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The Structure and Function of Oestrogens. VII* The Use of (9,12,12-$^2H_3$)- and (11ξ,12,12-$^2H_3$) Oestradiol in a Test of the Quinone Methide Hypothesis for Oestrogen Action

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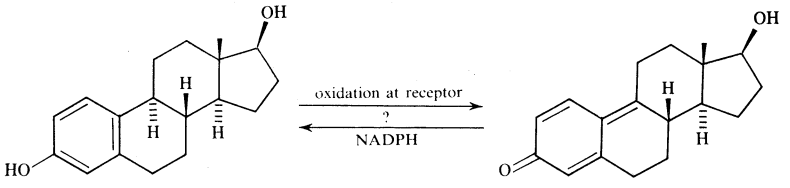
Abstract

Ovariectomized mice were injected intravaginally with a physiological dose of (9,12,12-$^2H_3$)oestradiol (3), and a control group was similarly injected with (11ξ,12,12-$^2H_3$)oestradiol (4). Gas–liquid chromatography/mass spectrometry (g.l.c./m.s.) analysis of the oestradiols recovered from the vaginae of the two sets of mice showed that the content and distribution of deuterium were the same as in the respective pure trideuterated oestradiols (3) and (4). This proved conclusively that the 9α-hydrogen of oestradiol is not exchanged during residence in and stimulation of the vagina. It therefore appears unlikely that reversible quinone methide formation in oestradiol is the trigger mechanism for stimulation of RNA synthesis, *unless* a hydrogen transfer relay system permits repetitive removal and replacement of the hydrogen atom at C9 during the oxidation–reduction cycle.

Introduction

The quinone methide hypothesis for the mechanism of action of oestrogens at the molecular level has been outlined previously (Collins and Matthews 1979; Collins *et al.* 1979; Collins and Stone 1983). Briefly, it is proposed that at the site of action in the target organs the phenolic moiety of an oestrogen is oxidized with removal of a benzylic hydrogen atom to give the corresponding quinone methide; rapid reduction of this by a biological source of hydride such as NADPH would regenerate the original phenolic oestrogen. The feedback from the rapid localized



(1) (2) Scheme 1

consumption of oxidant and/or reductant may then be responsible for the local increase in RNA synthesis observed in the target organs. Thus, for oestradiol (1), oxidation with removal of the 9α-hydrogen atom would give the corresponding quinone methide (2); hydride reduction of this would regenerate oestradiol (1) (Scheme 1).

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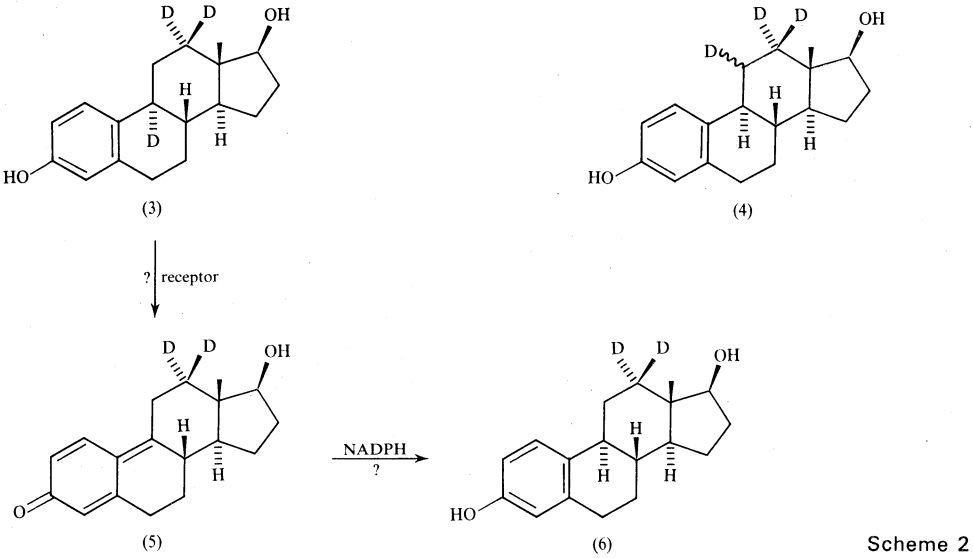
The intravaginal administration of a mixture of *meso*-(2-^{14}C)hexoestrol (Collins and Hobbs 1983) and *meso*-(2,3,4,5-$^{3}H_4$)hexoestrol in ovariectomized mice, and extraction of the excised organs showed no significant change in the $^{14}C:^{3}H$ ratio in the recovered oestrogen (Collins and Stone 1983). This experiment failed to support the quinone methide hypothesis, but a disparity between the specific radioactivity of the two labelled species left the interpretation in some doubt.



Scheme 2

We now describe the use of the two specifically trideuterated oestradiols (3) and (4) in a definitive test for exchangeability of the 9α-hydrogen of oestradiol in the mouse vagina. A physiological dose of (9,12,12-$^{2}H_3$)oestradiol (3, Scheme 2) was administered intravaginally to ovariectomized mice which were killed after 20 min. Following a chromatographic fractionation sequence, the deuterium content of the oestradiol in the purified extract was determined by gas–liquid chromatography/mass spectrometry (g.l.c./m.s.). A loss of the 9α-deuterium in (3) to give the dideuterated species (6) via (5) (Scheme 2) would indicate that the quinone methide cycle was operative. (11ξ,12,12-$^{2}H_3$)Oestradiol (4) was given to a second set of animals as a reference control, so that the possibility of loss of deuterium from the 12-position could be excluded in the first experiment. Trideuterated species of oestradiol were used so that the parent molecular ion in the mass spectrum was clearly beyond interference by the natural abundance M+1 and M+2 ions due to any unlabelled endogenous oestradiol present in the tissues.

Steroid hormones specifically labelled with radioactive isotopes have provided powerful probes in studies of biotransformations, but where very minute physiological doses of steroid hormones must be used, detection and characterization rest heavily on the non-specific criteria of radioactivity and chromatography R_F values. On the other hand, the combination of specific deuterium labelling and g.l.c./m.s. yields a characteristic mass spectrum as well as a g.l.c. retention time, thereby simultaneously providing positive chemical identification as well as quantification. The use of g.l.c./m.s. in the present project necessitated prior preparation of a highly

enriched oestradiol fraction from the vaginal tissue extract. The purification of picogram amounts of steroids requires the use of highly inert chromatographic media (Nyström and Sjövall 1975). Lipophilic derivatives of Sephadex fulfill these criteria. A selective method for purification of oestrogens from uterine tissue prior to g.l.c./m.s. analysis has been described previously (Axelson *et al.* 1981*b*). Oestrogens are isolated by filtration of the biological extract through the lipophilic strong cation exchanger, sulfohydroxypropyl Sephadex LH-20 (SP-LH-20), followed by chromatography on the anion exchanger, triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20). This purification method was used in the present study, but since the lipid content of the extracts was high, major non-polar and polar contaminants which may have interfered with ion-exchange chromatography were first removed by reversed-phase chromatography on Lipidex derivatives. Details of these chromatographic steps have been discussed previously (Axelson *et al.* 1974*b*, 1981*a*; Dyfverman and Sjövall 1978).

Materials and Methods

Chromatographic Materials

Lipidex 5000 and Lipidex 1000 (Packard Instruments, Downers Grove, Ill., U.S.A.) were extensively washed prior to use (Axelson *et al.* 1981*b*). SP-LH-20, 1·3 m-equiv/g, and TEAP-LH-20, 0·8 m-equiv/g, were prepared as previously described (Axelson and Sjövall 1979; Axelson *et al.* 1981*b*), and washed prior to use (Axelson *et al.* 1981*a*).

Isotopically Labelled Oestradiols

The preparation of (9,12,12-$^2H_3$)oestradiol (3) with the isotopic composition $^2H_4$, 12·2; $^2H_3$, 75·2; $^2H_2$, 8·0; $^2H_1$, 3·0; $^2H_0$, 1·7% and of (11ξ,12,12-$^2H_3$)oestradiol (4) with the isotopic composition $^2H_4$, 6·8; $^2H_3$, 83·2; $^2H_2$, 5·5; $^2H_1$, 2·9; $^2H_0$, 1·6%, has been described elsewhere (Collins 1983). The oestrogenic activity of the trideuterated oestradiols (3) and (4) was virtually identical with that of natural oestradiol, as shown by the tetrazolium assay (Martin 1960).

Tritium-labelled (1,2,6,7-$^3H_4$)oestradiol (3–4 TBq/mmol) was obtained from Radiochemical Centre, Amersham, England, and was used as supplied.

Experiments with Animals

Randomly bred mice of the QS strain were ovariectomized at 6–8 weeks of age and primed with a dose of oestradiol-17β 5–7 days before use.

One set of 250 mice (group I) was injected intravaginally with 100 pg per mouse of (9,12,12-$^2H_3$)-oestradiol (3) in 5μl of 0·9% (w/v) NaCl, and another set of 200 mice (group II) was similarly injected with the same dose of (11ξ,12,12-$^2H_3$)oestradiol (4). The animals were killed 20 min after injection and the vaginae from each group were removed and separately kept for no more than 30 min on ice until extraction.

Analytical Procedures

Extraction

Each set of vaginae was homogenized in ethanol (100 ml), and the homogenates were stirred overnight at 2°C, filtered, and the residues were extracted twice more with ethanol (100 ml). Evaporation of the extract from each group in vacuum gave residues of 276·4 mg (group I) and 420·7 mg (group II); treatment of these with dichloromethane:ethanol (1:1 v/v) (15 ml) yielded the final extracts of 273·6 and 417·4 mg, respectively, which were used in the separation procedure described below. (Further extraction of the tissue residues from the ethanol extraction with toluene:ethanol (1:1) gave an additional 45–48 mg of extractives, but these were not used for analysis.)

The dried extracts were redissolved in dichloromethane:ethanol (1:1 v/v) and aliquots (1:4) were transferred to centrifuge tubes. Tritium-labelled oestradiol (10^4 cpm = 40 pg) was added for recovery experiments.

Reversed-phase chromatography

The extracts were evaporated almost to dryness under a stream of nitrogen, and redissolved in 2 ml of methanol:water:chloroform (9:1:2 v/v). Following centrifugation for 5 min in a bench centrifuge, the supernatants were applied to columns of Lipidex 5000 (4·0 g, 20 by 0·8 cm) packed in methanol:water:chloroform (9:1:2 v/v) (Axelson *et al.* 1974*b*). The columns were eluted with the same solvent, and the first 20 ml of effluent containing the oestradiols was collected in round-bottomed flasks. Under these conditions non-polar contaminants are retained on the column (Axelson *et al.* 1974*b*). The solvent was evaporated until only aqueous solutions were present in the flasks (*c.* 2 ml), and additional water (3 ml) was added to each flask. These solutions were passed through columns of Lipidex 1000 (1 g, 5 by 0·8 cm) packed in water (Dyfverman and Sjövall 1978). Polar contaminants were washed from the columns with water (15 ml) prior to elution of the oestradiols with methanol (15 ml).

Ion-exchange chromatography (*Axelson* et al. *1981*a, *1981*b)

The methanolic eluates were passed through columns of the lipophilic, strong cation exchanger SP-LH-20 in the H^+ form, packed in methanol (0·2 g, 5 by 0·4 cm). The columns were rinsed with methanol (5 ml), and the effluents (about 20 ml) were applied to columns of the lipophilic, strong anion exchanger TEAP-LH-20 in the OH^- form (0·1 g, 4 by 0·4 cm), packed in methanol. Neutral compounds were washed from the columns with methanol (3 ml) and methanol:chloroform (1:1 v/v) (3 ml). Phenolic compounds, including the oestradiols, still sorbed on the ion exchanger were then selectively eluted with methanol (7 ml) saturated with CO_2. These fractions were taken to dryness in vacuum and transferred to small centrifuge tubes with methanol.

Derivatization

The samples were taken to dryness under N_2 and were treated with 100 μl of a mixture of trimethylchlorosilane, hexamethyldisilazane and pyridine (1:2:3 v/v). Trimethylsilylation was completed after 30 min at 60°C, and the reagents were removed under a stream of nitrogen. The derivatives were dissolved in hexane (50 μl) for g.l.c./m.s. analysis.

Gas chromatography–mass spectrometry analysis

A modified LKB 9000 instrument was used with a 25 m by 0·3 mm open-tubular glass capillary column coated with SE-30 and connected to the ion source via a single-stage adjustable jet separator (Axelson and Sjövall 1977). The column temperature was about 275° and the temperatures of the molecule separator and ion source were 260 and 310°C respectively. The energy of the bombarding electrons was 22·5 eV, the ionizing current was 60 μA, and the accelerating voltage was 3·5 kV. Partial mass spectra (7 a.m.u., m/z 415–421 in 2 s) were obtained with repetitive accelerating voltage scanning and a data sampling rate of 10 kHz. Intensity readings were bunched to give 20 values/a.m.u., which were recorded on magnetic tape. This mode was employed in carrying out accurate determinations of isotope content. Data were evaluated by off-line processing on an IBM 1800 computer (Axelson *et al.* 1974*a*). The oestradiol equivalent in the aliquot of the biological samples injected was about 200–400 pg, and that for the pure reference compounds was 1–10 ng. The retention time for oestradiol di-TMS ether was about 7 min. Duplicate g.l.c./m.s. analyses were made of the biological samples and reference compounds.

Results and Discussion

Quantitative g.l.c./m.s. analysis showed that about 70% of the trideuterated oestradiols (3) and (4) administered to the animals was recovered in the vaginal extracts. Tritium-monitoring of the eluates from each chromatographic column used in the isolation-purification process showed that the recovery of the added

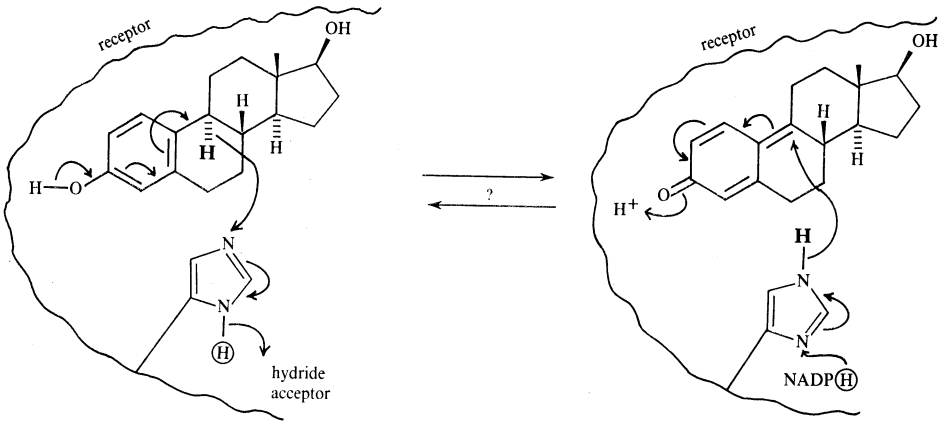
tritiated oestradiol was about 75% for the whole procedure. The purity of the final samples was high, and the equivalent of 2–5 vaginae could be injected onto the capillary column without overloading. Furthermore, no interfering peaks in the total ion current chromatogram were observed at the time of elution of oestradiol from the gas chromatographic column.

Table 1. G.l.c./m.s. analysis of the deuterium isotope distribution in the labelled oestradiols recovered after administration of (9,12,12-$^2H_3$)- and (11ξ,12,12-$^2H_3$)oestradiol in mice

The column headed with the *m/z* value of 416 corresponds to the percentage of undeuterated oestradiol molecules detected as the 3,17β-di-TMS ether, and the headings 417, 418, 419 and 420 correspond to the 3,17β-di-TMS ethers on the mono-, di-, tri- and tetradeuterated oestradiols respectively

Sample	*m/z* 416:	Ion current intensity (%)[A] 417	418	419	420
Vaginal extract I[B]	9·9	2·7	6·8	100·0	12·4
	9·1	4·8	9·7	100·0	12·0
Mean	9·5	3·8	8·3	100·0	12·2
Pure (9,12,12-$^2H_3$)-	2·2	3·9	10·4	100·0	15·9
oestradiol (3)	2·2	4·0	10·8	100·0	16·5
Mean	2·2	4·0	10·6	100·0	16·2
Vaginal extract II[C]	2·5	1·7	7·2	100·0	10·3
	3·4	2·3	7·3	100·0	11·8
Mean	2·9	2·0	7·2	100·0	11·1
Pure (11ξ,12,12-$^2H_3$)-	2·0	3·5	6·5	100·0	8·7
oestradiol (4)	1·8	3·5	6·8	100·0	7·7
Mean	1·9	3·5	6·6	100·0	8·2

[A] The ion current for *m/z* 419, the molecular weight of the trideuterated oestradiol di-TMS ether, was set to 100%.
[B] From the animals of group I dosed with (9,12,12-$^2H_3$)oestradiol (3).
[C] From the animals of group II dosed with (11ξ,12,12-$^2H_3$)oestradiol (4).



Scheme 3

The measured values of the deuterium isotope excess after correction for normal isotope distribution are presented in Table 1. It is clear from the table that the deuterium content of the labelled oestradiols recovered from both sets of animals is

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