Interconversion of 16-Epioestril and Oestril by Avian Liver Tissue *in vitro*

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(Received 3 October 1966)

1. [16-¹⁴C]16-Epioestril and [6,7-³H₂]oestril were incubated both simultaneously and separately at 40° with hen liver homogenates obtained from birds in the laying stage. 2. Purification and identification of products was made by ether extraction, Girard separation, Celite partition and paper chromatography and crystallization to constant specific activity both with and without derivative formation. Percentage conversions of the radioactive 16-epioestril and oestril were calculated from these specific activities. 3. Oestril yielded 16-epioestril as early as 5 min. after exposure of steroid to the tissue. No production of oestril from 16-epioestril was detectable in this time. 4. Considerably more 16-epioestril was formed from oestril than oestril by the reverse reaction after 90 min. of incubation. 5. 16-Oxo-oestradiol-17 β appeared in all cases to be the major identified intermediate in the interconversion of 16-epioestril and oestril.

The endogenous metabolic pattern of steroidal hormones of the more common test animals has not been well defined. One species, however, about which considerable information has been made available in recent years is the fowl. It is remarkable that the laying hen possesses an oestrogen pattern which qualitatively bears a very strong resemblance to that of the human. Thus Ainsworth, Carter & Common (1962), on intravenous administration of [16-¹⁴C]oestrone (3-hydroxy[16-¹⁴C]-oestra-1,3,5-trien-17-one) to a laying hen recognized, chromatographically, radioactive oestradiol-17 β (oestra-1,3,5-triene-3,17 β -diol), oestril (oestra-1,3,5-triene-3,16 α ,17 β -triol) and oestrone in the urine. Other radioactive metabolites detected were 16-epioestril (oestra-1,3,5-triene-3,16 β ,17 β -triol), 17-epioestril (oestra-1,3,5-triene-3,16 α ,17 α -triol), 16-oxo-oestradiol-17 β (3,17 β -dihydroxyoestra-1,3,5-trien-16-one) and 16-oxo-oestrone (3-hydroxyoestra-1,3,5-triene-16,17-dione). Previously MacRae, Dale & Common (1960) had demonstrated the conversion of [16-¹⁴C]-oestril into radioactive 16-oxo-oestradiol-17 β and 16-epioestril in laying hens. No additional radioactive conversion product was detected in the urine. Although the above compounds were identified from chromatographic evidence, oestrone (Ainsworth & Common, 1962), oestradiol-17 β (MacRae, Zaharia & Common, 1959) and 16-epioestril (Hertelendy & Common, 1964) have also been fully characterized in crystalline form

from hen urine or faeces or both. No oestril has, however, been isolated.

Intravenous injection of labelled oestradiol-17 β into the laying bird resulted in a radioactive oestril/16-epioestril ratio in excreta which was considerably lower than that found in human urine, where oestril is a major metabolite (MacRae & Common, 1960; Marrian & Bauld, 1955; Hobkirk, Nilsen & Purre, 1966). After intravenous administration of 16-oxo-[16-¹⁴C]oestradiol-17 β to the non-laying fowl, Ainsworth & Common (1963) identified only radioactive 16-oxo-oestradiol-17 β and 16-epioestril in the urine. This again suggested the equilibrium conditions in the hen to be such that 16-epioestril is formed at the expense of oestril.

On the other hand, Mitchell & Hobkirk (1959) demonstrated a significant conversion of radioactive oestradiol-17 β into labelled oestril by hen liver slices *in vitro*. Recently Ozon & Breuer (1965), by incubating 16-oxo-oestradiol-17 β with liver slices from chicks of 10–20 days of age and immature pullets, have shown both 16-epioestril and oestril to be formed in the ratio 3:2 in favour of the former metabolite. They also demonstrated oestril to be an incubation product of 16 α -hydroxyoestrone (3,16 α -dihydroxyoestra-1,3,5-trien-17-one) and of oestradiol-17 β .

To obtain a more complete understanding of the interconversion of oestril and 16-epioestril *in vitro*, these two compounds, in radioactive form, have been incubated with liver preparations from

the laying hen and their metabolites investigated as described below.

EXPERIMENTAL

Materials

NAD, NADP and glucose 6-phosphate were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. ATP was obtained as the disodium salt from Sigma Chemical Co., St Louis, Mo., U.S.A. 16-Oxo-oestradiol-17 β was acquired from Steraloids Inc., Pawling, N.Y., U.S.A. 16-Epioestril was purchased from Organon Inc., W. Orange, N.J., U.S.A., and oestril was a gift from Parke, Davis and Co., Ann Arbor, Mich., U.S.A. Dr A. E. Kellie of the Courtauld Institute of Biochemistry, The Middlesex Hospital, London, W. 1, donated the 16 α -hydroxyoestrone.

[16-¹⁴C]16-Epioestril was prepared by chemical reduction of 16-oxo[16-¹⁴C]oestradiol-17 β of specific activity 0.04 μ C/ μ g. as described by Hobkirk (1963). Successive crystallizations with authentic carrier steroid of a sample of the material eluted from a Celite partition column showed it to be 99% [16-¹⁴C]16-epioestril. It was used without further purification. The 16-oxo[16-¹⁴C]oestradiol-17 β was donated by Dr M. Levitz, New York University, New York, N.Y., U.S.A. [6,7-³H₂]Oestril of specific activity 52 μ C/ μ g., purchased from New England Nuclear Corp., Boston, Mass., U.S.A., was purified by column partition chromatography and shown to be at least 98% pure by crystallization with pure unlabelled oestril.

All other reagents were of suitable chemical grade and were purified where necessary by published techniques (Givner, Bauld & Vagi, 1960). Final distillation of organic solvents was done in all-glass apparatus and these solvents were stored in brown glass bottles.

Celite 535 (Johns-Manville Corp. New York, N.Y., U.S.A.) was purified, prepared and tested as described by Bauld & Greenway (1957).

Methods

Assay of radioactivity. All measurement of radioactivity was performed by liquid-scintillation spectrometry in a Nuclear-Chicago Corp. no. 6725 dual-channel instrument operating at an efficiency of 66% and 33% for ¹⁴C and ³H respectively. In double-label counting less than 1% of ³H appeared in the ¹⁴C channel while approx. 15% of ¹⁴C appeared in the ³H channel. The radioactivity for all samples was determined to within 5% probable error. The material to be counted was dried in glass counting vials and dissolved in 0.2 ml. of methanol before dilution with 10 ml. of toluene containing 0.3% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene. Calculation of ¹⁴C and ³H from double-labelled samples was done according to the method of Okita, Kabara, Richardson & LeRoy (1957).

Preparation of homogenate. This was carried out as partially described by Mitchell & Hobkirk (1959) and Lucis (1965). Livers from White Rock hens, in the laying period at the time of decapitation, were excised, rinsed free of blood, weighed and immediately used. Krebs-Ringer phosphate solution, pH 7.4 (Krebs & Henseleit, 1932), modified to contain K₂HPO₄ (0.1 M) rather than Na₂HPO₄ (0.1 M) and fortified with 0.04 M-nicotinamide, was used in the proportion of 1 ml./100 mg. wet wt. of whole liver

tissue. The tissue was homogenized for 30 sec. in a VirTis 45 homogenizer.

Simultaneous incubation of ¹⁴C- and ³H-labelled substrates. To a series of 20 ml. liquid-scintillation spectrometer vials (Nuclear-Chicago Corp., Toronto, Canada) were added 9.1 $\times 10^5$ counts/min. of [6,7-³H₂]oestril and 9.9 $\times 10^5$ counts/min. of [16-¹⁴C]16-epioestril in methanol. In each case an appropriate amount of unlabelled pure standard steroid, also dissolved in methanol, was added to give a final weight of 50 μ g. of each substrate. Propylene glycol (0.1 ml.) was added to each vial and the methanol completely evaporated in a gentle stream of air. In addition to the substrates, cofactors were added as follows: NAD (2.3 μ moles), NADP (2 μ moles), glucose 6-phosphate (15 μ moles), ATP (10 μ moles) and MgCl₂ (279 μ moles).

To each of the vials was added 5 ml. of the above liver homogenate and incubation was carried out at 40° in an atmosphere of O₂ + CO₂ (95:5) with regular mechanical agitation in a Dubnoff metabolic incubator. Times of incubation varied from 5 to 90 min. Blanks were identical except for boiling of the homogenate for 20 min. before addition to the incubation mixture.

Extraction of free radioactive material from the incubation mixture. After incubation, 100 μ g. each of unlabelled oestril, 16-epioestril, 16-oxo-oestradiol-17 β and 16 α -hydroxyoestrone were added to the incubation medium as carriers. Simultaneously the protein of the mixture was precipitated by the addition of 50 ml. of acetone. The mixture was kept overnight at room temperature (24°) to complete the extraction. The acetone was removed by filtration (Whatman no. 31 filter paper) and the tissue residue rinsed with additional acetone (four 40 ml. portions). The washings and acetone extract were combined with 50 ml. of distilled water and the acetone was evaporated under reduced pressure. The aqueous residue was then extracted with peroxide-free ether (four 60 ml. portions). The ether was evaporated off and the extracts were further dried by placing over anhydrous CaCl₂ in a vacuum desiccator overnight. The aqueous portions of each sample were stored for later extraction of conjugated material.

Purification and characterization of the radioactive incubation products. The dried ether extracts were subjected to a modified Girard separation for 18 hr. at room temperature (24°) as described by Givner *et al.* (1960). The ketonic and non-ketonic fractions, after ether extraction, were evaporated to dryness, dissolved in methanol and a sample (one-hundredth) from each was reserved for assay of radioactivity. The remaining material was chromatographed on Celite partition columns according to the method of Givner *et al.* (1960). This resulted in the separation of fractions containing oestril, 16-epioestril and ring-D α -ketolic oestrogens. [The last fraction would consist of any 16 α -hydroxyoestrone, 16 β -hydroxyoestrone (3,16 β -dihydroxyoestra-1,3,5-trien-17-one) and 16-oxo-oestradiol-17 β formed during incubation.] Radioactive eluates corresponding to each of these chromatographic fractions were evaporated to dryness and redissolved in methanol (3.0 ml.). One-tenth of each was retained for counting. A larger sample (two-thirds) was taken for further purification and identification.

Successive crystallization of radioactive 16-epioestril and oestril. The eluates obtained from the partition columns were submitted to a final step for the achievement of radiochemical purity. Pure crystalline unlabelled carrier of the

appropriate type was added to all 16-epioestril and oestril fractions and the mixtures were acetylated in pyridine and acetic anhydride overnight. The samples were successively crystallized several times from methanol-water. The specific activities of the crystals (approx. 1 mg. accurately weighed was counted in each case) became constant and equal to that of the mother liquor on the second crystallization.

Characterization of the radioactive ring-D α -ketolic fraction. The ring-D α -ketols were examined by descending paper chromatography in a toluene-propylene glycol solvent system at 24° (Hobkirk *et al.* 1966). Whatman no. 42 paper was impregnated with propylene glycol-methanol (1:1, v/v), blotted to remove excess of solvent and dried in air

sufficiently long to remove methanol before chromatography. The ring-D α -ketolic fraction from the Celite column was applied to the starting line with small volumes of methanol along with pure 16 α -hydroxyoestrone (40 μ g.) and 16-oxo-oestradiol-17 β (40 μ g.) as standards. The paper was developed for 4 days (overflow collected) in a jar previously saturated with toluene for 24 hr. Radioactive areas corresponding to standards were eluted with methanol overnight. The eluates were filtered through sintered glass, evaporated and redissolved in methanol (4.0 ml.). One-tenth was retained for counting. The remaining nine-tenths of the radioactive fractions eluted from the paper was transferred to centrifuge tubes and crystallized with pure unlabelled carrier from acetonitrile as described by Levitz,

Scheme 1. Typical distribution of radioactivity during different stages of extraction and purification of incubation products. An example from a [16-¹⁴C]16-epioestril + [6,7-³H₂]oestril experiment after 90 min. incubation is shown (see the Methods section). Figures are given as percentages of incubated radioactivity.

Spitzer & Twombly (1956). The crystals were of constant specific activity and equal to that of the mother liquor on the second recrystallization. From the specific activities thus obtained and knowing the weights of carrier used, percentage conversions of the $[6,7-^3\text{H}_2]\text{oestriol}$ and $[16-^{14}\text{C}]\text{-}16\text{-epioestriol}$ were calculated.

Extraction of possible conjugates. Although conjugation in itself was not of major interest, the residual aqueous fraction after ether extraction of free radioactivity was treated as follows. The water fraction was gently heated to remove any ether, cooled, 3M-NaCl added and the pH adjusted to 2. After extraction with 3 vol. of ethyl acetate (Burstein & Lieberman, 1958) the extracts were dried over Na_2SO_4 and evaporated *in vacuo*. The dry residue was dissolved in methanol (5.0 ml.) and a sample removed for assay of radioactivity. The degree of 'conjugation' in this tissue system was so low (0.5–2.0% of incubated substrate) that extraction of this fraction was dispensed with in later experiments.

Additional incubations. Confirmation of results obtained from the experiment outlined above was sought by carrying out further incubations with only a single substrate in any one experiment and additional times of incubation. Two series of incubations of this type were done. In the first of these 1.0×10^8 counts/min. of $[6,7-^3\text{H}_2]\text{oestriol}$ plus $50\text{ }\mu\text{g.}$ of unlabelled oestriol were incubated for 1, 5, 30 and 90 min. Secondly, 8.6×10^5 counts/min. of $[16-^{14}\text{C}]\text{16-epioestriol}$ plus $50\text{ }\mu\text{g.}$ of unlabelled 16-epioestriol were incubated for 90 min. only. Suitable blanks were carried out in each case. All other experimental details were identical with those of the double-labelled study described above. The three series of experiments outlined were performed on different occasions with livers excised from three individual birds. In all cases duplicate incubations were performed at each different incubation time.

RESULTS

The distribution of radioactivity obtained in a typical double-labelled experiment is shown in Scheme 1. Significant amounts of activity were associated with the 16-epioestriol, oestriol and ring-D α -ketolic fractions. As shown in Table 1, virtually all of the radioactivity in the two triol fractions remained associated with the carriers during crystallization. However, on subfractionation of the ring-D α -ketolic fraction, in no case was radioactivity found in the position of $16\alpha\text{-hydroxy-oestrone}$ or $16\beta\text{-hydroxyoestrone}$. In all experiments, 16-oxo-oestradiol- 17β eluted from chromatograms and crystallized as typified by Table 1 was the main metabolite. It accounted for 61–79% of the total ^3H of the ring-D α -ketolic fraction after $[6,7-^3\text{H}_2]\text{oestriol}$ incubation at various times and 65–100% of the ketolic ^{14}C when $[16-^{14}\text{C}]\text{16-epioestriol}$ was the substrate. The rest of the radioactivity remained on the starting line of the chromatograms and was not further investigated. Table 2 indicates that oestriol may be metabolized to 16-epioestriol very rapidly. In the first 5 min. of incubation (Expt. A) 15.9–17.5% of the oestriol was transformed into the latter metabolite. After

Table 1. Successive crystallization of radioactive incubation products of hen liver homogenate

Oestriol				16-Epioestriol				16-Oxo-oestradiol- 17β			
Purification step		Sp. activity (counts/min./mg.)		Wt. (mg.)	16-Epioestriol		Purification step	Wt. (mg.)	16-Oxo-oestradiol- 17β		Purification step
		^{14}C	^3H		14C	^3H			14C	^3H	
Celite-column pool + 20 mg. of carrier	Acetylation with acetic anhydride and pyridine	13.3	4210	20.0	4960	15140	Celite-column pool + 20 mg. of carrier	20.0	20350	9200	Paper-chromato- graphic pool + 20 mg. of carrier
		5.7	6260								
		9.2	4020								
Mother liquor 1	Crystals 1	3.9	4090				Mother liquor 2	1.6	20180	8820	Crystals 2
		5.5	4020								
Mother liquor 2	Crystals 2						Mother liquor 3	12.8	20390	9050	Mother liquor 2
Mother liquor 3	Crystals 3						Crystals 1	16.4	20060	9080	Crystals 1
Crystals 1	Mother liquor 1						Mother liquor 1	3.1	20000	8880	Mother liquor 1
Crystals 2	Mother liquor 2						Crystals 2	14.8	20370	8870	Crystals 2
Mother liquor 2	Crystals 3						Crystals 3	1.6	20180	8820	Mother liquor 2
Crystals 3	Mother liquor 3						Crystals 3	12.8	20390	9050	Mother liquor 3

A typical purification of radioactive oestriol triacetate, 16-epioestriol triacetate and 16-oxo-oestradiol- 17β is shown. Recrystallization of the acetates was done from methanol-water and the 16-oxo-oestradiol- 17β was crystallized from acetonitrile. The example was from a $[16-^{14}\text{C}]\text{16-epioestriol} + [6,7-^3\text{H}_2]\text{-oestriol}$ incubation at 90 min. Experimental details are given in the text. Weights and specific activities are in terms of the free steroids.

30 and 90 min. incubation periods the amount of 16-epioestril had decreased but still remained significantly high. This decrease was not reproducible on repetition of the study with liver tissue obtained from another bird. Nevertheless, 5.2–6.2% of 16-epioestril was again formed after 5 min. of exposure of oestril to the tissue (Table 2, Expt. B). After 90 min. of incubation 19.5% of oestril had been metabolized to the epimer. In all cases a decrease in the percentage recovery of unchanged oestril accompanied increased 16-

epioestril production. Material identified as 16-oxo-oestradiol-17 β derived from oestril also increased with enhanced 16-epioestril formation.

When 16-epioestril was the substrate (Table 3), duplicate 5 min. incubations yielded no detectable oestril. Considerable conversion (8.1–8.6%) occurred at 90 min., however. A repeat of this experiment on another occasion with a new liver preparation again yielded oestril (Table 3, Expt. C), but less in this case (2.7–3.1% at 90 min.). It is noteworthy that 16-oxo-oestradiol-17 β appeared

Table 2. *Percentage conversion of [6,7-³H₂]oestril into labelled 16-epioestril and 16-oxo-oestradiol-17 β at various times of incubation*

Incubation, extraction and purification of products were performed as described in the text. The percentage of substrate converted and unchanged was calculated from the product of the specific activity of crystals 2 (cf. Table 1) and the weight of carrier (20 mg.) added. Expts. A and B designate separate incubations with livers from different laying hens; (1) and (2) represent duplicate incubations of the same preparation.

Expt. no.	Time of incubation (min.)	Conversion into 16-epioestril (%)	Conversion into 16-oxo-oestradiol-17 β (%)	Unchanged substrate (%)
A (1)	1	1.7	0.06	31.9
(2)	1	2.5	0.07	33.0
A (1)	5	17.5	0.10	17.7
(2)	5	15.9	0.09	18.1
A (1)	30	5.6	—	—
(2)	30	6.0	—	—
A (1)	90	8.1	1.2	9.4
(2)	90	8.3	1.4	9.8
A (1)	Blank	2.2	0.04	40.2
(2)	Blank	2.6	0.06	32.9
B (1)	5	6.2	0.14	56.6
(2)	5	5.2	0.12	57.0
B (1)	90	19.5	0.82	30.3
(2)	90	19.5	0.84	28.9
B (1)	Blank	1.7	0.01	52.8
(2)	Blank	2.2	0.02	53.2

Table 3. *Percentage conversion of [16-¹⁴C]16-epioestril into labelled oestril and 16-oxo-oestradiol-17 β at various times of incubation*

Incubation was performed for 5 and 90 min. only. All other details are as given in Table 2. Expts. B and C designate separate incubations with livers from different laying hens; (1) and (2) represent duplicate incubations of the same preparation.

Expt. no.	Time of incubation (min.)	Conversion into oestril (%)	Conversion into 16-oxo-oestradiol-17 β (%)	Unchanged substrate (%)
B (1)	5	0	0.35	79.5
(2)	5	0	0.40	69.9
B (1)	90	8.1	1.2	41.2
(2)	90	8.6	1.4	40.1
B (1)	Blank	0	0.02	71.3
(2)	Blank	0	0.03	72.2
C (1)	90	2.7	—	39.4
(2)	90	3.1	9.3	43.0
C (1)	Blank	0	0.15	50.0
(2)	Blank	0	0.21	53.2

quantitatively related to increased product formation once more, with one exception. 16-Epioestriol incubation at 90 min. with tissue from one bird resulted in 1.2–1.4% 16-oxo-oestradiol-17 β formation. Tissue obtained from another bird, however, yielded 9.3% of the ketol under the same conditions. As product identification was of primary interest in this work such discrepancies between liver activities of different birds were not too disturbing.

It is noted that consistently higher amounts of 16-oxo-oestradiol-17 β were recovered when 16-epioestriol was metabolized to oestriol than during the reverse reaction. Also, it appears that a larger fraction of the ring-D α -ketolic compounds was identifiable as 16-oxo-oestradiol-17 β when 16-epioestriol was the substrate than on oestriol incubation. Oestriol appeared to yield a larger percentage of more polar unidentified material in this fraction.

DISCUSSION

The above findings in relation to the interconversion of oestriol and 16-epioestriol by liver homogenates from the laying hen supplement in part the studies *in vivo* of MacRae *et al.* (1960) and Ainsworth & Common (1963). As mentioned above, these authors showed 16-epioestriol to be the major triol excreted by the fowl whereas oestriol was either scarce or undetectable.

Considerably more 16-epioestriol was formed *in vitro* from oestriol than oestriol by the reverse reaction, at least under the experimental conditions employed. Further, the 16 α -to-16 β epimerizing systems appeared to be more rapid-acting, converting oestriol into 16-epioestriol almost immediately on contact of substrate and tissue. The 16 β -to-16 α epimerization did not appear nearly so active. This evidence *in vitro* appears to be in agreement with the results of Common and co-workers. These authors, however, did not detect any oestriol formation from 16-epioestriol *in vivo*. The present work has shown this pathway to exist *in vitro*. We have also shown that 16-oxo-oestradiol-17 β appears in all cases to be the major identified intermediate in oestrogen triol interconversion, a fact that has partially been borne out by Ozon & Breuer (1965) on incubation of 16-oxo-oestradiol-17 β by hen livers and isolation of both epimers.

It should be indicated that recovery of radioactivity in these experiments never reached 100%. Blank values in Tables 2 and 3 indicate cases where only 50% or less of the material incubated was extractable in unchanged form. Such a low recovery may have resulted from the cumulative effect of tissue binding, experimental loss at different stages of purification and other factors resulting in underestimation of the degree of metabolism.

In conclusion, it must be stated that up to the present time only a few tissues in a limited number of species have been shown to metabolize oestrogen triols to a significant degree. King (1960) indicated phenolic triol interconversion by rat kidney preparations. Hobkirk, Nilsen & Belenkie (1965) have demonstrated the dehydrogenation of oestriol to 16-oxo-oestradiol-17 β by cat erythrocytes and considerable dehydrogenation of 16-epioestriol by rat erythrocytes. The latter has also been reported by Portius & Repke (1960). The results of the present work now show that homogenates prepared from the livers of laying hens are also capable of effecting such conversions.

We thank Dr N. Nikolaiczuk, MacDonald College of McGill University, Montreal, Canada, for providing suitable liver tissue from laying hens. Grateful acknowledgement is made to the Banting Research Foundation, Toronto, Canada, and the Medical Research Council of Canada for financial aid. H.R.R. is a candidate for the degree of Doctor of Philosophy in Biochemistry, McGill University, Montreal, Canada. R.H. is a Research Associate of the Medical Research Council of Canada.

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