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PHYSICAL ASSOCIATION OF OESTROGENS AND OTHER STEROIDS WITH DNA

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Abstract—^{14}C-Labelled mestranol, 3-*O*-methyl oestrone and cholesterol-5α,6α-oxide have been prepared. Along with the natural oestrogens, E_1 and E_2, and other steroids, these compounds have been used to determine the extent of their physical association with DNA. Analysis of binding both by equilibrium solubilization and by caesium chloride density gradient centrifugation showed the same relative order of binding: mestranol > 3-*O*-methyl oestrone > oestrone > cholic acid > cholesterol-5α,6α-oxide > progesterone, testosterone > oestradiol. DNA from *Micrococcus lysodeikticus* showed a higher affinity for cholic acid than did calf thymus DNA, while pretreatment of the latter with proteinase K somewhat reduced the level of physical binding of oestrone.

There is a well-established connection between the administration of oestrogens and the subsequent, reproducible development of tumours in many organ sites for several susceptible mammalian species [1]. Causal evidence has now been gathered to the extent that both synthetic oestrogens and the endogenous hormones have been implicated in malignant changes *in homo* both for hormone-sensitive and hormone-insensitive tissue [2].

In the light of growing acceptance that DNA is the cellular target for the majority of chemical carcinogens [3], Ts'o and Lu began physical binding studies, using equilibrium dialysis to determine affinity constants between nucleic acids and various carbocyclic compounds [4]. They observed that the binding of hydrocarbons and steroids decreased in the series phenanthrene > testosterone > oestradiol > diethylstilboestrol > naphthalene. Moreover, binding to RNA was about half that to DNA. Goldberg and Atchley subsequently showed that hormones, including E_1‡ and E_2, bind physically to DNA from human placental nuclei causing a change in its melting profile that is consistent with a weakening of interstrand bonds [5].

Using data from equilibrium dialysis studies on the binding of some steroid hormones to poly G and to DNA, Cohen *et al.* later concluded that steroids with two oxygenated functional groups are necessary for binding and that it involves hydrogen bonding to purines, probably to the 2-amino group of guanine [6]. Hydrogen bonding has also been invoked to describe the interaction of steroidal quaternary ammonium salts to DNA [7].

This present study seeks to explore the relative extent of physical association of DNA with a range of steroids preparatory to an examination of chemically- and enzymatically-mediated, covalent binding of steroids to DNA [8, 9]. The endogenous oestrogens E_1 and E_2 have been examined along with oestrogen ethers 3-*O*-methyl oestrone and mestranol—the latter being a widely used oestrogenic component in contraceptive formulations. The range of steroids was widened to include cholic acid and cholesterol-5α,6α-oxide—both steroids linked to the aetiology of colonic cancer [10].

MATERIALS AND METHODS

DNA was supplied by Sigma (London) Chemical Co. Ltd. (Poole, Dorset, U.K.) (*Micrococcus lysodeikticus* Type XI, average molecular weight $>10^6$) and Koch Light Ltd. (Colnbrook, Bucks., U.K.) (calf thymus, average molecular weight $>10^6$). Proteinase K from *Tritirachium album* was supplied by Sigma substantially free of DNase and RNase. Oestrone, oestradiol, cholesterol, cholic acid, progesterone and testosterone were also supplied by Sigma. [4-^{14}C]Oestrone, [4-^{14}C]oestradiol, [4-^{14}C]cholesterol, [carboxyl-^{14}C]cholic acid, [4-^{14}C]progesterone, [4-^{14}C]testosterone, and [*O*-methyl-^{14}C]methyl methanesulphonate were purchased from the Radiochemical Centre (Amersham, Bucks., U.K.). Mestranol [11], 3-*O*-methyl oestrone [12] and cholesterol-5α,6α-oxide [13] were synthesized according to published procedures.

Synthesis of [4-^{14}C]*cholesterol-5*α,6α-oxide. A solution of [4-^{14}C]cholesterol (40 μCi, specific activity 57 mCi/mmole) and carrier cholesterol (15.4 mg, 40 μmole) in dichloromethane (5 ml) was treated with a solution of bicarbonate-washed *m*-chloroperbenzoic acid (17.2 mg, 100 μmole). [4-^{14}C] Cholesterol-5α,6α-oxide was obtained pure by preparative TLC (Kieselgel 60, ethyl acetate–light petroleum, 1:3 v/v, R_f 0.4) to give the product, 16.5 mg (93%), specific activity 4 mCi/mmole.

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‡ Abbreviations: E_1, oestrone; E_2, oestradiol; poly G, polyriboguanylic acid; mestranol, 3-*O*-methyl 17-α-ethynyloestradiol; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulphate; HMP, hydrogen monophosphate; C.t., calf thymus; *M.l.*, *Micrococcus lysodeikticus*.

Synthesis of 3-O-[^{14}C]*methyl oestrone.* Oestrone (6.8 mg, 25 μmole) was dissolved in absolute ethanol (0.5 ml) containing sodium ethoxide (25 μmole) and stirred under nitrogen gas. [^{14}C]Methyl methanesulphonate (50 μCi, 30 μmole) was added and the mixture stirred 5 hr. The solid formed was collected by filtration, washed with cold ethanol, and crystallized from methanol to give the product, 4.7 mg (70%), specific activity 1.65 mCi/mmole, identical with authentic material by TLC (Kieselgel 60, light petroleum–acetone, 4:1 v/v, R_f 0.45).

Synthesis of [O-*methyl*-^{14}C]*mestranol.* Using the above procedure, 17-α-ethynyloestradiol (6.75 mg, 23 μmole) [14] was methylated to give the product, 4.5 mg (67%), specific activity 0.4 mCi/mmole, identical with authentic mestranol by TLC (Kieselgel 60, light petroleum–acetone, 4:1 v/v, R_f 0.29).

Proteinase K treatment of DNA. Calf thymus DNA (100 mg) was added to Tris–HCl buffer (50 ml, pH 7.0, 10 mM) containing EDTA (5 mM), NaCl (0.4 M) and SDS (0.5% w/v). A small amount of toluene (*ca* 0.05% v/v) was added and the DNA dissolved at 4° in the dark by placing the flask on an inclined, slowly rotating turntable.

Proteinase K (5 mg) was added and the mixture incubated at 37° for 18 hr then treated with phenol reagent (50 ml). DNA was precipitated from the aqueous phase by addition of 1 vol. of saturated ethanolic ammonium chloride. The fibrous precipitate was washed twice with ethanol, twice with ether, and air-dried. The DNA was redissolved in HMP buffer (to a final concentration of 2.5 mg/ml). Folin–Ciocalteu analysis [15] showed this DNA to be protein-free. Untreated calf thymus DNA contained up to 2% protein.

Preparation of DNA–steroid physical complexes. For each of the nine compounds, ^{14}C-labelled material (10 μCi) was cochromatographed with carrier steroid (10 μmole) on TLC. The steroid was eluted with benzene, evaporated in a nitrogen stream, and dissolved in pure dioxan (500 μl). This solution was dispersed into a solution of DNA (50 mg) in HMP buffer (20 ml, 7.5 mM phosphate, 5×10^{-4} M EDTA, pH 6.8) and the solution equilibrated in the dark for 72 hr at 4° in a flask on an inclined, slowly rotating turntable. The DNA solution was filtered slowly through Whatman No. 1 paper under gravity and maintained at 4°. Aliquots were removed before and after filtration for radioactive assay [16].

Caesium chloride gradient centrifugation. An aliquot (200 μl) of the filtered DNA–steroid physical complex was gently dispersed into a CsCl solution (4 ml, 1 g/ml, refractive index 1.392) contained in a 5 ml polypropylene or polyallomer centrifuge tube. The solution was overlayered with paraffin oil and centrifuged at 33,000 rpm for 40 hr at 5° in a Spinco Ultracentrifuge fitted with a SW 50.1 swingout rotor (six tubes, balanced in pairs, run concurrently).

After centrifugation, the tube bottoms were pierced and fractions (20 drops) collected, diluted with water (to 1 ml), and assayed for DNA (E_{260nm}) and for radioactivity (Packard Tricarb 3380).

Washing with organic solvents. An aliquot (100 μl) of the DNA–steroid physical complex was treated with saturated ethanolic ammonium acetate (1 vol.)

Table 1. Physical association of steroids with DNA by equilibrium solubilization

Steroid	DNA	Physical complex, ^{14}C dpm: Before filtration	Physical complex, ^{14}C dpm: After filtration	% ^{14}C remaining after filtration	^{14}C cpm after washing	Mmole steroid/mole base pair in physical complex
Mestranol	C.t.	99,435	93,821	94.3	10	118.5
3-*O*-Methyl oestrone	C.t.	116,206	70,290	60.5	12	88.8
Oestrone	C.t.	98,357	53,983	54.9	12	68.2
Cholic acid	C.t.	94,167	28,065	29.8	9	35.4
Cholesterol oxide	C.t.	95,033	15,797	16.6	15	19.9
Progesterone	C.t.	98,488	8768	9.2	12	12.5
Testosterone	C.t.	98,678	8727	8.8	15	10.8
Oestradiol	C.t.	95,837	4498	4.7	10	5.7
Cholic acid	*M.l.*	94,395	40,732	43.1	8	51.4
Cholesterol oxide	*M.l.*	93,650	18,005	19.2	14	22.7
Oestrone	dp C.t.	99,712	45,999	46.1	15	57.3

Samples were counted for 10 min, efficiencies (50–55%) were determined by automatic external standardisation. C.t., Calf thymus; *M.l.*, *Micrococcus lysodeikticus*; dp C.t., deproteinated calf thymus DNA. Dpm are average values from two to four individual experiments after discarding experiments deviating by more than 10%.

and the precipitated DNA pellet washed three times with absolute ethanol and twice with ether and air-dried. The pellet was dissolved in HMP buffer (100 μl) and assayed for radioactivity.

RESULTS

The relative solubilization of the nine steroids by aqueous DNA solution was determined under saturation conditions by equilibration with excess of each of the steroids and separation of the excess solid. The results provided (Table 1) are typical of two to four independent determinations. The extent of dissolution of a standard amount of steroid (10 μCi, 10 μmole) in the fixed volume of DNA solution (20 ml) varied from 5% for oestradiol to 95% for mestranol. In all cases, precipitation of the DNA followed by systematic washing with ethanol and with ether removed essentially all radioactivity from the DNA sample.

Further characterization of the DNA–steroid complex was carried out by caesium chloride density ultracentrifugation. The profiles of distribution of steroidal radioactivity and of absorbance at 260 nm for DNA are illustrated for mestranol (Fig. 1a) and are representative of the whole series.

The total amount of radioactivity recovered in the DNA band was recorded for each steroid ranging from 27 to 76% (Table 2). The data given relate to experiments using polyallomer or polypropylene tubes. Rather larger losses of steroid were experienced using cellulose nitrate centrifuge tubes.

For cholic acid and cholesterol-5α,6α-oxide, both of the binding experiments were also carried out using *Micrococcus lysodeikticus* DNA. The distribution of radioactivity across the CsCl gradient is illustrated for the epoxide (Fig. 1b); the same pattern was observed for cholic acid. *M.l.* DNA consistently showed a 50% enhancement of binding of cholic acid over C.t. DNA while the corresponding increase for cholesterol-5α,6α-oxide was small and not significant relative to the variation between repeated experiments.

The binding of oestrone to calf thymus DNA which had been deproteinated by proteinase K pretreatment was determined. The data (Tables 1 and 2) show a reduction in binding of some 10–15%.

The extent of physical association of cholesterol-5α,6α-oxide with C.t. DNA was essentially invariant for incubation periods up to 3 days at 2–4°. However, at 37° the extent of association increased with duration of incubation up to 5 days but was largely covalent in nature since the steroidal radioactivity could not be removed from the DNA by precipitation and washing with organic solvents [17].

DISCUSSION

The dispersion of an aromatic hydrocarbon in DNA solution and separation of the excess, solid hydrocarbon is a well-proven method for the formation of physical complexes between DNA and a wide range of polycyclic aromatic hydrocarbons [4, 16] which has also been used for steroids [4, 9]. Its manifest simplicity belies the fact that it gives relatively reproducible results which can be used for

Table 2. Physical association of steroids with DNA by caesium chloride density centrifugation

Steroid	DNA	Activity added to CsCl gradient, ^{14}C dpm	Activity on gradient associated with DNA, ^{14}C dpm	Activity retained by DNA (%)	Mmole steroid/mole base pair in physical complex	Weak binding (eqm.–grad.)*
Mestranol	C.t.	187,642	138,936	74.0	87.6 (74%)	30.9 (26%)
3-*O*-Methyl oestrone	C.t.	140,580	102,096	72.6	65.0 (73.2%)	23.8 (26.8%)
Oestrone	C.t.	107,966	47,827	44.3	30.2 (44.3%)	38.0 (55.7%)
Cholic acid	C.t.	56,130	39,243	69.9	24.7 (69.8%)	10.7 (30.2%)
Cholesterol oxide	C.t.	31,594	10,465	33.1	6.6 (33.2%)	13.3 (66.8%)
Progesterone	C.t.	19,770	14,740	73.1	4.6 (36.8%)	7.9 (63.2%)
Testosterone	C.t.	18,964	15,366	76.0	4.8 (44.4%)	6.0 (55.6%)
Oestradiol	C.t.	8996	2447	27.2	1.5 (26.3%)	4.2 (73.7%)
Cholic acid	*M.l.*	81,464	58,840	72.2	37.1 (72.2%)	14.3 (27.8%)
Cholesterol oxide	*M.l.*	36,010	13,425	37.3	8.5 (37.4%)	14.2 (62.6%)
Oestrone	dp C.t.	114,205	41,879	36.7	25.0 (43.6%)	32.3 (56.4%)

C.t., Calf thymus; *M.l.*, *Micrococcus lysodeikticus*; dp C.t., deproteinated calf thymus DNA. Samples were counted for 10 min, efficiencies (50–55%) were determined by automatic external standardization. Dpm are average values from two to four individual experiments after discarding experiments deviating by more than 10%.

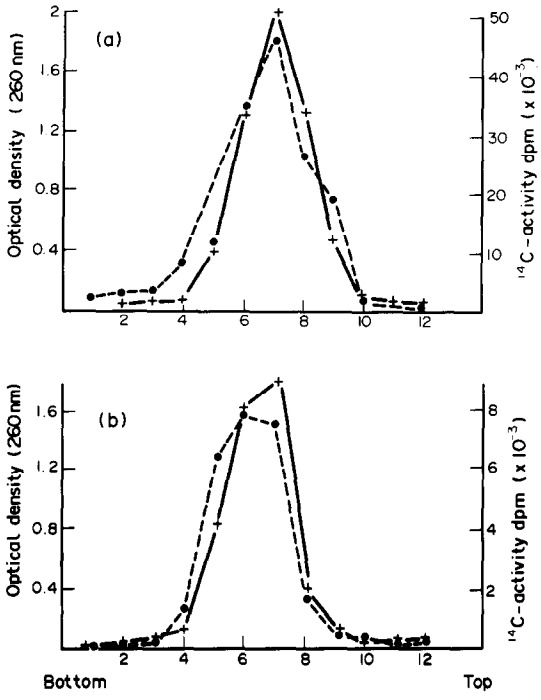

Fig. 1. Distribution of steroidal radioactivity (—+—) and DNA light absorption at 260 nm (---●---) across caesium chloride density gradients as a function of fraction number for mestranol (a) with calf thymus DNA and for cholesterol-5α,6α-oxide with *Micrococcus lysodeikticus* DNA (b).

comparative purposes: though its prime utility is to provide a suitable substrate for chemical or metabolic conversion into covalent complexes.

The present study establishes a relative order of physical association for nine steroids: mestranol > 3-*O*-methyl oestrone > oestrone > cholic acid > cholesterol-5α,6α-oxide > progesterone, testosterone > cholesterol > oestradiol (Table 1). The extent of solubilization for mestranol corresponds to one molecule of steroid for every ten base pairs and falls to one-twentieth of this value for oestradiol. This level of solubility, 5 mM–240 μM, can be compared to the solubility of these steroids in water, typically $<5 \times 10^{-5}$ M and further diminished by salting-out effects of buffer solutions [18].

The nature of the DNA–steroid complexes was characterized in two ways. First, it was shown to be exclusively physical in nature since precipitation of the DNA and washing of the pellet with ethanol and then ether removed essentially all the steroidal radioactivity. Second, the use of caesium chloride gradient ultracentrifugation separated DNA from other substances by virtue of its buoyant density. In every case, the coincidence of the steroidal radioactivity with the ultraviolet absorption of the DNA across the gradients established the intimacy of the DNA–steroid physical complex (Figs. 1a and 1b).

Some dissociation of steroid from DNA was invariably observed as a result of ultracentrifugation. While some profiles (e.g. Fig. 1b) show a small accretion of radioactivity towards the top of the density gradient, it appeared that most of the loss is attributable in part to absorption of steroid by the plastic centrifuge tubes—most marked for cellulose nitrate—and in part to its extraction into the paraffin oil overlayer. This behaviour could be simulated by removal of a part of the radioactivity from the physical complexes by extraction into ligroin or benzene.

The extent of physical association was calculated from the radioactivity of steroid in the DNA-containing fractions (Table 2). It is significant that precisely the same relative binding order results as for the equilibrium solubilization data, though there is now a sixty-fold range of binding from mestranol to oestradiol. However, the proportion of solubilized steroid which does not band with DNA on the gradient varied from 26% for mestranol to 74% for oestradiol (Table 2). These data might suggest that weaker physical binding of steroids to DNA, which is saturated by the equilibrium solubilization process, can be differentiated from stronger physical binding by the gradient ultracentrifugation analysis. It is to be noted that this weaker binding is the minor component of the physical association of DNA with mestranol, 3-*O*-methyl oestrone and cholic acid which, along with oestrone, clearly constitute the group of steroids which show a pattern of tighter binding to DNA than the remainder.

The effect of using a high G:C DNA from *Micrococcus lysodeikticus* was examined for cholic acid and for cholesterol-5α,6α-oxide. The data for the former steroid indicate that the physical binding of cholic acid responds favourably to G:C-rich DNA and the enhancement in binding of 50% corresponds quite well to the relative G:C content of *M.l.* and C.t. DNA (72 and 40%, respectively). On the other hand, the much smaller increase for cholesterol-5α,6α-oxide binding was not significantly different from the variation between repeated experiments.

Since DNA is often contaminated by tightly-bound protein, and as it has been suggested [19] that such protein may play a major role in binding endogenous chemicals to DNA, we compared the association of oestrone to C.t. DNA before and after its treatment with proteinase K. We consistently observed a fall in the association of oestrone with protein-free DNA but, as this never exceeded 20%, we deemed it inappropriate to employ this laborious technique more generally.

The variety of structural features present in the nine steroids used in this work makes rationalization of the physical binding data exceedingly difficult. The clearest deduction is that methylation of the 3-hydroxyl group in the oestrogens has a beneficial influence on physical binding. Since this group must be un-ionized at pH 6.8, its influence in lowering the physical binding to DNA must be attributed to hydrogen bond donation, most likely to solvent, and is most marked for oestradiol. It would appear that any hydrogen bonding* involving the steroidal C-3 oxygen must involve DNA as the H-bond donor.

* Hydrogen bonding has also been discussed by Cohen *et al.* [6] using data from equilibrium dialysis studies. This technique identified less than one binding site per 10^4 nucleotide base pairs and gave a binding order testosterone > oestrone, oestradiol > 3-*O*-methyl oestradiol > 3-*O*-methyl oestrone.

Intercalation of molecules into DNA has usually been identified with planar, aromatic species [20] which can be accommodated by expanding the 'vertical' interbase separation from the 3.4 Å in B-DNA to about 6.8 Å [21]. It is conceivable that rings A and B of the oestrogens might be intercalated into DNA and thereby account for part of the interaction of these most strongly associated steroids. However, it is clear that this mode of association must be inappropriate for the four steroids having a 10-β-methyl group, making them some 6 Å thick, and even more so for the *cis*-fused A:B ring junction of cholic acid.

Patel has recently proposed three separate modes of physical binding between antibiotics and DNA, namely intercalation, wedge binding and groove binding [22]. It is possible that one of the two latter modes might explain the binding to DNA of irehdiamine, a diaminosteroid which causes kinks to form in the DNA [23, 24].

The extent of binding for the more strongly associated steroids approaches one molecule per ten base pairs, which clearly exceeds the availability of wedge-binding sites for native DNA. It must be concluded that for these species, and especially for cholic acid, some form of groove binding is responsible for their physical association with DNA. The relative contributions of hydrogen bonding and hydrophobic interactions to this association are beyond the scope of this work but might be explored by other physical techniques.

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