Arch Toxicol (1984) 55: 97–103

Interaction of estrone and estradiol with DNA and protein of liver and kidney in rat and hamster in vivo and in vitro*

Max Caviezel, Werner K. Lutz, Urs Minini, and Christian Schlatter

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, CH-8603 Schwerzenbach, Switzerland

Abstract. [6,7-^{3}H] Estrone (E) and [6,7-^{3}H]estradiol-17β (E_2) have been synthesized by reduction of 6-dehydroestrone and 6-dehydroestradiol with tritium gas. Tritiated E and E_2 were administered by oral gavage to female rats and to male and female hamsters on a dose level of about 300 µg/kg (54 mCi/kg). After 8 h, the liver was excised from the rats; liver and kidneys were taken from the hamsters. DNA was purified either directly from an organ homogenate or via chromatin. The radioactivity in the DNA was expressed in the units of the Covalent Binding Index, CBI = (µmol chemical bound per mol DNA-P)/(mmol chemical administered per kg b.w.). Rat liver DNA isolated via chromatin exhibited the very low values of 0.08 and 0.09 for E and E_2, respectively. The respective figures in hamster liver were 0.08 and 0.11 in females and 0.21 and 0.18 in the males. DNA isolated from the kidney revealed a detectable radioactivity only in the female, with values of 0.03 and 0.05 for E and E_2, respectively. The values for male hamster kidney were < 0.01 for both hormones. The minute radioactivity detectable in the DNA samples does not represent covalent binding to DNA, however, as indicated by two sets of control experiments. (A) Analysis by HPLC of the nucleosides prepared by enzyme digest of liver DNA isolated directly or via chromatin did not reveal any consistent peak which could have been attributed to a nucleoside-steroid adduct. (B) All DNA radioactivity could be due to protein contaminations, because the specific activity of chromatin protein was determined to be more than 3,000 times higher than of DNA. The high affinity of the hormone to protein was also demonstrated by in vitro incubations, where it could be shown that the specific activity of DNA and protein was essentially proportional to the concentration of radiolabelled hormone in the organ homogenate, regardless of whether the animal was treated or whether the hormone was added in vitro to the homogenate.

Carcinogens acting by covalent DNA binding can be classified according to potency on the basis of the Covalent Binding Index. Values of $10^3 - 10^4$ have been found for potent, 10^2 for moderate, and $1-10$ for weak carcinogens. Since estrone is moderately carcinogenic for the kidney of the male hamster, a CBI of about 100 would be expected. The actually measured limit of detection of 0.01 places covalent DNA binding among the highly unlikely mechanisms of action. Similar considerations can be made for the liver where any true covalent DNA binding must be below a level of 0.01. It is concluded that an observable tumor induction by estrone or estradiol is unlikely to be due to DNA binding.

Key words: Estrogen – Hormone – Carcinogenesis – DNA binding – Protein binding – Estrone – Estradiol

Introduction

Although there is no doubt about the importance of steroid estrogen hormones in carcinogenesis (IARC 1974, 1979), the mechanism of action is under debate. Three possibilities are being discussed, (i) a covalent binding of chemically reactive metabolites to DNA (Blackburn et al. 1977; Bolt and Kappus 1974; Jaggi et al. 1978; Pelkonen et al. 1978; Tsibris and McGuire 1977), (ii) the generation of DNA-damaging oxygen species generated during catechol-quinone oxidation processes (Cerutti 1978; Mitchell et al. 1982), and (iii) non-genotoxic mechanisms involving some aspect of the hormonal activity (Cameron et al. 1982; Henderson et al. 1982; Weisburger and Williams 1981; Yager and Yager 1980). It is also possible that the effect observed is a result of a combination of the various single activities, each of which having a different relative weight in different target cell types.

This laboratory has been involved in attempts to quantify the covalent DNA binding of various chemical carcinogens (Lutz 1979), and a correlation of tumorigenic vs. DNA-binding potencies in vivo (Lutz 1982) seems to allow to assess whether a given hepato-carcinogenicity in the rat can be based upon DNA binding or whether other mechanism are more likely to be responsible (Lutz et al. 1982; Sagelsdorff et al. 1983).

In 1978, we reported on non-extractable radioactivity on liver DNA of rats treated with tritiated ethinylestradiol (EE) and estrone (E). Although the apparent binding potency was as much as 10^4 times lower than that of aflatoxin B_1 the data indicated that a minute genotoxicity of these estrogens mediated by DNA binding in vivo could not be excluded (Jaggi et al. 1978). The experimental techniques available at that time did not allow, however, the definite proof of a DNA-hormone adduct on such a low level of binding. With semi-preparative HPLC methods becoming available, we have taken up this line again, essentially in the search for endogenous DNA damage. We therefore radiosynthesized [^{3}H]estrone (E) and [^{3}H]estradiol (E_2), administered up to 10 mCi [^{3}H] to rats and hamsters and purified DNA from livers and kidneys by different

* Paper presented at the Satellite Symposium of the European Society of Toxicology, Rome, March 29, 1983

Offprint requests to: C. Schlatter

procedures known to produce a DNA with varying degrees of protein contamination. Enzymatic hydrolysis of the samples to deoxyribonucleosides was performed to investigate whether the radioactivity which was measurable in all samples could be due to a nucleoside-estrogen adduct.

Materials and methods

Chemicals and materials. 6-Dehydroestradiol (1,3,5,(10),6-estratetraen-3,17β-diol) and 6-dehydroestrone (1,3,5,(10),6-estratetraen-3-ol,17-one) were purchased from Research Plus Steroid, Denville, NJ. Carrier-free tritium gas was from The Radiochemical Centre, Amersham, England. L-[4,5-^{3}H(N)]Lysine with a specific activity of 74.6 Ci/mmol was from New England Nuclear, Boston, MA. Copper oxide (wire form) and palladium/coal (10%) catalyst were from Fluka, Buchs, Switzerland, and tlc-alu-silicagel-60 plates with concentrating area, were from Merck, Darmstadt, FRG. Nonidet P 40 (NP 40) was from BDH Chemicals, Poole, England; Hydroxylapatite (HA) DNA-Grade Bio-Gel HTP was from Bio Rad, Richmond CA; Dialysis tubing (Viking type 20/32 molecular weight exclusion 12,000–14,000 Daltons) was from Union Carbide, Chicago, IL. Deoxyribonuclease I (E.C. 3.1.4.5) from bovine pancreas, phosphodiesterase I (E.C. 3.1.4.1) from crotalus atrox venom, alkaline phosphatase III (E.C. 3.1.3.1) from *Escherichia coli,* reference nucleosides and reference hormones were from Sigma, St. Louis, MO. All other chemicals were from Merck, Darmstadt, FRG, or from Fluka, Buchs, Switzerland, and were of analytical grade.

Synthesis of [6,7-^{3}H]estrone (E) and [6,7-^{3}H]estradiol (E_2). E and E_2 were radiosynthesized by reducing a mixture of 6-dehydroestrone (0.093 mmol) and 6-dehydroestradiol (0.070 mmol) according to Merrill and Vernice (1970) with 0.149 mmol carrier-free tritium gas in 2 ml ethyl acetate containing 31.5 mg 10% Pd/C catalyst in a 10 ml round-bottom flask for 2 h at 20° C on a high vacuum line. The remaining educt was reduced with hydrogen gas, the unreacted tritium/hydrogen mixture was combusted over copper oxide at about 500° C and lyophilized into a break-seal ampoule. The reaction yielded 25.2 mg (0.093 mmol) E and 19.0 mg (0.070 mmol) E_2, with a specific activity of 48 Ci/mmol. Identity was checked by thin-layer-chromatography and by cocristallization with unlabelled hormones. Immediately before use, the radiolabelled hormones were purified by 2-dimensional thin-layer-chromatography on silica gel with dichloromethane : ethanol (100 : 7.5) used for the first dimension. The R_F values were 0.57 and 0.43 for E and E_2, respectively. The appropiate strips were cut out and developed with ethanol ($R_F = 1.0$ for both hormones). The hormones were eluted from the spots with ethanol and stored overnight at 4° C. The radiochemical purity was determined 1 h before administration to the animals and was always better than 98%.

Animals and treatments. Male and female golden Syrian hamsters [Kfm-Ham/s] (90–120 g) were from Madörin Kleintierfarm, Füllinsdorf, Switzerland; Sprague Dawley rats [Iva: SIV 50.SD] (200–250 g) from Ivanovas, Kisslegg im Allgäu, FRG. The animals had free access to tap water and laboratory chow (No. 21-343-7 Klingental Mühle AG, Basel, Switzerland). E and E_2 was administered by oral gavage in about 200 µl ethanol on dose levels given in the Tables. Urine was collected and frozen at −20° C. Eight hours later, the animals were killed by open heart puncture under ether anaesthesia, and liver and kidneys were excised.

Isolation of DNA. DNA was isolated in two ways: *(a) directly* from a liver homogenate blended under denaturing conditions in lysing medium (0.24 M sodium phosphate, 8 M urea, 0.01 M EDTA, 1% sodium dodecylsulfate (SDS), pH 6.8, (Viviani and Lutz 1978), *(b) via chromatin* which was prepared by homogenizing the liver in three volumes 75 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl pH 7.8 in a Potter-Elvehjem-type teflon homogenizer and precipitation with the non-ionic detergent NP 40 according to Yaneva and Dessev (1976). This pellet contained about 2–3 mg DNA and 20–30 mg protein per gram liver and was blended in lysing medium. The further purification of the DNA from a) and b) followed the standard method used in our laboratory (Sagelsdorff et al. 1983) which is based on a deproteination with phenol : chloroform : isoamyl alcohol (PCI) and extensive washing after adsorption to a hydroxylapatite column. After desorption with high ionic strength (0.48 M in phosphate) and dialysis against 0.2 M sodium chloride, DNA was precipitated with two volumes of ethanol, stored overnight at −20° C, dried on aspirator vacuum and dissolved in 10 mM $MgCl_2$, 10 mM Tris buffer pH 7.0. The amount of DNA was determined on the basis of an absorbance of 20 for a solution of 1 mg/ml (Viviani and Lutz 1978). An aliquot of the DNA (0.4–1 mg) was used for liquid scintillation counting.

Isolation of chromatin protein. One milliliter of the first PCI extract was shaken with 2 ml 1% SDS in 14 mM sodium phosphate pH 6.8. Protein was precipitated with 25 ml acetone, redissolved and reprecipitated five times. Finally, the protein sample was diluted 1 : 10 with destilled water, precipitated with about two volumes of acetone at −20° C, redissolved in 2 ml 1% SDS, and diluted with water 1 : 10. The amount of protein was determined with the Folin reagent. One milliliter (about 0.5 mg protein) was used for liquid scintillation counting.

Contamination of purified DNA with protein. Two male rats (250 g) were given by oral gavage 7 mCi/kg L-[^{3}H]lysine. After 4 h, DNA and chromatin protein was isolated via chromatin from the livers. The specific protein activity was 44,100 and 45,200 dpm/mg and the DNA was radiolabelled to give 95.4 and 108 dpm/mg. Nucleoside analysis showed that the natural nucleosides were not radiolabelled.

HPLC analysis of the nucleosides. DNA (1–2 mg/ml) was digested enzymatically by the method described (Sagelsdorff et al. 1983). The deoxynucleosides were separated by HPLC with a water to methanol gradient (0–10% in 5 min, 10% for 5 min, 10–100% in 40 min) using a HPLC system consisting of a semipreparative column (250 mm · 8 mm ID) Lichrosorb RP 18 equipped with two HPLC pumps (Kontron LC pump 410) controlled by a Kontron programmer 200, for generating a linear gradient of two eluants (Kontron, Zürich, Switzerland). Fractions of 2 min (7 ml) were collected and counted for radioactivity after addition of about 1 ml MeOH to the fractions no. 12–15 to avoid phase separation after addition of Insta-Gel.

HPLC of estrogen metabolites. The supernatant of the first acetone precipitation of chromatin protein from the PCI phase was dried under a stream of nitrogen and dissolved in the same

buffer used for DNA. Urine samples were diluted with DNA-buffer to a specific activity of about 1,000 dpm/ml. Reference hormone samples estrone, estradiol, estriol were dissolved in the same buffer. All samples were analyzed by the HPLC system used for the separation of nucleosides.

Control experiments. No. 1. Binding of radiolabelled metabolites to DNA occuring only during the isolation procedure was checked by incubating the supernatant of the first chromatin precipitation (derived from rat No. 3 and 6) with an unlabelled chromatin pellet from a control rat. DNA was then isolated according to method b).

No. 2. Non-enzymatic binding of [^{3}H]estrone to DNA was checked by incubating for 2 h at room temperature 1 mCi hormone with a homogenate of 4 g liver prepared (i) by blending in 25 ml lysing medium and (ii) by the homogenization in 20 ml as used for chromatin precipitation. DNA was isolated (i) directly and (ii) via chromatin, and exhibited a specific activity of 900 and 40 dpm/mg, respectively. The former value was about one hundred times higher than the value reported by Jaggi et al. (1978) from a similar control experiment.

Background radioactivity of DNA and of nucleoside fractions was determined from samples obtained from animals which were held in parallel to the treated ones and which did not receive radiolabelled compounds.

Liquid scintillation counting, calculations and statistics. Samples were counted in Packard high performance glass vials in a Packard Tri Carb 460 CD liquid scintillation counter in 10 ml Insta-Gel (Packard Instruments, Downers Grove, IL).

The specific radioactivity of DNA was normalized by the radioactivity dose administered:

$$CBI' = (dpm/mg\ DNA)/(dpm/kg\ body\ weight)\,.$$

This value was converted to molar units of the Covalent Binding Index CBI = (μmol chemical bound/mol DNA-P)/(mmol chemical applied/kg b.w.) (Lutz 1979), by multiplication of the CBI' with $309 \cdot 10^6$ on the basis of an average molecular weight of 309 for a deoxyribonucleotide in DNA.

The limit of detection for radioactivity in a DNA sample was calculated on the level of two standard deviations (SD). One SD was determined to be 0.5 cpm as derived from the standard deviation determined within each of 16 pairs of background counts of DNA isolated from untreated animals (Kaiser 1965).

The limit of detection for radioactivity in a nucleoside analysis was calculated on the basis of the total variability due to statistical counting error and fluctuations due to vial, scintillation cocktail, counter, external radiation and composition of the sample of each fraction as compiled from five nucleoside analyses of control DNA digests and is shown in the Figures on a level of two SD as a shaded area.

Results

Experiments with female rats

Table 1 summarizes the results obtained for the radioactivity in liver DNA and chromatin protein, 8 h after oral administration of tritiated estrogen hormones to female rats. All liver DNA samples were radiolabelled to an extent which can be called minimal if it is considered that each animal received of the order of 10 mCi of [^{3}H]radiolabel. No difference between E- and E_2-treatment was observable, the mean values for the activity of DNA isolated via chromatin, and expressed after normalization by the dose administered in CBI units, being 0.082 ± 0.033 and 0.086 ± 0.014 for E and E_2, respectively. These values were by a factor of 13 lower than the values published earlier (Jaggi et al. 1978) obtained after direct isolation of DNA from liver homogenate. One main difference between the two isolation procedures is the level of protein contamination. The direct DNA isolation as used in the early study yielded a protein contamination of 0.5% as derived from experiments with [^{35}S]methionine incorporated into protein (Lutz 1979). DNA isolated via chromatin and use of [^{3}H]lysine as a protein precursor resulted in a value of about 0.2%.

Table 1. Binding of tritium radioactivity to liver DNA of female rats, 8 h after oral administration of [^{3}H]estrone and [^{3}H]estradiol

Hormone	Estrone		Estrone		Estrone	Estrone	Estradiol	Estradiol	Estradiol	Estradiol
Animal no./body weight (g)	1/204		2/211		3/205	Control exp. no. 1	4/203	5/204	6/223	Control exp. no. 1
Dose										
μg/kg	303		324		475		277	270	492	
mCi/kg	54		58		84		49	48	87	
Organ	Liver		Liver		Liver	Chromatin	Liver	Liver	Liver	Chromatin
[^{3}H] Activity (% of dose)	3.7		3.0		3.0	Supernat. of rat no. 3	2.3	2.6	2.6	Supernat. of rat no. 6
DNA										
Method of isolation	Chrom.	Direct	Chrom.	Direct	Chrom.	Chrom.	Chrom.	Chrom.	Chrom.	Chrom.
Total act. (cpm)	19.2	25.3	19.7	35.9	44.5	27.5	19.8	22.3	40.3	34.1
Background (cpm)	14.7	14.7	14.7	14.7	20.9	20.3	14.7	14.7	20.9	20.3
Spec. act. (dpm/mg)	23.0	59.3	27.3	109.0[a]	72.4[a]	39.2	24.5	31.5	59.0	52.5
CBI units	0.06	0.15	0.07	0.26	0.12	–	0.07	0.09	0.10	–
Chromatin protein Specific activity (dpm/mg)	$6.5 \cdot 10^5$		$5.4 \cdot 10^5$		$2.5 \cdot 10^5$	$1.9 \cdot 10^5$	$2.7 \cdot 10^5$	$3.5 \cdot 10^5$	$1.65 \cdot 10^5$	$1.5 \cdot 10^5$

[a] An aliquot of this DNA sample was used for an HPLC analysis of deoxynucleosides (see Fig. 1)

Taking into account the relative abundance of methionine or lysine in a protein fraction likely to become a contaminant of DNA, a difference by factors of 2–3 (Table 1) and 13 (early vs. present study) are indeed of the same order.

Because it is assumed that steroid estrogen hormones exert their hormonal activity on the DNA level via protein receptor binding (Brotherton 1976), the protein activity could be very high and the protein contamination of DNA could become crucial. The determination of the specific activity of chromatin protein (Table 1, bottom line) shows that this is the case. Specific activities were $3 \cdot 10^3$ to $3 \cdot 10^4$ times higher in protein than in DNA. DNA radioactivities are therefore most probably due to traces of contaminating protein, and the higher apparent CBI values obtained via direct DNA isolation (Jaggi et al. 1978; and data given in Table 1) are easily unterstood. Although the protein contamination is reason enough to explain the DNA radioactivity, DNA aliquots were digested enzymatically to nucleosides, and the nucleosides were analyzed by HPLC in a search for potential nucleoside-estrone metabolite adducts (Fig. 1). Both DNA isolated directly and via chromatin were analysed. The distribution of radioactivity over the elution profile was completely different except for fractions 2 and 5 + 6 which were radiolabelled in both samples. A nucleoside-steroid adduct expected to be more lipophilic than the natural nucleosides and to elute in later fractions could not therefore be located. The radioactivity did not either coelute with the natural nucleosides. This is an indication that no incorporation of tritium from tritiated water or other small radiolabelled breakdown products into DNA had occurred during biosynthesis. The DNA radioactivity must therefore rather be due to contaminations by metabolites and/or contaminating protein present in a qualitatively different way dependent on the method of DNA isolation.

In order to verify that radioactivity eluting in fractions 2 and 5 + 6 could be due to metabolites, the organic extract (PCI phase) of chromatin and a diluted urine sample were loaded on the HPLC system. Figure 2 with the respective elution profiles shows that both samples contained radiolabelled material eluting in these early fractions.

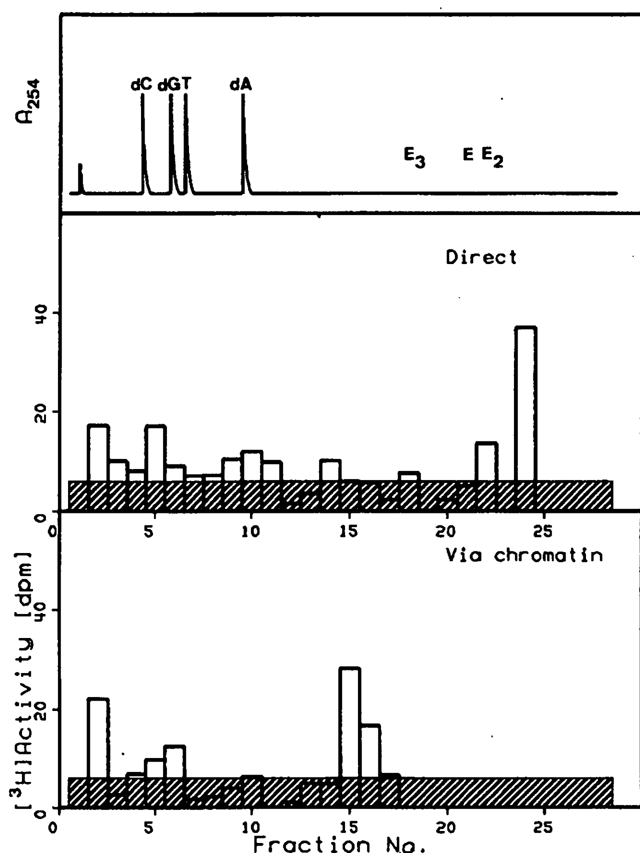

Fig. 1. Optical density *(top)* and radioactivity profile *(center and bottom)* of HPLC analyses of DNA samples isolated directly *(center)* or via chromatin *(bottom)* from the liver of female rats, 8 h after oral administration of [6,7-^{3}H]estrone. dC, dG, T, dA = natural deoxyribonucleosides. E, E_2, E_3 = elution area for estrone, estradiol and estriol. *Center:* Enzyme digest of 2.1 mg DNA loaded, containing 229 dpm; [^{3}H]recovery 88%. *Bottom:* 2.5 mg DNA containing 180 dpm; [^{3}H]recovery 109%

Control incubations. If the DNA radioactivity is primarily a function of protein not completely removed from the DNA, such a non-covalent interaction should also be formed if an unlabelled control chromatin pellet is resuspended in the radioactive supernatant of a chromatin precipitation obtained in a main experiment. The results of this control experiment no. 1 given in Table 1 show that the specific activity of DNA and chromatin protein isolated from the unlabelled control pellet was almost as high as the macromolecules derived from the animals which had been administered E or E_2. The specific activity of the control *protein* was so high after this in vitro incubation that as little as 0.03% contamination on the DNA could fully account for the radioactivity measured in the DNA.

Control experiment no. 2 substantiated the hypothesis that the difference between the specific activity of DNA samples obtained by different isolation procedures is due to different

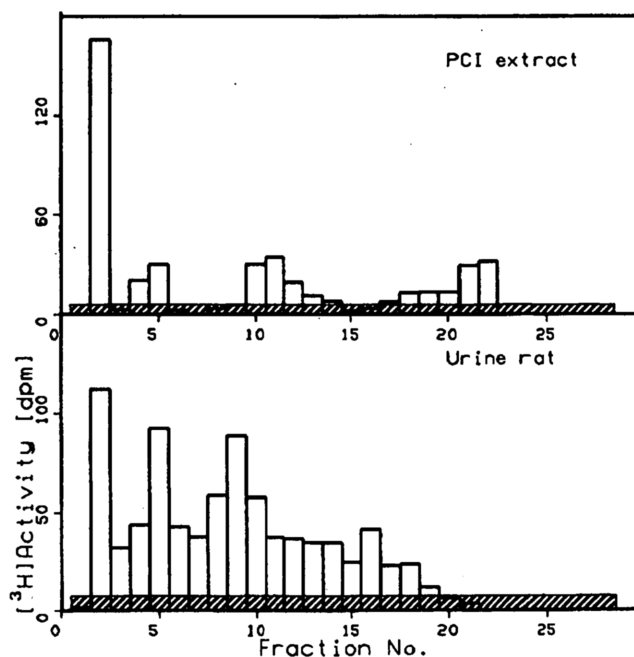

Fig. 2. Radioactivity profile *(center and bottom)* of HPLC analyses of the organic extract (with PCI) obtained during the isolation of DNA from chromatin *(center)* and of urine collected from an E-treated female rat. The HPLC system was the same as used for the analysis of enzyme-digested DNA

contaminations by non-covalently bound estrogen. There was a 20-fold difference in the specific activity of the DNA purified directly or via chromatin after incubation of liver homogenate with [^{3}H]E, the absolute values being equal to or even higher than those found after treatment of animals if the specific activities are adjusted to the same radioactivity levels in the homogenate.

Additional control experiments not to be reported in detail here have shown that each DNA purification step reduced the specific activity only by a certain factor from non-covalently bound radioactivity so that, for a given procedure and a given compound, the amount of radioactivity present in the homogenate ultimately determines the residual DNA radioactivity.

Experiments with male and female hamsters. The kidney of the male (but not the female) hamster is known to be a target organ for estrogen carcinogenicity (IARC 1979). If a reactive metabolite is formed in the male hamster kidney able to bind to DNA in substantial amounts, a difference between males and females should be observable. The data obtained after oral administration of E and E_2 are compiled in Tables 2 and 3, respectively. Similarly to the rat, all *liver* DNA samples were slightly radiolabelled, and there was, again, no difference observable between E and E_2.

Among the DNA samples isolated from the *kidneys*, a minute radioactivity was detectable only in the females. Kidney DNA isolated from male hamsters did not contain any measurable radioactivity on a limit of detection of 3.2 dpm per scintillation vial. This finding is in contrast to what could have happened if the susceptibility of the male to tumor induction was reflected by higher DNA binding. The difference between males and females does not go in parallel with the radioactivity present in the whole kidney homogenate. There, the value for the male was two to three times higher than for the female. A working hypothesis to explain this finding will be given in the Discussion section.

Discussion

Our findings make it highly unlikely that a genotoxic activity of estrone and estradiol mediated by DNA binding is a mechanism of tumorigenic activity. The high level of protein-bound radioactivity can easily explain all radioactivity measured on a DNA sample.

The high level of protein binding could be achieved by non-covalent binding to a specific hormone receptor. This protein could in turn bind to specific regions in the DNA (Baskevitch et al. 1983; Brotherton 1976) and be the reason for the apparent DNA binding of hormones. In addition, *covalent*

Table 2. Binding of tritium radioactivity to liver and kidney DNA of male and female hamsters, 8 h after oral administration of [^{3}H]estrone

Sex; no.	Male 1		Male 2		Female 3		Female 4	
Body weight (g)	120		120		136		128	
Dose								
µg/kg	261		269		347		354	
mCi/kg	46.5		47.9		61.9		63.0	
[^{3}H] Activity in total whole blood (% of dose)	1.01		1.05		0.50		0.56	
Organ	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
[^{3}H] Activity (% of dose)	5.1	0.22	5.6	0.21	1.5	0.08	1.6	0.09
DNA isolated via chromatin								
Total act. (cpm)	29.5	16.8	29.7	17.2	20.9	21.6	25.6	19.8
Background (cpm)	15.9	16.5	15.4	16.5	15.9	16.5	15.4	16.5
Spec. act. (dpm/mg)	69.6	< 3.2	76.5	< 3.2	29.1	13.5	39.5	11.2
CBI units	0.21	< 0.01	0.22	< 0.009	0.07	0.03	0.09	0.025

Table 3. Binding of tritium radioactivity to liver and kidney DNA of male and female hamsters, 8 h after oral administration of [^{3}H]estradiol

Sex; no.	Male 1		Male 2		Female 3		Female 4	
Body weight (g)	119		111		143		148	
Dose								
µg/kg	289		314		222		213	
mCi/kg	51.2		55.5		39.2		37.6	
Organ	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
[^{3}H] Activity (% of dose)	6.3	0.26	7.0	0.32	1.8	0.11	2.4	0.13
DNA isolated via chromatin								
Total act. (cpm)	26.7	16.5	28.8	15.8	20.1	19.4	22.6	20.1
Background (cpm)	15.9	16.5	15.4	16.5	15.9	16.5	15.4	16.5
Spec. act. (dpm/mg)	71.5	< 4.5	69.3	< 3.2	22.7	11.3	38.1	12.4
CBI units	0.19	< 0.009	0.17	< 0.008	0.08	0.04	0.14	0.05

interactions with protein cannot be excluded. Estrogen hormones are to a certain extent oxidized to catechols and quinones, compounds which are slightly electrophilic and can react with thiol (-SH) groups of cysteine and its derivates. For a reaction of quinones with DNA, i.e., with centers of much lower nucleophilicity, very specific substituents and configurations seem to be required such as those found in certain antineoplastic antibiotics of natural origin (Moore 1977). No chemical data are, however, available to show a covalent interaction of simple benzoquinones with DNA.

A number of reports deal with the binding of estrogens to protein and DNA in vitro (Bucala et al. 1982; Kappus et al. 1973; Kappus and Bolt 1976; Maggs et al. 1982; Marks and Hecker 1969; Refs. in the Introduction) in the presence of some drug-metabolizing system. The radioactivity isolated with these DNA samples can always be attributed to protein contaminations because the data are comparable to our in vitro control incubations if the specific activity of the macromolecules is put into relation to the radioactivity incubated. A more extensive purification of the DNA by CsCl gradient for instance revealed that the radioactivity did not coelute with the optical density of the DNA band (Duncan and Brookes, 1979).

Biological test systems for genotoxicity in general produced negative results, for instance in the Ames test (Lang and Redmann 1979), in mutagenicity tests in mammalian cells (Drevon et al. 1981), in a DNA repair assay in vitro or alkaline elution test in vivo (Yager and Fifield 1982) or in a test on initiating capabilities in the Solt-Farber system (Schuppler et al. 1983).

Our data do not exclude a genotoxic mechanism of action in general. Evidence is increasing on the importance of oxygen radicals in tumor initiation and first stage promotion and it is postulated that quinone-hydroquinone oxidation-reduction processes can generate reactive oxygen species able to hydroxylate DNA. This type of indirect genotoxicity goes unobserved if DNA is investigated for bound radiolabelled test compound and it is conceivable that the oxidation of estrone and estradiol to catechol and the corresponding quinone could result in indirect DNA damage. So far, there is no indication that this mechanism of action is operative in the liver. Yager and Fifield (1982) did not detect any DNA damage induced by estrogens either in vivo as assessed by alkaline elution or in primary hepatocyte culture as assessed by DNA repair synthesis. Nevertheless, this type of indirect genotoxicity could become particularly important in cells which do not have much estrogen receptor protein but some drug-metabolizing capacity. This situation could be encountered in the male hamster kidney, a target for estrogen carcinogenicity. Possibly because estrogens are bound only weakly to protein in this organ in the male, we were able to isolate a DNA free of radioactivity although the whole-organ radioactivity was even higher than in the females where DNA radioactivity was detectable.

Malignant renal tumors were found in 15 out of 15 male hamsters treated with a 20 mg pellet of E_2 subcutaneously every 21 weeks (IARC 1979). An average estimated dose of as little as 5 μmol/kg/day as used in this treatment therefore led to a 100% tumor incidence. A correlation of tumorigenic potencies and CBI for DNA of the target organ (Lutz 1982) shows that CBI of $> 10^2$ would be required, if covalent binding to DNA was the mechanism of carcinogenic action. The actual CBI for DNA of the male hamster kidney was $< 10^{-2}$. The discrepancy by a factor of $> 10^4$ is an additional indication that mechanisms other than DNA binding must be responsible for the respective tumor formation. It must therefore be concluded that DNA binding is an unlikely mechanism of tumorigenic action of estrone or estradiol.

Acknowledgement. Supported in part by grant no. 3.842.0.81 of the Swiss National Science Foundation.

References

Baskevitch PP, Vignon F, Bousquet C, Rochefort H (1983) Increased DNA binding of the estrogen receptor in an estrogen-resistant mammary cancer. Cancer Res 43: 2290–2297

Blackburn MG, Orgee L, Williams GM (1977) Oxidative bonding of natural oestrogens to DNA by chemical and metabolic means. J Chem Soc Chem Commun 11: 386–387

Bolt HM, Kappus H (1974) Irreversible binding of ethynyl-estradiol metabolites to protein and nucleic acids as catalyzed by rat liver microsomes and mushroom tyrosinase. J Steroid Biochem 5: 179–184

Brotherton J (1976) Sex hormone pharmacology. Academic Press, London, pp 479–481

Bucala R, Fishman J, Cerami A (1982) Formation of covalent adducts between cortisol and 16-hydroxyestrone and protein: Possible role in the pathogenesis of cortisol toxicity and systemic lupus erythematosus. Proc Natl Acad Sci USA 79: 3320–3324

Cameron RG, Imaida K, Tsuda H, Ito N (1982) Promotive effects of steroids and bile acids on hepatocarcinogenesis initiated by diethylnitrosamine. Cancer Res 42: 2426–2428

Cerutti P (1978) Repairable damge in DNA. In: Hanawalt P, Friedberg E, Fox C (eds) DNA repair mechanism. Academic Press, New York, pp 1–14

Drevon C, Piccoli C, Montesano R (1981) Mutagenicity assays of estrogenic hormones in mammalian cells. Mutat Res 89: 83–90

Duncan SJ, Brookes P (1979) Lack of oestradiol-DNA binding in mouse embryo cell cultures or following rat-liver microsomal metabolism. Cancer Lett 6: 351–355

Henderson BE, Ross RK, Pike MC, Casagrande JT (1982) Endogenous hormones as a major factor in human cancer. Cancer Res 42: 3232–3239

Jaggi W, Lutz WK, Schlatter C (1978) Covalent binding of ethinylestradiol and estrone to rat liver DNA in vivo. Chem Biol Interact 23: 13–18

IARC (1974) Monographs on the evaluation of carcinogenic risk of chemicals to man, sex hormones, vol 6. International Agency for Research on Cancer, Lyon

IARC (1979) Monographs on the evaluation of the carcinogenic risk of chemicals to humans, sex hormones, vol 21. International Agency for Research on Cancer, Lyon

Kaiser H (1965) Zum Problem der Nachweisgrenze. Z Anal Chem 209: 1–19

Kappus H, Bolt HM, Remmer H (1973) Irreversible protein binding of metabolites of ethynylestradiol in vivo and in vitro. Steroids 22: 203–225

Kappus H, Bolt HM (1976) Irreversible protein binding of norethisterone (norethindrone) epoxide. Steroids 27: 29–45

Lang R, Redmann U (1979) Non-mutagenicity of some sex hormones in the Ames Salmonella/microsome mutagenicity test. Mutat Res 67: 361–365

Lutz WK (1979) In vivo covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. Mutat Res 65: 289–356

Lutz WK (1982) Constitutive and carcinogen-derived DNA binding as a basis for the assessment of potency of chemical carcinogens. In: Snyder R, Parke DV, Kocsis J, Jollow DJ, Gibson GG (eds) Biological reactive intermediates, chemical mechanism and biological effects. Plenum Press, New York, pp 1349–1365

Lutz WK, Jaggi W, Schlatter C (1982) Covalent binding of diethylstilbestrol to DNA in rat and hamster liver and kidney. Chem Biol Interact 42: 251–257

Maggs JL, Grabowski PS, Rose ME, Park BK (1982) The biotransformation of 17α-ethynyl[^{3}H]estradiol in the rat- irreversible binding and biliary metabolites. Xenobiotica 12: 657–668

Marks F, Hecker E (1969) Metabolism and mechanism of action of oestrogens. XII. Structure and mechanism of formation of water-soluble and protein-bound metabolites of oestrone in rat-liver microsomes in vitro and in vivo. Biochim Biophys Acta 187: 250–265

Merrill EJ, Vernice GG (1970) Synthesis of [6,7-^{3}H]-17-ethinylestradiol and [6,7-^{3}H]-17-ethinylestradiol-cyclopentyl-1-^{14}C-ether. J Labelled Compds 6: 266–275

Mitchell JR, Hughes H, Lauterburg BH, Smith CV (1982) Chemical nature of reactive intermediates as determinant of toxicologic responses. Drug Metab Rev 13: 539–553

Moore HW (1977) Bioactivation as a model design for bioreductive alkylation. Science 197: 527–532

Pelkonen O, Boobis AR, Nebert DW (1978) Genetic differences in the binding of reactive carcinogenic metabolites to DNA. Carcinogenesis 3: 383–400

Sagelsdorff P, Lutz WK, Schlatter C (1983) The relevance of covalent binding to mouse liver DNA to the carcinogenic action of hexachlorocyclohexane isomers. Carcinogenesis 4: 1267–1273

Schuppler J, Dammé J, Schulte-Hermann R (1983) Assay of some endogenous and synthetic sex steroids for tumor-initiating activity in rat liver using the Solt-Farber system. Carcinogenesis 4: 239–241

Tsibris JCM, McGuire P (1977) Microsomal activation of estrogens and binding to nucleic acids and proteins. Biochem Biophys Res Commun 78: 411–417

Viviani A, Lutz WK (1978) Modulation of the binding of the carcinogen benzo(a)pyrene to rat liver DNA in vivo by selective induction of microsomal and nuclear aryl hydrocarbon hydroxylase activity. Cancer Res 38: 4640–4644

Weisburger JH, Williams GM (1981) Carcinogen testing: Current problems and new approaches. Science 214: 401–407

Yager DJ and Yager R (1980) Oral contraceptive steroids as promotors of hepatocarcinogenesis in female Sprague-Dawley rats. Cancer Res 40: 3680–3685

Yager DJ, Fifield DS (1982) Lack of hepatogenotoxicity of oral contraceptive steroids. Carcinogenesis 3: 625–628

Yaneva M, Dessev G (1976) Isolation and properties of structurated chromatin from Guerin Ascites tumor and rat liver. Eur J Biochem 66: 535–542

Received August 17, 1983/Accepted December 8, 1983