The Inhibition of Deoxyribonucleic Acid Nucleotidyltransferase by Stilboestrol Derivatives

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The inhibition by diethylstilboestrol of DNA nucleotidyltransferase isolated from calf thymus was studied. The inhibition exercised by diethylstilboestrol appears to obey competitive kinetics with respect to DNA primer. The activities of both replicative and terminal enzymes were affected to the same extent. There was no evidence of binding between DNA and diethylstilboestrol. A comparative study of the inhibitory effects of some stilboestrol derivatives is presented. The alkyl substitution in the $\alpha\alpha'$ -positions seem to alter the inhibitory effect of these compounds: dimethylstilboestrol was more inhibitory than stilbene, and diethylstilboestrol was more inhibitory than dimethylstilboestrol. Hexoestrol, in which the $\alpha\alpha'$ -ethylenic linkage is saturated, was the most effective inhibitor.

The androgen-dependence of human prostatic cancer was established by the classical investigations by Huggins & Hodges (1941). Treatment of prostatic cancer by anti-androgen therapy such as castration or diethylstilboestrol administration followed from these experiments. The oestrogen-dependent nature of breast carcinoma has also resulted in extensive trials of anti-oestrogenic compounds such as 17 β -hydroxy-2 α -methyl-5 α -androstan-3-one (Blackburn & Childs, 1959). Studies on the molecular basis of steroid action must therefore provide valuable information on the biochemical control mechanisms associated with hormone-dependent tumours. Though investigations concerned with the effects of steroid hormones on RNA polymerase and RNA synthesis have been reported (Liao & Williams-Ashman, 1962; Wilson, 1962; Hamilton, 1964), little attention has been directed towards the hormonal control of DNA synthesis. However, Fahmy, Griffiths, Mahler & Williams (1967) have shown that certain oestrogens stimulate *in vitro* the isolated DNA polymerase enzyme system (DNA nucleotidyltransferase, EC 2.7.7.7) prepared from calf thymus gland. Since diethylstilboestrol inhibits the oestrogen-stimulated mitotic activity of mouse vagina (Emmens, Cox & Martin, 1962), it was therefore decided to investigate the effect of this compound and those with related structures on the DNA polymerase enzyme system. It is noteworthy that Mangan, Neal & Williams (1967) have reported that diethylstilboestrol inhibited both RNA polymerase activity

and the incorporation of amino acids by ribonucleoprotein particles isolated from prostatic tissue.

MATERIALS AND METHODS

Chemicals. Deoxy-GTP, deoxy-CTP, deoxy-ATP and [^3H]TTP* (specific radioactivity 2.0 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. The dimethylstilboestrol was a gift from Dr L. Martin, Imperial Cancer Research Fund, London, W.C. 2, and the dienoestrol was provided by Dr O. Morton, Medical Director, B.D.H. (Research) Ltd., London, W. 1. Hexoestrol, diethylstilboestrol and stilbene were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Enzyme studies. The enzyme preparation used in this investigation was the semi-purified F_2 fraction isolated from calf thymus by the procedure of Shepherd & Keir (1966). The enzyme was stored in sealed 2.0-ml. ampoules at -20° . The [^3H]TTP was made up in water and diluted with non-labelled TTP to provide a specific radioactivity of 10×10^6 – 12×10^6 counts/min./ μmole . The enzyme (200–250 μg . of protein) was incubated at 37° for 60 min. (unless otherwise stated) with 50 m μmoles of [^3H]TTP in 255 μl . of a medium containing 5 μmoles of tris-HCl buffer, pH 7.5, 15 μmoles of KCl, 100 m μmoles of EDTA, 1 μmole of MgCl_2 , 2 μmoles of 2-mercaptoethanol and 50 μg . (unless stated otherwise) of thermally denatured DNA. For the determination of the

* Abbreviations: TTP, thymidine 5'-triphosphate; TMP, thymidine 5'-monophosphate.

replicative enzyme activity, the medium contained 50 μ moles each of deoxy-ATP, deoxy-CTP and deoxy-GTP. These triphosphates were omitted from the terminal enzyme assay. Either 5 μ l. of ethanol or one of the compounds under investigation in this volume of ethanol was added to the reaction mixture.

Protein content of the enzyme preparation was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Radioactivity measurement. Samples (50 μ l.) were withdrawn at zero time and after 60 min. placed on fibre-glass 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. of scintillation liquid [473 ml. of toluene, 25 ml. of Hyamine hydroxide, 100 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 2 g. of 1,5-diphenyloxazole] and heated for 10 min. at 57°. The radioactivity was measured with a Nuclear-Chicago model 6860 liquid-scintillation spectrometer.

RESULTS

Inhibition of DNA polymerase by diethylstilboestrol. The relationship between DNA polymerase activity (expressed as μ moles of [3 H]TMP incorporated/mg. of protein) and the concentration of diethylstilboestrol in the incubation medium is shown in Fig. 1. A linear relationship was observed between 2 and 6 μ g. of diethylstilboestrol. Insolubility of diethylstilboestrol occurs at concentrations above 15 μ g./reaction mixture.

Inhibition of DNA polymerase activity by diethylstilboestrol at various DNA primer concentrations. The activity of DNA polymerase was determined at various concentrations of DNA primer, with and without the presence of diethylstilboestrol (2.5 μ g. in the reaction medium). The results are shown in Fig. 2. A small amount of [3 H]TMP (0.3 μ mole/mg. of protein) was incorporated at zero concentration of DNA primer. This was not removed by subsequent washings with ice-cold 5% trichloroacetic acid and could be due to enzyme contamination with DNA from thymus tissue. This was taken into account in the results illustrated in Fig. 2 by subtracting this amount from all the data presented in Fig. 2.

The data of Fig. 2 were redrawn as a double-reciprocal plot (Lineweaver & Burk, 1934) shown in Fig. 3. Linear plots were obtained for the control and diethylstilboestrol-inhibited assays. The two plots intersected on the vertical axis, suggesting that diethylstilboestrol exerts its effect by competitively inhibiting the enzyme.

Inhibition of DNA polymerase activity by compounds related to diethylstilboestrol. Table 1 summarizes the results of the comparisons made between the compounds investigated. The compounds were each added in 5 μ l. of ethanol to provide a final concentration of 40 μ M in the incubation medium.

Fig. 1. Inhibition of DNA polymerase activity by diethylstilboestrol. The reaction mixture was prepared according to the method of Fahmy *et al.* (1967). A 50 μ g. sample of thermally denatured DNA (calf thymus), 200 μ g. of protein of a partially purified DNA polymerase isolated from calf thymus, [3 H]TTP (specific radioactivity 10×10^6 counts/min./ μ mole) and the indicated amounts of diethylstilboestrol were added. Incubation was at 37° for 2 hr.

Fig. 2. Inhibition of DNA polymerase activity by diethylstilboestrol at various concentrations of DNA primer. The assay conditions were as described in Fig. 1 with the following exceptions: thermally denatured DNA was included at the concentrations shown and the specific radioactivity of [3 H]TTP was 12×10^6 counts/min./ μ mole. Incubation was at 37° for 2 hr. ○, Control incubation without diethylstilboestrol; ●, incubation with 2.5 μ g. of diethylstilboestrol added.

Inhibition of terminal DNA polymerase by diethylstilboestrol. Partially purified preparations of DNA polymerase from calf thymus have revealed

Fig. 3. Competitive inhibition of DNA polymerase activity by diethylstilboestrol. Allowance was made for the small amount of $[^3\text{H}]\text{TMP}$ incorporated in the absence of DNA primer. The values were plotted according to the reciprocal method of Lineweaver & Burk (1934). Initial velocity, v , is expressed as $\text{m}\mu\text{moles}$ of $[^3\text{H}]\text{TMP}$ incorporated/mg. of protein; substrate concentration, $[\text{S}]$, is expressed as $\mu\text{g.}$ of thermally denatured DNA primer/reaction mixture. ○, Control incubation without diethylstilboestrol; ●, incubation with $2.5\mu\text{g.}$ of diethylstilboestrol added.

the presence of two distinct enzymes. The replicative enzyme gives optimum activity with the four triphosphates. The terminal enzyme utilizes only one triphosphate (Keir & Smith, 1963), extensively incorporating TMP only into the terminal positions of the DNA primer (Keir, Shepherd & Hay, 1963). The inhibitory effect of diethylstilboestrol on both enzymes over a 3 hr. period is illustrated in Fig. 4. The activity of the terminal enzyme in the preparation was 15–20% of the activity of the replicative system. Both enzymes were inhibited to the same extent by diethylstilboestrol. The concentration of ethanol used in these experiments had no effect on the activity of either the replicative or terminal enzymes.

DISCUSSION

The present investigation clearly demonstrates the inhibition *in vitro* by diethylstilboestrol of the activity of DNA polymerase isolated from calf thymus gland. The degree of inhibition varied from one enzyme preparation to another, as observed by Keir, Omura & Shepherd (1963) when they reported inhibition of DNA polymerase activity by actinomycin D. It is noteworthy that Mangan *et al.* (1967) demonstrated the inhibition by diethylstilboestrol of the RNA polymerase activity of nuclei isolated from rat prostate. Since both DNA polymerase and RNA polymerase utilize

Table 1. Relation between chemical structure and degree of inhibition

The reaction mixture was as in the experiments in Fig. 1, but the compounds studied were at a final concentration of $40\mu\text{M}$ in the incubation medium. Percentage inhibitions are expressed as means $\pm$ s.d. of four experiments.

Compound	Inhibition (%)
9 ± 1
Stilbene
9 ± 1
4,4'-Dihydroxystilbene
13 ± 1
Dimethylstilboestrol
14 ± 1
Dienoestrol
26 ± 2
Diethylstilboestrol
38 ± 2
Hexoestrol	

DNA as a primer in the system, it might be suggested that diethylstilboestrol exercises its inhibitory action either by interference with the DNA primer that is common to both enzymes, or by interfering with the binding of DNA to the active site on the enzymes. Preliminary observations, not reported, suggest that the u.v.-absorption characteristics of the DNA are not altered by the presence of diethylstilboestrol. Binding may not therefore occur, though actinomycin D exerts its inhibitory effect by binding to the guanine residues of the DNA primer (Reich, Goldberg & Rabinowitz, 1962; Goldberg, Rabinowitz & Reich, 1962).

The introduction of oestrogen administration for the treatment of carcinoma of the prostate (Huggins, Scott & Hodges, 1941) was based on experiments showing that the growth of prostatic cancer was androgen-dependent and that oestrogens antagonized the androgenic action. It is not completely

Fig. 4. Action of diethylstilboestrol on the activities of the replicative and terminal DNA polymerase systems. The assay conditions differed from those in Fig. 1 in that the reaction mixture contained 250 μ g. of enzyme protein. —, Replicative enzyme activity; $\circ$, standard assay; $\bullet$, standard assay + 5 μ g. of diethylstilboestrol., Activity of the terminal enzyme assayed in the absence of deoxy-ATP, deoxy-CTP and deoxy-GTP: Δ , uninhibited enzyme system; $\blacktriangle$, system to which 5 μ g. of diethylstilboestrol was added.

understood, however, whether oestrogens exercise this antagonism via the pituitary, thereby decreasing testosterone secretion from the testis, by a direct effect on the testis or by inhibiting some mechanism at the site of action in the prostate. Slaunwhite, Sandberg, Jackson & Staubitz (1962) have reported that the activity of the 17β -hydroxy steroid dehydrogenase enzyme system converting androstenedione into testosterone is low in testicular tissue removed from patients with prostatic cancer who had been treated with diethylstilboestrol. This capacity to synthesize testosterone can be restored by administration of human chorionic gonadotrophin concomitantly with diethylstilboestrol, thus being consistent with an effect of oestrogen on testicular function mediated through the pituitary. Further, plasma testosterone and luteinizing hormone concentrations are decreased by diethylstilboestrol therapy (Alder *et al.* 1968). There is also evidence that oestrogens have a direct effect on the testis (Samuels, Short & Huseby, 1964), but the results now reported on DNA polymerase and those of Mangan *et al.* (1967) on RNA polymerase suggest that there may also be a direct biochemical effect of diethylstilboestrol in the prostatic tissue. This is supported by the observation of a selective uptake of radioactivity in the prostate after the intraperitoneal administration of [3 H]diethylstilboestrol (Mangan *et al.* 1967). No identification of the radioactive material in the tissues was attempted, however.

The relationship between the structure of the stilboestrol derivative and the degree of inhibition of DNA polymerase (Table 1) is of interest. The observation that diethylstilboestrol more efficiently inhibits the system than dimethylstilboestrol indicates that the groups attached to the central ethylene linkage are effective in the binding of inhibitor to enzyme. Though the *p*-hydroxyl groups may also aid this binding, it has been shown that stilbene inhibited DNA polymerase nearly as effectively as did dihydroxystilbene. Hexoestrol, the reduced form of diethylstilboestrol, showed the greatest inhibitory action of the compounds studied.

Diethylstilboestrol has therefore been shown to inhibit the activity of the DNA polymerase enzyme system isolated from calf thymus. Though actinomycin also inhibits this system, the effectiveness of diethylstilboestrol as an anti-androgenic compound may be due at least in part to its selective uptake by prostatic tissue. It is noteworthy that 17β -hydroxy- 2α -methyl- 5α -androstan-3-one, which has been used as an anti-oestrogen in breast cancer and prevents the uptake of oestrogen by human breast tissue (Deshpande, Jensen, Bulbrook, Berne & Ellis, 1967), also inhibits the DNA polymerase by 10% (A. R. Fahmy & K. Griffiths, unpublished work). Hexoestrol, the most effective of the inhibitors studied, is also concentrated by hormone-responsive human breast tumours to a greater extent than by the unresponsive type of tumour (Folca, Glascock & Irvine, 1961).

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