The Effect of Castration on the Ribonucleic Acid Metabolism of an Experimental Prostatic Tumour

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1. The incorporation of [^{14}C]phenylalanine into the protein of microsomes obtained from an androgen-dependent transplantable prostatic tumour has been investigated. 2. The incorporation of [^{14}C]ATP into the RNA of the nuclei from the same tumour has also been examined. 3. The castration of the host animal depresses the incorporation of both labelled compounds on subsequent incubation *in vitro*. 4. The uptake of phenylalanine can be markedly stimulated by polyuridylic acid only in tumours obtained from castrated host animals. 5. The tumour RNA-polymerase activity appears to be dependent on the concentrations of androgens in the host animal.

The prostate gland is one of the best examples of a strongly androgen-dependent tissue. It has been established that androgens control the growth of the prostate, at least in part, by their influence on RNA synthesis in the gland. Evidence has been presented that male steroid hormones control the rate of messenger-RNA synthesis (Liao & Williams-Ashman, 1962) and that the response is mediated via the nuclear RNA polymerase (DNA-dependent ribonucleotide triphosphate-ribonucleic acid nucleotidyltransferase: EC 2.7.7.6) (Hancock, Zelis, Shaw & Williams-Ashman, 1962). Little is known about the biochemical response of prostatic tumours to androgens, although the arrest of such carcinomas by castration alone or with combined oestrogen therapy is a successful clinical treatment.

The evaluation of the metabolic processes by which androgens regulate the growth of prostatic tumours has been severely hindered by the shortage of suitable hormone-responsive transplantable tumours. Induction of prostatic carcinomas in experimental animals by chemical means produces tumours that do not generally show the marked hormone dependency of the parent tissue, e.g. the methylcholanthrene-induced tumours described by Dunning, Curtis & Segaloff (1946).

Experiments have been carried out on an androgen-dependent prostatic tumour to establish whether a partial explanation of the steroid action could be found at the level of messenger-RNA synthesis, as in the non-malignant tissue. Messenger-RNA synthesis has been measured indirectly (e.g. Liao & Williams-Ashman, 1962; Pedersen &

Hultin, 1963) by using a protein-synthesizing system and following the incorporation of [^{14}C]phenylalanine into peptide linkage. The response of the system to added polyU† can be used as an indication of the amount of natural messenger RNA present in the system. Alternatively, the synthesis of RNA (including messenger RNA) may be followed by measurement of the activity of RNA polymerase in the nuclear fraction of tissue homogenates (e.g. Weiss, 1960; Widnell & Tata, 1964). The preparation of uncontaminated nuclei from solid tumours presents considerable practical difficulties, unless special homogenization equipment is used, such as that designed by Emanuel & Chaikoff (1957). A combination of such methods used *in vitro* has been used in the present investigation.

MATERIALS AND METHODS

Chemicals. ATP, CTP, GTP and UTP were purchased from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Phosphoenolpyruvate (sodium salt) and pyruvate kinase were products of C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Solutions of the phosphate-containing compounds were titrated to pH 7.8 with *N*-NaOH before use. PolyU was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., and L-amino acids were obtained from British Drug Houses Ltd., Poole, Dorset. L-[U- ^{14}C]phenylalanine (282 mc/m-mole) and [^{14}C]AMP (8 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. The [^{14}C]AMP was converted enzymically into [^{14}C]ATP in 43% yield by the method of Ochoa, Burma, Kröger & Weill (1961). Puromycin was the product of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Actinomycin D was a generous gift from Merck,

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† Abbreviation: polyU, polyuridylic acid.

Sharp and Dohme, Rahway, N.J., U.S.A. Hyamine 10-X (Hopkin and Williams Ltd., Chadwell Heath, Essex) was converted into the hydroxide form by the method of Eisenberg (1958).

Chemical analysis. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard. RNA was determined by the method of Meijbaum (1939), with highly polymerized yeast RNA as reference; DNA was assayed by the procedure of Burton (1956), with highly polymerized calf-thymus DNA as standard.

Prostatic tumour. The prostatic carcinoma used in these experiments was developed by the implantation of 7,12-dimethylbenz[*a*]anthracene into the ventral prostate glands of male Sprague-Dawley rats. Pellets were made by mixing the carcinogen with twice its weight of paraffin wax (m.p. approx. 50°) and heating until the wax just melted. The mixture was thoroughly agitated, poured into appropriate moulds and allowed to cool. Pellets (about 100 mg.) were implanted surgically into the ventral prostate glands of a group of eight rats that were then maintained under normal laboratory conditions. After about 4 months three of the animals developed palpable growths in the region of the prostate gland. The animals were killed and the least necrotic parts of the tumours were surgically transplanted into the livers of male Sprague-Dawley rats (approx. 200 g.). Tumours were thereafter transplanted into the spleens of rats. At 4-5 weeks after transplantation the tumours had reached a palpable size and the animals were castrated or sham-operated via the scrotal route under ether anaesthesia. Groups of 9-12 tumours (each weighing 1.2-3 g.) were used 7-12 days after the operations. The tumours showed a marked androgen-dependency in the early transplantations, since castration resulted in a 40-45% decrease in the average weight of the tumours. After repeated passage through many transplantations, however, the tumours grew more slowly, became progressively necrotic and showed virtually no relationship to the androgenic status of the host. The experiments under discussion were all carried out on prostatic tumours from the early transplantations, where the hormone-dependency was consistently maintained.

Preparation of nuclear fractions. The outer areas of the tumours were carefully dissected free of the central necrotic tissue and cooled in ice. The pooled material was homogenized at 2° in 4 vol. of 0.25 M-sucrose by using a motor-driven Teflon pestle (Tri-R Instruments, Jamaica, N.Y., U.S.A.) at 1200 rev./min. for a total homogenization time of 45 sec. The crude homogenate was centrifuged at 700g for 5 min. and the resultant residue was rehomogenized for a further 30 sec. in 15 ml. of 0.25 M-sucrose. The homogenate was layered over an equal volume of 0.33 M-sucrose containing $MgCl_2$ (1 mM) and centrifuged at 800g for 10 min. The pellet of nuclei and cell debris was suspended in 20 ml. of 0.25 M-sucrose containing $MgCl_2$ (1 mM) (medium A) and centrifuged at 150g for 10 min. After the careful withdrawal of the supernatant, the purified nuclei were collected by further centrifuging at 700g for 5 min. The collected nuclei were gently resuspended in 15 ml. of medium A and the final purification procedure was repeated. The final nuclear preparation was stored in 2.0 ml. of medium A.

Extracts of the nuclear preparations and of the whole tumour tissue were prepared by the method of Schneider (1945). From the DNA content of these extracts, 35% of the original tumour DNA was recovered in the final prepara-

tion. The low yield of purified nuclei is a reflection of incomplete homogenization of the tumour; without exception, a heavy sediment of undegraded cellular material and debris followed the first low-speed purification step.

Preparation of microsomes and pH5 enzyme fractions. These were prepared as described by Mainwaring & Williams (1965) for uterine tumour material. The washed microsomes were suspended in 2.5 ml. of a medium containing sucrose (0.15 M), $MgCl_2$ (10 mM), KCl (25 mM) and tris buffer (35 mM) adjusted to pH 7.8 with HCl. The pH5 enzyme fraction was purified by passage through Sephadex G-25, equilibrated with a medium containing tris (10 mM), $MgCl_2$ (10 mM), KCl (5 mM) and β -mercaptoethanol (5 mM), adjusted to pH 7.8 with HCl. In certain preliminary experiments, whole cell sap from the 105000g supernatant was used after purification by gel filtration.

Assay of RNA polymerase in the tumour nuclei. The assay system contained 0.3 μ mole each of CTP, GTP and UTP, 0.03 μ mole of [^{14}C]ATP, 3 μ moles of NaF, 2.5 μ moles of β -mercaptoethanol, 60 μ moles of tris-HCl buffer, pH 8.1, 15 μ moles of KCl and 0.20 ml. of nuclear suspension in a total volume of 0.5 ml. The final $MgCl_2$ concentration was adjusted to 5 mM. After incubation in air for 15 min. at 37°, the reactions were stopped by the addition of 3.0 ml. of ice-cold 0.8 N-HClO₄, together with 0.2 ml. of yeast RNA solution (10 mg./ml.). The precipitates were collected by centrifuging at 2° and washed twice with ice-cold 0.3 N-HClO₄, and finally twice in cold ethanol-ether (3:1, v/v). The extraction of the labelled RNA from the precipitates was carried out as described by Mainwaring & Williams (1965). The final extracts were dissolved in 0.5 ml. of *m*-Hyamine hydroxide and added to 10 ml. of scintillation medium (Bray, 1960). Radioactivity was measured by liquid-scintillation spectrometry at a counting efficiency of 65% (determined with [^{14}C]AMP under identical conditions).

Incorporation of [^{14}C]phenylalanine into peptides. The system contained 2 μ moles of ATP, 0.4 μ mole of GTP, 10 μ moles of phosphoenolpyruvate, 50 μ g. of pyruvate kinase, 10 m μ moles (0.1 μ C) of L-[^{14}C]phenylalanine, 10 m μ moles of each of the remaining 19 naturally occurring L-amino acids, 0.3 ml. of pH5 fraction (or whole cell sap) and 0.25 ml. of microsomal suspension in a final volume of 1.0 ml. The final $MgCl_2$ concentration was adjusted to 12 mM. Incubations were carried out in air at 37° for 1 hr. The collection, washing and counting of the radioactive protein precipitates were carried out as described by Mainwaring & Williams (1965).

RESULTS

Incorporation of [^{14}C]phenylalanine into the protein of prostatic-tumour microsomes. The incorporation system was based on the experiments summarized in Table 1, with whole cell sap or the pH5 fraction as the source of activating enzymes and transfer RNA. Certain similarities are apparent between the experimental results in the two cases: (a) an energy-generating system is essential for activity; (b) the systems are sensitive to puromycin (Yarmolinsky & de la Haba, 1959); (c) purification of the enzymes by gel filtration is necessary for maximum incorporation of labelled phenylalanine.

Table 1. *Requirements for the incorporation of [14 C]phenylalanine with microsomes and either whole cell sap or pH 5 fraction*

The microsomes, whole cell sap and pH5 fraction were prepared as described in the Materials and Methods section from tumours carried in normal male rats. The standard incorporation system contained 2 μ moles of ATP, 0.4 μ mole of GTP, 10 μ moles of phosphoenolpyruvate, 50 μ g. of pyruvate kinase, 10 μ moles (0.1 μ C) of L-[14 C]phenylalanine, 10 μ moles of each of the remaining naturally occurring L-amino acids, 0.3 ml. of either (a) whole cell sap (4.4 mg. of protein) or (b) pH5 fraction (3.2 mg. of protein) and 0.25 ml. of microsomal suspension (240 μ g. of microsomal RNA) in a total volume of 1.0 ml. The final MgCl₂ concentration was adjusted to 12 mM. Incubations were for 1 hr. at 37°. Results are expressed as counts/min./250 μ g. of microsomal RNA.

System	[14 C]Phenylalanine incorporated	
	(a) Whole cell sap	(b) pH5 fraction
Complete system	661	854
With untreated cell sap or pH5 fraction	150	306
Without ATP, phosphoenolpyruvate, pyruvate kinase	70	101
Without GTP	679	681
Without cell sap or pH5 fraction	31	41
With final MgCl ₂ concn. 5 mM	—	436
With final MgCl ₂ concn. 10 mM	—	649
With added puromycin (30 μ g.)	55	62
With added polyU (50 μ g.)	445	1104
Preincubation of polyU + cell sap or pH5 fraction, then added to complete system	320	1019

The low-molecular-weight inhibitor(s) has not been identified, but the presence of such material has been demonstrated in other tumour systems (Mainwaring & Williams, 1965). A marginal requirement for GTP was found only with the pH5 enzyme fraction. In marked contrast, however, was the behaviour of the two systems to added polyU. With the whole cell sap as the source of enzymes, the activity of the system actually decreased in the presence of polyU; with pH5 fractions, the synthetic messenger RNA caused a 20% increase in the incorporation of radioactivity. Degradation of synthetic polyribonucleotides by whole cell sap has been described by other workers, e.g. in *Escherichia coli* (Tissières & Watson, 1962) and Ehrlich ascites tumour (Pedersen & Hultin, 1963). It is uncertain whether such a degradation affords a satisfactory explanation of the above phenomenon, but preincubation of the polyU with whole cell sap before addition to the system resulted in a similar decrease in activity. This was not the case in a similar preincubation with the pH5 fraction but the stimulation of incorporation was confirmed. If degrada-

Fig. 1. Incorporation of [14 C]phenylalanine into protein (a) with microsomes and pH5 fraction from sham-operated animals, (b) with microsomes from castrated animals and pH5 enzymes from sham-operated animals. The constituents of the assay system are as described in the Materials and Methods section. Incorporation is expressed as counts/min./mg. of microsomal RNA. O, No polyU; $\blacktriangle$, 25 μ g. of polyU added; $\blacksquare$, 50 μ g. of polyU added.

tion of the polyU is the factor implicit in these findings, it suggests that the digestion products are strongly inhibitory to the system. The pH5 fraction was used exclusively in the subsequent studies.

Comparison of the activities of tumour microsomes from castrated and sham-operated rats. Figs. 1(a) and 1(b) illustrate the incorporation of phenylalanine into the microsomal protein of tumours carried in control (sham-operated) and castrated rats respectively. The pH5 enzyme fraction from

the control group was used in both cases. Since the yield of tumour microsomal RNA/g. of tumour was generally lower in the castrated group, the results have been corrected to give the radioactivity incorporated per unit amount of microsomal RNA.

The tumour microsomes from the castrated group were much more responsive to polyU than those derived from the controls. Without the synthetic messenger RNA, the system from castrated animals possessed low activity.

The direct addition of testosterone propionate to the system derived from the group of castrated animals did not enhance the activity of the protein-synthesizing system.

Synthesis of RNA in the tumour nuclear preparations. The conditions necessary for the synthesis of RNA in the isolated tumour nuclei are summarized in Table 2. With nuclei obtained from the control group (Table 2a) the incorporation of [14 C]ATP into RNA requires the presence of the remaining nitrogenous bases in the form of the 5'-triphosphates and the presence of both Mg^{2+} and β -mercaptoethanol. The incorporation of radioactivity was suppressed by the presence of ribonuclease to a background level. Actinomycin D, known to inhibit RNA polymerase by combination with the necessary DNA template (Cavalieri & Nemchin, 1964), was a potent inhibitor of the enzyme. In these respects, the requirements are similar to RNA-polymerase preparations obtained from other sources (Weiss, 1960; Gorski, 1964).

Under standard conditions, the tumour nuclei from castrated animals have a decreased ability to synthesize RNA *in vitro* (Table 2b). These results suggest that castration of the tumour-bearing animals decreases the activity of the tumour RNA polymerase.

DISCUSSION

The experimental results are consistent with the view that the RNA synthesis in the prostatic tumour is dependent on physiological concentrations of androgens. This is indicated by the marked response of the tumour microsomal system to polyU after castration of the host animals. The control group showed only a small response to polyU, and Silverman, Liao & Williams-Ashman (1963) and Liao & Williams-Ashman (1962) have reported similar results with an incorporation system isolated from normal ventral rat prostate gland. The confirmatory experiments on the synthesis *in vitro* of RNA in the tumour nuclei suggest that a relationship exists between the synthesis of tumour RNA and the hormonal status of the animal. This is in agreement with the findings of Hancock *et al.* (1962) with aggregate RNA-polymerase preparations from normal prostatic nuclei.

There will almost certainly be changes in the concentrations of other hormones besides androgens after castration. The striking dependence of the normal prostate gland on physiological concentrations of androgens and the similar behaviour of the prostatic tumour does, however, suggest that the recorded effects were due to androgen depletion rather than to changes in the concentrations of other humoral agencies. Androgens may control the growth of the normal prostate gland by means of other metabolic parameters in addition to RNA synthesis. Control of the number of mitochondria (Edelman, Brendler, Zorngniotti & Edelman, 1963), the rate of fatty acid synthesis (Nyden & Williams-Ashman, 1953) and the permeability of membranes may all be implicated. Thus only a partial explanation of the response to androgens of the prostatic tumour would be expected at the level of RNA synthesis.

Even assuming this to be so, it leaves unsolved one of the biggest problems pertinent to the understanding of the biochemistry of the prostate gland and prostatic carcinomas. Adrion (1922), Francks (1954) and others have distinguished two distinct areas in the human prostate. They are distinctive in gross structure, tendency to neoplastic change and response to steroid hormones (both androgens and oestrogens). Thus a meaningful interpretation of the effects of androgens and other hormones on prostatic tissues must be related to characteristic

Table 2. Incorporation of [14 C]ATP into RNA of tumour nuclei

The nuclei (110–140 μ g. of DNA) were obtained as described in the Materials and Methods section from sham-operated or castrated male rats. The complete system contained 0.3 μ mole each of CTP, GTP and UTP, 0.03 μ mole (0.05 μ c) of [14 C]ATP, 3 μ moles of NaF, 2.5 μ moles of β -mercaptoethanol, 15 μ moles of KCl, 60 μ moles of tris-HCl buffer, pH 8.1, and 0.20 ml. of nuclear suspension in a total volume of 0.5 ml. The final $MgCl_2$ concentration was adjusted to 5 mM. Incubations were for 15 min. at 37°. Results are expressed as counts/min./125 μ g. of DNA.

System	[14 C]ATP incorporated
(a) Control group	
Complete system	591
Without CTP, GTP, UTP	64
Without GTP	96
Without β -mercaptoethanol	263
With final $MgCl_2$ concn. 1 mM	146
With ribonuclease (50 μ g.)	50
With actinomycin D (30 μ g.)	40
(b) Castrated group	
Complete system	336
Without CTP, GTP, UTP	62
With actinomycin D (30 μ g.)	31

biochemical differences in the two morphological areas. This would involve more selective sampling of tissues than that employed in the present study.

The mechanisms by which the androgens regulate the activity of prostatic RNA polymerase has not been established so far. In a somewhat parallel set of circumstances in uterus, Talwar, Segal, Evans & Davidson (1964) have postulated that the response of uterine RNA polymerase to oestrogens involves de-repression of an enzyme. Such a mechanism may also be present in the prostate.

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