The Effect of Oophorectomy on the Ribonucleic Acid Metabolism of a Transplantable Uterine Tumour

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1. The incorporation of [^{14}C]phenylalanine and of [^3H]uridine into the Guerin T8 uterine carcinoma has been examined. 2. The addition *in vitro* of polyuridylic acid caused an increase in the incorporation of labelled phenylalanine into tumour microsomes obtained from both oophorectomized and intact animals. 3. Oophorectomy of tumour-bearing animals decreased the specific activity due to the uptake *in vivo* of [^3H]uridine into both the soluble and insoluble RNA of the tumour. 4. Actinomycin D caused a decrease in specific activity of RNA in the tumour *in vivo*, and puromycin inhibited uptake *in vitro* into tumour microsomes.

Oophorectomy has often been found to be beneficial in the clinical treatment of certain uterine carcinomas. Oestrogens have a profound effect on the growth of normal uterine tissue, and in the early stages of proliferation the hormones promote a marked increase in the imbibition of water and in the phospholipid and RNA content of the cells. The early stimulation of uterine RNA metabolism has evoked considerable interest, especially the messenger-like RNA synthesized in the nucleus under the priming influence of DNA (Wilson, 1963; Gorski, 1964). It was therefore decided to determine whether oestrogens had similar effects on the RNA metabolism of a steroid-dependent uterine carcinoma, since any such information could have considerable importance from the therapeutic standpoint.

To study RNA polymerase (EC 2.7.7.6) directly, *in vitro*, it is necessary to prepare very clean preparations of nuclei (Gorski, 1964; Widnell & Tata, 1964). Considerable difficulties are encountered in the preparation of clean nuclei from solid tumours owing to the extreme resistance of the tumour matrix to conventional homogenization (see review by Dounce, 1963). However, the presence of messenger RNA can be demonstrated *in vitro* in a cell-free system composed of microsomes, supernatant enzymes and necessary cofactors. In such a system, the response to added synthetic polyribonucleotides with messenger properties can be used as an indirect measure of the natural messenger RNA present in the preparations (e.g. Liao & Williams-Ashman, 1962). Greenman &

Kenney (1964) reported that the incorporation of [^{14}C]phenylalanine into uterine ribosomes was decreased after oophorectomy. Further, the ribosomal system from oophorectomized animals was more responsive to polyU† than uterine ribosomes from untreated control animals. The administration of oestradiol-17 β to oophorectomized animals restored the activity *in vitro* of the uterine ribosomes, but decreased the response to polyU. It has proved much easier to prepare clean microsomes rather than clean nuclei from solid uterine tumours, and the indirect method for the demonstration of messenger RNA has been used in the present investigation. Further, the modified RNA extraction procedure of Kidson, Kirby & Ralph (1963) has made it possible to carry out confirmatory experiments, *in vivo*, on the synthesis of messenger RNA in the uterine tumour.

MATERIALS AND METHODS

Uterine tumour. The tumour line used in these experiments was the Guerin T8 carcinoma, which originally arose spontaneously in the rat uterus. One such tumour line maintained in this Laboratory has been found to have a decreased rate of growth after oophorectomy of the host animals. The tumour has been carried by transplantation into the spleen of female Sprague-Dawley rats (150–250 g.) (Williams, 1965). After 10 days the tumour weighed approx. 4–5 g. and the animals were then oophorectomized under ether anaesthesia or sham-operated. The animals were killed by cervical fracture 7 days later and the tumours (7–10 g.) were removed.

Chemicals. ATP and GTP (sodium salts) were obtained from P.-L. Biochemicals Inc., Milwaukee, Wis., U.S.A. Pyruvate kinase, phosphoenolpyruvate (sodium salt) and UDP were products of C. F. Boehringer und Soehne

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† Abbreviation: polyU, polyuridylic acid.

G.m.b.H., Mannheim, Germany. Solutions of the phosphate-containing compounds were titrated to pH 7.5 with 0.1N-NaOH before use. L-Amino acids and human serum albumin were obtained from British Drug Houses Ltd., Poole, Dorset. PolyU was synthesized from UDP by using the polynucleotide phosphorylase of *Micrococcus lysodeikticus* (Steiner & Beers, 1961) or was a product of Koch-Light Laboratories Ltd., Colnbrook, Bucks. L-[U-¹⁴C]-Phenylalanine (282 mc/m-mole) and [G-³H]uridine (1.22 c/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Puromycin was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Actinomycin D was the generous gift of Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Hyamine 10-X was a product of Hopkin and Williams Ltd., Chadwell Heath, Essex, and was converted into the OH⁻ form by the ion-exchange method of Eisenberg (1958). Yeast RNA was obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A.

Fractionation of the tumours. The tumours were cooled on crushed ice and the solid tissue was dissected free from obviously necrotic material. The tumour was minced with scissors and sliced with a McIlwain tissue chopper (H. Mickle Ltd., Gomshall, Surrey) at a blade setting of 0.15 mm. The tissue was homogenized in 2.5 vol. of an aqueous medium (medium A) containing sucrose (0.15 M), MgCl₂ (10 mM), KCl (25 mM) and tris (35 mM) adjusted to pH 7.8 (25°) with HCl (Rendi & Hultin, 1960). Homogenization was performed in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (Tri-R Instruments, Jamaica, N.Y., U.S.A.) at a speed of 2000 rev./min. Debris, nuclei and mitochondria were removed by centrifuging the homogenate at 12000g for 10 min. The supernatant was centrifuged at 105000g for 1 hr. and the soluble fraction was carefully withdrawn from the microsomal pellet. The pellet was suspended in 15 ml. of medium A and gently homogenized by hand in an all-glass homogenizer. The washed microsomes were collected by centrifugation at 105000g for 35 min. and finally dispersed in 4 ml. of medium A. The subsequent removal of lipoprotein with iso-octane as performed by Campbell, Cooper & Hicks (1964) was found in our experiments to be unnecessary as it had no effect on the assay, and was therefore omitted.

Portions of clear 105000g supernatant from tumours from the sham-operated group of animals were diluted with 2 vol. of a medium (medium B) containing tris (10 mM), MgCl₂ (10 mM), KCl (10 mM) and β-mercaptoethanol (6 mM), adjusted to pH 7.8 with HCl. The solution was titrated to pH 5.1 with N-acetic acid and the precipitate was collected by centrifugation at 10000g for 5 min. The precipitate was suspended in 5 ml. of medium B and rendered completely soluble by readjustment of the pH to 7.8 with dilute tris buffer, pH 9.0. The solution (pH 5 enzyme fraction) was passed through a 1.6 cm. × 20 cm. column of Sephadex G-25 equilibrated with medium B, to remove low-molecular-weight inhibitors (Mansbridge & Korner, 1963).

Assay system in vitro. The assay system contained the following components in a total volume of 1.0 ml.: 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate, 50 μg. of pyruvate kinase, 0.5 μmole of GTP, 10 μmoles (0.1 μC) of L-[¹⁴C]phenylalanine, 10 μmoles of each of the other 19 naturally occurring amino acids, 0.4 ml. of pH 5 fraction and 0.25 ml. of microsomal suspension. The final MgCl₂ concentration was adjusted to 10 mM. This is similar to that used by Campbell *et al.* (1964) in a study of the effects of

polyU on rat-liver microsomes and by Greenman & Kenney (1964) in their work on rat uterine ribosomes. The tubes were incubated in air at 37° and the reactions were initiated by the addition of the microsomal suspension. A zero-time control was used in all experiments in which trichloroacetic acid was added to a final concentration of 5% (w/v) before the microsomes and a small correction was applied to all count rates. The reactions were terminated by the addition of trichloroacetic acid and the protein precipitates were collected by centrifugation. The precipitates were washed twice with cold 5% (w/v) trichloroacetic acid and then heated at 90° for 15 min. in trichloroacetic acid solution. After thorough cooling in ice, the protein precipitates were collected on Millipore filters, 5 cm. diam. (Oxo Ltd., London, E.C.4), and washed several times with distilled water. The membranes were dried under low heat and placed in counting vials with 10 ml. of scintillator containing 5 g. of 2,5-diphenyloxazole and 0.3 g. of *p*-bis-(5-phenyloxazol-2-yl)benzene/l. of toluene. The samples were counted on a liquid-scintillation spectrometer (Isotope Developments Ltd., Reading, Berks.) at 42% counting efficiency.

Incorporation in vivo of uridine into tumour RNA. These experiments were based on those of Kidson *et al.* (1963) and Kidson & Kirby (1964). [³H]Uridine was injected intraperitoneally at various time-intervals before the killing of the animals by cervical fracture. The tumours were immediately removed and frozen on solid CO₂. The actual total times for incorporation were determined from the injection time to the freezing of the tumours. To achieve a base-line activity of RNA polymerase, actinomycin D was injected intraperitoneally into a control group of animals before the injection of [³H]uridine. Pooled tissue samples from at least four tumours (non-necrotic tissue, total 5 g.) was subjected to the differential RNA extraction procedure of Kidson *et al.* (1963). Ribosomal and transfer RNA were first extracted with 90% (w/v) phenol containing 0.5% (w/v) of naphthalene-1,5-disulphonic acid and 0.1% (w/v) of 8-hydroxyquinoline. After centrifugation, the ribosomal and transfer RNA was precipitated from the aqueous phase with 2 vol. of ethanol. The insoluble interface material was then further extracted with 90% phenol containing 1% (w/v) of 8-hydroxyquinoline and 6% (w/v) of 4-amino-salicylic acid. The messenger RNA and DNA were precipitated from the aqueous phase with ethanol. The RNA-containing precipitates were washed several times with cold 65% (v/v) ethanol and hydrolysed for 16 hr. at 37° in 0.3N-KOH (Schmidt & Thannhauser, 1945). After treatment with excess of HClO₄ in ice, residual protein and DNA were removed by centrifugation. Each supernatant was carefully titrated to pH 7.0 with KOH and allowed to stand in the cold. Insoluble KClO₄ was removed by centrifugation and the final supernatant, containing RNA nucleotides, was made up to a final volume of 5.0 ml. Samples were evaporated to dryness *in vacuo*, dissolved in 0.5 ml. of M-Hyamine hydroxide and added to 10 ml. of scintillation medium (Bray, 1960). Radioactivity was determined by liquid-scintillation spectrometry at a counting efficiency of 18% (determined with tritiated water standards under identical conditions).

Chemical determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with human serum albumin as a standard. The protein concentration (mg./ml.) was also given by 1.5E₂₈₀ - 0.75E₂₆₀. Paper-electrophoresis experiments were performed in the

Shandon Universal electrophoresis apparatus Mk. II with 0.1M-tris-HCl buffer, pH 7.0.

RNA was determined by the orcinol method of Meijbaum (1939), with yeast RNA as a standard.

RESULTS

Characterization of the tumour amino acid-incorporating system. Preliminary experiments were devised to determine the requirements for the incorporation of [14 C]phenylalanine into protein with the tumour microsome preparations. The results are presented in Table 1. The system was completely dependent on the presence of an energy-generating source, but only slightly stimulated by GTP. The optimum concentration of Mg^{2+} was 10mM. Puromycin, which is known to inhibit the transfer of activated amino acids from aminoacyl-transfer RNA into protein (Yarmolinsky & de la Haba, 1959), was a potent inhibitor of the system. The pH5 fractions contained a potent low-molecular-weight inhibitor that was readily removed by gel filtration. Samples of the low-molecular-weight material were concentrated by freeze-drying and, when added to a system containing purified pH5 enzymes, the inhibition was again observed. The nature of this material is unknown, but it possessed an E_{280}/E_{260} ratio approx. 1.3–1.4 and gave rise to two weak ninhydrin-positive

bands after low-voltage paper electrophoresis. PolyU stimulated the incorporation of [14 C]phenylalanine into protein.

Comparison of the activities of the tumour microsome preparations from intact and oophorectomized animals. The yield of tumour microsomes from the oophorectomized animals, in terms of microsomal RNA, was somewhat lower than that from the sham-operated controls. Thus, to make a direct comparison between the activity of the microsomes from tumours from oophorectomized animals and the control group, the radioactivity incorporated was related to the microsomal RNA content. Tumour pH5 enzymes prepared from the control group were used in both cases. This was to ensure that any differences in activity were not due to lower concentrations of transfer RNA or activating enzymes in the supernatant tumour fractions from the oophorectomized animals, but were related to changes in the microsomes themselves, especially the composition of the microsomal RNA.

The tumour microsomes from sham-operated animals responded less readily to polyU than did those from oophorectomized animals, although the change in rate, rather than the extent, of the reactions was responsible for this difference. Fig. 1 shows the uptake of [14 C]phenylalanine into microsomes from sham-operated animals, and Fig. 2 shows similar results with microsomes from oophorectomized animals. The uptake as compared with the control level, for 50 μ g. of

Table 1. *Characterization of the tumour amino acid-incorporating system*

The microsomal and pH5 fractions were prepared as described in the Materials and Methods section from tumours carried in normal female rats. The complete system contained 2 μ moles of ATP, 0.5 μ mole of GTP, 10 μ moles of phosphoenolpyruvate, 50 μ g. of pyruvate kinase, 10 μ moles (0.1 μ mol) of L-[14 C]phenylalanine, 10 μ moles of each of the remaining naturally occurring L-amino acids, 0.4 ml. of pH5 fraction (3.6 mg. of protein) and 0.25 ml. of microsomal suspension (190 μ g. of microsomal RNA, 490 μ g. of protein) in a final volume of 1.0 ml. The final $MgCl_2$ concentration was adjusted to 10 mM. Incubation was for 1 hr. at 37°.

System	[14 C]Phenylalanine incorporated (counts/min.)
Complete	649
With final $MgCl_2$ concn. 5 mM	201
With final $MgCl_2$ concn. 15 mM	423
Without ATP, phosphoenolpyruvate and pyruvate kinase	42
Without GTP	583
Without pH5 fraction	37
With added puromycin (30 μ g.)	61
With added polyU (50 μ g.)	838
With untreated pH5 enzymes (3.7 mg. of protein)	290
With treated pH5 enzymes + low-mol.-wt. fraction	382

Fig. 1. Incorporation of [14 C]phenylalanine into protein with microsomes 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./250 μ g. of microsomal RNA. ○, PolyU absent; ▲, 25 μ g. of polyU present; ■, 50 μ g. of polyU present.

Fig. 2. Incorporation of [^{14}C]phenylalanine into protein with microsomes from oophorectomized animals and pH5 enzymes from sham-operated controls. The constituents of the assay system are as described in the Materials and Methods section. Incorporation is expressed as counts/min./250 μg . of microsomal RNA. ○, PolyU absent; ▲, 25 μg . of polyU present; ■, 50 μg . of polyU present.

polyU at 60 min. after addition, was increased by about 50 and 200% respectively. In the presence of polyU the tumours from oestrogen-depleted animals approached the activity found in the control group.

Incorporation in vivo of [^3H]uridine into tumour RNA in oophorectomized and intact animals. The incorporation of [^3H]uridine into tumour RNA after different times is presented in Table 2. The specific activity of the interface RNA was very much higher than that of the aqueous RNA, especially at short labelling times. The yield of interface RNA was approx. 7–10% of the recovery of aqueous RNA; a similar proportion was reported by Kidson *et al.* (1963) for rat liver. The results are consistent with the presence of a high-turnover RNA in the interface material, compared with the aqueous RNA fraction. In all cases, the specific activity of the interface and aqueous RNA fractions extracted from tumours in the sham-operated group was higher than that from the oophorectomized animals. The ratios of specific activities varied with the time of incorporation but for aqueous fraction the ratio of sham-operated group to the oophorectomized group was about 6:1 at 10 min. and 2:1 at 30 min. The corresponding ratios for the interface material were 2:1 and 4:1 respectively.

DISCUSSION

PolyU stimulated the incorporation of [^{14}C]phenylalanine into the uterine-tumour microsomes, but not to the dramatic extent reported by Green-

Table 2. Incorporation of [^3H]uridine into the aqueous and interface RNA fractions of tumour from oophorectomized and sham-operated animals

[^3H]Uridine (200 μC ; 3.0 μmoles) was injected with final times of 10, 30 and 120 min.; 1 hr. before the [^3H]uridine injections, one-third of the rats in each group were given an injection of 250 μg . of actinomycin D to serve as a base line for RNA polymerase. The RNA fractions were extracted and hydrolysed as described in the Materials and Methods section and the specific activities are given as counts/min./mg. of RNA. The yield of aqueous RNA/5 g. wet wt. of tumour was 20 ± 2 mg. for the sham-operated group and 16 ± 1 mg. for the oophorectomized group. Interface RNA was 7–10% of the aqueous RNA in both cases.

Incorporation time (min.)	Specific activity of RNA			
	Oophorectomized group		Sham-operated group	
	Test	Control	Test	Control
(a) Aqueous RNA fractions				
10	71	50	193	61
30	402	96	801	122
120	166	142	907	146
(b) Interface RNA fractions				
10	1319	240	3010	307
30	607	272	1590	316

man & Kenney (1964) for normal uterine ribosomes prepared with the use of deoxycholate, and with 105 000g supernatant as a source of activating enzymes and transfer RNA. It is unlikely that the different means of preparation is reflected in the degree of stimulation by polyU. Campbell & Cooper (1963), for example, found that the stimulation of liver microsomes by polyU is much greater than with liver ribonucleoprotein particles in identical conditions of incubation. Greenman & Kenney (1964) reported that their preparations contained high concentrations of ribonuclease and it is possible that, despite the use of ribonuclease inhibitors, there was a depletion of messenger RNA during the preliminary isolation of the microsomes before deoxycholate treatment. Such a depletion would result in a more marked response to polyU. This would be in accordance with the results of Fessenden, Cairncross & Moldave (1963), who reported an increased stimulation of liver ribonucleoprotein particles by polyU if the particles were first incubated with pH 5 supernatant.

It has been demonstrated that the addition of polyU has a greater stimulatory effect on the incorporation of [^{14}C]phenylalanine into tumour microsomes from oophorectomized rats than into

microsomes prepared from sham-operated controls. In the absence of synthetic messenger RNA, the tumour microsomes from the control group have a much greater activity. The present concept of protein synthesis is that maximum activity is found when the ribosomes are present as aggregated complexes or polysomes. This has been postulated for ribosomes from both normal tissues (e.g. Warner, Knopf & Rich, 1963) and neoplastic tissues (Penman, Scherrer, Becker & Darnell, 1963; White, Pederson & Hultin, 1963). The aggregates appear to be made up of ribosomes attached to a thin strand, which is probably messenger RNA (Warner, Rich & Hall, 1962). Thus an explanation of the present results with tumour microsomes and their response to polyU could be that oophorectomy impaired the synthesis of endogenous messenger RNA, with a resultant loss in the number of bound ribosomes. Such preparations would respond to synthetic messenger RNA and the incorporating ability of the system would be re-established. Greenman & Kenney (1964) reported that, after oophorectomy, there was a loss of incorporation into normal uterine ribosomes and that the lowered activity was associated with an increased response to polyU. However, certain modifications to the general polysome concept have recently been proposed. Munro, Jackson & Korner (1964), for example, have reported that single unbound ribosomes have a certain synthetic capacity and hence there are certain reservations in accepting evidence, from work *in vitro* alone, about the concentrations of messenger RNA in the tumour microsomal preparations.

The labelling experiments *in vivo* demonstrate that oophorectomy decreases the specific activity of the interface and aqueous RNA extracted from the uterine tumour. Kidson *et al.* (1963) have presented striking evidence that the interface RNA has the characteristics of messenger RNA, namely high turnover rate, heterogeneity, association with DNA and a different fractionation pattern in countercurrent distribution when compared with aqueous RNA. The latter fraction has the turnover rate and the centrifugation pattern of ribosomal and transfer RNA.

The high turnover rate and sensitivity to actinomycin D (Reich, Franklin, Shatkin & Tatum, 1962) suggest that the tumour interface RNA contains the messenger RNA fraction. The control of the synthesis of uterine-tumour messenger RNA by physiological concentrations of oestrogens is in accordance with results from the normal tissue (Gorski, 1964). Further, the concentrations of tumour ribosomal RNA and transfer RNA are affected by oophorectomy, and the synthesis of these RNA fractions is also sensitive to actinomycin D. Moore & Hamilton (1964) have reported that

the formation of uterine ribosomal RNA is decreased in oophorectomized rats and further decreased by low concentrations of actinomycin D.

Thus it appears that the uterine tumour used in these investigations responds to oestrogens in a manner similar to the normal tissue. The mechanism of the response in the normal uterus probably involves de-repression of RNA polymerase (Talwar, Segal, Evans & Davidson, 1964), but this possibility has not been investigated in the present work.

It has been assumed that the effects of oophorectomy on the tumour have been due to oestrogen deprivation rather than to other changes in hormone concentrations after surgery. This seems a valid assumption in view of the similar behaviour between the uterine tumour and normal uterine tissue and the proven dependence of the latter on physiological concentrations of oestrogens.

It should also be stated that the tumour line used in these experiments may represent a rather specialized case. This tumour is strongly oestrogen-dependent, whereas many similar Guerin tumours are less responsive to oestrogens. Consequently, it would seem that the surgical removal of the ovaries may not decrease the growth of experimental uterine tumours in all cases. Human uterine tumours also appear to have a varied response to oestrogens, and therefore a combination of oophorectomy and anti-metabolite therapy (such as 6-azauridine; Šorm & Keilova, 1958) may prove more effective than the effects of oestrogen deprivation alone.

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