The Metabolism of Testosterone and Dihydrotestosterone in an Androgen-Dependent Tumour

A POSSIBLE CORRELATION BETWEEN DIHYDROTESTOSTERONE AND TUMOUR GROWTH *IN VIVO*

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The effects of dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one) and testosterone on the growth of the androgen-dependent Shionogi SC-115 tumour in mice have been compared and the metabolites in the tumour arising from each steroid have been identified. After the transfer of SC-115 tumour cells to castrated male mice, treatment of the recipients with dihydrotestosterone produced a striking proliferative response that enabled earlier tumour detection and led to a higher tumour incidence than obtained with testosterone. At short intervals after the intravenous injection of 200 μ Ci of [1,2-³H]testosterone the amounts of radioactivity in tumour, muscle and seminal vesicles were almost equal. The metabolism of [1,2-³H]testosterone in tumour and muscle was slight in comparison with the extensive metabolism in seminal vesicles. Whereas up to 7% of the total neutral steroid recovered from whole tumour tissue and isolated nuclei was in the form of [1,2-³H]dihydrotestosterone, the amount of this compound in the corresponding preparations from seminal vesicles was several times greater. When the metabolism of [1,2-³H]dihydrotestosterone in tumour tissue was studied, it was found that more than 60% of the total neutral steroid in both cytoplasm and nuclei consisted of [1,2-³H]dihydrotestosterone. Thus much higher intracellular concentrations of dihydrotestosterone occurred with the administration of this steroid than with testosterone. Tumour cell proliferation was suppressed by oestradiol and the amount of androgen in nuclei was significantly decreased by high doses of this hormone.

The atrophy of accessory genital glands in castrated adult males is easily reversed by the administration of testosterone (Price & Williams-Ashman, 1961). It is clear that the regeneration of tissue under these conditions represents the summation of a number of anabolic processes, including DNA, RNA and protein synthesis, at the biochemical level (Butler & Schade, 1958; Kochakian & Harrison, 1962; Williams-Ashman *et al.*, 1964; Coffey *et al.*, 1968) and cell hypertrophy and hyperplasia at the cytological level (Burkhart, 1942; Price & Williams-Ashman, 1961). Although the information on the specific role of testosterone in promoting these effects is incomplete, recent experimental work has afforded considerable evidence linking the biological activity of testosterone in certain target tissues to the formation of dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one) in cytoplasm and to the uptake of dihydrotestosterone by nuclei. For example, studies on a variety of androgen-sensitive tissues suggest that metabolism of testosterone to dihydrotestosterone is characteristic of the response of some types of end organ to an androgenic stimulus (Farnsworth & Brown, 1962; Bruchovsky & Wilson, 1968; Tveter &

Aakvaag, 1969; Shimazaki *et al.*, 1969; Wilson & Walker, 1969; Wilson & Gloyna, 1970; Bardin *et al.*, 1970). Deviations from the usual kinetics of dihydrotestosterone formation have been found in several pathological states such as testicular feminization (Northcutt *et al.*, 1969; Mauvais-Jarvis *et al.*, 1969; Wilson & Walker, 1969), benign prostatic hypertrophy (Gloyna & Wilson, 1969; Siiteri & Wilson, 1970; Gloyna *et al.*, 1970; Giorgi *et al.*, 1971) and acne vulgaris (Sansone & Reisner, 1971).

In addition to studies on the metabolism of testosterone, considerable attention has been focused on the identification of metabolites appearing in the target-cell nucleus, as this structure is believed to be the chief site of steroid hormone action (Tomkins *et al.*, 1969; Dofuku *et al.*, 1971; Gehring *et al.*, 1971; Steggle *et al.*, 1971). Since it is now well established that nuclei from prostate (Bruchovsky & Wilson, 1968; Anderson & Liao, 1968) and seminal vesicles (Fang *et al.*, 1969) as well as other tissues (Bullock & Bardin, 1970) preferentially accumulate dihydrotestosterone, the potential significance of this steroid in promoting the action of testosterone is obvious. Nevertheless, in spite of the abundant circumstantial

evidence on the relevance of dihydrotestosterone to the androgenic effect, it is uncertain whether this hormone completely mimics the action of testosterone. Indeed, in tissue-culture experiments with prostate, the cytological changes produced by each hormone were found to be different (Baulieu *et al.*, 1968). Although these results *in vitro* suggest that dihydrotestosterone specifically promotes the proliferation of target cells (Baulieu, 1970) the evidence for a parallel selective effect on mitotic activity *in vivo* is scant. One approach to the investigation of the effects of dihydrotestosterone on cell division *in vivo* is to examine the relationship of dihydrotestosterone to the growth of androgen-dependent tissue in which a significant proportion of cells continuously divide. A suitable tissue for such studies appears to be the Shionogi SC-115 neoplasm (Minesita & Yamaguchi, 1964). This mouse mammary adenocarcinoma grows only in males and is remarkably sensitive to changes in the hormonal milieu: tumour growth is inhibited or abolished by castration, by the administration of oestradiol-17 β , and reversal of these effects is readily achieved with testosterone (Minesita & Yamaguchi, 1964, 1965; Meakin, 1968). Further, results demonstrating the presence of a small amount of dihydrotestosterone in the tumour tissue shortly after the administration of testosterone (Yamaguchi *et al.*, 1971) are compatible with the presumed correlation between dihydrotestosterone and cell division. The chief aim of the present study was the clarification of the role of dihydrotestosterone in governing the proliferation of the experimental tumour. To obtain information on this question, the transformation products of testosterone were identified and compared with those in seminal vesicles, a normal target tissue, and with those in muscle, a non-target tissue. The metabolic fate of dihydrotestosterone in the tumour nuclei and cytoplasm was also determined.

Materials and Methods

Experimental animals

Dr. Tetuo Minesita, of the Aburahi Laboratories, Koko-cho, Japan, supplied this laboratory with sufficient animals to establish a colony of DD/S strain mice carrying the SC-115 carcinoma. Male mice were anaesthetized with ether and castrated through a scrotal incision. The preparation of tumour tissue for transplantation was carried out in the following manner. Tumours weighing 5–10g were dissected free of subcutaneous tissue, rinsed in ice-cold phosphate-buffered saline (Dulbecco & Vogt, 1954) and transferred to a cup-shaped receptacle constructed of stainless-steel screen (30 mesh; 0.55mm grid). A preliminary mincing of tissue was carried out with scissors and then the mince was forced through the screen with a Teflon pestle. Tissue fragments were collected in a beaker containing ice-cold tissue-

culture medium (CMRL 1415-ATM, Connaught Medical Research Laboratories, Toronto, Canada), transferred in suspension to a 50ml graduated centrifuge tube and sedimented at 25g for 1 min in an MSE Multex centrifuge. After centrifugation the volume of tumour tissue was measured and the sample was diluted with CMRL 1415-ATM to give a concentration of 0.1 or 0.2ml of tumour tissue/ml of medium. Each animal was injected subcutaneously above the thoracic spine with 1ml of such a preparation.

Preparation of hormones for injection

Testosterone, dihydrotestosterone and oestradiol were dissolved in medium to give a final concentration of 0.4mg/0.25ml. Two types of injection solutions were tried: sesame oil–chloroform (39:1, v/v) and water–ethanol–polyoxyethylene sorbitan monopalmitate (57:20:3, by vol.). The latter was used in the majority of experiments described in later sections of this paper. Further trials with other media suggested that an aqueous medium (Dorfman & Kincl, 1963) consisting of NaCl (0.9%), polyoxyethylene sorbitan monopalmitate (0.4%), CM-cellulose (0.5%) and benzyl alcohol (0.9%) is the most satisfactory. Hormone injections were started 1 day after injection of tumour cells and then repeated three times weekly until 12 doses were given. Thus each animal received 4.8mg of appropriate steroid during the course of treatment.

Homogenization of tissue

All tumour tissue was first broken up by the screening procedure already described. The tumour fragments were next suspended in 50ml of tris–HCl buffer, pH 7.0 (10mM), containing EDTA (0.05mM), MgCl₂ (5mM), mercaptoethanol (0.5mM) and NaCl (150mM), and centrifuged at 25g for 1 min in an MSE Multex centrifuge. Three cycles of this operation were required to free the tissue of erythrocytes. For experiments on whole tissue, the final pellet was resuspended in 20ml of water and homogenized in a Dounce apparatus with 25 strokes of a large-clearance pestle and 10 strokes of a small-clearance pestle. The homogenate was then extracted with 100ml of chloroform–methanol (2:1, v/v).

Fractionation of the tumour into cytoplasmic and nuclear samples was accomplished by similar techniques except that the final pellet was resuspended in 40ml of 0.25M-sucrose. As before, the tissue was disrupted in a glass homogenizer and then sedimented in a Sorvall RC 2-B Superspeed refrigerated centrifuge at 250g for 10 min (SS-34 rotor, r_{av} 5.4cm). The supernatant (cytoplasmic fraction) was decanted and extracted with chloroform–methanol (2:1, v/v) and the pellet was resuspended in 25 ml of 0.88M-sucrose containing 0.5mM-CaCl₂. This suspension was

layered over a discontinuous sucrose density gradient consisting of 5 ml of 2.2M-sucrose and 5 ml of 1.8M-sucrose, both containing 0.5 mM-CaCl₂. Samples were centrifuged at 53000g_{av.} for 90 min (SW-27 rotor, $r_{av.}$ 11.56 cm) in a Beckman-Spinco L2-65B ultracentrifuge. The temperature setting was 0–4°C. At the completion of centrifugation the supernatant was decanted and the pellet (nuclear fraction) was re-suspended in 2 ml of tris-HCl buffer, pH 7.0 (10 mM), containing EDTA (0.05 mM), MgCl₂ (5 mM), mercapto-ethanol (0.5 mM) and NaCl (50 mM). Portions were taken for counting of nuclei and for measurement of radioactivity and the remaining sample was then extracted with 10 ml of chloroform-methanol (2:1, v/v).

Slight variations of this procedure were introduced to facilitate the preparation of other experimental tissues. Seminal vesicles were combined with prostate and epididymal tissues to increase the amount of starting material. Nevertheless, the seminal vesicles constituted the major part of the sample thus obtained and for convenience the pooled tissues are referred to as seminal vesicles in following descriptions. To prepare the seminal vesicles for homogenization the tissue was chopped with scissors, chopped again with an automatic tissue slicer, and finally grated through the stainless-steel screen. The resulting tissue mince was then easily processed through the various steps described for the preparation of cytoplasm and nuclei from tumour.

Thigh muscle was chopped with scissors and minced in a high-speed tissue blender to render it suitable for subsequent homogenization steps and extractions.

Counting of nuclei. Nuclei were diluted in buffer containing Methyl Blue and counted with a haemocytometer.

Extraction of steroids from tissue

Chloroform-methanol (2:1, v/v; 5 vol.) was added to the appropriate fractions. Flasks and tubes containing the samples were flushed with N₂, stoppered and agitated on a rotary shaker for 20 min at 400 rev./min. Next the emulsion was centrifuged at 400g for 10 min in an MSE Multex centrifuge. The upper aqueous phase was again shaken with a volume of upper-phase solvent (chloroform-methanol-water, 3:38:47, by vol.) equal to the volume of aqueous phase removed (Folch *et al.*, 1957). Centrifugation was used to separate the phases and the cycle was repeated once more. The final chloroform-methanol phase was passed through a Teflon filter (pore size, 5 µm). A stream of N₂ was used to dry the filtrate.

Preparation of extracts for analysis

Mallinckrodt silicic acid no. 2847 (Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.) was

prepared as described by Horning (1964). To separate neutral steroids from total lipids a column (1 cm × 10 cm) was prepared with a slurry of 100–200-mesh silicic acid in chloroform-methanol (4:1, v/v). The column was washed with 50 ml of solvent mixture and then with chloroform. After the tissue extract was applied to the column in a small volume of chloroform, neutral steroids were eluted with 150 ml of chloroform. The eluate was dried and redissolved in chloroform in preparation for analysis by t.l.c.

Fig. 1. Induction of tumour growth

Male mice were castrated 50 days before the beginning of the experiment. These animals were divided into three groups of 12–16 mice each and all animals including those in a fourth non-castrated group were injected subcutaneously with 0.2 ml of tumour tissue. One group of castrated mice served as a control, whereas the second and third groups received 5 mg of testosterone or dihydrotestosterone in 12 doses over a period of 4 weeks. The mice were examined at 2–3 day intervals for the presence of tumour masses. Lumps were not considered significant unless the diameter was greater than 0.5 cm. Because of the slow disappearance of the tumour inoculum in uninjected castrated males no attempt was made to establish the final incidence of tumours in this group until week 5 of the experimental period. Incidence of tumours: ●, 50 day castrated mice treated with dihydrotestosterone; ○, intact control mice; △, 50 day castrated mice treated with testosterone; ▲, 50 day castrated controls; ----, average incidence of tumours in 50 day castrated controls from combined results of three experiments (41 mice).

Fig. 2. Effect of tumour size on the incorporation of [1,2-³H]testosterone

Male mice with tumours of various sizes were each injected with 200 μ Ci of [1,2-³H]testosterone (specific radioactivity 5mCi/0.0282mg) and 15–30min later the animals were killed. Tumours were weighed, forced through a wire screen as described in the Materials and Methods section and then homogenized in a ball-type Dounce homogenizer. Portions of the homogenate were dissolved in Bio-Solv scintillation fluid and the radioactivity was measured. The results are shown as a plot of radioactivity (d.p.m.) versus weight of tumour.

Further purification of the extract was necessary, however, before it was suitable for analysis by g.l.c. For this purpose short silicic acid columns were used as described previously (Vihko, 1966). After the removal of cholesterol by this method the final eluate was dried in a conical glass vial under a stream of N₂. Appropriate steroid standards were added and a sample of the mixture was removed for analysis by g.l.c.

Chromatography

Thin-layer chromatography. Glass plates (20cm × 20cm) were coated with a suspension of Silica Gel H (Merck, Brinkmann Instruments, Toronto, Canada) in water (30g/72ml) or aluminium oxide, neutral, type T (Merck), in water (30g/42ml). The thickness of the coating was 250 μ m. The plates were activated

at 100°C for 1h. Separations of androstanediol (5 α -androsterane-3 α ,17 β -diol), androstanedione (5 α -androsterane-3,17-dione), androstenedione (4-androstene-3,17-dione), dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one) and testosterone were achieved on silica gel with one development of the plate in chloroform–methanol (49:1, v/v). The respective *R_F* values were 0.35, 0.85, 0.79, 0.59, 0.51. Aluminium oxide plates were always developed twice in methylene chloride–ether (9:1, v/v) to obtain separations of androsterone (3 α -hydroxy-5 α -androstan-17-one; *R_F* 0.22), from dihydrotestosterone (*R_F* 0.51). Finally, good resolution of all six steroids was obtained on aluminium oxide plates developed once or twice in cyclohexane–ethyl acetate–acetic acid (60:40:0.1, by vol.). Appropriate standards were added to each extract before the application of the sample to the plate. On completion of chromatography the plates were dried and sprayed with a solution of Morin (0.01%, w/v, 2',3,4',5,7-pentahydroxyflavone in methanol; Merck) and examined under u.v. light. After the positions of the standard had been noted the silica gel was transferred in sections to counting vials. Methanol (1ml) and 10ml of 0.4% diphenyl-oxazole in toluene were added to each vial and the samples were assayed for radioactivity.

Gas-liquid chromatography. Analysis of steroids by this method was accomplished with an F & M model 402 gas chromatograph (Hewlett Packard Analytical Instruments, Pointe Claire, Quebec, Canada) equipped with a flame-ionization detector. Steroids were recovered by means of an effluent splitter designed in this laboratory. Under the conditions of operation, the recovery of steroid was always 80% or greater of the theoretical maximum. Glass columns (210cm × 2mm) were packed with Gas-Chrom Q, 100–200 mesh (Applied Science Laboratories, State College, Pa., U.S.A.), coated with 3% QF-1. Operating temperatures were as follows: oven, 210°C; flash heater, 250°C; detector, 230°C; the flow rate of carrier gas (argon) was 80ml/min. Samples were collected in Teflon tubing 15cm long and after the completion of a run each tube was flushed with 10ml of scintillation fluid. Retention times (min) for androstanediol, androsterone, dihydrotestosterone, testosterone, androstanedione and androstenedione were 4.1, 6.9, 9.3, 14.5, 16.5, and 25.2 respectively. Resolution of 3 α ,17 β - and 3 β ,17 β -androstanediol and of androsterone and 3-epiandrosterone (3 β -hydroxy-5 α -androstan-17-one) could be accomplished with this procedure.

Radioactive materials

Both [1,2-³H]testosterone (5mCi/0.0282mg) and [1,2-³H]dihydrotestosterone (1mCi/6.0 μ g) were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.). Purity was checked by t.l.c. and g.l.c.

and varied between 93% and 95% in several tests. Solutions for intravenous injections were prepared as follows: to each 200 μ l of ethanol-benzene solution containing radioactive steroid was added 20 μ l of 5% (v/v) polyoxyethylene sorbitan monopalmitate and the solution was dried under N_2 . The dry preparation was then dissolved in a suitable amount of water containing 5% (v/v) ethanol. Each mouse was injected with 250 μ l of such a solution.

Liquid-scintillation counting

Liquid-scintillation counting was carried out with either a diphenyloxazole-toluene solution (4g of diphenyloxazole/litre of toluene) or a diphenyloxazole-toluene solution that contained toluene (1 litre), diphenyloxazole (6g), water (75ml) and Bio-Solv (BBS-3; 116g; Beckman Instruments, Scientific Instruments Division, Fullerton, Calif., U.S.A.). Samples were deposited in glass vials and the radioactivity was measured in an automatic liquid-scintillation system. External standardization was used to correct for quench and all results were normalized to d.p.m.

Results

Induction of tumour growth by testosterone and dihydrotestosterone

The growth of tumour in groups of castrated mice treated with testosterone and dihydrotestosterone was compared with the growth of tumour in intact control groups. Mice castrated 50 days previously were implanted with tumour tissue and given a total of 5 mg of steroid in 12 doses over a period of 4 weeks. From the results shown in Fig. 1, it is clear that the rate of tumour appearance in the group treated with dihydrotestosterone exceeded the rate in the intact controls and in the group receiving testosterone. The final incidence of tumours in the castrated groups was 25%, somewhat higher than the expected incidence of 14% (broken line), which was the average value obtained from several experiments. These results indicate that testosterone is less effective than dihydrotestosterone in supporting tumour development when injected in equal doses. Further, the tumours which grew in response to injections of dihydrotestosterone enlarged quickly and attained a volume several times greater than the volume reached with testosterone treatments. The tumours in the intact mice enlarged slowly but eventually grew to large proportions. Experiments were next carried out to determine whether the growth of tumour in intact mice and in testosterone-treated castrates might be explained by the formation of dihydrotestosterone in the tumour.

Incorporation of radioactivity after the administration of [1,2- 3H]testosterone

To select favourable conditions for subsequent studies *in vivo*, the effect of tumour size and of time on the incorporation of androgen was studied. Mice with tumours of various sizes were injected intravenously with 200 μ Ci of [1,2- 3H]testosterone and 15–30 min later the radioactivity in each tumour was measured. The results (Fig. 2) indicate that the incorporation of radioactivity increases with tumour weight and thus suggest an advantage to the use of larger tumours. Accordingly, tumours weighing 5–10 g were used in all further experiments as a routine.

The influence of time on the incorporation of steroid was studied next. Pairs of mice, each with a tumour of appropriate size, received an intravenous dose of 200 μ Ci of [1,2- 3H]testosterone. After this injection, the mice were killed at different times up to 60 min and the radioactivity in each tumour was

Fig. 3. Effect of time on the incorporation of [1,2- 3H]testosterone

Pairs of tumour-bearing mice were injected intravenously with 200 μ Ci of [1,2- 3H]testosterone (specific radioactivity 5 mCi/0.0282 mg). The mice were killed at 1, 5, 15, 30, 45 and 60 min after the injections. The plasma, seminal vesicles and thigh muscles from each pair of animals were combined; the tumours were processed individually. Homogenates were prepared as described in the Materials and Methods section and assayed for radioactivity. The results obtained for each pair of tumours were averaged. Radioactivity recovered: ○, plasma; △, muscle; ▲, seminal vesicles; ●, tumour.

Scheme 1. *Purification of neutral steroids*

measured. The plasma, seminal vesicles and muscle from each pair of mice were combined and the radioactivity in the individual tissue fractions was determined (Fig. 3). From the time of the earliest measurement, the radioactivity in the seminal vesicles and tumour remained almost constant at values of 5000000 and 2500000 d.p.m./g respectively. The values for muscle and plasma fell within this range after 15 min. There was no striking difference in the uptake of radioactivity by the various tissues, and it was not possible to demonstrate an optimum period of incorporation. Consequently, 30 min was chosen as a convenient interval in several experiments to be described later.

Extraction of steroids from tissues

Since neoplastic tissue obtained from host mice is often haemorrhagic, the removal of erythrocytes is a mandatory preliminary step towards various biochemical and cytological determinations. If the original tumour mince is suspended in iso-osmotic buffer, centrifugation at a low speed results in the sedimentation of tumour fragments and erythrocytes remain in suspension. During this procedure, however, a large proportion of radioactivity in the initial tumour mass is recovered in the supernatant fraction as well. Hence several experiments were performed to ascertain whether the cell-separation method caused any significant change in the distribution of neutral

metabolites. The outline of the analysis carried out on the supernatant and pellet fractions is shown in Scheme 1 and the corresponding results are shown in Table 1. With every tissue studied, the major proportion of polar metabolites was recovered in water-soluble fraction 1. This fraction, when it was derived from tumour and muscle, contained a large amount of neutral metabolites. The quantity of polar metabolites in the three tissue fractions was substantial, as is evident from the radioactivity recovered in water-soluble fraction 2 and the radioactivity in polar compounds detected by chromatography. The final recovery of neutral steroids in relation to the total starting radioactivity was 4, 14 and 1% for tumour, seminal vesicles and muscle respectively. The results of a t.l.c. analysis to estimate the identity of the neutral metabolites in water-soluble fraction 1 and in the tissue fraction are listed in Table 2. Testosterone was the principal metabolite in both fractions from tumour and muscle, whereas considerably less was found in the parallel fractions from seminal vesicles. From an examination of the relative amount of testosterone and the other androgens, dihydrotestosterone-androsterone, androstenediol, androstenedione and androstane-dione, in tumour and seminal vesicles it is evident that there is a reasonable correlation between the types of androgens found in water-soluble fraction 1 and in the tissue isolated by centrifugation. The correlation is weaker with muscle, particularly in the proportion of radioactivity in the dihydrotestosterone-androsterone compartment. Thus Tables 1 and 2 indicate that the procedure outlined in Scheme 1 may be used to obtain a represen-

tative sample of neutral steroids from the tissue fractions of both tumour and seminal vesicles. Although the comparable muscle fraction is less likely to provide an accurate picture of the distribution of neutral metabolites, the relative enrichment of the combined dihydrotestosterone-androsterone product was thought to be a sufficient reason to pursue further investigations on this tissue.

Time-sequence studies on the recovery of neutral metabolites in tissue fractions

A detailed study of the appearance of neutral metabolites in the tissue fraction of tumour, seminal vesicles and muscle was carried out to determine whether the retention of metabolites in dividing tissue compares with the retention in a target tissue or with that in a non-target tissue. Pairs of tumour-bearing mice were injected with 200 μ Ci of [1,2- 3 H]-testosterone. At intervals from 1 to 60 min the mice were killed and the plasma, tumours, thigh muscles and seminal vesicles were removed from each animal. Except for the tumours, the various tissue samples from each pair of mice were combined and analysed according to the procedure described in Scheme I. Tumours were processed individually and the final results from each duplicate were averaged. The recovery of radioactivity in water-soluble fractions 1 and 2, in the chloroform-methanol extract and in the final post-chromatography fraction is shown in Fig. 4. The difference between the radioactivity in the final fraction and the radioactivity in the chloroform-

Table 1. *Distribution of radioactivity in extracts of tumour, seminal vesicles and muscle*

Groups of two or three normal or tumour-bearing mice were injected with 200 μ Ci of [1,2- 3 H]testosterone and 30 min later the animals were killed. In separate experiments tumours, seminal vesicles and thigh muscles were removed, homogenized and extracted as described in Scheme 1. After this procedure the amount of radioactivity in each fraction was measured.

Tissue	$10^{-4} \times$ Radioactivity (d.p.m.)						
	In tissue Total	In aqueous tissue extract		In tissue fragments			
		Water-soluble fraction 1		Water-soluble fraction 2	Compounds removed by chromatography		Steroids retained by tissue
		Polar	Neutral		Polar	Neutral	
Tumour	1745.0	1131.3	219.2	56.0	49.1	67.9	4
Seminal vesicles	351.0	143.1	14.5	45.2	36.7	48.7	14
Muscle	299.8	101.0	117.3	5.4	2.3	2.8	1

Table 2. *Preliminary identification of androgen metabolites in neutral steroid extracts*

The extracts of neutral steroids from each water-soluble fraction 1 and from each tissue fraction described in Table 1 were analysed by t.l.c. The relative amount of each steroid identified is expressed as a percentage of the total radioactivity in the neutral steroid fraction. No attempt was made to separate dihydrotestosterone from androsterone. The radioactivity defined as 'other' represents material recovered at the origin and in positions that did not correspond to any of the standards.

Origin	Identity					Other
	Testosterone	Dihydro-testosterone-androsterone	Androstane-diol	Androstene-dione	Androstane-dione	
Tumour						
Water-soluble fraction 1	73	4	6	9	2	6
Tissue	69	3	2	3	1	22
Seminal vesicles						
Water-soluble fraction 1	27	25	30	4	2	12
Tissue	13	24	24	6	5	28
Muscle						
Water-soluble fraction 1	90	1	2	1	0	6
Tissue	52	17	7	5	1	18

methanol extract yields an estimate of the polar compounds in the latter preparation that are removed during the chromatography procedure. From the series of curves presented in Fig. 4, three points are clear. First, there was a continuous decline in the recovery of neutral steroids with time such that at 30 min these were decreased to 5% or less of the total radioactivity and at 60 min to approx. 1% in plasma, muscle and seminal vesicles and to 3% in tumour. Secondly, the decline in neutral metabolites was accompanied by an upward change in the quantity of polar metabolites in each tissue as is evident from the increasing percentage of radioactivity recovered in water-soluble fractions 1 and 2 and from the larger portions of radioactivity extracted by the chromatography step. Thirdly, no striking differences in the tissue retention of neutral metabolites were revealed by these experiments.

Identification of neutral metabolites in tissue fractions

The extracts containing neutral metabolites were next analysed by t.l.c. and g.l.c. and the results are summarized in Figs. 5 and 6. Testosterone was the chief androgen in the neutral fraction from plasma, tumour and muscle, forming 50% or more of the total radioactivity in this fraction at all times. Conversely, in the same fraction from seminal vesicles, testosterone was a relatively minor metabo-

lite contributing 5% to the total radioactivity at 1 min and 20% at 30 min. Dihydrotestosterone was present at 2% or less in the plasma and muscle fractions, at 5% or less in the tumour fraction, but in the seminal vesicles fraction the amount was considerably greater, varying between 10 and 20%. The percentage of androstane-diol was much higher in the last-named fraction than in any of the others. Except in one or two instances, the other androgens (androstenedione, androstane-dione and androsterone) tended to increase with time in all tissues. Since the initial amounts of dihydrotestosterone, androstane-diol, androsterone and androstane-dione were higher in seminal vesicles than in plasma, tumour and muscle, it is probable that the steroids found in seminal vesicles were partly or wholly synthesized in this tissue. Similarly, since the initial percentage of dihydrotestosterone in tumour was greater than the initial percentage in plasma and muscle, it appears that the tumour may independently synthesize dihydrotestosterone from testosterone. The presence of an active-transport system, which could also account for these results, has not been excluded. Finally, the rapid increase in the amount of androsterone in muscle (Fig. 6b) is noteworthy. The attainment of substantial amounts of this steroid probably accounts for the large amount of radioactivity in the dihydrotestosterone-androsterone recorded for muscle in Table 2.

Fig. 4. Recovery of neutral metabolites of $[1,2-^3\text{H}]$ -testosterone

Pairs of tumour-bearing mice were injected intravenously with $200\mu\text{Ci}$ of $[1,2-^3\text{H}]$ testosterone. At intervals from 1 to 60 min later the mice were killed and various tissues were removed from each animal. The plasma, seminal vesicles and thigh muscles were combined whereas tumours were treated individually. The tissue samples were processed according to Scheme 1. Duplicate results from the tumour samples were averaged. Radioactivity recovered: ●, water-soluble fraction 1; ○, water-soluble fraction 2; △, chloroform-methanol extract; ▲, post-chromatography fraction containing purified neutral metabolites. (a) Plasma; (b) tumour; (c) muscle; (d) seminal vesicles.

Incorporation of radioactivity by nuclei after the administration of $[1,2-^3\text{H}]$ testosterone and $[1,2-^3\text{H}]$ -dihydrotestosterone

Although formation of the androgen dihydrotestosterone takes place in seminal vesicles, it is evident that the tumour accumulates various androgens including a significant amount of dihydrotestosterone. The relative amounts of testosterone and dihydrotestosterone in the two tissues are widely different but it is possible that the differences are less marked in nuclei, the presumed site of action of these steroids. Accordingly, further studies were carried out to compare the nuclear uptake of both compounds. First, the radioactivity in nuclei from three

Fig. 5. Identification of neutral metabolites in tissue fractions from plasma and tumour

The purified extracts of neutral steroids obtained as described in the legend to Fig. 4 were analysed by t.l.c. and g.l.c. Although the two methods yielded similar results only those obtained by t.l.c. are shown. In addition to the radioactivity recovered in the steroids indicated, small amounts were often located at the origin and in other areas that did not correspond to the position of the standard compounds. Androstane- $3\beta,17\beta$ -diol was identified in trace amounts. The principal metabolites in plasma (a and b) and tumour (c and d) were: ○, testosterone; ●, dihydrotestosterone; □, androstane- $3\alpha,17\beta$ -diol; ▲, androstenedione; △, androstanedione; ■, androsterone.

tumours was compared at 5, 30 and 60 min after the administration of $[1,2-^3\text{H}]$ testosterone. Table 3 shows that the incorporation was maximal at 30 min. Next, the same amount of $[1,2-^3\text{H}]$ testosterone was injected into each of five normal mice and after a 30 min interval nuclei were isolated from the combined seminal vesicles. Nuclear radioactivity was then measured and in two such experiments the average value shown in Table 3, column 2, was obtained. Finally, an equivalent dose of $[1,2-^3\text{H}]$ dihydrotestosterone was injected into two tumour-bearing mice and nuclei were recovered from both tumours. The average of the results is shown in Table 3, column 3. From the results in Table 3 and Table 5 (expt. 1), it is apparent that when mice were treated with $[1,2-^3\text{H}]$ -testosterone the amount of radioactivity in nuclei from tumour and seminal vesicles was identical at

30 min. Less radioactivity was recovered at this time in tumour nuclei from mice receiving $[1,2-^3\text{H}]$ -dihydrotestosterone. Moreover, in whole tumour

from the last-named group the radioactivity, at 1700000 d.p.m./g was also below that expected after the administration of labelled testosterone.

Identification of radioactive steroids in nuclei

Individual 30 min samples obtained in the experiments described in the previous section were extracted with chloroform-methanol, purified on silicic acid columns and analysed by t.l.c. and g.l.c. The results of several analyses are shown in Table 4. After the administration of $[1,2-^3\text{H}]$ testosterone the chief metabolite in tumour nuclei was testosterone, which constituted 88% of the neutral steroid fraction; in the same fraction from seminal vesicles nuclei only 17% was in the form of this steroid. The corresponding amounts of dihydrotestosterone were 4% in the fraction from tumour nuclei and 67% in the fraction from seminal vesicles nuclei. Thus the relative proportion of testosterone and dihydrotestosterone in tumour nuclei and seminal vesicles nuclei was almost reversed. Far more dihydrotestosterone was found in nuclei from target tissue than in those from tumour. When $[1,2-^3\text{H}]$ dihydrotestosterone was administered to tumour-bearing mice approx. 80% of the radioactivity in the nuclear extract was recovered in the form of dihydrotestosterone. A slightly lower percentage was found in the matching cytoplasmic fraction, as is also shown in Table 4. It is clear from the results presented in Tables 3 and 4 that, whereas the amount of radioactivity in tumour nuclei originating from $[1,2-^3\text{H}]$ testosterone may exceed the amount established with $[1,2-^3\text{H}]$ dihydrotestosterone, the small amount of metabolic conversion of $[1,2-^3\text{H}]$ testosterone is probably not sufficient to produce more than a low concentration of $[1,2-^3\text{H}]$ -dihydrotestosterone in nuclei. On the other hand,

Fig. 6. Identification of neutral metabolites in tissue fractions from muscle and seminal vesicles

Details of the analytical methods are given in the legend to Fig. 5. The principal metabolites in muscle (a and b) and seminal vesicles (c and d) were: $\circ$, testosterone; $\bullet$, dihydrotestosterone; $\square$, androstane- $3\alpha,17\beta$ -diol; $\blacktriangle$, androstenedione; $\triangle$, androstenedione; $\blacksquare$, androsterone.

Table 3. Incorporation of radioactive androgens by nuclei

Normal or tumour-bearing mice were injected intravenously with $200\mu\text{Ci}$ of $[1,2-^3\text{H}]$ testosterone (specific radioactivity $1\text{ mCi}/5.6\mu\text{g}$) or of $[1,2-^3\text{H}]$ dihydrotestosterone (specific radioactivity $1\text{ mCi}/6.0\mu\text{g}$) and animals were killed at the times shown. Five mice were used for each determination on seminal vesicles. Nuclei were purified as described in the Materials and Methods section and counted in a haemocytometer. As the slight difference in the specific radioactivity of the injected steroids can be disregarded, the radioactivity is expressed directly as d.p.m./nucleus.

Steroid injected	Tissue ...	$10^4 \times$ Radioactivity incorporated (d.p.m./nucleus)		
		Tumour Testosterone	Seminal vesicles Testosterone	Tumour Dihydrotestosterone
Time after injection of steroid (min)				
5		4.2		
30		9.0	12.7	3.3
60		6.5		

Table 4. *Identification of androgen metabolites in nuclei and cytoplasm*

Cytoplasm and nuclei containing radioactive androgens were extracted with chloroform-methanol (2:1, v/v). Neutral steroids were obtained in a pure form after the extracts were passed through columns of silicic acid once or twice. The purified metabolites were analysed by t.l.c. and g.l.c. The relative amount of each steroid identified is expressed as a percentage of the total radioactivity in the neutral steroid fraction. Radioactivity that did not correspond to any of the standard compounds is shown as 'other'.

Tissue	Steroid injected	Androgens recovered (% of total)						Other
		Dihydro-testosterone	Testosterone	Androstane-diol	Androsterone	Androstenedione	Androstane-dione	
Tumour nuclei	Testosterone	4	88	1	1	1		5
Seminal vesicles nuclei	Testosterone	67	17					16
Tumour nuclei	Dihydro-testosterone	82		2	4			12
Tumour cytoplasm	Dihydro-testosterone	67		7	6		5	15

with the administration of [1,2-³H]dihydrotestosterone the conversion is unnecessary and a much higher concentration of [1,2-³H]dihydrotestosterone is obtained.

Effects of oestradiol on the induction of tumours

The influence of oestradiol on tumour growth was studied in male and female mice. In one type of experiment intact male mice were implanted with tumour tissue and given a total of 5 mg of oestradiol in 12 doses over a period of 4 weeks; in the other type of experiment, female mice were implanted with tumours and given 5 mg of testosterone or dihydrotestosterone in divided doses over the same time-interval. The incidence of tumours in the three groups of mice is shown in Fig. 7. The broken line indicates the expected incidence in a group of normal male mice injected with tumour tissue alone. Not only was the appearance of tumours delayed in the male mice given oestradiol, but the final incidence of tumours was decreased by two-thirds. In female mice receiving dihydrotestosterone the final tumour incidence of 71% was far greater than the corresponding incidence in female mice treated with testosterone. In the absence of exogenous androgens, tumour growth in female mice was rare.

Effects of oestradiol on the metabolism of [1,2-³H]-testosterone

Whereas it is generally accepted that oestradiol decreases the testicular synthesis of testosterone by inhibiting the release of interstitial-cell-stimulating hormone from the pituitary, it has been postulated that the hormone may directly affect target tissues

by blocking the metabolism of testosterone (Farnsworth, 1969; Groom *et al.*, 1971; Leav *et al.*, 1971). Since a suppressive action of oestradiol on the formation of dihydrotestosterone in tumour would provide an attractive explanation for the oestrogenic inhibition of cell proliferation in this tissue, a search for such an effect was carried out in tumour-bearing animals treated with 25 µg of oestradiol for 0.5 or 1.5 h. Table 5 shows that the uptake of radioactivity by whole tumours in oestradiol-treated animals was similar to that in untreated controls. However, the radioactivity in nuclei from animals treated with oestradiol for 0.5 h was significantly lower than the radioactivity in nuclei from controls ($P < 0.01$), as was the radioactivity in nuclei from animals treated with oestradiol for 1.5 h ($P < 0.005$).

Cytoplasmic and nuclear fractions from tumour tissue were extracted with chloroform-methanol and the extracts were purified as described in the Materials and Methods section. An average of 60% of the nuclear radioactivity was recovered in the form of neutral steroids, whereas the proportion of cytoplasmic radioactivity recovered in this form was less than 20%. There was no difference in the relative amount of neutral steroids in similar samples from treated and control animals.

The identity of the androgens in the neutral steroid fractions as determined by t.l.c. and g.l.c. is shown in Table 6. It is clear from these results that the administration of oestradiol did not produce a significant decrease in the percentage of dihydrotestosterone in nuclei and cytoplasm, as might be expected if oestradiol inhibited the metabolism of testosterone. The relative percentage of each androgen, apart from dihydrotestosterone and testosterone, was lower in nuclei than in cytoplasm, thus suggesting

that the nuclear membrane in the tumour cells, as in normal cells, is impermeable to many androgenic steroids (Bruchovsky, 1971).

Discussion

Previous reports on the growth characteristics of the SC-115 tumour have indicated that several androgenic steroids in addition to testosterone will stimulate tumour-cell reproduction (Minesita & Yamaguchi, 1964, 1965). One of the androgens, 17 β -hydroxy-2-methylandrostan-3-one, a close analogue of dihydrotestosterone, was found to be more

Fig. 7. Effect of administration of oestradiol on the induction of tumour growth

Each animal in a group of 15 intact male mice was implanted with 0.2ml of tumour tissue and then treated with 5mg of oestradiol in 12 doses over a period of 4 weeks. Two groups of female mice were also implanted with tumour and treated with 5mg of either testosterone or 5 α -dihydrotestosterone over the same period. The mice were examined at 2–3 day intervals for the presence of tumour masses. Lumps were not considered significant unless the diameter was greater than 0.5cm. Because of the slow disappearance of the initial tumour inoculum in female mice no attempt was made to establish the final incidence of tumours in these groups until week 5 of the experimental period. Incidence of tumours: ▲, females treated with dihydrotestosterone; △, females treated with testosterone; ●, males treated with oestradiol; ----, expected incidence in normal males (see Fig. 1).

potent than testosterone in producing this effect (Minesita & Yamaguchi, 1964). In the present study it has been demonstrated that dihydrotestosterone itself induces a striking proliferative response resulting in earlier tumour detection and leading to both a higher tumour incidence and more extensive tumour enlargement than obtained with testosterone.

The results of studies on the metabolic fate of testosterone and dihydrotestosterone in established tumours are consistent with several provisional statements on the relationship of these steroids to cell division. Any consideration of the experimental results, however, must take into account the difficulty in correlating biochemical measurements on a large tumour, perhaps composed of heterogeneous cell populations, with the critical molecular events surrounding the process of cell division in a subpopulation of unknown size. Although the dependency of the initiation of tumour growth on the natural androgens, testosterone and dihydrotestosterone, is convincing, a similar absolute requirement for growth in an advanced tumour is less certain. If it is assumed that a steady supply of androgen is essential for cell multiplication, at least two observations on the behaviour of the tumour after the interruption of hormonal supply are perplexing. First, castration of a tumour-bearing host may produce regression that is only partial and which is followed later by renewed growth. Secondly, withdrawal of exogenous androgen in castrated animals carrying tumours of advanced size often has only a slight retarding effect on tumour growth. In both cases the failure to abolish tumour proliferation may be explained by the existence of two interacting cell populations, one responsive to hormonal manipulations, the second unresponsive. Another possibility is that once the tumour has reached a significant size the necrotic parts of the tissue mass together with the adrenal glands provide adequate hormone to support continued cell division. The occasional development of tumours in castrated males and in females may also be due to the ability of the adrenal glands to act as a source of androgens. Unfortunately these poorly understood phenomena restrict the complete interpretation of the significance of androgen metabolism and accumulation by the aging SC-115 tumour cell.

The effectiveness of dihydrotestosterone in promoting a proliferative response in tumour is clearly demonstrated in Fig. 1. Studies on the metabolism of dihydrotestosterone in mature tumours (Tables 3 and 4) indicate that after administration *in vivo* very little intracellular transformation of this compound takes place and thus dihydrotestosterone is the chief steroid to appear in both nuclei and cytoplasm.

The metabolism of exogenous testosterone in tumour is also slight and contrasts dramatically with the extensive metabolism in seminal vesicles (Figs. 5

Table 5. *Effects of oestradiol on the incorporation of radioactive androgens*

Two groups of three or four tumour-bearing mice were injected intravenously with 25 μ g of oestradiol either 1 h before or coincident with the injection of 200 μ Ci of [1,2- 3 H]testosterone. A third group of mice served as control and received radioactive testosterone only. At 30 min after the injection of the radioactive compound, the animals were killed and the tumours were homogenized. Cytoplasm and nuclei were prepared as described in the Materials and Methods section. The radioactivity in whole tumours and in nuclei was measured and the results are expressed as the mean $\pm$ S.E.M. for each experimental group: Expt. 1, control group; Expt. 2, animals treated with oestradiol for 0.5 h; Expt. 3, animals treated with oestradiol for 1.5 h.

Expt. no.	Oestradiol administered (μ g)	Duration of treatment (h)	$10^{-5} \times$ Radioactivity incorporated in tumour (d.p.m./g)	$10^4 \times$ Radioactivity incorporated in nuclei (d.p.m./nucleus)
1	0		22.2 ± 2.8 (4)	12.7 ± 1.3 (4)
2	25	0.5	24.1 ± 2.7 (4)	6.1 ± 1.4 (4)
3	25	1.5	22.0 ± 2.3 (3)	5.1 ± 0.3 (3)

Table 6. *Effects of oestradiol on the metabolism of [1,2- 3 H]testosterone*

Nuclear and cytoplasmic fractions were obtained as described in the legend to Table 5. These fractions were extracted with chloroform-methanol (2:1, v/v) and the extracts were purified by chromatography on silicic acid. Analysis of neutral steroids was carried out by t.l.c. and g.l.c. The relative amount of each steroid identified is expressed as a percentage of the total radioactivity in the neutral steroid fraction. The values in parentheses indicate the number of experiments performed to obtain the mean values listed. Expt. 1, Control group; Expt. 2, animals treated with oestradiol for 0.5 h; Expt. 3, animals treated with oestradiol for 1.5 h.

Expt. no.	Androgens recovered (% of total)						
	Dihydro-testosterone	Testosterone	Androstane-diol	Androsterone	Androstene-dione	Androstane-dione	Other
Nuclei							
1 (3)	4	88	1	1	1		5
2 (3)	6	87	1	1	1		4
3 (2)	5	89	1	1			4
Cytoplasm							
1 (4)	6	46	2	12	12	5	17
2 (3)	4	55	2	9	9	5	16
3 (3)	4	59	2	9	9	7	10

and 6). As a result, when testosterone is administered, it too is the predominant steroid at all times in intact tumour tissue (Fig. 5), and in tumour cytoplasm and nuclei (Table 4). In spite of the prevalence of testosterone, a small but significant amount of dihydrotestosterone is found in tumour, as has been reported previously (Yamaguchi *et al.*, 1971). From the rapid appearance of dihydrotestosterone in the neoplastic tissue it may be inferred that the formation of dihydrotestosterone takes place in the tissue itself, but presumably because of the limited metabolism of testosterone, the dihydrotestosterone/testosterone ratio is very much smaller in tumour nuclei than in seminal vesicles nuclei. Thus the response of tumour

to testosterone differs from that of seminal vesicles in two important respects: less metabolism of testosterone takes place in tumour and considerably less dihydrotestosterone is assimilated by tumour nuclei. The results on the relative amount of testosterone and dihydrotestosterone in tumour nuclei, however, would also be explained if one population of nuclei selectively incorporated dihydrotestosterone and another one incorporated testosterone. This possibility has not been excluded but seems unlikely.

In view of the potent effect of dihydrotestosterone on cell proliferation, the metabolism of testosterone to dihydrotestosterone affords an attractive explanation for the mitotic activity induced by testosterone.

If indeed the amount of dihydrotestosterone in nuclei is directly related to the size of the proliferative response, it can be expected that dihydrotestosterone would stimulate tumour growth more efficiently than testosterone. Clearly the higher concentration of dihydrotestosterone produced in nuclei after the administration of dihydrotestosterone would account for the large difference in potency (Tables 3 and 4).

The results presented in Fig. 7 demonstrate that tumour growth in males is inhibited by oestradiol. While this effect may be explained by the suppression of the release of pituitary interstitial-cell-stimulating hormone, two lines of evidence suggest that the tumour may be directly influenced by this hormone. First, as shown in Fig. 7, the incidence of tumours in females treated with exogenous dihydrotestosterone or testosterone is lower than the incidence in groups of castrated males receiving similar doses of androgen. (Fig. 1) Secondly, an inhibitory effect of oestradiol on tumour growth has been observed in castrated and androgenized males (Minesita & Yamaguchi, 1964). Evidence is also available from studies on prostate that suggests that oestradiol may interfere with the metabolism of testosterone (Farnsworth, 1969; Groom *et al.*, 1971; Leav *et al.*, 1971). Thus it was of interest to examine the conversion of testosterone in SC-115 tumour cells exposed to oestradiol for any changes in the formation of dihydrotestosterone that might be linked to the inhibition of tumour cell proliferation by oestrogen.

After the administration of oestradiol in doses 25 times greater than the experimental dose of radioactive testosterone, the incorporation of radioactivity was the same in whole tumours from treated and control mice (Table 5) but the amount of radioactivity in nuclei from treated animals was significantly below the amount in nuclei from controls. The absence of a significant decrease in the relative amount of dihydrotestosterone in nuclei and cytoplasm from the two groups suggests that the metabolism of testosterone was not affected by oestradiol. Higher doses and longer intervals of treatment may be required before any such change is detectable. Nevertheless, in view of the decrease in nuclear uptake of androgen brought about by oestradiol, and because it is difficult to imagine how this finding might be related to a fall in concentration of circulating gonadotrophin, it seems reasonable to suspect that SC-115 tumour cells are directly influenced by oestradiol.

In summary the evidence presented in this paper indicates that dihydrotestosterone is an effective promoter of cell division, as reflected in its ability to stimulate tumour growth. The hormone is found in nuclei after the administration of both testosterone and dihydrotestosterone, but a higher value is achieved with dihydrotestosterone, in keeping with

its greater potency. Whereas experiments with oestradiol failed to reveal any effects of this hormone on the metabolic formation of dihydrotestosterone, the amount of androgen in nuclei was significantly decreased by high doses of oestrogen. Although these findings are consistent with the view that the function of dihydrotestosterone *in vivo* is concerned with cell renewal, it remains unknown whether testosterone is simply a precursor of dihydrotestosterone.

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