THE EFFECT OF SOME STILBOESTROL COMPOUNDS ON DNA POLYMERASE FROM HUMAN PROSTATIC TISSUE

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ABSTRACT

The inhibition by diethylstilboestrol of DNA nucleotidyltransferase isolated from human hyperplastic and neoplastic prostatic tissue has been studied. Whereas diethylstilboestrol consistently inhibited the enzyme, oestradiol-17β often had little or no effect. Dihydrodibutylstilboestrol, the synthesis of which is described, was also found to consistently inhibit the DNA polymerase, which contrasted with the lack of effect observed with the cis and trans isomers of dibutylstilboestrol. Two of the enzyme preparations derived from hyperplastic prostates were stimulated by testosterone and 5α-dihydrotestosterone.

INTRODUCTION

Earlier studies of Fahmy, Griffiths, Mahler and Williams(1) showed that certain natural oestrogens stimulated in vitro the DNA polymerase enzyme system (DNA nucleotidyltransferase, EC2.7.7.7.) prepared from calf thymus gland. More recently, it was also shown by Fahmy and Griffiths (2) that diethylstilboestrol and some of its analogues inhibited the activity of this enzyme system. Since the androgen dependent nature of human prostatic cancer is well known and its treatment by diethylstilboestrol well established (3), it was decided to investigate the effects of diethylstilboestrol and other

steroid hormones on the DNA polymerase enzyme system prepared from these tissues. At the same time, the effect on these enzyme preparations of a newly synthesized dihydrodibutylstilboestrol was studied. A preliminary report of some of these experiments has been presented (4).

MATERIALS AND METHODS

Chemicals Deoxy-GTP, deoxy-CTP, deoxy-ATP and [^{3}H]thymidine 5'-triphosphate (TTP, specific activity 5.0 c/m-mole) were obtained from Schwarz BioResearch Inc., New York, N.Y., U.S.A. Non-radioactive TTP was purchased from Sigma (London) Chemical Co., London, S.W.6. Calf thymus DNA and all other A.R. reagents were supplied by British Drug Houses, Ltd., Poole, Dorset. Diethylstilboestrol deoxy-p-anisoin, and n-butylbromide were obtained from Koch-Light Laboratories, Ltd., Colnbrook, Bucks., England. Diethylstilboestrol diphosphate (Honvan) was a gift from Ward Blenkinsop and Co. Ltd., Wembley, London.

Tissues Prostatic tissue was cooled to 0°C after removal and retained at this temperature until processed in the laboratory 60 mins. later.

Enzyme studies The DNA polymerase preparations studied were those of the semi-purified F_2-fraction isolated by the procedure of Shepherd and Keir (5). Enzyme preparations were stored in sealed vials at -20°C. The enzyme (100-250 µg. protein) was incubated at 37°C for 2 hr. with 50 mµmoles of [^{3}H] TTP (specific activity 10 x 10^6 counts/min/µmole) in 255 µl. medium containing 5 µmoles tris HCl buffer, pH 7.5, 15 µmoles KCl, 100 mµmoles EDTA, 1 µmole $MgCl_2$, 2 µmoles 2-mercaptoethanol, and 50 mµmoles each of deoxy-GTP, deoxy-CTP and deoxy-ATP together with 50 µg. thermally denatured DNA. Either 5 µl. ethanol, or compounds in this volume of ethanol, were added to the reaction mixture. Protein content of the enzyme was determined by the method of Lowry et al(6).

Radioactivity determination Samples (50 µl.) were withdrawn at zero time and after 2 hr. placed on fibreglass disks (Whatman GF/C), washed in ice-cold 5% (w/v) trichloroacetic acid and dried with ethanol. The disks were placed in vials containing 10 ml. scintillation liquid (473 ml. toluene, 25 ml. hyamine hydroxide, 100 mg. 1,4-bis (5-phenyloxazol-2-yl) benzene, and 2 g. of 2,5-diphenyloxazole) and heated for 10 min. at 57°C. The radioactivity was measured with a Nuclear-Chicago

Model 6860 liquid scintillation spectrometer.

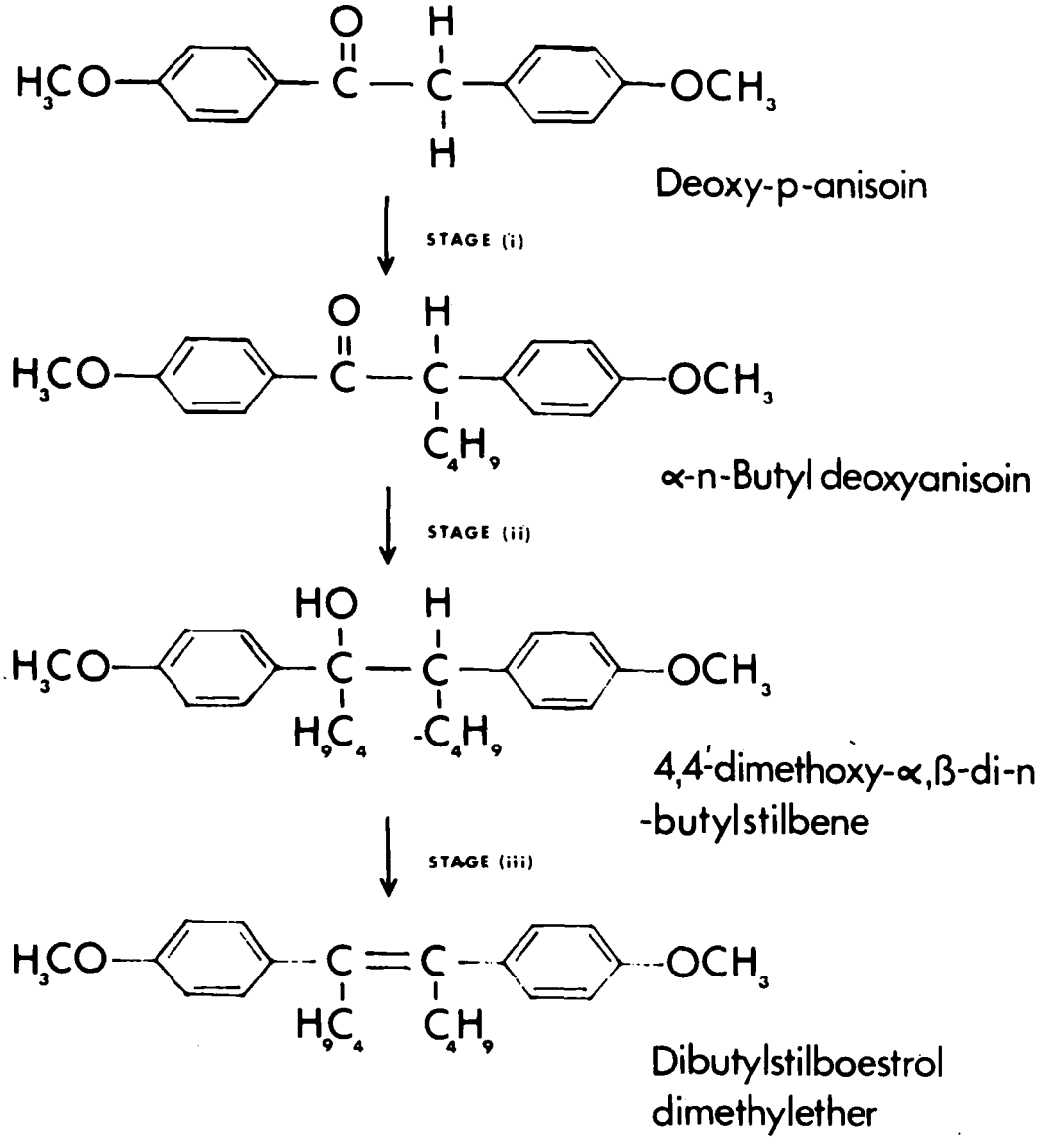
Chemical synthesis of Dibutylstilboestrol The method of Dodds et al (7) for the preparation of alkyl substituted stilboestrols was adopted with some modifications. The outline of the procedure was as follows (Fig. 1) Stage (i) Deoxy-p-anisoin was alkylated with n-butyl-bromide and sodium ethoxide, and (Stage ii) the product brought into reaction with a Grignard reagent (C_4H_9MgBr.) The resulting carbinol was then dehydrated using PBr_3 in $CHCl_3$ (Stage iii) with the formation of dibutylstilboestrol dimethylether. Dodds et al (7) effected demethylation of the ether by using alcoholic KOH in a constantly rotating autoclave at 200-210° (max. pressure approximately 15 atmospheres) for 24 hr. Moore (8) however, demethylated the ether by heating in alcoholic KOH in a sealed bomb at 210°C for 20 hr. Unsuccessful attempts to demethylate the ether with aluminium bromide were made (7). However, it has now been shown in these investigations that demethylation of the ether can be effected with boron tribromide (BBr_3).

Dibutylstilboestrol dimethylether (5 g.) was dissolved in 150 ml. dichloromethane and slowly added to a cold solution of BBr_3 (3.5 ml.) in 125 ml. dichloromethane and left overnight at room temperature. A $CaCl_2$ filled drying tube protected the reaction mixture from moisture. The mixture was then shaken with water to hydrolyse excess BBr_3 and boron complexes and the phenolic product collected by extraction into ether. The solvent was evaporated in a rotary film evaporator. The oily product was purified by distillation under vacuum and the fraction, distilling at 192-196°, 0.18 mm Hg., was collected. The yield was 3.2 g. of a pale yellow oil.

The purity of the compound was checked by chromatography on a thin layer of Merck silica gel G in the solvent system petroleum ether (60-80°) 75: acetone 25 (v/v) (system A). The principal component, (Rf. 0.28) had a faint pink fluorescence in UV-light (240 mμ) changing within 45 secs. exposure to yellow. A pale blue fluorescing material (Rf. 0.68) was identified as the dibutylstilboestrol dimethylether.

The oil was further purified by column chromatography on silica gel (100-200 mesh). The column (15 cm. length, 1 cm. diam.) was packed with silica gel (6 g.) prepared as a slurry with petroleum ether (60-80°). The column was equilibrated and washed with this solvent. The oil (0.2 g.) adsorbed on silica gel and suspended in petroleum ether,

Fig 1


Deoxy-p-anisoin
STAGE (i)
∝-n-Butyl deoxyanisoin
STAGE (ii)
4,4'-dimethoxy-∝,ß-di-n
-butylstilbene
STAGE (iii)
Dibutylstilboestrol
dimethylether

was layered carefully onto the column. Elution was carried out with a stepwise gradient elution with increasing concentrations of acetone in petroleum ether 60-80°C. The material appearing as a yellow band when viewed with UV-light, was eluted with 10% acetone in petroleum spirit. This material gave one spot on thin layer chromatography (Rf.0.28) in solvent system A and analysis indicated C,81.2%:H,8.8% ($C_{22}H_{28}O_2$ - C,81.5%:H8.6%).

On standing in $CHCl_3$ at room temperature white needle shaped crystals separated. These were filtered, recrystallized from $CHCl_3$ (MP 145-146°) and analysed. The analysis indicated C,81.6%:H,8.5%. The material had an Rf. of 0.28 on thin layer chromatography in the same solvent system A. Nuclear magnetic resonance studies indicated that this stable crystalline material was the trans-isomer of dibutylstilboestrol. The cis isomer was almost completely converted to the trans form by dissolving the oil in CCl4 containing 0.5% iodine and refluxing for 45 mins. The CCl4 and iodine were distilled and the residue crystallized from $CHCl_3$, this procedure providing a 90% yield.

Chemical synthesis of dihydrodibutylstilboestrol Dodds et al (7) hydrogenated diethylstilboestrol in acetone solution using a palladium catalyst. In the present study the dibutylstilboestrol was hydrogenated in acetic acid solution using a platinum catalyst. The crystalline compound that separated was recrystallized twice from benzene, (MP 165-166°C, uncorrected). Analysis indicated C,80.8%:H,9.1% ($C_{22}H_{30}O_2$ would be C,81.0%:H,9.2%). A MP of 167-168°C for the meso-form of dihydrodibutylstilboestrol has been reported (9).

Preparation of dipotassium diethylstilboestrol disulphate This sulphate was prepared by the method of Short and Oxley (10). Diethylstilboestrol (3 g.) and sulphamic acid (7 g.) in 20 ml. pyridine were heated at 100°C with stirring for 20 min. The separated solid was collected and recrystallized from 1N KOH. Subsequent crystallizations were made from hot water, and the white needle-like crystals dried *in vacuo* and P_2O_5 at 55°C for three days. Analysis indicated C,42.4%:H,3.9%:S,12.5%:K,15.3% ($C_{18}H_{18}O_8S_2K_2$ requires C,42.8%:H,3.6%:S,12.7%:K,15.5%).

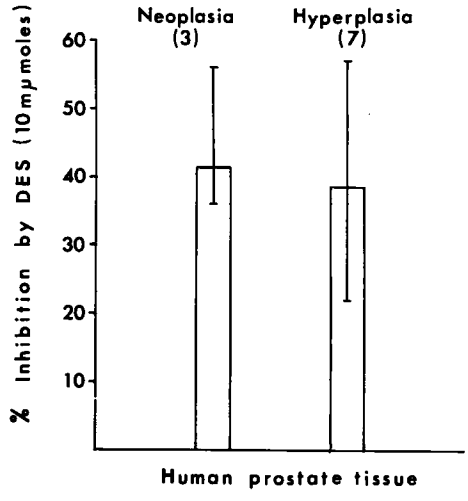
The compound had a Rf. of 0.6 when run on thin layers of silica gel G in the solvent system n-butanol, acetic acid,water: 4,1,1 (v/v).

RESULTS

Diethylstilboestrol was found to inhibit the DNA polymerase from both hyperplastic and neoplastic human prostatic tissue. Fig. 2 shows the average degree of inhibition of the enzyme system, and the range of this effect in a number of experiments. On average, there was a 40% inhibition of the enzyme from both types of tissue.

Fig 2

EFFECT OF DIETHYLSTILBOESTROL(DES) ON DNA POLYMERASE ISOLATED FROM PROSTATE TISSUE



The effect of 10 mμmoles diethylstilboestrol on the DNA polymerase activity of three neoplastic and seven hyperplastic human prostates, each experiment completed in triplicate. The range for different preparations is shown.

The effects of various stilboestrol analogues on the DNA polymerase is indicated in Table 1, which shows the relative inhibitory capacity of diethylstilboestrol and its reduced product hexoestrol, and also of dibutylstilboestrol and dihydrodibutylstilboestrol. It is interesting to note that whereas diethylstilboestrol and hexoestrol inhibited the enzyme to the same degree, dibutylstilboestrol generally had little effect. However, dihydrodibutylstilboestrol consistently and effectively inhibited the DNA polymerase.

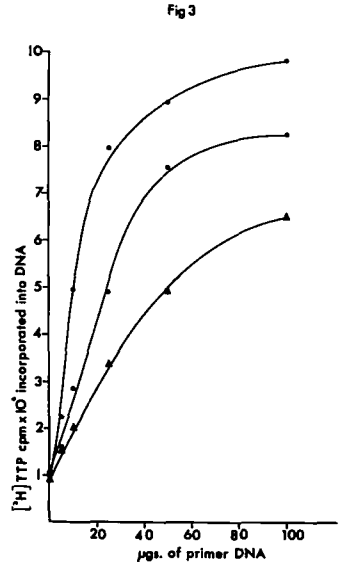
TABLE 1

THE EFFECT OF VARIOUS STILBOESTROL COMPOUNDS ON DNA-POLYMERASE ISOLATED FROM HUMAN PROSTATE TISSUE

DNA polymerase activity (cnts/min/mg. of protein)	Gland pathology	% Inhibition of DNA polymerase activity by the compound at 40 μM final concentration			
		Diethylstilboestrol	Hexoestrol	Dibutylstilboestrol	Dihydro-dibutylstilboestrol
7,523	Neoplasia	31	41	0	41
14,611	Neoplasia	45	40	30	33
8,713	Neoplasia	28	32	6	23
9,103	Hyperplasia	31	-	-	28
8,485	Hyperplasia	29	26	11	29

In fig. 3 the activity of this same enzyme was determined at various concentrations of DNA primer, with and without the presence of diethylstilboestrol and dihydrodibutylstilboestrol, (40 μM final concentration of both inhibitors).

A small amount of [^{3}H] TTP was incorporated at zero concentration of DNA primer. This was not removed by subsequent washings with ice-cold 5% trichloroacetic acid and is probably due to enzyme contamination with DNA from the prostatic tissue. Fig. 4 shows the effect of varying the concentration of inhibitor, both diethylstilboestrol and dihydrodibutylstilboestrol on a DNA polymerase preparation from hyperplastic prostate tissue.



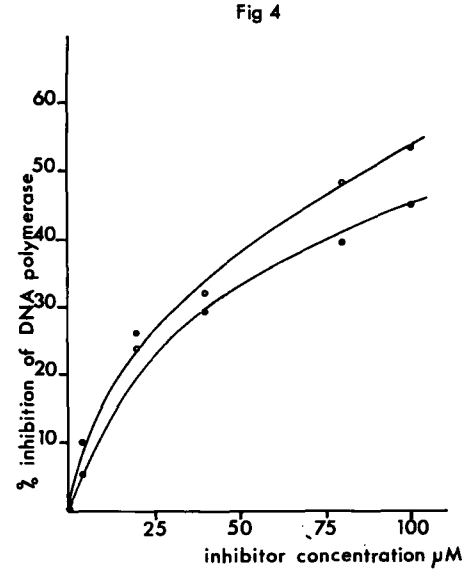


Fig. 3 Inhibition of DNA polymerase activity by diethylstilboestrol and dihydrodibutylstilboestrol at various concentrationsof DNA primer. The assay conditions are as described in methods except that thermally denatured DNA was included at the concentrations shown. Control incubation: ● incubation with diethylstilboestrol: ▲ (40 µM final concentrations), incubation with dihydrodibutylstilboestrol: o (40 µM final concentration). Fig. 4 Inhibition of DNA polymerase activity from hyperplastic prostatic tissue by diethylstilboestrol o , and by dihydrodibutylstilboestrol● added in amounts indicated.

The results shown in table 2 indicate that the phosphate and sulphate esters were less effective inhibitors of the DNA polymerase than the parent compound under these experimental conditions.

TABLE 2

THE EFFECT OF PHOSPHATE AND SULPHATE ESTERS OF DIETHYLSTILBOESTROL (DES) ON PROSTATIC DNA POLYMERASE

DNA polymerase activity cnts./min/mg. of protein	Gland pathology	% Inhibition of DNA polymerase activity		
		Diethylstilboestrol (40 μM)	Diethylstilboestrol -diphosphate (40 μM)	Diethylstilboestrol -disulphate (40 μM)
8,644	Neoplasia	30·7	16·0	—
7,052	Hyperplasia	51·2	10·5	8·0
8,614	Hyperplasia	33·0	9·5	7·0
6,727	Hyperplasia	22·4	6·0	—
8,485	Hyperplasia	29·5	11·3	9·7

Unlike the diethylstilboestrol, oestradiol-17β did not consistently inhibit the prostatic DNA polymerase (Table 3).

TABLE 3

THE EFFECT OF OESTRADIOL-17β ON PROSTATIC DNA POLYMERASE

DNA polymerase activity (cnts./min/mg. of protein)	Gland pathology	% Inhibition of DNA polymerase activity by oestradiol-17β, 40μM final concentration
11389	Hyperplasia	7·9
7,464	Hyperplasia	13·0
9,103	Hyperplasia	5·6
8,644	Neoplasia	0
14,611	Neoplasia	33·0
8,713	Neoplasia	2·5

This inhibitory effect of oestradiol-17β on the prostatic DNA polymerase is in contrast to the observed stimulation of the calf thymus enzyme previously described(1). Also of interest in these investigations, was the observed stimulation by 17β-hydroxyandrost-4-en-3-one (testosterone) and 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone) of two enzyme preparations derived from hyperplastic prostate tissue. At a final concentration of 40 μM, testosterone stimulated the enzyme systems by, respectively, 14 and 38% and 5α-dihydrotestosterone by 32 and 30%. Neither compound had any effect on the prostatic tumour tissue studied, and there was also no effect on the calf thymus enzyme system.

DISCUSSION

Studies on the molecular basis of steroid action must provide valuable information regarding the biochemical control mechanisms associated with hormone-dependent tumours. It would appear that the observed effects of testosterone *in vivo*, on the glands of reproduction (11 - 13) are dependent upon the preliminary synthesis of RNA in the cell nuclei of these organs (14 - 17), Liao and Williams-Ashman (18) have also described an increased activity of the RNA polymerase of rat prostate within an hour of testosterone administration to castrated animals. Little attention has however been

directed towards the hormonal control of DNA synthesis in prostatic tissue, although it has been shown (19) that the DNA polymerase of ventral prostate extracts from castrated rats was enhanced after testosterone administration.

The results of the present study demonstrate *in vitro*, that diethylstilboestrol inhibits the DNA polymerase extracts isolated from human neoplastic and hyperplastic prostatic tissue. Although the degree of inhibition varied from one DNA polymerase preparation to another, as did the inhibition of the calf thymus enzyme by actinomycin-D reported by Keir *et al* (20), diethylstilboestrol consistently inhibited the prostatic enzyme.

It is noteworthy that Mangan *et al* (21) demonstrated the inhibition by diethylstilboestrol of the RNA polymerase activity of nuclei isolated from rat prostate. Since DNA and RNA polymerase utilize DNA as a primer, it is possible that diethylstilboestrol exerts its inhibitory action by interfering with the primer or with the binding of the DNA to the active site on the enzymes. These results provide further support for the suggestion that at least part of the effectiveness of diethylstilboestrol in the treatment of carcinoma of the prostate may be due

to its direct biochemical effect on the prostatic tissue. Hexoestrol, the most effective of the inhibitors studied by Fahmy and Griffiths (2) on the calf thymus preparations also consistently inhibited the prostatic enzyme, and it is interesting that hexoestrol is concentrated by hormone responsive human breast tumours to a greater extent than by the unresponsive type of tumour (22).

The earlier observations of Fahmy and Griffiths (2) suggested an interesting relationship between the structure of the stilboestrol analogue and the degree of inhibition of DNA polymerase. The alkyl substitution in the aa'-positions altered the inhibitory effect, the larger the alkyl group, the greater was the degree of inhibition. Hexoestrol with the saturated aa'-ethylenic linkage was the most effective inhibitor.

Because of these results, the cis and trans isomers of dibutylstilboestrol were synthesized, and also the corresponding saturated compound dihydrodibutylstilboestrol. It was of particular interest to observe that in most instances the cis and trans isomers of dibutylstilboestrol had little effect on the prostatic DNA polymerase, whereas generally the reduced compound was equally as effective as diethylstilboestrol. Since dihydrodibutylstilboestrol has only 0.1% of the oestrogenic activity of diethylstilboestrol

(23) it will be of further interest to study its uptake and localisation in prostatic tissue, relative to diethylstilboestrol and also its effect on plasma testosterone and luteinizing hormone with regard to its possible use in the treatment of prostatic cancer. Diethylstilboestrol therapy decreases plasma testosterone and LH in man (24) and also decreases the activity of the testicular enzyme 17β-hydroxysteroid dehydrogenase converting androstenedione to testosterone (25).

The decreased effectiveness of the phosphate and sulphate esters of diethylstilboestrol would suggest that the free p-hydroxyl groups have some role in the inhibitory effect, as does the group substitution on the ethylene linkage (2). The treatment of prostatic carcinoma with diethylstilboestrol diphosphate (Honvan) probably depends upon the high phosphatase activity of the prostate, releasing the diethylstilboestrol within the target tissue.

Although oestradiol-17β did inhibit the enzyme in certain prostatic preparations, it had little or no effect on others. Furthermore, it was of interest to observe that oestradiol-17β inhibited the prostatic enzyme whereas previously (1) it was consistently found to stimulate the DNA polymerase from the calf thymus. Also of interest was the marked stimulation by <u>both</u> testosterone and 5α-dihydrotestosterone on the activity of two DNA polymerase enzymes

prepared from human hyperplastic prostatic tissue. These androgens did not stimulate all preparations from hyperplastic tissue, and had little or no effect on the enzyme isolated from prostatic tumour tissue, or on the calf thymus enzyme. Further investigations on the relationship between these observations and the histology of the tissue are continuing.

Studies are also in progress to further purify these prostate enzyme systems to eliminate the possibility that the variability in some of the hormone effects is due to the presence of contaminating nucleases.

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