Sulphation of Oestrone *in vitro* by Bovine Adrenal Tissue

By A. SNEDDON AND G. F. MARRIAN

Division of Chemistry and Biochemistry, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, W.C. 2

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In a preliminary paper on the isolation of 18-hydroxyoestrone from the urine of pregnant women, Loke, Watson & Marrian (1957) referred to experiments which suggested that this oestrone metabolite was formed in small yield on incubating oestrone with bovine-adrenal homogenates. Subsequent work by Loke (1958), in which attempts were made to isolate 18-hydroxyoestrone from such homogenates, gave inconclusive results. Accordingly it was decided to reinvestigate the metabolism *in vitro* of oestrone by bovine-adrenal preparations by using [16-¹⁴C]oestrone as substrate.

In preliminary experiments in which [16-¹⁴C]-oestrone was incubated with coarse minces of fresh bovine adrenocortical tissue, the recovery of the added radioactivity in ether or ethyl acetate extracts of the partially worked up incubation mixtures was very variable and sometimes as low as about 30%. Further investigation showed that the greater part of the unaccounted-for radioactivity could be extracted from aqueous media by butan-1-ol or, more conveniently, by ethyl acetate after saturation of the aqueous phase with sodium chloride. Subsequently this oestrone metabolite was fully characterized as oestrone sulphate.

Purdy, Engel & Oncley (1961) have shown that, after the administration of ¹⁴C-labelled oestradiol to non-pregnant women, the radioactivity in the plasma was largely present in oestrone sulphate. They also found that in the plasma of pregnant women the molar concentration of oestrone sulphate was about four times that of unconjugated oestrone. On the basis of these findings they concluded that 'oestrone sulphate is an important circulating estrogen in the human'. The present finding that the bovine adrenal cortex which, like the adrenal cortex in some other species, may be an oestrogen-secreting organ, can conjugate oestrone with sulphate is therefore of interest.

EXPERIMENTAL AND RESULTS

Materials

The Celite 535 (Johns Manville and Co. Ltd., London) used for column chromatography was purified by standing overnight in conc. HCl, washing with water until the washings were free from Cl⁻ ions and drying at 110°. The Al₂O₃ (Savory and Moore Ltd., London) used in column

chromatography was activated by heating at 100° under reduced pressure and then deactivated by the addition of 3% (by wt.) of water. Sephadex-G25 was purchased from Pharmacia, Uppsala, Sweden. Unwashed Whatman no. 1 paper was used for paper chromatography.

Oestrone was obtained from Schering A.-G., Berlin, Germany. In the incubation experiments it was used without further purification. For the reverse isotope-dilution experiment it was recrystallized twice from ethanol. [16-¹⁴C]Oestrone of activity 12.1 mc/mg. was purchased from The Radiochemical Centre, Amersham, Bucks., and was purified on a Celite column, with methanol-water (7:3, v/v) as stationary phase and benzene-hexane (1:1, v/v) as mobile phase. Sodium oestrone sulphate was obtained from the Medical Research Council's Steroid Reference Collection through the courtesy of Professor W. Klyne (Westfield College). It was purified by passage through a Celite-Al₂O₃ column by the method of Purdy *et al.* (1961).

All solvents were purified before use by distillation.

Incubation procedure

All incubations were carried out in 50 ml. portions of a modified Ca²⁺ ion-free Krebs-Ringer phosphate buffer having the following composition (final concentrations in parentheses): NaCl (96.0 mm); KCl (4.6 mm); KH₂PO₄ (1.16 mm); MgSO₄ (1.16 mm); NaHCO₃ (3.48 mm); sodium fumarate (8.21 mm); sodium L-glutamate (4.77 mm); glucose (11.2 mm); nicotinamide (5.92 mm); Na₂HPO₄ (10.8 mm); NaH₂PO₄ (2.7 mm). Before use the incubation medium was placed in a water bath at 37° and was gassed for 1 hr. with O₂ + CO₂ (95:5).

Pairs of adrenal glands, obtained from animals within a few minutes after slaughter, were transported to the Laboratory in a chilled container, dissected free from fat and cut open longitudinally. The medullae were removed as completely as possible, and the cortical tissue was minced in a chilled Latapie mincer. For each incubation 5 g. of mince was used.

To each incubation mixture was added 500 µg. of oestrone in 0.5 ml. of ethanol and 0.04 ml. of a standard solution of [16-¹⁴C]oestrone in ethanol corresponding to an accurately known rate of about 1.5 × 10⁵ counts/min.; the incubations were carried out for 1 hr. at 37° in an atmosphere of O₂ + CO₂ (95:5).

All incubations were commenced within 1 hr. of the death of the animal. Preliminary experiments, not reported in detail, showed that the conjugation of oestrone was greatly diminished when adrenals were used which had been left at room temperature for a few hours and then stored overnight at -20°. Presumably this was due to destruction of ATP which is involved in the synthesis of 'active sulphate' (Robbins & Lipmann, 1956).

Extraction and fractionation of incubation mixtures

Each incubation was terminated by the addition of 117 ml. of methanol so as to give a methanol concentration of 70% (v/v). After vigorous shaking the mixture was centrifuged for 10 min. and the supernatant decanted off. The residue was re-extracted with 167 ml. of aq. 70% (v/v) methanol, and the combined supernatants were washed four times with 0.5 vol. of hexane.

The aqueous-methanol phase was evaporated under reduced pressure to remove all methanol, and the aqueous residue diluted with an equal volume of water and extracted twice with equal volumes of ether. The ether extract was washed twice with small volumes of water, dried over Na_2SO_4 and evaporated to dryness.

The aqueous phase and washings from the ether extraction were combined and extracted twice with equal volumes of ethyl acetate. The extract was evaporated to dryness after washing with water and drying over Na_2SO_4 .

The aqueous phase and washings from the ethyl acetate extraction were combined, saturated with NaCl and extracted twice with equal volumes of ethyl acetate. The extract was dried over Na_2SO_4 without previous washing, evaporated to dryness, immediately redissolved in methanol to which a few drops of aqueous ammonia had been added and the solution stored at 0°.

In each experiment suitable samples of the ether extract, of the ethyl acetate extract and of the ethyl acetate extract after saturation with NaCl were plated, and the radioactivity was determined at infinite thinness by using the Nuclear-Chicago Autochange gas-flow counter with a thin end-window.

The results of six such experiments carried out in duplicate on the adrenals of four 6-year-old cows and two young steers are shown in Table 1. In each case extraction with ethyl acetate after the initial ether extraction removed only small addi-

tional proportions of the added radioactivity. However, further extraction with ethyl acetate after saturation of the aqueous phase with sodium chloride removed the greater part of the remaining radioactivity; at this stage of the work the assumption was made that this fraction of the radioactivity represented an oestrogen-conjugate fraction.

Purification of the oestrogen-conjugate fraction

The combined oestrogen-conjugate fraction from the above experiments, having a total radioactivity of 2.927×10^5 counts/min., was chromatographed on a Celite- Al_2O_3 column as described by Purdy *et al.* (1961) for the separation of oestrogen sulphates, glucosiduronates and phosphates. The sulphate fraction contained 94.5% of the radioactivity applied to the column.

An attempt to characterize the conjugate at this stage by paper chromatography failed because of heavy contamination with impurities that caused the spots to spread during the equilibration period and to streak on running.

Further purification was effected by partition between butan-1-ol and *n*-NaOH (cf. Schachter & Marrian, 1936, 1938), passage through an Al_2O_3 column in methanolic solution, and finally by fractionation on a column of Sephadex-G 25 with water as eluent as described by Beling (1961). In the last-named procedure 94.5% of the radioactivity applied to the column was eluted in a narrow band just behind a band of cloudy opalescent material. The oestrogen-conjugate fraction was recovered from the aqueous eluate from the column by extraction with ethyl acetate after saturation with NaCl and contained radioactivity equivalent to 1.91×10^5 counts/min. or 65% of that in the combined crude oestrogen-conjugate fraction.

Provisional identification of the oestrogen-conjugate fraction as oestrone sulphate by paper chromatography

Portions of the purified oestrogen-conjugate fraction, each containing radioactivity equivalent to 0.4×10^5 counts/min., were chromatographed on paper at 25° with the aq. ammonia (sp.gr. 0.88)-ethyl acetate-butan-1-ol system of Schneider & Lewbart (1956), referred to below as system 1, and the aq. ammonia (sp.gr. 0.88)-dibutyl ether-2-methylpropan-2-ol system of Schneider & Lewbart (1959), referred to below as system 2. On each paper authentic oestrone sulphate (100 $\mu\text{g.}$) was run as a standard.

Examination of paper chromatograms for radioactive areas on the Nuclear-Chicago Actigraph Chromatogram Scanner by using a gas-flow detector with a thin end-window showed that in the channels on which the oestrogen-conjugate fraction had been run there were main peaks of radioactivity having R_f values of 0.42 and 0.14 in systems 1 and 2 respectively, and lesser peaks having R_f values of about 0.90 and 0.80 respectively in these two systems.

These lesser peaks with the high R_f values were almost certainly due to oestrone liberated from the conjugate by hydrolysis before or during chromatography, since components with nearly identical

Table 1. Incubation of bovine-adrenocortical-minces with $[16\text{-}^{14}\text{C}]$ oestrone and the extractability of the radioactive metabolic products from aqueous media

Experimental details are given in the text.

Animal	Percentage of added radioactivity extracted		
	By ether	By ethyl acetate	By ethyl acetate after addition of NaCl
Cow	81.7	1.5	20.1
	77.5	5.0	12.8
Cow	77.5	0.9	14.7
	73.7	2.3	20.4
Cow	67.0	4.2	18.8
	94.5	2.7	14.3
Cow	42.8	0.7	44.7
	48.6	1.1	46.1
Steer	77.5	1.5	14.3
	79.6	1.4	11.8
Steer	77.5	1.5	25.6
	71.8	1.9	31.6

R_F values in the two systems respectively, which gave positive reactions with the Zimmermann reagent, positive reactions with the Folin-Ciocalteu reagent and negative tests for SO_4^{2-} ions after solvolysis *in situ* (see below), were detected on chromatograms of both the oestrogen-conjugate fraction and the authentic oestrone sulphate.

On staining chromatograms with the Zimmermann reagent by the method of Bush (1961) a main ketonic component with R_F values of 0.37 and 0.14 in systems 1 and 2 respectively was detected in the oestrogen-conjugate fraction. The corresponding R_F values for the main ketonic component in the authentic oestrone sulphate were 0.37 and 0.15.

On staining with the Folin-Ciocalteu reagent by the method of Mitchell & Davies (1954) no phenolic components could be detected in either the oestrogen-conjugate fraction or the authentic oestrone sulphate other than that believed to be free oestrone (see above). However, after solvolysis *in situ* by the dioxan-hydrochloric acid method of Schneider & Lewbart (1956), a phenolic component with R_F values of 0.38 and 0.14 in systems 1 and 2 respectively was detected in the oestrogen-conjugate fraction, and one with R_F values of 0.38 and 0.12 in the same two systems in the authentic oestrone sulphate.

After solvolysis *in situ* (see above) and by using the rhodozonc acid test for SO_4^{2-} ions as described by Schneider & Lewbart (1956), yellow spots with R_F values of 0.38 and 0.17 in systems 1 and 2 respectively appeared on the oestrogen-conjugate channels and ones with R_F values of 0.44 and 0.19 in the same two systems in the oestrone sulphate channels. These R_F values do not agree very well with those found in the other tests described above, although there was reasonably good agreement between the values for the oestrogen conjugate and the authentic oestrone sulphate in both systems. The reason for poor agreement of these R_F values with those obtained by other tests became apparent when it was found that SO_4^{2-} ions applied to a chromatogram paper spread and migrated downwards during a dummy solvolysis procedure sufficient to displace the centre of the rhodozonc acid-stained spot by as much as 2 cm. In view of this, and since, as described above, the oestrogen conjugate was eluted from a Celite-alumina column in the oestrogen sulphate fraction, there can be no doubt that the conjugate is a sulphate.

Identification of the oestrogen in the conjugate as oestrone

The paper-chromatographic tests described above provided strong evidence that the oestrogen conjugate is oestrone sulphate. However, since the sulphates of different ketonic oestrogens might not

necessarily have greatly differing R_F values in the two solvent systems employed, confirmatory evidence was sought for the presence of oestrone.

The main bulk of the oestrogen-conjugate fraction was solvolyzed by the method of Burstein & Lieberman (1958) as follows: After dissolution of the fraction in 10 ml. of water, 2 g. of NaCl and 0.06 ml. of 16N- H_2SO_4 were added and the solution was extracted with 10 ml. of ethyl acetate. The extract was maintained at 37° for 3 hr., washed once with 5 ml. of aq. 5% (w/v) NaHCO_3 , twice with 3 ml. of water, dried over Na_2SO_4 and evaporated to dryness.

One-twentieth of the dried material was chromatographed on paper at 25° in 'system B5' of Bush (1952) with oestrone, oestradiol-17 β , 16-oxo-oestradiol-17 β and 6 α -hydroxyoestrone as standards. The R_F values were as follows: unknown, 0.93; oestrone, 0.90 and 0.91; oestradiol-17 β , 0.81 and 0.79; 16-oxo-oestradiol-17 β , 0.65 and 0.62; 6 α -hydroxyoestrone, 0.45 and 0.42.

To the remainder of the solvolyzed material was added authentic oestrone (209.7 mg.), and the mixture was crystallized three times from ethanol. The yield after the third crystallization was 100.6 mg. After each crystallization portions of the material were plated and the radioactivity was determined in the Nuclear-Chicago Autochanger gas-flow counter. The counts/min./mg. after the three crystallizations were respectively 278, 308 and 263.

The thrice-crystallized material was then acetylated in the usual way with acetic anhydride and pyridine, and the crude acetate weighing 115.9 mg. was crystallized three times from hexane, yielding 40.1 mg. of final product. The counts/min./mg. after the three crystallizations were respectively 224, 245 and 256.

The paper-chromatographic evidence and that from the reverse isotope-dilution experiments shows conclusively that the oestrogen in the conjugate was indeed oestrone.

SUMMARY

1. Incubation of bovine-adrenocortical minces under aerobic conditions with [$^{16-14}\text{C}$]oestrone gave variable yields of a water-soluble radioactive oestrone metabolite, not extractable from aqueous solution by ether or by ethyl acetate, but extractable by the latter after saturation of the aqueous phase with sodium chloride, or by butan-1-ol.

2. On chromatography on Celite-alumina columns by the method of Purdy *et al.* (1961) the metabolite was eluted in the oestrogen sulphate fraction.

3. By paper chromatography in two systems devised by Schneider & Lewbart (1956, 1959) for

The peptide was hydrolysed partially by N-acetic acid at 100° for 2 hr. and completely when N-hydrochloric acid was used under the same condi-