Hormonal Steroids in the Tissue of Induced Rat Mammary Tumours

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The possible presence of steroids in the tissue of induced hormone-dependent rat mammary tumours was investigated. The method used involves a preliminary extraction of tumours followed by chemical separation and thin-layer chromatography. The identified compounds were cholesterol, androst-4-ene-3,17-dione, 5 β -androst-1-ene-3,17-dione, androsta-1,4-diene-3,17-dione and oestrone. This is the first report of the presence of these steroids in the tissue of an experimental tumour of a non-endocrine organ. In particular 5 β -androst-1-ene-3,17-dione has not previously been identified from natural sources.

The possibility that tumours of non-endocrine tissue may metabolize hormones has led us to study this subject in hormone-dependent mammary adenocarcinomas induced in rats by 7,12-dimethylbenz[*a*]anthracene (Huggins, Briziarelli & Sutton, 1959). An investigation of the enzyme constitution of these tumours suggested that their metabolism is mainly reductive. With oestrone and testosterone being used as substrates, these tumours have been found to contain an oestrone 17 β -hydroxy steroid oxidoreductase and testosterone Δ^4 -5 α -hydrogenase, 3-oxo steroid reductase and 17 β -hydroxy steroid oxidoreductase; the enzyme activities towards the substrate were higher than those of mammary tissue from pregnant rats and showed no qualitative metabolic differences (King, Gordon & Helfenstein, 1964; King, Panattoni, Gordon & Baker, 1965).

The capacity of dimethylbenzanthracene-induced rat mammary tumours to metabolize steroid hormone *in vitro* suggested an investigation of the possible presence of steroidal molecules in the tissue of this type of tumour.

The present study represents such an attempt, which has been pursued with a direct analysis of this type of tumour.

METHODS

Sprague-Dawley rats were each injected with a single intravenous dose of 3mg. of 7,12-dimethylbenz[*a*]anthracene suspended in 0.4ml. of an oil-in-water emulsion. When the induced mammary adenocarcinomas had attained a considerable size (60 days) the animals were killed with ether vapour and the tumours removed and kept at -40° until analysis.

Extraction procedure. A 400g. portion of tumour tissue, trimmed of fat and fibrous tissue, was minced and then homogenized. The homogenate was diluted with 1l. of

0.9% NaCl and heated in a boiling-water bath for 2hr. Ethanol was added to the cooled suspension in amount sufficient to give a final concentration of 80% (v/v). After one night in the refrigerator the suspension was filtered and the protein residue was extracted with chloroform-methanol (2:1, v/v). The ethanol and the chloroform-methanol solutions were combined and then evaporated *in vacuo*. The brown oil obtained was dissolved in chloroform (600ml.) and washed with water (600ml.) three times. The water washings were discarded and the chloroform fraction was dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*. The chloroform-extracted material weighed 11.76g. The protein residue was hydrolysed in 75ml. of 0.5M-HCl and 75ml. of dioxan for 24hr. at room temperature, neutralized with 2M-NaOH and partitioned between M-K₂CO₃ and ether-chloroform (3:1, v/v). The organic phase was washed twice with M-K₂CO₃ and three times with saturated NaCl solution after evaporation to dryness (Anliker, Rohr & Ruzicka, 1957). The weight of the final yellow oil was 0.40g.

Saponification. The fractions obtained from the chloroform extraction (11.76g.) and from the acid hydrolysis (0.40g.) were combined in 100ml. of ethanolic 5% (w/v) KOH and then were heated under a reflux condenser under a stream of N₂ for 2hr. The non-saponifiable matter was represented by 3.20g. of a yellow viscous material. The saponifiable matter (9g.) was partitioned twice between 50ml. of ether and 100ml. of M-K₂CO₃. After ether evaporation the residue weighed 1.60g. The M-K₂CO₃ solution was not examined; the ether residue was partitioned between light petroleum (b.p. 70–80°) and 70% (v/v) methanol. The methanol extract (0.24g.) was dissolved in dichloromethane and thoroughly washed with 2M-NaOH; this was acidified with conc. HCl and then extracted with dichloromethane, which, evaporated *in vacuo*, gave 0.40mg. of yellow substance (phenolic fraction).

Digitonin separation. The non-saponifiable matter (3.20g.) was treated with an ethanolic solution of digitonin and left in the refrigerator overnight (Varley, 1958). The digitonide complex was precipitated with ether and filtered. The ethanol-ether solution evaporated *in vacuo* gave 0.30g. of an oily residue. The digitonide complex was

decomposed by dry pyridine and furnished 2.7 g. of a crystalline product.

Separation of ketonic from non-ketonic fractions. The digitonin-non-precipitable fraction (0.30 g.) was separated into ketonic (200 mg.) and non-ketonic fractions (40 mg.) by means of Girard's reaction (Girard & Sandulesco, 1936).

Chromatography. The different residues were purified by partitioning between equal volumes of light petroleum (b.p. 40–60°) and aqueous methanol used in three different dilutions, 30%, 50% and 70% (Anliker *et al.* 1957). The light-petroleum layer was back-extracted with 70% methanol. The pooled methanol layers were re-extracted with light petroleum (b.p. 40–60°) and dried in a stream of N₂. The residues were individually run on special chromatographic plates (PSC-Fertigplatten Kieselgel F254; E. Merck A.-G., Darmstadt, Germany) and developed in chloroform-ether (9:1, v/v)-saturated tanks. The dried plates were examined under a short-wave (254 nm.) ultraviolet lamp; a narrow band on the edge of the plates was then sprayed with phosphomolybdic acid. The silica gel corresponding to the individual areas was scraped off the plate and eluted several times with dichloromethane, which was evaporated to dryness in a stream of N₂.

The residues obtained from the eluted chromatographic areas were submitted to thin-layer chromatography and to spectroscopic analyses, in comparison with known steroids. To facilitate separation and purification, 50 cm.-long plates were used. The chromatograms were carried out on fluorescent silica gel-coated plates in chloroform-ether (9:1, v/v). After being examined under the ultraviolet lamp, the spots were sprayed with a modified Zimmermann reagent for the identification of keto steroids (Bongiovanni, Eberlein & Thomas, 1957) and with conc. H₂SO₄-ethanol (1:1, v/v), which stains most of the steroids characteristically. The sprayed Chromatoplates were heated on a hot plate at 100°. The ultraviolet spectra were determined in ethanol; the infrared spectra were measured in carbon tetrachloride in a microcuvette.

Biological assay. Assays were performed on rats and mice of both sexes. Of several preliminary assays, this paper reports only the indicative experiments on the uterotrophic activity. Beginning at 20–23 days of age the animals were injected subcutaneously daily for three consecutive days with the test material dissolved in sesame oil. The animals were examined *post mortem* on the fourth day. The uterus, blotted dry, was weighed to the nearest 0.5 mg. (Dorfman, 1962).

RESULTS

Uterotrophic activity. The chloroform-extracted material and the phenolic oestrogen fraction of the

saponifiable matter raised the weight of mice uteri significantly above the values of untreated controls. The saponifiable matter (1 mg.) produced the strongest increase in weight of the uterus, which was 1.3 times that obtained with 0.4 µg. of oestrone (Table 1).

Cholesterol. The crystalline substance isolated from the digitonin-precipitable complex obtained from the non-saponifiable material was recrystallized from acetone, yielding a product of m.p. 147–149°, [α]_D –36.9° (chloroform); its *R_F* value was identical with that of pure cholesterol simultaneously run on thin-layer chromatography. The infrared spectrum gave an additional proof of the identity of the isolated substance with the standard cholesterol.

Androsta-1,4-diene-3,17-dione. A semicrystalline portion of the ketonic fraction (20 mg.) run on thin-layer chromatography showed the same mobility as authentic androsta-1,4-diene-3,17-dione; these spots became visible under the short-wave (254 nm.) ultraviolet lamp. They were Zimmermann-positive and stained orange in the sulphuric acid reaction. The preparative chromatography of the remaining fraction yielded a crystalline substance with m.p. 141–143°, undepressed by mixing the test material with authentic androsta-1,4-diene-3,17-dione (m.p. 145–146°). The ultraviolet spectra gave a maximum at 244 nm., as did the authentic compound. The infrared spectrum showed maxima at the same wavelengths as the authentic sample.

5 β -Androst-1-ene-3,17-dione. The partition chromatography of the ketonic fraction also separated an area from which a sticky material (9 mg.) was obtained. The *R_F* value of this material run on thin-layer chromatography was identical with that of 5 β -androst-1-ene-3,17-dione. The spots of both substances were visible in ultraviolet light; Zimmermann reaction was positive and in the sulphuric acid reaction the spots gave a pale-greenish colour. The ultraviolet spectra showed a maximum at 229–230 nm., compatible with that of authentic 5 β -androst-1-ene-3,17-dione. The bands of the infrared spectrum were in accordance with the presence of a Δ^1 -3-ketone and of a 17-ketone.

Table 1. *Uterotrophic activity of various fractions of material extracted from tumours*

Material administered	Total dose in 3 days (mg.)	No. of rats	Body wt. (g.)			Wt. of uterus (g.)
			Initial	Final	Difference	
Controls	—	5	8.2	10.2	2.0	7.4 ± 0.7
Oestrone	0.0004	5	9.8	11.8	2.0	29.6 ± 2.1
Chloroform-extracted material	20.0	5	8.6	10.6	2.0	13.2 ± 4.6
Non-saponifiable matter	20.0	5	7.6	9.3	1.7	20.9 ± 0.9
Saponifiable matter	1.0	5	7.7	9.8	2.1	38.4 ± 2.4

Androst-4-ene-3,17-dione. A small oily quantity of the ketonic fraction moved at the same rate as androst-4-ene-3,17-dione, and, like this, was visible under ultraviolet light. The spots of both compounds were stained by the Zimmermann reaction and became blue-green with the sulphuric acid reagent. The test compound gave absorption maxima compatible with those of androst-4-ene-3,17-dione (239–242 nm.). Only the presence of a Δ^4 -3-ketone could be clearly demonstrated with the infrared spectroscopy.

Oestrone. The phenolic fraction extracted from the saponified matter revealed a chromatographic spot with the same mobility as that obtained by parallel runs of oestrone. Neither of the spots absorbed ultraviolet light. The Zimmermann reaction was positive and the sulphuric acid reagent produced a yellow-orange color. The absorption maximum (279–280 nm.) was comparable with that of pure oestrone. The paucity of the material and contamination by extraneous substances interfered with the infrared analysis; the presence of the hydroxyl group and 17-ketone was clear, but the bands of the aromatic ring were feeble.

DISCUSSION

The results of this experiment indicate that steroid compounds are present in the tissue of fast-growing hormone-dependent mammary tumours induced in the rat by 7,12-dimethylbenz[*a*]-anthracene. The identified steroids were cholesterol, androsta-1,4-diene-3,17-dione, androst-4-ene-3,17-dione, 5 β -androst-1-ene-3,17-dione and oestrone. The first two of these products were obtained in pure crystalline form. Cholesterol was the compound isolated in greatest amounts. Its isolation was not surprising because tumours are known to have a high content of cholesterol (Schubert, 1956). It might be suspected that cholesterol was the precursor of the other steroids found in the tumours. The presence of an intermediate metabolite to prove this assumption could not be found. The presence of androsta-1,4-diene-3,17-dione was unexpected because there are only a few instances of its occurrence in Nature. Androsta-1,4-diene-3,17-dione was first isolated from the faeces of a pregnant cow as a metabolite of subcutaneously administered progesterone (Miller, Turner, Fukushima & Salamon, 1956). The metabolic conversion was also described for a group of bacteria that can dehydrogenate ring A of progesterone and other Δ^4 -3-oxo steroids to form the corresponding Δ^1 ,4-dien-3-one compounds (Vischer & Wettstein, 1953; Fried, Thoma & Klingsberg, 1953). The transformation of [14 C]progesterone into androsta-1,4-diene-3,17-dione has occurred also *in vitro* by

cystic ovary but not by normal bovine ovaries (Gawienowski, Lee & Marion, 1961).

The evidence for the presence of the other steroids was clear but not so complete because of the small amount of material and because the presence of impurities precluded the isolation in crystalline form. However, the evidence of infrared and ultraviolet spectra in addition to the mobility values in the thin-layer chromatograms leaves little doubt about the identity of the compounds. Androst-4-ene-3,17-dione has been identified in normal and neoplastic endocrine organs of humans and animals (Dorfman & Ungar, 1965). 5 α -Androst-1-ene-3,17-dione is a naturally occurring steroid isolated from human urine (Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948; Lieberman, Fukushima & Dobriner, 1950), but the isolation from natural sources of the 5 β -epimer found in this experiment has not previously been described.

The bioassay had suggested the presence of a substance with oestrogenic activity in the phenolic fraction of the saponifiable matter (Table 1). This was supported by the isolation from this fraction of a substance that appeared to be oestrone. This steroid has been found in tumours of endocrine tissue that normally produce oestrone, but not in tumours of non-endocrine organs such as the induced rat mammary carcinoma.

There is the possibility that Δ^1 -3-oxo derivatives isolated in the present experiment may be artifacts of dehydration produced during boiling and saponification. This would signify the presence in the tumours of the hydroxylated form of the Δ^1 -3-oxo derivatives, that is 1- or 2-hydroxy-androst-4-ene-3,17-dione and 1- or 2-hydroxy-5 β -androstane-3,17-dione. In this case one might assume that these steroids have been produced by a 1 β -hydroxylation such as that observed *in vivo* in man, and with guinea-pig liver *in vitro* (Schneider & Bhacca, 1966; Dixon, Jones & Pennington, 1968). If it is supposed that the tumour tissue examined contains the C-19 1 β -hydroxy steroids, these would require a successive oxidation in C-19 to be transformed into oestrogens. This, however, is in contrast with the biosynthetic pathway based on experimental data, according to which the oxygen attack at C-19 occurs before the elimination of 1 β -hydrogen (the 2 β -hydroxy isomer would offer less possibilities) by means of an enzymic mechanism (Morato, Hayano, Dorfman & Axelrod, 1961; Gual, Morato, Hayano, Gut & Dorfman, 1962; Townsley & Brodie, 1968).

It must be remembered that there exists a class of undefined dehydrogenases capable of introducing carbon-carbon double bonds and having characteristics such as to suggest a hydroxylation-dehydrogenation mechanism (Gurr & Bloch, 1966). Possibly then Δ^1 -3-oxo derivatives might be formed

in this way in the present experiment; therefore 5β -androst-1-ene-3,17-dione, 5β -androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione could be the true precursors of oestrone (Ryan, 1960). In this case the most likely formation of oestrone could proceed from 5β -androst-4-ene-3,17-dione and 5β -androst-1-ene-3,17-dione via the corresponding 19-hydroxy derivatives (Scheme 1).

The rapid rearrangement to the aromatic structure of these compounds as well as of 19-

hydroxyandrosta-1,4-diene-3,17-dione explains the failure to find these steroids (Ryan, 1960). With regard to androsta-1,4-diene-3,17-dione, injected into apparently healthy men, this steroid increases the excretion of urinary oestrogens as much as testosterone and 19-nortestosterone. It would seem that the loss of the 19-methyl group in 19-norsteroids does not constitute a primary condition for the aromatization of ring A (Breuer, 1962).

Scheme 1. Possible biosynthetic pathways to oestrone in the experiment described.

The fact that malignant neoplasms of non-endocrine organs frequently contain hormones raises the question whether these hormones have been taken up from the circulation or whether they are synthesized by the tumour tissue (Hobbs & Miller, 1966). As regards the present experiment, one cannot be certain of the validity of either hypothesis; the second does not appear to be completely unfounded.

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