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The Effects of Diethylstilboestrol and Castration on the Nucleic Acid and Protein Metabolism of Rat Prostate Gland

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1. Implantation of diethylstilboestrol pellets into adult male rats brings about similar biochemical changes in the nucleic acid and protein metabolism to castration. 2. The avidity of the prostate for labelled diethylstilboestrol was greater than that of the other organs examined. 3. Uptake of labelled diethylstilboestrol and testosterone into the nuclei of the rat prostate is greater in castrated rats than in controls. 4. Diethylstilboestrol appeared to be associated with the protein and not with the DNA fraction of the nucleus.

Carcinoma of the prostate in man is usually initially treated by castration, DES* administration or a combination of both. A widely held theory as to the mode of action of DES on the normal prostate is that it exerts its effect by suppression of androgen production or the gonadotrophic effect of the anterior pituitary (Fearon, 1961). However, the work of Huggins & Clark (1940) with androgen-maintained castrated dogs and of Goodwin, Rasmussen-Taxdal, Ferreira & Scott (1961) with androgen-maintained hypophysectomized dogs showed the suppression of prostatic secretion to be, at least in part, independent of both the testes and the pituitary. Lasnitzki (1963), using excised mouse prostate glands grown in tissue culture, has also shown that DES can act directly on the prostate. Williams-Ashman, Liao, Hancock, Jurkovitz & Silverman (1964) have extensively studied the effect of castration, and castration followed by androgen administration, on the nucleic acid and protein metabolism of the normal rat prostate. In view of the work of Williams-Ashman *et al.* (1964) linking the hormonal control of prostatic growth with changes in RNA and protein synthesis, and of the effect of DES on prostatic growth referred to above, we have investigated the biochemical changes involving prostatic nucleic acid and protein metabolism brought about by DES implantation into the normal adult male rat and compared them with those brought about by castration. We have also carried out preliminary investigations into the subcellular distribution of labelled DES in the prostate, and have compared these results with those obtained by using labelled testosterone.

* Abbreviations: DES, diethylstilboestrol; RNP, ribonucleoprotein; polyU, polyuridylic acid; polyUG, polyuridylylguanylic acid.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]DES (1·4c/m-mole), [1,2-$^3H_2$]testosterone (41·8c/m-mole), [8-^{14}C]ATP (14·2mc/m-mole) and L-[U-^{14}C]phenylalanine (222mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. DES and puromycin were supplied by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. ATP was a product of the Sigma (London) Chemical Co. Ltd., London, S.W. 6. Koch–Light Laboratories Ltd., Colnbrook, Bucks., supplied CTP, GTP, UTP, calf thymus DNA, bovine serum albumin and polyU. Pyruvate kinase, phosphoenolpyruvate (potassium salt) and yeast RNA were obtained from the C. F. Boehringer Corp. (London) Ltd., London, W. 5. Triton X-100 was a gift of Rohm and Haas Co. supplied by Lennig Chemicals Ltd., London, W.C. 1. Actinomycin D was a generous gift of Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Hyamine 10X was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, and Sephadex G-25 was supplied by Pharmacia (G.B.) Ltd., London, W. 13. Testosterone propionate was obtained from British Drug Houses Ltd., Poole, Dorset, as were orcinol and diphenylamine, which were recrystallized from benzene and from petroleum spirit containing fuller's earth respectively before use. All other chemicals were British Drug Houses Ltd. AnalaR grade.

Animals. Adult male Sprague–Dawley rats weighing approx. 200g. were used throughout. Bilateral orchidectomy was performed via the scrotal route under anaesthesia. DES and testosterone pellets weighing approx. 30mg. were made with a tablet press supplied by A. Gallenkamp and Co. Ltd., and placed subcutaneously in the animals' flanks, under ether anaesthesia, with an exploring trocar. When animals were killed, pellets were removed and dried to constant weight. Absorption of DES was calculated as the difference between initial and final pellet weights. Prostates were removed, blotted and weighed. In some experiments the prostate of each rat was exposed by means of a ventral incision, under anaesthesia, and labelled DES or testosterone was injected directly into one of the ventral lobes. The incision was closed with suture clips.

Then 1 hr. later the animals were killed and the prostates removed. Intraperitoneal injection of labelled DES or testosterone was used in some experiments.

Isolation of nuclei. Nuclei were prepared from prostate glands by essentially the method developed by Widnell & Tata (1964) for isolating nuclei from rat liver, but final centrifugation of the prostatic nuclei was done in 2·2 M-sucrose.

Isolation of RNP particles. RNP particles were isolated by method II of Silverman, Liao & Williams-Ashman (1963), but with Triton X-100 in place of sodium deoxycholate, as recommended by Hunter & Korner (1966).

Assays of enzymic activity. (*a*) RNA polymerase. Activities of the DNA-dependent enzyme, RNA polymerase (nucleoside triphosphate–RNA nucleotidyltransferase, EC 2.7.7.6), were determined with purified prostatic nuclei by essentially the method used by Liao, Leininger, Sagher & Barton (1965) and calculated as $\mu\mu$moles of [^{14}C]AMP incorporated/mg. of DNA. Preliminary experiments indicated the requirement for all four nucleoside triphosphates for enzyme activity as reported by Liao *et al.* (1965). Activities were measured at relatively low salt concentrations, since Gorski (1964) and Liao *et al.* (1965) have shown that the response of RNA polymerase activity to variations in hormone concentrations in uterus and prostate is minimized in the presence of high salt concentrations. Each reaction vessel contained, in a total volume of 0·5 ml., the following, which were added while the tubes stood in an ice bath: 60 μmoles of tris–HCl, pH 8·1; 3 μmoles of NaF; 2·5 μmoles of β-mercaptoethanol; 15 μmoles of KCl; 2·5 μmoles of $MgCl_2$; 0·3 μmole each of CTP, GTP and UTP; 0·03 μmole of [^{14}C]ATP. Nuclei containing about 100 μg. of DNA were added in 0·25 ml. and in some cases 10 μg. of actinomycin D was added. After 15 min. incubation at 37°, the tubes were chilled and 5 ml. of cold 10% (w/v) trichloroacetic acid was added. The precipitates were washed three times with 5 ml. of cold 5% trichloroacetic acid, once with 5 ml. of cold 95% (v/v) ethanol and once with 5 ml. of cold 5% trichloroacetic acid. The precipitates were taken up in four drops of 1 M-Hyamine 10X solution and kept overnight at 37°. Liquid scintillator (Bray, 1960) (7·0 ml.) was added and the sediment brought into solution with stirring. Counting was carried out in an I.D.L. Liquid Coincidence Scintillation Counter no. 2022. Counts were corrected for background. The incorporation that occurred in the presence of 10 μg. of actinomycin D was subtracted from the values obtained from the corresponding assays.

(*b*) Studies on amino acid incorporation *in vitro*. The incubation medium used to determine the ability of the RNP particles to incorporate amino acids contained the following in a total volume of 1·0 ml.: 60 μmoles of tris–HCl, pH 7·8; 45 μmoles of KCl; 11 μmoles of β-mercaptoethanol; 10 μmoles of $MgCl_2$; 1 μmole of ATP; 1 μmole of GTP; 9 μmoles of phosphoenolpyruvate; 50 μg. of pyruvate kinase; 0·2 μc of [^{14}C]phenylalanine; 10 μmoles of each of the other 19 naturally occurring amino acids, unlabelled. A sample (0·1 ml.) of the supernatant fraction from control rats, containing approx. 1 mg. of protein, was added to each reaction vessel. Low-molecular-weight components had previously been removed by Sephadex G-25 gel filtration in medium A of Silverman *et al.* (1963). About 300 μg. of particulate RNA was added to each vessel and 100 μg. of puromycin or 100 μg. of polyU added where appropriate. The incubation was for 45 min. at 37° (Liao & Williams-Ashman, 1962), and was stopped by the addition of 5 ml. of ice-cold 10% (w/v) $HClO_4$. The precipitates were washed three times with 5 ml. of ice-cold 5% (w/v) $HClO_4$, and then heated for 15 min. at 90° with 5 ml. of 5% $HClO_4$. They were then washed twice with 5 ml. of ice-cold 5% $HClO_4$ and treated as before. Counts were corrected for background. The incorporation that occurred in the presence of 100 μg. of puromycin was subtracted from the values obtained in the corresponding assays. The activities were calculated as counts/min. incorporated/300 μg. of particulate RNA, and expressed as percentages of the activities obtained with RNP particles from control animals.

Distribution of radioactive DES among various rat tissues after intraperitoneal injection. DES was injected into normal male rats. After 1 hr., the rats were killed and the testes, adrenals, pituitaries, gastrocnemius muscles and prostate glands removed. Homogenates of the various organs were made in medium A of Widnell & Tata (1964). Portions of these homogenates were dissolved by incubation in 1 M-Hyamine 10X at 37° for 24 hr., and the radioactivity was then determined after addition of liquid scintillator (Bray, 1960).

Distribution of radioactive DES and testosterone among subcellular components of the prostate gland. Prostatic homogenates were prepared in medium A by the method of Widnell & Tata (1964). After the initial centrifugation at 700*g* for 10 min., the pellet was used as a source of nuclei as described by Widnell & Tata (1964) with the modification to which reference has been made above. The supernatant was centrifuged at 100000*g* for 60 min. This gave a pellet, which is referred to as the combined mitochondrial and microsomal fraction, and the supernatant fraction. The two particulate fractions were washed twice by suspension in 10 ml. of cold medium B of Widnell & Tata (1964). The washing solution had been saturated with unlabelled DES or testosterone by shaking at 4° for 18 hr., followed by filtration to remove excess of DES or testosterone. Nuclei were resedimented at 500*g* after each washing. Microscopical examination revealed that no change in appearance of the nuclei resulted from this washing procedure. The combined mitochondrial and microsomal fraction was resedimented by centrifuging at 100000*g* for 1 hr. These washings were carried out to remove labelled DES or testosterone that was not firmly bound, although it is possible that this procedure might bring about some redistribution of radioactivity. Radioactivity determinations were carried out on these fractions. Portions of the fractions were dissolved in 1 M-Hyamine 10X by incubation at 37° for 24 hr. Radioactivity determinations were carried out after addition of aqueous scintillator (Bray, 1960).

Distribution of radioactive DES within the prostatic nuclei. Nuclei, isolated from two prostates after direct injections and after washing in unlabelled DES as above, were suspended in 1 ml. of a solution containing sucrose (0·32 M), $MgCl_2$ (2 mM) and potassium phosphate, pH 6·8 (1 mM) and disrupted by gentle homogenization in 7 ml. of 6·5 M-CsCl, containing trisodium EDTA (5 mM), as used by Sporn & Dingman (1966). The solution was subjected to centrifugation (Fisher, Cline & Anderson, 1964) in an 8 × 10 ml. angle-head MSE Superspeed 40 centrifuge at 85000*g* for 72 hr. at approx. 20°. Fractions (1 ml.) were collected with an MSE tube-piercer, and the extinction was measured at 260 mμ and 280 mμ in a Hilger and Watts Uvispek spectrophotometer. CsCl was removed by prolonged dialysis of each fraction against cold distilled water, and radioactivity,

protein and DNA determinations were carried out on each fraction.

Analytical ultracentrifugation of RNP particles. RNP particles prepared as above were analysed in a Spinco model E analytical ultracentrifuge at 35 600 rev./min. and 20°, and photographed by schlieren optics.

Chemical analyses. Samples of filtered whole homogenate, nuclei and ribosomal suspensions were precipitated and washed three times with 10 ml. of cold 0·2 N-$HClO_4$. Nucleic acids were twice extracted from the precipitates with 2 ml. of 0·5 N-$HClO_4$ at 75° for 10 min. DNA was determined in the combined supernatant by the method of Burton (1956) as modified by Giles & Myers (1965), with calf thymus DNA as standard. RNA was determined by the orcinol method of Mejbaum (1939), with yeast RNA as standard. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

RESULTS

Effect of DES on prostate weight and on nucleic acid and protein content. Fig. 1 shows the relationship between uptake by loss of DES from a subcutaneous implant and weight of prostate gland. Figs. 2(*a*) and 2(*b*) show the relationship between RNA, protein and DNA in the organ during the period of regression as shown in Fig. 1. For comparison, the effects of castration followed by testosterone implantation are included. The values in Figs. 2(*a*) and 2(*b*) are expressed as ratios of the concentrations of RNA and protein to those of DNA. The concentration of DNA has been shown by Butler & Schade (1958) and Liao (1965) and confirmed by ourselves, to increase slightly after castration. Liao (1965) reported DNA concentrations (mg./g. of tissue) of 1·99 for rats 70 hr. after castration and 1·04 for rats castrated, left for 22 hr. and then treated with androgen for 48 hr. In the present study, we observed that the DNA concentration (mg./g. of tissue) rose from an initial value of 1·53 to one of 1·96, 48 hr. after castration.

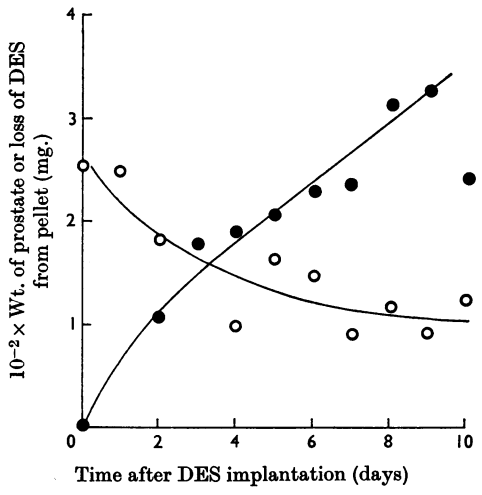

Fig. 1. Loss of DES from a subcutaneous implant and concurrent loss in weight of rat prostate. ●, Loss of DES; ○, loss in weight of prostate. Each weight is the average derived from two animals.

Effect of DES and castration on RNA polymerase, amino acid incorporation in vitro *and RNP particles.* The activity of RNA polymerase and the ability of RNP particles to incorporate amino acids after DES implantation are shown in Table 1. Results obtained from parallel experiments with intact rats, castrated rats and castrated rats implanted with testosterone pellets are included for comparison. This experiment has been repeated on several occasions. The relative enzymic activities observed have always been of the order shown in Fig. 1. It was found necessary to shorten the experimental period when it was required to isolate RNP particles from castrated animals, because of the rapid decline in yield of RNP particles after castration. At 3½ days after castration, it was found that the total yield of RNP particles had declined to 29% of that obtained from an equivalent weight of prostatic tissue excised from testosterone-treated castrated rats. This finding is in agreement with that reported by Williams-Ashman *et al.* (1964). In view of this decline, sedimentation patterns of particles from intact, castrated and DES-treated rats were examined. Figs. 3(*a*) and 3(*b*) show the patterns obtained from intact and castrated rats. DES treatment was found to give a similar result to castration. Fig. 3(*c*) shows the pattern resulting from the treatment of RNP particles from intact rats with 1 μg. of ribonuclease/ml. (Mansbridge & Korner, 1966).

Experiments with [^{3}H]*DES or* [^{3}H]*testosterone* in vivo. The presence of [^{3}H]DES in several tissues was examined after intraperitoneal injection (Table 2). Table 3 shows the subcellular distribution in the prostate of [^{3}H]DES or [^{3}H]testosterone after intraperitoneal injection. Table 4 shows that, after direct injections into the prostate, prostatic nuclei of castrated rats take up more [^{3}H]testosterone and [^{3}H]DES/mg. of DNA than those of control animals.

Sporn & Dingman (1966) have examined the intranuclear location of certain carcinogens by caesium chloride density-gradient centrifugation. Using a similar technique, we have examined the intranuclear localization of labelling after direct injection of [^{3}H]DES into the prostate of normal and castrated rats. Fig. 4 shows the results obtained in one experiment with normal rats. A similar pattern of results was observed when castrated rats were used.

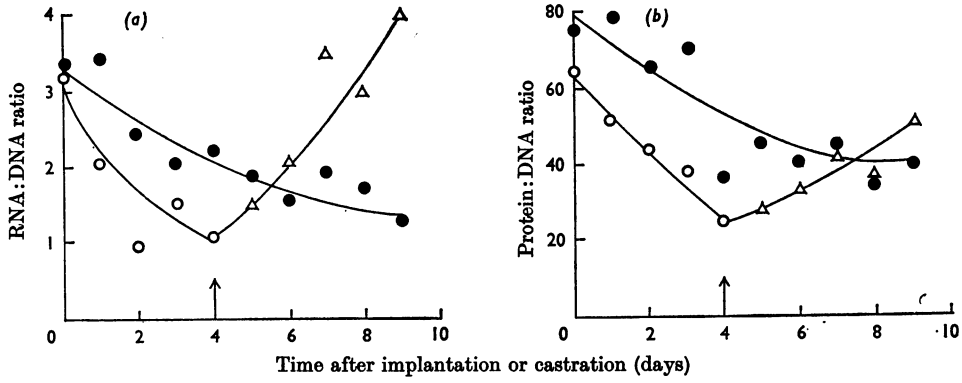

Fig. 2. Changes in (*a*) RNA:DNA ratio and (*b*) protein:DNA ratio of the rat prostate brought about by DES implantation, and by castration followed by testosterone implantation. ●, DES implanted; ○, castrated; △, castrated and testosterone implanted (testosterone was implanted 4 days after castration).

Table 1. *Effect of DES and castration on RNA polymerase activity and* [^{14}C]*phenylalanine incorporation in the prostate*

Details of assay systems are given in the text. Results are the averages of at least three determinations of pooled prostatic tissue from 12 rats/group. Duplicate experiments agreed within ±10%. RNA polymerase activity in the control was 333 $\mu\mu$moles of [^{14}C]AMP incorporated/15 min./mg. of DNA. Basal [^{14}C]phenylalanine incorporation *in vitro* in the control was 3800 counts/min./45 min. incubation/mg. of RNA.

	RNA polymerase activity (% of control)	[^{14}C]Phenylalanine incorporation *in vitro*	
		Basal (% of control)	Stimulation by polyU (% of corresponding basal values)
Control	100	100	218
DES	37*	39*	231*
Castrated rats	47†	62§	294§
Testosterone-treated castrated rats	148‡	100‖	228‖

* 3 days after DES implantation.
† 3 days after castration.
‡ 6 days after castration and 3 days after testosterone implantation.
§ 1 day after castration.
‖ 1 day after castration accompanied by testosterone implantation.

DISCUSSION

Liao & Williams-Ashman (1962) and Liao (1965), on the evidence of experiments in which castration caused a decreased ability of RNP particles to incorporate valine *in vitro*, which could be reversed by addition of polyUG, and the decrease in RNA polymerase activity after castration, have proposed that the fall in RNA polymerase activity results in a deficiency of messenger RNA, which in turn leads to impaired protein synthesis. They suggest that this is one of the main biochemical changes that results in regression of the prostate. Their results indicated a large stimulatory effect on valine incorporation on addition of polyUG to RNP particles prepared from castrated rats, but only a small effect on the activity of RNP particles prepared from testosterone-treated castrated animals. These findings have been frequently quoted (Frieden, 1964) in connexion with discussions of the nature of hormonal growth control mechanisms. However, later work on the stimulation of phenylalanine incorporation by polyU (Silverman, Liao & Williams-Ashman, 1963) yielded results similar to those obtained with RNP particles from castrated and testosterone-treated castrated rats in the present study (Table 1).

It appears that, although the percentage stimulation of phenylalanine incorporation on adding polyU to RNP particles from castrated rats is slightly higher than that observed with particles

from intact or testosterone-treated castrated rats, the activities of all particles are more than doubled, whatever the source. It would appear possible therefore that 'messenger RNA deficiency' is not the entire reason for protein loss and prostatic regression, since RNP particles prepared from the prostates of normal rats appear to be deficient in the messenger appropriate for phenylalanine incorporation. Another explanation for the loss of protein after castration is suggested by the finding, referred to in the Results section, of a dramatic decrease in the yield of RNP particles. It would appear that the changes in RNA polymerase activities in prostates observed after the various treatments used may be associated with the production of ribosomal rather than messenger RNA.

Recently, evidence has been presented in agreement with this (Liao, Barton & Lin, 1966; Liao, Lin

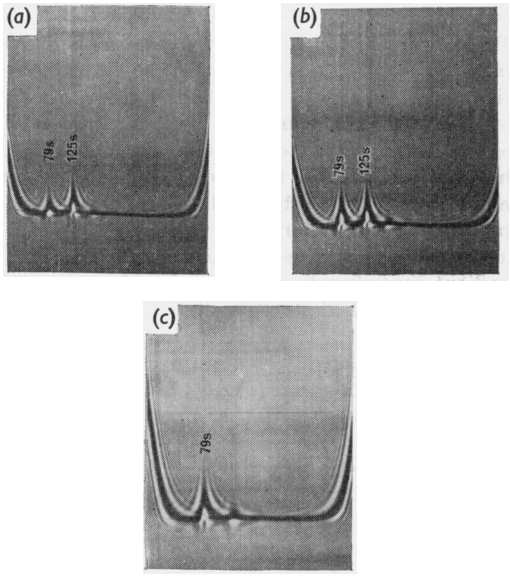

Fig. 3. Ultracentrifugation of prostatic RNP particles isolated from normal and castrated rats. (*a*) RNP particles from control rats. (*b*) RNP particles from castrated rats. (*c*) RNP particles from control rats, treated with 1 μg. of ribonuclease/ml. for 5 min. at 37°. Ultracentrifugation conditions were: 35 600 rev./min.; temperature 20°; approx. 1·5 mg. of protein/ml. in 0·35 M-sucrose–0·025 M-KCl–0·01 M-$MgCl_2$–0·035 M-tris–HCl, pH 7·5.

Table 2. *Distribution of radioactivity between various rat tissues after injection of* [^{3}H]*DES*

Measurements were made 1 hr. after intraperitoneal injection of 118 mμmoles of [^{3}H]DES.

Tissue	Distribution of DES ($\mu\mu$moles/organ)	($\mu\mu$moles/g. wet wt. of tissue)
Testis	71	22
Adrenal	20	59
Pituitary	2	41
Prostate	80	268
Gastrocnemius muscle		15

Table 3. *Subcellular distribution of radioactivity in the prostate after injection of* [^{3}H]*DES or* [^{3}H]-*testosterone*

Measurements were made 1 hr. after intraperitoneal injection of 118 mμmoles of [^{3}H]DES or 1 mμmole of [^{3}H]testosterone.

Fraction	% of radioactivity in the organ: DES-treated	Testosterone-treated
Supernatant	89	97
Mitochondrial+ microsomal	9	2
Nuclear	1	1

Table 4. *Nuclear content of DES or testosterone*

Measurements were made 1 hr. after direct injection into prostate of 50 mμmoles of [^{3}H]DES or 1 mμmole of [^{3}H]-testosterone. Results are the averages of three separate nuclear extracts, each prepared from two prostates. Triplicate radioactivity determinations were carried out on each nuclear sample. Results varied within ±5%.

	Nuclear content ($\mu\mu$moles/mg. of DNA): DES	Testosterone
Control rats	132	0·299
Castrated rats	278	2·135

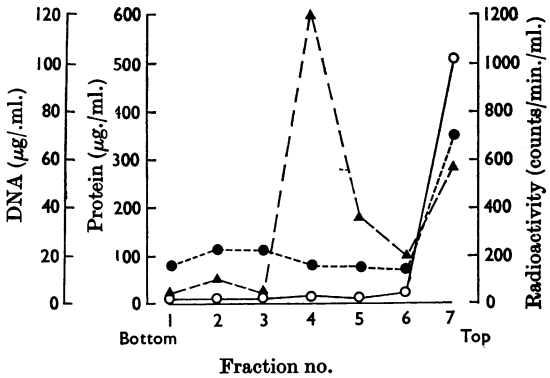

Fig. 4. CsCl-density-gradient centrifugation of normal prostatic nuclei after direct injection of [^{3}H]DES. ○, Protein; ▲, DNA; ●, radioactivity. Centrifugation conditions were: 85 000*g* for 72 hr.; temperature approx. 20°.

& Barton, 1966). Also, Widnell & Tata (1966) have shown that the product of RNA polymerase activity of rat liver nuclei, when examined in low salt concentrations, is ribosomal rather than messenger RNA. This theory is further supported by the sedimentation patterns given in Figs. 3(*a*), 3(*b*) and 3(*c*). Fig. 3(*a*) shows that normal prostatic tissue contains approximately equal concentrations of monomeric and dimeric ribosomes. This contrasts with the situation in the liver, in which, by using similar methods to those used in this study, it has been observed that the monomer population greatly exceeds that of the dimer (Dass & Bayley, 1965). A high proportion of dimers compared with monomers has been reported by Mansbridge & Korner (1966) as being present in the transplantable hepatoma 223. These authors suggested that the high dimer population in that tissue is due to an excess of messenger RNA, which largely prevented the available ribosomes from aggregating into larger polymers. The results given in Fig. 3(*c*) confirm that the dimers observed in the normal prostate consist of monomers associated by means of an RNA that is very susceptible to attack by low concentrations of ribonuclease.

Wettstein, Staehelin & Noll (1963) have suggested that the messenger RNA that links ribosomes together is the target for attack by low concentrations of ribonuclease. The results given in Fig. 3(*b*) show that the large fall in total ribosome population that follows castration is not accompanied by any great change in the dimer:monomer ratio. Munro, Jackson & Korner (1964) and Staehelin, Wettstein, Oura & Noll (1964) have reported that the partial degradation of polymers leads to an increase in the monomer rather than the dimer population. The results given in Fig. 3(*b*) would not appear to indicate that any extensive degradation of polymers to monomers has occurred as a result of castration. Further, the relatively high proportion of dimers still apparent after castration, would, according to the theory advanced by Mansbridge & Korner (1966), indicate that even in this physiological state there still exists in the prostate an excess of messenger RNA for the available ribosome population. The results in Table 1 indicate that the RNP particles obtained from DES-treated animals have undergone similar changes in incorporating ability to those exhibited by particles obtained from castrated animals. It must be stressed, however, that all the changes referred to above that result from DES implantation might be expected to occur in a regressing organ. Therefore, to gain further insight into the mechanism by which DES exerts its physiological effect, the experiments involving injection of [^{3}H]DES were carried out. Uptake of DES was found to take place into all the tissues examined (Table 2), with the prostate showing the largest accumulation. This finding is similar to the result obtained by Harding & Samuels (1962) for the uptake of labelled testosterone into various organs of the rat. Examination of the subcellular distribution of [^{3}H]DES and [^{3}H]testosterone (Table 3) indicated a predominance of the labelling in the supernatant fractions. However, although only a small uptake into the nuclear fractions was observed, this could be increased on castration of the animals (Table 4).

It therefore appears that not only are the biochemical changes brought about by DES implantation similar to those resulting from androgen withdrawal induced by castration, but that the avidity of the prostate for DES and its subsequent subcellular distribution is also similar to that observed with testosterone. In view of the striking similarities in the behaviour of DES and testosterone with regard to the prostate, it would appear probable that both DES and testosterone affect prostatic cells directly, although an additional effect via the endocrine glands cannot be ruled out. Wilson & Loeb (1965) have used caesium chloride-density-gradient centrifugation to examine the subnuclear location of labelled steroids. The results of experiments in which the intranuclear location of labelled DES was examined by caesium chloride-density-gradient centrifugation (Fig. 4) indicated that the label was associated with the upper protein fraction rather than the DNA fraction in the nuclei of both normal and castrated animals. The preliminary extensive washing of the nuclei in unlabelled DES solution, and the prolonged dialysis of the density-gradient fractions (necessary to remove caesium chloride before protein and other determinations), would seem to reduce the possibility of artifacts due to non-specific absorption of DES. However, the possibility of redistribution of labelled DES on treatment of the nuclei with high concentrations of caesium chloride cannot be ruled out from any study of this kind.

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