Oxidative and Photochemical Linkage of Diethylstilbestrol to DNA in Vitro

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SUMMARY

Diethylstilbestrol (DES) can be bound to DNA in vitro as a result of the action of long-wavelength UV irradiation or of several oxidizing systems. The covalently linked DES cannot be separated from the DNA by organic solvent extraction, gel filtration chromatography, or sucrose gradient ultracentrifugation. A level of binding of 1 molecule of DES per 1000 bases can be achieved by photochemical means and exceeded by iodine-promoted linkage. Analysis of the DES associated with the apurinic acid derived from the covalent DNA complex shows a 2:1 selectivity by DES for binding to purine rather than to pyrimidine bases. The particular effectiveness of iodine as a binding reagent is of special significance in view of the greatly enhanced carcinogenicity of a DES iodination product, diiododiethylstilbestrol, over that of the parent compound.

INTRODUCTION

DES4 is not only an established synthetic estrogenic hormone (8) but it also has an established history as a chemical carcinogen (10–12, 16). Continued interest in this latter activity has recently linked it with cancer of the vagina in adolescent girls (13).

There has been growing attention for some years to the covalent interactions of a wide range of chemical carcinogens with DNA (18), and particular emphasis has been placed on the coupling of polycyclic aromatic hydrocarbons to DNA (12). Correlations have been established for some hydrocarbons between their potency as carcinogens and their capacity to bind to DNA in living systems (4). However, DES has hitherto been reported only to form a physical complex with DNA (24), possibly of similar type to those involving polycyclic aromatic hydrocarbons (7).

It thus appeared opportune to explore the possible formation of a covalent linkage between DES and DNA in vitro such as might be promoted by the range of irradiative (5, 22, 25) and oxidative (14, 17, 19, 20) systems which are known to cause hydrocarbon binding to DNA. The work described here establishes high levels of DES binding to native and to denatured DNA as a result of the treatment of physical complexes either by UV irradiation or by iodine. It gives details of the preparation, purification, and partial characterization of the covalent complexes and provides kinetic and other information that indicates that the mechanism of the binding process is complex.

MATERIALS AND METHODS

Materials. DES-monoethyl-1-14C was purchased from the Radiochemical Centre, Amersham, England. Carrier DES and calf thymus DNA, of average molecular weight (106 daltons), were purchased from the Sigma Chemical London Co. Ltd., Kingston upon Thames, England. Solutions of native DNA were prepared by its slow dissolution in 10 mM phosphate buffer, pH 6.8, containing 1 mM EDTA to a final concentration of 6.7 mM at 4°. Solutions of denatured DNA were prepared by twice-repeated heating of solutions of native DNA for 10 min at 100° followed by rapid cooling in ice water for 30 min. Standard solutions of twice-recrystallized DES in pure benzene were stored at 1° in the dark. AnalaR grade 1,4-dioxan was purified by chromatography on activated alumina before use. Sephadex G-200 and G-10 filtration media were obtained from Pharmacia, AB, Uppsala, Sweden, and Unisolve I liquid scintillator was purchased from Koch-Light Laboratories Ltd., Colnbrook, England. All other materials were of AnalaR grade and glass-distilled water was used throughout.

DES:DNA Solutions. A solution of DES (10 μCi; 16.67 μmoles DES) in 10 ml benzene was evaporated under a slow stream of nitrogen and the solid was dissolved in 2 ml dioxan. Standard DNA solution (200 ml) was added, and the dispersion so formed was equilibrated at 4° in the dark for 3 days before filtration through Whatman No. 1 paper and storage at 1° in the dark prior to use.

General UV Irradiation Procedure. Aliquots (2.5 ml) of DES:DNA solution were irradiated in 1-cm-path length Pyrex cuvets at 25 cm from the point source of a Hanovia HA 100 high-pressure mercury arc fitted with filters to restrict transmission to the 310 to 400 nm band and focused onto an area approximately 8 mm in diameter.

General Oxidative Binding Procedure. Iodine oxidations were performed by the addition of a solution of iodine in 10 μl of 0.3 M dioxan to a 2.5-ml aliquot of the filtered
DES: DNA solution, which was then incubated in the dark at 20° for varying time intervals. A series of oxidations were performed on aliquots of stock solution of DES (1.62 x 10^-3 M) and native DNA (6.7 x 10^-3 M phosphate) using initial iodine concentrations in the range 2 x 10^4 to 5 x 10^-2 M. These aliquots were then incubated in the dark for 72 hr at 20° prior to assay.

Hydrogen peroxide-mediated binding of DES to DNA was investigated by the addition of aqueous hydrogen peroxide to the DES:DNA solution to a final concentration of 15 mM. In certain experiments, the hydrogen peroxide solution was augmented by the addition of ferrous sulfate to a final concentration of 1 mM.

The ascorbic acid-hydroxylating system was similar to that used by Lesko et al. (17) with final concentrations of 30 mM ascorbic acid; 2.8 mM ferrous sulfate, and 14 mM EDTA being incubated with the DES:DNA complex (3.4 mM DNA phosphorus) at 20°. Aliquots were removed at intervals up to 24 hr for assay.

**DNA Precipitation and Washing Procedure.** DNA was precipitated from aliquots of the above reaction mixtures (2.5 ml) by the addition of at least 5 volumes of ethanol saturated with ammonium acetate at room temperature. The fibrous precipitate was collected by centrifugation at 1000 x g for 2 min and washed 3 times with absolute ethanol and twice with ether. The DNA was redissolved in 1 ml of 10 mM phosphate buffer, pH 6.8 for analysis.

**Gel Filtration Chromatography and Radioactive Assay on Sephadex G-200.** The foregoing DNA solution (1 ml) was layered onto a column of Sephadex G-200 (28 x 1.5 cm) and eluted with phosphate buffer (10 mM) at a flow rate of 1 ml/10 min. The eluate was continuously monitored for UV absorbance at 254 nm and fractions (30 drops, 1.90 ml) were collected. A sample (1.0 ml) from each fraction was diluted with Unisolve I (14 ml) to give a clear, homogeneous solution the radioactivity of which was determined with a Packard Tri-Carb Model 3380 scintillation counter using automatic external standardization.

**Sucrose Gradient Ultracentrifugation of the DES:DNA Complex.** A sample of a washed DES:DNA complex (0.15 ml), prepared by centrifugation with 1.2 mM iodine for 20 min before washing, was layered onto a sucrose density gradient (5 ml; 5 to 20%, w/v) in a cellulose nitrate tube and then subjected to ultracentrifugation at 50,000 x g for 20 hr at 5° in a Spinco Model L ultracentrifuge fitted with an SW 50 swinging bucket rotor. Fractions (0.3 ml) were collected from the pierced tube and monitored for UV absorption at 260 nm in 1-cm path-length semimicro quartz cuvets before dilution to 1 ml with standard phosphate buffer and assaying for 14C radioactivity as before.

**Partial Hydrolysis of DES:DNA Complex.** A 1-ml aliquot of the same DES:DNA solution, washed after iodine treatment, was incubated with 2 ml formic acid (98%) at 30° for 17 hr (21). The solution was then cooled to -10° and neutralized by the slow addition of ice-cold ammonia solution (8 M). The resulting solution was layered onto a Sephadex G-10 column (86 x 1.5 cm) at 22° and developed with 500 ml of 10 mM phosphate buffer, pH 6.8, followed by 0.1 N hydrochloric acid (200 ml). The eluate was monitored for UV absorption, collected, and assayed for radioactivity as before.

**Reaction of DES with Iodine.** DES (268 mg) in dioxan solution (200 ml) was added to 10 liters of 10 mM phosphate buffer, pH 6.8, and 300 mg iodine were added. The mixture was stirred for 3 days during which time a like amount of iodine was further added. This solution was extracted with ether (3 x 100 ml) and the combined extracts washed with sodium thiosulfate solution (2 x 100 ml; 5%, w/v) and 100 ml water, dried, and evaporated. The crude product was partly resolved by thin-layer chromatography on silica plates (40 x 20 cm) developed with 80-100 petrol:ether (2:3). Bands were detected by UV absorption, collected, and extracted; and the products were analyzed by UV absorption and mass spectrometry using an AEI MS12 machine with a direct inlet probe at 150°.

**Reaction of DES Promoted by Long Wavelength Irradiation.** A solution of DES (14 mg) in glacial acetic acid (5 ml) was irradiated in a 2-cm-path length cuvet as above for 4 hr. The solution was diluted with water and neutralized, and the products were extracted with ether and purified by thin-layer chromatography on silica plates eluting with ether.

**RESULTS**

**Covalent Binding of DES to DNA.** The amount of DES that could not be removed from native or denatured DNA by precipitation and washing increased with increasing exposure to UV irradiation and approached a level of 120 molecules of DES per 10^6 base pairs for native DNA after some 2 hr irradiation. For denatured DNA there was no evidence of any saturation limit to this binding, which attained a level of 500 molecules of DES per 10^6 base pairs after 4 hr irradiation (Chart 1).

Similar behavior was observed for iodine-mediated binding of DES to native and to denatured DNA which in both cases was approximately 3 times as effective in overall linkage and reached a level of 1600 molecules of DES per 10^6 base pairs in the case of denatured DNA (Chart 2).

By contrast, the levels of binding of DES to denatured DNA as a result of the ascorbic acid-hydroxylating system or of hydrogen peroxide treatment augmented by ferrous iron were much lower, being some 40 and 80 molecules of DES, respectively, per 10^6 base pairs after optimum exposure, while hydrogen peroxide alone gave no detectable binding to denatured DNA (Chart 3). No significant binding of DES to native DNA was measured for any of these 3 systems.

The extent of binding of DES to native DNA showed a variable dependence on the concentration of iodine when studied over a 10^4-fold concentration range of the latter. From 0 to 10^{-3} M iodine, the extent of binding was simply proportional to the iodine concentration, which permitted the calculation of a theoretical relationship by linear regression analysis of the data (Chart 4). However, at the highest iodine concentrations used, the extent of binding of DES fell significantly below that predicted by this curve.
This is illustrated for a washed DES:DNA complex produced by UV irradiation (Chart 5). Although the DNA eluted as a broad band between Fractions 15 and 32, it showed almost complete coincidence with the pattern of elution of radioactivity. In a similar experiment using an unwashed aliquot of the same reaction mixture, the non-covalently linked DES was eluted from the column after the DNA band.

**Sucrose Gradient Ultracentrifugation.** A well-washed DES:DNA complex formed by iodine oxidation sedimented as a broad band on sucrose gradient ultracentrifugation, the DNA peak at Fraction 10 having a sedimentation coefficient \( s_{20,w} \) of 1.95 (Chart 6). Nonetheless, a good correlation is evident between the \(^{14}\text{C}\) radioactivity and the UV absorption at 260 nm for the fractions collected.

**Gel Filtration Analysis of a Depurinated DES:DNA Complex.** Depurination of DES:DNA complexes formed by either UV irradiation or iodine oxidation was effected by standard methods (21). Chromatography of the hydrolysis products on Sephadex G-10 showed that some 34% of the total radioactivity was eluted in coincidence with the apurinic acid (Chart 7), while the purine-containing fractions were devoid of radioactivity. The major part of the remaining activity could not be eluted from the column material simply by raising the ionic strength of the eluate, but it was recovered by extraction with hot ethanol.

**Products of Irradiation of DES with Long-Wavelength Light.**

A washed DES:DNA complex produced by exposure to a large excess of iodine for 72 hr (containing 1.2 moles of DES per \( 10^6 \) phosphate) was thoroughly dried in a vacuum. Semimicroanalysis showed some 4.8% iodine content in the sample while a control on a native DNA sample revealed some 6% iodine incorporation.

**Sephadex Gel Filtration.** Covalent complexes of native DNA and DES produced either by UV irradiation or by iodine oxidation were examined by gel filtration using Sephadex G-200. In no case was any separation of the \(^{14}\text{C}\) activity from the characteristic DNA UV absorption noted.
UV. Irradiation of DES in acetic acid gave a mixture of 2 products in addition to much unchanged starting material (Chart 8). The major one was formed in 30% yield as golden-yellow crystals and characterized by its absorption peaks at 406 and 287 nm as the hexahydro-3,6-dioxo-9,10-diethylphenanthrene (I) (9). The minor one, obtained in 2% yield, was identified as 9,10-diethyl-3,6-dihydroxyphenanthrene (II) on the basis of its spectral properties (15).

**Products of Interaction of Iodine and DES.** The mixture of crude products formed by exposure of DES to iodine in dilute aqueous solution proved to contain over a dozen components on analysis by thin-layer chromatography, and resolved fractions proved to be insufficiently stable for rigorous purification. Mass spectrometric examination of these materials was impeded by the high temperatures required for their volatilization but, nonetheless, showed that the principle components were polyiodinated derivatives of DES which contained up to 6 iodine atoms per molecule.

**DISCUSSION**

While DES has been reported as forming a weak, physical complex with denatured DNA (24) there is no previous evidence of any covalent attachment of this synthetic hormone to any nucleic acid species. The present results show unambiguously that such covalent binding can be formed between DES and DNA in native or, more readily, denatured forms.

The same criteria that have been widely applied to establish the covalent nature of the bonding of polycyclic aromatic hydrocarbons to DNA (1, 19, 20, 25) are satisfied by the properties of the complexes produced on exposure of DES:DNA mixtures to irradiation or to oxidation. Thus, in excess of 99.5% of the DES in such untreated mixtures can be washed away from the precipitated DNA by organic solvents yet a smooth growth in the amount of DES inseparable from the DNA follows upon either increasing dose of UV irradiation (Chart 1) or increasing duration of exposure to iodine solution (Chart 2). Moreover, the DES thus bound to the DNA cannot be removed by the force fields applied in gel filtration chromatography or in sucrose gradient ultracentrifugation. The results of experiments using the former technique (Chart 4) show an excellent correlation between the radioactivity profile associated with the DES and the UV absorption envelope of the DNA. Although the sucrose gradient ultracentrifugation gives a broad pattern of dispersion of the DNA sample, which is most likely a consequence of extensive shearing during the washing routine used, it gives a satisfactory correlation with the distribution of radioactivity in the gradient (Chart 5).

The 2 most effective means of establishing this covalent binding involve UV irradiation in the 310 to 400 nm band and iodine oxidation. These both lead to levels of bonding of DES to native and to denatured DNA which are directly comparable to those established for carcinogenic and non-carcinogenic polycyclic aromatic hydrocarbons (1). In particular, the effectiveness of iodine is most significant in view of the evidence that iodination both accelerates and enhances the carcinogenicity of DES (2, 3).

The bonding to DNA is more selective than that observed for aromatic hydrocarbons in 2 respects. First, the exposure of physical complexes of the hormone and nucleic acid to either the ascorbic acid:ferrous sulfate or hydrogen peroxide:ferrous sulfate systems resulted in only low levels of bonding of DES to DNA although both of these systems have been established as promoting moderately good levels of attachment of aromatic hydrocarbons to DNA (17, 19) (Chart 3). More specifically, hydrogen peroxide alone does not appear to cause any significant binding of DES to DNA although it is of proven effectiveness for several carcinogenic hydrocarbons (17).

Secondly, the covalent bonding of DES to native DNA shows a specificity for attachment to purine rather than to pyrimidine bases as revealed by the controlled hydrolysis of
the covalent DES : DNA complex to apurinic acid. While on present results this selectivity appears to be no more than 2:1 in favor of purines over pyrimidines, we have not observed any significant discrimination in the binding of anthrancene to DNA (23). Attempts to define the mechanism of attachment of DES to DNA bases have met with limited success. The results of the apurinic acid degradation suggest that the site of attachment is preferentially to the purine bases. The dose dependence of iodine-stimulated binding shows that it is for the most part directly proportional to the iodine concentration but also that there is a small, significant amount of covalent binding of DES to DNA which is independent of iodine and is of the order of $10^{-3}$ molecule of DES per DNA base pair (Chart 4). In addition, there appears to be a saturation of binding sites in the DNA which is attained at iodine concentrations around $10^{-2}$ M. However, the deviations from simple behavior at higher iodine concentrations must also be linked to progressive iodination of DNA bases, especially cytosine (6), and also to possible iodination of DES molecules attached to the DNA. Either or both of these phenomena are in accord with the observation of a significant iodine content in the DNA for which incorporation is not directly dependent on the presence of DES in the system. It thus seems reasonable to suggest that binding is a result of a net 2-electron oxidation of DES, rather than of its iodination, and that this leads to its preferential attachment to a purine residue in the DNA.

Although DES is transformed into the phenantrene derivatives I and II to a limited extent under UV irradiation conditions similar to those used for binding DES to DNA, the rate of formation of these materials appears to be insufficient to account for the photochemical attachment of DES to DNA. It thus seems more reasonable to attribute the covalent binding in this case to a photochemical oxidation involving electron expulsion from the stilbestrol, to be followed by covalent processes linking the DES to a purine base. Clearly, further investigations are required to establish further mechanistic details, the nature of the DES : purine complex, and the important question of the possible binding of DES to DNA in vivo.

REFERENCES

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