estrone (XLII) labelled with radioactive C^{14} within the steroid nucleus would be a very valuable compound for the study of the metabolism of the estrogens. Accordingly, the preparation of tagged estrone sufficiently high in radioactivity to serve this purpose was undertaken.

Two problems were immediately evident in this synthesis. First, C^{14} was available only in the elementary form of carbon dioxide. Its conversion to a specific reagent of high radioactivity, suitable for incorporation into the steroid ring, was necessary. Second, a synthesis was required using this reagent in which the overall yield would be of practical use.

Arndt and Eistert (18) have demonstrated the general application of the Arndt-Eistert synthesis (19) in the conversion of an acid to its next higher homolog. The acid chloride (II) of an acid (I) is added to an excess of diazomethane. The corresponding diazoketone (III) thus formed is then converted to the desired acid (VI). In addition, Eistert (20) postulated that the diazoketone loses nitrogen to form a

short-lived radical (IV) that rearranges to the ketene (V). Addition of the elements of water converts this ketene to the required acid. This may be shown diagramatically:



That such a rearrangement takes place has received substantial support from Huggett et al. (21). These workers synthesized heavy benzoic acid, labelled in the carboxyl group with C^{13}. Subjection of this compound to the Arndt-Eistert reaction (in the above equations, R = C_6H_5) afforded phenyl acetic acid-C^{13}. The heavy isotope was found in the carboxyl group of the phenyl acetic acid as shown by decarboxylation with copper chromite and quinoline. Thus the carbonyl carbon atom of the starting acid became the carbonyl carbon atom in the final product; the newly introduced carbon atom then occupied the position alpha to it.

This evidence has been confirmed recently by the use of C^{14} (22). Benzoic acid-C^{14} was prepared labelled in the carboxyl group (23). Conversion to phenyl acetic acid-C^{14} was carried out by an Arndt-Eistert synthesis. Formation of the amide followed by a Hoffman reaction in which the liberated carbon dioxide was found to contain the radioactivity again demonstrated that rearrangement had occurred.

A reverse procedure has been performed by another group of workers (24). 3(β)-Hydroxy-$\triangle^{5,6}$-etiobilienic acid (VIII, Chart I) was converted to its homologous acid (XV)*[x] using diazomethane-C^{13}. Ring closure to dehydroepiandrosterone acetate (VII)* was effected without loss of the heavy carbon. Since the pyrolysis involved the loss of a carbon atom and this carbon atom appeared to be the end one of the extended acetic acid side chain, the evidence again suggested that rearrangement occurred in the Arndt-Eistert reaction involved.

In 1938 Kuwada and Nakamura (25) reported the use of the Arndt-Eistert synthesis in the preparation of dehydroepiandrosterone acetate (VII). These workers used as a starting material 3(β)-hydroxy-$\triangle^{5,6}$-etiobilienic acid (VIII), arising

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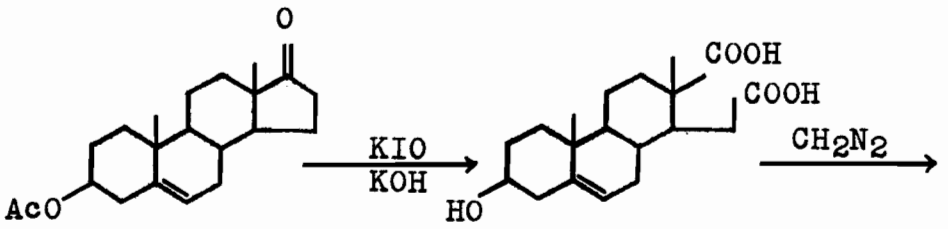
[x] The numbers of all tagged molecules are designated with an asterisk.

from the oxidation of cholesterol with chromate (26). More recently, Hershberg, Schwenk and Stahl (24) prepared dehydroepiandrosterone acetate-16-C^{13} (VII)* from this same starting acid, using diazomethane-C^{13}, through the identical intermediates, starting from dehydroepiandrosterone acetate itself. This was carried out to develop a useful method of labelling both the androgen and the estrogen series.

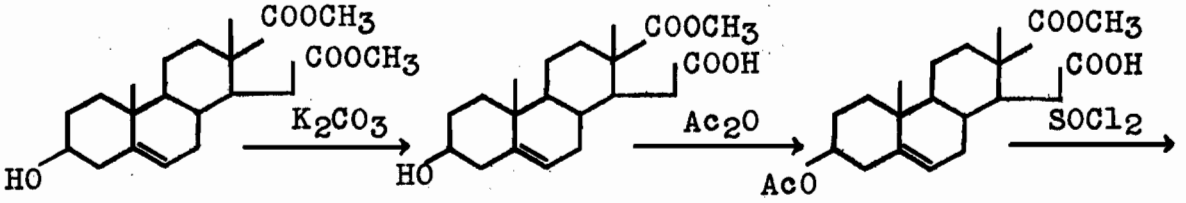
Chart I demonstrates this latter synthesis in schematic form. The 3(β)-hydroxy-$\Delta^{5,6}$-etiobilienic acid (VIII) arising from the oxidation of dehydroepiandrosterone acetate (VII) was esterified with excess diazomethane. The resulting dimethyl ester (IX) was then subjected to partial saponification in potassium carbonate solution, giving rise to the 3(β)-hydroxy-$\Delta^{5,6}$-tertiary monomethyl ester (X). This followed in accordance with the general rule that esters of primary carboxylic acids are hydrolyzed in preference to esters of tertiary carboxylic acids. Acetylation of the 3-hydroxyl group was followed by treatment with thionyl chloride, yielding the acid chloride of the 3-acetoxy-$\Delta^{5,6}$-tertiary monomethyl ester (XII). Addition of this acid chloride to three molar equivalents of diazomethane-C^{13} gave the corresponding diazoketone (XIII)*. Its rearrangement to the amide (XIV)* was effected with silver nitrate dissolved in aqueous ammonia; the amide (XIV)* was hydrolyzed to the free 3(β)-

CHART NO. I

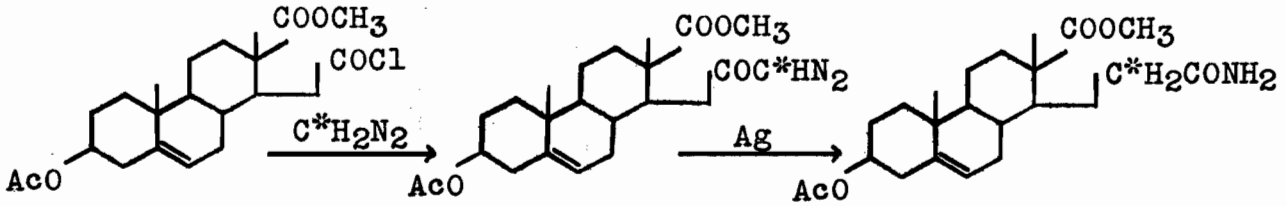
Hershberg, Schwenk and Stahl.



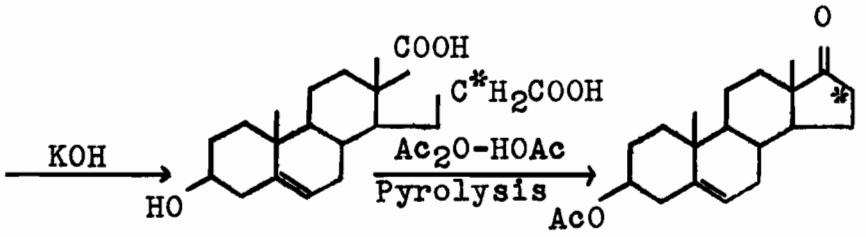
VII VIII



IX X XI



XII XIII XIV



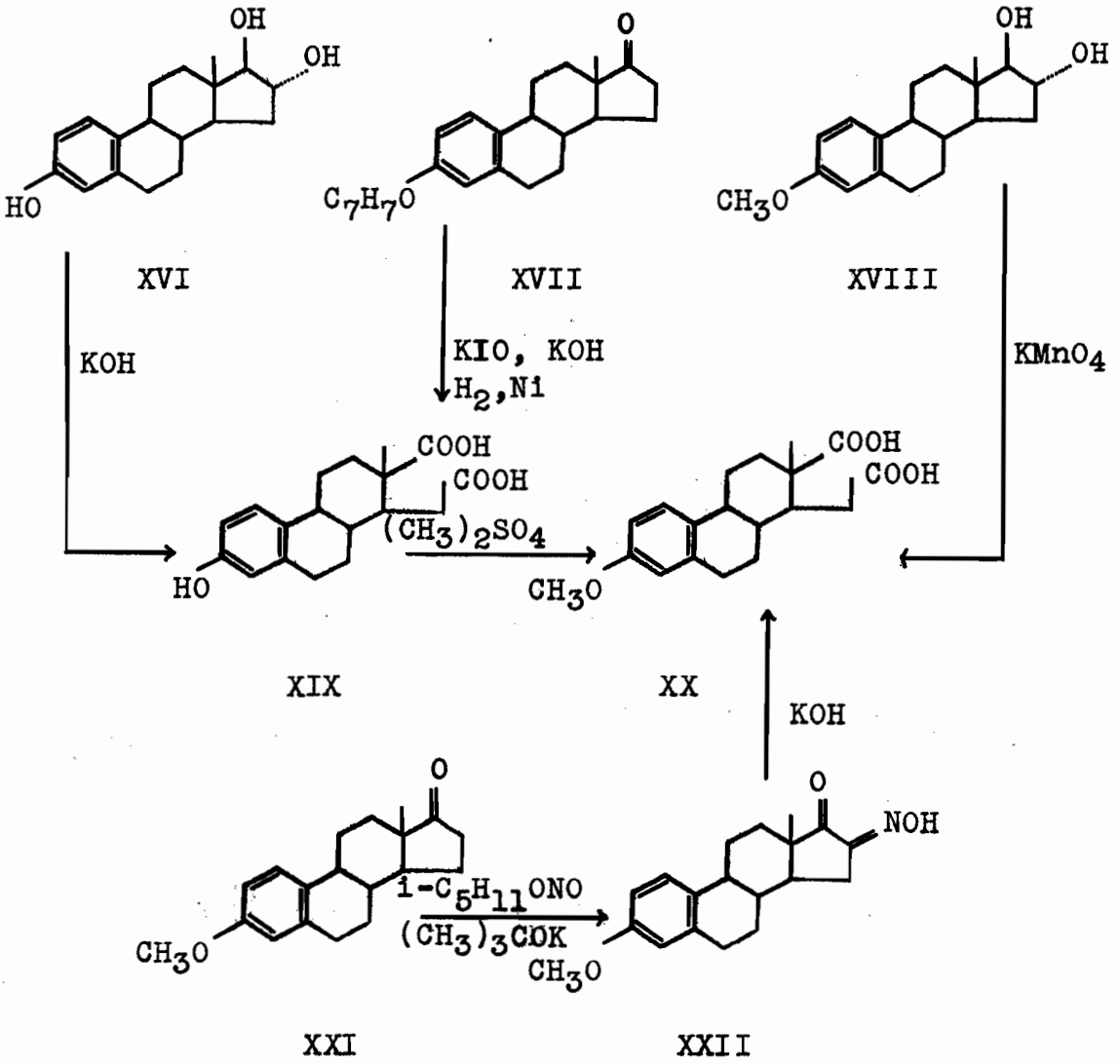
XV VII

hydroxy-$\Delta^{5,6}$-homodiacid (XV)*. Reacetylation of the 3-hydroxyl group, followed by pyrolysis, resulted in formation of dehydroepiandrosterone acetate (VII)*, labelled at position 16 with C^{13}.

Jamieson (27) has suggested the possibility of labelling estrone in ring D by a similar series of reactions. Basically, this involves three major operations; first, the opening of the D ring of estrone; second, the extension of the acetic acid side chain thus formed by one carbon atom, with either heavy C^{13} or radioactive C^{14}, followed by rearrangement; and third, ring closure to estrone again, incorporating this labelled carbon atom.

To effect this synthesis, the corresponding diacid in the estrone series, 16,17-seco-$\Delta^{1,3,5:10}$-estratriene-3-ol-16,17-dioic acid (XIX), hereafter referred to as marrianolic acid, is required. In 1932, Marrian and Haslewood (28) first isolated marrianolic acid by the oxidative fission of fused alkali on estriol (XVI). This was confirmed the following year by MacCorquodale, Thayer and Doisy (29). In addition, these authors formed the methyl ether of marrianolic acid (XX) using dimethyl sulphate in alkali and showed that it was identical with the oxidation product obtained by treating estriol-3 methyl ether (XVIII) with potassium permanganate (30). Another group

of workers (31) obtained marrianolic acid-3 methyl ether by treatment of the sixteen oximino derivative of estrone-3 methyl ether (XXII) with a solution of phosphorus pentachloride in acetyl chloride, followed by prolonged hydrolysis in alcoholic potassium hydroxide solution.



Potassium hypoiodite was first described by Miescher et al. for

oxidative cleavage of the D ring of dehydroepiandrosterone (32). These same workers have adapted this reagent to the estrogenic hormones (33, 34, 35, 36). Marrianolic acid (XIX) has been obtained in good yields by this procedure from the oxidation of the benzyl ether of estrone (XVII) (33). This low temperature conversion with potassium hypoiodite and the high temperature conversion with alkali fusion gave the same marrianolic acid stereoisomer.

Esters of marrianolic acid and similar acids of the more unsaturated members of the estrogenic series, have been subjected to the Arndt-Eistert synthesis. Litvan and Robinson (31) converted the tertiary monomethyl ester of marrianolic acid-3 methyl ether (XLVIII, Chart III) to 16a,17-seco-$\triangle^{1,3,5:10}$-estratriene-3-ol-16a,17-dioic acid dimethyl ester-3 methyl ether (LI), (subsequently referred to as homomarrianolic acid dimethyl ester-3 methyl ether). Their overall yield was approximately 1 per cent. However, more satisfactory results were obtained by Bachmann et al. who employed this method of extending the acetic acid side chains of corresponding tertiary monomethyl esters in the syntheses of equilenin (37) and estrone A (38) and related homodiacids (19). Similarly, in the total synthesis of estrone (39) and in related series (40, 41), this reaction has been employed with good yields.

Once the acetic acid side chain has been extended one carbon atom by the Arndt-Eistert synthesis, the open D ring becomes a 1,6-diacid and hence is capable of ring closure, according to Blanc's rule (42), to a cyclic ketone possessing five carbon atoms. Cyclizations have been accomplished by several methods. Bardhan (43) has effected ring closure by treatment of homomarrianolic acid-3 methyl ether (LII) with acetic anhydride and acetic acid. This same method was used in ring closure to dehydroepiandrosterone acetate (24, 25). More successful has been the use of lead carbonate, first applied by Litvan and Robinson (31) and later by Anner and Miescher (39) in their total synthesis of estrone. In the equilenin series, cyclization has been performed with sodium methylate in excellent yields (37).

The use of these various reactions in the preparation of tagged estrone is dependent upon the availability of the reagent, diazomethane (XXXII)*, labelled in its carbon atom. Concurrently, other workers in these laboratories have been engaged in the conversion of barium carbonate-C^{14} into simple radioactive molecules, capable of application in the tagging of various steroids (44, 46). An elegant method has been described for the preparation of sodium cyanide-C^{14} (XXVII)* (45). Carbonation of triphenylmethylsodium (XXIII) with carbon dioxide-C^{14} yields triphenylacetic acid-1-C^{14} (XXIV)*. Treatment of this tri-substituted acetic acid (XXIV)* with thionyl chloride

followed by ammonia affords triphenylacetamide-1-C^{14} (XXV)*, which is dehydrated to triphenylacetonitrile-1-C^{14} (XXVI)* by the action of phosphorus pentoxide. Hydrogenolysis of the nitrile (XXVI)* by treatment with sodium in alcohol yields sodium cyanide-C^{14} (XXVII)* and triphenylmethane (XXVIII).

$$(C_6H_5)_3\text{-CNa} \xrightarrow{C^*O_2} (C_6H_5)_3\text{-C-C}^*\text{OOH} \xrightarrow[NH_4OH]{SOCl_2} (C_6H_5)_3\text{-C-C}^*\text{ONH}_2$$

XXIII XXIV XXV

$$\xrightarrow{P_2O_5} (C_6H_5)_3\text{-C-C}^*\text{N} \xrightarrow{Na,EtOH} \text{NaC}^*\text{N} + (C_6H_5)_3\text{-CH}$$

XXVI XXVII XXVIII

The conversion of sodium cyanide-C^{14} (XXVII)* to diazomethane-C^{14} (XXXII)* has been reported (47). Reduction of sodium cyanide-C^{14} (XXVII)* with platinum catalyst in acetic acid affords methylamine-C^{14}, collected as the hydrochloride (XXIX)* following the addition of hydrochloric acid. Methylamine hydrochloride-C^{14} (XXIX)* is converted to diazomethane-C^{14} (XXXII)* in the usual manner, with one minor modification. Refluxing methylamine hydrochloride-C^{14} with urea yields N-methylurea-C^{14} (XXX)*, labelled in the methyl group. After the addition of sodium nitrite, ice and sulphuric acid are added, precipitating methyl labelled N-nitroso-N-methylurea (XXXI)*. In routine preparations, after the addition of sodium nitrite, the resulting solution is added to ice and sulphuric

acid, thus precipitating N-nitroso-N-methylurea (48).

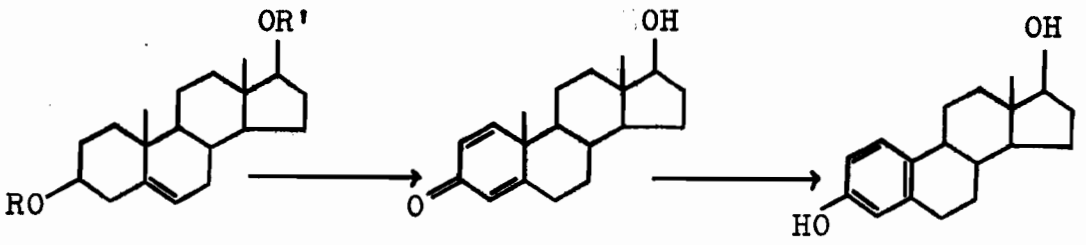
$$NaC^{*}N \longrightarrow C^{*}H_3\text{-}NH_2\cdot HCl \longrightarrow C^{*}H_3\text{-}NH\text{-}CO\text{-}NH_2$$

XXVII XXIX XXX

$$\longrightarrow C^{*}H_3\text{-}N(NO)\text{-}CO\text{-}NH_2 \longrightarrow C^{*}H_2N_2$$

XXXI XXXII

Other methods exist for the labelling of estrone and β-estradiol in the skeletal ring structure with C^{14}. Inhoffen and Zuhlsdorff (49), pioneer workers in the aromatization of ring A in the sterol skeleton, converted $\Delta^{1,4}$-androstadiene-17(β)-ol-3-one (XXXIV), prepared from cholesterol via Δ^{5}-androstene-3,17-diol-3,17-diacetate (XXXIII, R,R' = CH_3CO), into β-estradiol (XXXV). This was confirmed by Wilds and Djerassi (50) who prepared the intermediate $\Delta^{1,4}$-androstadiene-17(β)-ol-3-one from Δ^{5}-androstene-3,17-diol-3-acetate-17-benzoate (XXXIII, R = CH_3CO; R' = C_6H_5CO).



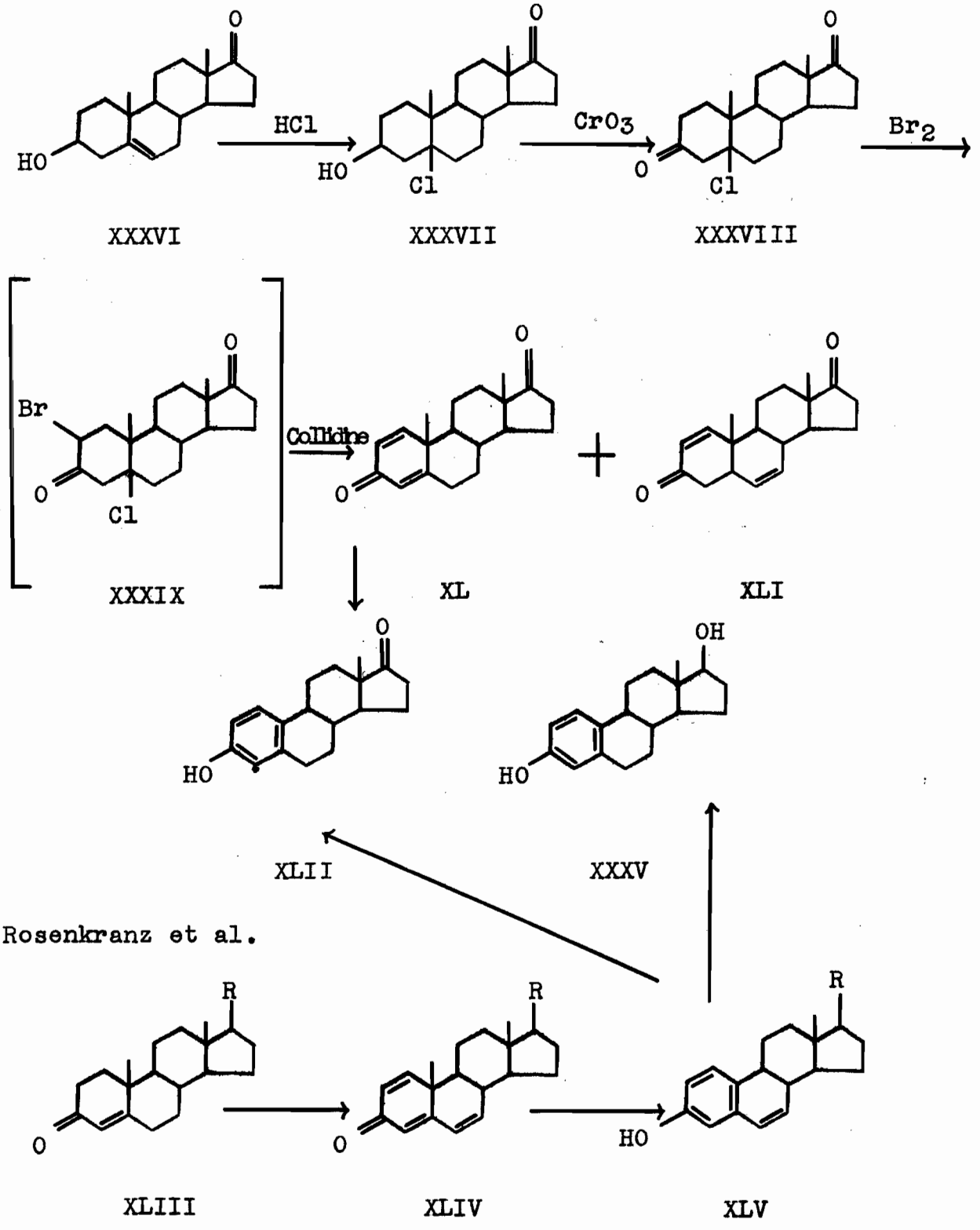
XXXIII XXXIV XXXV

More recently estrone has been synthesized from dehydroepiandrosterone (XXXVI, Chart II) (51) and Δ^4-androstene-3,17-dione (XLIII, R = O) (52) and β-estradiol from testosterone acetate (XLIII, R = CH_3COO) (52). Hershberg, Rubin and Schwenk (51) prepared 5-chloroandrostane-3-ol-17-one (XXXVII) by saturation of the double bond in dehydroepiandrosterone (XXXVI) with hydrogen chloride. Upon chromic acid oxidation, this 5-chloro derivative (XXXVII) was converted to 5-chloroandrostane-3,17-dione (XXXVIII), which on subsequent bromination afforded the probable structure 2-bromo-5-chloroandrostane-3,17-dione (XXXIX). Dehydrohalogenation of XXXIX with collidine yielded a mixture of $\Delta^{1,4}$- and $\Delta^{1,6}$-androstadiene-3,17-diones (XL and XLI). The former (XL) is aromatized to estrone (XLII).

Rosenkranz et al. (52) have prepared estrone and β-estradiol from the bromination of Δ^4-3 keto steroids. Dibromination of testosterone acetate (XLIII, R = CH_3COO) to the dibromo derivative followed by dehydrobromination with collidine afforded $\Delta^{1,4,6}$-androstatriene-17(β)-ol-3-one-17-acetate (XLIV, R = CH_3COO). Aromatization gave Δ^6-dehydro-estradiol-17-monoacetate (XLV, R = CH_3COO) which was hydrogenated to β-estradiol (XXXV). Starting with Δ^4-androstene-3,17-dione (XLIII, R = O) through the same reaction series, estrone (XLII) was obtained.

CHART NO. II

Hershberg, Rubin and Schwenk.



XXXVI XXXVII XXXVIII

XXXIX XL XLI

XLII XXXV

Rosenkranz et al.

XLIII XLIV XLV

Methods of producing ring A labelled sterols from sterols possessing a Δ^4-3 keto grouping in this ring have been described by Turner (53), Ziegler (54), and Fujimoto (55). Testosterone-3-C^{14} (53) and testosterone-4-C^{14} (53, 55) have been prepared. Ring A labelled β-estradiol and estrone could be obtained respectively from testosterone-3(or 4)-C^{14} (XLIII, R = OH)* and its oxidative derivative Δ^4-androstene-3,17-dione-3(or 4)-C^{14} (XLIII, R = O)*. Likewise, testosterone-3(or 4)-C^{14} could be converted to Δ^5-androstene-3,17-diol-3(or 4)-C^{14} (XXXIII, R,R' = H), the starting material of Inhoffen, and Wilds and Djerassi, by a reaction series similar to that reported for the conversion of Δ^4-cholestenone to cholesterol (56, 57, 58). Thus the preparation of ring A labelled estrogens is theoretically possible. However, the overall synthesis is of such length as to make micro scale work exceedingly difficult.

The use of radioactive diazomethane in the Arndt-Eistert synthesis offered a more promising method of labelling estrone with C^{14}. It was decided to explore this method in detail to achieve this end.

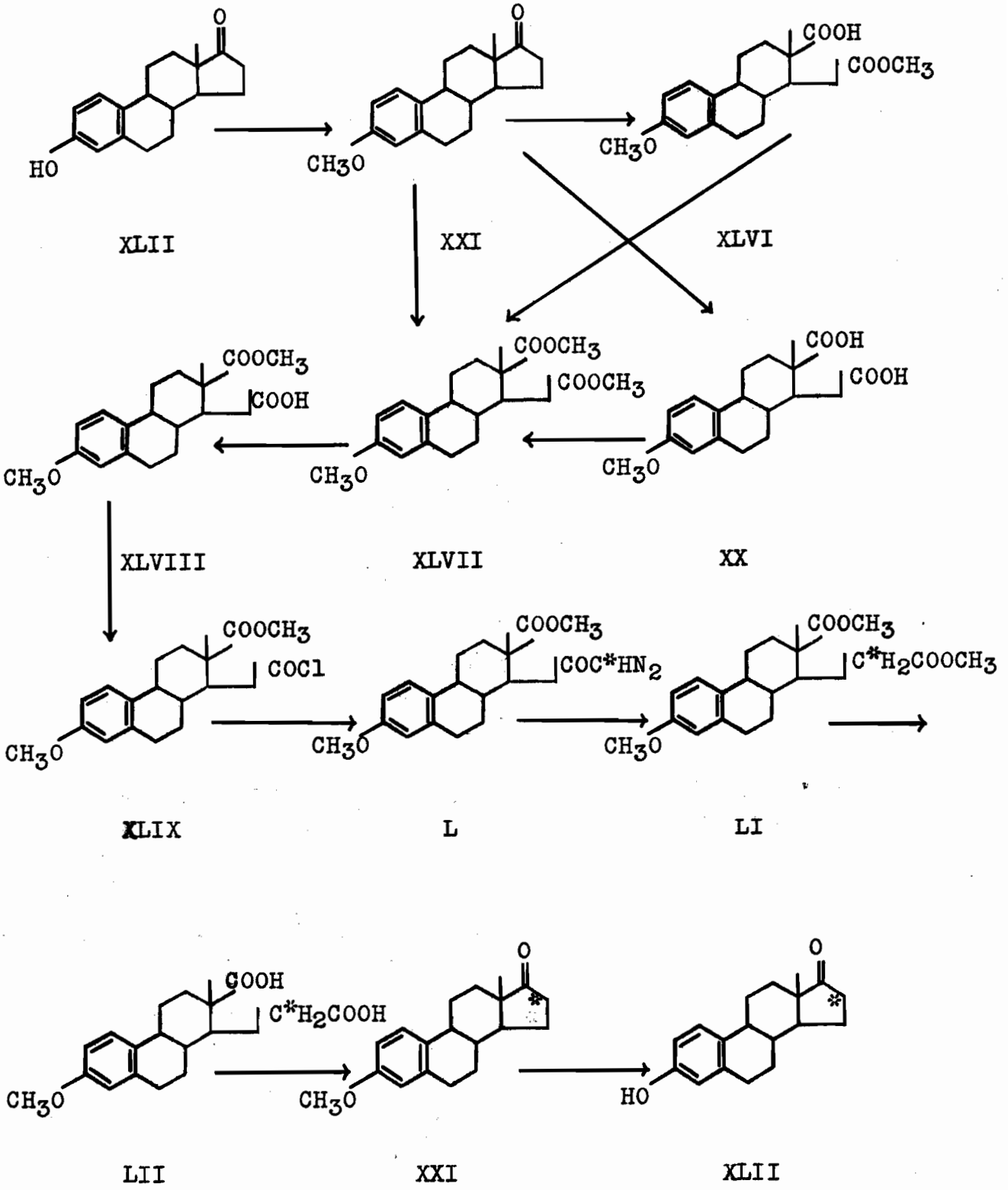
2. Discussion

The synthesis of estrone-16-C^{14} (XLII)* divided itself conveniently into three distinct sections. First, estrone was converted to marrianolic acid dimethyl ester-3 methyl ether (XLVII), the starting material required for the Arndt-Eistert reaction. Second, diazomethane-C^{14} (XXXII)* was prepared from barium carbonate-C^{14}. Finally, marrianolic acid dimethyl ester-3 methyl ether was lengthened to its homologous dimethyl ester (LI)* via the Arndt-Eistert reaction using diazomethane-C^{14}. This homolog (LI)* was then cyclized to estrone, labelled at position 16 with C^{14}.

In preparation for subsequent oxidation estrone was converted to estrone-3 methyl ether (XXI). Methylation of the phenolic hydroxyl group has been claimed to stabilize the aromatic nucleus against oxidation (30). Further, the purification of later intermediary compounds was facilitated by protection of this phenolic group.

Estrone was methylated with dimethyl sulphate in 30 per cent aqueous potassium hydroxide solution (59). This method has found general application (31, 43, 60). Although satisfactory for small quantities of estrone, larger amounts were handled in more dilute (25 per cent methanol) solutions of potassium hydroxide. Addition of dimethyl sulphate in proportions of ten

CHART NO. III


HO
O
CH3O
COOH
COOCH3
XLII
XXI
XLVI
COOCH3
COOH
XLVIII
XLVII
XX
COCl
COC*HN2
C*H2COOCH3
XLIX
L
LI
C*H2COOH
LII
XXI
XLII

millilitres per gram of estrone afforded consistent methylation in yields of 80-90 per cent, producing estrone-3 methyl ether (XXI), melting point 167-171°C. Following collection of the sparingly soluble methyl ether (XXI), a small quantity of unchanged estrone was recoverable from the alkaline medium.

Similarly, methylation with methyl iodide in sodium and ethanol yielded estrone-3 methyl ether (XXI) in equivalent yields. Niederl and Vogel (61) have reported the methylation of 2-nitroestrone by this method.

The oxidation of the benzyl ether of estrone (XVII) with hypoiodite in alkaline methanol described by Heer and Miescher (33) afforded crude marrianolic acid-3 benzyl ether in a yield of 93 per cent. The yield of purified product was not recorded. Investigations on the cleavage of estrone-3 methyl ether (XXI) with hypoiodite were carried out under essentially similar conditions as those used by the Swiss workers. In addition, the resulting alkaline oxidation mixtures were worked up in three separate ways.

The alkaline oxidation mixture was extracted directly with ether to yield a neutral fraction. The neutral fraction was partitioned with Girard's T reagent into ketonic and non-ketonic materials. The ketonic fraction resulted in isolation

of a small quantity of unchanged estrone. The non-ketonic fraction yielded crystals of marrianolic acid dimethyl ester-3 methyl ether (XLVII), melting point 74-75°C, in a yield of 29 per cent. The alkaline phase was then acidified and ether extracted. A 30 per cent yield of marrianolic acid primary monomethyl ester-3 methyl ether (XLVI), melting point 144-145°C, was obtained from the combined ether extracts. A small sample of these crystals was saponified in potassium hydroxide solution yielding marrianolic acid-3 methyl ether (XX), melting point 195-197°C. This diacid (XX) showed no depression of melting point on admixture with an authentic sample of marrianolic acid-3 methyl ether (XX). Esterification of the remaining primary monomethyl ester (XLVI) with diazomethane afforded crystals of the dimethyl ester (XLVII), melting at 74.5-75.5°C. Esterification of marrianolic acid-3 methyl ether (XX) was effected with 1 per cent sulphuric acid in methanol to yield the primary monomethyl ester (XLVI). Similarly, in the hypoiodite oxidation of dehydroepiandrosterone acetate (VII), the isolation of the primary monomethyl ester (24) and the dimethyl ester (24, 32) of 3(β)-hydroxy-$\Delta^{5,6}$-etiobilienic acid have been reported. Saponification without previous isolation gave 3(β)-hydroxy-$\Delta^{5,6}$-etiobilienic acid in 59 per cent yield.

Second, the alkaline oxidation mixture was acidified directly and ether extracted. The combined extracts were washed

with a dilute solution of thiosulphate to remove the free iodine. Esterification of the crude product was effected directly by the addition of an ethereal solution of diazomethane. This solution yielded crude marrianolic acid dimethyl ester-3 methyl ether (XLVII), melting at 60-70°C. Purification of this dimethyl ester (XLVII) by chromatography on alumina gave crystals of melting point 74°C. The yield was 48 per cent.

Finally, improved yields of the dimethyl ester (XLVII) were realized by proceeding in a manner similar to that reported by the Swiss investigators (33). The alkaline oxidation mixture was acidified directly and ether extracted. The free iodine present was removed by washings with a dilute solution of thiosulphate. Following removal of the solvent under reduced pressure, the resulting crude mixture was saponified directly. Marrianolic acid-3 methyl ether (XX), melting point 199-201°C, was obtained in 66 per cent yield. Esterification to the dimethyl ester (XLVII) with an ethereal solution of diazomethane proceeded in almost quantitative yield.

The preparation of diazomethane (XXXII)*, labelled in its carbon atom with C^{14}, from barium carbonate-C^{14} was a major problem of other workers in these laboratories. The successful completion of this task has been previously mentioned, and the reaction series outlined. (See pages 14 and 15.)

During the development of this synthesis, small amounts of unisolated, weakly radioactive N-nitroso-N-methylurea (XXXI)* became available. Since a reasonable estimate of these small quantities could not be determined, they were further diluted with inactive crystalline N-nitroso-N-methylurea, the resulting mixtures decomposed to diazomethane-C^{14} (XXXII)* and employed in Arndt-Eistert reactions. Subsequently, diazomethane-C^{14} was prepared from isolated crystalline radioactive N-nitroso-N-methylurea (XXXI)*.

Finally, diazomethane-C^{14} (XXXII)* was prepared as a confirmatory measure to the above synthesis, starting with 12.24 millicuries of radioactive barium carbonate. An overall yield of 69.6 per cent was obtained for the conversion of barium carbonate-C^{14} to sodium cyanide-C^{14} (XXVII)*, confirming the yield (68-72 per cent) claimed by Belleau and Heard (45), with substitution of the mercurimetric method (62) for the argentimetric method of determining cyanide. In addition, the apparatus used by Belleau (44) for the hydrogenolysis of triphenylacetonitrile-1-C^{14} (XXVI)* was modified, permitting any size hydrogenolysis to be carried out with normal laboratory apparatus. However, although the overall yield for the conversion of barium carbonate-C^{14} to sodium cyanide-C^{14} compared favourably as indicated, the hydrogenolysis of triphenylacetonitrile-1-C^{14} (XXVI)* proceeded in somewhat lesser yield (81.1 per cent) than the value (90 per cent) reported by the original workers (45).

In a subsequent hydrogenolysis of triphenylacetonitrile-1-C^{14}, prepared from 12.24 millicuries of barium carbonate-C^{14}, using this modified apparatus, Yates (17) obtained sodium cyanide-C^{14} in a yield of 78 per cent, with an overall yield of 67 per cent, based on barium carbonate-C^{14}. In both these syntheses, yields from the barium carbonate-C^{14} to the nitrile (XXVI)* were somewhat higher than claimed (45), and hence the overall yields of sodium cyanide-C^{14} (XXVII)* were unchanged.

Confirmation of the overall yield for the conversion of sodium cyanide-C^{14} (XXVII)* to diazomethane-C^{14} (XXXII)* was accomplished by an indirect route. The activities of certain intermediary compounds between barium carbonate-C^{14} and sodium cyanide-C^{14} (XXVII)* were determined on a millimole basis. Triphenylacetic acid-1-C^{14} (XXIV)* possessed an activity of 6.3 x 10^8 disintegrations per minute per millimole (d./min./mmole)[x]. Again the nitrile (XXVI)* was found to disintegrate at 5.85 x 10^8 d./min./mmole. The nitrile (XXVI)* caused serious contamination of the Geiger-Muller tube. Since this compound showed no physical changes during storage, the contamination presumably was due to sublimation of the nitrile (XXVI)* from the counting disc. The activity of sodium cyanide-C^{14} (XXVII)* cannot be determined directly (44). However, since dilution with inactive material was avoided, the activity of the radioactive cyanide

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[x] A description of counting techniques is presented in Part II.

must have been identical to that of the above mentioned intermediary compounds on a millimole basis.

Sodium cyanide-C^{14} (XXVII)* was converted to radioactive N-nitroso-N-methylurea-C^{14} (XXXI)* without isolation of the intermediary methylamine hydrochloride-C^{14} (XXIX)* or N-methylurea-C^{14} (XXX)* in sufficient purity for activity determinations. In addition, a 4:1 dilution at the N-methylurea-C^{14} stage with inactive reagent N-methylurea was carried out; the weight of the N-methylurea-C^{14} was estimated by assuming yields of 85 per cent for the reduction of sodium cyanide-C^{14} to methylamine hydrochloride-C^{14} (XXIX)* and 78 per cent for the conversion of the hydrochloride (XXIX)* to N-methylurea-C^{14} (XXX)*, as consistently obtained by Solomon (46) in inactive runs. Hence the activity of the N-nitroso-N-methylurea-C^{14} (XXXI)* was expected to be one-fifth of that found for the triphenylacetic acid-1-C^{14} (XXIV)* (6.3×10^8 d./min./mmole), which would have been approximately 1.3×10^8 d./min./mmole. Unfortunately, N-nitroso-N-methylurea-C^{14} was found to give inconsistent activity determinations, with values approximately one-tenth of the anticipated figure.

To determine an accurate activity for the N-nitroso-N-methylurea-C^{14}, diazomethane-C^{14} (XXXII)* was generated and used to prepare estrone-16-C^{14} (XLII)*. This radioactive

estrogen possessed an activity of 1.36 x 10^8 d./min./mmole. Further, a small portion of the recovered diazomethane-C^{14} was added to an excess of pure 3-keto-Δ^4-etiocholenic acid. The radioactive 3-keto-Δ^4-etiocholenic acid methyl ester obtained was purified by chromatography on activated alumina, and was found to possess an activity of 1.4 x 10^8 d./min./mmole.

Hence the yield for the conversion of sodium cyanide-C^{14} (XXVII)* to N-methylurea-C^{14} (XXX)* as mentioned above was confirmed. Further, N-methylurea-C^{14} was converted to N-nitroso-N-methylurea-C^{14} (XXXI)*, melting point 112-113°C, in an identical yield (85 per cent) as reported (46). Since N-nitroso-N-methylurea-C^{14} is convertible to diazomethane-C^{14} in approximately 80 per cent yield, as shown by titration of diazomethane produced from inactive N-nitroso-N-methylurea (to be discussed later), the conversion of sodium cyanide-C^{14} to diazomethane-C^{14} in an overall yield of 40 per cent was realized.

The synthesis of estrone (XLII) from the dimethyl ester of marrianolic acid-3 methyl ether (XLVII) is outlined in Chart III. Briefly, partial saponification of the dimethyl ester (XLVII) affords the tertiary monomethyl ester (XLVIII) which is converted to the corresponding acid chloride (XLIX). The diazoketone (L) is obtained by the addition of this acid chloride (XLIX) to excess diazomethane. Rearrangement to homo-

marrianolic acid dimethyl ester-3 methyl ether (LI) is followed by saponification to the free homodiacid (LII). The homodiacid (LII) is cyclized to estrone-3 methyl ether (XXI). Demethylation yields free estrone (XLII).

This synthesis has been reported by Anner and Miescher (39) in their total synthesis of estrone. Unfortunately, the various intermediates described were racemic mixtures, and were not separated into optically active isomers. The melting points of these intermediary compounds are strikingly different from those of the natural (+) series. Bachmann, Kushner and Stevenson (38) had previously described the synthesis of estrone A from racemic mixtures of marrianolic acid dimethyl ester-3 methyl ether (XLVII).

The Arndt-Eistert reaction was applied to optically active marrianolic acid tertiary monomethyl ester-3 methyl ether (XLVIII) by Litvan and Robinson (31) affording homomarrianolic acid dimethyl ester-3 methyl ether (LI) in about 1 per cent yield. The oily, tertiary monomethyl ester (XLVIII) was obtained by partial saponification of unisolated dimethyl ester (XLVII), derived by esterification of free marrianolic acid-3 methyl ether (XX) with diazomethane. Miescher et al. (63) obtained the oily tertiary monomethyl ester (XLVIII), $[\alpha]_D^{21} = +61^\circ \pm 4^\circ$, by this same procedure. The poor yield of the homo

dimethyl ester (LI) was attributed to regeneration of marrianolic acid-3 methyl ether (XX). Esterification of this free acid with diazomethane gave a mixture of contaminant esters, making crystallization extremely difficult. However, it was noticed that these workers used a maximum of two moles of diazomethane per mole of acid chloride. A minimum of three equivalents of diazomethane are recommended by Bachmann and Struve (19), since each molecule of acid chloride consumes two molecules of diazomethane. In agreement with Bachmann and Struve it was found that low amounts of diazomethane drastically reduced the yields in the Arndt-Eistert reaction.

Model experiments were carried out starting with pure marrianolic acid dimethyl ester-3 methyl ether (XLVII) of melting point 74-75°C. In general, the work described is a modification of that employed by Anner and Miescher in their total synthesis of estrone (39).

The partial hydrolysis of the dimethyl ester (XLVII) was investigated in an attempt to obtain the tertiary monomethyl ester (XLVIII) in crystalline state. Since primary esters of carboxylic acids are hydrolyzed more readily than tertiary esters, potassium carbonate was used to obtain preferential primary ester hydrolysis. The application of potassium carbonate for hydrolyses of this nature has been reported (24, 25, 40). Hydrolysis of marrianolic acid dimethyl ester-3 methyl

ether (XLVII) with potassium carbonate (3.5 equivalents) in 75 per cent methanol afforded a colourless oil in almost quantitative yield. This viscous oil could not be made to crystallize.

The isolation of marrianolic acid tertiary monomethyl ester in crystalline state has been reported by Miescher (33). An ethereal solution of marrianolic acid dimethyl ester was extracted with 2 N sodium hydroxide solution. After standing a short time the alkaline solution was acidified to yield the tertiary monomethyl ester. However, marrianolic acid dimethyl ester-3 methyl ether was not extractable from ether with 2 N sodium hydroxide solution. Presumably, the extraction of marrianolic acid dimethyl ester was due to the presence of the unprotected phenolic hydroxyl group. Upon standing, hydrolysis occurred in the cold alkaline solution. Various workers have successfully hydrolyzed primary esters in presence of tertiary esters by using equimolar or slightly greater than equimolar solutions of sodium hydroxide and potassium hydroxide (31, 37, 39, 64, 65). Marrianolic acid dimethyl ester-3 methyl ether (XLVII) was subjected to partial saponification by refluxing for two hours with 1.8 equivalents of methanolic potassium hydroxide. Following isolation of the acidic fraction, crystallization could not be induced. Further, refluxing the dimethyl ester (XLVII) for fifteen hours with 1.3 equivalents

of potassium hydroxide in 66 per cent methanol with subsequent chromatography using methyl silicate, failed to produce crystalline monomethyl ester (XLVIII). In the hands of Anner and Miescher (39) these saponification conditions afforded the crystalline racemic tertiary monomethyl ester (XLVIII). The oily, tertiary monomethyl ester (XLVIII) obtained from hydrolysis with potassium carbonate in 75 per cent methanol was used for the next step without further purification. In a later section crystalline tertiary monomethyl ester was isolated of melting point 99-104°C.

Two reagents, thionyl chloride and oxalyl chloride, find general application in the preparation of acid chlorides of steroidal carboxylic acids. Litvan and Robinson (31) formed the acid chloride of marrianolic acid tertiary monomethyl ester-3 methyl ether (XLVIII) by use of thionyl chloride. This reagent was used for acid chloride formations of other tertiary monomethyl esters in satisfactory yields (24, 25, 37, 38). Nevertheless, rearrangement has been demonstrated in the preparation of acid chlorides of mono-esters of simple organic diacids using thionyl chloride, increasing in amounts with the decreasing purity of the thionyl chloride and the higher temperature used (66, 67). On the other hand, oxalyl chloride caused no rearrangement (67). For this reason the acid chloride of marrianolic acid tertiary monomethyl ester-3 methyl ether (XLIX) was formed using oxalyl chloride.

The application of oxalyl chloride to the formation of acid chlorides of aromatic and aliphatic acids has been investigated in detail by Adams and Ulich (68), and excellently reviewed by Markley (69). Acid chlorides are formed by treatment of the free acid or its sodium salt with oxalyl chloride (68). Since this latter method is of more general applicability, the acid chloride of the tertiary monomethyl ester (XLVIII) was prepared by treatment of its sodium salt with oxalyl chloride. The sodium salt was formed by treatment of the free tertiary monomethyl ester (XLVIII) with a methanolic solution of 0.1 N sodium hydroxide using 95 per cent of the theoretical amount of alkali required. Slightly less than a theoretical amount of alkali was used to prevent possible charring of the sodium salt during the period of drying (six hours at 80°C in vacuo), following the evaporation of the solvent. However, it should be noted that Wilds and Shunk have used greater than theoretical amounts (70). A suspension of the dry sodium salt in benzene to which a few drops of pyridine were added, was frozen by immersion in an ice-salt bath and treated with a large excess (seven to eight mole equivalents) of oxalyl chloride, affording the acid chloride of the tertiary monomethyl ester (XLIX). The addition of trace quantities of pyridine was found to accelerate the decomposition of the double anhydride arising as an intermediate in the formation of acid chlorides, (68).

In the normal Arndt-Eistert reaction using steroidal acid chlorides, the excess of diazomethane employed is generally greater than the minimum three equivalents recommended (19). The desirability of maximum yield of diazoketone from a fixed quantity of acid chloride is stressed from the steroidal point of view with little emphasis on the excess diazomethane involved. The complete reverse holds true when diazomethane-C^{14} (XXXII)* is used. In this case a maximum yield of diazoketone from a fixed amount of diazomethane is required. The yield from the steroidal point of view is of secondary importance.

A closer inspection of the mechanism of the Arndt-Eistert reaction (19) immediately reveals the difficulty of achieving this goal. Addition of an acid chloride to diazomethane results in the formation of the corresponding diazoketone with the liberation of hydrogen chloride. The hydrogen chloride then reacts with additional diazomethane, forming methyl chloride. Hence two molecules of diazomethane are consumed per molecule of acid chloride. Further, if any of the hydrogen chloride is not destroyed by this reaction, then it may react with the previously formed diazoketone to give the ω-chloromethylketone.

$$RCOCL + CH_2N_2 \longrightarrow RCOCHN_2 + HCl$$

$$HCl + CH_2N_2 \longrightarrow CH_3Cl + N_2$$

$$RCOCHN_2 + HCl \longrightarrow RCOCH_2Cl + N_2$$

In the preparation of dehydroepiandrosterone acetate-16-C^{13} (VII)*, a sufficient quantity of diazomethane-C^{13} was employed to permit removal of an aliquot to determine the diazomethane-C^{13} concentration (24). From this value the molarity of diazomethane-C^{13} solution was calculated, and then three mole equivalents were used per mole of acid chloride in the subsequent Arndt-Eistert reaction (24). However, such a procedure was of an impractical nature when handling radioactive diazomethane. Both the minute amounts formed and the high value precluded any such treatment. Thus the conversion of inactive N-nitroso-N-methylurea (XXXI) to diazomethane was investigated to ascertain the exact yield of diazomethane obtainable in order to carry out Arndt-Eistert reactions with the desired mole equivalents of this reagent. N-nitroso-N-methylurea (XXXI) was prepared by the method of Arndt (48). Air drying for two to three hours at room temperature, followed by final drying under reduced pressure in the cold, afforded a crystalline product of melting point 123°C (with decomposition). N-nitroso-N-methylurea was converted to diazomethane by its addition to a mixture of ether and a solution of 80 per cent potassium hydroxide, cooled to -5° to -10°C by immersion in an ice-salt bath (48). A single addition was used since only small quantities of N-nitroso-N-methylurea were processed. After slow generation of the diazomethane for thirty minutes in the cold, the mixture was allowed to warm to room temperature; the volatile components were distilled by

heating the mixture to 45°C with warm water. The diazomethane content was determined by the use of benzoic acid as described by Fieser (71), and found to be 82 per cent of theoretical. Similarly, diazomethane prepared as above was dried and distilled, first from potassium hydroxide pellets, then from strips of sodium. The yield was 72 per cent. Occasionally, somewhat lower yields were obtained.

The Arndt-Eistert reaction with subsequent cyclization was explored to determine the best semi-micro scale method of obtaining estrone-3 methyl ether (XXI) with highest yield from the diazomethane used. The quantity of diazomethane was of necessity restricted in order to duplicate the succeeding radioactive runs.

Initially, Arndt-Eistert reactions (19) were carried out using five equivalents of dry diazomethane (calculated on a 70 per cent conversion from N-nitroso-N-methylurea). The diazoketone of marrianolic acid tertiary monomethyl ester-3 methyl ether (L) was isolated in crystalline state in a yield of 42 per cent. Its rearrangement (39, 40) to crystalline homomarrianolic acid dimethyl ester-3 methyl ether (36 per cent), was effected with silver oxide. Substantially improved percentage conversion (27-28 per cent) to the homo-dimethyl ester (LI) was obtained by direct rearrangement of the unisolated diazo-

ketone (L). Since the low yields of these various compounds obtained appeared to be due to the difficulties in their isolation in crystalline state, the reaction sequence from marrianolic acid dimethyl ester-3 methyl ether (XLVII) to the homodiacid (LII) was carried out without isolation of the intermediary compounds, using three equivalents of diazomethane (39). The crystalline homodiacid (LII) was obtained in 23 per cent yield. Cyclization (31, 39) of the crystalline homodiacid (LII) by pyrolysis of its lead salt afforded estrone-3 methyl ether (XXI) in 79 per cent yield. Previously, a solution of acetic anhydride in acetic acid (Blanc's reaction) had been employed (43). Additional estrone-3 methyl ether was obtained on cyclization of the residual oily mother liquor of the homodiacid (LII), giving a 41 per cent overall yield for the conversion of dimethyl ester (XLVII) to estrone-3 methyl ether (XXI). Similarly, the dimethyl ester (XLVII) was converted to estrone-3 methyl ether (XXI) directly using three equivalents of diazomethane in yields of 34-40 per cent.

Alternatively, estrone-3 methyl ether (XXI) was obtained in a much lower yield (15 per cent) directly from the homo-dimethyl ester (LI) by treatment with sodium methylate followed by decarboxylation of the unisolated intermediate 16-carbomethoxy estrone-3 methyl ether with concentrated hydrochloric and acetic acids, as described for equilenin (37, 72). Slight demethylation occurred in this latter step, but not to

the extent reported by Bachmann et al. (37).

The demethylation (39) of estrone-3 methyl ether (XXI) was effected by heating the methyl ether at 180-185°C with pyridine hydrochloride for four hours in an atmosphere of nitrogen. On cooling, a little dilute hydrochloric acid was added to dissolve the pyridine hydrochloride, and the precipitate filtered and recrystallized from methanol and water affording estrone (XLII) of melting point 258-262°C in a yield of 85 per cent. Acetylation (73) of a few milligrams of this estrone with acetic anhydride and pyridine in a ratio of 3:1, gave estrone acetate, of melting point 125-126°C. Repeating this same procedure and varying the time of heating, demethylations were found to be complete with two hours' heating. A further time reduction to one hour afforded a mixture of estrone (XLII) and its methyl ether (XXI).

The use of a solution of hydriodic acid in glacial acetic acid has been described for the demethylation of estrone-3 methyl ether (31). With estriol-3 methyl ether (XVIII), this technique afforded estriol (XVI) in amounts up to 64 per cent (60). In a somewhat similar manner a conversion of 70 per cent has been reported for the demethylation of $\Delta^{1,3,5:10}$-estratriene-3-ol-16-one-3 methyl ether with a mixture of hydrobromic and acetic acids (74).

The nitrogenous base, triethylamine, has been claimed to decrease the amount of diazomethane (XXXII) required in certain Arndt-Eistert reactions (75). This compound competes with diazomethane for the hydrogen chloride liberated when an acid chloride reacts with diazomethane (see page 33) and the formed triethylamine hydrochloride is precipitated from solution. Berenbom and Fones (75) converted benzoyl chloride to its corresponding diazoketone using approximately one equivalent of diazomethane and triethylamine. Yates (17) has shown the successful Arndt-Eistert conversion of 3-keto-Δ^4-etiocholenoyl chloride to 21-diazoprogesterone-21-C^{14} in the presence of triethylamine, using approximately 2.5 mole equivalents of diazomethane. The Arndt-Eistert extension of the acid chloride of the monomethyl ester of marrianolic acid-3 methyl ether (XLIX), affording estrone-3 methyl ether (19 per cent) was carried out using 1.5 mole equivalents of diazomethane and triethylamine. The triethylamine hydrochloride generated was filtered off and the work-up carried out as usual.

The preparation of radioactive estrone (XLII)* was carried out in a manner similar to that described above using radioactive diazomethane. Diazomethane-C^{14} (XXXII)*, calculated to contain approximately 400 microcuries of C^{14} per millimole, was generated from crystalline radioactive N-nitroso-N-methylurea, prepared from barium carbonate-C^{14} as previously described.

(See pages 24 to 26.) The reaction series was carried out starting from the dimethyl ester (XLVII) without isolation until the saponification of the radioactive homo-dimethyl ester (LI)* was completed. Crystals of estrone-3 methyl ether-16-C^{14} (XXI)*, melting point 167-171°C, exhibiting no depression of melting point on admixture with authentic estrone-3 methyl ether, were obtained in a yield of 32 per cent, based on the dimethyl ester (XLVII). Demethylation to estrone-16-C^{14} (XLII)*, melting point 257-262°C, possessing an activity of 1.36×10^8 d./min./mmole (Geiger counter) and 7.05×10^8 d./min./mmole (windowless flow gas counter) was effected in an 80 per cent yield. The compound was identified further by mixture melting point determination and reduction to β-estradiol-16-C^{14} (XXXV)* (described in Part I-B). The recovered excess diazomethane-C^{14} (XXXII)* was further processed in the presence of triethylamine yielding an additional 24 milligrams of estrone-3 methyl ether-16-C^{14}. The remaining excess diazomethane-C^{14} was then employed by another worker in these laboratories for other Arndt-Eistert reactions (17).

The overall yield from estrone (XLII) to estrone-16-C^{14} was approximately 15 per cent. This figure compares favourably with the 9.6 per cent reported for the preparation of dehydroepiandrosterone acetate-16-C^{13} (VII)* using a somewhat similar reaction series (24).

Initially, the diazomethane-C^{14} (XXXII)* was derived from unisolated weakly radioactive N-nitroso-N-methylurea (XXXI)* diluted with carrier N-nitroso-N-methylurea and employed in a molar ratio of steroid to diazomethane-C^{14} of approximately 1:3. The use of this diazomethane-C^{14} led to the isolation of unknown radioactive compounds; their identity was not pursued. Since normally diazomethane is prepared from isolated crystalline N-nitroso-N-methylurea, the preparation of N-nitroso-N-methylurea-C^{14} was altered by a dilution at the N-methylurea-C^{14} stage to increase the amount of radioactive N-nitroso-N-methylurea obtained, and hence permit its isolation. Crystalline radioactive N-nitroso-N-methylurea[x] (melting point 115-116°C) was prepared from two-fifths of a millicurie of barium carbonate-C^{14}, with an approximate 45 fold dilution of radioactivity in route. The resulting diazomethane-C^{14} afforded estrone-3 methyl ether-16-C^{14} (XXI)* of melting point 167-171°C. No depression of melting point was observed on admixture with authentic estrone-3 methyl ether. The yield was 18 per cent based on the diester (XLVII). Demethylation to estrone-16-C^{14}, melting point 257-262°C, activity 1.6 x 10^7 d./min./mmole (windowless flow gas counter) was effected in 74 per cent yield. The compound was further characterized by mixture melting point determination,

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[x] This N-nitroso-N-methylurea-C^{14} was prepared by Mr. S. Solomon.

bioassay[x], and polarographic constant[x].

A subsequent preparation from radioactive diazomethane[*] containing approximately 350 microcuries of C^{14} per millimole afforded estrone-16-C^{14}, melting point 259-262°C, of activity 5.26×10^8 d./min./mmole (windowless flow gas counter) in a yield of 18 per cent based on the diester (XLVII). Further, dilution of the crystalline residue afforded additional estrone-16-C^{14} of activity 3.7×10^7 d./min./mmole. An overall yield of 20.6 per cent was obtained based on C^{14} activity. The recovered diazomethane-C^{14} was diluted and reprocessed.

Table I (page 42) shows a comparison between the various compounds isolated in the course of this synthesis and those reported in the literature, including the corresponding racemates obtained by Anner and Miescher (39, 76) in their total synthesis of estrone.

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[x] Thanks are due to Dr. N. T. Werthessen for carrying out these determinations.

[*] This diazomethane-C^{14} was generated from N-nitroso-N-methyl-urea-C^{14} prepared by Dr. B. Belleau and Mr. S. Solomon.

TABLE I

COMPOUND	NATURAL OPTICALLY ACTIVE (+)		RACEMATES	
	Found	Literature	Natural C/D trans	C/D cis
Marrianolic acid-3 methyl ether (XX)	199-201°C $[\alpha]_D^{24}=+66°\pm4°$	200-201°C (29) 189-190°C (31) 192°C (30)	222-224°C	249-251°C
Marrianolic acid dimethyl ester-3 methyl ether (XLVII)	74.5-75.5°C $[\alpha]_D^{24}=+68°\pm4°$	oil (31, 63, 77)	95-96°C	91-93°C
Marrianolic acid primary methyl ester-3 methyl ether (XLVI)	147-149°C $[\alpha]_D^{28}=+84°\pm3°$	--	--	--
Marrianolic acid tertiary methyl ester-3 methyl ether (XLVIII)	100-105°C $[\alpha]_D^{27}=+71°\pm5°$	oil (31, 63) $[\alpha]_D^{21}=+61°\pm4°$ (63)	172-173°C	147-148°C
16-Diazo marrianolic acid tertiary methyl ester-3 methyl ether (L)	99-104°C	oil, (31)	133-134°C	oil
Homomarrianolic acid-3 methyl ether (LII)	252-254°C $[\alpha]_D^{24}=+78°\pm3°$	253°C (31) 251-252°C (43)	225-227°C	212-214°C
Homomarrianolic acid dimethyl ester-3 methyl ether (LI)	83-85°C	85°C (31)	95-96°C	--
Estrone-3 methyl ether (XXI)	169-171°C $[\alpha]_D^{24}=+171°\pm4°$	168.5-169.5°C (59) 171-172°C (77)	143-144°C	162-164°C
Estrone (XLII)	259-262°C $[\alpha]_D^{25}=+161°\pm5°$	262°C $[\alpha]_D^{24}=+163°\pm1°$ (78)	251-254°C	126-128°C

3. Experimental Work

All melting points were taken on a Fisher-Johns melting point apparatus, standardized using reference samples supplied by Arthur H. Thomas and Company for the Kofler hot stage. Values were corrected accordingly. The samples were viewed through a microscope.

The abbreviations listed below apply throughout all experimental sections of this thesis:

C	-	Centigrade
min.	-	minute
m.p.	-	melting point
%	-	per cent
g.	-	gram
mg.	-	milligram
ml.	-	millilitre
mm.	-	millimetre
mmole	-	millimole.

Unless otherwise stated, radioactive determinations were recorded on a Nuclear Instrument and Chemical Corporation 64-scaler unit working in conjunction with a Tracerlab windowless flow Q-gas counter or a Tracerlab TGC-2 Geiger Tube as designated.

(a) Inactive

(1) Preparation of estrone-3 methyl ether (XXI)

a. Dimethyl sulphate

Ten grams of estrone (XLII), m.p. 256-258°C, was dissolved in a solution of methanol (170 ml.), 30% potassium hydroxide solution (250 ml.) and water (300 ml.). To this vigorously stirred solution dimethyl sulphate (100 ml.) was added dropwise over 90 min. at room temperature. A white solid was precipitated during the course of this addition. The suspension was refluxed for 30 min., cooled to 5°C, and the precipitate collected; the precipitate was washed with 5% aqueous potassium hydroxide solution and water. Recrystallization was effected from methanol yielding 9.1 g. (86%) of estrone-3 methyl ether (XXI), m.p. 167-171°C, $[\alpha]_D^{24} = +171^\circ \pm 4^\circ$ (chloroform). The alkaline filtrate was acidified with concentrated hydrochloric acid to pH 2 (Congo indicator paper), extracted with ether and the ether extracts washed with water until washings were neutral. Following evaporation of the solvent, the residue was crystallized from methanol and water, affording 611 mg. of XLII, m.p. 255-260°C.

b. Methyl iodide

To sodium (0.15 g.; 0.0065 g.-atoms) dissolved in

ethanol (20 ml.) were added estrone (1 g.; 3.70 mmoles) and methyl iodide (10 ml.), and the solution refluxed 6 hours. The volatile components were removed under reduced pressure. A solution of 1.5 N sodium hydroxide (30 ml.) was added; the insoluble material was collected and washed with water. The precipitate was dissolved in ethyl acetate, treated with decolourizing charcoal for 15 min. with refluxing, filtered, the solvent evaporated, and the residue crystallized from methanol to yield 894 mg., (85.3%) of the methyl ether (XXI), m.p. 168-171°C. Acidification of the alkaline fraction gave a white precipitate. Following collection, the precipitate was crystallized from methanol and water. Unchanged XLII (42 mg.; 4%), m.p. 252-256°C, was recovered.

(2) Hypoiodite oxidation of estrone-3 methyl ether (XXI)

XXI (2.088 g.; 7.35 mmoles) was dissolved in methanol (500 ml.) with heating. Upon cooling, slight crystallization occurred. To this solution with continuous stirring were added dropwise, at approximately the same rate, iodine (5.31 g.; 20.9 mmoles) dissolved in methanol (53 ml.), and potassium hydroxide (7.54 g.) dissolved in methanol (35 ml.) and water (15 ml.), over 2 to 3 hours. At the beginning the iodine solution was kept in excess. After standing 15 hours at 5°C the bulk of the methanol was removed under reduced pressure. The alkaline solu-

tion was diluted with water and 1 N sodium hydroxide (50 ml.), extracted with ether and the combined ethereal extracts washed with 1 N sodium hydroxide and 5% sodium thiosulphate solutions (the latter to remove any iodine present), and lastly with water. The residue obtained on evaporation of the solvent was refluxed 20 min. with Girard's T reagent (2 g.) in glacial acetic acid (10 ml.) and methanol (3 ml.). The solution was diluted with ice and water and the pH adjusted to 6-7 by addition of 2 N sodium hydroxide. The solution was extracted with ether, the ethereal extracts washed with 2.5% sodium carbonate and water and the solvent removed under reduced pressure. This non-ketonic fraction, on crystallization from methanol, afforded 748 mg. (28.5%) of marrianolic acid dimethyl ester-3 methyl ether (XLVII), m.p. 74-75°C. The alkaline phase was acidified with concentrated hydrochloric acid and extracted with ether. The combined ethereal extracts were washed with 5% sodium thiosulphate and water, then extracted with 10% sodium carbonate (5 times). In the first extraction crystals of the sodium salt of marrianolic acid primary monomethyl ester-3 methyl ether were precipitated from the solution, dissolving on dilution with water. The carbonate extracts were acidified, ether extracted and the ethereal extracts washed with water. The solution was filtered through an alumina pad, the solvent evaporated and the resulting residue crystallized from ethyl acetate-pentane (1:4), yielding 740 mg. (30%) of XLVI, m.p. 144-145°C.

(3) Saponification of marrianolic acid primary monomethyl ester-3 methyl ether (XLVI)

A small quantity of XLVI (150 mg.) was refluxed in 10 ml. of a solution of potassium hydroxide (10 g.) in methanol (15 ml.) and water (30 ml.). The solution was diluted with water, acidified with concentrated hydrochloric acid and ether extracted. The ether extracts were washed with water until the water washings were neutral, then evaporated under reduced pressure. The resulting oil was crystallized from aqueous methanol, giving crystals of marrianolic acid-3 methyl ether (XX), m.p. 195-197°C. A mixed melt with authentic marrianolic acid-3 methyl ether exhibited no depression of melt.

(4) Methylation of marrianolic acid primary monomethyl ester-3 methyl ether (XLVI)

The remaining XLVI (590 mg.) was esterified with an ethereal solution of diazomethane to yield 570 mg. (93%) of XLVII, m.p. 74.5-75.5°C, crystallized from methanol.

(5) Partial methylation of marrianolic acid-3 methyl ether (XX)

XX (25 mg.; 0.075 mmoles) was dissolved in 2 ml. of 1% methanolic sulphuric acid, and allowed to stand at room tem-

perature for 20 hours. The solution was diluted with water, extracted with ether, and the combined ethereal extracts washed with 10% sodium bicarbonate, then extracted with 10% sodium carbonate solution (4 times). (In the first sodium carbonate extraction, the sodium salt of the primary monomethyl ester (XLVI) precipitated, dissolving on aqueous dilution.) The alkaline extracts were acidified with concentrated hydrochloric acid and ether extracted, affording 18 mg. (76%) of the monomethyl ester (XLVI), m.p. 147-149°C, from ethyl acetate-hexane.

(6) Hypoiodite oxidation of estrone-3 methyl ether (XXI) and esterification

XXI (2.716 g.; 9.56 mmoles) dissolved in methanol (800 ml.), was oxidized under identical conditions as previously described, using iodine (6.88 g.; 27.0 mmoles) in methanol (86 ml.) and potassium hydroxide (12.4 g.) in methanol (55 ml.) and water (24 ml.). After removal of most of the methanol under reduced pressure the solution was diluted with water, acidified with concentrated hydrochloric acid and extracted with ether. The ethereal extracts were washed with 10% aqueous sodium thiosulphate and water. An ethereal solution of diazomethane (from 20 g. of N-nitroso-N-methylurea) was added to the washed extracts and the mixture allowed to stand for 2 hours at room temperature. The excess diazomethane was destroyed with 10% aqueous acetic acid, the ethereal solution washed first with 1 N sodium hydroxide

solution, then with water until the washings were neutral, treated with decolourizing charcoal and filtered. Evaporation of the solvent afforded a viscous yellow oil, yielding impure XLVII, m.p. 60-70°C. This material was chromatographed on 50 g. of activated alumina. From this column was obtained a light-coloured oil (hexane-benzene 4:1), which was crystallized from methanol, yielding 1.654 g. (48%) of XLVII, m.p. 73-74°C.

(7) Hypoiodite oxidation of estrone-3 methyl ether (XXI) and saponification

XXI (3.934 g.; 13.8 mmoles) dissolved in methanol (1,200 ml.) was oxidized as above using iodine (10 g.; 39.3 mmoles) in methanol (125 ml.) and potassium hydroxide (18 g.) in methanol (80 ml.) and water (35 ml.). Following acidification, the ethereal extracts obtained were washed with 10% aqueous sodium thiosulphate and water, then evaporated to dryness, affording a red-brown oil. This material was saponified directly by refluxing for 3 hours with potassium hydroxide (10 g.) dissolved in methanol (15 ml.) and water (30 ml.). The saponification mixture was diluted with water, acidified with concentrated hydrochloric acid, extracted with ether, the ethereal extracts washed with water until the washings were neutral, treated with activated charcoal, filtered, and finally evaporated under reduced pressure. There remained a colourless oil which gave, on crystallization from

ethyl acetate, 3.048 g. (66.3%) of marrianolic acid-3 methyl ether (XX), m.p. 199-201°C $[\alpha]_D^{24} = +66° \pm 4°$ (c = 0.99% in chloroform).

(8) Esterification of marrianolic acid-3 methyl ether (XX)

XX (3.0 g.; 9.04 mmoles) was esterified in an ethereal solution of diazomethane (from 10 g. of N-nitroso-N-methylurea) affording 3.07 g. (95%) of XLVII, m.p. 74-75°C. Recrystallization of a small sample gave XLVII, m.p. 75-75.5°C, $[\alpha]_D^{24} = +68° \pm 4°$ (c = 0.94% in chloroform).

(9) Partial saponification of marrianolic acid dimethyl ester-3 methyl ether (XLVII)

XLVII (900 mg.; 2.5 mmoles) was refluxed for 5 hours in 31.5 ml. of 4% potassium carbonate (3.5 mole equivalents), dissolved in 75% methanol and 13.5 ml. of 75% methanol. The alkaline solution was diluted to 10 volumes with water, washed with three 50-ml. portions of ether, acidified with concentrated hydrochloric acid and extracted with ether. The combined extracts were washed with water until the washings were neutral, then evaporated under reduced pressure, yielding 808 mg. (93.4%) of XLVIII as a clear, colourless oil. This oily, tertiary monomethyl ester (XLVIII) could not be made to crystallize.

(10) Formation of the acid chloride of marrianolic acid tertiary monomethyl ester-3 methyl ether (XLIX)

XLVIII (808 mg.; 2.34 mmoles) was shaken in 20.2 ml. of 0.11 N methanolic sodium hydroxide (0.95 mole equivalent) until the clear oil was dissolved, and allowed to stand overnight. The methanol was evaporated under reduced pressure leaving a white, solid material. A sample of this substance did not melt under 300°C, but exhibited slight charring above 250°C. The sodium salt was dried *in vacuo* for 6 hours at 80°C (water bath) then slurried in anhydrous benzene (15 ml.) and pyridine (0.2 ml.), and the mixture frozen by immersion in an ice-salt bath. The addition of distilled oxalyl chloride (4 ml.) to the frozen mixture turned it a deep yellow colour; this was accompanied by the evolution of gas. Following retention in the cold for 30 min., liquefaction occurred and sodium chloride settled out. After a further period of 30 min. at room temperature, the volatile components were evaporated under reduced pressure at 25°C. To ensure complete evaporation of the chlorinating agent the residue was slurried in 5 ml. of dry benzene and again evaporated under reduced pressure. This treatment was repeated twice. The residue was further dried *in vacuo* at 0.1 mm. for 15 min. The acid chloride was dissolved in ether (75 ml.) with vigorous shaking, decanted from the sodium chloride into a dropping funnel and used directly for an Arndt-Eistert synthesis as described in "11".

(11) Formation of the diazoketone of marrianolic acid tertiary monomethyl ester-3 methyl ether (L)

N-Nitroso-N-methylurea (1.717 g.; 16.7 mmoles) was added to a flask containing a mixture of ether (25 ml.) and 80% aqueous potassium hydroxide (3.3 ml.), cooled to -5°C by immersion in an ice-salt bath and the mixture immediately set up for distillation. The outlet tube of the distillation apparatus was immersed in dry ether (15 ml.), cooled in an ice-salt bath. The flask and contents were kept cold for 30 min. during which time the ethereal layer gradually deepened in yellow colour. After standing a further 30 min. at room temperature, ether and diazomethane were distilled until the residual ether in the reaction flask became colourless. The ethereal solution of diazomethane was dried approximately 15 hours over potassium hydroxide pellets at 2°C, redistilled, further dried over sodium for 3 hours, then decanted.

The acid chloride (XLIX) (2.34 mmoles) as described in "10" was added dropwise over a period of 2-3 hours to this solution of diazomethane (11.65 mmoles), kept at -5°C and stirred magnetically. The mixture was stored overnight in the cold, allowed to stand for 2 hours at room temperature, then the volatile components removed by evaporation under reduced pressure at 20°C. The resulting yellow oil was taken up in

40 ml. of methanol, distilled from magnesium turnings. The oil obtained from 10 ml. of this solution evaporated to dryness under reduced pressure at room temperature, was crystallized from methanol, yielding 83.5 mg. (42%) of the diazoketone of marrianolic acid tertiary monomethyl ester-3 methyl ether (L), m.p. 99-104°C (with decomposition).

(12) Rearrangement of the diazoketone (L)

L (50 mg.) was dissolved in methanol (10 ml.). To this solution, kept at 50°C, freshly prepared dry silver oxide (75 mg.) was added in small portions over a 30-min. period. The mixture was refluxed for 1 hour, a further quantity of silver oxide (25 mg.) added and refluxing continued for another hour. After standing overnight, the mixture was filtered, the silver oxide well washed with methanol, and the resulting filtrate taken to dryness. The yellow oil was taken up in ether, the ethereal solution washed with 10% aqueous sodium carbonate and water, filtered, and evaporated to dryness. Crystallization was induced with methanol, affording 18 mg. (36%) of homomarrianolic acid dimethyl ester-3 methyl ether (LI), m.p. 83-85°C.

Similarly, the unisolated diazoketone (L) was treated with silver oxide (1 g.) affording 181 mg. (27.6%) of LI, m.p. 84-85°C. The residual mother liquor (370 mg.) was refluxed for 3

hours with potassium hydroxide (784 mg.) in methanol (10 ml.) and water (20 ml.). Following acidification and dilution to 10 volumes with water, the mixture was extracted with ether, the ethereal extracts washed with water and evaporated to dryness, and the resulting oil crystallized from ethyl acetate, yielding 28.6 mg. of homomarrianolic acid-3 methyl ether (LII), m.p. 252-254°C with softening at 245°C.

(13) Transformation of marrianolic acid dimethyl ester-3 methyl ether (XLVII) to homomarrianolic acid-3 methyl ether (LII) without isolation of intermediates

XLVII (500 mg.; 1.39 mmoles) was subjected to partial saponification with 17.5 ml. of 4% potassium carbonate in 75% methanol and 7.5 ml. of 75% methanol, yielding an acidic fraction (XLVIII) (445 mg.; 92.7%). The sodium salt was formed using 11.75 ml. of 0.104 N methanolic sodium hydroxide. The acid chloride (XLIX), prepared with 3 ml. of oxalyl chloride, was added to an ethereal solution of diazomethane (4.18 mmoles; from 603 mg. of N-nitroso-N-methylurea), and the diazoketone (L) rearranged with silver oxide (750 mg.) without isolation. Saponification of the rearranged product was effected with potassium hydroxide (1.01 g.) in methanol (10 ml.) and water (11 ml.), yielding 119.5 mg. (23%) of homomarrianolic acid-3 methyl ether (LII), m.p. 251-253°C. The residual oily mother liquor weighed 276 mg.

Similarly, XLVII (500 mg.; 1.39 mmoles) afforded 59.3 mg. (12.3%) of the homodiacid (LII) and a residual mother liquor (350 mg.) using diazomethane (2.1 mmoles) and reagent grade triethylamine (0.3 ml.). The triethylamine hydrochloride was removed by vacuum filtration, and the filtrate worked up as usual.

(14) Cyclization of homomarrianolic acid-3 methyl ether (LII)

LII (119.5 mg.) was intimately mixed with lead carbonate (120 mg.) and the mixture transferred to a pyrolysis tube. The mixture was heated in an air bath to 325°C and the estrone-3 methyl ether (XXI) thus formed distilled under high vacuum. Crystallization of the light yellow coloured oil from methanol yielded 77 mg. (78.6%) of XXI, m.p. 168-171°C; mixed m.p. with an authentic sample of estrone-3 methyl ether (m.p. 169-171°C) was found to be 168-171°C. The oily mother liquor (276 mg.) was dissolved in hot ethyl acetate (5 ml.), slurried with lead carbonate (276 mg.), and the solvent evaporated under a stream of nitrogen, with heating. Pyrolysis afforded 82 mg. of XXI, m.p. 166-170°C. Hence the overall conversion of the dimethyl ester (XLVII) to estrone-3 methyl ether (XXI) was 42.8%.

Similar treatment of the crystalline homodiacid (LII) and its residual mother liquor from the run using triethylamine

yielded 75 mg. (19%) of estrone-3 methyl ether (XXI).

(15) Demethylation of estrone-3 methyl ether (XXI)

XXI (102 mg.) was heated with pyridine hydrochloride (2 g.) in an atmosphere of nitrogen at 180-185°C in an oven for 4 hours. On cooling, 25 ml. of 5% hydrochloric acid was added; the precipitate was collected and washed with water. The precipitate was dissolved in hot methanol, treated with decolourizing charcoal, filtered, and crystallized from methanol and water, yielding 82 mg. (84.6%) of estrone (XLII), m.p. 257-260°C $[\alpha]_D^{26} = +161^\circ \pm 5^\circ$ (c = 1.01% in dioxane).

XLII (40 mg.) was heated at 95°C with pyridine (6 ml.) and acetic anhydride (2 ml.) for 5 hours. After standing overnight, the solution was poured into a mixture of ice and water, the precipitate was taken up in ether, the ethereal extracts washed with water and then evaporated under reduced pressure. Crystallization of the residue from aqueous acetone yielded crystals of estrone acetate (33 mg.; 72%), m.p. 126-128°C.

(16) Cyclization of homomarrianolic acid dimethyl ester-3 methyl ether (LI)

Sodium methylate (125 mg.; 2.3 mmoles) was added to

LI (238 mg.; 0.64 mmoles) dissolved in benzene (10 ml.), and the mixture refluxed for 2 hours in an atmosphere of nitrogen. Following the addition of glacial acetic acid (2 ml.) and 10% hydrochloric acid (5 ml.), the mixture was taken up in ether, the aqueous layer drawn off, and the organic layer washed with a solution of 10% sodium carbonate and water, affording a neutral fraction (198.7 mg.) giving a positive test to ferric chloride. This neutral fraction was refluxed with glacial acetic acid (12 ml.), concentrated hydrochloric acid (6 ml.) and water (1.5 ml.) for 10 hours in an atmosphere of nitrogen. The mixture was diluted to 10 volumes with water and ether extracted. The combined ethereal extracts were extracted with 10% aqueous sodium carbonate and 1 N sodium hydroxide solution, yielding acidic (122.7 mg.), phenolic (29.5 mg.), and neutral (32 mg.; 15%) fractions. The neutral fraction afforded 16.5 mg. of estrone-3 methyl ether (XXI), m.p. 168-172°C, from methanol. The phenolic fraction yielded a trace amount of estrone (XLII), m.p. 252-255°C from ethanol, while the acidic fraction gave free homodiacid (LII), m.p. 250-252°C, from acetone.

(b) Radioactive

(1) Preparation of triphenylacetic acid-1-C^{14} (XXIV)*

Sodium (2.3 g.; 0.1 g.-atoms) was introduced into a beaker, and covered with dry mineral oil (30 ml.). The mixture was heated gently until the sodium formed liquid balls. Mercury (230 g.) was added rapidly from a separatory funnel, inserted through a piece of cardboard placed upon the beaker. The mixture was carefully probed and the dirty mineral oil decanted. The amalgam was washed with additional mineral oil, dry hexane and absolute ether, and immediately added to triphenylchloromethane (12.6 g.; 45.2 mmoles), m.p. 110-113°C, dissolved in anhydrous ether (300 ml.) in an atmosphere of nitrogen. The mixture, cooled by the application of wet towels, was shaken for 6 hours. During the first 15 min. the ethereal layer developed a deep red-brown colour. The mixture was allowed to settle overnight. A 10 ml. sample of the ethereal layer was withdrawn, added to water and the resulting sodium hydroxide liberated by decomposition of the triphenylmethylsodium (XXIII) titrated with 0.100 N standard hydrochloric acid. The triphenylmethylsodium solution was found to be exactly 0.1 N. Hence the yield of XXIII from triphenylchloromethane was 66%.

The apparatus for the carbonation reaction was assembled exactly as described by Belleau (44). Barium carbonate-C^{14}

(600 mg.; 3.04 mmoles) containing 6.12 millicuries of radioactive carbon was introduced into the generator flask. The system was swept with nitrogen, previously scrubbed in a column of Drierite and Caroxite, then evacuated using a water pump. After isolation of the system the reaction vessel was cooled by immersion in liquid air. Carbon dioxide-C^{14} was released by the slow dropwise addition of 30% aqueous perchloric acid; the liberated gas was frozen to the walls of the reaction vessel. The reaction vessel was isolated from the system and removed from the liquid air. Immediately, 50 ml. of the 0.1 N solution of triphenylmethylsodium (XXIII) (1.6 molar proportions) was introduced into the reaction vessel and the mixture shaken vigorously for 2 min. A precipitate settled out of the deep red solution. The reaction vessel was then removed from the system and on addition of 10% aqueous ammonium hydroxide (50 ml.) the mixture became yellow.

The above procedure was repeated with another 600 mg. of barium carbonate-C^{14}. The two batches were combined, transferred to a separatory funnel, the alkaline layer drawn off and the yellowish ethereal layer extracted three times with 10% ammonium hydroxide solution. The combined aqueous extracts were washed once with ether, cooled by immersion in an ice-salt bath and acidified with concentrated hydrochloric acid. The precipitate was collected on a filter, washed with water

and dried in a vacuum oven at 70°C, affording 1571 mg. (90%, based on the barium carbonate-C^{14}) of triphenylacetic acid-1-C^{14} (XXIV)*, m.p. 266-269°C, of specific activity 6.3 x 10^8 d./min./mmole (Geiger-Muller tube).

(2) Formation of triphenylacetamide-1-C^{14} (XXV)*

Triphenylacetic acid-1-C^{14} (XXIV)* (1570 mg.; 5.45 mmoles) was refluxed in purified thionyl chloride (10 ml.) for 2.5 hours. The volatile components were removed under reduced pressure affording a yellowish crystalline acid chloride. Without further purification the acid chloride was pulverized under concentrated ammonia (20 ml.) and the mixture allowed to stand for 2 hours. The triphenylacetamide-1-C^{14} (XXV)* was collected on a filter, washed with water and dried. The yield of XXV*, m.p. 249.5-250°C, was 1506 mg. (96.2%).

(3) Dehydration of triphenylacetamide-1-C^{14} (XXV)*

To 1504 mg. (5.24 mmoles) of XXV* dissolved in 30 ml. of dry toluene was added phosphorus pentoxide (10 g.) and the mixture heated under reflux for 50 hours. The mixture was cooled and discharged by the cautious addition of ice and cold water. After further addition of water (total volume, 200 ml.), the aqueous phase was separated and extracted 6 times with benzene.

The combined organic extracts were washed with water, dried over potassium hydroxide pellets for 20 min., filtered, and finally evaporated to dryness in vacuo, yielding 1404 mg. (99%) of crude triphenylacetonitrile-1-C^{14} (XXVI)*, of observed activity 5.85 x 10^8 d./min./mmole (Geiger-Muller tube).

(4) Hydrogenolysis of triphenylacetonitrile-1-C^{14} (XXVI)*

Crude XXVI* (1400 mg.; 5.2 mmoles) was introduced into a three-necked round-bottomed flask and dissolved in absolute ethanol (25 ml.) with heating. The system was swept with dry carbon dioxide-free nitrogen (the inlet tube was immersed below the surface of the liquid), and the effluent gas from the reflux condenser bubbled through a 1 N solution of sodium hydroxide (5 ml.). Adjusting a slow, steady input of nitrogen, the solution was refluxed and sodium (2 g.; 0.087 g.-atoms) added in small pieces over a period of 1 hour. Refluxing was continued a further 30 min. and the solution permitted to cool to room temperature. The aqueous alkali from the trap was added to the reaction mixture, the three-necked flask was fitted with a dropping funnel and the condenser was set up for distillation so that its outlet protruded below the surface of a 1.035 N solution of sodium hydroxide (6.2 ml.; 1.2 molar equivalents calculated from the nitrile (XXVI)*), diluted with 10 ml. of distilled water. A rapid stream of nitrogen was bubb-

led into the alcoholic reaction mixture and 50% aqueous sulphuric acid (50 ml.) added dropwise from the dropping funnel, liberating hydrogen cyanide-C^{14}. The solution was boiled gently until 25 ml. of distillate was collected.

The aqueous alkaline solution containing the sodium cyanide-C^{14} (XXVII)* was washed with ether and diluted to 50 ml. in a volumetric flask by the addition of aqueous back-extractions of the ethereal washing. Titration of 1 ml. of this solution by the mecurimetric method (62) showed a total sodium cyanide-C^{14} (XXVII)* content of 206.8 mg. (69.6% based on barium carbonate-C^{14}; 81.1% based on triphenylacetonitrile-1-C^{14} (XXVI)*). The alkaline solution containing 202.6 mg. (4.13 mmoles) of XXVII* was evaporated under reduced pressure at 50°C in a long-necked, round-bottomed flask and finally dried at 0.1 mm. for 4 hours at room temperature.

(5) Hydrogenation of sodium cyanide-C^{14} (XXVII)*

The hydrogenation apparatus was assembled exactly as described by Solomon (46) and checked with a model run using inactive sodium cyanide. The system was evacuated with a water pump and the long-necked reaction flask containing the dry sodium cyanide-C^{14} (XXVII)* isolated from the system. The remainder of the system was filled with hydrogen, generated in a Kipp generator

and purified by bubbling through solutions of 20% hydrochloric acid, 20% sodium hydroxide, and saturated potassium permanganate.

Platinum oxide (85 mg.) and purified glacial acetic acid (5 ml.) were introduced into the reaction chamber; the chamber was then evacuated with a water pump. The catalyst was reduced with hydrogen until it became lumpy and mossy in appearance and the hydrogen uptake ceased. Approximately 1 hour was required. The shaking was stopped and an additional 5 ml. of glacial acetic acid and 0.96 ml. of concentrated hydrochloric acid were introduced. The reduced catalyst, acetic and hydrochloric acids, were transferred into the evacuated reaction flask by opening the intervening stop-cock and the apparatus shaken until the uptake of hydrogen ceased. The time required was 4.5 hours. The hydrogenation absorbed 230 ml. of hydrogen (123% of theoretical).

The reaction mixture was filtered and the catalyst well washed with dilute aqueous hydrochloric acid. The filtrate was evacuated <u>in vacuo</u>, affording solid methylamine hydrochloride-C^{14} (XXIX)*. The yield was assumed to be identical with the 85% consistently obtained by Solomon (46) in this reduction. Hence, 237 mg. (3.51 mmoles) of XXIX* was anticipated.

(6) Formation of N-methylurea-C^{14} (XXX)*

The methylamine hydrochloride-C^{14} (XXIX)* without further purification was refluxed for 3 hours with reagent urea (726 mg.; 12.1 mmoles) and water (0.85 ml.). The mixture was cooled to room temperature and diluted with inactive reagent N-methylurea (XXX) (808 mg.; 10.92 mmoles). This amount of XXX was estimated to give a dilution factor of 4:1 with respect to the radioactive XXX* formed, assuming a 78% conversion of methylamine hydrochloride-C^{14} (XXIX)* to XXX*.

(7) Preparation of N-nitroso-N-methylurea-C^{14} (XXXI)*

The total diluted N-methylurea-C^{14} (XXX)* (1010 mg.; 13.6 mmoles as calculated above) was cooled by immersion in an ice-salt bath (-5°C) and sodium nitrite (1.33 g.; 19.3 mmoles) and water (5.7 ml.) added. Cooled, concentrated sulphuric acid (0.669 ml.) and ice (7.25 g.) were mixed together, retained in an ice-salt bath and slowly added to this cooled mixture with vigorous stirring. The frothy, light yellow precipitate was collected on a Büchner filter funnel, washed with water (1 ml.) and dried in a vacuum desiccator in the cold. Crystals (1210 mg.) of N-nitroso-N-methylurea-C^{14} (XXXI)*, m.p. 112-113°C were obtained. The activity of this compound could not be determined by direct plating procedure.

(8) Generation of diazomethane-C^{14} (XXXII)*

N-Nitroso-N-methylurea-C^{14} (XXXI)* (1202 mg.; 11.5 mmoles) was added to a mixture of ether (27 ml.) and 80% potassium hydroxide solution (4 ml.), cooled to -5°C in an ice-salt bath, and the diazomethane-C^{14} collected and dried as previously described for inactive diazomethane (page 52).

An aliquot of diazomethane-C^{14} (taken from the excess recovered diazomethane-C^{14}, following the Arndt-Eistert reactions), was treated with excess 3-keto-Δ^4-etiocholenic acid. The resulting radioactive methyl ester was purified by chromatography on activated alumina and counted at 1.4×10^8 d./min./mmole (Geiger-Muller tube). This same figure applied to the diazomethane-C^{14}.

(9) Preparation of estrone-16-C^{14} (XLII)*

The acid chloride (XLIX) prepared from 555 mg. (1.6 mmoles) of the tertiary monomethyl ester (XLVIII) was added to diazomethane-C^{14}, (6.3 mmoles) prepared in "8", as described in section (a). The excess diazomethane-C^{14} was recovered by warming the ethereal solution with water (45°C); the distillation was continued until the distillate became colourless. The remaining volatile components were removed under reduced pressure

at 20°C, and the oily residue treated in the usual manner. A single crop (77 mg.) of homodiacid (LII)*, m.p. 248-252°C, was crystallized (ethyl acetate) for identification purposes. With authentic LII (m.p. 252-254°C) the m.p. of the mixture was 251-254°C. Cyclization of the crystalline homodiacid (LII)* and its residue afforded 48.3 mg. (76.4%) and 128.6 mg. (41.2%) of estrone-3 methyl ether (XXI)*, recrystallized from methanol to yield 153 mg. (32.3% based on the dimethyl ester (XLVII)) of XXI*, m.p. 167-171°C, unchanged on admixture with authentic XXI (m.p. 167-171°C). Demethylation afforded 115.6 mg. (80%) of estrone-16-C^{14} (XLII)*, m.p. 257-262°C, m.p. 259-262°C on admixture with authentic estrone (m.p. 259.5-262°C), of activity 5.03 x 10^5 d./min./mg. (Geiger-Muller tube) and 6.21 x 10^6 d./min./mg. (windowless flow gas counter). Similarly, the recovered diazomethane-C^{14} (approximately 2 mmoles) afforded 30 mg. of XXI* from 460 mg. (1.33 mmoles) of XLVIII. The excess diazomethane-C^{14} was again recovered and an aliquot used to determine its radioactivity, (see "8").

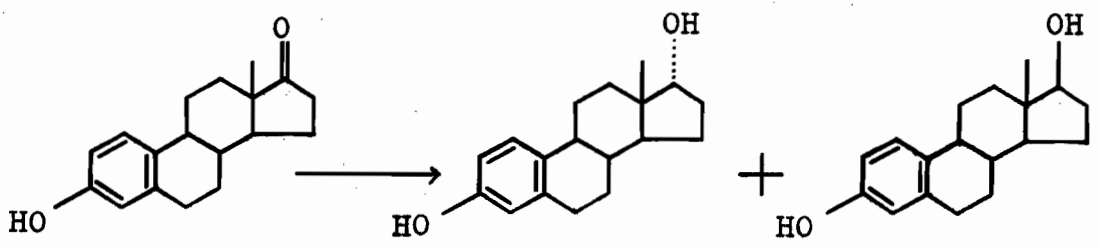
Similarly, diazomethane-C^{14} was prepared from 1163 mg. (11.3 mmoles) of N-nitroso-N-methylurea-C^{14} (m.p. 114-116°C). An aliquot (containing 4.5 mmoles) was employed using 464 mg. (1.34 mmoles) of the tertiary monomethyl ester (XLVIII). Crystalline homodiacid (LII)* (56.4 mg.) of m.p. 250-252°C was obtained. Cyclization afforded 101 mg. (25.4% based on the dimethyl ester

(XLVII)) of XXI*, m.p. 168-171°C, which was demethylated to 64 mg. (66%) of estrone-16-C^{14} (XLII)*, m.p. 259-262°C, counting at 1.9 x 10^6 d./min./mg., (windowless flow gas counter). LII*, XXI*, and XLII* showed no depression of m.p. on admixture with authentic samples of each. The crystalline residue was diluted with inactive estrone (200 mg.), affording 185 mg. of diluted XLII* of activity 1.37 x 10^5 d./min./mg.

B. THE REDUCTION OF ESTRONE-16-C^{14} TO β-ESTRADIOL-16-C^{14}

1. Introduction

The hydrogenation of estrone (XLII) to its dihydro derivatives, α-estradiol (LIII) and β-estradiol (XXXV), has been investigated by several groups of workers.



XLII LIII XXXV

Schwenk and Hildebrandt (79) first prepared both isomers in somewhat impure condition. They did not state the method used. Hydrogenation of estrone exclusively to β-estradiol was effected by Girard et al. (80) using sodium and alcohol, or catalytically with nickel in the cold. Similar results were obtained by Dirscherl (81) using catalytic hydrogenation with platinum oxide in neutral or alkaline media. However, both Raney nickel alloy in aqueous potassium hydroxide (82) and aluminum isopropylate with isopropyl alcohol (83), gave mixtures of the two isomers. In each case the biologically more active β-isomer predominated. This necessitated separation by fractional crystallization, by use of

digitonin (82, 84), which forms a sparingly soluble β-estradiol digitonide, or by use of urea (85), which gives an insoluble β-estradiol ureide. More recently, Grant and Seemann (86) have developed an elegant method of obtaining β-estradiol exclusively, working with the sulphate salt of estrone. A relatively new hydrogenating agent, lithium aluminum hydride (87), has been described by Nystrom and Brown (88). Ott and Murray (89) have employed this metal hydride to hydrogenate estrone (XLII) and estrone-3 methyl ether (XXI) solely to the 17(β)-diol (XXXV) and its methyl ether respectively. Although details of this work are lacking, they concluded that hydrogenation with lithium aluminum hydride yielded a product of excellent quality and quantity, the epimeric form of which was almost entirely as that obtained from reduction in neutral media, following the Auwers-Skita rule. The complete absence of the α-isomer (LIII) on reduction of estrone acetate has been reported (90). However, Levi (91) has found this hydrogenation to yield trace amounts of the α-isomer (LIII). Bachmann and Dreiding (77) have successfully hydrogenated with this reagent in the equilenin series, obtaining the β-dihydro derivative of equilenin-3 methyl ether in quantitative yield. However, equilenin itself was not reduced. It was decided to work out the best conditions for hydrogenation of estrone with lithium aluminum hydride, and apply these to the reduction of estrone-16-C^{14} (XLII)*.

2. Discussion

Model experiments were conducted to test the hydrogenation of estrone (XLII) with lithium aluminum hydride. Initial hydrogenation runs were carried out by addition of estrone dissolved in ether to an equal weight of lithium aluminum hydride, partially suspended in dry ethereal solution. This constituted a very large mole excess of the hydride, assuming one molecule of the hydride reacted with four molecules of ketone group (88), yet was half the excess used by Bachmann and Dreiding (77). Contrary to the publication issued by Metal Hydride Incorporated (92), lithium aluminum hydride was found to be only slightly soluble in ether, even though used in a finely powdered state. Considerable amounts remained as a fine suspension. Decomposition of the excess hydride with ice and water, followed by acidification and extraction, yielded β-estradiol in practically quantitative yields. No change in melting point was exhibited on mixed melting point with authentic β-estradiol (XXXV).

Since pure crystalline estrone was found to be relatively insoluble in anhydrous ether, the ether was replaced in this hydrogenation by dioxane, rather than preparing estrone acetate as described by other workers (90). The amount of solvent required was drastically less. The hydride was found to be relatively insoluble as reported (92) (100 milligrams/100 grams of dioxane). Decomposition of excess hydride was performed

with acetone, the volatile components removed under reduced pressure, acidified water added, and β-estradiol (XXXV) isolated by ethereal extraction.

The hydrogenation was repeated using a small volume of dry dioxane to dissolve the estrone, with subsequent addition of ether to increase the volume. Since only trace quantities of dioxane were present, decomposition of the excess hydride was accomplished with ice and water. (In the presence of larger quantities, loss of steroid might have occurred in the ethereal extraction step due to the solubility of dioxane in water.) Using this technique hydrogenations of estrone in amounts between 20 and 100 milligrams were carried out with practically quantitative yields.

The reduction of smaller amounts of estrone was effected by adding the powdered hydride directly to the dry ethereal solution of estrone. The hydrogenation proceeded readily, but yields were lower, due to the difficulty of crystallizing such small quantities.

The isolation of β-estradiol (XXXV) by treatment with urea, forming the ureide complex was tried using pure β-estradiol (85). The recovery of β-estradiol was found to be 75 per cent. Hence, as a precautionary measure, it was not considered desirable

to employ this purification procedure on the radioactive reduction product directly.

Radioactive estrone-16-C^{14} (XLII)* of activity 7.05 x 10^8 d./min./mmole was hydrogenated under the conditions described using dioxane and ether. After crystallization and collection of the first crop (76.5 per cent), the residual crystalline residue was diluted with inactive β-estradiol. A ureide separation (85) was performed to exclude any possible contamination by α-estradiol-16-C^{14} (LIII)*. Based on C^{14} activity, the yield of β-estradiol-16-C^{14} (XXXV)* was almost quantitative, amounting to 97.5 per cent.

Another hydrogenating agent, sodium boron hydride, has been recently described (93). This hydride possesses distinct advantages over lithium aluminum hydride, being both soluble and stable in cold water and methanol. The reduction of estrone and estrogen esters in methanolic solutions with this reagent have been reported (94). Sodium boron hydride was used to hydrogenate estrone in cold aqueous alkali with excellent results. A medium of cold alkali was considered preferable to one of methanol when reducing small quantities of estrone, since acidification rendered the steroid insoluble. With methanol, dilution with water to 50 per cent was employed (94).

3. Experimental Work

(1) Hydrogenation of estrone (XLII) in ethereal solution

Lithium aluminum hydride (100 mg.) was added to 50 ml. of anhydrous ether (stored over sodium) and refluxed gently, with magnetic stirring for 30 min. To this cooled suspension, with continued stirring, a solution of 100 mg. of estrone (XLII), m.p. 259.5-262°C, in anhydrous ether (125 ml.) was added over a period of 1 hour. The mixture was stirred a further 3 hours, decomposed by the cautious addition of ice and water, acidified with 20% hydrochloric acid and the two layers separated. The aqueous phase was extracted with ether (2 x 20 ml.), saturated with sodium chloride, and re-extracted with ether (2 x 20 ml.). The combined ethereal extracts were washed with water until washings were neutral, then evaporated under reduced pressure at 40°C. The crystalline residue (97 mg.) was recrystallized from aqueous ethanol, yielding a first crop of 88 mg. of β-estradiol (XXXV), m.p. 173-175°C. The crystalline residue of 19 mg. (m.p. 160-170°C) was not worked up further. Mixed melt with authentic β-estradiol (m.p. 176-177°C) gave a m.p. of 174-176°C.

Hydrogenation of XLII (10 mg.) dissolved in anhydrous ether (15 ml.) by the direct addition of lithium aluminum hydride (10 mg.), decomposition and extraction as before, following standing overnight, yielded a crystalline residue (9.2 mg.), crystallized from aqueous ethanol to give 5.6 mg. of XXXV, m.p. 171-176°C.

(2) Hydrogenation of estrone (XLII) in dioxane solution

Lithium aluminum hydride (100 mg.) was added to 20 ml. of dioxane (freshly distilled from sodium) and the suspension stirred for 30 min. at 60°C. On cooling, 99 mg. of XLII, m.p. 259.5-262°C, dissolved in dry dioxane (10 ml.) was introduced dropwise over 30 min. The solution was stirred for an additional 3 hours. Decomposition was effected by the addition of a little acetone. Removal of the volatile components under reduced pressure afforded a grey-white powder. Dilute hydrochloric acid was added and the mixture extracted with ether (2 x 30 ml.). The aqueous phase was saturated with sodium chloride and further extracted with ether (3 x 25 ml.). Combination of the ethereal extracts, followed by aqueous washing to neutrality and evaporation of the organic phase, led to a crystalline residue (98 mg.). Crystallization from aqueous ethanol gave 82 mg. of β-estradiol (XXXV), m.p. 173-176°C, and a crystalline residue (m.p. 159-168°C).

(3) Hydrogenation of estrone (XLII) in dioxane-ethereal solution

To a suspension of lithium aluminum hydride (60 mg.) in ether (30 ml.), refluxed with stirring for 30 min., a solution of XLII (60 mg.) in dioxane (2 ml.) and ether (25 ml.) was added dropwise over the period of 1 hour. Stirring was continued for a further 60 min., additional lithium aluminum hydride (10 mg.) was added, and stirring continued for 2 hours. Decomposition

with ice and water, acidification, and ether extraction as above yielded a crystalline residue (60 mg.). Crystallization from aqueous ethanol gave 42 mg. of XXXV (m.p. 174-176°C), and a crystalline residue of 17.5 mg., m.p. 160-170°C.

Similar handling of 20 mg. of XLII (m.p. 259.5-262°C) afforded a crystalline residue (20 mg.), yielding a first crop of 14.5 mg. of XXXV (173-176°C) and a crystalline residue of 5.5 mg., melting at 155-165°C.

(4) β-Estradiol ureide separation

49 mg. of β-estradiol (XXXV), m.p. 176-177°C, was dissolved in boiling methanol (5-6 ml.), saturated with urea at 2°C. Isolation of the ureide (46.5 mg.), m.p. 214°C, crystallized at 2°C, followed by decomposition with hot water, yielded 37 mg. of XXXV, (m.p. 176-177°C). The recovery was 75%.

(5) Hydrogenation of estrone-16-C^{14} (XLII)* in dioxane-ethereal solution

40 mg. of estrone-16-C^{14} (XLII)*, m.p. 257-262°C, activity 2.61 x 10^6 d./min./mg., was hydrogenated under the identical conditions as described above for dioxane-ethereal solution. The isolated crystalline residue (40 mg.) on crystal-

lization from ethanol and water, yielded a first crop of 30.7 mg. (76.5%) of β-estradiol-16-C^{14} (XXXV)*, m.p. 173-175°C. Mixed melt with authentic β-estradiol (m.p. 176-177°C) gave a m.p. of 173-176°C. To the radioactive crystalline residue was added 250 mg. of β-estradiol. The ureide was formed using boiling methanol (30 ml.) saturated with urea at 2°C. Filtration of the ureide, crystallized at 2°C, followed by decomposition with hot water yielded β-estradiol-16-C^{14} (190 mg.; 73%), which on crystallization from aqueous ethanol gave two crops (180 mg.), m.p. 175-176°C. This diluted β-estradiol-16-C^{14} had a specific activity of 83.7 x 10^3 d./min./mg., giving an activity of 21.7 x 10^6 d./min. for the total diluted β-estradiol-16-C^{14} (260 mg.). This was equivalent to 8.3 mg. (21%) of undiluted β-estradiol-16-C^{14} (XXXV)*. Thus the total yield on an activity basis was 97.5%.

(6) Hydrogenation of estrone (XLII) with sodium boron hydride

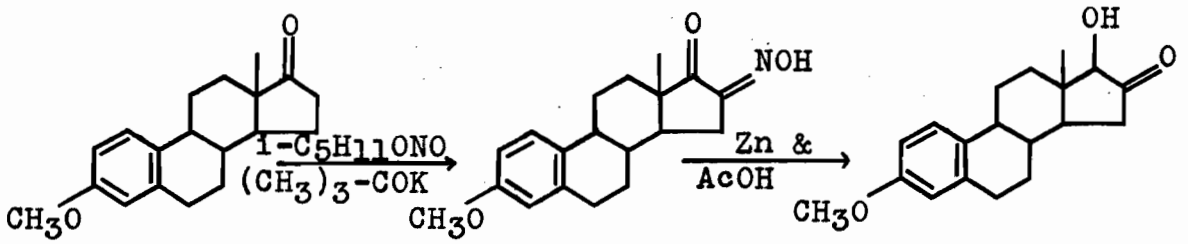
XLII (21 mg.), m.p. 256-258°C, was dissolved in 1.5 N sodium hydroxide solution (15-20 ml.) with heating, the solution cooled to 5°C, and sodium boron hydride (20 mg.) added. After standing overnight at 5°C, the solution was heated to destroy the excess hydride, cooled, acidified with 50% hydrochloric acid and 19.8 mg. (99%) of XXXV, m.p. 172-174°C, isolated by filtration.

Similarly, XLII (100 mg.) in 1.5 N sodium hydroxide (50 ml.) and methanol (5 ml.) afforded 96 mg. of XXXV, m.p. 172-175°C.

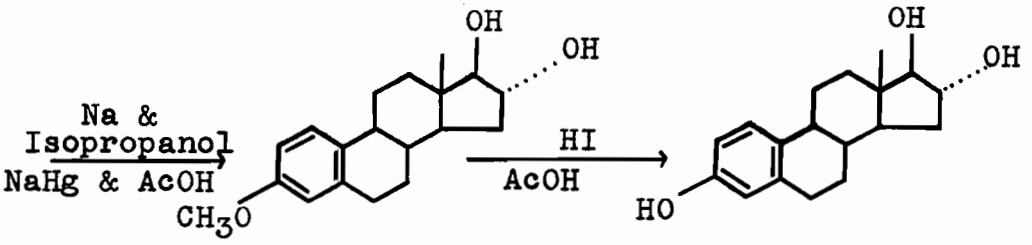
C. THE ATTEMPTED CONVERSION OF ESTRONE-16-C^{14} INTO ESTRIOL-16-C^{14}

1. Introduction

Processes for the conversion of estrone (XLII) into estriol (XVI) have been developed by Butenandt and Schäffler (95) and Huffman (96-98). The procedures used in these processes were of similar nature. Estrone-3 methyl ether (XXI) was converted into 16-oximinoestrone methyl ether (XXII) by treatment with isoamyl nitrite and potassium tertiary butylate (31). The former workers employed a modified technique, obtain-



XXI XXII LIIIa



XVIII XVI

ing somewhat higher yields than the original workers (31). The α-ketol (LIIIa) obtained following reduction of the 16-oximino

derivative (XXII) with zinc and acetic acid (99) was further reduced with sodium and isopropanol (95) or sodium amalgam (98), yielding estriol-3 methyl ether (XVIII). Evidence for the structure of the α-ketol (LIIIa) has been recently presented by Huffman and Lott (100, 101). Demethylation to estriol (XVI) was effected by treatment of the methyl ether (XVIII) (95) or its diacetate (98) with hydriodic acid in glacial acetic acid. Similar conditions were used previously by Litvan and Robinson (31) with estrone-3 methyl ether (XXI).

The comparative yields of these various steps are shown below:

	Butenandt and Schäffler	Huffman
Estrone-3 methyl ether (XXI)		
16-Oximinoestrone-3 methyl ether (XXII)	90%	75%
16-Keto β-estradiol-3 methyl ether (LIIIa)	57%	77%
Estriol-3 methyl ether (XVIII)	50%	53%
Estriol (XVI)	55%	40% - 64%

Further, Huffman has reported the conversion of the oximino derivative (XXII) into estriol-3 methyl ether (XVIII) in 46 per cent yield without isolation of the intermediary α-ketol (LIIIa); hence the yield from the methyl ether (XXI) to estriol (XVI) was 14-22 per cent. Butenandt and Schäffler reported a conversion of 15 per cent.

2. Discussion

Estrone-3 methyl ether-16-C^{14} (XXI)* was refluxed with potassium tertiary butylate in tertiary butyl alcohol (95) and nitrosated with isoamyl nitrite to crude 16-oximinoestrone-3 methyl ether-16-C^{14} (XXII)* in 71 per cent yield (31). A similar yield (75 per cent) has been reported by Huffman (60); both, however, were lower than the 75-88 per cent obtained by the original workers (31) and the 90 per cent recorded by Butenandt (95). Literature melting points for the purified 16-oximino derivative (XXII) have been reported at 161-162°C (31), 180-183°C (60), and 198°C (95), all with decomposition. Recrystallized XXII* was found to melt at 188-190°C, with decomposition.

The 16-oximino derivative (XXII)* was converted to estriol-3 methyl ether-16-C^{14} (XVIII)* exactly as described by Huffman (98). Stodola reduction of the oximino derivative (XXII)* with zinc and acetic acid to the α-ketol (LIIIa)*, and subsequent hydrogenation with sodium amalgam in ethanolic acetic acid without purification of the α-ketol (LIIIa)* afforded the crude methyl ether (XVIII)* in 56 per cent yield. However, serious loss was encountered on purification; 31 per cent of the methyl ether (XVIII)*, melting point 148-150°C, was obtained showing no depression of melt on admixture with authentic estriol-3 methyl ether (XVIII). A 46 per cent yield of the methyl ether (XVIII),

melting point 158-160°C, was reported by Huffman (98); Butenandt (95) obtained a melting point of 167-169°C following rigorous purification. An attempt to obtain weakly radioactive estriol-3 methyl ether (XVIII)* from estrone-3 methyl ether (XXI)* without purification of the oximino derivative (XXII)* or the α-ketol (LIIIa)*, afforded an unknown compound melting at 178-180°C in 24 per cent yield. On demethylation, crystals of melting point 293-294°C were obtained, depressing the melting point of authentic estriol by 15°C. Both these compounds gave negative tests for an α-ketol grouping with phosphomolybdic acid[x].

Demethylation of estriol-3 methyl ether-16-C^{14} (XVIII)* by heating with pyridine hydrochloride in an atmosphere of nitrogen for four hours afforded a crystalline compound (38 per cent) of melting point 255-258°C. Mixture melting point determination with authentic estriol (XVI) gave a 10°C depression of melt. A mixed melt with authentic estrone (XLII) showed no melting point depression. Evidently, this demethylation procedure removed the elements of water from the α-glycol present in ring D, yielding estrone.

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[x] Thanks are due to Dr. M. Saffran for carrying out these tests.

3. Experimental Work

(1) Preparation of 16-oximinoestrone-3 methyl ether-16-C^{14} (XXII)*

Potassium (300 mg.; 7.66 mmoles) was dissolved in tertiary butyl alcohol (20 ml.), estrone-3 methyl ether-16-C^{14} (XXI)* (600 mg.; 2.11 mmoles) of activity 9.7 x 10^3 d./min./mg. added, and the mixture refluxed for 1.5 hours. Some precipitation occurred as the solution was cooled. To this yellow suspension, magnetically stirred, was added isoamyl nitrite (0.9 ml.; 6.4 mmoles); the suspension turned red. The reaction mixture was stirred for 2 hours at room temperature, for 2.5 hours at 50°C, then an additional 15 hours at room temperature. All the above operations were carried out in an atmosphere of nitrogen. Following the addition of ice and water, the solution was extracted with ether and the aqueous phase acidified with concentrated acetic acid. The combined ethereal extracts were back-extracted 6 times with 0.1 N aqueous sodium hydroxide and the alkaline extracts acidified with acetic acid. The acidified solutions were allowed to stand for 6 hours at 5°C, the precipitates collected on a Büchner filter funnel and washed with water. Crude XXII* (471 mg.; 71%) was obtained; two crystallizations from aqueous ethanol afforded 272 mg. (41%) of XXII*, m.p. 188-190°C, with decomposition.

(2) Reduction of 16-oximinoestrone-3 methyl ether-16-C^{14} (XXII)* with zinc and acetic acid

To XXII* (272 mg.; 0.87 mmoles) covered with acetic acid (10 ml.) and water (0.6 ml.), was added zinc dust (800 mg.) and the mixture swirled in a water bath (45°C) until XXII* was dissolved. Water (9.4 ml.) was added, the mixture refluxed for 70 min., cooled to room temperature and the liquid decanted into a separatory funnel. The residual zinc was washed with small portions of benzene (100 ml.) and the benzene washings added to the aqueous acetic acid along with 1.5 N aqueous sodium hydroxide (80 ml.) and ether (200 ml.). The aqueous layer was drawn off and the organic phase washed successively with 0.5 N hydrochloric acid (2 x 50 ml.), 5% sodium bicarbonate (2 x 50 ml.), and water. The ether-benzene solution was evaporated in vacuo, affording a white, crystalline residue (LIIIa)*, m.p. 164-167°C.

(3) Reduction of 16-keto β-estradiol-3 methyl ether-16-C^{14} (LIIIa)* with sodium amalgam

LIIIa* was dissolved in ethanol (35 ml.) and acetic acid (3.5 ml.). To this solution maintained at 40°C in a water bath and efficiently swirled was added 2% sodium amalgam (70 g.) (for preparation see page 58) over a period of 1 hour. During the course of this addition sodium acetate was precipitated. At this point, additional 50% aqueous acetic acid (3.5 ml.)

was added. The mixture was transferred to a separatory funnel with ether and water, the mercury and aqueous layer drawn off, and the aqueous layer extracted with ether. The combined organic solvent was washed with water, 0.5 N sodium hydroxide (3 x 75 ml.), then water until washings were neutral, and finally evaporated to dryness. Crystallization of the residue from aqueous methanol afforded 149 mg. of XVIII*, m.p. 125-135°C. Two recrystallizations raised the m.p. of XVIII* (82 mg.; 31%) to 148-150°C. Mixed m.p. with estriol-3 methyl ether (XVIII) (m.p. 154-156°C) gave a melt of 150-154°C.

(4) Demethylation of estriol-3 methyl ether-16-C^{14} (XVIII)*

XVIII* (62 mg.) was heated with pyridine hydrochloride (1 g.) in an atmosphere of nitrogen at 180-185°C in an oven for 4 hours. On cooling, 5% aqueous hydrochloric acid (15 ml.) was added and the precipitate collected with ether (6 extractions). The combined ethereal extracts were washed with water, evaporated to dryness, and the crystalline residue recrystallized 3 times from methanol and water, yielding 22 mg. (38%) of estrone (XLII), m.p. 255-258°C. Mixed m.p. with authentic estrone (XLII) (m.p. 259-262°C) gave a m.p. of 257-261°C; with authentic estriol (XVI) (m.p. 280-282°C) a m.p. of 245-247°C.

D. THE CARBONYL CARBON ATOM ELIMINATION IN THE CYCLIZATION OF HOMOMARRIANOLIC ACID-3 METHYL ETHER

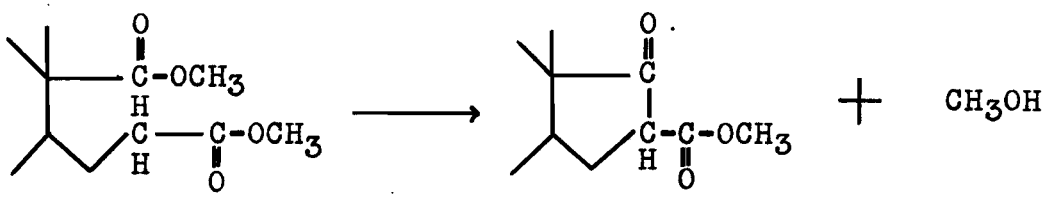
1. Introduction

Little doubt exists that rearrangement is an established criterion in the Arndt-Eistert extension of an organic acid to its next higher homolog (21, 22). The choice of the Arndt-Eistert reaction for extending the acetic acid side chains of the tertiary monomethyl esters of 3(β)-hydroxy-Δ^5-etiocholenic acid (X) and marrianolic acid-3 methyl ether (XLVIII) with C^{13} and C^{14} respectively was made for this very reason, since subsequent ring closure is known to occur with the loss of a carbon atom. This carbon atom was assumed to be the terminal one of this extended side chain, and hence without rearrangement the tagging atom would have been eliminated (24).

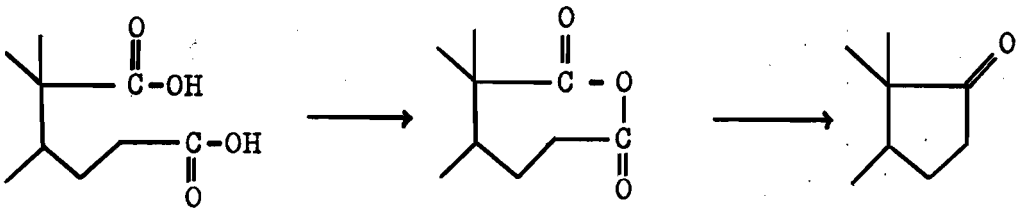
Although stability of the tertiary carboxyl group in comparison to the primary was anticipated in these cyclizations, the possibility exists that the tertiary carbonyl carbon atom, at least in part, may have been eliminated in the cyclization processes. Were it to be wholly eliminated, then rearrangement would not be a necessary criterion for this tagging of steroids in ring D, and hence other reactions involving less expensive tagging molecules such as sodium cyanide-C^{14} (XXVII)*, would

be applicable, with the conservation of C^{14}.

In the Dieckmann cyclization reaction (102), elimination of the primary carbonyl carbon atom definitely occurs, since ring closure takes place before the loss of the skeletal carbon atom.



However, such a clear cut reaction mechanism is not exhibited in the case of Blanc's cyclization (42), where previous anhydride formation occurs (24).



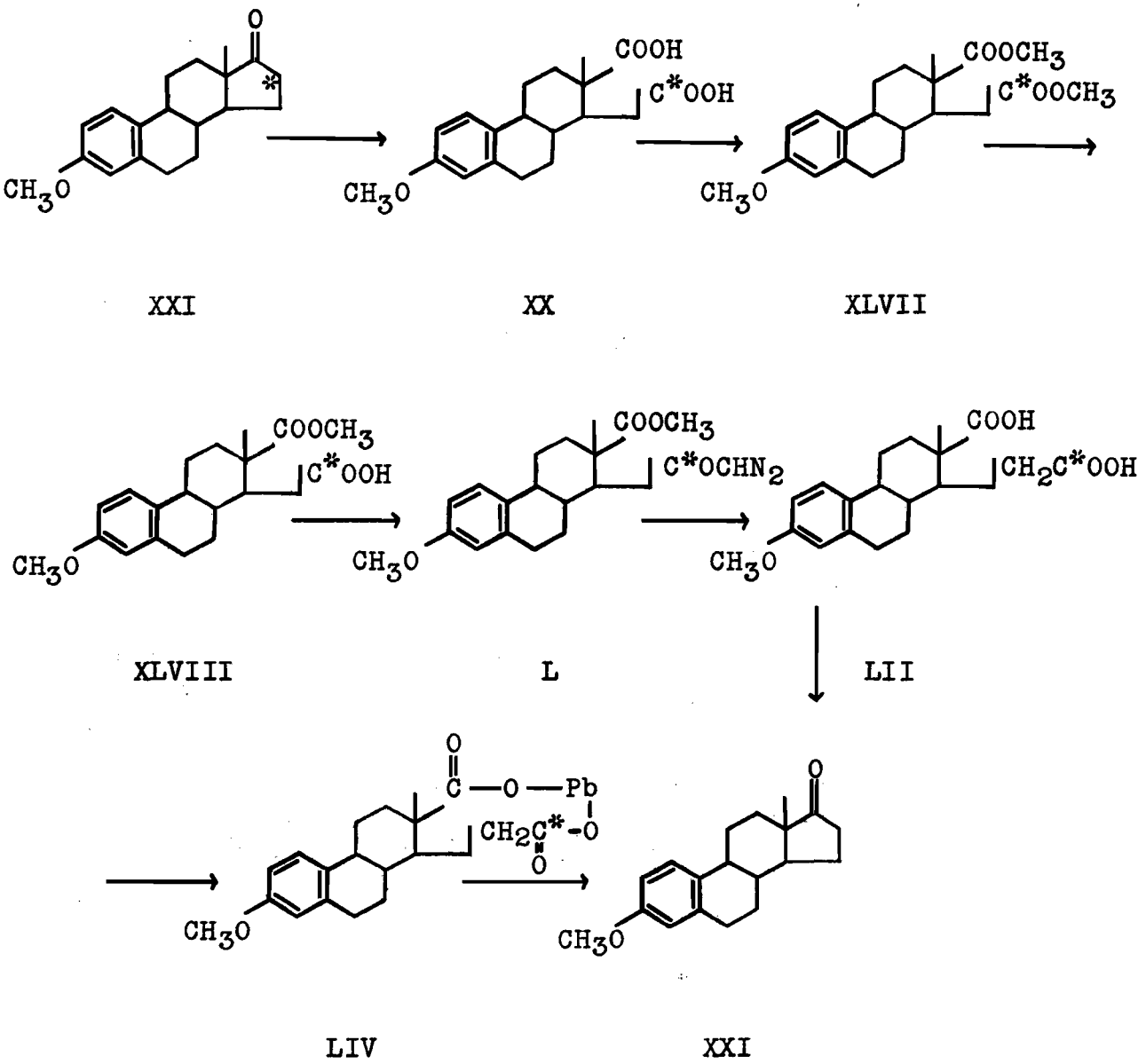
Similarly, in cyclization employing the lead salt of the homodiacid (LIV, Chart IV) the same uncertainty exists. The establishment of which carbon atom is lost in these latter two cyclizations is of considerable interest. Regardless of which occurs, the D ring thus formed by cyclization following an Arndt-Eistert extension with labelled diazomethane (XXXII)*, will have the labelled carbon atom at position 16.

2. Discussion

Estrone-3 methyl ether-16-C^{14} (XXI)*, of activity 9.2 x 10^5 d./min./mmole was oxidized with hypoiodite as previously described, affording marrianolic acid-3 methyl ether-16-C^{14} (XX)*. The yield of this diacid (XX)* was increased from the 66 per cent previously obtained by the subsequent treatment of the mother liquor with methanolic sodium hydroxide and isolation of additional diacid (XX)* (7 per cent) from the sodium salt formed (24). The dimethyl ester (XLVII)*, 9.7 x 10^5 d./min./mmole, formed by esterification of the diacid (XX)* with diazomethane, was saponified to the tertiary monomethyl ester (XLVIII)*, obtained in crystalline form by purification through its sodium salt. Esterification of this crystalline monomethyl ester (XLVIII)* with diazomethane yielded the dimethyl ester (XLVII)*. Unisolated monomethyl ester (XLVIII)* was subjected to the Arndt-Eistert extension using inactive diazomethane, yielding the homodiacid (LII)* (10.7 x 10^5 d./min./mmole). During these operations, the C^{14} previously located in the terminal carbon atom of the acetic acid side chain, was moved to the terminal carbonyl carbon atom of the extended propionic acid side chain.

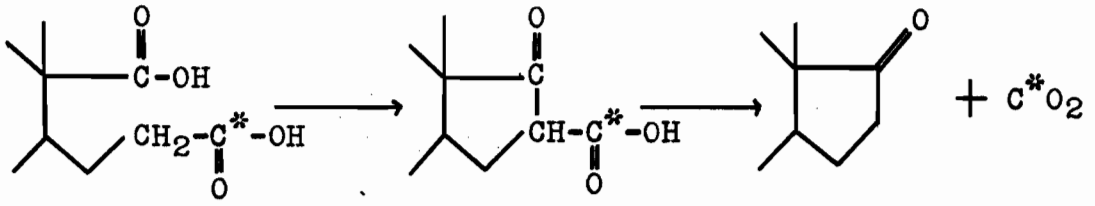
Cyclization of the non-isolated homodiacid (LII)* by pyrolysis with lead carbonate resulted in the formation of estrone-3 methyl ether (XXI)*, possessing an activity of 2.4 x

CHART NO. IV


O
*
CH3O
COOH
C*OOH
COOCH3
C*OOCH3
XXI
XX
XLVII
COOCH3
C*OOH
C*OCHN2
COOH
CH2C*OOH
XLVIII
L
LII
O
C—O—Pb
CH2C*-O
O
LIV
XXI

10^4 d./min./mmole. Since 97.5 per cent of the C^{14} activity was lost in comparison to the starting methyl ether (XXI)*, then cyclization proceeded with elimination of the primary carbonyl carbon atom.

The possibility existed that the lead salt (LIV)* did not form in this pyrolysis and the reaction might have proceeded in a fashion somewhat similar to the Dieckmann reaction (102).



Hence the lead salt of the homodiacid (LIV)* was formed by treatment of the free diacid (LII)* with methanolic basic lead acetate. Pyrolysis of this salt (LIV)* to estrone-3 methyl ether (XXI)* was carried out, thus eliminating the possibility of water splitting out, followed by decarboxylation of the acid formed.

3. Experimental Work

(1) Preparation of marrianolic acid-3 methyl ether-16-C^{14} (XX)*

Estrone-3 methyl ether-16-C^{14} (XXI)* (1.5 g.; 5.28 mmoles), m.p. 167-171°C, 3.2 x 10^3 d./min./mg., was oxidized to the diacid (XX)* under identical conditions as previously described. Following collection of the crystalline XX*, (1.178 g.; 67.3%), m.p. 197-199°C, the mother liquor was dissolved in methanol (10-15 ml.). A sodium hydroxide pellet was added and heated into solution, during which time simultaneous precipitation of the sodium salt of XX* occurred. The white precipitate was collected and washed with cold methanol. It failed to melt under 300°C, although slight decomposition was exhibited above 260°C. The sodium salt was dissolved in water and the free diacid (XX)* liberated by the addition of 20% hydrochloric acid. The collected precipitate was washed with water and crystallized from ethyl acetate, yielding 133 mg., (7.6%) of XX*, m.p. 196-199°C. The total yield of diacid (XX)* was 74.9%.

(2) Esterification of marrianolic acid-3 methyl ether-16-C^{14} (XX)*

The diacid (XX)* (1.178 g.; 3.54 mmoles) was esterified in an ethereal solution of diazomethane to yield dimethyl ester (XLVII)* (1.099 g.; 86.2%), m.p. 74-75°C, of activity 2.7 x 10^3 d./min./mg.

(3) Formation of marrianolic acid tertiary monomethyl ester-3 methyl ether-16-C^{14} (XLVIII)*

The dimethyl ester (XLVII)* (1 g.; 2.77 mmoles) was dissolved in 35 ml. of 4% potassium carbonate in 75% methanol, and 15 ml. of 75% methanol, and refluxed for 5 hours. After the usual work-up, a colourless oil (912 mg.) was obtained. This tertiary monomethyl ester (XLVIII)* could not be made to crystallize directly. The oily ester (XLVIII)* (228 mg.) was treated with 5.7 ml. of 0.110 N methanolic sodium hydroxide (0.95 mole equivalent), the volatile components were removed under reduced pressure, and the sodium salt dried in vacuo for 2 hours at 70°C. The sodium salt was washed with anhydrous ether (3 x 25 ml.); final traces of residual ether were evaporated under a stream of dry nitrogen. Slight decomposition but no melt of the sodium salt was exhibited under 300°C. The sodium salt was dissolved in water and the free acid (XLVIII)*, liberated as a colourless oily residue by the addition of hydrochloric acid, was washed with water. Crystallization was effected from aqueous methanol by allowing the solvent to evaporate slowly in the cold for several days. The crystals (121 mg.) of XLVIII* melted at 100-105°C, $[\alpha]_D^{27} = +71^\circ \pm 5^\circ$ (c = 0.98% in chloroform).

(4) Esterification of marrianolic acid tertiary monomethyl ester-3 methyl ether-16-C^{14} (XLVIII)*

Crystalline XLVIII* (25 mg.) was esterified in an ethereal solution of diazomethane, yielding 20 mg. of the dimethyl ester (XLVII)*, m.p. 70-74°C. Mixture m.p. determination with authentic dimethyl ester (XLVII), m.p. 74-75°C, showed a melt of 71-75°C.

(5) Preparation of homomarrianolic acid-3 methyl ether-16a-C^{14} (LII)*

The Arndt-Eistert extension of the oily tertiary half ester (XLVIII)* (684 mg.; 1.97 mmoles) was carried out as previously described. Without isolation the diazoketone was rearranged with silver oxide and the resulting homo-dimethyl ester (LI)* saponified to the homodiacid (LII)*. A single crop of LII* (48 mg.), m.p. 247-252°C, of activity 3.1 x 10^3 d./min./mg. was collected.

(6) Cyclization of homomarrianolic acid-3 methyl ether-16a-C^{14} (LII)*

The non-isolated homodiacid (LII)* (585 mg.) was mixed with an equal weight of lead carbonate and cyclized as usual.

The estrone-3 methyl ether (XXI)* resulting was dissolved in ether (100 ml.), the ethereal solution washed with 10% sodium carbonate, 5% hydrochloric acid and water, then evaporated under reduced pressure, yielding light yellow crystals of estrone-3 methyl ether (135 mg.), m.p. 164-167°C. The crystals were refluxed with charcoal in methanol, filtered, and recrystallized from methanol, yielding 115 mg. of XXI*, m.p. 170-172°C, of activity 85 d./min./mg.

(7) Formation of the lead salt of homomarrianolic acid-3 methyl ether-16a-C^{14} (LIV)*

The homodiacid (LII)* (40 mg.) was dissolved in hot methanol (6 ml.) and to this warm solution was added a few drops of a solution of basic lead acetate (5-10% lead acetate in 90% methanol). The lead salt (LIV)* (45 mg.) precipitated immediately and following collection was washed with methanol. It did not melt under 300°C, but manifested signs of charring above 250°C.

(8) Pyrolysis of the lead salt of homomarrianolic acid-3 methyl ether-16a-C^{14} (LIV)*

The lead salt (LIV)* (45 mg.) was pyrolyzed as usual to yield estrone-3 methyl ether (XXI)*, m.p. 170-172°C of activity 72 d./min./mg.

PART II

A. THE METABOLISM OF ESTRONE-16-C^{14}

1. Introduction

The preliminary investigations into the metabolism of estrone-16-C^{14} (XLII)* presented here serve as a sequel to previous studies carried out in these laboratories employing β-estradiol labelled with radioactive iodine (103). An excellent review on the metabolism of the estrogens is contained in the presentation of this former exploratory work (see also 104, 105); hence a further discourse is rendered redundant. Rather, the following comprises a critical survey of the newer concepts evolved in the metabolism of the estrogenic hormones through the use of isotopic tagging.

The usefulness of stably labelled estrogens has been recognized for considerable time. The most desirable tagging is the replacement of one or more carbon or stable hydrogen atoms of the desired estrogen with heavy or radioactive carbon or hydrogen respectively. In this manner the resulting labelled estrogen should be physiologically acceptable to the animal organism. Unfortunately, this method of tagging was not immediately realized and attention became focused upon the readily accessible radioactive halogens.

Prior to the advent of isotopically tagged estrogens, the fate of exogeneous estrogen was, in the main, unknown. Whether supplied to the organism in physiological or massive doses, in round figures 10 per cent (range 0-40 per cent) of the administered dose was identifiable in the urine without alteration of the original ring system (104, 105). This balance sheet was practically unaltered by the minute traces demonstrated in the feces by biological assay (105). The significance of the bile as an important medium of excretion of the estrogens was indicated by investigations using bile-fistula dogs. Cantarow et al. (106, 107) obtained almost quantitative recovery of injected estrogenic activity from the bile in two to three days (later findings showed considerably less activity (108, 109)). They postulated an enterohepatic circulation of the estrogens similar to that of the bile salts, small amounts of the steroid being lost to the feces with each circulation. An enterohepatic circulation was similarly suggested by Tschopp (110) for the distribution of bisdehydrodoisynolic acid in rats. Nevertheless, fecal excretion of estrogens in significant amounts was not demonstrated by these workers (106-110).

Recent investigations with estrogens tagged with radioactive halogens have lent support to this concept of estrogenic enterohepatic circulation. Distribution studies of equilin, brominated with radioactive bromine (8) in dogs, rabbits and

monkeys, and β-estradiol, iodinated with radioactive iodine (9) in mice have demonstrated high concentrations of radioactivity excreted into the intestine by way of the bile, and highly significant amounts of radioactivity eliminated in the feces. Urine excretion of the former as a conjugated phenolic steroid was indicated; partition of the fecal excretion of mice following injection of the latter suggested its elimination as acidic metabolites.

Other investigations have been carried out with synthetic estrogens labelled with radioactive bromine (7, 111). Distribution studies with triphenylethylene, labelled with radioactive bromine, have shown high concentrations of radioactivity recoverable in blood and accessory sex organs (7). Radioactive bromine labelled dimethylethylallenolic acid exhibited no measurable penetration into the hypophysis (111). The usefulness of labelled synthetic estrogens is uncertain since their metabolic transformations may in no way represent that of the natural estrogens.

While the results obtained from the application of estrogens tagged with radioactive halogens are of considerable importance, it must be remembered that these tagged molecules are generally inactive in a biological sense and their metabolic behaviour may be strikingly different from that of the biologic-

ally active, naturally occurring estrogens.

A more physiologically acceptable tagged estrogen, sodium estrone sulphate, labelled with radioactive sulphur, was rapidly and completely hydrolyzed by an enzyme in rat liver (112, 113); the radioactive sulphur was excreted in the feces and urine as inorganic sulphate. Further studies with this labelled estrogen are in progress using other tissues (114), since only the liver contains this hydrolyzing enzyme. 17-Methyl estradiol, labelled with C^{14} in this methyl group, has given a distribution picture similar to those of the radioactive halogenated estrogens (115). Although biologically active, this molecule is not a naturally occurring estrogen. The preparation of another excellently tagged estrogen, 6,7-deuterio-estrone acetate, has been recently described (116).

Preliminary exploratory investigations have been carried out employing estrone-16-C^{14} (XLII)*. These investigations served to substantiate certain observations arising through the use of physiologically less acceptable tagged estrogens; other observations were noted. Methods of handling this tagged steroid for future experimentation were determined.

2. Experimental Techniques

(a) Determination of radioactivity

(i) Labelled steroids - The radioactive determinations were recorded on a 64-scaler unit, operating in conjunction with a thin, mica window Geiger counter or a windowless flow Q-gas counter. Both counters were employed unshielded. When the Geiger counter was used the counting plates were placed at a constant fixed position below the mica window in order that the measured activities be comparable. The more sensitive Q-gas counter (4.5-5 times the Geiger counter) was employed in metabolic experiments where increased sensitivity was required.

All radioactive compounds, excepting barium carbonate, were plated on cleaned, copper discs (diameter - 25 mm.; area - 4.9 $cm.^2$) as infinitely thin layers, whence the absorption error may be neglected (117). The labelled compound was dissolved in analytical reagent methanol; aliquots of this solution were transferred to copper discs using 1 ml. pipettes. The entire surface was covered. The methanol was allowed to evaporate at room temperature, then the plates were heated at 50°C for 30 seconds and equilibrated to room temperature. A maximum of 10 micrograms of radioactive material was employed per plate. Normal values were between 1 and 3 micrograms,

giving weights of less than 1 microgram/cm.^{2} of surface area. Best pipetting was obtained by filling the pipette and adjusting the zero level with its tip just touching the surface of the radioactive solution. The pipette was withdrawn without touching the tip, an aliquot deposited on a disc and the pipette again withdrawn in identical fashion.

The activity of all plates was determined to within 1% probable error (118, 119), and this observed activity corrected for background radiation and the small coincidence error due to the resolving time of the instrument. This latter error amounted to 0.5% (Geiger counter) and 0.22% (Q-gas counter) per 1000 disintegrations for activities of less than 5000 and 9000 d./min. respectively (120, 121).

(ii) Radioactive barium carbonate - The assay of radioactivity in samples of barium carbonate has received considerable attention and has been excellently reviewed by Calvin et al. (122). A small quantity of radioactive barium carbonate, covered with 95% ethanol in a mortar (100 mg./ml. of ethanol) was ground for several minutes (122). The alcoholic slurry was allowed to settle for a few seconds, then the upper part pipetted into cleaned, weighed, aluminum dishes (area - 9 cm.^{2}) and the alcohol evaporated by heating with an infrared lamp. The dishes were tapped occasionally to ensure even plating of the carbonate. Final drying of the plates was effected by heating for 30 min.

at 110-120°C in an oven. After equilibration to room temperature the plates were weighed and the weight (in mg.) of carbonate per cm.^{2} determined. The activities were counted in the Q-gas counter for sufficient time to admit a probable error of less than 2% (119). The observed radioactivity was corrected for background radiation and also for self-absorption from a self-absorption graph prepared according to the directions of Yankwich et al. (117, 123). This graph exhibited close harmony to that described by Calvin et al. (122) for a windowless counter.

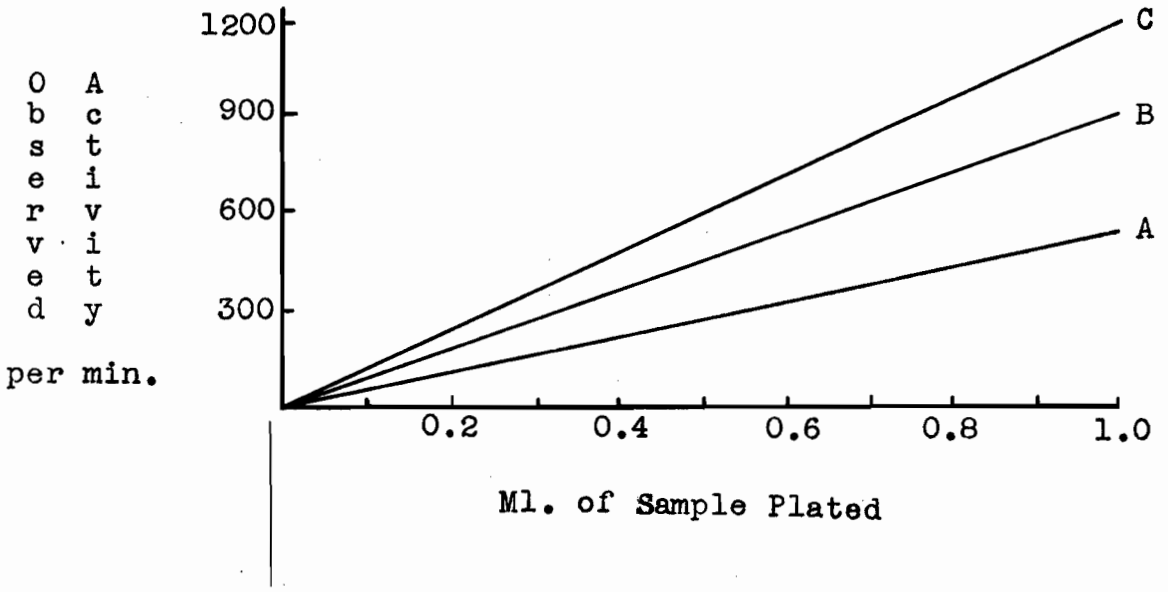
(iii) Radioactivity in urine, urinary and fecal extracts - In the course of the metabolic experiments the need arose for a reliable and rapid method of determining with reasonable accuracy (within 5%) the C^{14} activity in samples of urine and extracts of urine and feces. This problem has been realized by other investigators using C^{14} tagged molecules and was met generally by combustion of the sample and assay of the resulting radioactive carbon dioxide as barium carbonate-C^{14} (where standard procedures are well established (122, 124)). Unfortunately, with small amounts of C^{14}-containing urine and feces and extracts thereof, this procedure abolishes further partition studies of the radioactive materials in these various specimens.

The attempts of various workers to adapt the non-

combustion, direct counting technique of Yankwich and Weigl for C^{11}-containing samples to those containing C^{14} have been reviewed by Yates (17). More successful was the procedure of Hogness et al. (125) used in the metabolic studies of nicotinic acid-C^{14} (126, 127). These workers plated various biological fluids directly and estimated the extent of self-absorption of these plates by comparison with a series of calibration curves, carefully prepared for each individual fluid studied. This rigid control work renders difficult the activity determinations of different solutions of varying composition.

The following observations led to the development of a more simplified technique.

Urine collected from a mouse injected with estrone-16-C^{14} (XLII)* was diluted with distilled water. Plating and activity determinations of increasing amounts of this radioactive diluted urine gave a reproducible straight line, A, of constant slope, shown below.



On addition of a predetermined amount of C^{14} (as estrone-16-C^{14}) to an aliquot of this diluted urine and repetition of this plating and counting procedure, a second straight line, B, was obtained. The amount of added estrone-16-C^{14} was of negligible weight (less than 1 microgram). The observed activity at any point on B minus the activity at the corresponding point on A was found to be less than the known added activity of the estrone-16-C^{14} by a definite percentage, say "x%". Hence, "x%" of the added activity was absorbed by the plated material. Addition of an increased known amount of C^{14} to another aliquot of the diluted urine resulted in the derivation of line C. The activity at any point on C minus the corresponding activity on A again showed less than the known added C^{14} activity by this same percentage "x". The consistency of these observations was verified by identical treatment of several specimens of urine collected from different mice injected with radioactive estrone.

In general terms, addition of known amounts of C^{14} activity to diluted urine possessing radioactivity results in measured recovery of a constant fraction of the activity added. Hence the observed activity of the original diluted urine is 100 - x% of the activity present. The practical application of these observations may be realized in several ways. The following three methods were employed routinely in the investigations

with estrone-16-C^{14} (XLII)*, using standard solutions of this radioactive hormone. Reproducible results within 5% were obtained by these procedures.

The activity of estrone-16-C^{14} (XLII)* was determined by weighing about 1 mg. of the labelled steroid accurately on a microbalance and dissolving it in 5 ml. of distilled ethanol (volumetric flask). Appropriate dilution of an aliquot of this stock solution with ethanol and activity determination of this diluted solution as described in (i) gave an activity of 2.61 x 10^6 d./min./mg. for this labelled compound. From this stock solution standard solutions were made up in ethanol possessing the activities desired. In addition, a standard solution in chloroform was prepared by evaporating an aliquot of this stock solution of estrone-16-C^{14} and dissolving the residue in analytical reagent chloroform.

Method I

Two ml. of diluted urine, or aqueous extract of feces or organic solvents possessing C^{14} activity, and 1 ml. of distilled ethanol were mixed thoroughly. Plating and activity determinations of 3 aliquots (0.5-0.8 ml. each) of this solution were carried out as described in (i). The activity determinations were carried out for sufficient time to admit a

probable error of less than 2% (118, 119). The correction of this observed activity for self-absorption was obtained by mixing thoroughly another 2 ml. of this same specimen with 1 ml. of ethanolic standard solution of estrone-16-C^{14} with plating and activity determinations of identical aliquots as before. The corrected activity was calculated as follows:

Known activity of standard solution	1500 d./min./ml. (500 d./min./1/3 ml.)
Observed activity of specimen + alcohol	500 d./min./ml.
Observed activity of specimen + standard solution	900 d./min./ml.

$$\text{Absorption} = \frac{500 + 500 - 900}{500} = 20\%$$

Corrected value for specimen + alcohol = 500 x 1.2 = 600 d./min./ml.

Corrected value of original specimen = 600 x 1.5 = 900 d./min./ml.

Method II

This method employed two standard solutions of estrone-16-C^{14}, one possessing approximately twice the activity of the other.[x] Two solutions, made up by thoroughly mixing 2 ml. of the unknown aqueous radioactive solution with 1 ml. of each standard solution, were plated and the plates counted as in Method I. Calculations were made as follows:

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[x] Another member of these laboratories has developed concurrently a similar two-standard solution method (17).

Known activity of standard solution I · 1500 d./min./ml.

Known activity of standard solution II 3000 d./min./ml.

Observed activity of specimen + standard solution I 900 d./min./ml.

Observed activity of specimen + standard solution II 1300 d./min./ml.

Recovery of standard activity = $\frac{1300 - 900}{500}$ = 80%,

Therefore absorption = 20%.

Observed activity of specimen = 1300 - 800 = 500 d./min./ml.

or 900 - 400 = 500 d./min./ml.

Corrected value of specimen = 500 x 1.2 = 600 d./min./ml.

Corrected value of specimen alone = 600 x 1.5 = 900 d./min./ml.

Method III

An organic extract of radioactive urine or feces was evaporated to dryness under a stream of nitrogen at room temperature. Five ml. of chloroform was added and the flask stoppered and swirled for a few minutes. Aliquots (0.5, 0.7, and 0.8 ml.) of this solution were plated, dried and counted as in (i). The remaining chloroform solution (3 ml.) was evaporated under nitrogen, and 3 ml. of standard chloroform solution added. Activity determinations were performed on identical aliquots of this solution, and were as follows:

Known standard chloroform solution	1200 d./min./ml.
Observed activity in chloroform alone	600 d./min./ml.
Observed activity in standard chloroform solution	900 d./min./ml.

$$\text{Absorption} = \frac{400 - 300}{400} = 25\%.$$

Corrected value of whole extract = 5 x 600 x 1.25 = 3750 d./min.

(b) Administration of radioactive estrone

Estrone-16-C^{14} was administered in ethyl laurate containing 10% ethanol by subcutaneous route. A solution of the labelled steroid was prepared by the following procedure. Ten mg. of estrone-16-C^{14}, covered with 2.25 ml. of distilled ethyl laurate, was heated into solution and diluted with 0.25 ml. of ethanol. The addition of ethanol was found to prevent precipitation of the steroid from solution on standing. The weight of the solution was determined.

Each mouse was injected with 0.25 ml. of this solution, containing approximately 1 mg. of estrone-16-C^{14}; the accurate weight was determined by weighing the syringe and needle before and after injection. This solution possessed a strong leaking tendency, readily observable by the oily appearance of the hair around the site of injection. This difficulty was avoided by insertion of the needle through the gluteal

muscle of the anaesthetized animal (ether), depositing the solution of labelled steroid dorsally under the skin.

Estrone-16-C^{14} was administered also subcutaneously and intramuscularly as a fine suspension in 10% alcoholic, aqueous 5% "Tween 80" (polyoxyethylene sorbitan monooleate). Four mg. of labelled steroid was dissolved in 0.2 ml. of boiling alcohol and diluted with 1.8 ml. of 5% "Tween 80". Unfortunately, this suspension (1 mg./0.5 ml.) was not absorbed readily. Another surface activating agent, "Tween 20", afforded a solution of estrone under these identical conditions but was found to be toxic when administered to test animals.

(c) Collection of urine and feces

For collection of urine and feces from a group of mice a wire cage of 9.5 in. diameter was placed upon a fine copper screen, resting upon a glass dish. The floor of the cage was approximately 3/4 of an in. from the wire screen. The feces fell upon the screen from the cage and were collected twice daily and stored under a layer of ether at 5°C. The urine passed through the screen and was collected in the dish. To aid preservation of the urine the dish was rubbed with an alcoholic solution of thymol and allowed to dry prior to commencing the experiment. At the termination of the experiment, the cage,

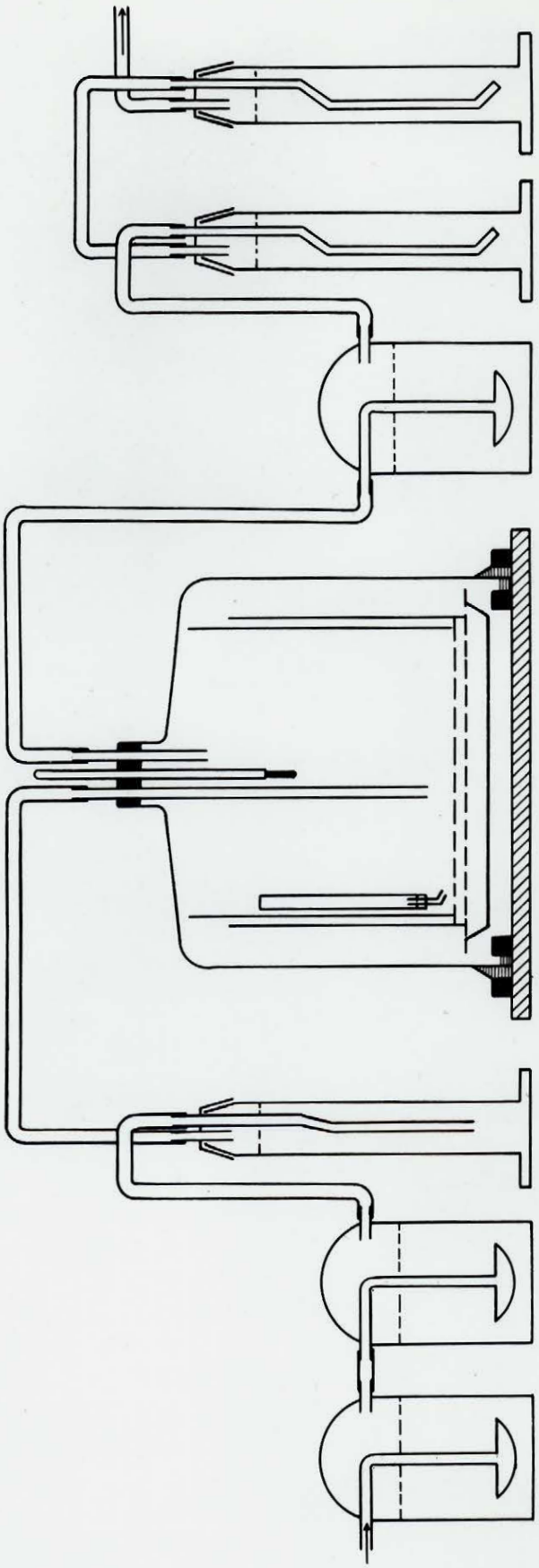
copper screen and dish were scrubbed thoroughly with distilled water and particles of food removed from the diluted urine by filtration. The diluted urine was stored at 5°C. Throughout the experiment the animals were fed morning and night and received water without restriction.

For short term experiments with a single animal, a circular cage was employed with outer edges below the wire floor tapering sharply inwards, forming a wide-mouthed funnel, then outwards again to the original width of the cage. A circular wire screen filled with food was placed in the middle of the cage over the orifice. Urine and feces were separated by the former running down the tapered walls and collecting at the bottom, and the latter falling from the lip into a small container. In similar fashion to the above, the feces and the diluted urine from the washed cage were stored at 5°C.

(d) Collection of respiratory carbon dioxide

When the collection of respiratory carbon dioxide from a group of animals was necessitated, the apparatus for isolation of urine and feces from mice, described in (c), was placed under a glass jar constructed of half a 5-gallon bottle as outlined in Figure I (page 109). This set-up was a modification of carbon dioxide collection apparatus previously described

FIGURE I



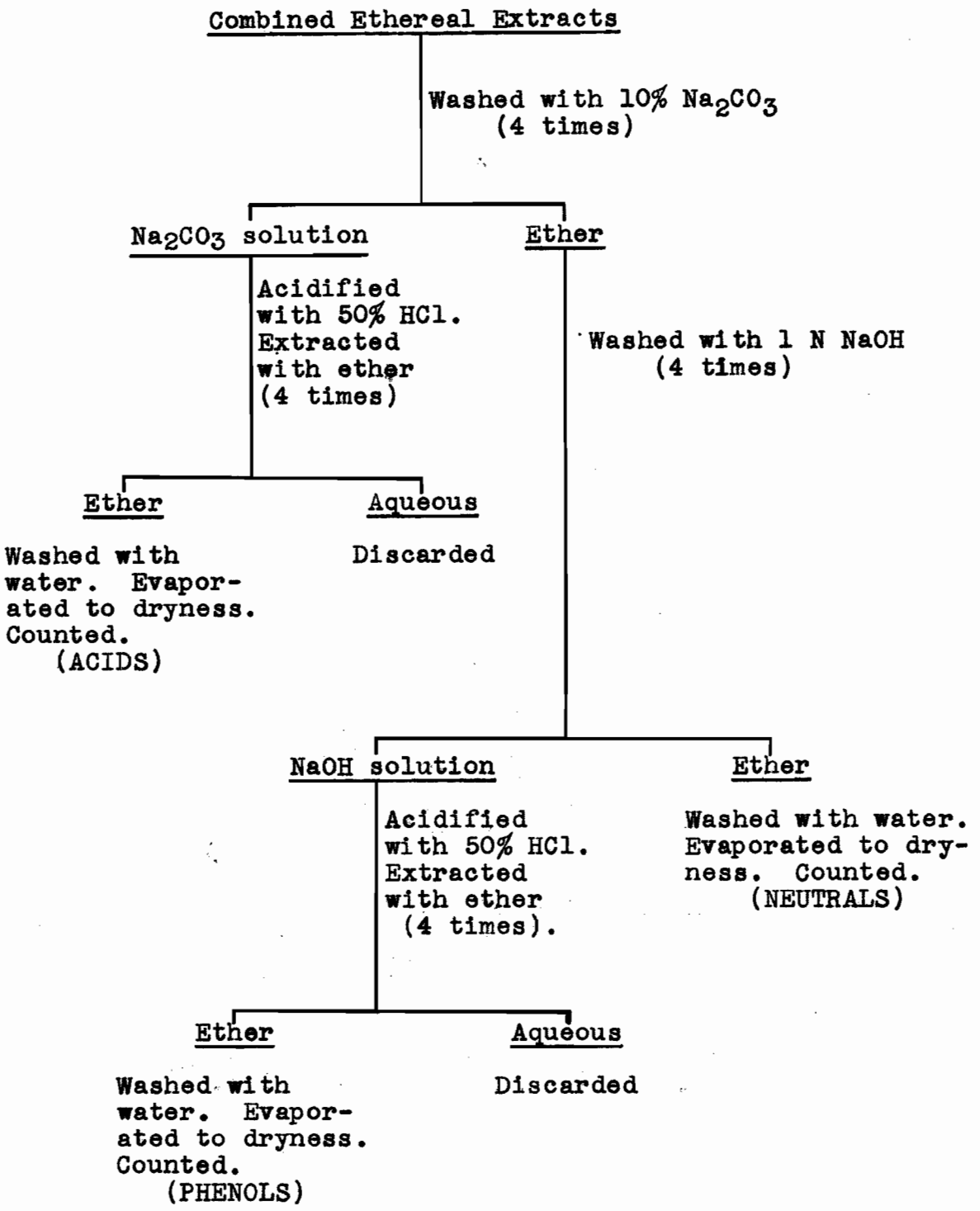
(128, 129). Two concentric rings of paraffin were placed on a heavy plate of glass and filled with thick petrolatum. The apparatus was placed in the middle of these rings on the glass plate (a piece of sheet metal was inserted around the inside of the cage to prevent the animals from climbing) and covered with the glass jar. The bottom edge of the jar was buried in the petrolatum. Air drawn into the sealed jar by means of a water pump at approximately 1 litre/min. was freed of carbon dioxide by passage through absorption bottles containing 55% sodium hydroxide and saturated barium hydroxide solutions, and adjusted to approximately 75% humidity by bubbling through a solution of saturated sodium chloride (128). Carbon dioxide expired from the animals was absorbed from the air withdrawn from the chamber by bubbling through absorption bottles of carbonate-free sodium hydroxide solutions. A final absorption bottle of saturated barium hydroxide solution served as an indicator for complete removal of carbon dioxide by the sodium hydroxide traps.

For collection of the respiratory carbon dioxide of five mice for 13 hours, the first carbon dioxide absorption bottle from the chamber was filled with 250 ml. of 55% sodium hydroxide solution and 600 ml. of water. The second absorption bottle contained 100 ml. of the same alkaline solution and 50 ml. of water. Following termination of the collection period the

alkaline solutions were combined and diluted to 1600 ml. with distilled water. To a 400-ml. aliquot was added 50 g. of ammonium carbonate, then 27.5 g. of barium chloride and the mixture shaken and stored for 15 hours at 5°C (130). The precipitated barium carbonate, collected on a sintered glass filter funnel and washed thoroughly with boiling water and dried at 70°C *in vacuo*, weighed 27.85 g. Activity measurements were performed as in (ii).

(e) Fractionation of diluted urine and feces

Control partitioning experiments with radioactive estrone and estradiol between various organic solvents and alkaline solutions were still in progress when this preliminary metabolic work was performed and hence were not usable in these investigations. Hence, a simplified partitioning scheme was employed, designed to fractionate radioactive organic extracts of diluted urine and feces into neutral, acidic, and phenolic fractions. This scheme is tabulated in diagrammatic form on the following page.



A fecal organic extract was prepared by stirring the crushed feces under a layer of ether for 30 min. The mixture

was allowed to stand until the ethereal layer was clear; the layer was removed. This procedure was repeated 6 times. In a similar manner, an aqueous extract of feces was obtained.

(f) Radioactivity of urinary urea

An aliquot of diluted urine was heated for 15 min. on a steam bath with activated charcoal, cooled, filtered, buffered with acetic acid buffer and treated with urease enzyme. The mixture was allowed to stand overnight. On addition of barium chloride a fine precipitate was obtained; the mixture was heated on a steam bath for 10 min., cooled to 5°C, and the precipitate collected and washed with hot water on a Büchner filter funnel. The barium carbonate was dried in vacuo at 70°C, and its radioactivity measured as in (ii).

(g) Tissue distribution experiments [x]

Two mammary tumor-bearing female mice (C3H strain) were injected with estrone-16-C^{14} in 10% alcoholic, aqueous 5% "Tween 80", one subcutaneously, the other intramuscularly in the left hind leg. After 12 hours, the animals were anaes-

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[x] The co-operation of Dr. C. P. Leblond and Mr. R. Greulich of the Department of Anatomy, McGill University, in these distribution studies, is gratefully acknowledged.

thetized with ether, and sacrificed by means of exsanguination from the inferior vena cava.

The various tissues were removed, weighed, and heated (boiling water bath) in 2 N sodium hydroxide solution (1 ml. of alkali per 50 mg. of tissue) until dissolution occurred. Tissues weighing less than 50 mg. were dissolved in 1 ml. of alkali. Blood plasma was dissolved in an equal volume of 4 N sodium hydroxide solution. One-half ml. aliquots of these alkaline solutions were plated on small watch glasses and dried in an oven at 40°C. The dried aliquots were assayed for radioactivity with a Geiger counter working in conjunction with a Berkley Decimal Scaler, Model 2000. The activity of the estrone-16-C^{14} was measured by mixing a measured aliquot of the excess solution for injection with a solution of liver brei in 2 N sodium hydroxide (50 mg./1 ml.). Plating and counting were performed as above.

In addition, another mouse (C3H strain) similarly injected and sacrificed after 12 hours, was used for the preparation of radioautographs of the major organs and tissues. Unfortunately, the development of these radioautographs requires several months. They will not be available for considerable time.

3. Results

(a) Metabolism of labelled estrone in the mouse

Five non-pregnant female mice, weighing between 24 and 40 g., were injected subcutaneously, each with approximately 1 mg. of estrone-16-C^{14} dissolved in 0.25 ml. of ethyl laurate containing 10% ethanol. The total administered dose was 4.91 mg., representing a gross injected activity of 13 x 10^6 d./min. An average weight loss of 1.4 g. per animal was noted at completion of the experiment.

The respiratory carbon dioxide was collected for the first 13 hours following injection. With conversion to barium carbonate-C^{14} (27.84 g.) of activity 11.6 d./min./mg., the carbon dioxide-C^{14} activity was found to be 2.14% of the administered radioactivity. This figure represented an average excretion of injected radioactivity of 0.164% per hour. Ninety-seven hours after the commencement of the experiment, a 3-hour collection period showed an excretion of radioactivity of 0.083% per hour; the activity of the barium carbonate-C^{14} (8.63 g.) was 3.74 d./min./mg.

The urine and feces were collected for a 6-day period. The former possessed 22% of the administered radioactivity of which 1.86% was extractable directly with ether. Hydrolysis

(autoclaving at 115°C and 15 lb. pressure for 30 min.) with 15 volumes % hydrochloric acid liberated 10.7% to ethereal extraction; 89.3% remained in the aqueous layer. Partitioning of this 10.7% into acids, phenols, and neutrals by extraction of the ethereal solution with 10% sodium carbonate and 1 N sodium hydroxide solutions gave 7.1%, 2.4% and 1.0% respectively in these fractions.

Removal of the radioactivity of this diluted urine was found to be practically quantitative (99.5%) on treatment with activated charcoal. The residual activity was found to be mainly radioactive urea, affording barium carbonate-C^{14} of activity 11.6 d./min./mg. By calculation, the urinary urea-C^{14} contained 0.13% of the administered radioactivity.

From the feces 20.45% of the injected activity was recoverable by washings with ether (2.15%) and water (18.4%). This figure does not represent a total radioactivity of the feces since qualitative measurement of the dried residue indicated considerable residual radioactivity. Hydrolysis (refluxing with 15 volumes % hydrochloric acid under toluene for 30 min.) rendered 12% of the radioactivity of the aqueous fraction ether extractable.

The animals were sacrificed on the seventh day from

the commencement of the experiment. Various radioautographs [x] were made and several organs and tissues examined for radioactivity. Radioactivity in measurable amount was located in the blood plasma alone; the kidney, liver, adrenal, thyroid, ovary, uterus and gall bladder were void. The feces collected on the seventh day were radioactive by qualitative examination.

(b) Fate and distribution of labelled estrone in the animal organism

Two tumor-bearing, female mice of the C3H strain were injected, one subcutaneously, the other intramuscularly, each with 1 mg. of estrone-16-C^{14} as a micro-crystalline suspension in 0.5 ml. of aqueous 5% "Tween 80" containing 10% ethanol. These injections resulted in very poor absorption of the labelled steroid, the major portion remaining at the site of injection after 12 hours when the animals were sacrificed. With the intramuscularly administered steroid 73% was recovered from the injection site; the skin and tissue around the site of the subcutaneously administered hormone were similarly highly radioactive.

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[x] This part of the experiment was performed by Mr. R. Greulich, Department of Anatomy, McGill University.

The tissues and excreta were examined for radioactivity and the results are shown in Tables II and III. With the animal subcutaneously injected (Table II) the activity remaining at the site of injection could not be measured accurately; hence the percentages of activity refer to the recovered activity.

The most significant observation in both cases was the high proportion of radioactivity excreted through the gastro-intestinal tract, amounting to 40% of the absorbed dose (intramuscular) and 80% of the recovered activity (subcutaneous). The only other significant activities were located in the urine (11% and 14%), liver (1% and 4%) and gall bladder (1%, subcutaneous), and tumor (0.6% and 3%). On a concentration basis (activity per mg. of tissue), the gall bladder showed high concentration of radioactivity. Although the fixation in the liver and tumor was an appreciable portion of the injected dose, the concentration of activity per mg. was not significant. No activity was discernible in the expiratory carbon dioxide from one of these animals, collected during the 12-hour period.

The procedure used for the plating and activity determination of tissues is realized now to be of limited application. Although satisfactory results are obtained with pure labelled estrone, the various tissues digested in the alkali prior to plating play an extremely important role in the

TABLE II

Distribution and Fate of Estrone-16-C^{14} in a Tumor-bearing C3H Female Mouse

All counts are expressed as disintegrations recorded/100 seconds using a lead-shielded, thin mica window Geiger counter.

Count injected (subcutaneous) 73,340
Total recoverable count 8,170 (11.2%)

	Total Count	Count per mg.	Recovered Dose %
Gastro-intestinal tract and excreta			
Salivary glands	7	0.08	
Stomach	35	0.14	0.43
Intestine	2888	1.38	35.35
Feces	3640		44.56
Urine	920		11.25
Viscera			
Liver	399	0.27	4.88
Gall bladder	97	6.3	1.19
Pancreas	7	0.04	0.09
Spleen	0		
Lung	8	0.04	0.1
Heart	2	0.02	
Bladder	5	0.27	0.06
Kidney	5	0.02	0.06
Brain	20		0.24
Endocrine and accessory sex organs			
Pituitary	1	0.8	
Ovary	0		
Adrenal	2	0.33	
Uterus	2	0.06	
Thyroid	9	3.0	
Blood			
Plasma	12		
Cells	9		0.11
Tumor	55	0.09	0.67

TABLE III

Distribution and Fate of Estrone-16-C^{14} in a Tumor-bearing C3H Female Mouse

All counts are expressed as disintegrations recorded/100 seconds using a lead-shielded, thin mica window Geiger counter.

Count injected (intramuscular)	50,350	
Unabsorbed	37,035	(73%)
Absorbed	13,315	(27%)

	Total Count	Count per mg.	Absorbed Dose %
Gastro-intestinal tract and excreta			
Salivary glands	8	0.05	
Stomach	85	0.37	0.6
Small intestine	2083	1.09	15.6
Large intestine	1297	1.54	9.7
Feces	1855	1.41	13.9
Urine	2258		16.9
Expired CO_2	0		
Viscera			
Liver	163	0.12	1.2
Gall bladder	2	0.08	
Pancreas	12	0.07	
Spleen	1	0.002	
Lung	28	0.13	0.2
Heart	25	0.18	0.2
Bladder	2	0.04	
Kidney	14	0.04	
Brain	12	0.03	
Endocrine and accessory sex organs			
Pituitary	0		
Ovary	2	0.07	
Adrenal	6	0.91	
Uterus	9	0.08	
Thyroid	6	6.0	
Blood			
Plasma	28		0.2
Cells	0		
Tumor	360	0.25	2.7

degree of self-absorption of C^{14} activity and cause erratic results. However, the percentages obtained serve as rough indicators of the radioactivity present.

From the diluted urines of these animals approximately 15% of the C^{14} activity was removable by ethereal extraction. Hydrolysis of the resulting aqueous phase afforded another 25% ether soluble. A further hydrolysis yielded an additional 2%. Thus 55-60% of the radioactivity remained ether non-extractable. Partitioning of the ether soluble activity prior to hydrolysis into acids, phenols, and neutrals, showed 8%, 59%, and 32% present in these respective fractions. Following hydrolysis of the aqueous layer these percentages were considerably altered showing 38%, 40% and 21% respectively.

The radioactivity of the feces, removable by ethereal extraction, was not increased appreciably by previous acidic hydrolytic treatment. Fractionation of the ether soluble C^{14} activity of feces (not hydrolyzed) showed the relative proportion of radioactivity for acids, phenols, and neutrals to be 27%, 22% and 52% respectively.

4. Discussion

Preliminary investigations have indicated two major routes of excretion of estrone-16-C^{14} and its metabolites, namely, the feces and urine. In addition, a third excretory route, the respiratory carbon dioxide, is implicated. Tissue distribution studies have revealed significant concentrations of radioactivity present in the large and small intestines, feces, urine, and in one instance, the gall bladder, following administration of this labelled steroid.

A six-day collection of urine and feces from a group of five mice injected with estrone-16-C^{14} resulted in urinary and fecal excretion of 22% and more than 20% of the administered C^{14} activity respectively. These results substantiate the previous observations with tagged estrogens which were foreign molecules to the animal organism. Radioactive iodine-labelled β-estradiol administered to mice had demonstrated a 35% fecal and 10% urinary elimination of this radioactivity in 10-12 hours (103); equilin, tagged with radioactive bromine, had given 40-50% combined urinary and fecal excretion in 2-6 hours following injection into rabbits (8). Similarly, C^{14} methyl labelled 17-methyl estradiol administered to rats had resulted mainly in fecal elimination of radioactivity, with a small amount found in the urine (115).

The respiratory carbon dioxide collected from these animals for the first 13 hours following injection contained 2.14% (0.164%/hour) of the administered radioactivity. A further collection 84 hours later revealed 0.249% (0.083%/hour) for a 3-hour period. If one were to assume that throughout the time elapsed between these two collection periods the elimination of C^{14} through the respiratory carbon dioxide was at least equal to the latter rate, then a minimum of approximately 10% of the administered C^{14} was eliminated via the respiratory carbon dioxide during the first 4 days.

That this assumption is valid and this calculated 10% was indeed a minimum percentage eliminated in the respiratory carbon dioxide is inferred by the isolation of barium carbonate-C^{14} of activity 11.6 d./min./mg. (2285 d./min./mmole) from the filtrate of the urine of these animals treated with charcoal, through the action of the enzyme urease followed by the addition of barium chloride. Since this treatment with charcoal removed 99.5% of the radioactivity present in the urine leaving a clear, colourless filtrate, the possible adsorption of C^{14} metabolites of estrone-16-C^{14} on the precipitated barium carbonate-C^{14} was excluded. Hence the urea affording this radioactive barium carbonate possessed an activity of 2285 d./min./mmole. The investigations of Mackenzie and du Vigneaud (131) with l-methionine containing C^{14} in the methyl group have

demonstrated that the carbon of urea is quantitatively derived from carbon dioxide. Hence the total carbon dioxide expired from these animals likewise should have contained 2285 d./min./mmole. By approximate calculation the respiratory carbon dioxide should have eliminated 20-25% of the administered C^{14}.

Only small quantities of the total radioactivity of this urine were removable by ether extraction. It has been stated that mice do not excrete steroids in urine (132). Unhydrolyzed urine yielded 1.86%; hydrolyzed urine gave 10.7%. The ether-soluble radioactive metabolites of the hydrolyzed urine were 71% extractable with 10% sodium carbonate solution, inferring acidic properties of these compounds. Surprisingly, the unhydrolyzed urines from mice (used in distribution studies) where the absorption of administered estrone-16-C^{14} was very poor afforded 15% of the radioactivity ether extractable and an additional 25% removable following hydrolysis. Approximately 40% of the radioactivity was extractable from ether with 10% sodium carbonate solution (acids), and a further 40% with subsequent 1 N sodium hydroxide solution (phenols). A second hydrolysis failed to render further significant quantities of radioactive metabolites ether extractable. This may infer a different handling of large and small doses of exogenous estrogen, although such differences have yet to be reported (105). Further, the respiratory carbon dioxide collected from one of these animals was

completely void of radioactivity. This is in marked contrast to the respiratory carbon dioxide collected from animals where the absorption of administered labelled steroid was practically quantitative.

In contrast, the hydrolyzed urine of a rabbit injected with radioactive bromine-labelled equilin lost 84% of its radioactivity on ethereal extraction (8). The chief urinary excretory product of exogenous estrone and β-estradiol in the rabbit is α-estradiol (133-135) and from β-estradiol up to 40% has been isolated from the urine (105). Although this estrogen may well be the urinary metabolite of equilin, Bauld and Heard (104) failed to obtain any recognizable conversion product following the administration to a rabbit of the more unsaturated estrogen, equilenin. Further, the ether extractable radioactivity (45%) of hydrolyzed urine from mice treated with radioactive iodine-labelled β-estradiol was removed practically quantitatively with 1 N sodium hydroxide solution (103).

The radioactivity (20% of administered C^{14}) contained in the feces of this group of animals afforded 2.15% to direct ethereal extraction and 18% to aqueous extraction. Hydrolysis of this aqueous extract liberated 12% of the radioactivity to ether, 88% remaining in aqueous solution. The extensive water solubility of these radioactive metabolites of feces (and urine)

is indicative of degradation of the original steroidal molecule. This view is further supported by the appearance of radioactivity in the respiratory carbon dioxide. Degradation of the estrogenic hormones through deep-seated oxidation has been postulated (104). However, the possibility that the labelled steroid may have been rendered water soluble by some other route has by no means been excluded. Investigations of the excretory products of progesterone-21-C^{14} have similarly shown large percentages of water soluble radioactive metabolites (17).

The ethereal extract of feces collected from mice where the injected estrone-16-C^{14} was absorbed poorly, afforded, on partitioning into acids, phenols and neutrals, 27%, 22% and 52% of the total extractable radioactivity in these respective fractions. By comparison, mice treated with radioactive iodo-estradiol gave more than 50% in the acidic fraction; the rest was contained in the phenolic fraction (103).

The tissue distribution of C^{14} following subcutaneous and intramuscular administration of estrone-16-C^{14} was somewhat limited by the poor absorption of the labelled steroid, injected as a micro-crystalline suspension in 10% alcoholic, aqueous "Tween 80". Decreased absorption was noted previously with radioactive iodo-estradiol where a detergent was used with the injected material (9). However, the high proportion of radio-

active metabolites excreted through the gastro-intestinal tract observed with β-estradiol, tagged with radioactive iodine (9), likewise was noted with estrone-16-C^{14}. Similar urinary excretions of radioactivity were obtained; one animal afforded a high concentration of C^{14} activity in the gall bladder as previously noted with the iodine-labelled estrogen (9).

Of the endocrine glands, only the thyroid exhibited high concentration of radioactivity per weight basis. However, the accuracy of these radioactive determinations was of uncertain reliability, since the observed activities recorded were barely above background activity. The ability of the thyroid to concentrate radioactivity following administration of radioactive iodo-estradiol had been previously noted (103); since the thyroid was known to pick up only inorganic iodide, this radioactivity was thought to infer the breakdown of iodo-estradiol in the body (9). The pituitary concentrations of radioactivity following administration of radioactive iodine-labelled β-estradiol (9) and progesterone-21-C^{14} (17, 132) were not confirmed with estrone-16-C^{14}.

The development of the many radioautographs prepared during these distribution studies is patiently awaited, since the location of radioactivity in the various organs and tissues should be discernible by this technique.

5. Experimental Work

(1) Determination of coincidence correction; the resolving times of the Geiger tube and windowless Q-gas counters

Two cleaned, copper discs[x] (area - 4.9 cm.^{2}) were carefully cut in half. To each half of one pair (S_1, S_2) was added a solution of radioactive steroid containing approximately 8000 d./min. (Geiger counter) and the half plates dried in the usual manner. The halves of the other disc were used as blank dummies (D_1, D_2). A pair of halves was placed upon a normal copper disc. The sequence of pair counting was D_1S_2, S_1D_2, D_1D_2, and S_1S_2; each activity determination was carried out for sufficient time to totalize approximately 200,000 disintegrations.

Values for the Geiger Counter

	Observed Activity (including background)	Observed Activity (minus background)	
D_1S_2	213184 d./24 min.	8832 d./min.	= N_1
S_1D_2	223872 d./24 min.	9281 d./min.	= N_2
D_1D_2	433549 d./25 min.	17295 d./min.	= N_1N_2
S_1S_2	46.4 d./min.		

$$\text{Resolving time } (T_r) = \frac{N_1 + N_2 - N_1N_2}{2(N_1 \times N_2)}$$

$$= 299 \text{ microseconds.}$$

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[x] All discs were dipped momentarily in a cleaning solution of technical sulphuric acid and sodium dichromate, immediately rinsed several times with water and distilled water and air dried.

A coincidence error of 0.5% per thousand disintegrations for activities up to 5000 d./min., was calculated from the data of Reid (121) using this T_r value.

A similar set of half plates was prepared for the windowless Q-gas counter.

Values for the Q-gas Counter

	Observed Activity (including background)	Observed Activity (minus background)	
D_1S_2	274722 d./36 min.	7320 d./min.	= N_1
S_1D_2	274370 d./35 min.	7528 d./min.	= N_2
D_1D_2	268107 d./18 min.	14583 d./min.	= N_1N_2
S_1S_2	311 d./min.		

T_r = 140 microseconds.

This T_r value gave a coincidence error of 0.22% per thousand disintegrations for activities up to 9000 d./min. (121).

(2) Determination of the absorption curve of radioactive barium carbonate

As previously described (page 99, (ii)), a series of plates was prepared from approximately 1 g. of weakly radioactive barium carbonate and assayed for radioactivity. The range of weights varied from 1.4 to 25 mg./cm.^{2}. In accordance with the procedure of Yankwich et al. (117, 123), the observed

Figure II

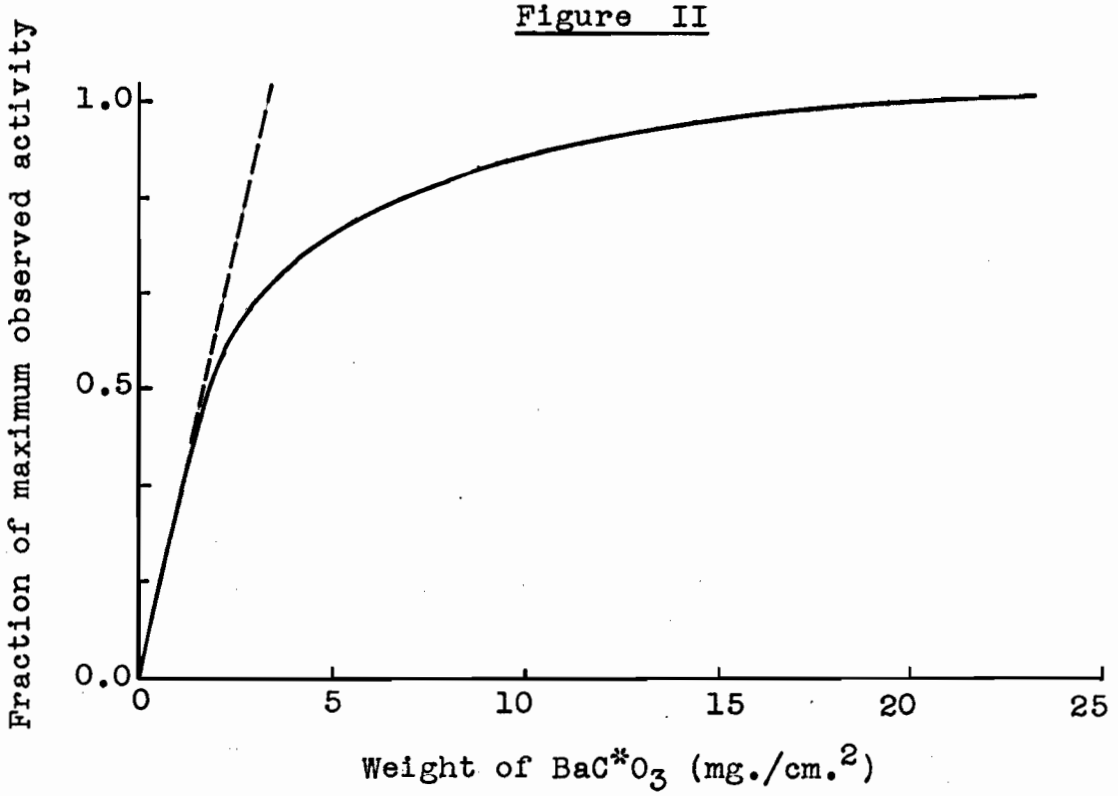
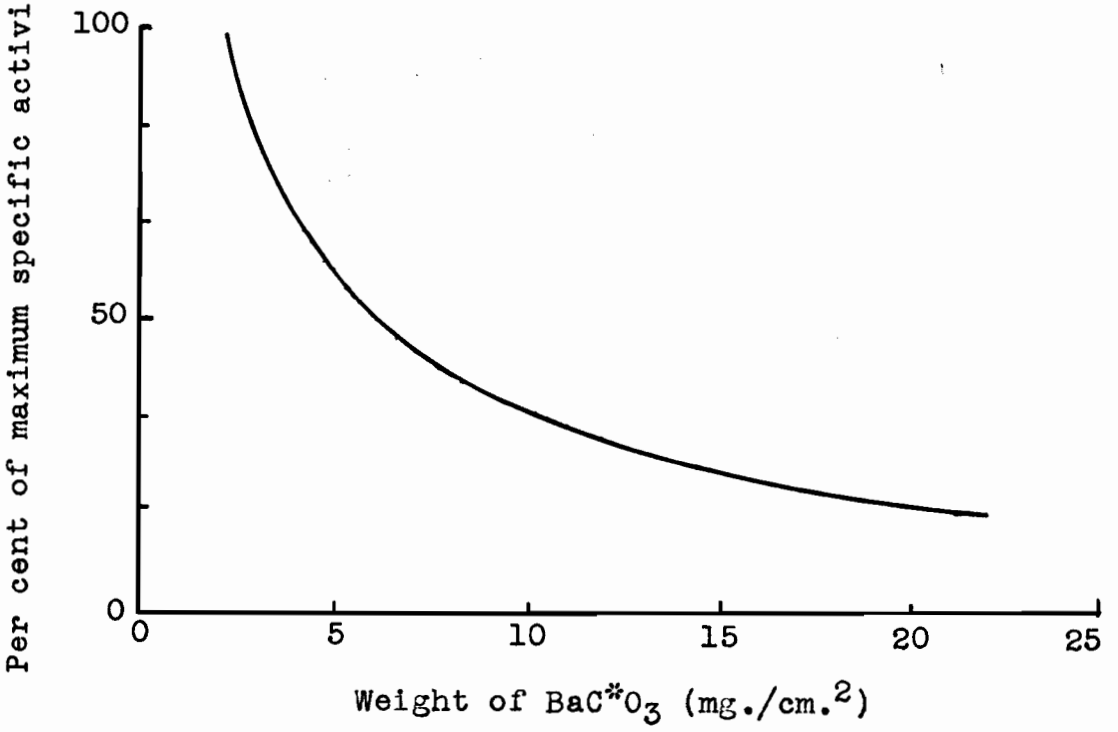


Figure III



activity of each plate was plotted as a fraction of the maximum observed activity, as shown in Figure II.

Figure III illustrates the absorption curve of radioactive barium carbonate and is derived from the preceding curve (Figure II). As the mass (i.e., thickness) of the barium carbonate plated was decreased, the observed self-absorption diminished until an almost linear relationship was obtained with sample thicknesses of less than 2 mg./cm.^{2}. Extrapolation of the almost linear portion of this curve gave the maximum specific activity obtainable for any given thickness measured at zero thickness. The ratio of the observed activity to this maximum specific activity was plotted (Figure III) for each determination on a percentage basis.

(3) Determination of activity of estrone-16-C^{14}

Estrone-16-C^{14} (0.922 mg.) was weighed accurately on a micro-balance into a 3-ml. volumetric flask and diluted to volume with distilled ethanol. A portion (1 ml.) of this solution was diluted to 50 ml. with ethanol. Three aliquots (each containing 5.532 micrograms) were plated and assayed for radioactivity as described in (i).

Geiger Counter

Sample No.	Observed Activity (Corrected as in (i)) d./min.
1	2760
2	2825
3	2775

Average = 2787 ± 45 (standard deviation = 22.5)

Calculated estrone-16-C^{14} activity = 5.03×10^5 d./min./mg.

Using the windowless flow Q-gas counter, 3 aliquots (each containing 1.106 micrograms) of a more dilute solution of this estrone-16-C^{14} afforded an activity of 2.61×10^6 d./min./mg.

Activities of other estrone-16-C^{14} preparations, as recorded throughout this thesis, were assayed in identical fashion.

SUMMARY

1. The application of the Arndt-Eistert synthesis to the preparation of estrone, labelled with C^{14} at position 16, was described.

2. The radioactive diazomethane used in these syntheses was prepared from barium carbonate-C^{14} through the intermediary compound, sodium cyanide-C^{14}; yields claimed by the original workers were substantiated.

3. A maximum yield of marrianolic acid dimethyl ester-3 methyl ether from the hypoiodite oxidation of estrone-3 methyl ether was obtained following the isolation of free marrianolic acid-3 methyl ether.

4. Estrone-16-C^{14} was hydrogenated to β-estradiol-16-C^{14}; estrone-3 methyl ether-16-C^{14} was converted to estriol-3 methyl ether-16-C^{14}. Demethylation of this latter methyl ether with pyridine hydrochloride proceeded with simultaneous dehydration to estrone-16-C^{14}.

5. Evidence was presented supporting a primary carbonyl carbon atom elimination in the cyclization of homomarrianolic acid-3 methyl ether to estrone-3 methyl ether.

6. A rapid and reliable direct-plating technique for the assay

of C^{14} activity in urine and organic extracts of urine and feces was recounted.

7. The preliminary metabolic experiments with estrone-16-C^{14} have confirmed the urine and feces as major excretory routes of labelled metabolites of naturally occurring, radioactively tagged estrogens. The major portion of these C^{14}-containing metabolites was of ether insoluble, water soluble nature, suggesting degradation of the original steroidal skeleton. The appearance of radioactivity in the respiratory carbon dioxide supported this hypothesis. In addition, a different handling of small and large doses of estrone-16-C^{14} was inferred.

CLAIMS TO ORIGINAL RESEARCH

The following abstracts from this thesis are claimed to be of an original nature.

1. The preparation of estrone-16-C^{14}.
2. An improved yield for the Arndt-Eistert extension of optically active marrianolic acid tertiary monomethyl ester-3 methyl ether to homomarrianolic acid dimethyl ester-3 methyl ether.
3. The isolation of the following crystalline compounds during these investigations:
   (a) marrianolic acid dimethyl ester-3 methyl ether (and 16-C^{14})
   (b) marrianolic acid primary monomethyl ester-3 methyl ether
   (c) diazoketone of marrianolic acid tertiary monomethyl ester-3 methyl ether
   (d) marrianolic acid tertiary monomethyl ester-3 methyl ether-16-C^{14}
   (e) marrianolic acid-3 methyl ether-16-C^{14}
   (f) homomarrianolic acid-3 methyl ether-16(and 16a)-C^{14}
   (g) estrone-3 methyl ether-16-C^{14}.
4. The hydrogenation of estrone-16-C^{14} to β-estradiol-16-C^{14}.
5. The conversion of estrone-3 methyl ether-16-C^{14} to estriol-3 methyl ether-16-C^{14}.

6. Demethylation and dehydration of estriol-3 methyl ether-16-C^{14} to estrone-16-C^{14} by treatment with pyridine hydrochloride.
7. Evidence supporting a primary carbonyl carbon atom elimination in the cyclization of homomarrianolic acid-3 methyl ether to estrone-3 methyl ether.
8. The development of a direct-plating technique for C^{14} assay in urine and organic extracts of urine and feces.
9. The elimination of C^{14} activity in feces, urine, and respiratory carbon dioxide following administration of estrone-16-C^{14}; the high water solubility of fecal and urinary C^{14} metabolites of estrone-16-C^{14}; observations supporting a difference in handling of small and large doses of estrone-16-C^{14} in mice.

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