Fig. 1. Hydrolysis of salep mannan by α -amylase preparations. (a) *A. oryzae* α -amylase: (1) crude enzyme; (2) once-crystallized enzyme; (3) four-times-crystallized enzyme. (b) *B. subtilis* α -amylase: (1) crude enzyme; (2) once-crystallized enzyme; (3) five-times-crystallized enzyme. (c) Human-salivary (○) and pig-pancreatic (●) α -amylases, crystallized thrice and twice respectively. Conditions were as described in the Experimental section.

170 mg. of glucose in 3 min., under the conditions of the activity determination (Fischer & Stein, 1961a). The reducing substances formed from mannan by the recrystallized fungal and bacterial amylases were equivalent only to about 1.5 mg. of mannose after 100 hr., and to about 0.5 mg. for the salivary and pancreatic enzymes. It is clear that most, if not all, of the hydrolysis of mannan caused by the crude *A. oryzae* and *B. subtilis* amylases is due not to α -amylase but to a separate

mannanase which is gradually removed as the amylase is purified. The recrystallized amylases are without significant action on the mannan.

SUMMARY

1. A reported hydrolytic attack of the α -amylases of *Aspergillus oryzae* and *Bacillus subtilis* on salep mannan was investigated. Crude preparations of the enzymes hydrolyse the polysaccharide but mannanase activity is lost when the enzymes are purified and crystallized. Several-times-crystallized specimens are without appreciable action on the mannan.

2. Crystalline α -amylases of human saliva and pig pancreas similarly have no appreciable action on the polysaccharide.

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The Biosynthesis and Metabolism of Oestrogens in the Rat

1. CONVERSION OF [4-¹⁴C]TESTOSTERONE INTO [¹⁴C]OESTRADIOL-17 β BY THE RAT OVARY

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Despite the fact that the rat is used extensively in endocrinological and biochemical research, little work has been published on the nature of the steroid hormone secretion in this animal, compared with man and other species. Thus, although Ketzer & Witt & Mitzner (1961) reported the identification of oestrone, oestradiol-17 α and oestradiol-17 β in the urine of the rat, there appears to be little or no other information on the secretion of oestrogen in this species.

The difficulty of determining steroids, particu-

larly oestrogens, in the body fluids of small rodents has hampered the interpretation of the role of endogenous sex hormones in carcinogenesis in these animals. Studies by Cronkite, Shellabarger, Bond & Lippincott (1960), however, suggest that the neoplastic response of breast tissue in the rat may be very dependent on the secretion of oestrogen by the ovary after whole-body irradiation.

One way of circumventing the difficulty of determining the secretion of small amounts of steroids is to measure the rate of biosynthesis of

the hormones under investigation from a radioactive precursor. The effect of applied variables, such as X-irradiation, on the rate of this biosynthesis may then be studied.

The conversion of androgens into oestrogens by ovarian tissue *in vitro* has been described for the human (e.g. Baggett, Engel, Savard & Dorfman, 1956; Wotiz, Davis, Lemon & Gut, 1956; Smith & Ryan, 1961) and for the dog (Hollander & Hollander, 1958). From these studies and many investigations *in vivo*, in these and other species, it has been speculated that this conversion represents a functional biosynthetic route *in vivo*.

We have studied, by reverse isotope dilution, the conversion of androgen into oestrogen by rat ovarian tissue *in vitro*. The present paper describes the biosynthesis and identification of [^{14}C]oestradiol-17 β derived from [^{14}C]testosterone as precursor. A preliminary account of this work has been given (Stitch, Oakey & Eccles, 1962). It cannot be assumed, however, that this metabolic process demonstrated *in vitro* represents a physiologically significant biosynthetic process in the rat *in vivo*.

MATERIALS AND METHODS

Materials

Solvents. All solvents, except where specified below, were of Analytical Reagent grade and were redistilled in all-glass apparatus. The middle fraction, of known boiling point, was collected. Ether was distilled from ferrous sulphate immediately before use. Hexane, free from aromatic hydrocarbons (British Drug Houses Ltd., Poole, Dorset), was of Laboratory Reagent grade and was redistilled before use.

Light petroleum (b.p. 80–100°) (British Drug Houses Ltd.) was used without further purification. Light petroleum (b.p. 90–102°) (ligroin, Laboratory Reagent grade; British Drug Houses Ltd.) was also used without further purification.

Propylene glycol (Laboratory Reagent grade; British Drug Houses Ltd.) was redistilled under reduced pressure.

Reagents. All inorganic and organic reagents, except where specified below, were of Analytical Reagent grade. Digitonin (British Drug Houses Ltd.) was purified by two recrystallizations from a mixture of methanol and diethyl ether. Dimethyl sulphate (General Purpose reagent; Hopkin and Williams Ltd., Chadwell Heath, Essex) was redistilled under reduced pressure before use.

Steroids. Oestrone [3-hydroxyoestra-1,3,5(10)-trien-17-one], supplied by L. Light and Co. Ltd., Colnbrook, Bucks., oestradiol-17 β [3,17 β -dihydroxyoestra-1,3,5(10)-triene] and oestradiol-17 α [3,17 α -dihydroxyoestra-1,3,5(10)-triene], both supplied by Schering A.G., Berlin, Germany, and testosterone (17 β -hydroxyandrost-4-en-3-one), supplied by Paines and Byrne Ltd., Greenford, Middlesex, and by Ciba Ltd., Horsham, Sussex, were all checked for chromatographic purity before use (Oakey, Stitch & Eccles, 1963). Oestradiol-17 β 3-methyl ether [3-methoxy-17 β -hydroxyoestra-1,3,5(10)-triene] was prepared from oestradiol-17 β with dimethyl sulphate (Brown, 1955). An

authentic specimen of oestradiol-17 β 3-methyl ether, for purposes of comparison by infrared spectroscopy, was supplied by Professor W. Klyne, M.R.C. Steroid Reference Collection, Westfield College, London, N.W. 3. Other steroids, used as carriers, were provided by Dr R. I. Dorfman, Worcester Foundation for Experimental Biology, Shrewsbury, Mass., U.S.A.

Chorionic gonadotrophin. Ampoules of 500 i.u., supplied by Paines and Byrne Ltd., were used.

Radioactive substrate. [^{14}C]Testosterone was supplied by The Radiochemical Centre, Amersham, Bucks. The material was stated, on supply, to have a specific activity of 21.5 mc/m-mole and radiochemical purity 100% (by dilution analysis). Examination of this material before use by paper chromatography in light petroleum (b.p. 90–102°)-propylene glycol (Savard, 1953) by the method of Stitch & Oakey (1961) showed that the bulk of the radioactivity accompanied the testosterone but revealed a trace (1%) of material with mobility identical with that of androst-4-ene-3,17-dione, and a small amount of radioactivity remaining on the starting line of the chromatogram.

Materials for chromatography. Whatman no. 1 filter paper sheets were 20 cm. $\times$ 50 cm. (selected for chromatography, W. R. Balston Ltd., and supplied by Reeve Angel and Co. Ltd., 9 Bridewell Place, London, E.C. 4). Celite 535 was supplied by Johns Manville and Co., 20 Albert Embankment, London, S.E. 11. The Celite was purified by treatment with HCl, washing and drying, as described by Bauld (1955). Alumina was supplied by Savory and Moore Ltd., 60 Welbeck Street, London, W. 1, and was deactivated and standardized by the procedure of Brown (1955).

Methods

Assay of ^{14}C -labelled materials. All radioassays were carried out by liquid-scintillation counting (Stitch, 1959).

Assay of carrier oestrogens. The three carrier oestrogens, oestrone, oestradiol-17 β and oestriol, from the incubation mixture, were determined, after chromatographic purification, by fluorimetry (Oakey *et al.* 1963).

Tissue. Eight female rats of the Harwell strain of Wistar origin were killed, without reference to their position in the oestrous cycle, by stunning and decapitation, at about 200 days of age. The ovaries were excised immediately, freed from adhering fat as far as possible, weighed and sliced freehand with scalpel and scissors to a thickness of about 0.5 mm. The weight of the combined ovaries was about 1.1 g.

Incubations. [^{14}C]Testosterone (4 μC) was evaporated to dryness, under a stream of nitrogen, in each of two 250 ml. round-bottomed flasks. The material in each flask was then suspended in propylene glycol (0.5 ml.). Krebs-Ringer phosphate buffer, pH 7.2 (40 ml.), containing glucose (1 mg./ml.) and chorionic gonadotrophin (500 i.u.), was then added. The tissue was divided between the flasks and incubation was continued at 37° with regular mechanical agitation for 5 hr.

Isolation of radioactive material from the incubation mixture. The distillation of all solvents from extracts and the concentration of solutions in organic solvents were carried out by distillation at a temperature not exceeding 60°, under reduced pressure and in a stream of nitrogen. After incubation, 1 mg. each of oestrone, oestradiol-17 β and oestriol were added to the incubation mixture. Testo-

sterone (5 mg.), and 1 mg. each of androst-4-ene-3,17-dione, androsterone (3 α -hydroxy-5 α -androst-17-one), dehydroepiandrosterone (3 β -hydroxy-androst-5-en-17-one) and progesterone (pregn-4-ene-3,20-dione), were added as carrier, and the protein of the incubation mixture was precipitated by the addition of 200 ml. of acetone. The incubation mixture was kept overnight at -20° and the acetone removed by distillation. The residual aqueous residue was extracted with ether (five portions of 50 ml.) and the radioactivity extracted was determined. The ether was reduced in volume to about 50 ml. and the concentrate was extracted with n -NaOH (four portions of 40 ml.). The alkaline extract, containing oestrogen carriers and associated radioactivity, after back-extraction with ether (two portions of 40 ml.), was adjusted to pH 7-8 with 11*N*-HCl (Engel, Slaunwhite, Carter & Nathanson, 1950), and the neutral solution was extracted with ether (five portions of 100 ml.). The combined ether extracts were washed with water (two portions of 50 ml.), dried over anhydrous sodium sulphate and reduced in volume to 100 ml. The radioactivity in this fraction was determined. The ether solution was distilled to dryness, the residue was dissolved in ethanol (0.5 ml.) and benzene, and the oestriol was separated from the oestrone and oestradiol by partition between the benzene and 75 mM- Na_2HPO_4 (Friedgood, Garst & Haagen-Smit, 1948).

RESULTS

The radioactivity obtained in the fractions during the chemical purification of the oestradiol-17 β is shown in Table 1.

Partition chromatography of oestrone-oestradiol fraction. A portion (one-twentyseventh) of the fraction containing the oestrone and oestradiol-17 β carriers was chromatographed on a column of Celite according to the method of Bauld (1955), but modified by using an initial elution with benzene-hexane (1:1, v/v) (30 ml.) followed by benzene (30 ml.) and benzene-ethylene dichloride (1:3, v/v) (50 ml.) and, finally, methanol (20 ml.), to elute further radioactivity from the column. The distribution of radioactivity and carrier oestrone and oestradiol-17 β from this column is shown in Fig. 1.

Isolation of oestradiol-17 β by partition chromatography. The remainder of the fraction containing the oestrone and oestradiol-17 β was evaporated to dryness. The material was then fractionated on a partition column (16 mm. $\times$ 21 mm.; 20 g. of Celite containing 16 ml. of 0.8*N*-sodium hydroxide) which was developed with benzene (200 ml.), benzene-ethylene dichloride (1:3, v/v) (180 ml.) and ethylene dichloride (180 ml.). Fractions of 10 ml. were collected and the position of the carrier oestrogen was determined fluorimetrically on portions (0.2 ml.) taken from alternate fractions. The fractions containing the eluted oestradiol-17 β carrier were combined, the amount of carrier and radioactivity determined, and, from these assays,

the specific activity with respect to oestradiol-17 β was calculated (Table 2).

Separation into α - and β -hydroxy fractions with digitonin. The purified oestradiol-17 β fraction from the preceding column was evaporated to dryness. Oestradiol-17 α (500 μg .) was added and separation of the mixture of oestradiol-17 β from

Table 1. *Distribution of radioactivity among fractions obtained during the purification of oestradiol-17 β*

Experimental details are given in the text. The coefficient of variation in the counting statistic was within 2%.

Purification stage	Radioactivity present	
	$10^{-4} \times$ Disintegrations/min.	Percentage of precursor
[4- ^{14}C]Testosterone precursor	1776	100
Non-phenolic	1370	77
Total phenols	51	2.88
'Oestrone + oestradiol' fraction	42	2.36
'Oestriol' fraction	12.60	0.71
Oestradiol-17 β after primary chromatographic isolation	2.95	0.16
β -Fraction after first precipitation with digitonin	1.26	0.07
β -Fraction after second precipitation with digitonin	0.49	0.03
α -Fraction after separation from β -fraction with digitonin	1.0	0.05

Fig. 1. Distribution of radioactivity, and carriers after partition chromatography of the phenolic extract of the incubation mixture, on Celite. Experimental details are given in the text. Peak 1 is oestrone, peak 2 is oestradiol-17 β . ●, Radioactivity; ○, carrier oestrogen. The histogram represents radioactivity eluted from the column with methanol.

oestradiol-17 α carriers was achieved with digitonin (Table 1). Aqueous methanol (60%, v/v) was used in place of ether for the precipitation of the β -steroid complex (Pearlman, Pearlman & Rakoff, 1954) since initial experiments confirmed that ether tends to break up the oestradiol-17 β -digitonin complex. The specific activity of the β -fraction was determined by assay of carrier and radioactivity (Table 2). The α -fraction remaining was evaporated to dryness, again treated with digitonin and gave a second small precipitate of radioactive material (Table 1).

Paper chromatography of oestradiol-17 β after precipitation with digitonin. To a portion of the β -fraction precipitated with digitonin and containing 11.5 μ g. of oestradiol-17 β , oestradiol-17 α carrier (10 μ g.) and further oestradiol-17 β carrier (8 μ g.) was added.

Examination by paper chromatography in the system light petroleum (b.p. 80–100°)–methanol–water (5:4:1, by vol.) (Oakey, 1961; Oakey *et al.* 1963) showed the absence of any radioactivity accompanying the oestradiol-17 α carrier, and the coincidence of the radioactivity with the oestradiol-17 β (Fig. 2). The position of the carrier oestrogens was shown, on a strip cut lengthwise from the centre of the paper, by dipping in a mixture of aq. 1% (w/v) ferric chloride and aq. 1% (w/v) potassium ferricyanide (Barton, Evans & Gardner, 1952). A photographic record of this was prepared (Fig. 2b) (Stitch & Oakey, 1961). Radioactivity on the remainder of the chromatogram was determined, by liquid-scintillation counting, of segments ($\frac{1}{2}$ in.) cut across the paper (Stitch & Oakey, 1961).

Serial purification I of oestradiol-17 β . Oestradiol-17 β carrier (20 mg. approx.) was added to a portion (one-fifth) of the first oestradiol-17 β fraction (containing 2520 14 C disintegrations/min. and 46 μ g. of oestradiol) obtained after purification with digitonin. The specific activity of this solution was determined by assay of the carrier and the radioactivity (Table 3). The solution was distilled to dryness and the crystalline residue recrystallized from hot aqueous ethanol (four recrystallizations) and from chloroform–hexane (two recrystallizations). The specific activity of the oestradiol-17 β as well as that of the residual liquors was determined at each stage of the six fractional recrystallizations. The constant specific activity obtained in the last four recrystallizations is shown in Table 3.

After the final recrystallization the material was chromatographed on a column of Celite (10 mm. $\times$ 140 mm.) with aqueous methanol as the stationary phase and eluted with benzene–hexane (9:11, v/v) (50 ml.), benzene–hexane (4:1, v/v) (80 ml.) and ethylene dichloride (50 ml.) (Oakey *et al.* 1963). Fractions of 3 ml. were collected. The radio-

activity was eluted with the carrier oestradiol-17 β and the specific activity of the fractions from the column compared with the specific activity obtained after recrystallization. There was no change

Table 2. *Purification of radioactive fractions containing oestradiol-17 β*

Experimental details are given in the text. The specific activity of the oestradiol-17 β fraction isolated chromatographically is expressed as disintegrations/min./mg. at subsequent stages of purification. The coefficient of variation in the counting statistic was within 2%.

Purification stage	$10^{-3} \times$ Specific activity (disintegrations/min./mg.)
Oestradiol pool after fractionation on celite	62.5
β -Fraction after precipitation with digitonin	54
Oestradiol-17 β 3-methyl ether after methylation	52
Fractions of peak radioactivity after chromatography of the methyl ether	61, 54.5, 69, 59

Fig. 2. Paper partition chromatography of the β -fraction. Oestradiol-17 α and oestradiol-17 β carriers were added to the radioactive β -fraction. Experimental details are given in the text. The material was loaded along the starting line of the paper and chromatographed in the system light petroleum (b.p. 80–100°)–methanol–water (5:4:1, by vol.). (a) The histogram shows the distribution of radioactivity on the chromatogram. (b) Photographic record of positions of carrier oestradiol-17 α ($E_{2\alpha}$) and oestradiol-17 β ($E_{2\beta}$) on a strip cut lengthwise from the centre of the chromatogram [aligned with (a)].

in the specific activity in the fractions of peak radioactivity on chromatography (Table 3).

Serial purification II of oestradiol-17 β . A portion (one-half) of the oestradiol-17 β from the digitonin separation (containing 6300 ^{14}C disintegrations/min. and 116 $\mu\text{g.}$ of oestradiol-17 β) was distilled to dryness. The residue was methylated and purified by the procedure described by Brown (1955), and the specific activity of the methylated oestradiol was calculated (Table 2) after assay of the carrier oestradiol-17 β 3-methyl ether and accompanying radioactivity.

Chromatography of oestradiol-17 β 3-methyl ether. The purified oestradiol-17 β 3-methyl ether was chromatographed on a column of alumina (23 mm. $\times$ 100 mm., containing 20 g. of alumina). Elution was carried out fractionally with benzene-light petroleum (b.p. 80–100°) (1:3, v/v) (20 ml.), benzene-light petroleum (2:3, v/v) (20 ml.) and benzene (200 ml.). Fractions of 3 ml. were collected and the position of carrier and radioactivity determined on portions of eluate. The peak of radioactivity eluted coincided precisely with the carrier oestradiol-17 β 3-methyl ether (Fig. 3a) and a plot of activity against effluent fraction volume across the peak showed no regression (Fig. 3b), strongly indicating the identity of the radioactivity with the carrier material.

The fractions containing the peak of radioactivity were combined, distilled to dryness and chromatographed on paper for 2.5 hr., in the system light petroleum (b.p. 90–102°)–methanol–water (25:24:1, by vol.). The position of carrier oestradiol-17 β 3-methyl ether and the radioactivity

Fig. 3. (a) Adsorption chromatography of the oestradiol-17 β 3-methyl ether on alumina. Experimental details are given in the text. ●, Distribution of radioactivity; ○, carrier oestradiol-17 β 3-methyl ether. (b) Specific activity of the fractions of peak radioactivity.

Table 3. *Serial purification I: recrystallization of oestradiol-17 β to constant specific activity*

Experimental details are given in the text. After the addition of 20 mg. of pure oestradiol-17 β , recrystallizations 1–4 were carried out from aqueous ethanol and recrystallizations 5 and 6 from chloroform–hexane. Purification stages 7 and 8 represent the fractions of peak radioactivity after chromatography (see the text). The coefficient of variation in the counting statistic was within 1.5%.

Purification stage	Specific activity (disintegrations/min./mg.)		
	Crystals	Residual liquors	Peak column fractions
Before recrystallization	116	—	—
1	120	140	—
2	116	128	—
3	100	117	—
4	101	123	—
5	99	113	—
6	101	110	—
7	—	—	101
8	—	—	108

Fig. 4. Paper chromatography of the oestradiol-17 β 3-methyl ether. Experimental details are given in the text. The fractions of peak radioactivity after chromatography on alumina (see Fig. 3a) were combined and applied along the starting line of the paper and chromatographed in the system light petroleum (b.p. 90–102°)–methanol–water (25:24:1, by vol.). (a) The histogram shows the distribution of radioactivity on the chromatogram. (b) Photographic record of the position of the carrier oestradiol-17 β 3-methyl ether (E_2M) on a strip cut lengthwise from the centre of the chromatogram [aligned with (a)].

were recorded by photography and liquid-scintillation counting (Stitch & Oakley, 1961; Oakley *et al.* 1963) (Fig. 4). The radioactivity ran precisely with the carrier oestradiol-17 β 3-methyl ether.

Chromatography of the α -fraction. The solution in aqueous methanol, containing the steroids not precipitable with digitonin, was evaporated to dryness and the residue extracted with warm ether (three portions of 5 ml.). The radioactivity was determined in this fraction (Table 1). To a portion containing 3000 ^{14}C disintegrations/min., oestradiol-17 β carrier (100 μg .) was added and the mixture chromatographed on paper for 48 hr. with light petroleum (b.p. 80–100°)–methanol–water (5:4:1, by vol.) (Oakley *et al.* 1963). The results showed the absence of any radioactivity in the region of the oestradiol-17 α carrier. A substantial amount of radioactivity accompanied the oestradiol-17 β carrier, indicating that precipitation of the β -steroid with digitonin was incomplete. Some radioactivity remained near the starting line and another small peak due to radioactive material less polar than the oestradiols was apparent.

Chromatography of non-phenolic fraction. The fraction remaining after extraction of the phenolic oestrogens with N-sodium hydroxide was examined by partition chromatography on paper by the Bush 'system A', light petroleum (b.p. 80–100°)–methanol–water (5:4:1, by vol.), the running time being 8.5 hr. The results showed that most of the radioactivity on the paper accompanied the testosterone carrier and that the remainder was mainly in the region corresponding to the androst-4-ene-3,17-dione.

DISCUSSION

The evidence described above shows beyond reasonable doubt that the ovary of the virgin female rat can metabolize [4- ^{14}C]testosterone to produce, among other compounds, [^{14}C]oestradiol-17 β . Since the position of the ^{14}C in the oestrogen molecule has not been located, proof of the direct conversion from testosterone, and the exclusion of biosynthesis from breakdown products of the precursor, is not available. This, however, is unlikely because of the large amount of unchanged testosterone that was recovered in the non-phenolic fraction and the relatively high incorporation of that portion of the testosterone that was, in fact, metabolized. Conversions such as have been described above should not necessarily be associated with any biological significance *in vivo* as representing a true pathway of oestrogen biosynthesis. Proof that oestradiol-17 β is secreted *in vivo*, by the ovary of the rat, and indeed, by the ovaries of many other species, is lacking. Such evidence has, however, been obtained for the human by Varangot & Cedard (1960), who demon-

strated higher concentrations of oestradiol-17 β in venous blood of the cannulated ovary than in the peripheral venous blood.

The claim by Ketz *et al.* (1961) that oestradiol-17 α is excreted by the rat is of interest, since we were unable to demonstrate the biosynthesis of this oestrogen *in vitro*. The oestradiol-17 α excreted may represent a metabolic end-product of oestradiol-17 β or oestrone. It has, for example, been shown by Breuer & Pangels (1960) that rabbit-liver slices can convert oestradiol-17 β into oestrone and oestradiol-17 α *in vitro*. Alternatively, the failure of the ovary to produce oestradiol-17 α from testosterone *in vitro* may indicate that aromatic conversion of the A ring of testosterone occurred without prior oxidation to androst-4-ene-3,17-dione. If the metabolic sequence had involved oxidation of testosterone to androst-4-ene-3,17-dione, followed by conversion into oestrone, reduction of this oestrogen might have yielded a proportion of the 17 α - as well as the 17 β -isomer in these circumstances. Fishman, Bradlow & Gallagher (1960) have shown, in the human, that oestrogen metabolism *in vivo* is largely oxidative, however, and conversion of oestradiol-17 β into oestrone is very rapid, whereas the reverse process is extremely slow. These authors conclude that, although oestriol, 2-methoxyoestrone and epi-oestradiol are derived from oestrone and not directly from oestradiol-17 β , oestrone itself is a direct metabolite of oestradiol-17 β .

Huang & Pearlman (1962) were unable to demonstrate the biosynthesis *in vitro* of oestrogen from [7- ^3H]androst-4-ene-3,17-dione by fully luteinized rat ovarian tissue. However, the value of 0.5 % maximum possible conversion described by these authors was higher than the conversion obtained by us using the ovaries from eight animals and with [4- ^{14}C]testosterone as precursor. The failure to demonstrate oestrogen biosynthesis from androst-4-ene-3,17-dione with luteinized ovarian tissue may indicate, in the rat at least, that the corpus luteum is not involved in oestrogen biosynthesis. Such a view is supported by the report of Falck (1959) that isolated luteal cells of the rat do not secrete oestrogen, the production of which is dependent on the joint action of combined cell systems of the ovary.

SUMMARY

1. The biosynthesis of oestradiol-17 β from [4- ^{14}C]testosterone by rat ovarian tissue has been demonstrated *in vitro*.

2. The specific activity of the oestradiol isolated was followed through multiple purifications by chromatography on columns and on paper, precipitation with digitonin, six recrystallizations in

two different solvent systems followed by partition chromatography, and by methylation, purification of the methyl ethers and chromatography in two successive systems.

3. The biosynthesis of at least five radioactive compounds in the phenolic fraction was indicated.

4. The lack of evidence for this biosynthesis as a significant biochemical process *in vivo* is emphasized.

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The Biosynthesis and Metabolism of Oestrogens in the Rat

2. THE CONVERSION OF [4-¹⁴C]TESTOSTERONE INTO [¹⁴C]OESTRADIOL-17 β BY THE OVARY AFTER WHOLE-BODY IRRADIATION

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Oestrous changes have been shown to persist in the vaginal smears of the rat for short periods after sterilizing doses (4400 r.) of X-irradiation and were followed, often, by periods of continuous vaginal cornification (Mandl & Zuckerman, 1956). This may indicate the ability of the X-irradiated ovary to produce oestrogen even in the absence of an organized follicular system. Slate & Bradbury (1962) observed no immediate irregularity in the vaginal cycles after X-irradiation, regardless of the dose. The continuation of cyclic vaginal change was never observed, however, when all follicular structures were destroyed after irradiation. It has been reported by Drips & Ford (1932) that even slight irradiation, one-tenth to one-fifteenth of the human erythema dose (less than 150 rads), caused temporary irregularity of the oestrous cycle in this

species. These authors noted, also, that the greatest disturbance of the subsequent oestrous cycles occurred when irradiation was carried out in the oestrous stage. With high doses of irradiation no cyclic activity was observed after the complete atrophy of the functioning structures of the ovary.

In mice, it was noted by Bischoff, Ulmann & Ingraham (1944) that oestrus was completely suppressed after 400 r. of X-irradiation and that the effect was irreversible during 13½ months of observation. These results are in contrast with those of Parkes (1926, 1927*a, b, c*), who observed regular oestrous cycles and behaviour up to 69 days after all follicles had been destroyed by irradiation. Nuzhdin, Shapiro, Petrova & Kitaeva (1955) reported that irradiation of the white mouse with doses as small as 50r. had a marked effect on