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The Protective Effect of Estradiol-17β against Polycyclic Hydrocarbon Cytotoxicity[1]

Arthur G. Schwartz[2]

Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

SUMMARY

7,12-Dimethylbenz(a)anthracene (DMBA) (10^{-6} M) inhibited the growth of cultured epithelial rat liver cells and depressed thymidine-^{3}H incorporation. In a series of methyl- and ethyl-substituted benz(a)anthracenes there was significant correlation between the reported carcinogenicity of the derivative and its capacity to inhibit acid-insoluble thymidine-^{3}H incorporation. Similar results were obtained with a diploid strain of rat lung fibroblasts, but a human cancer cell (HeLa) and a rat hepatoma (HTC) were insensitive to the cytotoxic effect of DMBA.

In contrast to DMBA, a series of diazo dyes of varying hepatocarcinogenicities did not significantly inhibit thymidine-^{3}H incorporation by liver epithelial cell cultures.

Estradiol-17β at 10^{-6} M protected liver and breast epithelial cultures from the inhibitory effect of DMBA on thymidine-^{3}H incorporation. The other steroids tested had little or no protective effect at 4×10^{-5} M. The possible relationship of this protective effect of estradiol-17β to clinical and epidemiological data in women is discussed.

INTRODUCTION

BA,[3] the parent compound for a group of active carcinogens, is itself noncarcinogenic (25). Methyl substitution at position 6, 7, 8, or 12 converts BA into an active carcinogen, while methyl substitution at other positions is ineffective (17, 25). Ethyl, or higher alkyl substitution, at any of the "active" positions is also ineffective (25). DMBA is one of the most potent chemical carcinogens known, while DEBA does not produce malignant tumors (25, 38).

On the basis of the close steric similarity of hydrocarbon carcinogens and steroid hormones, Yang *et al.* (47) have suggested that to be carcinogenic polynuclear hydrocarbons must be sterically similar to steroid hormones and that ethyl or higher alkyl substitution in the BA nucleus produces significant steric deviation from the steroid model. Along the same lines, there is considerable evidence that hydrocarbon carcinogens may act at the same site(s) in cells as steroid hormones (24, 26, 27, 29, 40, 45).

Several investigators have examined the effects of hydrocarbon carcinogens in cell cultures. Malignant transformation of embryonic hamster (5, 6, 15) and mouse ventral prostate (34) fibroblasts has been reported, as well as a cytotoxic effect on cultured rodent fibroblasts, manifested by reduced cloning efficiency and inhibition of growth (1, 2, 14, 21, 41, 43). Two carcinogens of another class, dimethylnitrosamine and *N*-nitrosomethylurea, have also been reported to be cytotoxic to normal human and hamster fibroblasts (23).

As here shown, treatment of cultures of rat liver epithelial cells or rat lung fibroblasts with DMBA has a marked cytotoxic effect, manifested by an inhibition of cell growth and a pronounced depression of acid-insoluble thymidine-^{3}H incorporation. In a series of methyl- and ethyl-substituted BA's there was significant correlation between the reported carcinogenicity of the compound and its capacity to inhibit acid-insoluble thymidine-^{3}H incorporation. Estradiol-17β protected the liver epithelial cells from the inhibitory effect, but other steroid hormones (progesterone, testosterone, hydrocortisone, and deoxycorticosterone) were inactive.

MATERIALS AND METHODS

DMBA, BA, the hydrochloride of DAB, and *p*-phenylazoaniline were obtained from Eastman Organic Chemicals, Rochester, N. Y. The 5-, 6-, 7-, and 8-methyl-BA's were generously supplied by Dr. M. Newman, Ohio State University; the 12-methyl, 7-ethyl, 12-ethyl, and 7,12-DEBA's were supplied by Dr. J. Pataki, University of Chicago; and 4'-methyl-DAB, 4'-ethyl-DAB, 2',3'-dimethyl-DAB, and *N*,*N*-diethyl-*p*-phenylazoaniline were from Dr. J. A. Miller, University of Wisconsin. Stock solutions at 3×10^{-2} and 3×10^{-3} M in DMSO (Eastman and Sigma Chemical Co., St. Louis, Mo.) were stored in aluminum foil-covered flasks at −25°. The final DMSO concentration was adjusted to 0.03% throughout, and control cultures received DMSO without hydrocarbon or diazo dye.

Steroid hormones (Calbiochem, La Jolla, Calif.) were stored at 4° as stock solutions in 95% ethanol. These were added to the cell cultures at a final concentration of 4×10^{-5}, 2×10^{-5}, 10^{-5}, or 10^{-6} M, and the final ethanol concentration was adjusted to either 0.07 or 0.13%. At the concentrations used, neither ethanol nor DMSO above had any discernible effect on cell growth or acid-insoluble thymidine-^{3}H incorporation.

Cell Lines. Two rat liver epithelial cell lines were estab-

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[2] Research Associate, supported by NIH Grant CA-AI 11992. Present address: The Fels Research Institute and Department of Microbiology, Temple University School of Medicine, Philadelphia, Pa. 19140.

[3] The abbreviations used are: BA, benz(a)anthracene; DMBA, 7,12-dimethylbenz(a)anthracene; DEBA, 7,12-diethylbenz(a)anthracene; DAB, 4-dimethylaminoazobenzene; DMSO, dimethyl sulfoxide.

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lished in this laboratory from the livers of an adult male (E-3) and female (G-1) Sprague-Dawley rat (manuscript in preparation). A rat lung fibroblast line (BL) was established from pooled lung tissue of 2 adult Sprague-Dawley female rats. The HTC line was established by Thompson *et al.* (42) from an ascites tumor derived from a solid hepatoma induced by feeding *N*,*N'*-2,7-fluorenylenebis-2,2,2-trifluoroacetamide to male Buffalo rats and was obtained from Dr. G. Tomkins. The HeLa cell line was derived from a human cervical carcinoma (22) and propagated in Dr. H. Eagle's laboratory for the past 15 years. All cell lines were propagated as monolayer cultures in Blake bottles in minimal essential medium (19) supplemented with 5% calf and 5% fetal calf serum.

Effect of DMBA on Cell Growth. From 100,000 to 200,000 cells of each cell line were inoculated into T-15 culture flasks (18) in minimal essential medium supplemented with 5% calf and 5% fetal calf serum, buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (3.75 mM), *N*,*N* - bis(2 - hydroxyethyl) - 2 - aminoethanesulfonic acid (2.5 mM), and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (2.5 mM) (20) adjusted to a final pH of 7.6. The cells were refed the following day. On the 3rd day after plating and at 48-hr intervals thereafter, the cultures received medium containing DMBA or control medium containing DMSO. All cultures were protected from light by covering with aluminum foil. Duplicate cultures were assayed for protein (37) on Days 3, 5, and 7.

Thymidine-^{3}H Incorporation. After 3 to 6 days of growth in normal medium, cells received medium containing a specific polycyclic hydrocarbon or diazo dye. Twenty-four hr later, replicate cultures were pulsed for 90 min with thymidine-^{3}H [Schwarz/Mann, Orangeburg, N. Y., (specific activity, 14 Ci/mmole) or New England Nuclear, Boston, Mass. (specific activity, 20 Ci/mmole)] at a concentration of 0.25 to 1.25 μCi/ml, and the acid-insoluble radioactivity per μg of protein was determined (11). Incorporation of acid-insoluble thymidine-^{3}H was linear for at least 3 hr under these conditions.

For determination of the effect of steroids on the DMBA-induced depression of thymidine-^{3}H incorporation, cells grown for 6 to 7 days in normal medium received medium containing either DMBA, a specific steroid, or the 2 in combination. The cultures were incubated for 24 hr and either pulsed with thymidine-^{3}H for 90 min or, alternatively, washed 2 times in normal medium, drained carefully, incubated for an additional 24 hr in normal medium, and then pulsed with thymidine-^{3}H. The acid-insoluble radioactivity per μg of protein was determined.

RESULTS

As shown in Chart 1, DMBA at 10^{-5} to 10^{-7} M inhibited the growth of the liver epithelial line (G-1) and rat lung fibroblast (BL) and was without effect on the growth of the 2 heteroploid lines, HTC and HeLa. Twenty-four-hr treatment with the carcinogen at these levels caused a marked depression in the rate of acid-insoluble thymidine-^{3}H incorporation in both G-1 and BL lines, with only a slight depression in the HeLa, and none in HTC (Table 1).

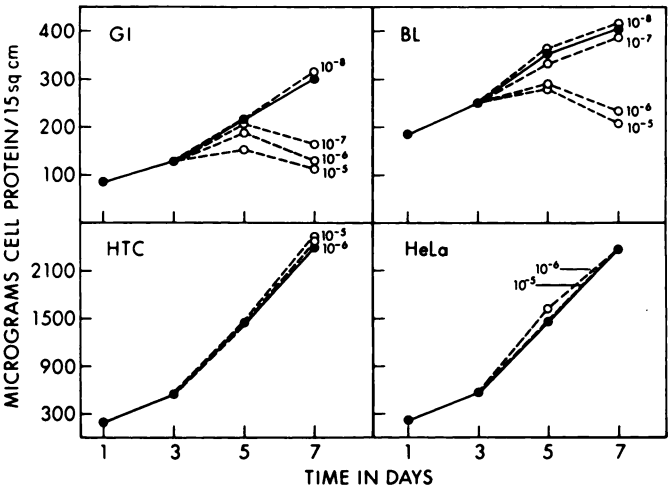

Chart 1. Effect of DMBA on growth of different cell lines. Three days after inoculation, cultures received DMBA (O- - -O) or control (●——●) media. *Points* are cell proteins in duplicate (averaged) T-15 cultures. DMBA concentrations (10^{-5} to 10^{-8}) are molar. The differences in cell proteins between duplicate cultures were generally less than 10%.

Table 1

Effect of DMBA on thymidine incorporation in various cell lines

Cells were grown for 6 days in normal medium and changed to DMBA-containing media for 24 hr. Cultures were pulsed with thymidine-^{3}H for 90 min. Acid-insoluble radioactivity is expressed as cpm/10 μg of protein. Values are averages of duplicate cultures, with a range of variation usually within 10%.

	Thymidine incorporation on Day 7 at indicated molar concentration of DMBA				
Cell line tested	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Normal					
Rat liver G1	1520	1250	690	160	200
Rat lung fibroblast BL	82	74	60	14	12
Cancer					
Rat hepatoma HTC	1110	1010	1310	1040	1060
Human cervical cancer HeLa	1590	1480	1440	1300	1110

In a series of methyl- and ethyl-substituted BA's there was a good correlation between the reported carcinogenicity of a specific compound and its capacity to inhibit thymidine-^{3}H incorporation in the rat liver cultures and fibroblasts (Chart 2; Table 2). Consistent with the markedly reduced carcinogenic activity of the ethyl-substituted derivatives, they inhibited thymidine-^{3}H incorporation to a lesser degree than the corresponding methyl-substituted compounds. This difference was more marked for the doubly substituted derivatives (DMBA *versus* DEBA) than for the monosubstituted compounds (7-methyl *versus* 7-ethyl and 12-methyl *versus* 12-ethyl).

Lack of Effect of Azo Dyes on Thymidine Incorporation. Unlike polycyclic hydrocarbons, which induce cancer in many different organs in the rat (46), aminoazobenzene dyes selectively induce liver cancer in this species (33). The most active carcinogen in the group, 2',3'-dimethyl-DAB, produced liver tumors in 10 of 10 rats in 1 month when fed in the diet at a level of 0.06% (7). As seen in Chart 3, these compounds, unlike the polycyclic hydrocarbon carcinogens, did not significantly depress acid-insoluble thymidine-^{3}H-incorporation in cultured liver epithelial cells at medium concentrations of 10^{-5} or 10^{-6} M.

Effect of Estradiol-17β. As previously mentioned, there is considerable evidence that polycyclic hydrocarbon carcinogens may act on the same site in cells as do steroid hormones. Estradiol-17β did in fact reverse the inhibiting effect of DMBA on acid-insoluble thymidine-^{3}H incorporation in cultured liver cells, and the degree of protection was proportional to the concentration of estradiol-17β (Table 3). The other steroids tested were without significant protective effect, with the exception of progesterone at the highest concentration. We have recently succeeded in establishing cultures of mouse breast epithelial cells using a modification of the procedure of McGrath *et al.* (30). In these cultures also estradiol-17β protected cells from the inhibitory effect of DMBA on thymidine-^{3}H incorporation (Table 4).

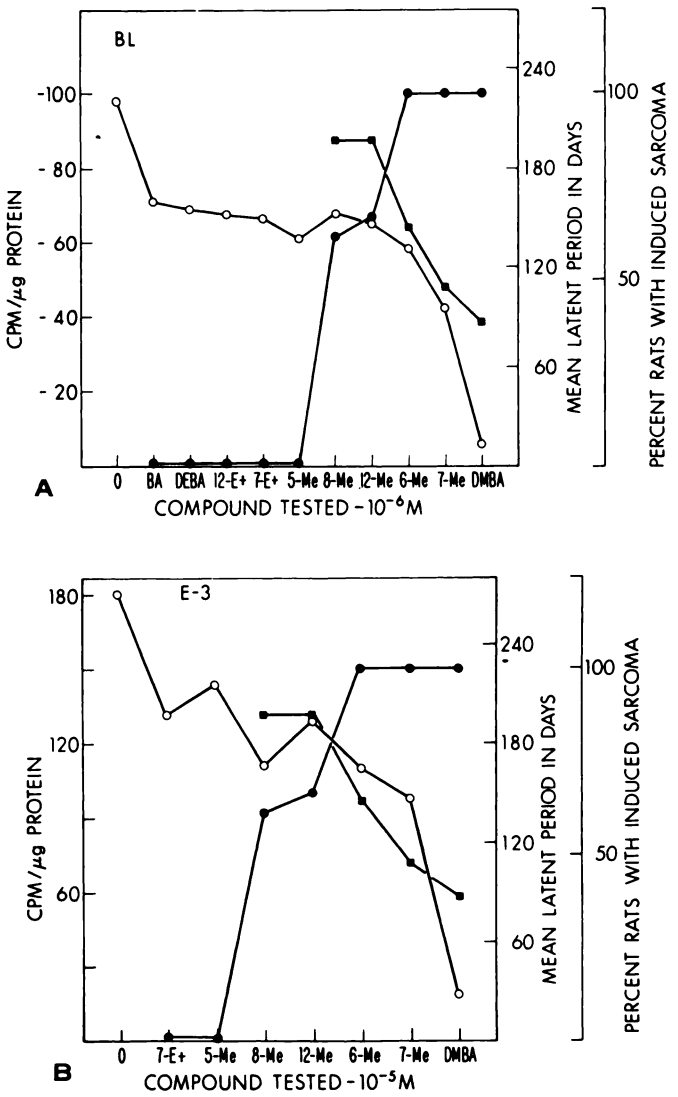

Chart 2. Correlation between cytotoxicity of polycyclic hydrocarbons and reported carcinogenicity [from data of Huggins *et al.* (25)]. Three-day (BL) (*A*) or 6-day (E-3) (*B*) cultures received media containing indicated polycyclic hydrocarbon at 10^{-6} (BL) or 10^{-5} M (E-3). Twenty-four hr later, the cultures were pulsed with thymidine-^{3}H for 90 min, and the acid-insoluble radioactivity per μg of protein was determined (O——O). Carcinogenicity is indicated as percentage of rats with induced sarcoma (●——●) and mean latent period in days (■——■). *Me*, methyl; *E+*, ethyl.

DISCUSSION

The capacity of a substituted BA to inhibit acid-insoluble thymidine-^{3}H incorporation in cultured rat liver epithelial cells and lung fibroblasts is apparently related to its capacity to induce cancer *in vivo*. This inhibition is not seen with the azo dye carcinogens, some of which are potent inducers of liver cancer in the rat. There is, however, evidence that the azo dye carcinogens are activated by the liver *in vivo* to the proximate carcinogens (31), and the failure of the cultured liver epithelial cells to respond to the azo carcinogens may reflect their inability to metabolize to the proximate carcinogen. Alternatively, this class of carcinogens may act through a different mechanism from the polycyclic hydrocarbons.

Gelboin *et al.* (21) have found that normal hamster and mouse fibroblasts were sensitive to the cytotoxic effect of benzo(*a*)pyrene while transformed fibroblasts were resistant. There was a good correlation between the sensitivity of a cell line to benzo(*a*)pyrene cytotoxicity and the levels of aryl hydrocarbon [benzo(*a*)pyrene] hydroxylase in cell homogenates, and they suggested that a metabolite of benzo(*a*)pyrene was the active cytotoxic agent. The insensitivity of, *e.g.*, HeLa and HTC cells to DMBA as here reported may thus reflect their inability to convert DMBA to a cytotoxic metabolite.

The present finding that estradiol-17β inhibits the cytotoxicity of DMBA may be related to some effects of the steroid *in vivo*. Bates (4) has found that male mice were more susceptible than female mice to skin tumorigenesis following the topical application of DMBA and that the susceptibility of female mice was significantly enhanced by castration. Further, the application of a tumor-initiat-

ing dose of DMBA during estrus or proestrus resulted in the appearance of fewer tumors than similar application during metestrus or diestrus. In keeping with these observations, Nebert *et al.* (36) have reported that estradiol-17β competitively inhibits polycyclic hydrocarbon hydroxylation by aryl hydrocarbon hydroxylase in mouse organ extracts. There is considerable evidence that polycyclic hydrocarbon carcinogens are metabolized by microsomal hydroxylating enzymes to active carcinogenic and cytotoxic metabolites (12, 13, 21, 28, 39). In a manner analogous to the postulated role of estrogen, 7, 8-benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase (44), reduces the rate of conversion of ^{3}H-labeled DMBA to water-soluble metabolites by hamster embryo cultures and protects these cells against the cytotoxic effect of DMBA (12, 13). 7, 8-Benzoflavone also protects against DMBA-induced skin tumorigenesis in mice (28).

Table 2

Concordant effect of methyl and ethyl substitution on thymidine incorporation in vitro and carcinogenicity in vivo

Experimental conditions are indicated in Chart 2. Values for thymidine incorporation are averages of duplicate cultures, with a range of variation within 10%. Counted samples contained from 59 to 89 μg of protein.

Compound	Thymidine incorporation (cpm/50 μg protein)	Carcinogenicity[a]	
		% rats with sarcoma	Mean latent period (days)
0 (control)	4900		
DMBA[b]	310	100	87
DEBA	4050	0	
7-Methyl-BA	1650	100	108
7-Ethyl-BA	3055	0	
12-Methyl-BA	2585	69	197
12-Ethyl-BA	3895	0	

[a] After Huggins *et al.* (25) (*cf.* Chart 3 legend).
[b] Hydrocarbons added at 10^{-5} M.

It is thus possible that estradiol-17β, like 7,8-benzoflavone, may inhibit the metabolism of polycyclic hydrocarbons to active carcinogenic and cytotoxic metabolites. We plan to investigate the effect of estradiol-17β on the rate of conversion of ^{3}H-labeled DMBA to water-soluble metabolites by cultured liver epithelial cells as well as its effect on *in vitro* aryl hydrocarbon hydroxylase activity in extracts of cultured liver cells.

Certain epidemiological and clinical data in women are

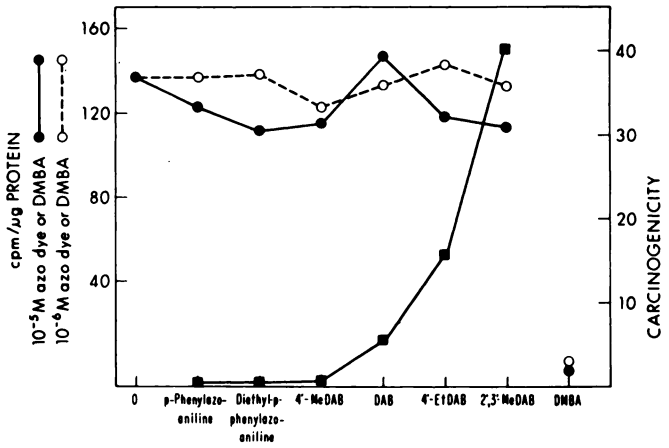

Chart 3. Lack of effect of diazo carcinogens on thymidine-^{3}H incorporation in E-3 cells. Six-day-old cultures received media containing diazo dye or DMBA at 10^{-5} or 10^{-6} M. After 24 hr the cells were pulsed with thymidine-^{3}H for 90 min and the acid-insoluble radioactivity per μg of protein was determined. Carcinogenicities of the azo dyes (■——■) are computed from the Miller formula (32) [Carcinogenicity = (6 × months fed × % tumors with test compound)/(months fed × % tumors with DAB), where the activity of (DAB) was arbitrarily designated at 6. Test compounds were fed at molar levels equivalent to 0.06% DAB.] and from the data of Miller and Miller (33), Brown and Hamdam (8), Brown and Kipp (9), and Brown (7). Me, methyl; Et, ethyl.

Table 3

Effect of steroids on DMBA-induced depression in thymidine-^{3}H incorporation in E-3

Acid-insoluble thymidine-^{3}H incorporation is expressed as cpm/600 μg of protein. Counted samples ranged from 340 to 670 μg of protein. Each determination is the mean of the values for 3 independent cell cultures (except as indicated) ± S.E.

Steroid	Concentration (M)	Incorporation	
		DMBA (4×10^{-7} M)	No DMBA
None		4140 ± 100[a]	65,000 ± 1230[a]
Estradiol-17β	10^{-6}	5320 ± 220[b]	63,600 ± 800
Estradiol-17β	10^{-5}	8120 ± 620[c]	61,200 ± 3400
Estradiol-17β	2×10^{-5}	10,000 ± 400[c]	41,830 ± 2390
Testosterone	10^{-6}	4510 ± 170	64,400 ± 1590
Testosterone	10^{-5}	4740 ± 170	61,730 ± 480
Testosterone	2×10^{-5}	4670 ± 255	59,170 ± 540
Progesterone	10^{-6}	4220 ± 100	67,400 ± 2530
Progesterone	10^{-5}	4820 ± 110	70,500 ± 475
Progesterone	2×10^{-5}	5990 ± 400[b]	71,930 ± 5270
Hydrocortisone	10^{-6}	3720 ± 280	58,230 ± 1800
Hydrocortisone	10^{-5}	3660 ± 70	63,070 ± 2600
Hydrocortisone	2×10^{-5}	3630 ± 300	63,670 ± 1190

[a] Mean of 4 cultures.
[b, c] Significantly higher than DMBA-treated cultures without steroid; $p < 0.005$ (Footnote *b*); $p < 0.001$ (Footnote *c*).

Table 4

Protective effect of estradiol-17β on DMBA-induced depression of thymidine incorporation in breast epithelial cells

Primary breast cells were grown for 5 days in minimal essential medium supplemented with 15% fetal calf serum and insulin, 10 μg/ml. Cultures then received media containing DMBA, a specific steroid, or the 2 in combination. Twenty-four hr later cultures were pulsed with thymidine-^{3}H for 90 min. Acid-insoluble radioactivity is expressed as cpm/10 μg of protein.

Steroid concentration (M)	Minor effect of steroids on thymidine incorporation (cpm/10 μg protein)		Reversal of DMBA inhibition[a] by estradiol-17β (cpm/10 μg protein)	
	Estradiol-17β	5α-Dihydrotestosterone	Estradiol-17β	5α-Dihydrotestosterone
0	1770	1770	212	212
10^{-6}	1690	1913	602	386
10^{-5}	1692	1873	677	279
4×10^{-5}	1327	1660	492[b]	311

[a] DMBA at 10^{-6} M reduced incorporation of thymidine from 1770 to 212 cpm/10 μg protein.
[b] Reflects inhibition in thymidine incorporation by estradiol-17β at 4×10^{-5} M.

consistent with the protective effect of estradiol-17β on the DMBA-induced cytotoxicity that we have observed in cultured cells and its possible protective effect against DMBA-induced skin tumorigenesis in mice. The plasma estradiol-17β concentration declines in women with age (3), while the incidence of most cancers rises progressively (16). Burch and Byrd (10) found that, although estrogens administered postoperatively to hysterectomized women had no effect on the incidence of breast cancer, the incidence of other types of cancers was markedly reduced. Similarly, it has been reported (35) that none of 120 women treated with estrogens for osteoporosis developed cancers during 601 person-years of estrogen therapy, although 5 to 6 cases might have been expected from morbidity data.

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REFERENCES

1. Alfred, L. J., Donovan, P. J., Baker, M. S., and Di Paolo, J. A. Protection of Cultured Hamster Embryonic Cells from 7,12-Dimethylbenz(a)anthracene Cytotoxicity and the Induced Synthesis of Aryl Hydroxylase. Cancer Res., *29:* 1805-1809, 1969.
2. Alfred, L. J., Globerson, A., Berwald, Y., and Prehn, R. T. Differential Toxicity Response of Normal and Neoplastic Cells *in vitro* to 3,4-Benzopyrene and 3-Methylcholanthrene. Brit. J. Cancer, *18:* 159-164, 1964.
3. Baird, D. T., and Guevara, H. Concentration of Unconjugated Estrone and Estradiol in Peripheral Plasma in Non-Pregnant Women throughout the Menstrual Cycle, Castrate and Postmenopausal Women and in Men. J. Clin. Endocrinol., *29:* 149-156, 1969.
4. Bates, R. R. Sex Hormones and Skin Tumorigenesis. 1. Effect of the Estrous Cycle and Castration on Tumorigenesis by 7,12-Dimethylbenz(a)anthracene. J. Natl. Cancer Inst., *41:* 559-563, 1968.
5. Berwald, Y., and Sachs, L. *In Vitro* Transformation with Chemical Carcinogens. Nature, *200:* 1182-1184, 1963.
6. Berwald, Y., and Sachs, L. *In Vitro* Transformation of Normal Cells to Tumor Cells by Carcinogenic Hydrocarbons. J. Natl. Cancer Inst., *35:* 641-657, 1965.
7. Brown, E. V. The Hepatocarcinogenicity of Some Disubstituted 4-Dimethylaminoazobenzenes. J. Med. Chem., *11:* 1234-1235, 1968.
8. Brown, E. V., and Hamdam, A. A. The Hepatocarcinogenicities of a Series of 4'-alkyl-4-Dimethylaminoazobenzenes. J. Natl. Cancer Inst., *27:* 663-666, 1961.
9. Brown, E. V., and Kipp, W. H. A Comparison of Electron Density and Hepatocarcinogenic Activity for Various Derivatives of 4-Dimethylaminoazobenzene. Cancer Res., *29:* 1341-1344, 1969.
10. Burch, J. C., and Byrd, B. F. Effects of Long-Term Administration of Estrogen on the Occurrence of Mammary Cancer in Women. Ann. Surg., *174:* 414-418, 1971.
11. Ceccarini, C., and Eagle, H. pH as a Determinant of Cellular Growth and Contact Inhibition. Proc. Natl. Acad. Sci. U. S., *68:* 229-233, 1971.
12. Diamond, L. and Gelboin, H. V. Alpha-naphthoflavone: An Inhibitor of Hydrocarbon Cytotoxicity and Microsomal Hydroxylase. Science, *166:* 1023-1025, 1969.
13. Diamond, L., McFall, R., Miller, J., and Gelboin, H. V. The Effects of Two Isomeric Benzoflavones on Aryl Hydrocarbon Hydroxylase and the Toxicity and Carcinogenicity of Polycyclic Hydrocarbons. Cancer Res., *32:* 731-736, 1972.
14. Diamond, L., Sordet, C., and Rothblatt, G. H. The Metabolism of 7,12-Dimethylbenz(a)anthracene in Cell Cultures. Intern. J. Cancer, *3:* 838-849, 1968.
15. Di Paolo, J. A., Nelson, R. L., and Donovan, P. J. Morphological, Oncogenic, and Karyological Characteristics of Syrian Hamster Embryo Cells Transformed *in vitro* by Carcinogenic Polycyclic Hydrocarbons. Cancer Res., *31:* 1118-1127, 1971.
16. Doll, R., Muir, C., and Waterhouse, J. Cancer Incidence in Five Continents, Vol. 11. Berlin: Springer-Verlag, 1970.
17. Dunning, W. F., and Curtis, M. R. Relative Carcinogenic Activity of Monomethyl Derivatives of Benz(a)anthracene in Fischer Line 344 Rats. J. Natl. Cancer Inst., *25:* 387-391, 1960.
18. Eagle, H. The Specific Amino Acid Requirements of a Human Carcinoma Cell (Strain HeLa) in Tissue Culture. J. Exptl. Med., *102:* 37-48, 1955.
19. Eagle, H. Amino Acid Metabolism in Mammalian Cell Cultures. Science, *130:* 432-437, 1959.
20. Eagle, H. Buffer Combinations for Mammalian Cell Culture. Science, *174:* 500-503, 1971.
21. Gelboin, H. V., Huberman, E., and Sachs, L. Enzymatic Hydroxyla-

tion of Benzopyrene and Its Relationship to Cytotoxicity. Proc. Natl. Acad. Sci. U. S., *64:* 118-1194, 1969.
22. Gey, G. O., Coffman, W. D., and Kubicek, M. T. Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium. Cancer Res., *12:* 264-265, 1952.
23. Huberman, E., Traut, M., and Sachs, L. Cell Susceptibility to the Cytotoxic Effect of the Carcinogens Dimethylnitrosamine and *N*-Nitrosomethylurea. J. Natl. Cancer Inst., *44:* 395-402, 1970.
24. Huggins, C. B., and Morii, G. Selective Adrenal Necrosis and Apoplexy Induced by 7,12-Dimethylbenz(a)anthracene. J. Exptl. Med., *114:* 741-759, 1961.
25. Huggins, C. B., Pataki, J., and Harvey, R. C. Geometry of Carcinogenic Polycyclic Hydrocarbons. Proc. Natl. Acad. Sci. U. S., *58:* 2253-2260, 1967.
26. Inhoffen, H. H. The Relationship of Natural Steroids to Carcinogenic Aromatic Compounds. *In:* J. W. Cook (ed.), Progress in Organic Chemistry, pp. 131-155. New York: Academic Press, Inc., 1953.
27. Jull, J. W. Hormonal Mechanisms in Carcinogenesis. Can. Cancer Conf., *6:* 109-123, 1966.
28. Kinoshita, N., and Gelboin, H. V. Aryl Hydrocarbon Hydroxylase and Polycyclic Hydrocarbon Tumorigenesis: Effect of the Enzyme Inhibitor 7,8-Benzoflavone on Tumorigenesis and Macromolecule Binding. Proc. Natl. Acad. Sci. U. S., *69:* 824-828, 1972.
29. Loeb, L. Estrogenic Hormones and Carcinogenesis. J. Am. Med. Assoc., *104:* 1597-1601, 1935.
30. McGrath, C. M., Nandi, S., and Young, L. Relationship between Organization of Mammary Tumors and the Ability of Tumor Cells to Replicate Mammary Tumor Virus and to Recognize Growth-Inhibitory Contact Signals *in vitro*. J. Virol. *9:* 367-376, 1972.
31. Miller, J. A. Carcinogenesis by Chemicals: An Overview—G. H. A. Clowes Memorial Lecture. Cancer Res., *30:* 559-576, 1970.
32. Miller, J. A., and Miller, E. C. The Carcinogenicity of Certain derivatives of *p*-Dimethylaminoazobenzene in the Rat. J. Exptl. Med., *87:* 139-156, 1948.
33. Miller, J. A., and Miller, E. C. The Carcinogenic Aminoazo Dyes. Advan. Cancer Res., *1:* 339-396, 1953.
34. Mondal, S., and Heidelberger, C. *In Vitro* Malignant Transformation by Methylcholanthrene of the Progeny of Single Cells Derived from C3H Mouse Prostate. Proc. Natl. Acad. Sci. U. S., *65:* 219-225, 1970.
35. Mustacchi, P., and Gordon, G. S. Frequency of Cancer in Estrogen Treated Osteoporotic Women. *In:* A. Segaloff (ed.), Breast Cancer, pp. 163-169. St. Louis: C. V. Mosby, 1958.
36. Nebert, N. W., Bausserman, L. L., and Bates, R. R. Effect of 17-β Estradiol and Testosterone on Aryl Hydrocarbon Hydroxylase Activity in Mouse Tissues *In Vivo* and in Cell Culture. Intern. J. Cancer, *6:* 470-480, 1970.
37. Oyama, V. I., and Eagle, H. Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin Ciocalteu). Proc. Soc. Exptl. Biol. Med., *91:* 305-307, 1956.
38. Pataki, J., and Huggins, C. Relation of Methyl and Ethyl Substitution of Benz(a)anthracene to Carcinogenicity. *In:* E. D. Bergmann and B. Pullman (eds.), Physicochemical Mechanisms of Carcinogenesis: The Jerusalem Symposia on Quantum Chemistry and Biochemistry, Vol. 1, pp. 64-71. New York: Academic Press, Inc., 1969.
39. Selkirk, J. K., Huberman, E., and Heidelberger, C. An Epoxide Is an Intermediate in the Microsomal Metabolism of the Chemical Carcinogen, Dibenz(a,h)Anthracene. Biochem Biophys. Res. Commun., *43:* 1010-1016, 1971.
40. Singer, S., and Litwack, G. Identity of Corticosteroid Binder I with the Macromolecule Binding 3-Methylcholanthrene in Liver Cytosol *In Vivo*. Cancer Res., *31:* 1364-1368, 1971.
41. Starikova, V. B., and Vasiliev, J. M. Action of 7,12-Dimethylbenz-(alpha)anthracene on the Mitotic Activity of Normal and Malignant Rat Fibroblasts *in Vitro*. Nature, *195:* 42-43, 1962.
42. Thompson, E. B., Tomkins, G. M., and Curran, J. F. Induction of Tyrosine 2-Ketoglutarate Transaminase by Steroid Hormones in a Newly Established Tissue Culture Cell Line. Proc. Natl. Acad. Sci. U. S., *56:* 296-303, 1966.
43. Vasiliev, J. M., and Gerelstein, V. I. Sensitivity of Normal and Neoplastic Cells to the Damaging Action of Carcinogenic Substances: A Review. J. Natl. Cancer Inst., *31:* 1123-1151, 1963.
44. Wiebel, F. J., Leutz, J. C., Diamond, L., and Gelboin, H. V. Aryl Hydrocarbon (Benzo(a)pyrene) Hydroxylase in Microsomes from Rat Tissues: Differential Inhibition and Stimulation by Benzoflavones and Organic Solvents. Arch. Biochem. Biophys., *144:* 78-86, 1971.
45. Williams, D. J., and Rabin, B. R. Disruption by Carcinogens of the Hormone Dependent Association of Membranes with Polysomes. Nature, *232:* 102-105, 1971.
46. Willis, R. Pathology of Tumors. London: Butterworths, 1967.
47. Yang, N. C., Castro, A. J., Lewis, M., and Wong, T. W. Polynuclear Aromatic Hydrocarbons, Steroids, and Carcinogenesis. Science, *134:* 386-387, 1961.