[CANCER RESEARCH 46, 2771-2774, June 1986]

Effects of Steroid Hormones and Antisteroids on Alkaline Phosphatase Activity in Human Endometrial Cancer Cells (Ishikawa Line)[1]

Christian F. Holinka,[2] Hiroki Hata, Hiroyuki Kuramoto, and Erlio Gurpide

Department of Obstetrics, Gynecology and Reproductive Science, Mount Sinai School of Medicine, New York, New York 10029 [C. F. H., H. H., E. G.], and Department of Obstetrics and Gynecology, Kitasato University, Kanagawa-Ken, Japan [H. K.]

ABSTRACT

Alkaline phosphatase activity in human endometrial cancer cells of the estrogen-responsive Ishikawa line was markedly stimulated (3–20-fold in 4 days) by estrogens, 5α-dihydrotestosterone, and dehydroepiandrosterone but not by testosterone, medroxyprogesterone acetate, glucocorticoids, several peptide hormones, prostaglandins, or growth factors. Maximum responses to estradiol were obtained at concentrations between 10^{-9} and 10^{-7} M; at 10^{-8} M estradiol, the highest activity was reached 48–72 h after addition of the hormone. A linear relationship between enzyme activity at 48 h and the length of exposure to the hormone was observed. Dibutyryl cyclic guanosine 3′:5′-monophosphate, but not dibutyryl cyclic adenosine 3′:5′-monophosphate enhanced alkaline phosphatase activity and acted synergistically with estradiol. *trans*-4-Monohydroxytamoxifen completely antagonized the stimulatory effect of estradiol and had no agonistic activity. Dihydrotestosterone and dehydroepiandrosterone appear to exert their effects, at least in part, by interacting with estrogen receptors, since the simultaneous presence in the medium of monohydroxytamoxifen abolished their influence on alkaline phosphatase activity. The specific antiandrogen monohydroxyflutamide partially antagonized the effect of these hormones, suggesting that their action involved androgenic mechanisms as well.

Exposure to elevated temperature and to specific inhibitors identified alkaline phosphatase of Ishikawa cells as a placental-type isoenzyme, thus contrasting with the nonplacental type found in glandular epithelial cells of normal endometrium and in another human endometrial cancer cell line, HEC-50. This study extends our previous observations of estrogen responsiveness in the Ishikawa cell line. In addition to the previously reported stimulatory effects on growth and progesterone receptor levels, we are now describing the stimulation by estrogens and C_{19} steroids of an enzyme, alkaline phosphatase, which can be used as a convenient end point to examine mechanisms of hormonal action.

INTRODUCTION

It has been well established that some mammary and endometrial tumors are hormone responsive and may therefore be susceptible to hormonal therapy. However, experimental studies of hormonal effects on endometrial tumors have thus far been limited by the absence of an endometrial cell line clearly responsive to estrogens at concentrations approximating physiological levels.

We recently demonstrated estrogen responsiveness in a human cell line derived from a well-differentiated endometrial adenocarcinoma (Ishikawa cells), established by Nishida and coworkers at Tsukuba University, Ibaraki, Japan. Added to the culture medium of these cells, estradiol enhanced growth and substantially stimulated specific binding of progesterone. These effects were counteracted by OHTAM[3] (1). In the present report, we describe that estradiol and several other estrogens greatly enhance the activity of alkaline phosphatase, an enzyme known to be regulated by ovarian hormones in the nonpregnant and pregnant rodent and monkey uterus (2–5). In addition, we show stimulation of the enzyme by the C_{19} steroids DHT and DHEA. To define the mechanisms of action of these compounds, we used the antiandrogen OHFL in addition to OHTAM. The results obtained suggest that the stimulation of alkaline phosphatase by the C_{19} steroids is mediated by estrogen receptors as well as androgen receptors.

MATERIALS AND METHODS

Ishikawa cells were routinely cultured in minimum essential medium (Eagle's) containing Earle's salts (GIBCO, Grand Island, NY) and 15% FBS (GIBCO) treated with activated charcoal to remove steroid hormones. Unless otherwise specified, this was the medium in which all cells were cultured. To remove endogenous steroid hormones, the serum was treated as follows. FBS (100 ml), mixed with 0.25 g activated charcoal (Sigma Chemical Co., St. Louis, MO) and 0.025 g dextran (clinical grade; Sigma), was stirred at 56°C for 30 min and centrifuged to separate the dextran-coated charcoal pellet; the supernatant was then subjected to the same treatment at 37°C. Thereafter, the charcoal-dextran treated FBS was filtered through a 20-μm sterilization unit (Nalge Co., Rochester, NY) and stored at −20°C. The cells were kept in plastic culture dishes (Falcon Plastics, Los Angeles, CA) and harvested by brief exposure to trypsin (0.05%)-EDTA (0.02%) (Flow Laboratories, McLean, VA) at 37°C. After inhibition of the action of trypsin by addition of 7.5% charcoal-dextran treated FBS in minimum essential medium and pelleting, the cells were resuspended in culture medium and plated in 10-cm culture dishes at the required densities, usually 2.5×10^6 cells. Chemicals, hormones, and other compounds added to the medium to test alkaline phosphatase responsiveness of cell cultures were obtained from commercial sources at the highest available purity. Hydroxyflutamide was generously supplied by the Schering Corp., Bloomfield, NJ.

Alkaline phosphatase activity was assayed by a method involving the hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol at pH 10.4 and the spectrophotometric determination of the product at 400 nm, as described in detail elsewhere (2). Proteins were determined by the method of Lowry *et al.* (6) and DNA was quantitated by the method of Burton (7).

RESULTS

Addition of estradiol to culture media of Ishikawa cells at a concentration of 10^{-8} M greatly increased alkaline phosphatase activities in these cells. As illustrated in Fig. 1, responses were observed both in low-density and high-density cultures, containing approximately 260 and 440 μg DNA/10-cm dish, respectively. The levels of enzyme activities ranged between 5 and 8 times those found in control dishes and the patterns were similar when expressed per mg protein or per mg DNA.

The stimulation of alkaline phosphatase activity was dose dependent. As illustrated in Fig. 2, maximum activities after 96 h of exposure to estradiol were observed at a dose range of 10^{-9} to 10^{-7} M. Fig. 2 also reveals a substantial enhancement of alkaline phosphatase activities by addition of 0.5 mM Bt_2 cGMP to the culture medium. We observed this effect in three additional experiments at concentrations of 1 mM Bt_2 cGMP. In contrast, 1 mM Bt_2 cAMP had no effect on enzyme activity in the presence or absence of estradiol.

Although Bt_2 cGMP stimulated alkaline phosphatase to a small extent in the absence of estradiol, its effect in the presence of the hormone suggests that Bt_2 cGMP and estradiol acted synergistically. Since butyrate has been reported to stimulate alkaline phosphatase activity (8), we performed control exper-

Received 11/13/85; revised 2/18/86; accepted 2/21/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant HD 07197, awarded by the National Institute of Child Health and Human Development, and Grant CA 15648, awarded by the National Cancer Institute.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: OHTAM, *trans*-4-monohydroxytamoxifen; DHT, 5α-dihydrotestosterone; DHEA, dehydroepiandrosterone; OHFL, hydroxyflutamide; Bt_2 cAMP, dibutyryl cyclic AMP; Bt_2 cGMP, dibutyryl cyclic GMP; FBS, fetal bovine serum.

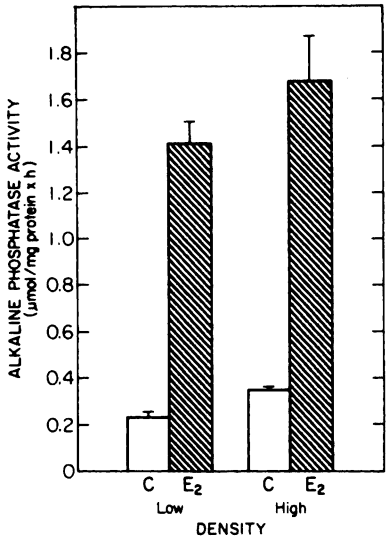

Fig. 1. Increases in alkaline phosphatase activities in low- and high-density cultures of Ishikawa cells. Cells were harvested from confluent dishes and plated into 10-cm culture dishes in a 1:12 split for low-density cultures and a 1:2 split for high-density cultures in the presence or absence of estradiol (10^{-8} M). Medium was changed 2 days thereafter and alkaline phosphatase activity was measured on day 4, as described in "Materials and Methods." The values represent means ± SEM of three dishes, assayed in triplicate. *C*, control; E_2, estradiol.

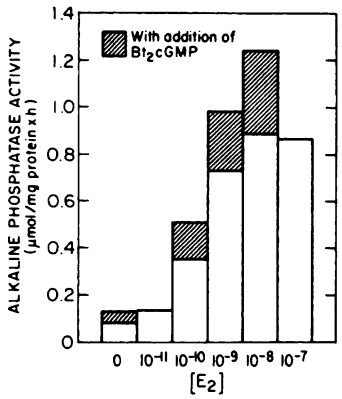

Fig. 2. Dose response of alkaline phosphatase stimulation by estradiol (E_2) (□) and its enhancement by Bt_2 cGMP. Cells were plated at a density of 2.5 × 10^6/10-cm dish in the presence of estradiol at the indicated concentrations. Parallel dishes were assayed containing 0.5 mM Bt_2 cGMP in addition to estradiol at different concentrations. Medium was changed 2 days after plating and enzyme activity was determined on day 4. The values represent means of two dishes assayed in triplicate. Individual values were within 10% of the mean.

iments to exclude the possibility that butyrate impurities present in the Bt_2 cGMP used in these studies may have contributed to the Bt_2 cGMP effects. Addition of butyrate to culture media at a concentration range from 0.25 to 25 μM, in the presence or absence of estradiol, did not alter enzyme activities.

To define the time course of increases in alkaline phosphatase activity, we measured the enzyme over a period of 4 days after addition of estradiol to the cultures. As illustrated in Fig. 3, alkaline phosphatase activity was increased by about 7-fold on day 2. Maximum levels of approximately 10 times those measured in control cultures were reached on day 3. The magnitude of enzyme stimulation was proportional to the length of hormone exposure. As is apparent from Fig. 4, the presence of estradiol for only 3 h doubled the enzyme activity measured 48 h after initial exposure of cells to the hormone. The activities increased in a linear fashion with increasing periods during which estradiol was present in the culture medium.

We next examined a series of hormones besides estradiol in order to define the specificity of enzyme responsiveness in Ishikawa cells. As illustrated in Table 1, estriol (10^{-8}, 10^{-6} M), ethynylestradiol (10^{-8} M), DHT (10^{-6} M), and DHEA (10^{-6} M)

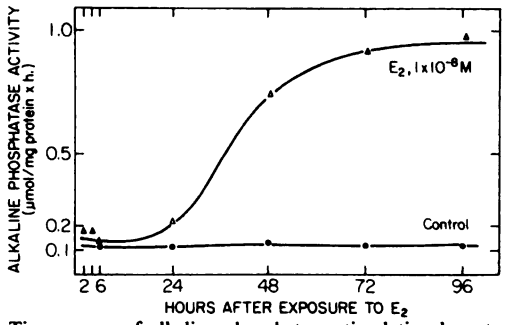

Fig. 3. Time course of alkaline phosphatase stimulation by estradiol (E_2). Cells were plated at a density of 2.5 × 10^6/10-cm dish and kept in hormone-free medium for 3 days. The medium was then replaced by fresh medium in the presence or absence of estradiol (10^{-8} M). Alkaline phosphatase activity was measured at the indicated periods. Values represent the means of two dishes assayed in triplicate. Individual values were within 10% of the mean.

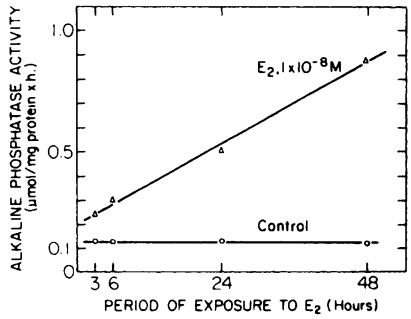

Fig. 4. Linear relationship between length of exposure to estradiol (E_2) and alkaline phosphatase activity 48 h after initial hormone exposure. Cells were plated at a density of 2.5 × 10^6/10-cm dish in hormone-free medium. The medium was replaced 3 days thereafter by fresh medium in the presence or absence of estradiol (10^{-8} M) and removed after 3, 6, or 24 h, as indicated. At that time, the dishes were washed thoroughly in hormone-free medium and kept in hormone-free medium to the time of enzyme assay 48 h after initial exposure to estradiol. Values represent the means of 2 dishes assayed in triplicate. Individual values were within 7% of the mean.

Table 1 *Effects of natural and synthetic steroid hormones and of monohydroxytamoxifen on alkaline phosphatase activity of Ishikawa cells*

Experiment	Addition to medium	Concentration (M)	Alkaline phosphatase activity (μmol/mg protein/h)	Fold increase
1	None		0.144	
	Estradiol	10^{-8}	1.17	8.1
	Estriol	10^{-8}	1.00	6.9
	Estriol	10^{-6}	1.05	7.3
	MPA[a]	10^{-6}	0.152	1.1
	Prednisolone	10^{-6}	0.172	1.2
	OHTAM	10^{-8}	0.191	1.3
	OHTAM	10^{-6}	0.186	1.3
2	None		0.172	
	Estradiol	10^{-8}	1.21	7.0
	MPA	10^{-6}	0.209	1.2
	Testosterone	10^{-6}	0.283	1.6
	Dexamethasone	10^{-6}	0.231	1.3
	Estradiol + MPA	$10^{-8} + 10^{-6}$	1.49	8.7
	Estradiol + OHTAM	$10^{-8} + 10^{-6}$	0.162	0.9
3	None		0.076	
	Estradiol	10^{-8}	1.55	20
	Estriol	10^{-8}	1.23	16
	Estetrol	10^{-8}	0.785	10
	Ethynylestradiol	10^{-8}	1.27	17
	DHT	10^{-6}	0.994	13
	DHEA	10^{-6}	1.40	18
4	None		0.095	
	Estradiol	10^{-8}	0.896	9.4
	Estrone	10^{-8}	0.515	5.4
	17α-Estradiol	10^{-8}	0.563	5.9

[a] MPA, medroxyprogesterone acetate.

increased the enzyme activities to levels comparable to those produced by estradiol (10^{-8} M). Estetrol [1,3,5(10)-estratriene-3,15α,16α,17β-tetrol]; (10^{-8} M), estrone (10^{-8} M), and 17α-estradiol (10^{-8} M) stimulated the enzyme with about one-half the potency of estradiol (10^{-8} M). Prednisolone (10^{-6} M), a compound which has been demonstrated to stimulate strongly the alkaline phosphatase activity in HeLa cells (9), dexamethasone (10^{-6} M), MPA (10^{-6} M), and testosterone (10^{-6} M) had no effect on the basal activity of the enzyme. Monohydroxytamoxifen (10^{-6} M) acted as a pure estrogen antagonist and completely abolished the estradiol (10^{-8} M)-stimulated alkaline phosphatase activity without exhibiting any estrogenic effects at 10^{-8} or 10^{-6} M concentrations. Medroxyprogesterone acetate (10^{-6} M) did not antagonize the effects of estradiol (10^{-8} M).

A number of other compounds, tested under the same experimental conditions, did not affect the enzyme. Among those were insulin (10 μg/ml), prolactin (250 ng/ml), epidermal growth factor (100 ng/ml), prostaglandin E_2 (10 μg/ml), prostaglandin $F_{2\alpha}$ (10 μg/ml), 12-*O*-tetradecanoylphorbol-13-acetate (0.32 μM), and nordihydroguaiaretic acid (50 μM), an inhibitor of arachidonic acid metabolism.

Since the stimulation of alkaline phosphatase by DHT and DHEA, shown in Table 1, may have occurred by interaction of these compounds with the estrogen as well as the androgen receptor, we designed experiments to selectively block each receptor by addition of the antiestrogen OHTAM (10^{-6} M) and the antiandrogen OHFL (10^{-4} M) to the culture medium. As illustrated in Fig. 5, OHTAM and OHFL by themselves did not alter enzyme activities. Added together with DHT or DHEA, however, OHTAM completely counteracted the effects of these compounds on stimulation of alkaline phosphatase, and OHFL reduced their stimulatory effect by 60 to 75%.

Using inhibitors generally recognized to distinguish organ-specific alkaline phosphatase isoenzymes, such as those of placenta, intestine, liver, or bone (10), we found that alkaline phosphatase activity of Ishikawa cells did not change on exposure to elevated temperature (57°C for 15 min) and was resistant to homoarginine (8 mM). In cultures exposed to estradiol, the enzyme was inhibited by about 40% in the presence of phenylalanine (5 mM), as illustrated in Table 2. These patterns characterize the placental group of organ-specific alkaline phosphatases (10).

Table 2 *Alkaline phosphatase isoenzymes in endometrial cells*

	Control activity (μmol/mg protein/h)	% of inhibition		
		Heat (57°C, 15 min)	Phenyl-alanine (5 mM)	Homo-arginine (8 mM)
Ishikawa cells[a]				
Control	0.140	8	0	0
Estradiol (10^{-8} M)	0.730	0	40	0
Endometrial glands[b]				
Proliferative	5.8 (n = 4)	78 (n = 1)	30 (n = 2)	85 (n = 2)
Secretory	1.0 (n = 4)	90 (n = 1)	8 (n = 3)	
Placenta[c]	20.4	0	75	5

[a] The values represent means of 2 separate experiments performed in triplicate. The cultures were exposed to estradiol for 4–6 days.
[b] The values for endometrial glands are presented for comparison and were taken from Ref. 12. Inhibitory patterns of whole endometrial tissues, also presented in Ref. 12, were similar to those of endometrial glands.
[c] Values for percentage of inhibition taken from Ref. 9. Control activity: Holinka and Gurpide, unpublished data.

DISCUSSION

Alkaline phosphatase provided us with a convenient and sensitive end point to investigate the responsiveness of human endometrial cancer cells to several hormones. As demonstrated in our results, estriol produced increases in the activity of this enzyme similar to those observed with estradiol at the same concentration (10^{-8} M). A comparable potency of estradiol and estriol has also been observed in studies of estrogenic stimulation of prostaglandin $F_{2\alpha}$ in cultured fragments of human endometrium (11). The observation of partial estrogenic effects of estetrol, added to the culture medium at 10^{-8} M, extends to human cells our previous finding in the rodent uterus, where estetrol produced increases in weight, total protein, and progesterone receptors (12).

Interestingly, exposure of cultures to estradiol for intervals as short as 3 h sufficed to double enzyme activities 48 h after hormone addition. The proportionality between the length of exposure to estradiol and the magnitude of enzyme increases suggests that the period of time during which estradiol was present in the culture medium was the major factor determining the increases in enzyme activities and that residual intracellular estradiol after medium change played a negligible role.

Addition to the medium of DHT or DHEA, but not of testosterone, produced large increases in alkaline phosphatase activity. The absence of testosterone effects may therefore indicate that DHT is the active metabolite and, further, that activities of 5α-reductase, the enzyme converting testosterone to DHT, may be low or absent in these cells. Simultaneous addition of OHTAM to the culture medium at equimolar concentrations completely blocked the action of DHEA and DHT. When added at a 100-fold molar excess, it reversed the stimulatory effect of estradiol. Hydroxyflutamide, at 10^{-4} M levels, largely, but not entirely, counteracted the effects of DHEA and DHT. Apparently, the C_{19} compounds interacted under these experimental conditions with both the androgen and the estrogen receptors. Partial interaction of OHTAM with the androgen receptor may be necessary to explain the complete inhibition of DHEA and DHT effects on alkaline phosphatase activity in Ishikawa cells, if OHFL is considered not to bind to the estrogen receptor.

Our results on the effects of temperature and of compounds that are commonly used to identify organ-specific isoenzymes permit several interesting conclusions: (*a*) Ishikawa cells, a transformed cell line originating from glandular epithelial cells of endometrium, express a placental-type alkaline phosphatase isoenzyme, in contrast to the nonplacental isoenzyme identified in human endometrium (13, 14) and in endometrial glandular epithelial cells (13); (*b*) the expression of the placental-type alkaline phosphatase is not a fixed property of endometrial adenocarcinoma cells, since HEC-50 cells, another human endometrial adenocarcinoma cell line, have the nonplacental type characteristic of normal human endometrium (14); (*c*) the estradiol-stimulated component of alkaline phosphatase, but not

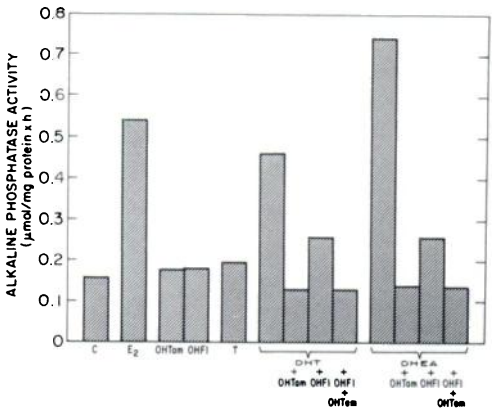

Fig. 5. Effects of estradiol and C_{19} steroids on alkaline phosphatase activity and antagonistic action of hydroxytamoxifen and hydroxyflutamide. Cells were plated at a density of 2.5 × 10^4/10-cm dish in hormone-free medium or in the presence of compounds as indicated. Medium was changed 2 days thereafter and alkaline phosphatase activity was assayed on day 4. Values represent the means of two dishes assayed in triplicate. Compounds were added at these concentrations: estradiol, 10^{-8} M; OHTAM, 10^{-6} M; OHFL, 10^{-4} M; testosterone, 10^{-6} M; DHEA, 10^{-6} M. Individual values were within 20% of the mean. *C*, control; E_2, estradiol; *T*, testosterone.

the basal levels, appeared to be sensitive to inhibition in our experiments. This finding suggests that estradiol stimulated an isoenzyme different from the basal enzyme, a possibility that remains to be experimentally examined.

The synergistic enhancement of responsiveness to estradiol when the hormone was added to the medium together with Bt_2 cGMP is interesting in view of the increase in the number of specific estrogen-binding sites by cGMP that has been reported by Fleming et al. (15, 16).

REFERENCES

1. Holinka, C. F., Hata, H., Kuramoto, H., and Gurpide, E. Responses to estradiol in a human endometrial carcinoma cell line (Ishikawa). J. Steroid Biochem., in press, 1986.
2. Holinka, C. F., and Finch, C.E. Age-related changes in the decidual response of the C57BL/6J mouse uterus. Biol. Reprod., *16:* 385–393, 1977.
3. Holinka, C. F., Hetland, M. D., and Finch, C. E. The response to a single dose of estradiol in the uterus of ovariectomized C57BL/6J mice during aging. Biol. Reprod., *17:* 262–264, 1977.
4. Holinka, C. F., and Gurpide, E. *In vivo* effects of estetrol on the immature rat uterus. Biol. Reprod., *20:* 242–246, 1979.
5. Atkinson, W. B., and Engle, E. T. Studies on endometrial alkaline phosphatase during the human menstrual cycle and in the hormone-treated monkey. Endocrinology, *40:* 327–333, 1947.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.
7. Burton, K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., *65:* 315–323, 1956.
8. Koyama, H., and Ono, T. Induction by short-chain fatty acids of alkaline phosphatase activity in cultured mammalian cells. J. Cell. Physiol., *88:* 49–56, 1976.
9. Cox, R. P., and McLeod, C. M. Regulation of alkaline phosphatase in human cell cultures. Cold Spring Harbor Symp. Quant. Biol., *29:* 233–251, 1964.
10. Fishman, W. H. Perspectives on alkaline phosphatase isoenzymes. Am. J. Med., *56:* 617–650, 1974.
11. Schatz, F., Markiewicz, L., and Gurpide, E. Effects of estriol on $PGF_{2\alpha}$ output by human endometrium and endometrial cells. J. Steroid Biochem., *20:* 999–1003, 1984.
12. Holinka, C. F., Bressler, R. S., Zehr, D. R., and Gurpide, E. Comparison of effects of estetrol and tamoxifen with those of estriol and estradiol on the immature rat uterus. Biol. Reprod., *22:* 913–926, 1980.
13. Holinka, C. F., and Gurpide, E. Hormone-related enzymatic activities in normal and cancer cells of human endometrium. J. Steroid Biochem., *15:* 183–192, 1981.
14. Kuramoto, H., Tamura, S., and Notake, Y. Establishment of a cell line of human endometrial adenocarcinoma *in vitro*. Am. J. Obstet. Gynecol., *114:* 1012–1019, 1972.
15. Fleming, H., Blumenthal, R., and Gurpide, E. Rapid changes in specific estrogen binding elicited by cGMP and cAMP in cytosol from human endometrial cells. Proc. Natl. Acad. Sci. USA, *80:* 2486–2490, 1983.
16. Fleming, H., Bumenthal, R., and Gurpide, E. Characteristics of cyclic nucleotide dependent regulation of cytoplasmic estradiol binders in cultured endometrial and breast cells. J. Steroid Biochem., *20:* 5–9, 1984.