smaller particles but did not solubilize them. Much less gangliosides were extracted after ultrasonic treatment of homogenates containing nuclei.

5. The effect of added agents and various treatments to homogenates on the quantity of gangliosides extracted from particulate and supernatant fractions was studied.

6. Addition of protamine or histone completely prevented the extraction of gangliosides.

7. A comparison of the affinity of subcellular fractions for basic proteins with the content of gangliosides was made. The fractions containing most of the tissue gangliosides showed the greatest affinity for basic proteins except a fraction rich in myelin fragments.

8. The probable localization of gangliosides in cell membranes is discussed.

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Oestriol Metabolism by Rat- and Rabbit-Liver Slices

ISOLATION OF 2-METHOXYOESTRIOL AND 2-HYDROXYOESTRIOL

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When Marrian, Loke, Watson & Panattoni (1957) isolated 16 α -hydroxyoestrone (3:16 α -dihydroxy-oestra-1:3:5-trien-17-one) from pregnancy urine, they suggested that this compound might be the intermediate in the conversion of oestrone (3-hydroxyoestra-1:3:5-trien-17-one) into oestriol (oestra-1:3:5-triene-3:16 α :17 β -triol). To test this suggestion, experiments were started to see if liver preparations could convert 16 α -hydroxyoestrone into oestriol. Rat-liver slices could readily bring about this reduction but during the experiments the observation was made that the yield of oestriol

was dependent on the gas phase above the incubation medium. When incubation was under air the yield of oestriol was 10 % but increased to 22 % on carrying out the incubation under nitrogen (R. J. B. King & G. F. Marrian, unpublished results). A number of other workers have found that oestrogen metabolism by rat-liver preparations is influenced by the gas phase used during the incubation (Ryan & Engel, 1953; Szego, 1953; Riegel & Mueller, 1954).

A number of new oestrogen derivatives have been isolated from biological material in recent years. These include 6' α '-hydroxyoestradiol-17 β (oestra-1:3:5-triene-3:6' α '-17 β -triol) and 6-oxo-oestradiol-17 β (3:17 β -dihydroxyoestra-1:3:5-trien-6-one)

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(Mueller & Rumney, 1957), 18-hydroxyoestrone (3:18-dihydroxyoestra-1:3:5-trien-17-one) (Loke, Marrian, Johnson, Mayer & Cameron, 1958) and 2-methoxy derivatives of oestrone (Kraychy & Gallagher, 1957; Engel, Baggett & Carter, 1957; Loke & Marrian, 1958), oestradiol-17 β (oestra-1:3:5-triene-3:17 β -diol) (Frandsen, 1959) and oestriol (Fishman & Gallagher, 1958). Furthermore, Levitz, Spitzer & Twombly (1958) have shown that [16-¹⁴C]oestriol injected into women is in part excreted in the urine as 16-oxo-oestradiol-17 β (3:17 β -dihydroxyoestra-1:3:5-trien-16-one) and 16-*epi*-oestriol (oestra-1:3:5-triene-3:16 β :17 β -triol). In view of the number of possible metabolites, the oxidative metabolism of oestriol by liver preparations was further investigated. This paper describes the isolation and characterization of 2-hydroxy-oestriol (oestra-1:3:5-triene-2:3:16 α :17 β -tetrol) and 2-methoxyoestriol (2-methoxyoestra-1:3:5-triene-3:16 α :17 β -triol) by incubating oestriol with rat- and rabbit-liver slices.

A preliminary account of the work published in this and the following paper has been presented to The Biochemical Society (King, 1960).

RESULTS

Oestriol metabolism of rat-liver slices and detection of two phenolic metabolites

The recovery of oestriol after incubation with rat-liver slices for 1 hr. in Krebs-Ringer phosphate was 55, 42 and 13 % when incubated under nitrogen, air and oxygen respectively. The nitrogen used was unpurified commercial material and probably contained traces of oxygen. This would suggest that the amount of oestriol metabolized under completely anaerobic conditions may be even less than shown here.

One stage of the extraction procedure used in this preliminary experiment involved the extraction of the phenols into *n*-NaOH. As certain of the recently discovered oestrogen metabolites are alkali-labile (see Layne & Marrian, 1958), the alkali-treatment was omitted from the extraction procedure used in all the subsequent experiments.

When oestriol was incubated with liver slices under oxygen and the ether extract of this incubation was chromatographed on paper in the formamide-CHCl₃ system for 11 hr., two phenolic metabolites were detected. They were both non-ketonic. Metabolite I had a mobility intermediate between that of 16-*epi*oestriol and 17-*epi*oestriol (oestra-1:3:5-triene-3:16 α :17 α -triol), whereas metabolite II was more polar than oestriol and did not migrate from the origin even after development in this system for 48 hr. Metabolite II was anomalous in that it developed the Folin & Ciocalteu blue colour before the paper was suspended in the ammonia tank.

In the system acetic acid-water-ethylene dichloride (70:30:100, by vol.), I had the same mobility as 16-*epi*oestriol whereas II was more polar than oestriol.

Loke (1958) has detected a compound in the phenolic ketonic fraction of late-pregnancy urine which he suggested was 2-methoxy-16 α -hydroxy-oestrone (2-methoxy-3:16 α -dihydroxyoestra-1:3:5-triene-17-one). On reduction of this compound with sodium borohydride, he obtained a product which, on his hypothesis, was 2-methoxyoestriol. This reduction product had the same chromatographic behaviour as I. This suggested that II might be 2-hydroxyoestriol.

Isolation of 2-methoxyoestriol after incubation of oestriol with rat-liver slices

Oestriol was incubated with rat-liver slices under oxygen in Krebs-Ringer phosphate solution, pH 7.4, for 4 hr., the medium was homogenized, extracted with ether and metabolite I separated from oestriol by paper chromatography in formamide-CHCl₃. After elution with methanol, I was considerably purified by discontinuous gradient elution from an alumina column with increasing amounts of methanol in benzene. The material eluted with 3 % of methanol in benzene was crystallized from methanol-benzene and characterized as 2-methoxyoestriol by comparison of the melting point, optical rotation and infrared spectrum with the values quoted for 2-methoxyoestriol (Fishman & Gallagher, 1958).

The yield of crystalline 2-methoxyoestriol was 15.4 mg. from 550 mg. of oestriol. The yield of II was insufficient for complete characterization.

Isolation of 2-hydroxyoestriol after incubation of oestriol with rabbit-liver slices

Rabbit-liver slices could produce appreciable amounts of II but only trace amounts of I; ox-liver slices produced neither of these two metabolites. To facilitate the isolation of reasonable amounts of II, rabbit-liver slices were employed.

Metabolite II is unstable under oxidative conditions, especially in alkaline solution, and consequently anaerobic conditions were used wherever possible. II was isolated by ether extraction and chromatography on paper in acetic acid-water-ethylene dichloride (70:30:100, by vol.). After elution with methanol, II was exhaustively leached with ethyl acetate and the combined ethyl acetate-soluble material crystallized from aqueous 50 % acetone. It was identified as 2-hydroxyoestriol by comparison of the melting point, ultraviolet and infrared spectra with those of authentic 2-hydroxyoestriol. From 190 mg. of oestriol, 1.3 mg. of crystalline 2-hydroxyoestriol was obtained.

Table 1. *Formation of 2-methoxyoestriol and 2-hydroxyoestriol by rat-liver slices*

Rat-liver slices (400 mg.), 4 ml. of Krebs-Ringer phosphate, pH 7.4, 200 μ g. of oestriol, in 0.05 ml. of propylene glycol, were incubated under oxygen for 2 hr. at 37°. The figures are given as percentages of oestriol added.

	Oestriol recovered	2-Methoxy-oestriol formed	2-Hydroxy-oestriol formed	Total recovery
Slices boiled 1 min. before incubation	70	1	0	71
Viable slices (oestriol added just before extraction)	70	0	0	70
Viable slices	5	10	18	33
No tissue (oestriol added just before extraction)	72	0	0	72

Quantitative study of the conversion of oestriol into 2-hydroxyoestriol and 2-methoxyoestriol by rat-liver slices

Results of this experiment are shown in Table 1. Even though synthetic 6' α' -hydroxyoestriol (oestra-1:3:5-triene-3:6' α' :16 α :17 β -tetrol) and 6-oxo-oestriol (3:16 α :17 β -trihydroxyoestra-1:3:5-triene-6-one) (Marrian & Sneddon, 1960) were available, no trace of either of these compounds could be detected.

It can be seen from Table 1 that about 40% of the added oestriol has been converted into undetected metabolites. Crepy (1947) has shown that guinea-pig- and rabbit-liver slices are capable of forming oestriol glucuronide. As this might account for some of the lost oestriol, the aqueous phase, after ether extraction of the free oestrogens, was extracted twice with 50 ml. portions of butanol and the butanol was evaporated to dryness under vacuum. The residue was hydrolysed with β -glucuronidase for 48 hr. and then extracted with ether and the free steroids were estimated as before. No oestriol, 2-methoxyoestriol or 2-hydroxyoestriol was liberated by this procedure.

EXPERIMENTAL

Preparation of liver slices and incubation medium. Rats and rabbits were killed by a blow on the back of the neck. The livers were rapidly removed and kept on ice until used. In the preparative-scale experiments the tissue slices were prepared with a Stadie-Riggs hand microtome, but in the other experiments the slices were cut free-hand to a thickness of about 0.5 mm. The slices were washed in ice-cold Krebs-Ringer phosphate, blotted dry and weighed.

Krebs-Ringer phosphate, pH 7.4, was prepared by the method of Krebs & Henseleit (1932). It was made up fresh from its component solutions for each experiment. The required gas was bubbled through this solution for at least 30 min. before the experiment was started. In the quantitative studies the gas was played on the surface of the incubation medium for 30 sec. and the tubes were immediately stoppered.

Paper chromatography. All papers were washed for 72 hr. in a Soxhlet apparatus with methanol- CHCl_3 (1:1, v/v) and

Table 2. *Chromatographic behaviour of a number of oestriol derivatives in acetic acid-water-ethylene dichloride (70:30:100, by vol.)*

Compound	R_F
16- <i>epi</i> Oestriol	0.7
Oestriol	0.6
6-Oxo-oestriol	0.4
2-Hydroxyoestriol	0.2
6' α' -Hydroxyoestriol	0.1

then dried at 37°. Chromatography was carried out in a thermostatically controlled room at $20 \pm 2^\circ$.

Analytical and preparative-scale chromatograms with formamide- CHCl_3 were carried out as described by Layne & Marrian (1958).

For the quantitative separation and isolation of 2-hydroxyoestriol the material was dissolved in methanol and transferred to Whatman 3 MM paper. After equilibration overnight in the chromatography tank, the paper was developed for about 3 hr. with acetic acid-water-ethylene dichloride (70:30:100, by vol.). About 10 μ g. of standard 2-hydroxyoestriol was run on each paper. This strip was cut out, sprayed with diluted Folin & Ciocalteu reagent and suspended in a tank containing NH_3 solution to locate the steroid. The corresponding areas of the main part of each paper were cut out and eluted with methanol in the apparatus designed by Saffran & Jarman (1960). The methanol was evaporated under nitrogen. The remainder of the paper was sprayed with Folin & Ciocalteu reagent to confirm that no oestriol was contaminating the 2-hydroxyoestriol area. When this system was used on a preparative scale, the material was applied to a large number of 14 cm. $\times$ 35 cm. strips of 3 MM paper as a band along the origin.

The average R_F of other oestrogen derivatives in this system with Whatman 3 MM paper are given in Table 2 (Whatman no. 1 paper may also be used). To obtain reproducible results with this solvent system rigid standardization of the procedure is essential.

Other chromatographic methods. Celite columns (1 cm. $\times$ 10 cm.), with the system methanol-water-ethylene dichloride (70:30:100, by vol.), were prepared by the method of Bauld (1955). In this system 2-methoxyoestriol is eluted with 4-11 ml. and oestriol with 11-35 ml. of ethylene dichloride. Alumina (Savory and Moore Ltd.) was used.

Melting points. All melting points were determined in sealed evacuated tubes and are uncorrected for emergent stem.

Girard reaction. The dry residue was dissolved in 1 ml. of ethanol and 1 ml. of acetic acid. Girard T reagent (5–10 mg.) (Girard & Sandulesco, 1936) was added and the solution allowed to stand overnight at room temperature. The vessel was chilled and 90% neutralized with cold N -NaOH. About 2 g. of NaCl was added and the volume made up to 25 ml. with cold water. This was extracted three times with 25 ml. of ether and the combined ether extract was washed once with 5 ml. of water. The ether was further washed twice with 5 ml. of 5% (w/v) $NaHCO_3$ and once with 5 ml. of water. The ether (non-ketonic) fraction was evaporated to dryness under vacuum.

The aqueous phase was acidified with 6 ml. of conc. HCl and left at room temperature for 1 hr. to hydrolyse the Girard complex. The solution was then extracted with ether and washed with $NaHCO_3$ and water as before to give the ketonic fraction.

Steroids. The oestriol, 16-epioestriol, 6-oxo-oestriol and 6 α -hydroxyoestriol were generous gifts from Dr G. F. Marrian, F.R.S. The 2-hydroxyoestriol was synthesized by Dr M. M. Coombes by a modification of the method used by Fishman (1958) to prepare 2-methoxyoestrogen derivatives. 2-Hydroxyoestradiol-17 β (oestra-1:3:5-triene-2:3:17 β -triol) was donated by Dr G. C. Mueller. The oestriol was added to the incubation flasks as a solution in propylene glycol.

Solvents. Ether (A.R.) was distilled once just before use. Other solvents and Celite were purified by the methods described by Bauld (1953).

β -Glucuronidase hydrolysis. To each flask were added 5 ml. of m -acetate buffer, pH 4.6, and 5×10^3 units (Fishman, Springer & Brunetti, 1948) of soluble β -glucuronidase obtained from *Patella vulgata* (Dodgson & Spencer, 1953). The flasks were stoppered and incubated at 37° for 2 days.

Estimation of steroids. Oestriol was estimated by means of the Kober reaction as modified by Brown (1952) and Bauld (1954). A modification of the method of Davies & Mitchell (1954) was used to estimate 2-hydroxyoestriol. m -Sodium carbonate (1.5 ml.) and 1.5 ml. of Folin & Ciocalteu reagent (diluted 1:4 with water) were added to the dry residue and incubated at 37° for 20 min. The resulting blue colour was read in a Unicam SP. 600 spectrophotometer at 745 $m\mu$.

When 2-methoxyoestriol is heated in boiling water for 4 min. with Bauld's (1954) 'oestriol reagent' and then cooled in cold water, the resulting red colour can be used to estimate the 2-methoxyoestriol. The colour has an absorption maximum at 522 $m\mu$, is stable for at least 20 min. at room temperature and obeys Beer's Law up to a concentration of 40 $\mu g.$ of steroid/3 ml. of reagent.

Extraction of steroids. In the preliminary experiments the following extraction procedure was used. The incubation medium plus liver slices were homogenized, 5 ml. of 2*N*-HCl was added, and the mixture extracted three times with 50 ml. of ether. The combined ether extract was washed twice with 20 ml. of 5% (w/v) $NaHCO_3$ and then extracted three times with 20 ml. of N -NaOH. The alkali was partly neutralized with 10 ml. of 5*N*- H_2SO_4 and then brought to pH 8–10 by bubbling CO_2 through the solution. The pH was checked with phenolphthalein indicator paper. Sodium chloride (10 g.) was added to prevent emulsification and the mixture extracted three times with 50 ml. of ether. The

ether extract was washed twice with 20 ml. of water and then evaporated to dryness under vacuum.

In all of the other experiments the $NaHCO_3$ wash and phenol separation were omitted so that the initial ether extract was evaporated to dryness directly.

Separation of 2-methoxyoestriol, oestriol and 2-hydroxyoestriol

The dry ether-soluble residue was dissolved in 2.5 ml. of methanol and a 1 ml. fraction of this used to estimate 2-hydroxyoestriol as described above. Another 1 ml. portion was evaporated to dryness on a warm-water bath with the aid of an air blast. The contaminating pigment was removed from this residue by discontinuous gradient elution from an alumina column (0.7 cm. $\times$ 6 cm.) deactivated with 2% water. The material eluted with 20 ml. of 1% methanol in benzene was discarded. The solvent was then changed to 4% methanol in benzene and 50 ml. of this solvent collected as the combined 2-methoxyoestriol and oestriol fraction. This was evaporated to dryness on a warm-water bath and the residue was dissolved in 2.5 ml. of ethylene dichloride. A portion (2 ml.) of this was transferred to a Celite column to separate the 2-methoxyoestriol and oestriol as described above. All of the 2-hydroxyoestriol is destroyed by this procedure.

Regular 70–75% recoveries of added oestriol could be obtained by this method, and the recovery of 2-methoxyoestriol was almost quantitative.

Large-scale isolation of metabolites I and II and their identification as 2-methoxyoestriol and 2-hydroxyoestriol

Oestriol (150 mg.) dissolved in 35 ml. of propylene glycol was incubated with 125 g. of rat-liver slices in 1.2 l. of Krebs–Ringer phosphate, pH 7.4, for 4 hr. The incubations were carried out under oxygen in a series of 250 ml. stoppered conical flasks. The flasks were reoxygenated after 2 hr. At the end of the incubation the contents of all the flasks were combined and homogenized in a Waring Blender. Hydrochloric acid (250 ml.; 2*N*) was added and the mixture extracted four times with 1 l. of ether. The ether extract was evaporated to dryness on a boiling-water bath. The resulting brown oil was extracted four times with 30 ml. of aqueous 70% methanol and the methanol was washed twice with 30 ml. of *n*-hexane. The aqueous methanol was evaporated to dryness under vacuum. The resulting brown oil was transferred to a large number of Whatman no. 42 papers and chromatographed in the formamide- $CHCl_3$ system (about 15 mg. of oil/15 cm.-wide paper) for 19 hr. The mobile phase which had run off the bottom of the papers was collected and evaporated to dryness under vacuum. This was called the 'eluate fraction'.

A complete separation of I from oestriol was achieved by this method, but the separation of oestriol and metabolite II was not satisfactory. The fraction containing II was rechromatographed in the same system for 60 hr. to remove the oestriol. Metabolites I and II were eluted with methanol to yield 25 mg. of I and 6 mg. of II. Another 400 mg. of oestriol was processed in the same way to give a further 71 mg. of I and 27 mg. of II.

Metabolite I was contaminated with a greenish-brown pigment which was removed by alumina chromatography.

The alumina was deactivated with 2% of water and a 1.5 cm. $\times$ 5 cm. column was prepared in benzene. Half of I was transferred to this column with benzene and eluted with increasing amounts of methanol in benzene. Portions (50 ml. each) of 0.5, 1.0 and 1.5% of methanol in benzene did not elute any of metabolite I but it was all eluted with 250 ml. of 3% of methanol in benzene. The other half of I was treated in the same way and the two parts were combined to yield 54 mg. of a yellowish oil. It was leached twice with 0.1 ml. of acetone to remove some of the yellow pigment. Two crystallizations from methanol-benzene did not completely remove an oily impurity but a further leaching with two portions of 0.1 ml. of acetone and subsequent crystallization from methanol-benzene gave 15.4 mg. of white crystals. These had m.p. 213–215°; $[\alpha]_D^{25} + 81.1^\circ$ in ethanol. The values quoted by Fishman & Gallagher (1958) for 2-methoxyoestriol are: m.p. 215–218°; $[\alpha]_D^{25} + 83^\circ$ in ethanol. The infrared spectrum of I was identical with that of 2-methoxyoestriol. The u.v. spectrum of I (ethanol $\lambda_{\max}$ 287 m μ , ϵ 3980; $\lambda_{\min}$ 253 m μ , ϵ 676) differed slightly from that of 2-methoxyoestriol (ethanol $\lambda_{\max}$ 286 m μ , ϵ 3700; $\lambda_{\min}$ 253 m μ , ϵ 350). This could be due to a trace impurity.

The batch of II obtained with rat-liver slices was impure and did not contain sufficient material to allow a complete characterization. This material was partially purified by precipitation from aqueous ethanol and was used for the preliminary experiments on the structure of this metabolite. The u.v.-absorption curve in ethanol showed a maximum at 287–289 m μ and a minimum at 254 m μ , which suggested that it was related to 2-methoxyoestriol. The colour reactions with Folin & Ciocalteu reagent, ammoniacal silver nitrate and 10% (w/v) ethylenediamine in aqueous ammonia (ammonia-water, 2:8) (Weil-Malherbe & Bone, 1957) were all compatible with II being a catechol. It was unstable under oxidative conditions, especially in alkali.

To facilitate the isolation of II, female rabbit-liver slices were used to prepare the next batch of II. The steroid:tissue:salt solution ratios were the same as had been used in the experiments with rats.

The incubation medium was extracted as before but the separation of oestriol and II was achieved by chromatography on a number of sheets of Whatman 3 MM paper in acetic acid-water-ethylene dichloride (70:30:100, by vol.) for 4 hr. The fraction containing II was eluted with methanol and rechromatographed in the same system for 5 hr. From 192 mg. of oestriol, 120 mg. of crude II was obtained as a dark-brown solid. This was exhaustively leached with 1 ml. portions of ethyl acetate and the ethyl acetate-soluble material was taken to dryness under nitrogen. The pale-yellow solid was washed three times with 0.5 ml. of ethyl acetate to remove most of the contaminating pigment. This left 19 mg. of white solid. This was crystallized twice from aqueous 50% methanol at 0°, the crystals being washed three times with 0.1 ml. of the same solvent after each crystallization. This gave 1.3 mg. of white crystals, which had m.p. 267–269° with shrinkage at 263°. The mixed m.p. with 2-hydroxyoestriol (m.p. 269–271°) was 265–268°.

The infrared spectrum of this material was virtually identical with that of authentic 2-hydroxyoestriol and the difference could be explained by the presence of a small amount of ketonic material absorbing at 1750 cm.⁻¹

Metabolite II obtained with rabbit-liver slices had the

same chromatographic behaviour, colour reactions, instability to alkali and u.v.-absorption curve as the material obtained with rat liver.

Attempted detection of other phenolic oestriol metabolites

The 'eluante fraction' obtained from 100 mg. of oestriol was chromatographed in formamide-CHCl₃ for 6 hr. No 16 α -hydroxyoestrone or 16-oxo-oestradiol-17 β could be detected.

Any 16-epioestriol which might have been formed from oestriol would have been isolated with the 2-methoxyoestriol. The mother liquors from the isolation of 2-methoxyoestriol were combined to give 40 mg. of brown oil. Although 16-epioestriol and 2-methoxyoestriol cannot be separated adequately by chromatographic methods, acetonide formation would distinguish these two compounds. The method used was that of H. J. Blair & J. B. Brown (personal communication). The brown oil was dissolved in 1 ml. of methanol plus 1 ml. of acetone. Two drops of conc. HCl were added and the solution was left at room temperature for 1 hr. The solution was evaporated to dryness under vacuum and the residue dissolved in 20 ml. of 0.1N-NaOH. This was extracted three times with 20 ml. portions of CHCl₃ and the CHCl₃ washed twice with 10 ml. of water, the water washings being added to the alkaline phase. The CHCl₃ was dried with Na₂SO₄ and evaporated to dryness under vacuum to yield 5.7 mg. of 'acetonide fraction'.

Carbon dioxide was bubbled through the alkali until neutral to phenolphthalein and then extracted three times with 20 ml. portions of ether. The ether was washed twice with 20 ml. of water and evaporated to dryness to give 23.3 mg. of 2-methoxyoestriol fraction.

A portion (500 μ g.) of the 'acetonide fraction' was hydrolysed with 10 ml. of 5% (v/v) HCl on a boiling-water bath for 30 min. The hydrolysate was extracted three times with 30 ml. portions of ether and the ether washed once with 20 ml. of water. The ether was evaporated to dryness to give the 'hydrolysed acetonide fraction'. Portions (30 μ g.) of the hydrolysed and unhydrolysed acetonide fractions were chromatographed in formamide-CHCl₃ for 11 hr. Both fractions ran as single compounds with the same mobility as 2-methoxyoestriol even though there was a separation of standard 2-methoxyoestriol from standard 16-epioestriol.

The appearance of 2-methoxyoestriol in the 'acetonide fraction' is probably explained by the observation of Engel *et al.* (1957) that the 2-methoxy derivative of oestrone is not completely extracted from toluene by N-NaOH.

Although the chromatographic systems used could separate oestriol, 6-oxo-oestriol, 2-hydroxyoestriol and 6 α' -hydroxyoestriol from each other, no 6-oxo-oestriol or 6 α' -hydroxyoestriol could be detected.

DISCUSSION

This demonstration of a 2-methoxylating system in rat and rabbit liver implicates this organ as the site of formation of the urinary 2-methoxyoestrogen derivatives. Such derivatives of oestrone (Kraychy & Gallagher, 1957; Engel *et al.* 1957;

Loke & Marrian, 1958), oestradiol-17 β (Frandsen, 1959), oestradiol-17 α (oestra-1:3:5-triene-3:17 α -diol) (Stimmel, 1959), oestriol (Fishman & Gallagher, 1958) and possibly 16 α -hydroxyoestrone and 16-oxo-oestradiol-17 β (Loke, 1958) have been isolated from human urine under various conditions, which would indicate that 2-methoxylation is a fairly general metabolic reaction.

The isolation of 2-hydroxy- and 2-methoxy-oestriol suggests that methoxylation is a two-stage process, the initial 2-hydroxylation being followed by methylation of the 2-hydroxyl group. This is supported by the experiments with cell-free preparations described in the following paper and by the conversion of 2-hydroxyoestradiol-17 β into 2-methoxyoestrone (2-methoxy-3-hydroxyoestra-1:3:5-trien-17-one) *in vivo* (Axelrod, Rao & Goldzieher, 1960).

To date no 2-hydroxylated oestrogens have been isolated from urine, but Garst & Friedgood (1952) have put forward some slender evidence that such compounds might in fact be present in urine and it is known that after the injection of [16-¹⁴C]oestradiol-17 β (Beer & Gallagher, 1955) or [16-¹⁴C]-oestrone (Midgeon, Wall & Bertrand, 1959) into humans, an appreciable amount of the urinary radioactivity is associated with a fraction more polar than oestriol.

It can be seen from the figures quoted in Table 1 that an appreciable amount of the added oestriol has been 'lost'. This might be due to the formation of ether-soluble, non-phenolic compounds such as 2:3-dimethoxyoestra-1:3:5-triene:16 α :17 β -diol or ring A saturated derivatives.

It seems more probable, however, that the oestriol was metabolized to ether-insoluble compounds. A number of workers (Szego, 1953; Riegel & Mueller, 1954; Sandberg, Slaunwhite & Antoniadis, 1957) have shown that oestrone and oestradiol-17 β can be irreversibly bound to liver proteins, and it would seem likely that a similar reaction is occurring with oestriol in the experiments described in this paper. Bhargava, Hadler & Heidelberger (1955) have demonstrated that the protein-binding of 1:2:5:6-dibenzanthracene results in the rupture of one of the anthracene rings to produce a muconic acid derivative. It may be that oestrogens can be bound via ring A in a similar manner.

Crepay (1947) has shown that guinea-pig and rabbit-liver slices can form oestriol glucuronide from oestriol, but no such conjugates could be detected in the experiments described in this paper. This is in agreement with the findings of Lieberman, Tagnon & Schulman (1952).

SUMMARY

1. The metabolism of oestriol by rat-liver slices has been shown to be stimulated by oxygen and

inhibited by nitrogen. These results have led to the detection of two previously unknown metabolites of oestriol *in vitro*. These have been characterized as 2-methoxyoestriol and 2-hydroxyoestriol by melting points and infrared spectrometry. The 2-methoxyoestriol has been further characterized by optical rotation and ultraviolet spectroscopy.

2. No other phenolic metabolites of oestriol could be detected.

3. With rat-liver slices, the yields of 2-methoxyoestriol and 2-hydroxyoestriol are 10 and 18% respectively. Rabbit-liver slices produce more of the 2-hydroxy compounds but less 2-methoxyoestriol than does rat liver, whereas ox liver produces neither of these metabolites.

4. With rat-liver slices, about 40% of the oestriol is unaccounted for. No glucuronides could be detected. The possible fate of this oestriol is discussed.

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Metabolism of Oestriol *in vitro*

COFACTOR REQUIREMENTS FOR THE FORMATION OF 2-HYDROXYOESTRIOL AND 2-METHOXYOESTRIOL

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The conversion of oestriol into 2-hydroxyoestriol and 2-methoxyoestriol by rat- and rabbit-liver slices described in the preceding paper indicated a new metabolic pathway for oestrogens *in vitro*. The results suggested that the oestriol was hydroxylated in the 2 position and this hydroxyl group was then methylated to form 2-methoxyoestriol.

A number of papers (reviewed by Grant, 1956*a*) have described the hydroxylation of neutral steroids *in vitro*, and Mueller & Rumney (1957) have studied the 6' α '-hydroxylation of oestradiol-17 β by rat- and mouse-liver microsomal preparations. These hydroxylations all appear to require molecular oxygen and reduced pyridine nucleotide. The 2-hydroxylation of oestriol represents a major difference from these reactions in that the hydroxyl group is entering a benzenoid ring structure. Brodie *et al.* (1955) have shown that the hydroxylation of aromatic compounds such as acetanilide also requires oxygen and reduced triphosphopyridine nucleotide, so that some similarity exists between the hydroxylation of aromatic and aliphatic compounds. However, Kaufman (1959) has shown that a tetrahydrofolic acid derivative is required for the conversion of phenylalanine into tyrosine,

whereas this vitamin has no effect on the 11 β -hydroxylation of 11-deoxycorticosterone (Tomkins, Curran & Michael, 1958).

The *O*-methylation of catechols is well known (Axelrod & Tomchick, 1958; Pellerin & D'Ionio, 1958), and, like *N*-methylation (Cantoni, 1951; Cantoni & Vignos, 1954), the methyl group can be derived from methionine via *S*-adenosylmethionine.

This paper describes the cofactor requirements of the rat-liver 2-methoxylating system.

EXPERIMENTAL

Enzyme preparations and methods

The rats were killed by a blow on the back of the neck. Liver and kidney homogenates were prepared by mincing the tissue in a Latapie mincer to remove connective tissue and then homogenizing with cold 0.25M-sucrose in a glass homogenizer fitted with a nylon pestle. The ovaries were disrupted in a 0.8 cm. $\times$ 15 cm. glass tube with a hand-operated metal plunger; usually, 20% (w/v) homogenates were used. The uteri were slit longitudinally and the inside surface was blotted dry with filter paper. They were then cut into small pieces and finely minced with a Mickle automatic slicer. This brei was added to the appropriate volume of 0.25M-sucrose to give a 20% (w/v) suspension.

When required, the cell debris and nuclei were removed by centrifuging for 10 min. at 700*g* at 0° in a MSE refrigerated centrifuge and the mitochondria by centrifuging

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