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Estrogen-induced endogenous DNA adduction: Possible mechanism of hormonal cancer

(estradiol/synthetic estrogens/renal carcinoma/Syrian hamster/^{32}P-labeling analysis)

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ABSTRACT **In animals and humans, estrogens are able to induce cancer in susceptible target organs, but the mechanism(s) of estrogen-induced carcinogenesis has not been elucidated. A well-known animal model is the development of renal carcinoma in estrogen-treated Syrian hamsters. Previous work demonstrated the presence of covalent DNA addition products (adducts) in premalignant kidneys of hamsters exposed to the synthetic estrogen, diethylstilbestrol, a known human carcinogen. In the present study, the natural hormone, 17β-estradiol, and several synthetic steroid and stilbene estrogens were examined by a ^{32}P-postlabeling assay for their capacity to cause covalent DNA alterations in hamster kidney. Chronic exposure to each of the estrogens tested led to the gradual formation of five chromatographically distinct unusual nucleotides specifically in kidney DNA. Irrespective of the estrogen used, chromatograms exhibited identical mobilities of each of these adducts in seven different systems on PEI-cellulose anion-exchange TLC, in three different conditions on reversed-phase TLC, and in one system on silica gel partition TLC. Therefore, the DNA adducts observed did not contain moieties derived from the structurally diverse estrogens. It is concluded that each of the estrogens induced the binding of the same unknown endogenous compound (or compounds) to target tissue DNA. This novel property of estrogens is postulated to play a key role in hormone-induced malignancy.**

The formation of covalent DNA addition products (adducts) is generally accepted as a key feature of the initiation of carcinogenesis by "genotoxic" chemicals—chemicals that are able to damage genetic material (1, 2). Unless the modified DNA nucleotides are promptly repaired, miscoding may ensue upon DNA replication, leading to point mutations, activation of oncogenes, and chromosomal alterations (3). A number of short-term tests have been developed recently to detect genotoxic activity of chemicals (4–6). Also, DNA adduct formation has been shown *in vivo* in experimental animal test systems with many chemical carcinogens of diverse structure (1, 2, 7). However, a number of important carcinogens (8) exist that do not fit this description and therefore have been classified as nongenotoxic carcinogens (9). While their mechanism of action has not as yet been defined, some of them [such as estrogens (10–13) and the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (14, 15)] have been shown to promote transformation—i.e., they facilitate the expression of neoplastic properties of previously initiated susceptible cells. Estrogens were found to be negative in short-term assays for the induction of gene mutations, irrespective of whether this was measured in prokaryotic (16, 17) or eukaryotic (18, 39) cells. On the other hand, some synthetic estrogens, such as diethylstilbestrol (DES) (19), have been reported to elicit chromosomal aberrations in cultured cells without inducing point mutations, but the mechanism of this effect has not been elucidated. In view of the extensive use of compounds with estrogenic activity in human medicine (20, 21) and in agriculture (22) and the occurrence of estrogenic compounds as contaminants in food (22, 23), it is important to define how these compounds cause cancer.

A central question to be addressed in this context is whether or not estrogens, like the majority of chemical carcinogens, induce covalent DNA alterations in the target tissue of carcinogenesis *in vivo*. In the present study, experiments were carried out to search for adduct formation in an established animal model for estrogen-induced cancer—i.e., the induction of renal carcinoma in the Syrian hamster (24). This model was chosen because a large number of natural and synthetic estrogens of diverse structure are known to induce renal carcinoma in 80–100% of the animals within 6–9 months after s.c. estrogen implantation (25). The tumors formed are malignant, as shown by their invasiveness and ability to metastasize and to kill the host; they are initially estrogen-dependent but acquire autonomy after serial transplantations (24, 26). In a previous paper (27), DES, a known animal and human carcinogen (28), was shown to induce DNA adduct formation in the kidneys of hamsters but not in liver, a nontarget tissue. The DES-induced renal DNA adducts developed gradually over a period of several months, preceding tumorigenesis, with a peak at 5 months. The present study has explored renal DNA adduct formation with the natural hormone, 17β-estradiol, and other carcinogenic estrogens.

MATERIALS AND METHODS

Chemicals. 17β-Estradiol, DES, hexestrol, progesterone, deoxycorticosterone acetate, and cholesterol were obtained from Sigma. 11β-Methoxy-17α-ethinylestradiol was a gift of J. P. Raynaud (Roussel Uclaf, Paris). 11β-Methyl-17α-ethinylestradiol and 11β-ethyl-17α-ethinylestradiol were gifts of F. Colton (Searle, Chicago). Impurities in the estrogens were not detected by mass spectrometry.

Materials and chemicals required for the ^{32}P-labeling adduct assay were the same as described before (7, 27, 29, 30). Preparation and use of the PEI-cellulose thin-layer chromatograms have been outlined before (7, 27, 29, 30). Reversed-phase (C_{18}) TLC plates were KC18 octadecylsilane (Whatman). Silica gel F-254 plates were purchased from EM Laboratories (Elmsford, NY).

Induction of Renal Carcinoma. Male Syrian hamsters (4–6 weeks old, obtained from Harlan Sprague–Dawley, Houston, TX) received two s.c. implants (31 mg of 17β-estradiol or 25 mg of hexestrol each plus 10% cholesterol). Three months later, two additional implants were given. Groups of three or four hamsters were killed 1, 3, 5, and 7 months after the initial

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Abbreviations: DES, diethylstilbestrol; 2-D, two-dimensional.

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hormone implant, and organs were immediately excised and frozen. Organs from hamsters treated with the synthetic steroid estrogens, 11β-methyl-17α-ethinylestradiol, 11β-ethyl-17α-ethinylestradiol, and 11β-methoxy-17α-ethinylestradiol, respectively (one 25-mg implant with 10% added cholesterol each at time zero and at 3 months), were taken at 9 months. At 7 months (17β-estradiol and hexestrol) or 9 months (17α-ethinylestradiol derivatives), kidneys but no other tissues contained tumors, some as large as 0.5–1.0 cm in diameter. Untreated and cholesterol-treated hamsters showed no tumors.

^{32}P-Labeling and Two-Dimensional (2-D) Mapping of Adducts. DNA was isolated by a solvent extraction procedure (31) from kidney, liver, spleen, lung, and heart of estrogen-treated and control hamsters. For the detection of covalent DNA adducts, a highly sensitive ^{32}P-postlabeling assay recently developed in one of our laboratories (7, 29, 30, 32) was used. DNA (4 μg) was digested to deoxyribonucleoside 3′-monophosphates, which were ^{32}P-labeled in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase (32). After removal of the normal nucleotides from the labeled digests by PEI-cellulose TLC in solvent I (Table 1), the adducts were separated by 2-D TLC in solvent II for the first dimension (Figs. 1 and 2) and in solvent III (Fig. 1) or IV (Fig. 2) for the second dimension. To remove nonspecific radioactive background, the chromatograms were given a final development with solvent V (32). For cochromatography, labeled digests corresponding to 4 μg each of the individual DNAs were mixed before application. ^{32}P-labeled adducts were located by autoradiography for 2–3 days at −80°C with DuPont Lightning Plus intensifying screens.

Rechromatography of Adducts. For further characterization of the most intensely labeled adducts, autoradiographically located adduct fractions were excised from replicate maps, and the adducts were extracted with 4 M ammonia for 20 min by a minor modification of a published technique (33). Pooled eluates were evaporated, and adducts were dissolved in 50–100 μl of water. Aliquots (20–50 dpm) were applied in 3 to 5 μl fractions, respectively, to PEI-cellulose, C_{18} reversed-phase (prewashed successively with solvents X, XI, and XII), and silica gel (prewashed with solvent XV) thin layers. The chromatograms were developed in solvents II, III, VI–IX (PEI-cellulose), XII–XIV (C_{18}), and XV (silica gel). Autoradiography was performed for 4–6 days. The stability of the adducts during extraction was ascertained by comparing the mobility of extracted adducts in solvents II and III with that of adducts that were rechromatographed without extraction by means of a contact transfer technique (33). Long-term stability of adducts a, b, and d under alkaline conditions was ascertained by incubating aliquots of extracts containing these compounds at 23°C for 15 hr with 4 M ammonia, followed by chromatography on C_{18} and silica gel thin layers in solvents XII and XV, respectively

RESULTS

Hamster kidney DNA was analyzed by ^{32}P-postlabeling assay (29, 30, 32). In this assay, deoxyribomononucleotides in enzymatic DNA digests are ^{32}P-labeled via T4 polynucleotide kinase-catalyzed [^{32}P]phosphate transfer from [γ-^{32}P]ATP, and evidence for the presence of adducts is obtained by the appearance of extra spots on PEI-cellulose anion-exchange thin-layer chromatograms of the ^{32}P-labeled digests, as detected by autoradiography. This method enables the detection of 1 aromatic adduct in ≈10^9 normal DNA nucleotides—i.e., ≈10 adducts per mammalian genome (32). When the ^{32}P-postlabeling assay was applied to kidney DNA from hamsters treated with 17β-estradiol for 5 months, the results shown in Figs. 1*B* and 2*B* were obtained. Kidney DNA from cholesterol-treated animals gave the autoradiograms shown in Figs. 1*A* and 2*A*. The analyses displayed in Figs. 1 and 2 differed only in the chromatographic conditions (see legends). Five extra spots (a–e) were detected in kidney DNA of 17β-estradiol-treated hamsters but not in kidney DNA from cholesterol-treated or untreated (not shown) hamsters. The pattern of background radioactivity displayed by both control and estrogen-exposed samples (Figs. 1 and 2) was not derived from the DNA digestion products. The total level of DNA modification was estimated by scintillation counting (7, 29, 30) to be 0.8–2 adducts in 10^7 DNA nucleotides at 5 months. Adduct levels at 3 and 7 months were lower by factors of 2–3, whereas only adducts a and d could be detected in kidney DNA after 1 month of 17β-estradiol exposure. DNA preparations from liver, spleen, lung, and heart of estrogen-exposed hamsters gave maps identical to those shown in Figs. 1*A* and 2*A* and thus were free of estrogen-induced DNA alterations. The results show that a natural hormone, 17β-estradiol, is able to elicit target organ-specific covalent DNA modifications *in vivo*.

In the course of these experiments, it was noted that the fingerprints of 17β-estradiol-exposed kidney DNA resembled those of kidney DNA from hamsters treated with the structurally different stilbene estrogens DES (Figs. 1*B* and 2*B*) and hexestrol (Fig. 1*G*). To explore this phenomenon further, ^{32}P-labeled digests of kidney DNAs from hamsters treated with 17β-estradiol or DES were mixed and cochromatographed under different conditions. Figs. 1*D* and 2*D* show that spots a–e were not separated with three different

Table 1. Chromatographic conditions for ^{32}P analysis of estrogen-induced DNA adducts

Solvent number	Solvent composition	TLC phase
I	1 M sodium phosphate, pH 6.8	PEI
II	3.8 M lithium formate/6.8 M urea, pH 3.4	PEI
III	0.64 M lithium chloride/0.4 M Tris·HCl/6.8 M urea, pH 8.0	PEI
IV	0.7 M sodium phosphate/7 M urea, pH 6.4	PEI
V	1.7 M sodium phosphate, pH 6.0	PEI
VI	2-Propanol/4 M ammonia, 2.8:2.2 (vol/vol)	PEI
VII	2-Propanol/4 M ammonia, 3.1:1.9 (vol/vol)	PEI
VIII	2-Propanol/3 M ammonium formate, pH 3.6, 1:1 (vol/vol)	PEI
IX	Ethanol/3 M ammonium formate, pH 3.6, 1:1 (vol/vol)	PEI
X	0.4 M ammonium formate, pH 6.2	C_{18} RP
XI	2-Propanol/0.4 M ammonium formate, pH 6.2, 1:1 (vol/vol)	C_{18} RP
XII	2-Propanol/2-butoxyethanol/0.4 M ammonium formate, pH 6.2/concentrated ammonia, 4:1:14.3:2.4 (vol/vol)	C_{18} RP
XIII	2-Propanol/0.2 M ammonium formate, pH 6.2, 1.5:3.5 (vol/vol)	C_{18} RP
XIV	2-Propanol/0.2 M ammonium formate, pH 6.2, 2:3 (vol/vol)	C_{18} RP
XV	2-Propanol/4 M ammonia, 11:9 (vol/vol)	Silica

RP, reversed phase.

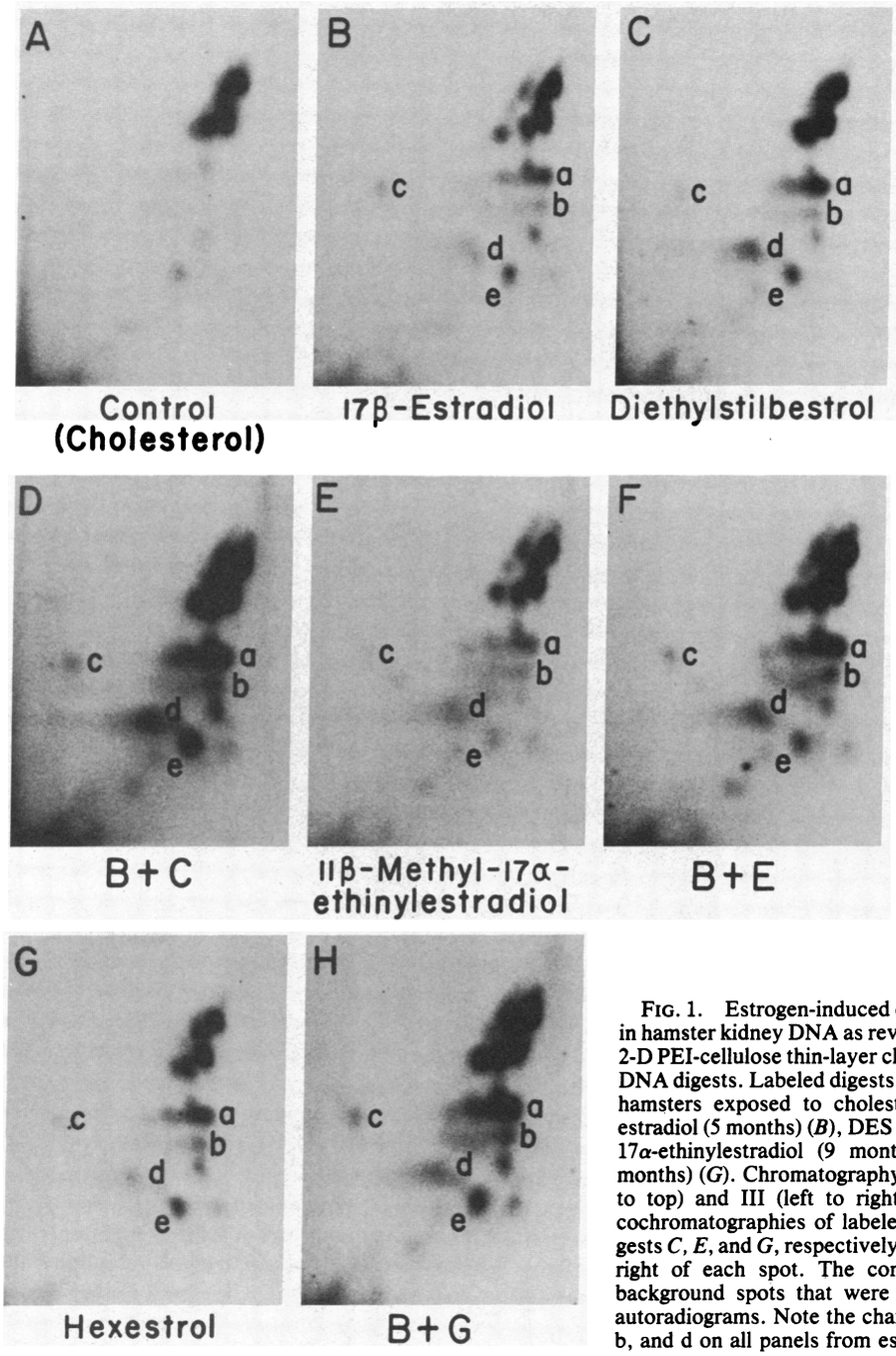

FIG. 1. Estrogen-induced covalent DNA adducts (a–e) in hamster kidney DNA as revealed by autoradiography of 2-D PEI-cellulose thin-layer chromatograms of ^{32}P-labeled DNA digests. Labeled digests were derived from DNAs of hamsters exposed to cholesterol (5 months) (*A*), 17β-estradiol (5 months) (*B*), DES (5 months) (*C*), 11β-methyl-17α-ethinylestradiol (9 months) (*E*), and hexestrol (7 months) (*G*). Chromatography was in solvents II (bottom to top) and III (left to right). *D*, *F*, and *H* represent cochromatographies of labeled digest *B* with labeled digests *C*, *E*, and *G*, respectively. Adducts are lettered to the right of each spot. The control sample (*A*) exhibited background spots that were also present on the other autoradiograms. Note the characteristic shape of spots a, b, and d on all panels from estrogen-exposed DNA.

solvents on 2-D PEI-cellulose TLC (Table 2, experiments 1 and 2), a technique known to resolve a great number of carcinogen–DNA adducts (7, 29, 30, 32). Likewise, the characteristic 17β-estradiol-induced DNA adducts were not resolved under these conditions from adducts formed in kidney DNA of hamsters treated with the other stilbene and steroid estrogens tested—i.e., hexestrol (Fig. 1*H*), 11β-methyl-17α-ethinylestradiol (Figs. 1 *E* and *F* and 2 *E* and *F*), 11β-ethyl-17α-ethinylestradiol (not shown), and 11β-methoxy-17α-ethinylestradiol (not shown), respectively.

In view of the structural diversity of the stilbene and steroid estrogens (Fig. 3), the identity of chromatographic patterns was surprising, suggesting the possible structural identity of the corresponding modified nucleotides. To prove this possibility, the major adducts a, b, and d were isolated from the 2-D maps and rechromatographed in a total of eight different solvents on PEI-cellulose, C_{18} reversed-phase, and silica gel thin layers (Table 2). Adducts c and e were not analyzed in this way because of insufficient radioactivity. In these experiments, adducts derived from individual modified DNA preparations and from mixtures of two such preparations were studied. In each case, chromatographic identity of the 17β-estradiol-induced adducts a, b, and d with the corresponding adducts elicited by the other estrogens tested was established. An example is shown in Fig. 4. In addition to identical chromatographic mobilities, the modified nucleotides resembled each other in terms of spot shapes (Figs. 1 and 4), relative intensities of labeling (Figs. 1 and 2), and total number detected. Also, compounds a, b, and d, independent of their origin, were completely stable during extraction and upon incubation in 4 M ammonia at 23°C for 15 hr as revealed by C_{18} and silica gel TLC in solvents XII and XV, respectively (autoradiograms not shown). All of these results provided strong evidence that each of the stilbene and steroid estrogens examined led to the gradual formation of an identical set of DNA adducts in the target organ of carcinogenesis.

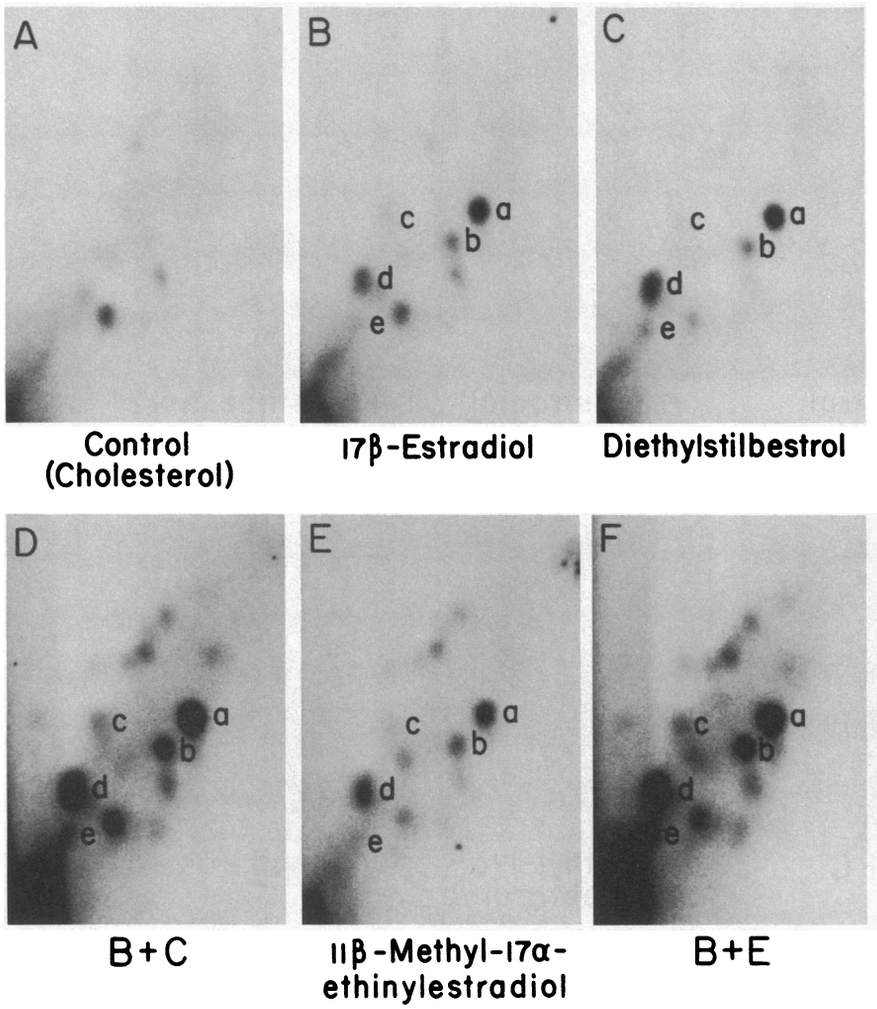

FIG. 2. Estrogen-induced covalent DNA adducts chromatographed under conditions differing from those of Fig. 1. Solvent IV was used for the second dimension of the PEI-cellulose chromatogram.

DISCUSSION

This work demonstrates that the natural estrogen, 17β-estradiol, as well as synthetic stilbene and steroid estrogens elicit covalent DNA alterations specifically in target tissue of carcinogenesis (hamster kidney). The DNA alterations are estrogen-specific, since no adducts were induced in hamster kidney by other steroid hormones such as progesterone or deoxycorticosterone acetate (unpublished results). The unexpected finding that the structurally diverse estrogens (Fig. 3) induce an identical set of covalently modified nucleotides

Table 2. Chromatographic evidence for identity of ^{32}P-labeled DNA adducts induced by structurally diverse estrogens

Experiment number	Chromatographic solvents	TLC Phase
1*	II + III (2-D)	PEI
2†	II + IV (2-D)	PEI
3	VI	PEI
4	VII	PEI
5	VIII	PEI
6	IX	PEI
7‡	XII	C_{18}
8	XIII	C_{18}
9	XIV	C_{18}
10§	XV	Silica

In experiments 1 and 2, ^{32}P-labeled DNA digest was analyzed; in all other experiments, purified ^{32}P-labeled adducts were chromatographed.
*See Fig. 1.
†See Fig. 2.
‡See Fig. 4 *Left*.
§See Fig. 4 *Right*.

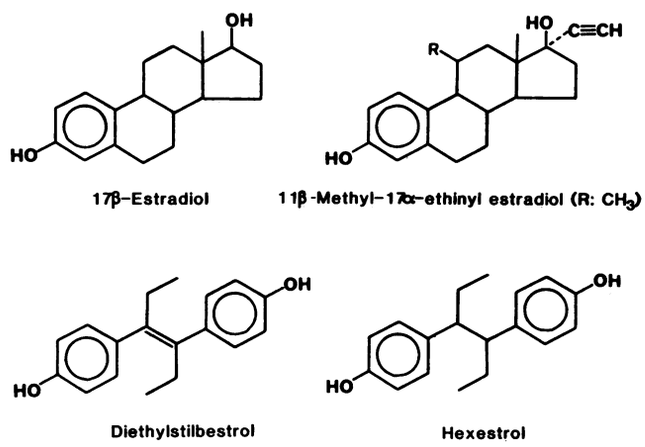

FIG. 3. Structures of estrogens investigated. Structures of 11β-ethyl-17α-ethinylestradiol (R = CH_2CH_3) and 11β-methoxy-17α-ethinylestradiol (R = OCH_3) are analogous to that of 11β-methyl-17α-ethinylestradiol as shown.

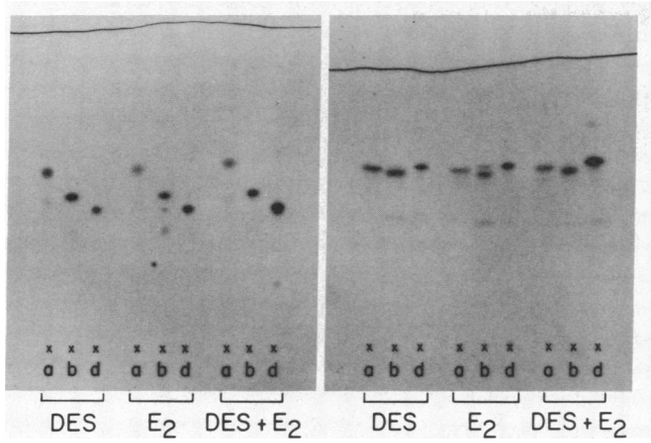

FIG. 4. Rechromatography of estrogen-induced ^{32}P-labeled DNA adducts. The major adducts (a, b, and d, respectively, see Fig. 2) were isolated from 2-D PEI-cellulose TLC maps, then rechromatographed on C_{18} reversed-phase TLC plates in solvent XII (*Left*) and on silica gel TLC sheets in solvent XV (*Right*). Adducts were derived from DNA of hamsters treated with DES or 17β-estradiol (E_2) or from a mixture of these DNAs (DES + E_2, see Fig. 2*D*).

in target tissue DNA indicates a novel mechanism of DNA adduction. It is proposed that exogenous estrogens or their metabolites do not themselves bind covalently to DNA but rather induce the formation of an unknown DNA-reactive compound (or compounds) in target tissue. The structural diversity of the estrogens examined makes it highly unlikely that the adduct moieties are derived from identical metabolic fragments of these compounds. The proposed mechanism of DNA binding is distinct from the established mechanism of DNA adduction, which involves direct electrophilic addition to DNA of carcinogens or their metabolites (34). While the structures of the estrogen-induced DNA-reactive compound(s) and of the resulting adducts remain to be determined, the chromatographic behavior of the modified nucleotides suggests the presence of bulky unsaturated or aromatic moieties. The nucleotide attachment sites of these residues remain to be identified. Alkali stability implies that the adduct moieties probably are not attached to N-7 or C-8 of guanine (35). Adducts containing small aliphatic groups would not have been resolved, since the conditions used here are selective for bulky lipophilic and aromatic nucleotide derivatives (7, 30). The indirect nature of estrogen-induced DNA alteration explains previous failures of *in vitro* (36, 37) or *in vivo* (38) experiments to isolate estrogen–DNA adducts. None of the estrogens tested gave any additional spots on the autoradiograms. Therefore, the adducts probably did not contain moieties derived from the administered estrogens. The possibility that the exogenous estrogens modified cellular metabolism so that endogenous estrogens were converted to DNA-reactive metabolites was not ruled out, however.

Organ-specific covalent DNA adduction in hamster kidney suggests that the observed DNA alterations play a role in the overall process of estrogen-induced carcinogenesis. Whether the proposed mechanism of indirect DNA adduction also applies to hormone-induced cancers in other tissues and species is currently being investigated. The results presented here provide evidence that structurally diverse estrogens exhibit highly specific genotoxic activities in the intact mammalian organism, yet may all act through the same molecular mechanism to induce cancer in a target organ.

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