Biochemical Pharmacology of Antiestrogen Action

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I. Introduction

NONSTEROIDAL antiestrogens have been available as clinically useful drugs for more than 20 years. One of the first compounds to enter clinical trial, Clomid [MRL41, a mixture of the E and Z geometric isomers of 2 chloro-1-(4β diethyl aminoethoxyphenyl) 1,2 diphenylethylene] is successfully used, in short courses of 5 to 10 days, for the induction of ovulation in subfertile women (5, 116, 118, 140). It should be pointed out, though, that this application of antiestrogens is particularly ironic because these drugs are potent antifertility agents in laboratory animals and were originally planned to be introduced as postcoital contraceptives (83). Nolvadex [tamoxifen, ICI 46,474, Z-1(4β dimethylaminoethoxyphenyl) 1,2 diphenylbut-1-ene citrate] is also used in some countries for the induction of ovulation (107, 192, 323); however, its major clinical application is in the treatment of breast cancer.

In 1896, Beatson first demonstrated that some patients with advanced breast cancer will respond to oophorectomy (18). This original observation has been adequately confirmed and it seems that about one-third of patients with breast cancer will respond to this form of treatment. Similarly, about one-third of postmenopausal patients will respond to adrenalectomy; however, in this case, it is the source of androgens that is being removed. Androgens are converted to estrogens by peripheral aromatizing enzyme systems (219).

The finding that a receptor molecule for estrogen is present in varying concentrations in breast tumors (95, 151, 196, 203, 274) reinforced the hypothesis (100, 145) that the determination of estrogen binding might be useful as a predictive test to preselect patients for endocrine therapy. Ten years ago (28) the usefulness of the estrogen receptor assay to determine hormone respon-

siveness was evaluated. This world-wide study was carefully monitored and the clinical case studies were independently assessed and correlated with hormone receptor binding determinations. Approximately 60% of patients with estrogen receptor positive (>10 femtomol/mg of cytosol protein) breast tumors responded to endocrine therapy, whereas less than 10% of patients with estrogen receptor negative tumors (<10 femtomol/mg cytosol protein) responded.

It is generally believed that estrogen can directly stimulate the growth of breast cancer; therefore, therapy with antiestrogens that block the binding of estradiol to the estrogen receptor (ER) provides a logical medical approach to prevent estrogen action. This strategy avoids the risks of ablative surgery and, in the case of adrenalectomy, the long-term side effects of steroid replacement.

The nonsteroidal antiestrogens nafoxidine (25, 89, 130), enclomiphene (126, 128), and tamoxifen (62, 314) were all tested in phase I and phase II clinical trials. The objective response rate of each of the antiestrogens was similar (~30%) but only tamoxifen has a low incidence of side effects (207, 232) and is at present the only antiestrogen available for the treatment of breast cancer. The results of these early clinical trials for the treatment of advanced breast cancer have been reviewed (127, 207, 232).

In recent years, attention has focused upon the use of tamoxifen as an adjuvant therapy following mastectomy. The rationale for this application is that the drug is relatively nontoxic compared to cytotoxic chemotherapy, and continuous therapy with antiestrogens might prevent the recurrence of hormone-sensitive disease. Laboratory studies with carcinogen-induced mammary tumor models tend to support this application (156, 160). Two approaches to the clinical evaluation of antihormone therapy are being investigated: either the use of tamoxifen alone as adjuvant therapy (17, 245, 251, 264) or tamoxifen in combination with a cytotoxic chemotherapeutic regimen (98, 99, 138). A preliminary analysis of the ongoing combination hormono/chemotherapy trials is encouraging (98, 99) with a reduction in the recurrence rate of postmenopausal patients who had tumors with high levels of estrogen receptor. However, to maintain the gains achieved in the 1- or 2-year treatment trials (17, 99), studies are underway to continue tamoxifen therapy for up to 5 years (311). Tamoxifen may be a tumorstatic rather than a tumoricidal agent (242, 293) so that long treatment regimens should be required to prevent cells from being recruited to divide.

The ubiquitous use of tamoxifen in breast cancer therapy has focused attention upon the development of new antiestrogens, an area of research that is currently attracting much interest. Additionally, a better understanding of antiestrogen action will not only define the mechanism of estrogen-controlled growth but also help

to identify further targets that might be vulnerable to new drugs.

There are several earlier reviews on the pharmacology and antitumor activity of antiestrogens (127, 154, 161, 178, 294, 297) that the reader might consult to obtain a broader understanding of this class of drugs. The aim of this review is to present a synthesis of the recent results and ideas on the biochemical and molecular pharmacology of the nonsteroidal antiestrogens. Special consideration will be given to metabolism, receptor-mediated mechanisms of action, and structure-activity relationships. However, it is important to review briefly the development and general pharmacology of antiestrogens in order to demonstrate the complexities encountered with a simple definition such as "antiestrogen."

II. Historical Development

The pioneering studies by Dodds and coworkers established the structure-activity relationships for nonsteroidal estrogens (69, 70, 72). Diethylstilbestrol (DES) (figure 1) was the most potent compound found (71, 73). Further structure-activity studies demonstrated that substituted triphenylethylenes are also estrogenic (256) but compared to DES these derivatives tend to be long-acting (258). α,αDi(p-ethoxyphenyl)-β-bromoethylene (DBE) and trianisylchloroethylene (TACE) are very long-acting estrogens (257, 307, 308) (figure 1), an effect that has been attributed to their ability to form depots in body fat (117, 255, 308).

Early attempts to develop estrogen antagonists focused on physiological antagonism with androgens and proges-
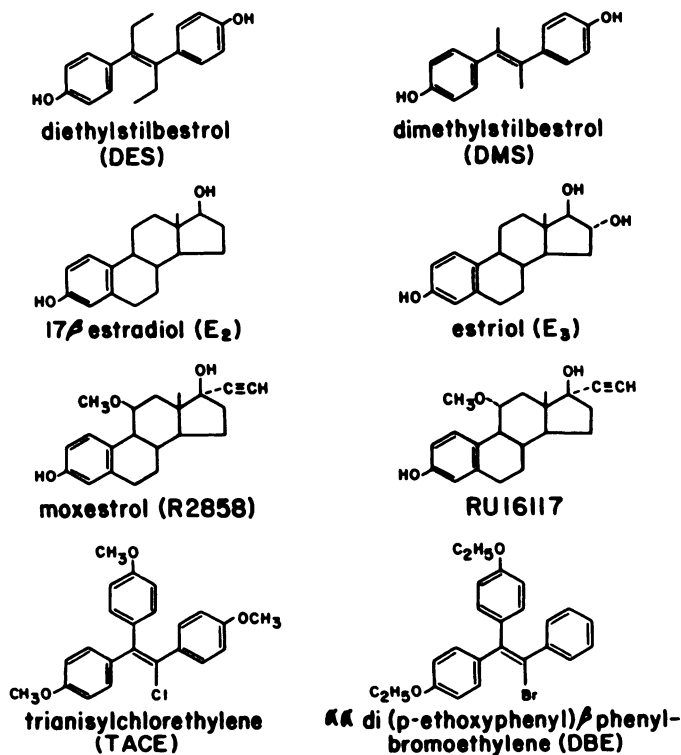

FIG. 1. Steroidal and nonsteroidal estrogens.

togens (79). The first clues that direct antagonism of estrogen action was possible occurred with the finding that the simultaneous administration of dimethylstilbestrol (DMS) (figure 1) and estradiol into the ovariectomized mouse vagina prevents the full vagina cornification that is produced with estradiol alone (85, 86). A similar result (49) can be obtained with 3,3′, 5,5′ tetramethyl α,β-diethylstilbestrol, a compound that reduces the duration of [³H]estradiol binding in vaginal tissues (50). However, neither of these compounds is an antiestrogen when administered systemically (49, 85).

Lerner and coworkers (210) described the pharmacological properties of the first systemically active, nonsteroidal antiestrogen, ethamoxytriphetol (MER25) (figure 2). The compound is virtually devoid of estrogenic activity in a wide variety of species (mice, rats, rabbits, chickens, and monkeys) and is an estrogen antagonist, but with low a potency. An exciting pharmacological property of MER25 and the related compound MRL37 (figure 2) is their antifertility actions in laboratory animals (14, 44, 282), an observation that stimulated a search for more potent agents for clinical application. Clomiphene (known originally as chloramiphene and MRL41) (132, 283), nafoxidine (U-11,100A) (75), nitromifene (CI628 or CN-55,945-27) (37), and tamoxifen (ICI 46,474) (122, 123) (figures 2, 3) are all the result of that search, but clinical application as postcoital contraceptives was found to be inappropriate. In the late 1960s enthusiasm for continued research by the pharmaceutical industry waned. In India, however, structure-activity relationship studies continued with an investigation of
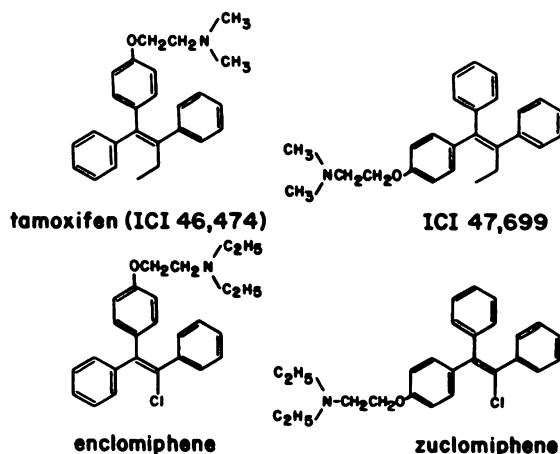

FIG. 3. The geometric isomers of substituted triphenylethylenes.

indene and chroman derivatives related to nafoxidine. The agent, centchroman (figure 2), was developed for clinical testing as a postcoital contraceptive (175).

The successful introduction of tamoxifen for the treatment of breast cancer (232, 314) provided the incentive for a renewed investigation of the structure-activity relationships of antiestrogens. This time the potential application is as antitumor agents for hormone-dependent disease. Trioxifene (152) (figure 2) is a compound related to nafoxidine but diverges from the general triphenylethylene structure by the introduction of a ketone that links the phenyl ring with the alkylaminoethoxy side chain to the rest of the molecule. The general pharmacology of trioxifene is very similar to that of tamoxifen (21, 165, 265). Phase II clinical trials have shown activity in the treatment of breast cancer (294) but the drug is not generally available for therapy.

The finding that a metabolite of tamoxifen, 4-hydroxytamoxifen (also called monohydroxytamoxifen or metabolite B) (figure 4), is a potent antiestrogen in the rat with a binding affinity for the ER equivalent to that of estradiol (159), stimulated a search for compounds with potential use as new research tools and anticancer agents. The hydroxylated metabolites of several antiestrogens are now known to have higher potency than their parent compounds (125). The hydroxylated triphenylethylene H-1285 (figure 4) is an interesting derivative because its binding affinity for the ER is reportedly 10 times higher than that of estradiol (271). These compounds are, however, all partial agonists in rat uterine assays in vivo. It can be argued that the ideal antitumor agent should have negligible estrogen agonist activity and be a potent antagonist with a high affinity for the ER. To this end, two novel antiestrogens, LY117018 (21) and LY156758 (23) (figure 4) have been introduced. Both compounds have a high affinity for the ER and low estrogenic activity in tests in vivo. Antitumor activity is observed in vivo (59) and in vitro (280), but no clinical trials have been reported that evaluate their efficacy in patients with breast cancer.
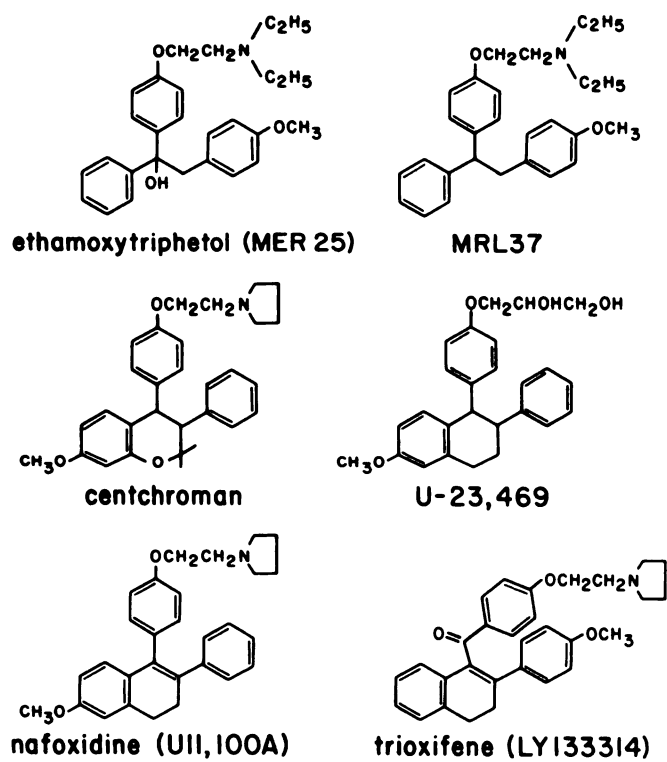
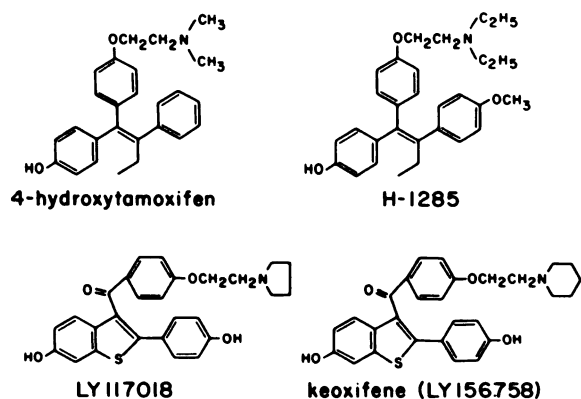

FIG. 2. Nonsteroidal antiestrogens.

FIG. 4. Hydroxylated nonsteroidal antiestrogens with a high binding affinity for the estrogen receptor.

In reviewing the historical development of antiestrogens, it is possible to define two types of antiestrogens that function through an ER-mediated mechanism.

III. General Classification of Antiestrogens

A. Estrogens with a Rapid Dissociation Rate from the Estrogen Receptor

Dimethylstilbestrol (DMS) was the first example of this type of antiestrogen. Studies with radiolabeled DMS have demonstrated an interaction with the estrogen receptor (39); however, continual exposure of target tissues to the compound produces a full agonist response (222). A similar situation occurs with the steroids, estriol and RU16117 (figure 1). Estriol exhibits a low binding affinity for the ER (194) and in short-term tests, produces an inhibition of estradiol-stimulated increases in uterine weight (55). Indeed, estriol is apparently sufficiently "antiestrogenic" to inhibit the induction of hormone-dependent rat mammary tumors with dimethylbenzanthracene (DMBA) (208). This led Lemon (208) to propose that this action might be important for the protection of patients from developing breast cancer. Thus, high levels of circulating estriol would be beneficial. Unfortunately, continual exposure of target tissues to estriol produces full estrogenic effects (218); only intermittent administration of estriol produces partial estrogenic action and blocks the effects of estradiol administered intermittently (53, 55). RU16117 also inhibits carcinogenesis with DMBA (187) and inhibits the growth of established DMBA-induced tumors (186). However, again RU16117 has a low affinity for the ER and a rapid dissociation rate. This property, which contrasts with the high binding affinity of the isomer R2858 (figure 1), prompted Bouton and Raynaud (31) to suggest that rapid dissociation of a ligand from the receptor could account for antiestrogenic activity. Unfortunately, this principle cannot be extrapolated to the triphenylethylene-type of antiestrogens.

B. Triphenylethylene Derivatives

These compounds all have a structural similarity to the estrogen triphenylethylene but are distinguished by a strategically substituted side chain, usually an alkylaminoethoxy group. However, a glyceryl side chain can be effective and is present in compounds like U-23,469 (figure 2). Most of these antiestrogens have a low affinity for the ER in vitro, so Korenman (195) suggested that this factor was important to explain their mode of action. However, triphenylethylene antiestrogens with a correctly positioned phenolic hydroxyl groups have an affinity equivalent to that of estradiol (159). Therefore, the structure of the drug, rather than its receptor affinity, became of primary importance for future study. The mechanism of action of antiestrogens is believed to be related to their structure whereas their potency is related to their relative affinity for the ER (211).

IV. General Pharmacology

An antiestrogen is usually identified and classified as a compound that will inhibit the vaginal cornification produced by estradiol in ovariectomized rats or will inhibit the increase in uterine weight produced by estradiol in immature rats. Typical results from a uterine weight test are shown in figure 5. Two active compounds of different potencies, tamoxifen and trioxifene, are compared for their ability to inhibit, in a dose-related manner, the uterotrophic effects of simultaneously administered estradiol. The compound LY126412, trioxifene without an alkylaminoethoxy side chain, is inactive at the doses tested.
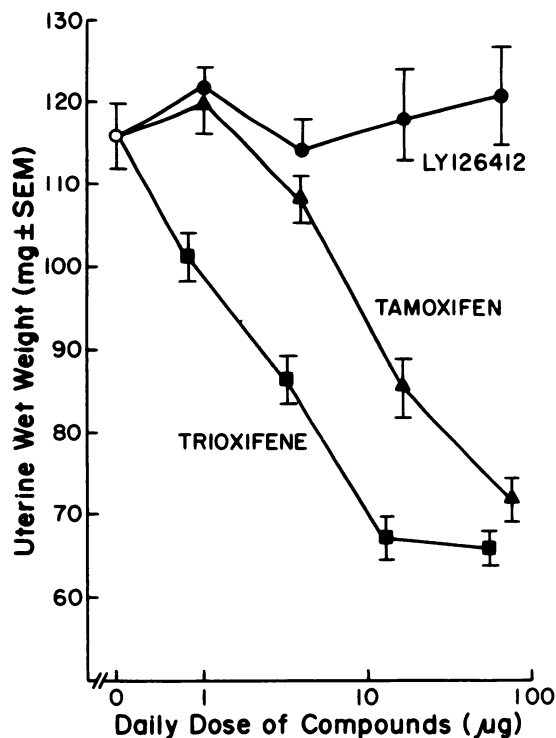

FIG. 5. The antiestrogenic effect of trioxifene, tamoxifen, and LY 126412 in the 4-day immature rat uterine weight test. Increasing daily doses of the compounds were administered with a standard dose of estradiol-17β (0.32 μg) and compared with estradiol alone. Eight rats per group. Data from Jordan and Gosden (165).

The nonsteroidal antiestrogens are competitive inhibitors of estrogen-induced increases in uterine weight. Increasing doses of estradiol can reverse the antiestrogenic action of MER25 in the mouse uterus (209) and the antiestrogenic action of LY117018 and 4-hydroxytamoxifen in the immature rat uterus (166). Similar competition or "rescue" experiments have been performed in vitro. The suppressive effects of an antiestrogen on cell growth can be reversed by the addition of excess estradiol to the culture medium (212, 216).

The triphenylethylene-types of antiestrogens are partial agonists with regard to inducing the growth of the immature rat uterus. However, the uterus is a heterogeneous mixture of cell types that respond differentially to the antiestrogens. The luminal epithelial cells are fully stimulated to an increase in size which is indistinguishable from a full estrogen-stimulated increase (47, 52). However, the hypertrophy that occurs as a result of the triphenylethylenes is not associated with either an increase in [³H]thymidine incorporation (176) or cell division (68). The stromal and myometrial cells are stimulated to a lesser extent by the triphenylethylene derivatives. It is interesting to note, however, that the antiestrogen LY117018 (166), which is only weakly uterotrophic in the rat, does not stimulate a large increase in the size of luminal epithelial cells.

The pharmacology of the so-called antiestrogens, however, is extremely complex and, at times, rather inconsistent. The drugs have different properties in different estrogen target tissues and different species. For this reason it has been difficult to establish a unifying mechanism of action for the compounds under all test conditions. It is therefore important to establish the validity of using an "antiestrogen" in a particular system before concluding that the effect observed results from an antagonism of estrogen action.

Part of the confusion with the definition and description of antiestrogens has developed from the early interpretation of the pharmacological properties of the geometric isomers of clomiphene and tamoxifen (figure 3). Originally the *cis* geometric isomer of clomiphene (isomer B) was shown to have antiestrogenic (67, 244, 284) and antitumor (281) properties in the rat, while the *trans* geometric isomer (isomer A) was primarily estrogenic (67, 284). In contrast, tamoxifen, the *trans* isomer of a substituted triphenylethylene is an antiestrogen (121, 169) in the rat with antitumor properties (161) whereas the *cis* isomer ICI47,699 is an estrogen (121, 169). The structure of ICI47,699 as the *cis* isomer was confirmed by x-ray crystallography (189), but in the light of new analytical information, the supposed *cis* and *trans* isomers of clomiphene were renamed enclomiphene and zuclomiphene, with an acknowledgment that the original designation was incorrect. Special attention should be paid to this particular point because an investigator may inadvertently obtain mislabeled samples of the isomers

(277). Enclomiphene (*trans* isomer) is a partial agonist with antiestrogenic activity in the immature rat whereas zuclomiphene (*cis* isomer) is an estrogen (52, 169).

The biological activity of the geometric isomers of 4-hydroxytamoxifen and CI628 has been evaluated and both isomers appear to be antiestrogenic (169). The Z isomers (related to the *trans* structure of tamoxifen) are very potent compared with the E isomers, but it is possible that fractional conversion from a compound with low biological activity to one with high antiestrogenic potency can cloud the pharmacological assessment of the E(*cis*) isomer. The isomers of hydroxylated triphenylethylenes are unstable in solution and in a recent study with the tritium-labeled E isomer of 4-hydroxytamoxifen, conversion to the Z isomer occurred under cell culture conditions (182).

Different species have different degrees of agonist and antagonist actions to nonsteroidal antiestrogens in their target tissues. This particularly important aspect of the pharmacology of antiestrogens will be considered in conjunction with their duration of action and metabolism.

A. Species Differences

MER25 is considered to be uniformly antiestrogenic in all species (210) although very slight estrogenic actions have been noted by some workers (60, 327). Compounds based upon triphenylethylene are more potent antiestrogens in the rat but they are also partial agonists (37, 122). In the mouse, mixed results are reported depending upon the test system and the compound tested. In the immature mouse uterine weight test, tamoxifen is a full agonist (306) with no detectable antiestrogenic activity (305). Similarly, in ovariectomized mice, the systemic administration of tamoxifen in an Allen-Doisy test results in full vaginal cornification (121); however, this response is dose- and time-dependent. A large dose (3 mg) of tamoxifen causes an initial stimulation of the vagina epithelium, but the cornification is transient and the animals develop a leukocytic smear that is refractory to estrogen stimulation for several weeks (84, 153). The vagina is hypertrophied (223) but the changes are different than those observed during continued estrogen administration (294). In contrast, 3,4-dihydroxytamoxifen (162) and LY117018 (21, 167) are both partial agonists with antiestrogenic properties in overiectomized mouse uterine weight tests. Both compounds also have less estrogenic activity in the immature rat and antiestrogenic activity is retained (21, 159, 166). Therefore, antiestrogens can be designed with high potency and low intrinsic activity as estrogens.

In the chick oviduct (20, 291, 295) and liver (41, 200), the triphenylethylene-based compounds are uniformly antiestrogenic with virtually no detectable agonist actions.

B. Duration of Action

Studies with radiolabeled antiestrogens have demonstrated long biological half-lives in animals (102, 158, 181) and man (103). The prolonged biological half-lives of triphenylethylene antiestrogens is not surprising since the related estrogenic derivatives DBE (257) and TACE (117) (figure 1) have a long duration of action which has been ascribed to their high solubility in body fat. Although the high lipophylicity of nonsteroidal antiestrogens might contribute to their long duration of action, the ubiquitous presence of so-called "antiestrogen binding components," in many (193, 290, 298), if not all, tissues will also serve to retard metabolism and excretion. The binding components have a very high affinity for triphenylethylene antiestrogens with alkylaminoethoxy side chains (290); triphenylethylenes without the side chain do not appear to bind (290). Another contribution to extended biological activity is the enterohepatic recirculation of metabolites. The primary route of excretion of the conjugated metabolites of tamoxifen is via the bile duct; however, studies in the rat and dog have demonstrated reabsorption of hydrolyzed metabolites.

The hydroxylated metabolite of tamoxifen, 4-hydroxytamoxifen, would be expected to have reduced lipophylicity and an increased sensitivity to metabolic conjugation. The affinity of 4-hydroxytamoxifen for "antiestrogen binding components" is reduced compared with tamoxifen (290). These factors might explain the shorter duration of action of 4-hydroxytamoxifen compared to tamoxifen (155). Similarly, the hydroxylated antiestrogen CI680M has a short action compared to its methyl ether CI680 (97).

The polyhydroxylated compounds LY117018 and LY156758 have a short duration of action in vivo (167; V. C. Jordan, B. Gosden, and E. M. Cormier, unpublished observation). Although LY117018 has a high affinity for estrogen receptors (24) there is a reduced affinity for "antiestrogen binding components" of the rat (290, 326). Clearly this will facilitate a rapid metabolism and excretion.

V. Metabolism

The clinical use of antiestrogens has focused much attention upon their pharmacokinetics and metabolism. Two different experimental approaches have been taken: (a) the development of specific analytical techniques to study serum levels of tamoxifen and its metabolites in patients; and (b) the synthesis of radiolabeled antiestrogens to study metabolism in animals.

A. Metabolites of Tamoxifen

Fromson and coworkers (102, 103) were the first to describe the metabolism of tamoxifen in laboratory animals and women. Initially a range of metabolites (A–F) was characterized in laboratory animals by the isolation of radiolabeled metabolites of ^{14}C-labeled tamoxifen us-

ing thin-layer chromatography (TLC). R_fs were compared with synthetic standards and the structure of some metabolites were confirmed by gas chromatography/mass spectrometry (GC/MS) (metabolites B and E). Initially, metabolite B (4-hydroxytamoxifen) was believed to be the major metabolite in man (103); however, there is now adequate evidence to prove that N-desmethyltamoxifen (known also as metabolite X) is the major metabolite (3). The misidentification occurred because 4-hydroxytamoxifen and N-desmethyltamoxifen have the same R_f value by TLC with the particular solvent system used (benzene/triethylamine). The known metabolites of tamoxifen in animals and man are shown in figure 6. Recently, two other metabolites of tamoxifen have been identified in patient sera: metabolite Y (13, 157, 188) (a deaminated derivative of tamoxifen (figure 6)) and metabolite Z (188) (the didemethyl derivative of tamoxifen). Kemp and coworkers (188) have suggested that tamoxifen is first converted to N-desmethyltamoxifen, then to didesmethyltamoxifen (primary amine), and finally to metabolite Y (primary alcohol). The mechanism for this final conversion has not been elucidated.

B. Analytical Techniques

The analytical techniques that are available to detect tamoxifen and its metabolites in patient sera are compared in table 1. The TLC and HPLC methods both depend upon the conversion of triphenylethylenes to fluorescent phenanthrenes for their detection. The reaction, originally described by Mallory et al. (220), was adapted by Sternson and coworkers (229) to identify tamoxifen and 4-hydroxytamoxifen in plasma samples.
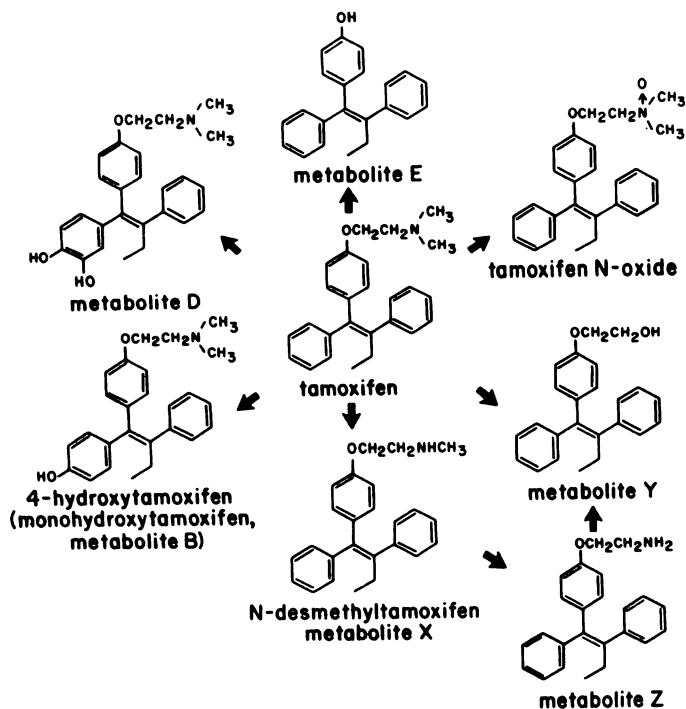

FIG. 6. Metabolites of tamoxifen in animals and man.

TABLE 1
Comparison of the assay methods available to measure the concentration of tamoxifen and its metabolites in biological fluids

Assay Method	Compound Identified (ref.)	Sensitivity (per ml)
TLC*	1. Tamoxifen (4)	2.5 ng
	2. Tamoxifen, N-desmethyltamoxifen, metabolite Y and metabolite Z (188)	ND
HPLC	1. Tamoxifen, 4-hydroxytamoxifen (229)	1 ng
	2. Tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen (111)	0.1 ng
	3. Tamoxifen, N-desmethyltamoxifen (34), metabolite Y (34)	<5 ng
	4. Tamoxifen, N-desmethyltamoxifen, 4-hydroxytamoxifen (36)	<1 ng
GC/MS	1. Tamoxifen (106)	ND
	2. Tamoxifen, 4-hydroxytamoxifen (65)	1 ng
	3. Tamoxifen 4-hydroxytamoxifen N-desmethyltamoxifen (64)	ND

* TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; ND, not determined.

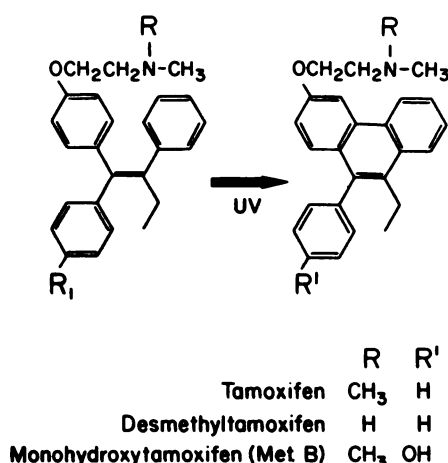|	R	R'
Tamoxifen	CH₃	H
Desmethyltamoxifen	H	H
Monohydroxytamoxifen (Met B)	CH₃	OH

FIG. 7. The conversion of triphenylethylenes to fluorescent phenanthrenes by ultraviolet (UV) activation.

The triphenylethylenes are first extracted with diethylether and converted to phenanthrenes (figure 7) before chromatography. The methodology has been improved and modified (111) but suffers from no internal standardization, the large amounts of serum used for assay, and the poor control that is available during the ultraviolet activation reaction. Recent developments have used internal standards, postcolumn fluorescent activation (34), and preliminary purification from interfering substances by using Sep-Pack C₁₈ cartridge (Water Assoc., Milford, MA) (36). It should also be pointed out that the extraction methodology recommended by Golander and Sternson (111) converts 50% of the 4-hydroxytamoxifen to its phenanthrene derivative. Clearly ether extraction should be avoided if postcolumn fluorescence

activation is to be used since this will lead to an underestimate of 4-hydroxytamoxifen.

C. Pharmacokinetics

Each of the assay procedures has been used to monitor the pharmacokinetics of tamoxifen, and in some cases, its metabolites, in patients. Overall, the studies confirm that tamoxifen has a long biological half-life and readily accumulates to steady-state levels upon repeated administration. A single oral dose of 10 mg of tamoxifen produces peak serum levels of 20 to 30 ng of tamoxifen/ml within 3 to 6 hr, but patient variation is very large (92). Nevertheless, continuous therapy with either 10 mg bid (92) or 20 mg bid (240) produces a steady state in serum within 4 weeks. The administration of loading doses (92, 322) has been recommended to raise the level of drug in the blood rapidly, followed by daily maintenance doses of 20 mg. As yet, no therapeutic benefit has been reported for this approach.

In general, the serum levels of N-desmethyltamoxifen are between 50% and 100% above the levels observed with tamoxifen, but again the patient-to-patient variation is very great. Patients taking 20 mg of tamoxifen bid achieve a steady state for tamoxifen at 4 weeks and for N-desmethyltamoxifen at 8 weeks. These data have been used to calculate approximate biological half-lives of 7 and 14 days for tamoxifen and N-desmethyltamoxifen, respectively (246). Metabolite Y (157) and 4-hydroxytamoxifen (64, 65, 91) are both minor metabolites of tamoxifen although it should be borne in mind that 4-hydroxytamoxifen has a binding affinity for human breast tumor estrogen receptors that is about 50 to 100 times greater than that of tamoxifen (91). Examples of the range of blood or serum concentration to be expected with various treatment regimens are illustrated in table 2.

TABLE 2
Comparison of the concentration of tamoxifen and its metabolites in patient blood during therapy for breast cancer.

Dose	Compound Measured (ref.)	Concentration (ng/ml)
10 mg bid	Tamoxifen	167 (143–197)*
	4-hydroxytamoxifen (65)	3 (2–5)
10 mg bid	Tamoxifen	113 (77–189)†
	N-desmethyltamoxifen	204 (163–265)
	Metabolite Y (157)	18 (5–49)
20 mg bid	Tamoxifen	300 (270–520)*
	N-desmethyltamoxifen	462 (210–761)
	4-hydroxytamoxifen (64)	7 (3–11)
20 mg bid	Tamoxifen	310 (164–494)†
	N-desmethyltamoxifen	481 (300–851)
	Metabolite Y (188)	49 (22–136)
20 mg/m² bid after loading doses	Tamoxifen	163 (95–240)
	N-desmethyltamoxifen	289 (187–325)
	4-hydroxytamoxifen (91)	10 (4–21)

* Plasma.

† Serum.

D. Metabolism by Laboratory Animals in Vivo

The first report (102) of the metabolism of ^{14}C-labeled tamoxifen in rat, mouse, rhesus monkey, and dog identified conversion to 4-hydroxytamoxifen as a significant metabolic pathway in all species. The catechol, metabolite D, is present as a glucuronide in the feces of all species studied and metabolite E, tamoxifen without the aminoethoxyside chain, is found as a minor metabolite in dog bile.

Recently the use of the tritiated antiestrogens, tamoxifen (30) U-23,469 (304), CI628 (nitromifene) (181), and LN1643 (28), have all confirmed that a primary metabolic transformation is from the parent drug to the phenolic derivative. The antiestrogens with a methoxy group are demethylated [analogous to the conversion of mestranol to ethinyl estradiol (9)] and those with an unsubstituted ring are para hydroxylated. These reactions are illustrated in figure 8 with U-23,469 and LN1643. The conversion of the phenolic derivative increases the affinity of the compound for the estrogen receptor and the hydroxylated metabolites concentrate in the estrogen target tissues (30, 181).

Conflicting results have been obtained for the metabolism of tamoxifen in the chicken. Borgna and Rochefort (30) demonstrated the conversion of tamoxifen to 4-hydroxytamoxifen in vivo and in vitro. Indeed the metabolism of tamoxifen to 4-hydroxytamoxifen by chick liver was used to prepare the first sample of [^{3}H]4-hydroxytamoxifen for subsequent estrogen receptor studies (29). In contrast, Binart and coworkers (20) did not observe 4-hydroxytamoxifen as a metabolite of tamoxifen in serum. It is possible that the technique of ether extraction used by the latter workers to isolate tamoxifen metabolites resulted in the degradation of 4-hydroxytamoxifen (111).

Large doses of tamoxifen or enclomiphene have been used to demonstrate metabolism to 4-hydroxytamoxifen (32) or 4-hydroxyenclomiphene (269) in rats. Nevertheless, sev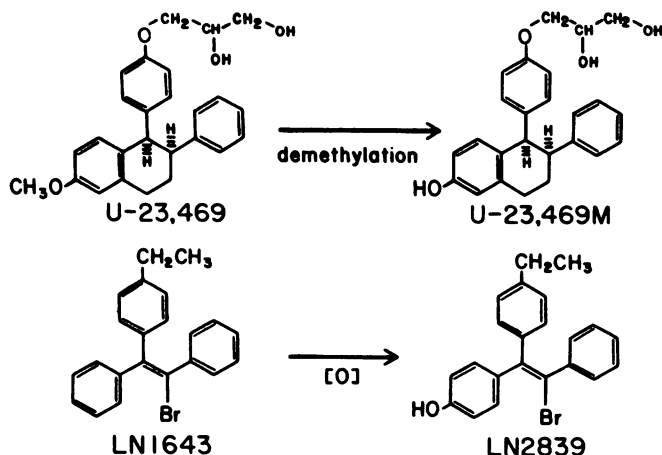eral different polar metabolic products of radio-labeled tamoxifen have been observed but, as yet, these have not been characterized. Borgna and Rochefort (30) described a polar metabolite, M$_2$, which is found to accumulate in the nuclear compartment of rat uteri 24 and 48 hr after the administration of tamoxifen. Furthermore, several polar metabolites of tamoxifen were observed by Robertson and coworkers (254). The same group compared the metabolism of tamoxifen and its cis isomer, ICI 47,699, in vivo and in vitro. No dramatic metabolic differences were observed between the isomers; however, an unknown metabolite was observed in the cytosols of ICI 47,699-treated rat uteri. Further study may be warranted since the single dose (5 μg) of ICI 47,699 selected for the study may have been inappropriate, as it appears to be at the lower end of the dose response curve.

E. Metabolism in Vitro

There are very few reports that describe the metabolism of antiestrogens in vitro. However, the studies that have been completed are rather interesting and several novel metabolites have been identified. Borgna and Rochefort (30) found that tamoxifen can be converted to 4-hydroxytamoxifen in liver, chicken oviduct, and lamb uterus in vitro but rat uterus, dimethylbenz[a]anthracene-induced rat mammary tumor and MCF7 cells do not transform tamoxifen significantly. An earlier study (134) had shown that [^{3}H]tamoxifen was not metabolized by MCF-7 cells but an unknown compound was observed to appear in the media. This may have been a breakdown product of tamoxifen rather than a metabolite. The observation illustrates that the triphenylethylene derivatives are potentially unstable.

Rat liver microsomes convert tamoxifen to 4-hydroxytamoxifen and N-desmethyltamoxifen (101). ICI 47,699 is also apparently hydroxylated and demethylated but a high concentration of unidentified polar metabolites are observed (254). The N-oxide of tamoxifen (figure 6) has been identified (101) as a metabolite of tamoxifen if microsomes from phenobarbital-treated rats are used. The N-desmethyl metabolite of tamoxifen was positively identified as the major metabolite and 4-hydroxytamoxifen (based upon HPLC retention times alone) is considered to be a minor metabolite. Mass spectral identification of 4-hydroxytamoxifen was not possible, but ether was used for extraction so there is a strong possibility that the 4-hydroxytamoxifen is converted to phenanthrenes during extraction (111). Tamoxifen N-oxide has antiproliferative actions against MCF-7 breast cancer cells in culture (15).

Although the metabolism of [^{3}H]CI628 (nitromifene) has been extensively investigated in vivo (181), recent reports (266, 268) of the metabolism of ^{14}C-labeled nitromifene in vitro, in the presence of rat cecal contents, have resulted in the identification of three novel metabolites (figure 9). Ruenitz and Bagley (266) have proposed that the $\alpha\alpha$ diarylacetophenone (III) results from the

FIG. 8. Metabolic activation of nonsteroidal antiestrogens to phenolic derivatives with a high affinity for the estrogen receptor.
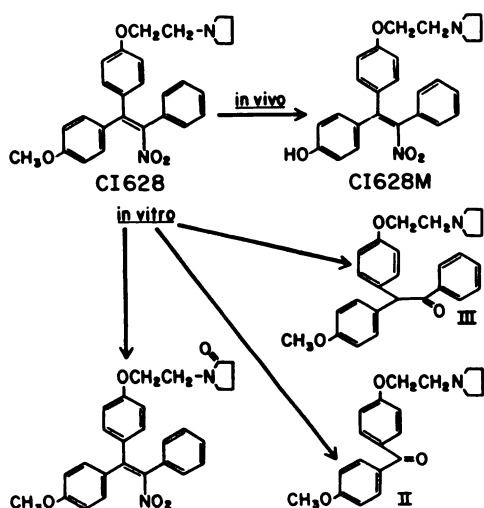
FIG. 9. Metabolism of CI 628 (nitromifene) in vivo and in vitro.

reduction of the nitro group of nitromifene to an amino group followed by enolization and hydrolysis to the ketone. The oxidation of the pyrrolidine ring of nitromifene is probably analogous to the oxidation of nicotine to conitine (139) and tremorine to oxotremorine (45). It has been pointed out that the ring-oxidized metabolite of nitromifene is less active as an antiestrogen, but more active as an estrogen than the parent compound (268). However, the biological activity was assessed in vivo, thereby exposing the molecule to further potential transformation. The result, may, therefore, not reflect the intrinsic efficacy of the parent compound at all.

VI. Models of Estrogen Action

The reason for the target site (uterus, vagina, pituitary gland) specificity of estrogens remained obscure until the synthesis of radiolabeled compounds. The first studies with ^{14}C-labeled DES in vivo established that it is rapidly (within 21 hr) excreted via the bile duct (120, 312), but no selective binding by uterine tissue was observed because the specific activity of the compound was too low. [^{3}H]Hexestrol, produced by the catalytic tritiation/reduction of DES, by Glascock working with Sir Charles Dodds (70), was used to demonstrate the selective accumulation of radioactivity by the reproductive organs of immature female goats and sheep (110). However, the synthesis and extensive study of the distribution of [^{3}H] estradiol in the immature rat by Jensen and Jacobson (147), established the concept of target tissue-specific binding by a physiologically active estrogen. Their work laid the foundation for all the subsequent studies involving the mechanism of action of estrogens.

The differential centrifugation of homogenates prepared from rat uteri prebound with [^{3}H]estradiol in vivo, demonstrated the subcellular distribution of radioactivity in both the nuclear/myofibrillar fraction and cytosol (the soluble proteins in the supernatant after sedimentation at $105,000 \times g$ for 1 hr) (238, 299). These studies indicated that [^{3}H]estradiol binds with stereospecificity

to a discrete protein within the cytosol and nuclear fractions. The cytosol protein was originally identified as a macromolecule that sediments at 9.5S on sucrose density gradients (309), but later studies reclassified the sedimentation value at 8S (260). The finding that [^{3}H] estradiol can specifically bind to the ER in vitro (310) was a fundamental observation for all the subsequent research and application of the methodology to breast cancer research.

Although the cytosol ER sediments at approximately 8S, the ER extracted from the nuclear-myofibrillar fraction with 0.4 M KCl sediments at 5S on gradients containing KCl (148). The cytosolic estrogen receptor was found to be disaggregated by 0.4 M KCl into 4S subunits (87, 146). There was, however, some disagreement as to whether the subunits of the cytosolic receptor sedimented at 5S (197) so that they were similar to the nuclear complex. It should be pointed out though that the precise sedimentation coefficient of the nuclear receptor is questionable since it can be isolated at any S value from 4S to 6S (109).

Gorski et al. (112) and Jensen et al. (149) independently developed a similar subcellular model to describe the early events involved in the expression of estrogen action. Blood-borne estradiol freely diffuses into all cells but is prevented from leaving the cells of an estrogen target tissue because the steroids bind, with a high affinity ($K_d = 0.7$ nM), to the cytoplasmic ER protein. The resulting steroid-receptor complex then moves into the nucleus (translocation) where it is concentrated for subsequent interaction with nuclear components. To explain the observation that 5S ER is extracted from uterine nuclei after the administration of [^{3}H]estradiol in vivo, but the 8S cytoplasmic ER is dissociated into 4S units by KCl in vitro (150), Jensen suggested that there is a temperature-dependent "transformation" of the ER complex from 4S to 5S before translocation to the nuclear compartment (33, 148). An extensive study of the transformation of ER in cytosol has been made by Notides (239, 241, 318). These careful studies correlate the conversion of the ER from a 4S to a 5S form in vitro with a decrease in the dissociation rate of estradiol from the ER, i.e., an increase in affinity. Recently, this change in affinity has been correlated with a positive cooperativity of estradiol interactions with the ER (240); however, it is not known whether this type of interaction occurs in vivo.

Studies on the subsequent interaction of the receptor complex with sites within the nucleus and the eventual fate of the steroid and the protein are extremely controversial. On the basis of the events observed with MCF-7 breast cancer cells in culture, it has been suggested that nuclear estradiol-ER complexes are almost all destroyed or "processed" over a 5-hr period in the continual presence of estradiol (135). This event has been implicated as the signal for the eventual synthesis of progesterone

receptor (137). The site within the nucleus that regulates these biochemical events is unknown, but an interaction of receptor complexes with G-C containing base pairs in the DNA may be involved because actinomycin D, in high concentrations, prevents processing (136). While this hypothesis is extremely attractive, it unfortunately does not appear to be able to be extrapolated to explain estrogen action in all target tissues. Continuous estrogen exposure in the rat does not induce a loss of uterine receptor (144).

Although the eventual fate of the ER within the nucleus is unknown, two potential pathways have been proposed. It is possible that the receptor complex is eventually destroyed within the nuclear compartment and the sensitivity of the tissue is maintained by resynthesis of ER in the cytoplasm (276). Alternatively, the ligand dissociates from the complex in the nucleus and the free receptor re-partitions to the cytoplasmic compartment to bind more ligand (177). These issues are unresolved and require further investigation. Nevertheless, the model 1 illustrated in figure 10 points out many of the features of estrogen action that have become accepted as the early events involved in the mechanism of action of estradiol in a target tissue.

There are, however, an increasing number of observations that are inconsistent with the classical two-step hypothesis. Some reports (214, 215) have challenged the cytoplasmic site for the "transformation" reaction and have suggested that translocation of the 4S ER precedes transformation to the 5S form in the nucleus. Indeed, the concept of translocation of ER from the cytoplasm to the nucleus has never been adequately proven, although the hypothesis is generally accepted. Several studies have suggested that unfilled ER is present in the nuclei of MCF-7 breast cancer cells (285, 328) and of myometrial cells of the rat uterus (42, 227, 285). While, as has been suggested (81), these results may be artifacts of the methodologies used to prepare impure nuclei, it is also reasonable to propose that rigorous attempts to purify nuclei may only serve to lyse the nuclear membrane and permit receptor to leach out.

There are two recent innovative approaches to the question of where unoccupied ER is located within the cell. Monoclonal (114) and polyclonal (113) antibodies raised to the ER are powerful tools for the detection of ER without the necessity of steroid binding. The results have proved to be controversial. A polyclonal antibody, raised to human breast tumor ER, and tagged with a fluorescent dye has been used to demonstrate both cytoplasmic and nuclear ER, but more importantly, translocation of ER to the nuclear compartment in the presence of estrogen and antiestrogen (249). In contrast, results with monoclonal antibodies linked to a horse radish peroxidase enzyme system have demonstrated ER only in the nuclear compartment (190) of breast cancer cells. The other approach (319) has been to enucleate
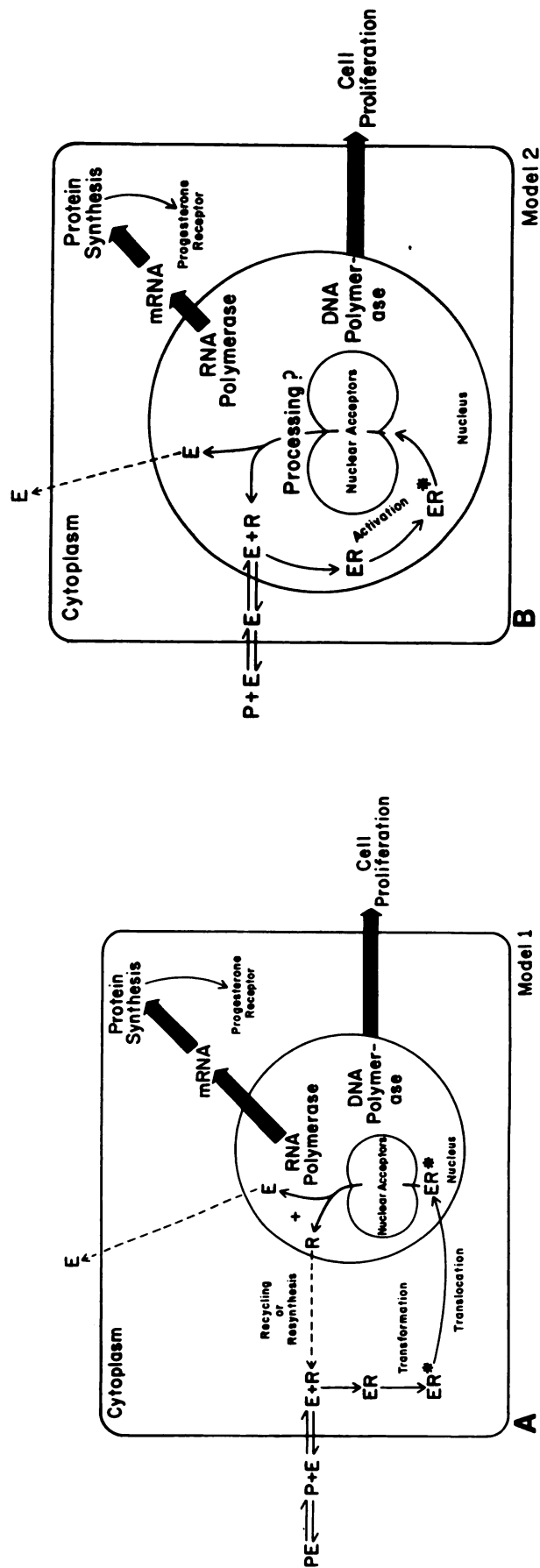
FIG. 10. A and B, models for the subcellular mechanism of estrogen (E) action. E dissociates from plasma proteins (P) and diffuses into target tissue cells. E binds to estrogen receptors (R) in the cytoplasm (model 1) or the nucleus (model 2). The estrogen receptor complex (ER) is activated (*) (transformation) before initiating the events associated with estrogen action: activation of RNA polymerase and subsequently protein synthesis and DNA polymerase and cell division. As yet, the fate of the nuclear ER complex is unknown.

ER-containing GH$_3$ rat pituitary tumor cells with cyto-chalasin B. Separation of cells into nucleoplasts and cytoplast on Percoll gradients containing cytochalasin B results in most of the unoccupied receptor being located in the nucleoplast faction. Although it is possible that each of these studies is generating a discrete artifactual result, the simplified model 2 of estrogen action illustrated in figure 10 should also be considered to be representative of subcellular events in vivo.

The nonsteroidal antiestrogens produce some interesting results that could be interpreted to support the simplified model of estrogen action rather than the traditional two-step hypothesis. The administration of estradiol, or an antiestrogen with a high affinity for the ER like 4-hydroxytamoxifen, to immature rats causes an increase in uterine wet weight and an elevation of progesterone receptor levels (68). The effects are associated with a re-compartmentalization of estrogen receptors from the cytosol to the nucleus and this is believed to be a requirement before estrogen-stimulated effects can occur (model I). However, antiestrogens with a low affinity for the ER that are unlikely to be metabolically activated, cause uterine growth, and an increase in progesterone receptor but, apparently, do not cause localization of receptor complexes in the nucleus compartment (168). One explanation is that the ER has a predominantly nuclear location in vivo, but when the cell is disrupted in vitro, the unoccupied receptor "leaks out" of the damaged nuclear membrane and appears as a cytosolic protein (figure 11). This "leakage" is prevented by the binding of any high affinity ligand to the ER so that the

complex is retained in the nucleus during cell disruption. In contrast, the low affinity ligands that are not metabolized to high affinity compound in vivo can bind to the ER in the nucleus to elicit estrogenic response but their rapid dissociation during homogenization allows the unoccupied receptor to leak out of the nucleus. Thus the majority of the receptor appears to be in the cytosol fraction after treatment with these compounds even though estrogenic stimulation occurs.

A further element to be considered in this complicated picture is the heterogeneity of nuclear estrogen receptors (54). Two classes have been described: type I, the classical estrogen receptor, and type II which may be found either in cytosol (54, 88) or the nuclear compartment (54, 88, 224) but whose distribution is unaffected by estrogen. The type II sites have low affinity for estradiol (K_d 30 nM) and appear to have high capacity. Their function is unknown; however, an increase in type II sites has been suggested to be an intermediate step in the mechanism by which estradiol causes uterine growth (54, 226). Recently, a natural ligand has been provisionally identified (225).

VII. Antiestrogen Action

A. Studies in Vivo

The dose-related inhibition of estradiol-stimulated uterine weight produced by antiestrogen in the rat (163, 174) is correlated with an ability to inhibit the binding of [^{3}H]estradiol in vivo (172). Antiestrogen administration following the binding of [^{3}H]estradiol by estrogen target tissues also rapidly reverses the estrogen binding (figure 12). This property of the nonsteroidal antiestrogens, however, does not completely explain antiestrogen action. Antiestrogens, which are estrogens in the mouse, also inhibit the binding of [^{3}H]estradiol in mouse estrogen target tissues (153).

Rochefort et al. (263) provided the first evidence that antiestrogen can apparently translocate the ER to the nucleus in vivo. The development (10) of [^{3}H]estradiol exchange assay to identify filled nuclear ER sites has proved to be a powerful technique with which to study the cellular "distribution" and kinetics of ER in vivo following antiestrogen administration. Most antiestrogens studied (38, 51, 163, 179) appear to translocate ER to the nucleus. Large doses of antiestrogens produce a long-term depletion of the rat uterine cytoplasmic ER pool (51, 57). Clark and coworkers (51) first suggested that the antiestrogen-ER complex remains in the nucleus for a prolonged period producing a specific inhibition of cytoplasmic ER resynthesis (57). As a result, the tissue is refractory to any subsequent estrogenic stimuli. This attractive theory gained support (179) but nafoxidine was shown to replenish ER (38) and it now seems clear that the ability of antiestrogens to produce a prolonged depletion of the cytoplasmic ER pool is a reflection of their long biological half lives. Under these circum-
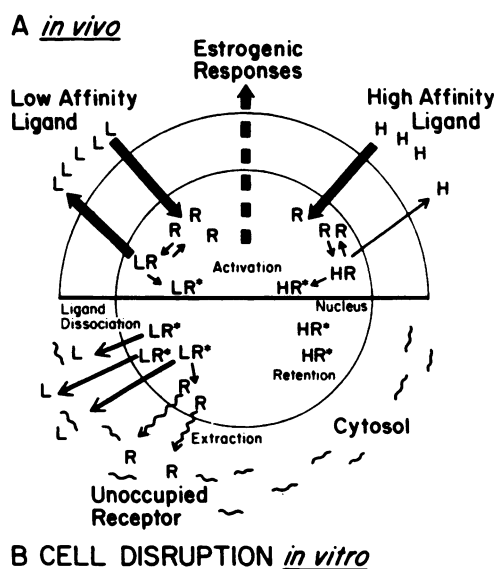
FIG. 11. A functional model for estrogen action. High affinity ligand H) enters the cell and binds to the estrogen receptor (R) in the nucleus to produce an activated complex HR* and estrogenic responses. During cell disruption, this complex is retained in the nucleus. Low affinity ligands (L) enter the cell and bind to nuclear receptor to produce an activated complex LR*. During homogenization in vitro, the LR* complex dissociates and unoccupied R, and presumably ligand, leaks out of the nucleus into the cytosolic fraction.

FIG. 12. The effect of an injection of 20 μg LY 117018 (sc in 0.1 ml of peanut oil) on the levels of radioactivity in immature rat uterus (O), vagina (△) and pituitary glands (□). The LY 117018-treated groups injected 2 h after an injection of 10 μCi (0.04 μg) [6,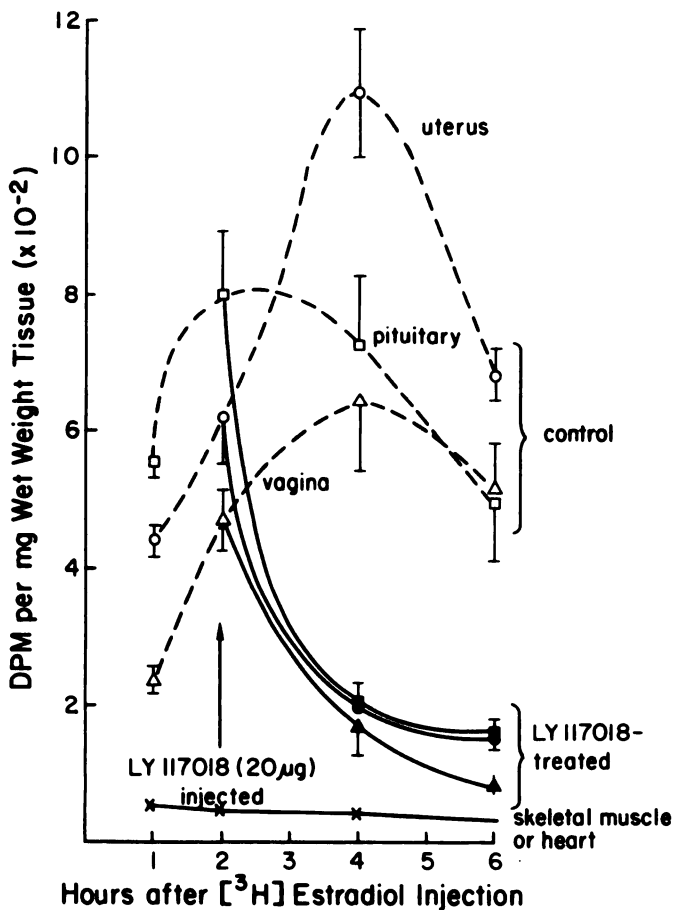7³H]estradiol in 0.1 ml of peanut oil. Control groups were injected with peanut oil alone. Ten rats per group. Tissue radioactivity was determined by using a Tricarb tissue oxidizer. Data from Jordan and Gosden (166).

stances, any resynthesized cytoplasmic receptor is translocated immediately to the nuclear compartment by ligands from the blood. Evidence can be presented to demonstrate that prolonged depletion of the cytoplasmic ER pool is not a primary antiestrogenic mechanism. This effect can be duplicated by the administration of long-acting estrogens which deplete the cytoplasmic receptor pool but stimulate full tissue growth (53, 174, 180). On the other hand, tamoxifen produces, as might be expected, a depletion of the cytoplasmic ER pool in a dose-related manner (174), but small doses of tamoxifen can inhibit estrogen action without dramatically altering ER levels in the cytoplasm (163). A similar result can be obtained with tamoxifen in the ovariectomized rate (198).

Progress in understanding nuclear effects of antiestrogens is retarded because the mechanism whereby estrogen stimulated uterine growth is poorly understood. The idea that true growth results from a strong association (salt resistant) of the ER complex with nuclear "acceptors" (56) was followed by reports that antiestrogen-ER

complexes are only loosely bound by the nuclear matrix (16, 163). Studies also indicate (16) that estrogen and antiestrogen may bind to separate sites in the nucleus. In assessing the relevance of these studies, it should be pointed out that the estrogenic and antiestrogenic geometric isomers of clomiphene can be salt extracted from nuclei to the same extent (270). It is possible though that the low affinity ligand dissociates from the complex and the unoccupied receptor readily extracts. Thus one effect (as previously suggested in section VI) that aids nuclear extraction would be ligand dissociation rather than the physicochemical properties of the complex. Ruh and Ruh (272) have shown, however, that the high affinity antiestrogen H1285-estrogen receptor complex is readily extracted from nuclei, indicating that nuclear estrogen and antiestrogen receptor complexes may indeed have different binding characteristics within the nucleus.

Antiestrogen action in the uterus is based upon the inhibition of estrogen-stimulated uterine growth. However, the situation is complicated by the ability of antiestrogens to initiate progesterone receptor synthesis (68, 191, 201) which is considered to be an estrogenic response (96). It is this mixture of estrogenic and antiestrogenic effects, produced by the triphenylethylene type of antiestrogens in vivo, that makes a unifying theory for antiestrogen action in vivo rather difficult.

B. Studies in Vitro

Much of the research on the subcellular aspects of antiestrogen action has focused upon human breast cancer cell lines. The most widely studied, the MCF-7 cell line, was cultivated from a pleural effusion derived from a patient with breast cancer (288). Estrogen, androgen, progesterone, and glucocorticoid receptors are all present in MCF-7 cells (133) and estrogen increases cell numbers (184, 217). Following the localization of the estradiol-ER complex in the nucleus, a rapid destruction or processing has been described (135, 137) and correlated with the subsequent appearance of cytoplasmic progesterone receptor. Of interest is the observation that nafoxidine receptor complexes are not processed and do not provoke the appearance of progesterone receptor (134). Tamoxifen seems to fall into an intermediate category. Tamoxifen receptor complexes undergo processing and stimulate progesterone receptor appearance at low concentrations of the antiestrogens, but at high concentrations all growth activities cease (134). The concept of processing is further complicated though by the finding (76) that low concentrations of antiestrogens with high affinity for the estrogen receptor stimulate progesterone receptor synthesis in MCF-7 cells, whereas antiestrogens with a low affinity do not.

The essence of "processing" is that the antiestrogen receptor complex is resistant to some, as yet undetermined, biochemical event that destroys receptor. This results in an accumulation of receptor complexes. Linking this proposition with the concept of Clark et al. (57)

that antiestrogens inhibit receptor resynthesis, one would expect there to be a stagnation of the receptor dynamics within the nucleus. Recent studies suggest, however, a much more complex situation (78). Nuclear ER synthesis and turnover has been measured in MCF-7 breast cancer cells by a density shift technique. Cells are incubated in medium supplemented with $^{13}C,^{15}N,^{2}H$ amino acids (dense amino acids) and a shift is monitored from "old light" (pre-existing) to "new dense" (newly synthesized) receptors by velocity sedimentation on 0.4 M KCl 5% to 20% sucrose gradients prepared in buffered deuterium oxide. The unoccupied nuclear receptor was found to have a half-life of 4.47 ± 0.26 hr, but nuclear receptors occupied with estradiol, CI628 or nafoxidine were found to have half-lives of 3.00 ± 0.38 hr, 4.9 ± 0.66 hr, and 3.43 ± 0.37 hr, respectively. Clearly, the receptor is turned over in the presence or absence of binding ligands and there appears to be no distinction between estrogens or antiestrogens.

Antiestrogens produce inconsistent effects with regard to progesterone receptor synthesis, but several other intracellular or secreted proteins are regulated by estrogens and antiestrogens. Estrogen stimulates the secretion of a 52 K dalton protein (originally identified as a 46 K dalton protein) from MCF-7 cells (320). Tamoxifen and 4-hydroxytamoxifen prevent estrogen-stimulated synthesis of the 52 K dalton protein and have no agonist activity (321). Similarly, estrogen stimulates the synthesis of specific, intracellular, proteins with molecular weights of 24 K and 36 K whereas nafoxidine is inactive (80). The function of each of the intracellular or secreted proteins is unknown, but research is aimed at establishing a link between their synthesis and estrogen-stimulated cell division.

Tamoxifen and nafoxidine alone produce an inhibitory effect on [^{3}H]thymidine incorporation (217) and DNA polymerase activity (82) as well as causing a reduction in DNA content of cultures (61) and cell numbers (61, 217). This inhibitory effect on MCF-7 cell growth is not a simple cytotoxic action of the drug since it can be readily reversed by addition of estradiol to the culture media (216). Studies of cell cycle kinetics show that at concentrations of 2 to 6 μM in the culture, tamoxifen reduces the proportion of cells in S phase and increases the number of cells in G_1 (242, 293). At 10 μM, tamoxifen causes cell death within 48 hr. Similar inhibitory effects of tamoxifen on [^{3}H]thymidine incorporation and/or cell numbers have been described for two other estrogen-responsive cell lines. CG-5 is a variant of MCF-7 which is claimed to be highly sensitive to estrogen; tamoxifen not only inhibits the growth of CG-5 cells but also potentiates the inhibitory effects of progestins (141). The ZR-75-1 cell line has the advantage that it grows in defined media. In the absence of estradiol, tamoxifen causes cell death, an effect that can be reversed by estrogen provided it is not later than 48 hr after the

antiestrogen (7, 8). It is also interesting to note that, unlike MCF-7 cells, tamoxifen has no effect on progesterone receptor synthesis in the ZR-75-1 cell line (6).

There is reasonable evidence that antiestrogens produce their inhibitory effects on growth in the cell lines via an estrogen receptor mechanism. However, very high concentrations of antiestrogens inhibit cell growth and this cannot be "reversed" with estrogen (115). There is also some evidence that tamoxifen will inhibit growth of mammary cancer cells that do not have estrogen receptors (115). While this effect could be modulated via an "antiestrogen binding protein" (298), the concentrations required to produce an effect (>7.5 μM) and the affinity of tamoxifen for the binding site (1 nM) seems to argue against this correlation. In this regard it may be important to consider the recent report by Lam (199) who has shown that tamoxifen, in the 1 to 10 μM range, is a potent inhibitor of calmodulin action. Since calmodulin has been implicated in the control of cell proliferation (43, 143), these observations warrant further investigation.

VIII. Radiolabeled Antiestrogens

Several nonsteroidal antiestrogens have been synthesized in tritium-labeled form to aid in an understanding of their metabolism and binding characteristics to ER. The compounds that are now commercially available are illustrated in figure 13; however, several compounds have been synthesized by individual investigators. In Dr. John A. Katzenellenbogen's laboratory, tritium-labeled U23,469 (304), CI628 (181), CI628M (185), the *cis* and *trans* isomers of tamoxifen and 4-hydroxytamoxifen
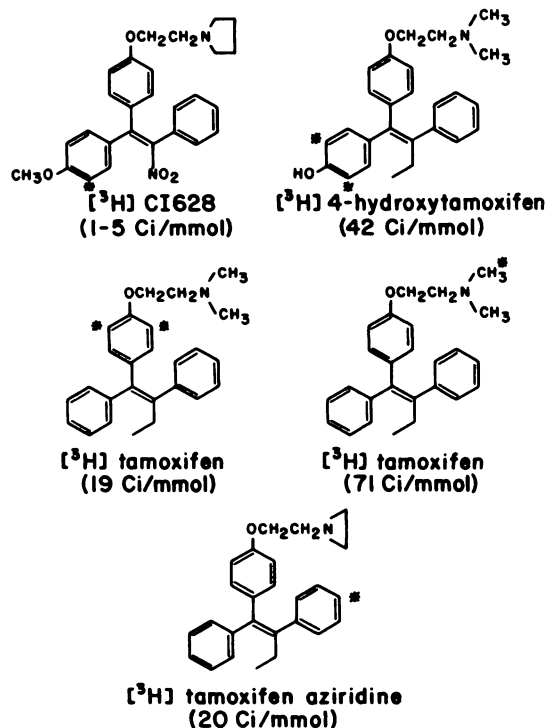

FIG. 13. Commercially available radiolabeled antiestrogens.

(252), and tamoxifen aziridine (184) were successfully synthesized and in Dr. Thomas S. Ruh's laboratory, tritium-labeled H1285 was synthesized (271, 272). Although most [³H]antiestrogens have been shown to be metabolically activated to hydroxylated derivatives before binding in target tissues (30, 181), [³H]4-hydroxytamoxifen binds directly to rat estrogen target tissues in vivo (158).

The studies that describe the interaction of tritium-labeled antiestrogens with the ER and other, nonestrogen receptor binding sites will be reviewed.

A. The Estrogen Receptor

The studies with [³H]tamoxifen (40, 173) and [³H] CI628 (181) demonstrate estrogen-specific binding of antiestrogens to the 8S estrogen receptor from immature rat uteri. The antiestrogens tamoxifen and CI628 have a rapid rate of dissociation from the receptor (40, 181) so that accurate studies of the interaction of tamoxifen and CI628 with the ER are particularly difficult. Borgna and Rochefort (29) prepared the first sample of [³H]4-hydroxytamoxifen by the metabolic oxidation of [³H]tamoxifen in chicken liver slices. This material was used to confirm the high binding affinity of 4-hydroxytamoxifen for the ER and describe the physical properties of estradiol and 4-hydroxytamoxifen ER complexes (29). A similar study has been reported with the compound CI628M (183). There are, however, several recent reports (77, 90, 260, 301) that describe specific differences in the physicochemical properties of estradiol and 4-hydroxytamoxifen ER complexes. By documenting these differences, it is hoped that changes in the receptor complex might ultimately explain the differences in the pharmacology of agonists and antagonists.

Estradiol rapidly dissociates from the untransformed receptor, whereas the dissociation is slowed from the heat transformed receptor. Molybdate ions appear to prevent the transformation of the estradiol-ER complex and estradiol rapidly dissociates from the receptor in the presence of molybdate. In contrast, 4-hydroxytamoxifen dissociates slowly from both the transformed and untransformed receptor and is unaffected by molybdate. Rochefort and Borgna (260) suggest that antiestrogens produce a change in the ER discretely different than that produced by estradiol. A similar conclusion has been reached as a result of studies to describe the interaction of human breast tumor ER with a polyclonal antibody raised to the calf uterine ER in the goat (113). Preincubation of antibody with cystolic ER impairs the subsequent binding of [³H]estradiol and reduces the affinity of the ligand-receptor protein interaction. However, the binding of 4-hydroxytamoxifen to the receptor is unimpaired by equivalent concentrations of the antibody. Higher concentrations of antibody can, however, substantially inhibit antiestrogen binding. In contrast, the interaction of the antibody with preformed estradiol and 4-hydroxytamoxifen ER complexes does not affect the

binding of the ligands. The model illustrated in figure 14 has been proposed to explain these observations (301).

Estradiol initially interacts with the ligand binding site on the receptor which then induces an activation or transformation reaction that locks the steroid into the receptor complex. The antiestrogen, because of its shape, wedges into the binding site on the receptor and prevents the full range of conformational changes required for receptor activation. The polyclonal antibody may interact with the unfilled or resting receptor to prevent the conformational changes that subsequently occur to lock estradiol into the receptor. However, the antiestrogen can still wedge into the proposed binding site, but because the ligand has a multipoint attachment to the receptor, it does not require further conformational changes to produce high affinity binding. It is suggested that once the conformational changes occur to lock the steroid into the receptor then the antibody is unable to reverse the process.

Monoclonal antibodies raised to the ER (114) are known to interact at different sites on the protein. Antibody D547 (raised to the extranuclear receptor from MCF-7 cells), which interacts at a site far removed from the ligand binding site, binds equally with estradiol and 4-hydroxytamoxifen receptor complexes from human breast tumor cytosols (300). There are no differences whether D547 is preincubated with the receptor before the ligand or incubated with the receptor complex. Thus, the antibody D547 does not discriminate between estrogen and antiestrogen receptor complexes. The new research tool will, however, be useful for immunohistochemical experiments to study the fate of agonist and antagonist receptor complexes in tumor cells. In contrast, the monoclonal antibody B36, developed from the calf uterine nuclear ER, can apparently discriminate between estradiol- or 4-hydroxytamoxifen-receptor complexes (262).

Finally, the interaction of estradiol and antiestrogen-(4-hydroxytamoxifen or CI628M) ER complexes with either DNA or polynucleotides has been reported by several laboratories. Initial studies showed no differences between the binding of estradiol or 4-hydroxytamoxifen with polynucleotide-cellulose columns (236) or sheered calf thymus DNA (29). Recently, it has been shown that estradiol-ER complexes bind more tightly to calf thymus DNA than antiestrogen receptor complexes (90). There is a possibility that this finding indicates that there is an alteration in the charge distribution on the receptor when an estrogen or antiestrogen is located at the binding site.

The technique of sucrose density gradient analysis has been used to demonstrate sedimentation differences between estradiol and antiestrogen-ER complexes. Small, but consistent differences are observed with chick oviduct ER (108) and human breast tumor ER in the presence of KCl (300) or polyclonal antibodies (301) but the biological significance of these observations is un-

Proposed Model

Estrogen Binding | Ligand Locking by Changes in Tertiary Structure | Biologically Active Complex
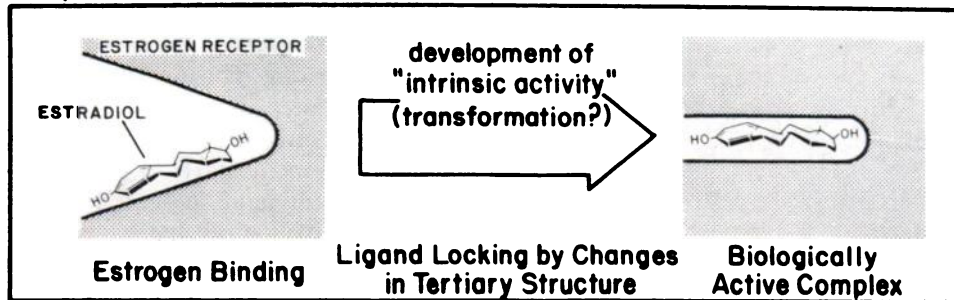
Effect of Ab Preincubation
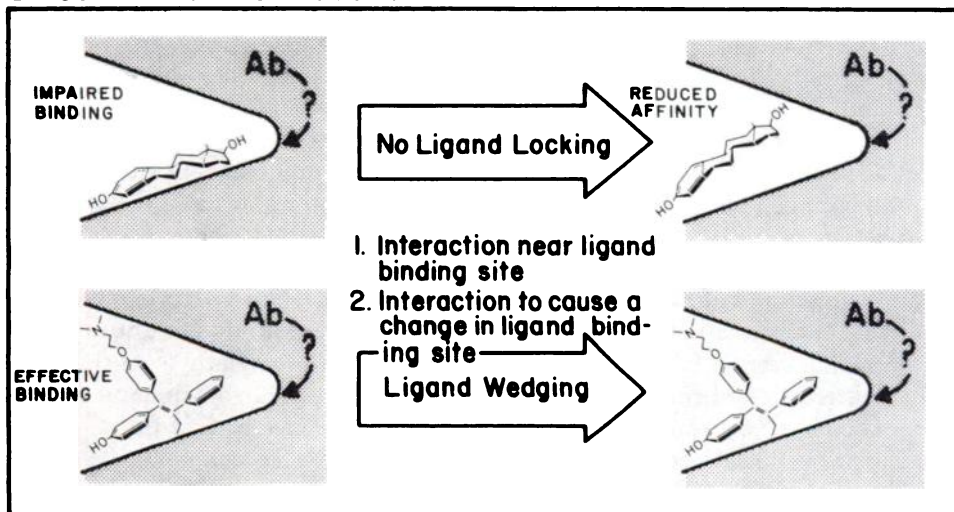
Effect of Ab After Ligand Binding

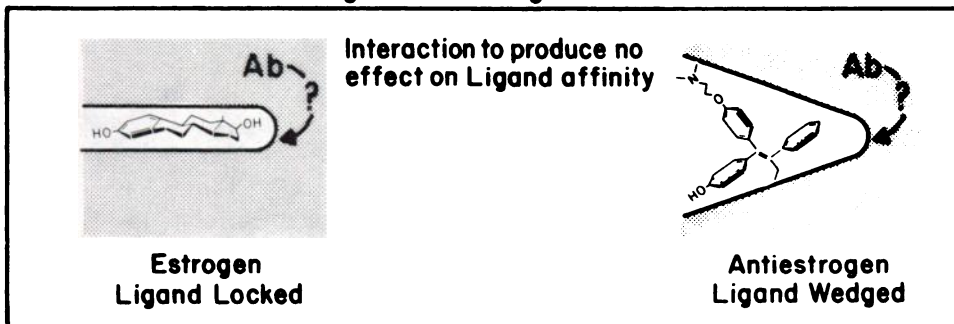Estrogen Ligand Locked | Antiestrogen Ligand Wedged

FIG. 14. Effect of a polyclonal antibody (Ab) to the estrogen receptor, raised in the goat, on the binding of estradiol and 4-hydroxytamoxifen to the ligand-binding site on the estrogen receptor (301).

known. High salt (0.4 to 0.6 M KCl) nuclear extracts from MCF-7 breast cancer cells that have been incubated with either [^{3}H]estradiol or [^{3}H]4-hydroxytamoxifen contain a 4S [^{3}H]estradiol ER complex but a 5S [^{3}H]4-hydroxytamoxifen estrogen receptor complex (77). These original findings have been confirmed (303); however, the results are not identical for all cell lines. Similar experiments with GH$_3$ rat pituitary tumor cells have shown that the nuclear estrogen and antiestrogen receptor complexes are both 5S (302, 303). Ruh and Ruh (272) have demonstrated that receptor complexes extracted from rat uteri after exposure to [^{3}H]H1285 or [^{3}H]estra-

diol are a mixture of 4S and 5S complexes although the heavier complex predominates in antiestrogen-treated rats. Attardi (12) has suggested that differences in sedimentation characteristics of nuclear estrogens and antiestrogen receptor complexes from rat uteri are the result of sensitivities to proteases. Thus different cell systems may have different amounts of protease enzyme systems. The unusual, though consistent (77, 237, 303), finding of a 4S [^{3}H]estradiol-estrogen receptor complex extracted from MCF-7 cells may result from the dissociation of 5S complexes promoted by divalent cations (230). However, the change in the estrogen receptor that

is produced by antiestrogens which enables the complex to resist degradation, is the subject of considerable interest.

B. Antiestrogen Binding Sites

The synthesis of radiolabeled antiestrogens permitted the study of other proteins that specifcally bind antiestrogen. The first report by Sutherland and Foo (292) described the interaction of tritium-labeled tamoxifen and CI628 with rat uterine and chick oviduct cytosol. In the rat uterus, CI628, tamoxifen, and estradiol bound to a similar number of saturable binding sites and estradiol could completely inhibit the binding of [³H]antiestrogens to these sites. In contrast, high affinity, saturable antiestrogen binding sites in chick oviduct, present at three times the concentration of estradiol binding sites and estradiol could only partially inhibit the binding of [³H] antiestrogens. Subsequent studies (296, 298) identified antiestrogen binding sites in the cytosols of ER positive breast tumors and several estrogen target tissues including immature rat uterus. The antiestrogen binding site in rat uterus (K_d approximately 1 nM) is only observed if 90% to 95% of ER is occupied by prior treatment with estradiol in vivo (233). The concentrations of antiestrogen binding sites in rat uterine cytosol fluctuates during the estrous cycle and is more resistant to thermal denaturation than the ER (94). However, some controversy now surrounds the exact subcellular location, identification and function of the site. Some laboratories (261) are unable to identify antiestrogen binding sites in 100,000 × g supernatants (cytosols) whereas others can (119, 164, 193). The target site specificity is also controversial as ER negative tumors (164, 231) and all human tissues tested have antiestrogen binding sites (193). Extensive studies in the rat (290) have identified the microsomal fraction of tissues to contain the highest concentration of antiestrogen binding sites. The liver is particularly rich in the sites (290, 325). Antiestrogen-sensitive binding sites in uterus, vagina and liver have been described for the immature rat in vivo (158). Furthermore, a triphenylethylene-antiestrogen binding site is present on rat low density lipoprotein (LDL) (K_d 28 nM) which is distinct from the binding site in liver (326). An endogenous ligand (58) is present in boiled ethanol extracts of rat liver that prevents the binding of [³H]tamoxifen to both LDL and liver preparations. The "ligand" has not as yet been characterized, and its physiological role is unknown.

There is general agreement about the structural specificity of "antiestrogen binding" sites (119, 290, 298, 325). The steroids estradiol, progesterone, testosterone, dihydrotestosterone, or hydrocortisone do not affect the binding of [³H]tamoxifen. Nonpolar antiestrogens, tamoxifen, CI628, enclomiphene, and nafoxidine, have a high affinity for the binding sites, but polar antiestrogens, 4-hydroxytamoxifen LY 117018 or LY 156758, have a lower affinity (290, 326). Interestingly enough, an "LY 117018-

binding component" of rabbit and rat uterine cytosols has recently been described (313) that appears to be specific for this particular compound. The relevance of this observation to the mechanism of antiestrogen action is unknown.

The alkylaminoethoxy side chain of tamoxifen (315) and enclomiphene (234) is important for high affinity binding; minor modifications in length or removal are generally detrimental but substitution of the amino group in tamoxifen with various unsaturated amine ring system increases affinity for the binding sites (290).

C. Antiestrogen Binding Sites: Biological Function

The ubiquitous distribution of antiestrogen binding sites (193) tends to argue against their central role in the regulation of estrogen-dependent events. Antiestrogen action in vivo (166, 209) and in vitro (61, 216) is generally reversible with estradiol. Therefore, if by definition, estrogen can only compete with antiestrogens for the estrogen receptor and not antiestrogen binding sites, then a single mode of action appears to be operating.

The estrogenic and antiestrogenic properties of ligands appear to correlate with relative binding affinities for the estrogen receptor and not antiestrogen binding sites (51, 235). The triphenylethylenes ICI 47,699 (cis isomer of tamoxifen) and zuclomiphene (cis isomer of enclomiphene) both have high affinity for "antiestrogen binding site" (290) but the compounds are weak estrogens rather than antiestrogens. Structural derivatives of clomiphene (235) and the metabolites of tamoxifen (61, 250) control the growth of MCF-7 breast cancer cells at concentrations consistent with their relative affinities for the estrogen receptor. In particular, the potent antiestrogen, LY 117018, has a high affinity for the ER and is a potent agent for the control of MCF-7 breast cancer cell growth (280) but has a low affinity for the antiestrogen binding site (290, 326).

Breast cancer cell lines have been studied extensively in an attempt to correlate levels of antiestrogen binding sites with inhibition of cell growth by antiestrogens. Faye and coworkers (93) have described a tamoxifen-resistant cell line RT × 6 derived from MCF-7 that has ER levels equivalent to those observed in wild-type MCF-7 cells but the tamoxifen-resistant cells contain very low levels of antiestrogen binding sites. In contrast, Miller and Katzenellenbogen (231) have compared three breast cancer cell lines MCF-7, T47D, and MD-MB-231 that contain similar levels of antiestrogen binding sites but high, low, and undetectable levels of ER, respectively. Tamoxifen inhibits the growth of the cells depending upon the presence of the ER.

Sutherland and coworkers (115) have shown that high concentrations (>5 μM) of antiestrogens that inhibit the growth of breast cancer cells cannot be reversed by estrogens. It is possible that this might represent a specific method of controlling the growth of cells by a nonestrogen receptor-mediated mechanism. At present,

however, it is unclear why such a high concentration of antiestrogen (at the limit of solubility of the compounds) is required to activate an antitumor mechanism via a binding site with a K_d of 1 nM.

Black and Goode (22) have proposed that tamoxifen may produce some of its *estrogenic* effects in the rat uterus via the "antiestrogen binding site." This conclusion is based upon the finding that LY 117018 has low affinity for antiestrogen binding sites and a high affinity for the ER, but while LY 117018 can inhibit estrogen action in the uterus, high doses (1 mg/rat) of LY 117018 are unable to inhibit the uterotropic effect of tamoxifen (1 mg/rat). However, recent studies (167, 316) demonstrate that LY 117018 can inhibit the uterotropic effects of tamoxifen if the correct dosage ratios are used.

Finally, one could suggest that the antiestrogen binding protein does not have a positive biological function per se, but may have an adverse effect on the expressions of the pharmacological actions of tamoxifen. If some breast tumors have larger concentrations of antiestrogen binding sites (or perhaps it is induced during therapy with tamoxifen) than others, then the drug may preferentially bind to the high affinity sites (K_d 1 nM) rather than block the ER (K_d =80 nM). Under these biochemical circumstances, the hormone-dependent tumors might continue to grow in the face of tamoxifen therapy. Indeed, treatment of patients that have failed tamoxifen therapy with aminoglutethimide has resulted in a substantial number of second objective responses (124, 131). Aminoglutethimide is considered to be an inhibitor of aromatizing enzyme systems that convert androstenedione to estrone. Hormone-dependent disease is controlled by preventing estrogen synthesis rather than preventing estrogen action in the tumor (275). It may, therefore, be possible to predict patients that can respond readily to tamoxifen therapy by an assessment of "antiestrogen binding sites" in a tumor: a high level of sites would prevent tamoxifen from blocking estradiol binding to the receptor. In fact, a preliminary report from Bloom and Fishman (26) suggests that they may have already developed such a test. The response of patients to tamoxifen was correlated to the ability of tamoxifen to inhibit the binding of [³H]estradiol to tumor ER. Those patients whose tumor was ER positive with estradiol as a competitor for [³H]estradiol binding, but was refractory to competition with tamoxifen, did not respond to tamoxifen therapy. Clearly, future research could establish the validity of this hypothesis.

IX. Antiestrogenic Mechanisms: Summary

It is now appropriate to summarize many of the subcellular effects observed with antiestrogens in vitro (figure 15). Although various exceptions have been mentioned previously, antiestrogens, in general, regulate estrogen-stimulated prolactin synthesis (211, 212), progesterone receptor production (76, 134), 24 K, 36 K (80), and 52 K (320, 321) protein synthsis and [³H]thymidine

incorporation (216), DNA increases (82), and the cell cycle (G₁ block) of estrogen-sensitive cells (242, 293). These effects may be caused by a variety of mechanisms. Blood-borne antiestrogen [possibly bound to low density lipoprotein (326)] dissociates from carrier proteins and diffuses into all tissues. In an estrogen target tissue, an antiestrogen can bind to ER (29), antiestrogen binding sites (290, 298, 315), or, as recently reported, calmodulin (199). The interaction equilibria that are established depend upon the relative binding affinities (RBA) of the antiestrogen from the proteins. Tamoxifen has a high binding affinity for the antiestrogen binding sites (K_d 1 nM, RBA = 100, E₂ RBA = 0), but a low binding affinity for the estrogen receptor ($K_d \simeq 80$ nM, RBA = 5, E₂ RBA = 100). In contrast, LY117018 has a low binding affinity for the antiestrogen binding site (RBA $\simeq$ <1) but a high affinity for the estrogen receptor (RBA > 100). On the one hand, it is possible that interaction of antiestrogens with either antiestrogen binding sites or calmodulin (or both) could affect the cell cycle. On the other hand, low concentrations of estrogens can reverse the inhibitory effects of antiestrogens in most model systems, although this is difficult with high concentrations (7.5×10^{-6} M) of antiestrogen.

Tamoxifen is a potent inhibitor of calmodulin-mediated phosphodiesterase (199). The other recognized inhibitors of calmodulin are major tranquilizers, e.g., trifluperazine, which incidentally are efficient inhibitors of [³H]tamoxifen binding to rat liver "antiestrogen binding sites" (S. D. Lyman and V. C. Jordan, unpublished observation) and inhibit the colony formation of breast cancer cells (317). Calmodulin is believed to be intimately involved in cell division (43, 324) and inhibitors of calmodulin, such as trifluoperazine, will produce a block in the G₁ phase of the cell cycle (143).

Trifluoperazine recently has been shown to prevent the binding of iodinated epidermal growth factor to neoplastic, but not normal, cells in culture (27). These fascinating observations may provide alternative mechanisms to explain the antiproliferative actions of tamoxifen in either ER positive or negative cells in culture (115). It is, therefore, possible to envision that breast tumors that contain a heterogeneous mixture of ER positive and negative cells may be influenced by tamoxifen to: (a) control cell-cell communication by modulation of the action of growth factors; or (b) control ER-mediated events.

Most studies at present have focused upon the interaction of antiestrogens with the ER: (a) Antiestrogens inhibit the binding of [³H]estradiol to the ER (287). [³H] Antiestrogens bind directly to the ER (29, 40, 173, 181). (b) Studies with radiolabeled estrogens and antiestrogens demonstrate that the ligands interact with the receptor in different ways (262, 301). Estrogens and antiestrogens may have a different method of "activating" receptors (260). (c) Differences in the size of nuclear estrogen and
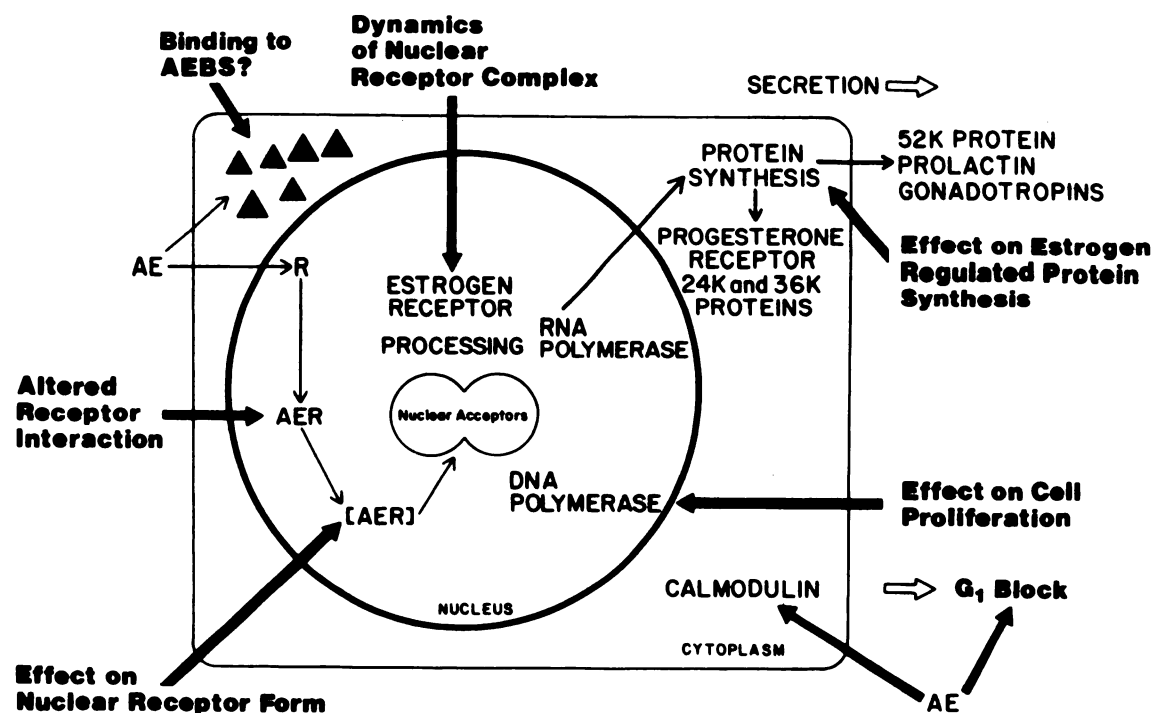
FIG. 15. Potential mechanisms of action of antiestrogens (AE).

antiestrogen receptor complexes have been noted (77, 303). (d) Differences in the interaction of estrogen and antiestrogen receptor complexes with DNA have been described (90). This may be related to the observation that antiestrogen receptor complexes are more easily extracted from nuclei by 0.4 M KCl than estradiol-ER complexes (270). (e) The concentration of estradiol-ER complexes extracted with 0.4 M KCl decrease over the first 6 hr of estrogen exposure ("processing") whereas antiestrogen-ER complexes do not (135–137, 303). (f) ER resynthesis was believed to be impaired by antiestrogen (57); however, the replenishment of receptor in the presence of estrogen and antiestrogen has been found to be similar (78).

The differences in the physicochemical properties of the estrogen or antiestrogen receptor complexes may reflect differences in charge distribution or tertiary changes in protein structure. Valuable insights into the differences in agonist and antagonist receptor complexes can be obtained by a study of structure activity relationships. Studies in vivo and in vitro will be considered to develop a hypothetical model for antiestrogen action via an interaction with the ER.

X. Structure-Activity Relationships (SAR)

The structure-activity relationships studies of antiestrogens are a natural extrapolation of the work completed by Dodds and Emmens with nonsteroidal estrogens in the 1930s, -40s and -50s (69, 83). The structure-activity relationships of estrogens have been reviewed (171).

Terenius (306) compared the agonist and antagonist activities of a broad range of antiestrogens (tamoxifen, enclomiphene, CI628, nafoxidine, MRL37, MER25) in

the 3-day immature mouse uterine weight test. However, the agonist activity of the triphenylethylene-based antiestrogens in the mouse (121, 174) make SAR studies impractical. Most of the early studies were undertaken to determine the antifertility activity of compounds in rats and mice (63, 204–206). As a result, it is often difficult to evaluate the impact of the estrogenic and antiestrogenic components of a test compound's pharmacology from the results of antifertility experiments.

With regard to specific compound groups, the interpretation of the early studies are further complicated by either misidentification (244) of geometric isomers of triphenylethylene derivatives or the testing of mixtures of geometric isomers (63). Since the pharmacological properties are often opposing (52, 121, 169), this approach to structure-activity analysis is inappropriate. Nevertheless, with these reservations, several studies will be considered to illustrate the important structural features of a compound necessary for it to exert antiestrogenic activity in vivo and in vitro.

A. Studies in Vivo

Simple hydroxylated indenes (273, 286), that are superficially related to the structure of DES are potent estrogens. The structure-activity relationships of the indene nucleus have been investigated in the search for potent antifertility relationships (204) (figure 16). The 6-methoxy group is an advantage for activity but potent antifertility activity is determined by the substituted amine ethoxy side chain. Optimal activity is observed with the pyrrolidino side chain (IND 1, figure 16) and other substituted side chains (IND 2, 3, 4) have reduced activity. A morpholino side chain (IND 5) produces a
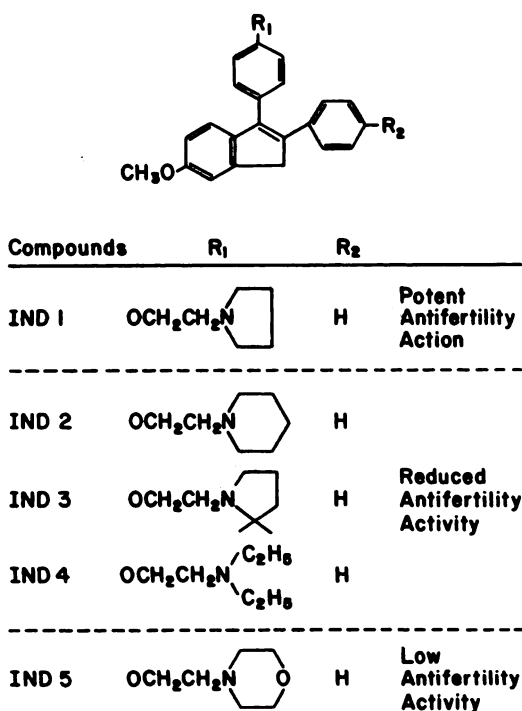
FIG. 16. The relative antifertility activity of substituted indenes in the rat. Data adapted from Lednicer et al. (204).
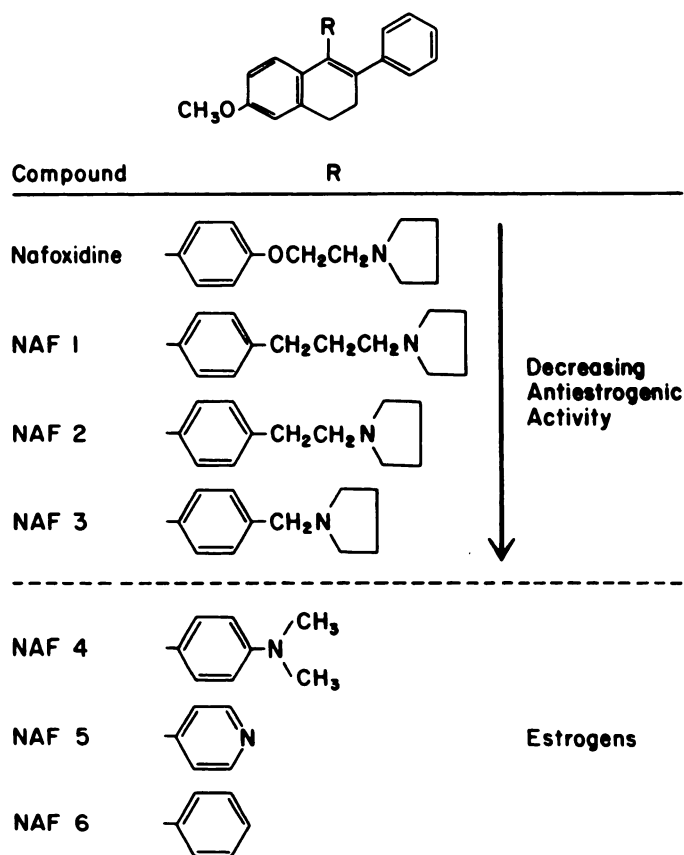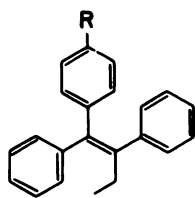

FIG. 17. The relative antiestrogenic activity of substituted 3,4-dihydronaphthalenes in immature rats. Data adapted from Lednicer et al. (205, 206).

compound with approximately 1% of the activity of IND 1 with the pyrrolidino side chain. In the same study, Lednicer and coworkers (204) showed that the 6 phenol of IND 4 had approximately 5% of the potency of the methoxy compound. As previously discussed in section IV B, a hydroxylated derivative might be expected to have a shorter duration of action so that larger doses will be required to maintain adequate drug levels. Recently a hydroxylated indene derivative (figure 21:2) has been shown to possess antitumor activity (278), although it is not clear whether the compound is a weak estrogen that has a short biological half life, or the compound has the ability to induce a receptor complex with low intrinsic activity.

The 3,4-dihydronaphthalenes further exemplify the importance of the substituted side chain for optimal activity (figure 17). Nafoxidine is the most potent compound of the series although the ether oxygen of the side chain can be replaced by carbon with very little loss of potency. However, decrease in the length of the side chain (NAF 1-3) reduces the antiestrogenic potency and, in fact, removal of the side chain (NAF 6) results in the complete loss of antagonist activity. The resulting compounds are estrogens (204–206). These observations led Lednicer et al. (206) to suggest that a basic group, at a given position in space is required to obtain a molecule with estrogen antagonist activity. This point of view is further supported by the observation that dimethylation ortho to the aminoethoxyside chain in MER25 (48) and tamoxifen (1) reduces antiestrogen activity and receptor

binding, respectively. The methyl substitutions reduce the number of positions in space that the side chain can adopt.

The importance of the side chain for the antiestrogen activity of tamoxifen has been studied. Removal of the side chain to produce the phenol (metabolite E) destroys antiestrogen activity and increases estrogenic activity (165). Robertson and coworkers (253) have tested a series of compounds related to tamoxifen but with side chains of differing basicity (figure 18). They concluded that the optimal interactions of the side chain with the receptor were unlikely to be ionic but rather hydrogen bonding between the side chain and amino acids of the receptor. Recently a series of fixed-ring tricyclic derivatives of tamoxifen and 4-hydroxytamoxifen with different side chains (figure 19) was tested for estrogenic and antiestrogenic activity (2). The phenolic hydroxyl is an advantage for binding to the ER but a relatively planar (oxygen-containing) tricyclic ring system is a disadvantage. The alkylaminoethoxy side chain confers optimal antiestrogen activity in the hydroxy dibenzo[a,e]cyclo-octenes. A glyceryl side chain causes a decrease in antagonist activity, whereas replacement with an allyl side chain causes a loss of antiestrogenic activity. This finding is particularly interesting since the compound LN 1643 and LN 2839 with only an ethyl side chain is an antiestrogen

Compound	R	
Tamoxifen	-OCH$_2$CH$_2$N(CH$_3$)(CH$_3$)	
TAM 1	-OCH$_2$CH$_2$N(pyrrolidine)	Equivalent Antiestrogenic Activity
TAM 2	-OCH$_2$CH$_2$N(piperazine)N-CH$_3$	
TAM 3	-OCH$_2$CH$_2$N(piperidine)	
TAM 4	-OCH$_2$CH$_2$N(C$_2$H$_5$)(C$_2$H$_5$)	Reduced Antiestrogenic Activity
TAM 5	-OCH$_2$CH$_2$N(morpholine)	
Metabolite Y	-OCH$_2$CH$_2$OH	
TAM 6	-OCH$_2$CH$_2$N(pyrroline)	Loss of Antiestrogenic Activity

FIG. 18. The effect of different side chains on the antiestrogenic activity of tamoxifen. Data adapted from Robertson et al. (253).

R = H or OH
R$_1$ = -OCH$_2$CH$_2$N(CH$_3$)(CH$_3$)
       -OCH$_2$CHCH$_2$OH
               |
               OH
       -OCH$_2$CH=CH$_2$
X = O, S, SCH$_2$ or CH$_2$CH$_2$

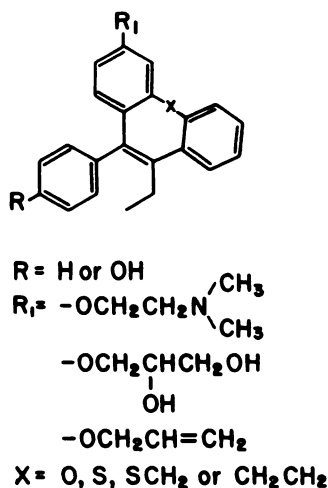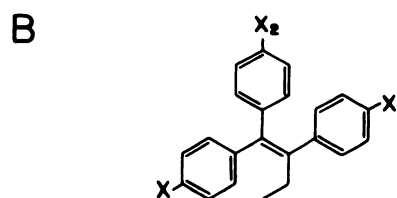FIG. 19. The structure of fixed ring derivatives of triphenylethylene with different side chains.

with antitumor properties in vivo (74) and in vitro (28). It is possible that the ether oxygen linking the allyl side chain to the phenyl ring flexes the side chain out of the area of interaction on the receptor that is required to prevent estrogen action. Alternatively, the allyl side chain may be sensitive to cleavage so that an estrogenic phenol is produced.

The substitution of a 4-phenolic hydroxyl in tamoxifen confers potent antiestrogen activity and very high binding affinity for the ER (159). The catechol derivative of tamoxifen (3,4-dihydroxytamoxifen, figure 20) also has a high binding affinity for the ER, but is a weak antiestrogen (159). Of interest though, is the finding that 3,4-dihydroxytamoxifen has no estrogenic properties in the 3-day immature rat uterine weight test (159). However, 4-hydroxytamoxifen and 3-hydroxytamoxifen are partial agonists (267), so it is possible that the dihydroxylated metabolite is able to inhibit estradiol from binding to the receptor, but is short acting because it is metabolized and excreted too quickly to exert a sustained biological

A
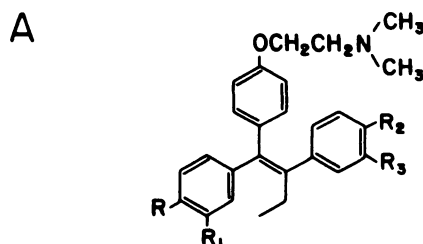
Compound	R	R$_1$	R$_2$	R$_3$
4-OH TAMOXIFEN	OH	H	H	H
3,4 diOH TAM	OH	OH	H	H
3-OH TAM	H	OH	H	H
4'-OH TAM	H	H	OH	H
3'-OH TAM	H	H	H	OH

B

Compound	X	X$_1$	X$_2$	Antitumor Activity
triphenylbut-1-ene (TPB)	H	H	H	NS
TPB 1	CH$_3$CO(O)	H	H	Weak
TPB 2	CH$_3$CO(O)	CH$_3$CO(O)	H	Potent
TPB 3	H	CH$_3$CO(O)	H	Weak
TPB 4	CH$_3$CO(O)	H	CH$_3$CO(O)	Potent
TPB 5	H	CH$_3$CO(O)	CH$_3$CO(O)	Weak

FIG. 20. The formula of hydroxylated derivatives of tamoxifen (part A) and antitumor activity of acetoxy derivatives of triphenylbut-1-ene (part B). Antitumor activity was determined in athymic mice implanted with human breast tumors. Data adapted from Schneider et al. (279)

response. Ruenitz et al. (267) have shown that the 4 position for substitution with a hydroxyl is ideal for both receptor binding and antiestrogenic activity; other substituted positions produce compounds with reduced receptor binding and antiestrogenic activity. A similar relationship between estrogen receptor binding and antitumor in vivo can be made in a group of acetoxysubstituted derivatives of 1,1,2-triphenylbut-1-ene (figure 20). It is an advantage to have an acetoxy group in position X (figure 20, TBP 2 and 4) (which is equivalent to the 4-hydroxy group in 4-hydroxytamoxifen) for receptor binding. Antitumor activity was assessed against the growth of ER positive human breast tumors in athymic mice (279). However, it is difficult to determine, in vivo, whether the action of the compounds to control tumor growth is the result of estrogenic or antiestrogenic action. The acetyl groups will almost certainly by hydrolyzed in vivo to present the tumor with potentially estrogenic triphenylethylenes (171). It is known, however, that high dose estrogen therapy can be used to control the growth of breast cancer (130, 142). A related diacetoxy compound, cyclofenyl (figure 21:5), is a full agonist in rat and mouse uterine weight tests (105), but is able to control the growth of dimethylbenzanthracene-induced rat mammary carcinomata (66). However, deacetylation of cyclofenyl and introduction of a single pyrrolidinoethoxy side chain (figure 21:6) produces a partial agonist with antietrogenic activity in the rat (105).

The cyclohexane ring system in the cyclofenyl molecule indicates that only a small spacing group is necessary to occupy the ER binding site rather than a stilbene-like system. Indeed, a substituted cyclopropane system will suffice as a skeleton for compounds with high affinity for the ER and estrogenic activity (221). An example of a *trans* substituted compound is ilustrated in figure 21:7. However, of some significance is the finding that the *cis* substituted derivative, known as analog II (figure 21:8), has low estrogenic activity in the mouse uterine weight test and weak antiestrogenic activity (248) and antitumor activity against dimethylbenzanthracene-induced tumors (247). The chlorine atoms are important as spacing groups because their removal destroys all biological (estrogenic and antiestrogenic) activity. This finding is quite significant when the biological properties of other simple chlorinated compounds are considered. Chlorine atoms are probably required as spacing groups in the estrogenic insecticide methoxychlor[2,2 bis (p-methoxy-phenyl)-1,1,1-trichloroethane] which is demethylated in vivo to the bisphenolic compound (35). This metabolic activation is probably necessary to permit receptor binding in the target tissue (243).

B. *Studies in Vitro*

Structure-activity relationship studies in vivo are complicated by the potential metabolism of the compound and the relative pharmacokinetics of the parent compound and its metabolites. An antiestrogen may be me-
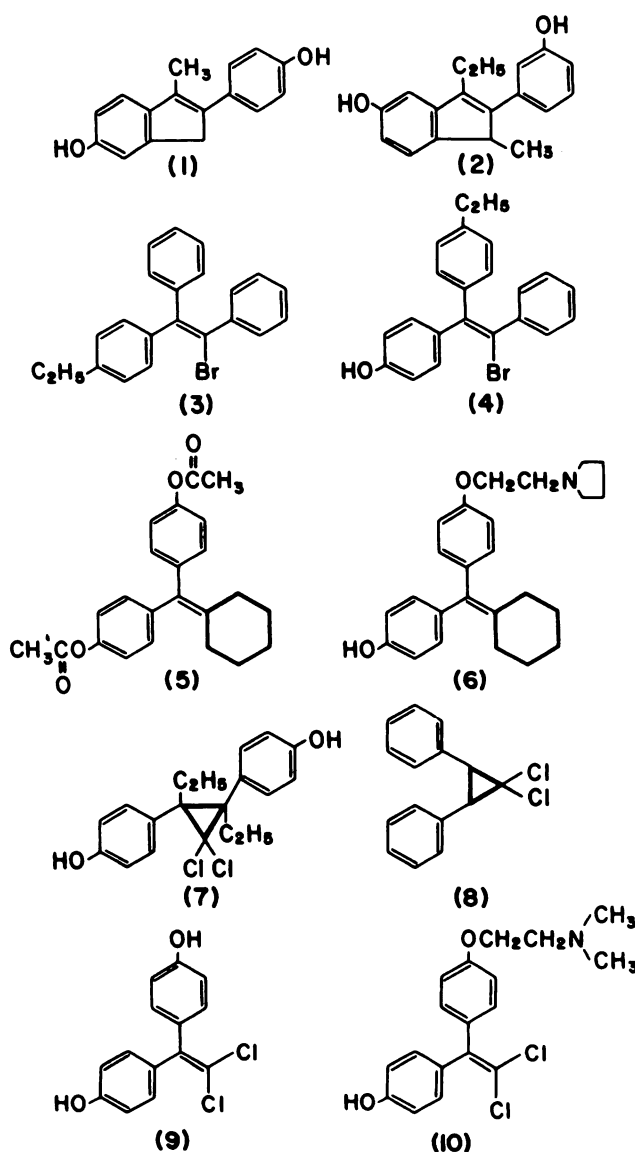
FIG. 21. Nonsteroidal estrogens and antiestrogens.

tabolized to a mixture of estrogens and antiestrogens which interact in different proportions at single or multiple receptor sites within a given target tissue. Structure-activity relationship studies in vitro can circumvent some of these problems and dissect out the action of the parent compound and its metabolites.

Studies of antiestrogens in vitro are restricted by the limited number of estrogen-responsive test systems. However, two systems have been studied: (a) the growth of MCF-7 breast cancer cells; and (b) estrogen-stimulated prolactin synthesis by primary cell cultures of immature rat pituitary glands.

The MCF-7 breast cancer cell system has been used to determine the effects of tamoxifen and its metabolites on cell proliferation (61). As might be expected from the relative binding affinity for the receptor, 4-hydroxytamoxifen is a more potent inhibitor than tamoxifen (61); however, the results with N-desmethyltamoxifen are more controversial. Coezy and coworkers (61) report the

metabolite has a low potency as an antiproliferative agent, whereas Reddel and coworkers (250) have demonstrated that concentrations of N-desmethyltamoxifen as high as 10 μM are extremely effective, apparently more so than either tamoxifen or 4-hydroxytamoxifen. The *cis* geometric isomer of tamoxifen, ICI 47,699, is ineffective as an antiproliferative agent (61) and stimulates MCF-7 cell proliferation (182). However, the *cis* geometric isomer of enclomiphene, zuclomiphene, is apparently extremely effective as an antiproliferative agent at a concentration of 5 μM (235). This observation is of interest because a similar cytotoxic effect for zuclomiphene has been reported in the prolactin-synthesis assay (211).

The antitumor activity of several clomiphene analogs has been tested: 9,599 (mono de-ethylated enclomiphene), 6,866 (enclomiphene with an addition of carbon in the aminoethoxy side chain), and 10,222 (enclomiphene with nitrogen substituted for the ether oxygen of the side chain) (235). At low concentrations (0.25 to 1.0 μM), where the growth-inhibitory effects are reversed by estradiol, the relative antitumor activity (6,866 > 10,222 > enclomiphene > 9,599) was in the same order as their relative binding affinities for the ER. There appears to be no correlation of antitumor activity with affinity for the antiestrogen binding sites.

A similar structure-activity study has been undertaken with a small series of substituted bromotriphenylethylenes: LN 1643 (figure 8), LN 2299 (*cis* isomer of LN 1643, figure 21.3), LN 2839 (the hydroxy metabolite of 1643, figure 21.4), and LN 2833 (LN 1643 with a secondary alcohol substitution in the ethyl side chain). Antitumor activity is directly correlated with the binding affinity for the receptor: 4-hydroxytamoxifen > LN 2839 > LN 1643 (28). LN 2299 and 2839 have very low affinities for the receptor but no antitumor effects have been reported (28). LN 2299 is an estrogen of equivalent potency to zuclomiphene in the prolactin synthesis assays and LN 2833 is a weak antiestrogen (V. C. Jordan and M. E. Lieberman, unpublished observations).

It is fair to point out that MCF-7 breast cancer cells in culture have not been extensively used to study structure activity relationships; however, LeClercq and coworkers (202) have recently presented some interesting SAR data. The bis phenolic dichloroethylene (figure 21:9) is estrogenic and able to reverse the antitumor action of nafoxidine. As might be predicted, substitution of one phenolic group with an alkylaminoethyl side chain (figure 21:10) reduces estrogenic activity but increases antiproliferative properties. The structural similarity of these compounds with cyclofenyl and analog II is illustrated in figure 21.

Overall the MCF-7 system for assay appears to produce results very similar to the prolactin synthesis assay which indicates that the compounds might have a common mechanism of action via the ER.

Primary cultures of rat pituitary cells respond to physiological concentrations of estradiol by a specific increase in prolactin synthesis (213). This model system for estrogen action has been validated for the study of structure-activity relationships within groups of nonsteroidal estrogens and antiestrogens. The antiestrogens, tamoxifen and 4-hydroxytamoxifen, inhibit estradiol-stimulated prolactin synthesis (212). Their potencies are consistent with their relative binding affinities for the ER; 4-hydroxytamoxifen is 30 times more potent than tamoxifen. To avoid the possibility that tamoxifen is metabolically activated to 4-hydroxytamoxifen in vitro, several *para* substituted derivatives of tamoxifen (*p*-methyl-, *p*-chloro-, and *p*-fluoro-) that are unlikely to be metabolized to 4-hydroxytamoxifen (9) have been tested. The substitution does not affect the binding of the compounds to the estrogen receptor (9, 212), and the derivatives of tamoxifen inhibit estradiol-stimulated prolactin synthesis consistent with their relative binding affinities for the receptor. Although it is an advantage for tamoxifen to be metabolized to 4-hydroxytamoxifen, it is clearly not a requirement for antiestrogenic activity (9, 212).

Antiestrogen action in the pituitary cells has been shown to be both competitive and reversible with the addition of excess estradiol (212). Furthermore, a series of known estrogens and antiestrogens has been tested in the system to establish its usefulness for structure-activity relationship studies (211). The biological potency of the binding ligands is directly related to their relative binding affinity for the ER. The relative potency of estrogens to stimulate prolactin synthesis was diethylstilbestrol; $\approx$ estradiol > ICI 77,949 (tamoxifen without its dimethyl aminoethane side chain) > ICI 47,699 (*cis* isomer of tamoxifen) $\approx$ zuclomiphene (*cis* isomer ofenclomiphene). The relative potencies of antiestrogens to inhibit estradiol-stimulated prolactin synthesis was *trans* 4-hydroxytamoxifen $\approx$ LY 117018 > trioxifene > enclomiphene $\approx$ tamoxifen. The compound LY 126412 (trioxifene without the side chain) does not interact with ER up to test concentrations of 10^{-6} M or exhibit estrogenic or antiestrogenic properties with the prolactin synthesis assay. The compound is also inactive in vivo (figure 5).
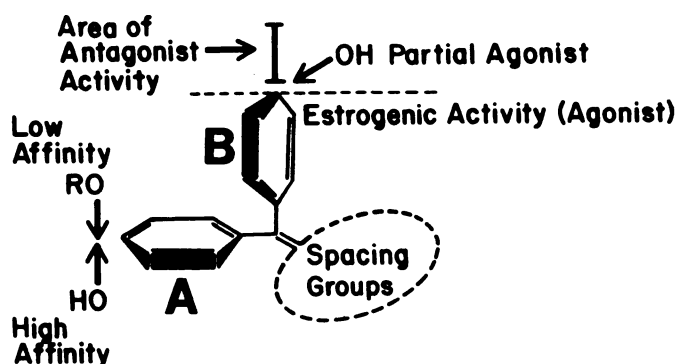

FIG. 22. A general ligand model to describe the structural requirement to control biological activity in vitro.
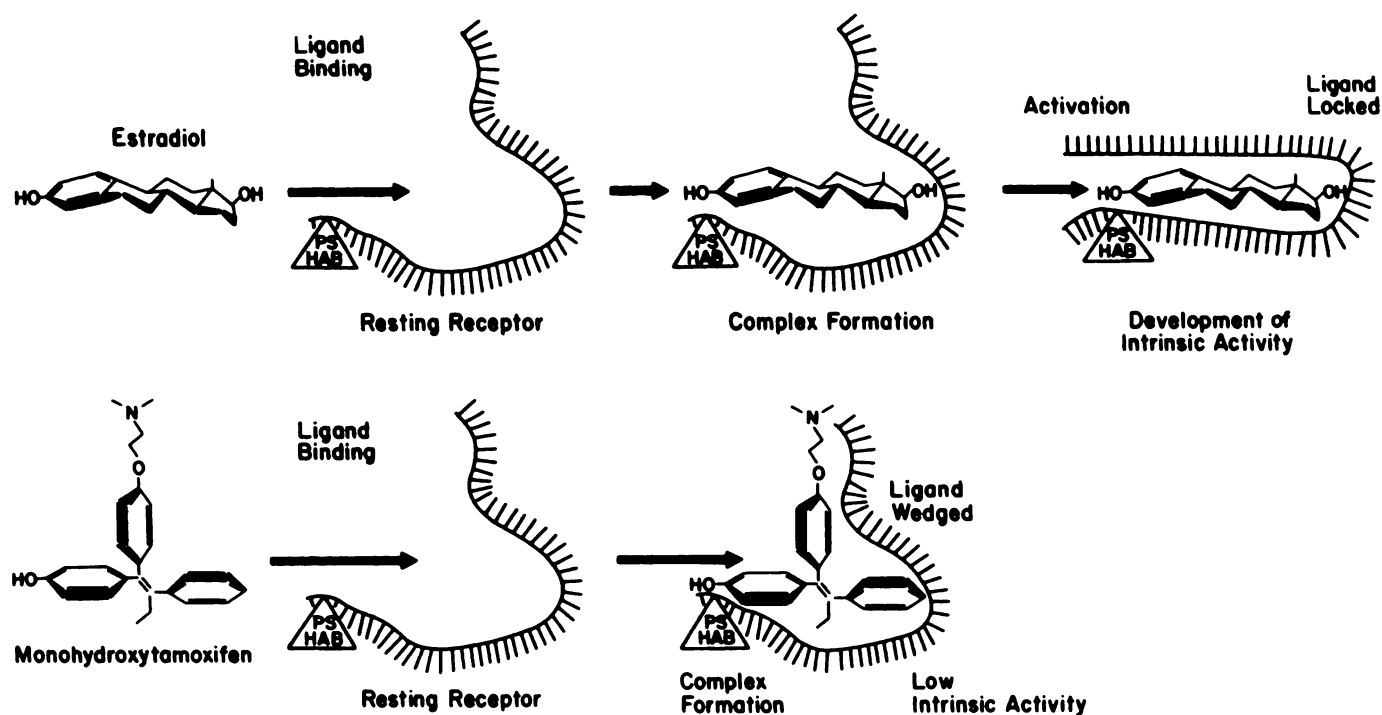
FIG. 23. Hypothetic models to describe the binding of estradiol or 4-hydroxytamoxifen with the ligand binding site on the estrogen. Estrogen can induce a conformational change in the receptor to lock the ligand into the receptor whereas the antiestrogen prevents these changes from occurring.

The prolactin-synthesis assay system is currently being used to evaluate the structural requirements for estrogen and antiestrogen action. The compounds in figure 20 are proving to be of particular interest. 4-Hydroxytamoxifen is the optimal substitution for tamoxifen; however, 3,4-hydroxytamoxifen, though it has a high binding affinity for the estrogen receptor (159), has a low potency as an antiestrogen in vitro because it is unstable. Nevertheless, much increased antiestrogenic potency is observed if the assay in vitro is conducted in media containing ascorbic acid, as an antioxidant and U-0521, an inhibitor of catechol orthomethyltransferase (COMT). The COMT inhibitor, U-0521, is known to stabilize the assay of catechol estrogens in vitro (129).

The substituted triphenylbut-1-enes are uniformally estrogenic as long as X_2 remains unoccupied (figure 20). What is particularly interesting though, is that *bis* substitution at X and X_2 (TPB4) with acetoxy groups produced an antiestrogen. However, the deacetylated compound is a partial agonist with antiestrogenic properties (170). A similar relationship occurs with cyclofenyl (compound 5, figure 21).

Overall, compounds can be classified into three categories based upon their structure (170). Antiestrogens have a side chain extending away from the binding site. Partial agonists have a *bis* phenolic structure and agonists are unsubstituted. Based upon these observations, the general structure-activity relationships in vitro can be described.

C. The Antiestrogenic Ligand

When considering the design of an antiestrogen, several features are dominant (figure 22). However, as with other drugs, the substituents that determine potency are different from those that determine pharmacological activity.

1. There is a broad range of compounds that bind to the ER and produce an estrogenic response in vivo (171).

2. The dimensions of the ER binding site are specific and precise. DES is a good example of a simple compound with a high affinity for the ER and potent estrogenic activity.

3. A phenolic hydroxy, equivalent to the C_3 phenol of estradiol, is extremely important for high affinity binding to the ER. This structural feature permits a variety of "spacing groups" to occupy the receptor binding site (phenyl, cyclohexane).

4. Alkyl ethers on ring A (figure 22) have a decreased affinity for the receptor, but an increased duration of action in vivo.

5. Depending upon the substitutions, triphenyl ethylenes can possess estrogenic or antiestrogenic activity. The increased potency of the triphenylethylene-type of antiestrogens (tamoxifen) compared to the less rigid triphenylethane (MRL37) and triphenylethanol (MER25) derivatives is at the cost of increased estrogenic activity since all the triphenyl ethylene derivatives are partial agonists.

6. Substitution of spacing groups (phenyl) with either

OH or OCH$_3$ does not have a major impact upon potency or pharmacological activity.

7. Substitutions on phenyl ring B governs pharmacological activity. Compounds without substitution are estrogens in vivo and in vitro: (a) A *para* hydroxy on ring B predicts estrogenic activity in vivo but partial agonist activity in vitro. (b) Extention of an alkylaminoethoxy or glyceryl side chain on ring B predicts partial agonist and antagonist properties in vivo but complete antagonist activity in vitro. (c) An acetoxy side chain on ring B predicts agonist activity in vivo (possibly metabolic activation to the phenols) but antiestrogenic activity in vitro. (d) An allyloxy side chain on ring B reduces antiestrogenic activity in vivo and in vitro compared with the alkylaminoethoxy side chain. (e) A *para* ethyl substitution on ring B predicts antagonist activity in vitro.

D. Application of Drug Receptor Theories

In their simplist form, the current theories of drug interaction with receptors are based upon the fundamental studies by Clark (46) and Gaddum (104) who suggested that the response to a drug is proportional to the number of receptors occupied. However, the occupation theory was modified by Stephenson (289) and Ariens and Simonis (11) into two steps: receptor binding (dependent upon the affinity of the drug for the receptor) followed by the production of a response (dependent upon the efficiency of intrinsic activity of the drug receptor complex). Thus, within a series of nonsteroidal estrogens (i.e., the intrinsic activity $\alpha = 1$), but which have pro-

gressively lower affinities for the estrogen receptor than estradiol, their log dose-response curves in an assay system will be progressively shifted to the right of estradiol's curve. However, for a group of compounds with intrinsic activities progressively less than 1.0, the maximal responses in their log dose-response curves will be progressively lower. These are partial agonists. However, the ideal antagonist would have a high affinity for the estrogen receptor but would have an intrinsic activity of zero. This ideal has been achieved with antiestrogens used in assay systems in vitro; therefore, the receptor interaction of estradiol or an antiestrogen such as 4-hydroxytamoxifen can be represented in the hypothetical scheme in figure 23. Estradiol first interacts via the C$_3$-phenolic group with a phenolic site on the receptor which then directs the steroid to the correct position at the binding site on the protein. The initial binding step is followed by a change in the tertiary structure of the protein that locks the steroid into the receptor; this change develops the intrinsic activity of the receptor complex. The antiestrogen 4-hydroxytamoxifen binds with high affinity via the interaction of the phenolic group with the phenolic site on the receptor. However, the tertiary changes in the receptor necessary to develop intrinsic activity in the complex are prevented by the alkylaminoethoxy side chain.

Based upon structure-activity relationship studies, a hypothetical model of the ligand interaction with the estrogen receptor binding site has been developed to describe the structural features necessary to initiate or to inhibit prolactin synthesis in vitro (211). Among the triphenylethylenes, compounds that have *cis* and *trans* geometric isomers are extremely important for the development of a ligand-receptor model because the isomeric molecules encompass estrogenic and antiestrogenic actions. Examples of the *trans* isomers, i.e., tamoxifen and enclomiphene are antiestrogens with zero intrinsic activity, whereas the *cis* isomers ICI 47,699 and zuclomiphene are estrogens with an intrinsic activity of 1.

To describe the interaction of the geometric isomers with the estrogen receptor, the *trans* stilbene-like structure of tamoxifen and enclomiphene could sit loosely at the binding site with low affinity binding so that the phenyl ring substituted with the *p*-alkylaminoethoxy side chain is projected away from the binding site (figure 24). The estrogenic ligands, zuclomiphene and ICI 47,699, with their low affinity for the ER, can create a *trans* stilbene-like structure with the *para* substituted phenyl ring. In this binding state, the aminoethoxy side chain would lie next to the phenolic site on the receptor with a weak interaction through the ether oxygen (figure 24). There would be no interaction of the side chain with a hypothetical antiestrogen region of the receptor and, as a result, no inhibition of estrogen action. The tertiary
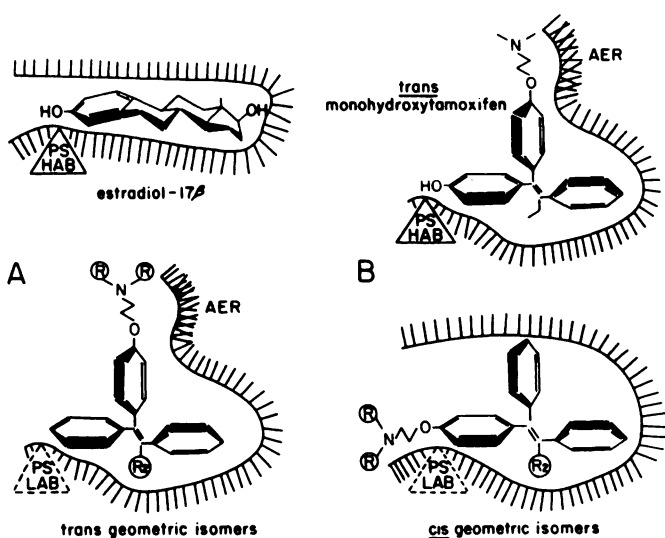
FIG. 24. Hypothetical models for estrogenic and antiestrogenic ligands binding to the estrogen receptor. Estradiol-17β is anchored at a phenolic site (PS) with high affinity binding (HAB). *trans* Monohydroxytamoxifen has the same high affinity binding but this antiestrogenic ligand binds to the receptor site so that the alkylaminoethoxy side chain can interact with a hypothetical antiestrogen region (AER) on the protein. Compounds without a phenolic hydroxyl have low affinity binding (LAB). The *trans* and *cis* geometric isomers refer to: (A) Tamoxifen (R = CH$_3$, R$_2$ = C$_2$H$_5$) and enclomiphene (R = C$_2$H$_5$, R$_2$ = Cl); (B) ICI 47,699 (R = CH$_3$, R = C$_2$H$_5$) and zuclomiphene (R = C$_2$H$_5$, R$_2$ = Cl).
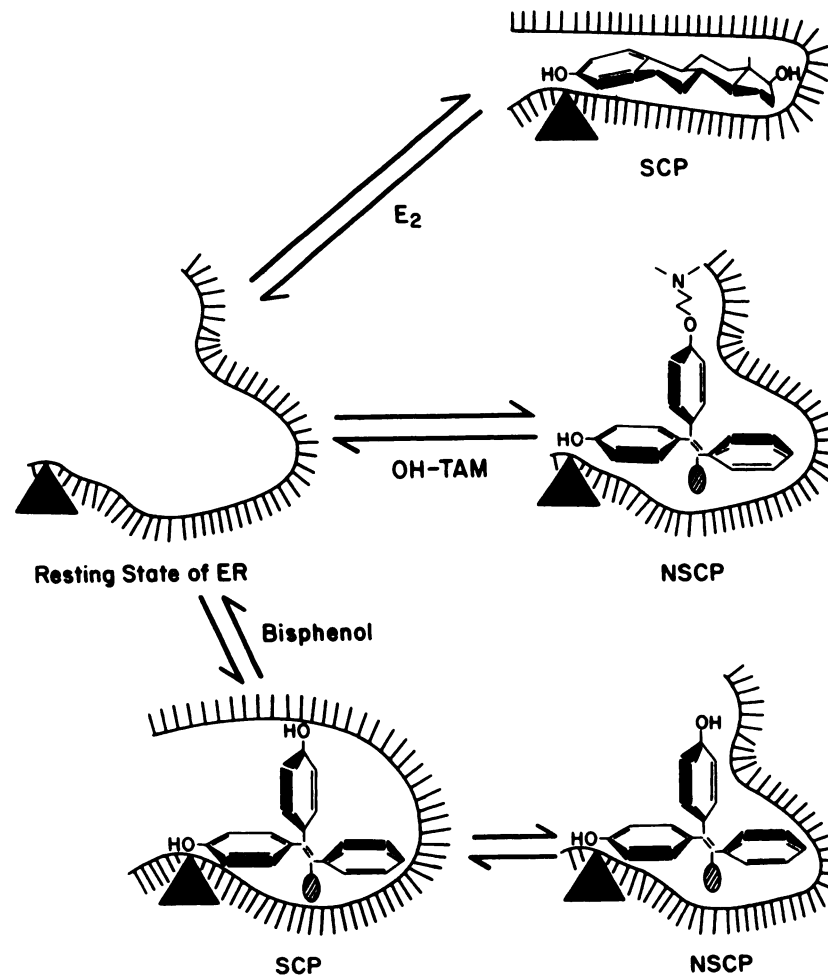
FIG. 25. Adaptation of Belleau's macromolecular perturbation theory (19) to describe the interaction of agonists, antagonists, and partial agonists with the estrogen receptor (ER). The phenol group on the ligand interacts with the phenolic site on the ER (closed triangle) and produce a high affinity interaction if the geometry of the ligand is correct. Estradiol (E₂), an agonist, induces a specific conformational perturbation (SCP) whereas 4-hydroxytamoxifen (OHTAM), antagonist, only induces a nonspecific conformational perturbation (NSCP). Bisphenol (partial agonist) produces a mixture of SCP and NSCP in the ER.

changes that are necessary to develop a high intrinsic activity for the complex can occur unimpeded.

The *geminal bis para*hydroxyphenyl compounds (deacetylated cyclofenyl, bisphenol) that are partial agonists in vitro are particularly interesting. Belleau's macromolecular perturbation theory (19), which was originally proposed to explain agonist, partial agonist, and antagonist activity of drugs at the muscarinic cholinergic receptor, may be used to explain partial agonists in terms of the ER model. According to Belleau's hypothesis, anagonist binds to the receptor and induces a specific conformational perturbation (SCP). An antagonist, on the other hand, binds to the receptor, produces a nonspecific conformational perturbation (NSCP), but the complex has zero intrinsic activity. Between these extremes, a partial agonist binds to the receptor and produces an equilibrium mixture of agonist and antagonist receptor complexes. Applying these definitions to the ER (Figure 25), estradiol binds with high affinity to the resting receptor and induces a SCP which results in the

ligand being locked into the binding site. 4-Hydroxytamoxifen (antagonist) wedges into the resting receptor and only produces a NSCP. Bisphenol (partial agonist) interacts at the ligand binding site, but while some of the receptors can be induced to lock the ligand into the protein, other ligand interactions are only able to induce a NSCP in the complex.

These hypothetical views of the interaction of the ER with agonists and antagonists may gain some substance through studies of the molecular biology of the estrogen receptor. If, in the future, the gene that controls ER synthesis can be cloned and large quantities of receptor prepared, then biophysical studies of receptor complexes with estradiol or 4-hydroxytamoxifen may provide clues to the 3-dimensional folding of the protein. Indeed, knowledge of the amino acid sequence of the ER and a description of peptide fragments near the ligand binding site obtained with alkylating compounds like tamoxifen aziridine (184) will permit the development of a precise molecular model for drug receptor interaction. In the

short term, though, a computer model of the receptor site is possible by extensive structure-activity relationship studies in vitro in parallel with receptor binding studies.

XI. Concluding Comments: Unresolved Issues

The past 5 years have seen many important advances in the understanding of estrogen and antiestrogen action. Progress has been facilitated by the introduction of radiolabeled antiestrogens with a high specific activity and high binding affinity for the ER, antibodies (monoclonal and polyclonal) raised to the ER, and renewed interest in structure-activity relationships with assay systems in vitro. A unifying theory of antiestrogen action is, however, impractical because there are several unexplained observations with antiestrogens that require further study: (a) The species differences in the pharmacology of antiestrogens is perplexing. While it is possible that the triphenylethylene-type antiestrogens (tamoxifen) are metabolized to estrogens in rodents, no convincing evidence has been presented to show metabolic differences between chickens and rodents. (b) Most antiestrogens exhibit agonist or partial agonist actions in vivo, but in vitro the compounds usually have zero intrinsic efficacy. The reason for this is unknown. (c) Tamoxifen binds to the so-called "antiestrogen binding site" with precise structual specificity and high affinity. The binding site requires definition biochemically and its physiological role needs to be established.

Finally, it is perhaps naive to believe that a clear view of the mechanism of action of antiestrogens can be described when the molecular mechanism of estrogen-controlled protein synthesis and cell division is as yet unknown. Antiestrogens will prove to be useful tools to probe estrogen action and to provide valuable comparative information to establish a molecular mechanism for both estrogens and antiestrogens.

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