Estrogens in the Environment

John A. McLachlan, Editor

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Foreword

This book is derived from the Symposium on "Estrogens in the Environment" sponsored by the National Institute of Environmental Health Sciences and held in Raleigh, North Carolina in September, 1979. The objectives of the Symposium, stated quite simply, were to determine what an estrogen is and how it works, and what effect estrogenic substances might have on human health. These objectives seemed timely since many chemicals with diverse chemical structures, some of which are common environmental contaminants, have been endowed with "estrogenic" properties.

To accomplish these goals, internationally recognized investigators in the fields of endocrinology, toxicology and environmental health, as well as representatives of federal agencies involved with the environment and human health, were invited to participate in the Symposium. The discussions were open and instructive and, where possible, have been included in the book. I hope that their inclusion will help recapture the excitement of the meeting.

New information presented at the Symposium included: Structure-activity relationships of steroid hormones and estrogenic xenobiotics determined by X-ray crystallographic and biochemical methods; the role of metabolism of natural and synthetic estrogens in hormone action; alternative models for the mechanism of action of estrogens at the biochemical, cellular and organ levels; detailed analyses of the hormonal activity of halogenated hydrocarbons; ecosystem dynamics of estrogenic substances including mycotoxins; potential routes of environmental, occupational and therapeutic exposure to estrogenic chemicals related to human health.

Interaction between investigators in different disciplines was stimulating and opened new avenues for future research. As such, this book should provide a focal point for workers in a wide variety of fields such as chemistry, endocrinology, pharmacology, toxicology, as well as obstetrics and gynecology, agricultural sciences, occupational and environmental health sciences, and epidemiology. Moreover, the data presented throughout the book show how limited our knowledge in this broad area is and should caution, as well as provide information for, those charged with applying these data in regulatory situations.

I want to thank the other members of the scientific committee, Dr. Kenneth S. Korach and James C. Lamb, IV, for their help in organizing the Symposium. They, Ms. Retha R. Newbold, and others were, in large part, responsible for the meeting's success. I am also indebted to the individual participants of the

Symposium for their cooperation, and I am especially grateful to the distinguished scientists who presided over the five Symposium sessions for their willingness to summarize the complex problems we were addressing. The question raised many years ago by Sir Charles Dodds concerning the structural diversity of estrogenic chemicals remains unanswered. We hope, however, that this volume moves us closer to that goal.

John A. McLachlan, Ph.D.
November, 1979

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MODELS OF ESTROGENIC-HORMONE ACTION

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In the 20 years since Elwood Jensen introduced experimental systems in which estrogen interaction with target tissues could be studied, a great deal of progress has been made. Receptors have been isolated and partially characterized. Specific regions of target cell genomes which are known to respond to estrogens now have been isolated, cloned and characterized to the extent that even the nucleotide sequences are largely known. Although much has been achieved, it is also important to define what is not known. This in turn will point out the directions that must be taken in future research efforts.

What is the function of the steroid-receptor complex remains the most crucial of these unaswered questions. While we know in several cases that increases in the specific products of certain genes occur in response to hormone treatment, we have no idea how this comes about. Furthermore, the cellular response to estrogen is invariably more complex than an effect on one or even several specific gene loci.

A second major question is how steroid receptor concentrations themselves are regulated. This includes developmental changes, as well as changes in receptor levels that occur during exposure to the hormone. This involves the depletion of cytoplasmic receptor, its translocation to the nucleus and the replenishment of cytoplasmic receptor.

An additional area of major interest, is the physical changes in the receptor that occur during its interaction with its ligand. Whether or not such changes are due to stabilization of different equilibrium states or induced conformational changes, we need to determine if all ligands cause essentially the same changes, or whether the chemistry of the ligand is important. Currently we know that chemistry of the ligand is essential in defining its affinity for the receptor and its dissociation rate. This in turn has drastic effects on the long term response to the hormone. However, information is needed on whether ligand chemistry has other effects on the receptor.

This discussion will focus on the first of the questions raised above. Current models of steroid hormone action have a general pattern in which the receptor is found initially in the cytoplasm and upon interacting with the steroid becomes "activated" (Figure 1). The activated steroid moves into the nucleus where it associates with the chromatin, either the DNA or chromosomal proteins, or both. While this model is compatible with much of the existing data, it does not provide a satisfactory explanation of how these hormones with their receptors function. Furthermore, there is evidence

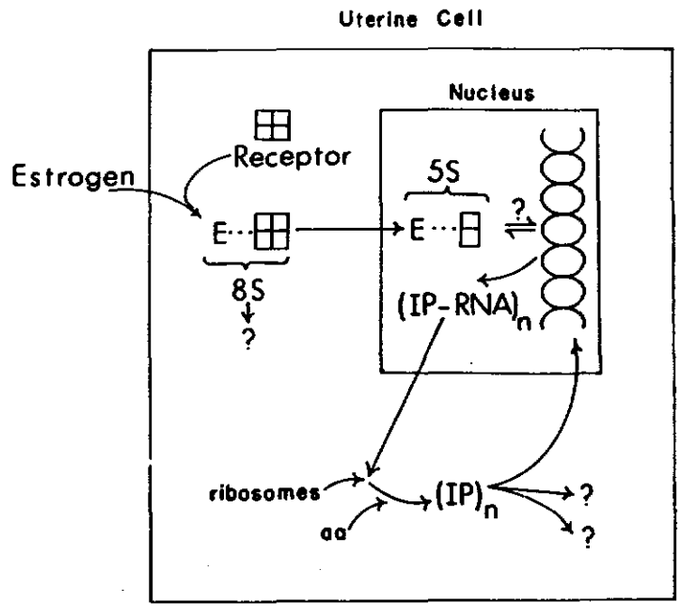

Figure 1

that does not fit neatly into this model. Since we have in the past outlined many of our reservations about current models, I would like to confine my remarks in this paper to some recent reports in the literature and work in our own laboratory.

The experimental system used in our laboratory historically has been the immature rat uterus. In the last few years, however, we have extended our studies to the pituitary. These studies have demonstrated that estrogen can regulate pituitary cells directly. Miller et al.[1] demonstrated that FSH production in primary cell cultures of ovine pituitaries was inhibited by 10^{-10} to 10^{-8} M estradiol-17β. At the same time, in the same cultures, TSH production was stimulated.[2] In both cases, removal of estrogen results in rapid reversal of the effect. We have extended these studies to the lactotrophs, which are the pituitary source of prolactin. Estrogen stimulates prolactin production in cultured rat pituitary cells 3 to 5 fold, similar to the increase seen in vivo.[3] Further, these changes in prolactin production appear to be due to increased prolactin biosynthesis, which in turn appears to be due to increased preprolactin mRNA concentrations present in the estrogen treated cells.[4,5]

At first glance these data seem to fit our steroid action model, i.e., estrogen is stimulating the production of a specific mRNA. However, it must be remembered that at the same time the lactotroph is being stimulated, estrogen also is stimulating thyrotrophs and inhibiting FSH producing cells. The receptor binding and translocation in all these cell types appears to be similar, although there is not much data on this subject. One is forced, therefore, to come up with a model in which a common receptor mechanism results in varied end point activities.

In a similar vein, McKnight[6] has recently reported an in vitro system in which chicken oviduct minces respond to the addition of estrogen and progesterone. The classical responses of increased ovalbumin and conalbumin syntheses and their respective mRNAs to estrogen in the oviduct now have been demonstrated in this in vitro system. There are curious differences in the time course of these responses and their dose response. Conalbumin synthesis appears to be a more sensitive response that is detected earlier than ovalbumin synthesis. It should be noted that these responses all depend on the use of a primed oviduct tissue. In the immature unprimed chick, the oviduct will not initiate immediately the synthesis of egg white proteins in response to estrogen. Estrogen priming for several

days causes cellular changes which permit egg white protein synthesis. Thus, in the chick oviduct, estrogen has two types of responses depending on the differentiated state of the cells. Again, the estrogen-receptor complex has not been reported to be different in these two states. Any general model of steroid action must therfore fit both of these differentiated states.

Another steroid hormone class, the glucocorticoids, has been shown to have a similarly broad range of apparent genomic effects. Ringold and Yamamoto[7] have shown that the synthetic glucocorticoid, dexamethasone stimulates the expression of a virus that has been integrated into the target cell's genome. Thus, a new genomic component appears to be readily regulated even though present at different integration sites in the genome.

Another interesting and related observation has been made by Ivarie and O'Farrell.[8] Two rat hepatoma cell lines in culture were treated with dexamethasone and the newly synthesized proteins of these cells were analyzed by the two dimensional gel electrophoresis systems developed by O'Farrell.[9] The dexamethasone treated cells showed changes in synthesis of a limited number of the many proteins that can be resolved by two-dimensional electrophoresis. The dexamethasone dependent proteins have been termed by Ivarie and O'Farrell as "domains." A domain may include proteins whose synthesis is increased, as well as those that are decreased. Out of more than 1000 proteins resolved in each cell line, only 7 proteins in one cell line and 8 proteins in the other were inducible consistently. A point of particular importance is that only one protein, tyrosine amino transferase, was common to both steroid induced domains of the two cell lines.

Thus, with two steroid hormones in several cell or organ culture systems, we see a very heterogenous response to apparently homogenous receptors. While there have been some suggestions of heterogeneity of receptors, the bulk of the evidence suggests that for any class of steroids the target cell receptors are homogenous. Perhaps the best evidence comes from the genetic studies of Yamamoto[10] and Bourgeois.[11] Their data suggests that the glucocorticoid receptor represents the product of a single gene.

What kind of a model can one propose that will explain the observations discussed above? It is my contention that interaction with one genomic site is highly improbable. Binding of a steroid-receptor to only one sequence of DNA cannot explain the heterogeneity of responses unless this single genomic

site in turn can control a variety of other genomic sites. A number of the steroid responses have been characterized as being primary responses, i.e., genomic responses that do not depend on prior protein synthesis. Therefore, the concept of a single gene induction as seen commonly in prokaryote systems does not present a useful model for steroid induced tissue responses.

An alternative model is that repetitive sequence of DNA might be recognized by the steroid receptor. The repetitive sequences would in turn be associated with a variety of unique sequences of DNA that would code for specific mRNAs. The repetitive sequence would act in a manner analogous to promoter regions of induced genes in prokaryotes. Various other chromosomal proteins might bind also to the repetitive or its associated unique sequence DNA in various differentiated states. These other protein-DNA interactions would determine whether or not a particular gene would interact with or respond to the steroid-receptor complex. The differential affinity of the steroid-receptor for its specific DNAs versus non-specific DNA interactions might not be readily detectable with present methodology as pointed out by Yamamoto and Alberts.[12] Studies to isolate potential regulatory sequences of DNA are underway in several labs. O'Malley[13] and Chambon[14] have reported the isolation and cloning of the oviduct gene. If promoter or other regulatory regions are adjacent to the coding regions of the gene it should be possible to directly determine if steroid-receptor complexes bind with higher affinity to these specific DNA sequences. Recent work on eukaryote gene structure now has been extended to the ovalbumin gene system by Chambon[14] and O'Malley.[13] The complexity of the transcribed region of the gene raises the potential that regulatory regions may be complex and not necessarily close to the coding sequences.

Another possible model of steroid-receptor regulation of nuclear function is that the steroid-receptor complex does not interact directly with the genome itself. Regulation of gene expression in animal cells appears to be more complex than originally believed. As pointed out earlier, studies with the hemoglobin gene and the virus SV40 have indicated that these genes themselves are more complex than originally thought with coding and non-coding "intervening sequences" interspersed. Furthermore, the evidence in these systems is that these genes initially are transcribed into large precursor RNAs which ultimately must be cleaved and then spliced to make the mature messenger RNA found in the cytoplasm. Several reports over the past few years have presented data indicating that transcription may not be the

ly site of regulation, but that regulation of what RNA is processed and rmitted to exit from the nucleus, may be equally or more important.[15] is may be especially important with mRNAs for regulatory proteins esent in small quantities.[16] With our new knowledge of the role of RNA ocessing, it is easy to conjure up new sites for regulating RNA processing d ultimately exit from the nucleus.

Furthermore, we know that modification of chromatin structure by modification of chromosomal proteins also would have major effects on gene expression. us, there are a large number of potential sites of action in the nucleus r steroid receptors. Such a model mechanism must work on nuclear components isting prior to the steroid receptor's entry into the nucleus. Therefore, vestigating newly synthesized proteins after hormone administration will scribe only the end points of gene expression and not its regulation.

Protein derivitization, such as phosphorylation, acetylation, methylation d adenylation have all been described. Several studies have been done with fects of steroids, including reports of increased phosphorylation of nuclear oteins after estrogen treatment. However, in most cases the methods used re capable only of detecting gross changes in proteins present in great undance. It would seem more likely that changes will involve proteins esent in smaller numbers than can be detected readily by column chromagraphy or single dimension gel electrophoresis. It will be necessary to e other methodology such as two-dimensional gels in which many proteins n be resolved.

It is well to remember that the steroid receptors themselves represent latively small populations of molecules; approximately 10,000 - 20,000 per ll, or 10^{-8} M if randomly distributed in target tissues. Under physiological nditions, 1,000 to 5,000 steroid receptors present in the nucleus has a eat physiological effect. Changes in similarly small populations of her nuclear proteins thus can be expected to be important.

Thus, its seems that future research must define a more encompassing model steroid-receptor regulation. This model must explain how an apparent mogeneous steroid receptor system involving translocation of the receptor to e nucleus gives rise to a very heterogenous variety of responses depending the state of the differentiation of the target cells.

ACKNOWLEDGMENTS

This work was supported by the College of Agricultural and Life Sciences, National Institutes of Health Grants HD 08192 and CA 18110, Ford Foundation Grant 630-0505A and by Grant No. 5 T32 HD07007-03 awarded by the National Institutes of Child Health and Development (DHEW).

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McLachlan, ed. Estrogens in the Environment

MOLECULAR BASIS OF ESTROGENICITY: X-RAY CRYSTALLOGRAPHIC STUDIES

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INTRODUCTION

From a careful examination of the molecular features of the numerous natural and synthetic products that have estrogenic or antiestrogenic properties it should be possible to identify those structural features that are responsible for receptor binding and hormonal function. However this task is complicated by uncertainties concerning solubility, transport, metabolism, and site of action of the compounds found to behave as estrogenic agonists or antagonists. An antiestrogen may act by competing for the target receptor site, the active site of a metabolizing enzyme, or a receptor in a feedback mechanism. Alternatively they may induce or inhibit the synthesis of other hormones or proteins that alter the course of action of endogenous estrogens.

If a specific receptor having a single estrogen binding site can be isolated and purified it should be possible to determine the relative binding affinities of a series of estrogen agonists and antagonists and the structural features responsible for binding should become apparent.

STRUCTURAL COMPARISONS

The crystallographically observed molecular structures of a number of natural and synthetic estrogens and antiestrogens will be compared, and an effort will be made to identify those features that determine binding and activity. The compounds examined will include phytoestrogens, mycotoxins and pesticides that have been demonstrated to exhibit estrogenicity.

Estradiol and Estrone

The three-dimensional structure of estradiol observed in the crystal structure of the hemihydrate[1] is illustrated in the stereo diagram (Figure 1). This is just one of three crystallographic determinations of the conformation of estradiol. The others include 1:1 complexes with urea[2] and propanol.[3] A superposition of two molecules of estradiol (Figure 2a) illustrates that despite variation in the immediate environment in the crystal complexes the estradiol conformation has limited variability. Crystallographic data on estrone[4] (Figure 2b) for which there are also three crystal forms containing

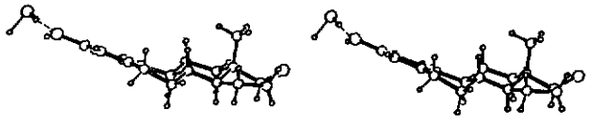

Fig. 1. Stereoview of the crystallographically observed conformation of estradiol.

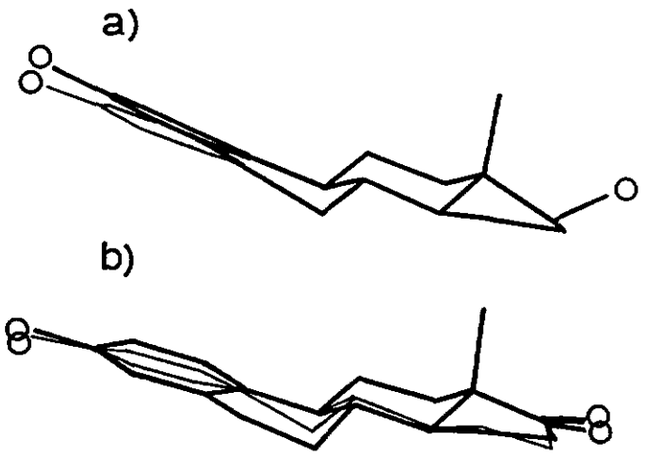

Fig. 2. Comparison of conformations of three crystallographically independent observations of estradiol (a) and two crystallographically independent observations of estrone (b). Overlap of the C and D rings has been maximized using a least-squares program, FITMOL, developed by G. D. Smith of the Medical Foundation of Buffalo, Inc.

four crystallographically independent molecules* show it to be a more flexible molecule than estradiol.

Most of the conformational flexibility in the 1,3,5(10)-estratriene backbone is located in the B-ring region. The B rings of all three estradiol molecules and three of the four estrone molecules are in 7α,8β-half chair conformations in which atoms C(9), C(10), C(5) and C(6) are coplanar and atoms C(7) and C(8) are equidistant from that plane on opposite sides. The remaining estrone molecule is found in an 8β-sofa conformation in which atoms C(9), C(10), C(5), C(6) and C(7) are coplanar and C(8) is on the β side of that plane.

*Some crystal forms have two or more independent molecules in the unit cell.

Analysis of conformational data on these and 21 other 1,3,5(10)-estratriene structures[5] illustrates that exocyclic nonbonded interactions play a decisive role in determining overall steroid conformation. This is particularly true of interactions across the A/C- and B/D-bay regions of the steroid backbone. Interactions between C(1) and C(11) stabilize the 7α,8β-half chair conformation of the flexible B ring in most 1,3,5(10)-estratrienes. 4-Bromo substitution and C(14)–C(15) dehydrogenation introduce strains at the C(6) and C(7) positions that are relieved by a shift in B-ring conformation to the 8β-sofa form. In estrone, estriol, and epiestradiol the 8β-sofa conformation is of comparable stability and, under favorable circumstances, conformational isomers will be co-crystallized apparently as a result of long range conformational effects involving interactions between C(12) and O(17). D.H.R. Barton[7] first described such long range effects in condensation reactions and called the phenomena conformational transmission.

Legrand *et al.*[6] have reported solution data further illustrating that changes in the D ring of estra-1,3,5(10)-trienes may be transmitted to the A ring. They found that the pKa's of estrone and estradiol in methanol differ by 0.10 pK units and that estrone is more acidic. This pK difference is consistent with the observed hydrogen bonding pattern in the crystal (Figure 3), a pattern that appears to require co-crystallization of solvent in crystals of estradiol. The three different crystal forms of estrone are all unsolvated, whereas an unsolvated crystal form of estradiol has not been isolated to date. In the solid state the more acidic estrone freely contributes its hydrogen atom to form a single hydrogen bond. Estradiol, however, always acts as a

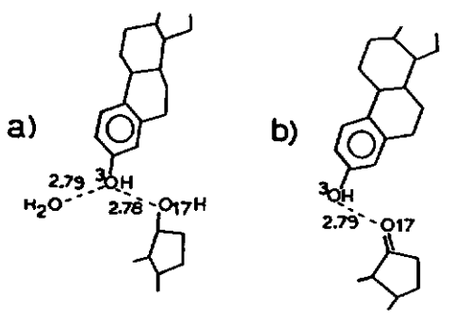

Fig. 3. Hydrogen bonding in crystals of (a) estradiol hemihydrate and (b) estrone. The C(3)-hydroxyl of estradiol acts as a hydrogen bond donor and acceptor while that of estrone acts as a donor only.

hydrogen bond donor and acceptor thus retaining a full hydrogen of its own. The donor hydrogen bond comes from the co-crystallized solvent. It seems reasonable to assume that these observed differences in overall conformation, conformational flexibility, pK values and affinity for solvent may play an important role in determining the considerable difference in affinity of estradiol and estrone for the estrogenic receptor.

Diphenylethylenes

The structural features most widely regarded as important to estrogenicity are a phenolic ring and two hydroxyl groups appropriately arranged relative to one another. A comparison of estradiol with one of the earliest synthetic estrogens, diethylstilbestrol, contributed to the development of this model. Early workers were overly enthusiastic in their efforts to illustrate a structural similarity between estradiol and diethylstilbestrol (DES), and they commonly drew DES as illustrated in Figure 4a in order to maximize its similarity to estradiol.[8] The crystal structures of anhydrous DES[9] and DES complexed[10] with various solvents provide valuable information on the flexibility of the molecule. The phenyl rings of DES are not coplanar and the ethyl groups do not lie in the plane of the rings. Anhydrous DES (Figure 4b) has a center of inversion at the midpoint of the C-C bond.

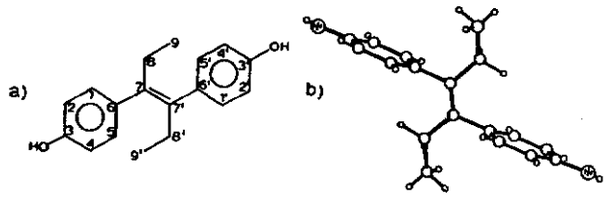

Fig. 4. (a) Diethylstilbestrol (DES) drawn to maximize its superficial resemblance to estradiol and (b) DES conformation as observed in the anhydrous crystal form.

The DES molecules found in several crystal complexes do not possess this symmetry. Busetta *et al.*[11] have illustrated that asymmetric DES more closely resembles natural estradiol in overall shape and hydrogen bonding (Figure 5). Although both hydroxyls act as hydrogen bond donors and acceptors in all crystal forms of DES, the geometry of the hydrogen bonds to one of the phenol rings in the asymmetric form more nearly resembles the hydrogen bonding in the estradiol complexes.[11]

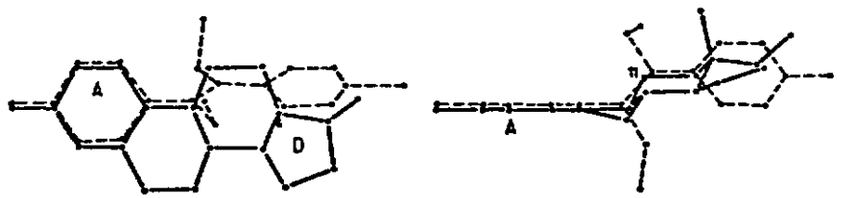

Fig. 5[11] Superposition drawing of acentric DES and estradiol from Busetta *et al.*

Numerous efforts have been directed toward correlating estrogenic activity with the distance between the terminal oxygens. In 1950, Keasling and Schueler mistakenly reported the interatomic distance between the terminal oxygens on diethylstilbestrol (DES) and estradiol as being approximately 14.5Å[12]. The crystallographically observed distances between the terminal oxygens in DES and estradiol are 12.1Å and 10.9Å respectively. The propensity of estradiol to form hydrogen bonds to water or other solvents and the 1.2Å disparity between the terminal oxygen distances in estradiol and DES suggest that water may play a significant role in linking estradiol to the receptor protein. The distance between O(3) and a hydrogen bonded oxygen in estradiol hydrate is 12.1Å, identical to the O-O distance in DES. The overall conformational match between the two molecules is shown in Figure 6. The dimethyl analogue of DES is reported to be inactive and has been known to crystallize in the centrosymmetric form (similar to Figure 4b) only, supporting Hospital's proposal that the asymetric form is bound to the receptor. A tetrafluorinated derivative of DES is reported to have reduced binding affinity yet retain some activity.[13] The reduced binding could be a result of direct interaction of the fluorines or conformational change associated with the substitution. To date the structure of this compound has not been reported.

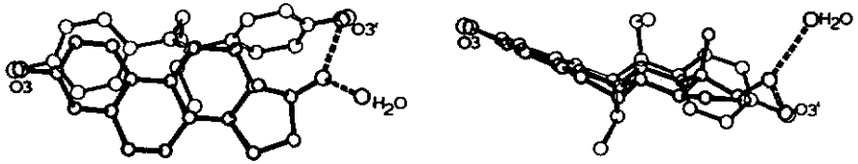

Fig. 6. Superposition drawing of solvated estradiol and DES, maximizing relative positioning of hydrophylic groups and hydrophobic bulk. $O3'$ is the hydroxyl group of an adjacent molecule in crystals of estradiol. Hydrogen bonds are indicated as (= = = = = =).

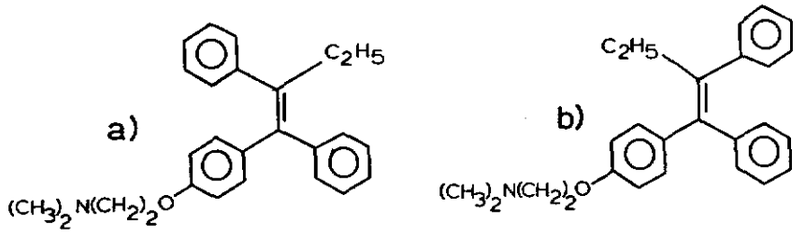

Fig. 7. Chemical drawings of (a) *trans* and (b) *cis*-tamoxifen.

Triphenylethylenes

Many *trans* triphenylethylenes including the *trans* isomer of tamoxifen (Figure 7a),[14] clomiphene,[15] and broparestrol[16] have been found to be anti-estrogenic. *Trans*-tamoxifen, 1-*p*-(2-dimethylaminoethoxy-phenyl)1,2-*trans*-diphenylbut-1-ene, is reported to be weakly estrogenic as well. In contrast the *cis* isomer of tamoxifen (Figure 7b) behaves like a conventional estrogen. X-Ray crystal structure results on these four compounds[15,16,17,18] illustrate that the triphenyl rings are inclined 50-60° with respect to the ethylene linkage (Table 1). The crystallographically observed conformations of *cis*[18] and *trans* tamoxifen are compared with that of the acentric form of DES in Figure 8.

If the *trans* phenyl rings of DES are important for its activity, and since it is *cis*-tamoxifen that is estrogenic, the $O(CH_2)_2N(CH_3)_2$ substituted ring and the phenyl *trans* to it in *cis*-tamoxifen would appear to be the rings that function in an analogous way to those of DES. In accordance with such a model the $O(CH_3)_2N(CH_3)_2$ substituted ring would simulate either the steroid A ring or D ring. In Figure 9, we have elected to consider the substituted tamoxifen ring as corresponding in function to the A ring at the binding site. This is the simplest possible model that might account for the similarity in receptor binding of tamoxifen and estradiol. The development of a model would be simplified if one of the triphenyl rings were phenolic. It has been suggested that the C(1) phenyl ring is metabolized *in vivo* to produce such a ring.[19] Other authors[20] dispute these findings and report the principal metabolite of tamoxifen to be an N-desmethyl derivative. There has also been speculation about possible hydroxylation of the other phenyl rings.

Hydroxytamoxifen,[*trans*-1(*p*-dimethylamineethoxyphenyl)1(*p*-hydroxyphenyl)2-phenylbut-1-ene] (Figure 10a) has been synthesized and is reported to bind to

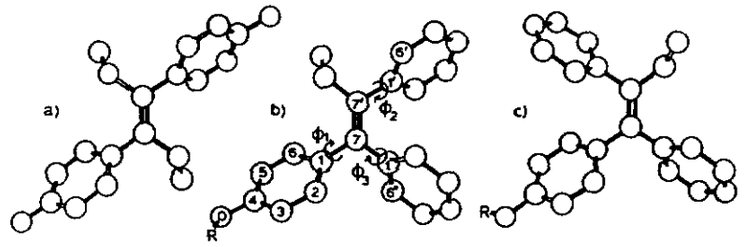

Fig. 8. A comparison of the conformations of (a) DES, (b) *cis*-tamoxifen, and (c) *trans*-tamoxifen viewed perpendicular to the plane of the ethylene group. R = $O(CH_2)_2N(CH_3)_2$.

TABLE 1

THE TRIPHENYLETHYLENE RING CONFORMATIONS

	ϕ_1 [a]	ϕ_2 [b]	ϕ_3 [c]
cis-tamoxifen			
molecule 1	51°	-126°	-130°
molecule 2	59	-131	-134
trans-tamoxifen	48	55	-116
trans-clomiphene	54	57	-129
trans-broparestrol	42	51	-119

Note: Atomic numbering is illustrated in Figure 8B.

[a] ϕ_1 = C(6)-C(1)-C(7)-C(7')

[b] ϕ_2 = C(6')-C(1')-C(7')-C(7)

[c] ϕ_3 = C(6")-C(1")-C(7)-C(7')

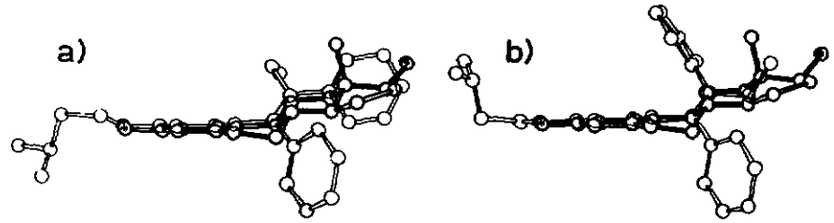

Fig. 9. Superposition drawings of estradiol and (a) estrogenic *cis* tamoxifen and (b) antiestrogenic *trans* tamoxifen assuming correspondence in receptor interaction of the substituted phenyl ring of tamoxifen and the A-ring of estradiol. The estrogenic isomer is observed to more nearly approximate the shape of estradiol.

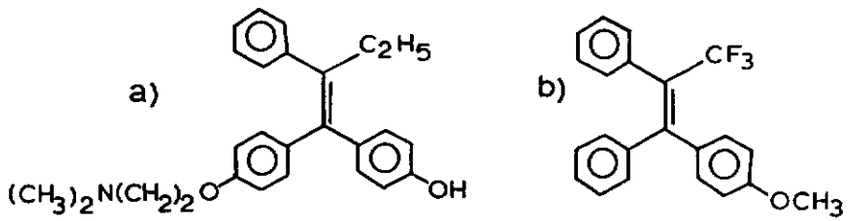

Fig. 10. Chemical drawings of (a) hydroxytamoxifen and (b) *trans*-*p*-methoxy-α-phenyl-α'-trifluoromethylstilbene.

the calf uterine estrogen receptor with affinity equal to that of estradiol and to be a potent antiestrogen.[21] Potent antifertility activity is also reported for *trans*-*p*-methoxy-α-phenyl-α'-trifluoromethylstilbene[22] (Figure 10b). If the methoxy group in the latter compound were metabolized to the hydroxy both of these potent antiestrogens could be expected to have two phenyl rings in nearly the same conformation as DES, with one of them hydroxylated. The antiestrogen behavior might then be attributed to either (1) the absences of a hydroxyl on the *trans* ring, or (2) the interference of the third ring with a protein interaction essential to activity. These data suggest that the $O(CH_2)_2N(CH_3)_2$ bearing ring does not simulate the steroid A ring (as suggested in Figure 9). This disparity might be resolved by assuming that the estrogenic and antiestrogenic triphenylethylenes bind differently at the receptor (different rings mimic the A ring in each case), or that estrogenic and antiestrogenic receptors differ.[23]

Another tricyclic compound found to have great estrogenic activity is cyclofenil (Figure 11a). Busetta has proposed that none of the phenyl rings of cyclofenil mimics the steroid A ring, but that the interactions of cyclofenil with the receptor resemble those of *cis* tamoxifen[11] (Figure 11b).

Phytoestrogens

Phytoestrogens are plant substances found to have estrogenic properties. They include coumestrol,[24] mirestrol[25] and genistein[24] (Figure 12). Coumestrol and mirestrol have ring structures comparable to that of a steroid, and coumestrol is constrained by unsaturation to have a nearly planar conformation. The connectivity and conformation of mirestrol was determined in one of the earliest X-ray studies of a steroid.[26] The comparison of the plant estrogen mirestrol, the natural estrogen estradiol, and the synthetic estrogen DES

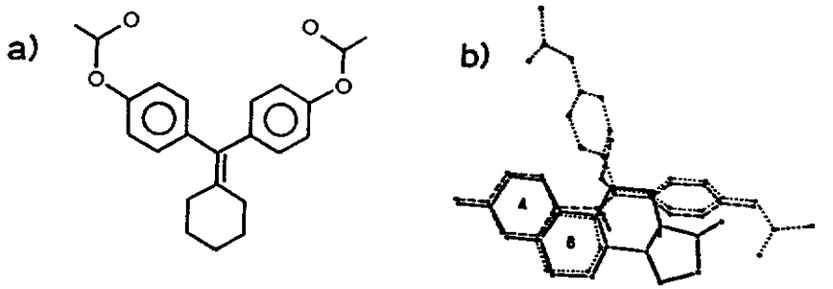

Fig. 11. (a) Chemical drawing of cyclofenil. (b) Superposition of estradiol (————), *cis*-tamoxifen (– – – –), and cyclofenil (· · · · · ·).

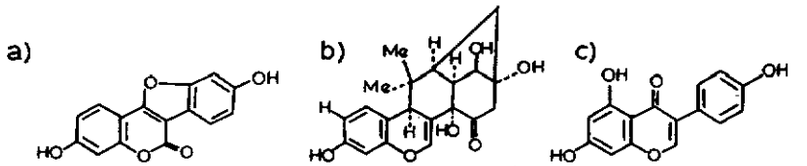

Fig. 12. (a) Coumestrol, (b) mirestrol, and (c) genistein are representative phytoestrogens.

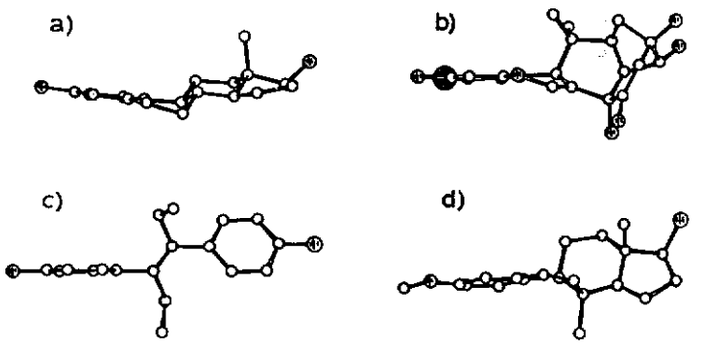

Fig. 13. A comparison of the overall conformation of (a) estradiol, (b) a brominated derivative of mirestrol, (c) DES, and (d) a modified steroid 3-methoxy-8α-methyl-1,3,5(10),6-estratetraen-17β-yl bromoacetate, emphasizing similarities of the A-ring region.

(Figure 13), bolsters the argument that one phenyl ring of DES mimics the A ring of the other two compounds and suggests the importance of the A ring to receptor binding. Change in configuration at C(8) can also produce a highly estrogenic steroid having an overall conformation[27] similar to that of bromomirestrol (Figure 13d).

Indenestrol and indanestrol (Figure 14), compounds synthesized as DES analogues, bear a close structural resemblance to the phytoestrogen genistein. Indenestrol has more than 100-fold greater binding affinity for the estrogen receptor than does indanestrol.[28] The crystal structure determination of the less active indanestrol[29] reveals that the three hydrogen substituents on the five membered ring are on the same face of the ring (Figure 15a). Because of steric hindrance the ethyl and methyl substituents take up equatorial orientations and the phenyl group is in an axial orientation relative to the fused rings. The overall conformation of indanestrol which is compared with that of estradiol in Figure 15b illustrates that the molecule is dramatically bent as a result of the axial orientation of the phenyl substituent. In the more active indenestrol the double bond in the five membered ring can be expected to produce a much flatter molecule.

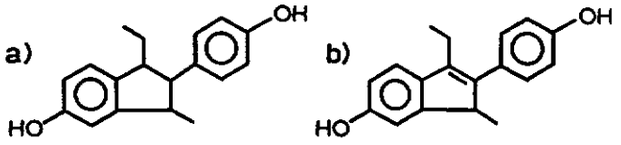

Fig. 14. Chemical drawings of (a) indanestrol and (b) indenestrol A.

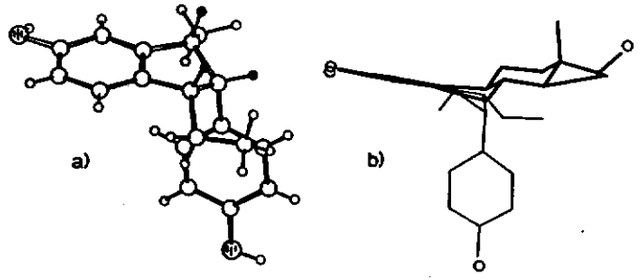

Fig. 15. (a) Perspective view of indanestrol illustrating the orientation of the hydrogen substituents on the five membered ring. (b) Superposition drawing of estradiol (darker) and indanestrol assuming that the fused rings mimic the steroid A and B rings.

Mycotoxins

Fungal toxins present in feeds cause various diseases in animals and may be carried further in the food chain. Zearalenone* (Figure 16), a mycotoxin produced by *Fusarium graminearum* is the cause of estrogenic syndrome in swine.[30] A number of analogues and naturally occurring derivatives of zearalenone have been tested for estrogenic activity. Zearalenone has a phenolic ring and a carbonyl group that might conceivably occupy positions in space corresponding to these groups in estrone. Examination of the crystallographically determined conformations of *trans*-zearalenone[31] and its 8'-hydroxy derivative[32] and their comparison with estradiol allow testing of such a hypothesis. The endocyclic torsion angles of the 14-membered rings which define the conformation of zearalenone and its 8'-hydroxy derivative are compared in Figure 16. The 8'-hydroxy substituent induces significant rearrangement of the 14-membered ring but the overall conformation of the two molecules remains similar as shown in Figure 17.

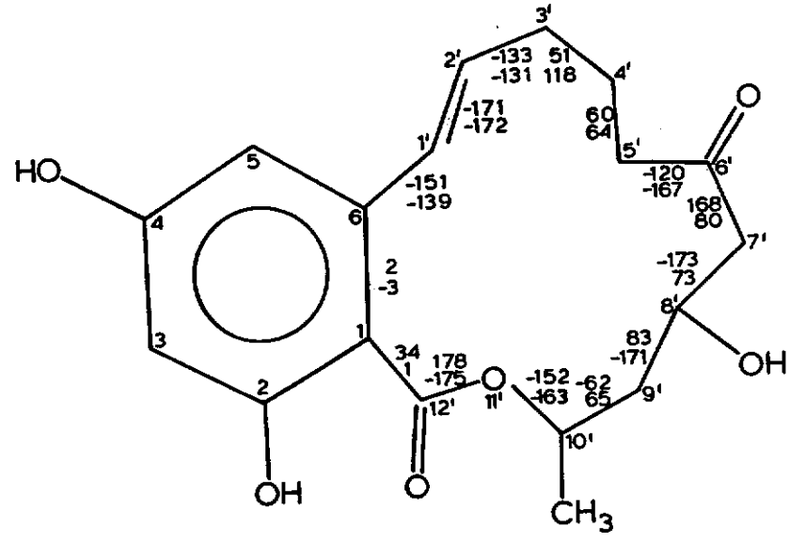

Fig. 16. Atomic numbering and the torsion angles of the 14-membered rings in *trans*-zearalenone (above) and its 8'-hydroxy derivative (below).

*6-(10-Hydroxy-6-oxo-*trans*-1-undecenyl-β-resorcyclic acid lactone.

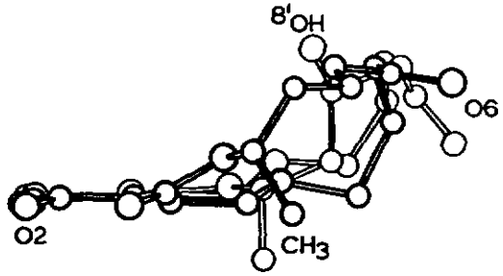

Fig. 17. Comparison of overall conformations of *trans*-zearalenone (darker) and its 8'-hydroxy derivative when viewed along a vector from C(2) to C(4) in the phenolic ring.

The greatest similarity in the observed conformations of 8'-hydroxy-*trans*-zearalenone and estradiol is obtained when the phenyl rings are nearly superimposed without hydroxyl overlap as shown in Figure 18. It is worth noting that while the phenyl ring hydroxyls are not overlapping, their locations are close enough to one another to allow hydrogen bond formation to the same site. The relative location of hydrogen bonded water of solvation in 8'-hydroxy zearalenone and estradiol hemihydrate are also shown in Figure 18. If the relative orientations of zearalenone and estradiol when bound to estrogen receptor are as depicted in Figure 18, the 6-oxo group could act as a hydrogen bond acceptor whereas the 17β-hydroxy of estradiol can act as a hydrogen bond donor and acceptor.

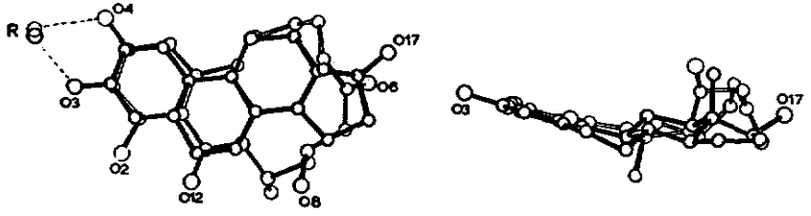

Fig. 18. Superposition drawing of estradiol and 8'-hydroxy zearalenone illustrating similarities in overall conformation and possible hydrogen bonding to the same receptor site (R).

Pesticides

The estrogenic activity of *o,p'*-DDT and certain other analogs of DDT has been well documented.[33] Of particular interest is the fact that inhibition of estradiol binding is found with *o,p'*-DDT but not *p,p'*-DDE.[34] The analogue *o,p'*-DDD has been described as a more potent inhibitor of the human placental 17β-hydroxysteroid dehydrogenase than many other structural analogues, non-steroidal estrogens, and antiestrogens,[35] In addition, McBlain, Lewin and Wolfe[36] have shown that the (-)isomer of *o,p'*-DDT is a far more potent estrogen than the (+)isomer.

The crystal structures of the racemic mixture of *o,p'*-DDT containing two molecules in the asymmetric unit,[37] the (-)isomer of *o,p'*-DDT,[38] and *p,p'*-DDT[37] have been reported. The conformations of the molecules are illustrated in Figure 19 and the relative orientations of the phenyl rings in the four structures are defined by the torsion angles listed in Table 2.

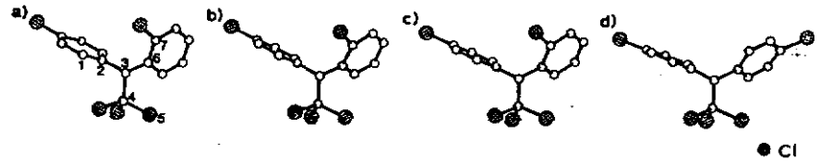

Fig. 19. Three crystallographically independent observations of the conformation of (-)isomer of *o,p'*-DDT (a, b, and c) and a single observation of *p,p'*-DDT (d). Two of the isomers of *o,p'*-DDT (a and b) are co-crystallized in the racemic mixture.

TABLE 2

THE CONFORMATION OF *o,p'*-DDT and *p,p'*-DDT

	ϕ_1[a]	ϕ_2[b]	ϕ_3[c]
o,p'-DDT			
molecule 1	87.4°	177.8°	76.3°
molecule 2	94.8	175.5	85.7
(-)*o,p'*-DDT	94.2	178.2	83.3
p,p'-DDT	92.1	174.3	94.8

Note: See figure 19 for numbering scheme.

[a] ϕ_1 = C(1)-C(2)-C(3)-C(4)

[b] ϕ_2 = C(2)-C(3)-C(4)-Cl(5)

[c] ϕ_3 = C(2)-C(3)-C(6)-C(7)

Although the crystal structure of DDE has not been reported, the ethylene linkage is certain to produce a much flatter molecule and this difference in shape accounts for some of the difference in activity. McBlain proposed that the R form of *o,p'*-DDT more nearly resembles estradiol in shape than the S form and that the (-)isomer must be the R form. The crystal structure determination revealed that the more active (-)isomer is indeed the R form.[38] In Figure 20a the *p* substituted phenyl ring of (-)*o,p'*-DDT is superimposed on the steroid A ring and the *p*-chlorine atom and the 3-hydroxy substituent are aligned.

The estrogenic activity of the DDT analogue methoxychlor is probably due to its metabolism to {[2,2-bis(*p*-hydroxyphenyl-1,1,1-trichloroethane}(HPTE)} Figure 21a) which has been shown to compete for the estrogen receptor.[39] The conformation of HPTE can be expected to resemble *p,p'*-DDT rather than *o,p'*-DDT. In Figure 20b, *p,p'*-DDT is oriented to permit maximum overlap of the *p*-substituents of the phenyl rings with the O(3) and O(17) hydroxyls of estradiol as well as overlap of the hydrophobic middle of the two structures.

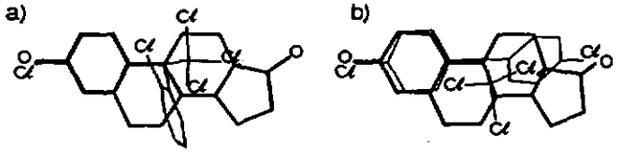

Fig. 20. (a) Superposition drawing of estradiol and (-)*o,p'*-DDT with phenyl ring overlap maximized by a least-squares procedure (FITMOL). (b) Superposition drawing of estradiol and *p,p'*-DDT in which the correspondence between the *p*-chloride substituents in the DDT and O(3) and O(17) of estradiol as well as the hydrophobic bulk distribution is maximized.

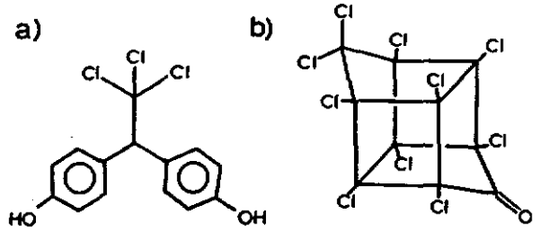

Fig. 21. (a) HPTE, the pesticide having the greatest similarity to estradiol, and (b) kepone, a pesticide competitor for the estrogen receptor bearing no apparent resemblance to estradiol.

Another pesticide that is reported to have estrogenic activity is kepone.[40] Since kepone (Figure 21b) has a cage-like structure and no phenolic rings, it is difficult to establish any structural similarity to the natural estrogens.

MECHANISM OF BINDING AND ACTIVITY

The only structural element common to all of the estrogens and antiestrogens discussed here (with the exception of kepone) is a phenyl ring, and there is ample evidence that a phenol ring is a more potent competitor for the estrogen receptor. Furthermore, Mueller[41] has shown that simple alkyl phenols can prevent binding of estradiol and displace the prebound hormone from the estrogen receptor of uterine cytosol. Tetrahydronaphthol (Figure 22a), an analog of the A and B rings of estradiol, is highly effective in preventing forward binding of estradiol. *p-sec*-Amyl phenol (Figure 22b) with a flexible alkyl chain corresponding to the B ring of estradiol is highly effective at 0°C in displacing estradiol which has been prebound by the receptor.

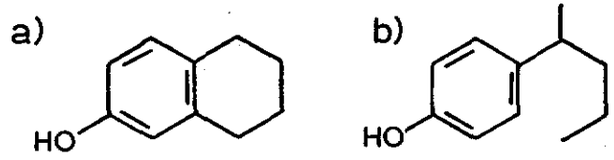

Fig. 22. Simple alkylphenols that prevent binding of estradiol and/or displace prebound hormones: (a) tetrahydronaphthol and (b) *p-sec*-amyl phenol.

On the basis of analysis of steroids having the highest affinity for the uterine progesterone receptor, we have previously proposed that the steroid A ring is primarily responsible for progesterone receptor binding and that structural differences in the D-ring region appear to be responsible for determining activity or distinguishing agonism from antagonism.[42] On the basis of the structural data presented above, it appears plausible that a phenolic A ring having a propensity to behave as a hydrogen bond acceptor as well as donor may be the most important determinant of high affinity binding to the estrogen receptor (Figure 23). The composition and orientation of the region of the estrogen and antiestrogen corresponding to the D ring of estradiol will govern subsequent hormonal events such as conformational change in the receptor, stabilization of a possible dimeric form of the receptor, and interaction with chromatin or DNA (Figure 24)[42,43] in the nucleus. Structures

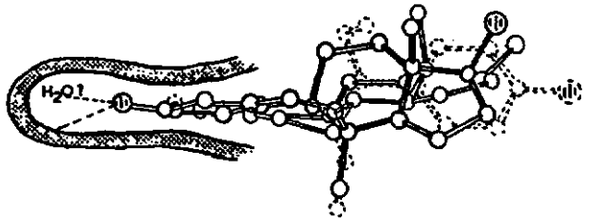

Fig. 23. Model for estrogen receptor binding in which the phenolic ring has an intimate association with the receptor and differences in variable D-ring region will control events subsequent to binding that govern activity.

having D rings or *pseudo* D rings suited by chemical makeup, overall shape, and spatial location to promote the required interaction would behave as agonists whereas structures lacking these features would behave as antagonists.

Clark, Pasko, and Peck find that long term nuclear retention of receptors is required for estrogen regulation of transcriptional events associated with uterine growth.[44] Horowitz and McGuire suggest that nuclear processing steps may be essential for the function of estrogenic compounds as inducers of progesterone receptor and that such a step is partially or completely impaired in antiestrogens.[45] Tseng and Gurpides have found that only phenolic steroids possessing a 17β-hydroxy group compete with estradiol for nuclear binding.[46] These observations are consistent with a model incorporating A-ring control of receptor binding and D-ring control of subsequent (nuclear) events.

SUMMARY

Some of the tentative conclusions that can be drawn from this analysis of the crystallographically observed conformations of estrogens and antiestrogens include the following:

(1) The greater binding affinity of estradiol over estrone may be due in part to a pK difference of the C(3)-hydroxyl that is caused by long range conformational transmission associated with C(17) substitution.

(2) The C(3) hydroxyl may act as a hydrogen bond donor and acceptor in the active site.

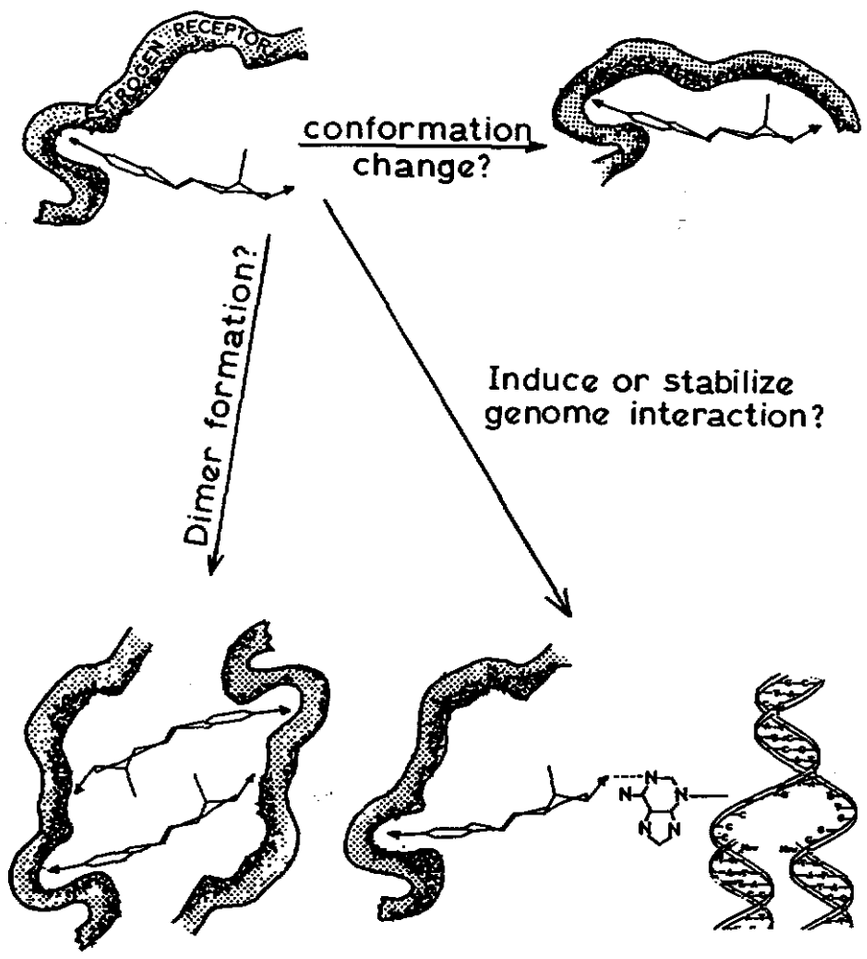

Fig. 24. Some of the possible roles that the D-ring region may play in controlling estrogenic behavior. Absence of the appropriate D-ring feature could cause antagonism.

(3) Asymmetric DES has a spatial arrangement similar to estradiol.

(4) Structural comparison of DES and estradiol suggests that water may play an important role in estradiol receptor interactions.

(5) If the *trans* phenyl rings of DES are essential to activity then the $O(CH_2)_2N(CH_3)_2$ bearing ring of *cis*-tamoxifen may mimic one of them in its interaction with the receptor.

(6) The triphenylethylenes of Figure 10 suggest that the $O(CH_2)_2N(CH_3)_2$ bearing ring of *cis*-tamoxifen does not mimic the steroid A ring or that estrogens and antiestrogens may bind differently to the same site or to different sites.

(7) The phytoestrogens and myrotoxins demonstrate structural similarity in the A-ring regions and diversity of the "D-ring" regions.

(8) A phenolic ring that can mimic the A ring of estradiol appears to be sufficient to permit effective competition for binding to estrogen receptors.

(9) Considerable variation at the D-ring region of steroids or comparable regions in nonsteroidal estrogens is compatible with high affinity binding and may differentiate between agonist and antagonist behavior.

ACKNOWLEDGEMENTS

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DISCUSSION

NAFTOLIN: The use of estrogen binding as synonymous with estrogen actions is hazardous and should be avoided.

DUAX: Your point is well taken. Nevertheless, most of the compounds reported to have significant estrogenic activity do exhibit significant binding to the estrogen receptor. This suggests that binding is required, but not sufficient for activity. This opens the door to the possibility of a pure antagonist that would bind to the receptor but lack the structural feature responsible for activity. I was attempting to determine whether any common structural feature could be identified as being essential for binding. Our proposal that the "D"-ring region may be the controlling factor in determining the degree of agonist and antagonist behavior through mediating events subsequent to receptor binding is meant to be highly speculative.

MUELLER: If one imagines that the estrogen enters with the "A"-ring and becomes bound on the "D"-ring (leaving the receptor's "A"-ring site open), is there anything in the crystallographic data which defines limitations for the "D"-ring binding interactions? I ask this question because substitutions on the "D"-ring of estradiol have such a dramatic influence on the binding of the steroid even though the "A"-, "B"-, and "C"-rings are the same and the phenol binding site of the receptor appears to be open to such compounds as p-sec amyl phenol when there is estradiol already bound.

DUAX: The variability of the "D"-ring region in steroids that bind to the estrogen receptor and the absence of a "D"-ring-like group in most non-steroidal estrogens make it difficult to detect the presence of a characteristic "D"-ring that may contribute to binding. It is possible that the dramatic effect that "D"-ring substitutions have upon binding affinity may result from long-range conformational effects transmitted to the "A"-ring, such as the influence of the 17-substituent upon the pK of the C(3) hydroxyl that Legrand reported. In regard to the ability of p-sec amyl phenol to bind to the phenol site when estradiol is prebound, it may be that even at 0°C, there is some equilibrium between free and bound estradiol and that some exchange with the excess p-sec amyl phenol is possible.

OFNER: In support of Dr. Mueller's emphasis of the importance of the "D"-ring in structure-activity relationships, I should like to mention our report (Ofner et al., Cancer Chemotherapy Reports 16:285, 1962) of a large difference in the estrogenic activities of 16α(very potent)- and 16β(low potency)-fluoro estradiol-17β bioassayed as the products of the placental aromatization of 16α(weak androgen)- and 16β(5 x 16α-F-steroid in chick comb potency)-fluoro-4-androstene-3,17-dione.

DUAX: We would suggest that "D"-ring substituents could influence receptor binding by the mechanism of long-range conformational effects transmitted to the "A"-ring and could influence activity by direct participation in the hormone receptor interactions and changes that follow initial binding. We would be interested in undertaking the crystal structure analysis of 16α- and 16β-fluoro derivatives of estradiol-17β in order to examine the nature of electronic properties and hydrogen bonding patterns that might occur in the crystals.

PATHRE: I have a couple of comments regarding your work on zearalenone. We have examined the ^{13}C-NMR spectra of zearalenone and its derivatives and found that there is no change in the chemical shifts of carbon atoms with respect to temperature (the lowest temperature, -120°C), which, I think, is consistent with your conclusions that in zearalenone the overall conformation of the molecule remains the same. Also, we found, as your model predicts, that removal of the 4-OH group in zearalenone results in a decrease in its uterotropic activity. Have you considered whether possible conformational differences exist between the solid state and solution state of these estrogens? Solvent interactions present in solution may not be observable by the structures observed in the solid state.

DUAX: I am pleased to learn that your solution spectral studies indicate the presence of a stable molecular conformation and that the 4-hydroxyl is as important to binding as our model predicts. It is always possible that a molecular conformation observed in an isolated crystal structure determination may differ from the conformation of that molecule in solution. For this reason it is important to analyze the data from as many closely related structures as possible and to study polymorphic forms of the same compound. Our analysis of crystallographic data on over 300 estranes, androstanes, and pregnanes collected in the Atlas of Steroid Structure indicates that, in general, the structures observed in the crystal are at, or very near, minimum energy conformations and that crystal packing forces have little or no influence on conformation. Furthermore, the fact that many crystals, including zearalenone, contain solvent of crystallization further suggests that the intermolecular interactions observed in the crystal, such as hydrogen bonds, have analogous interactions in solution. In the past, we have been able to resolve ambiguities in solution spectral interpretation and unambiguously demonstrate a correspondence between steroid conformation in solid and solution. Studies in our laboratory of more flexible molecules such as thyroid hormones, polypeptide hormones, and prostaglandins indicate that this correspondence between structure in solid and solution occurs for these compounds as well.

METCALF: Is it possible that the facile hydration of chlordecone (Kepone) provides a clue to the affinity of this compound for the estrogen receptor in a manner analogous to the mechanism you have suggested for solvated estradiol or estrone?

DUAX: That is a very good suggestion. If Kepone is energetically stabilized by solvation, it is possible that a tightly bound water molecule in the plane of the carbonyl group could play a role analogous to that of the 3-hydroxyl of estradiol.

McLachlan, ed. Estrogens in the Environment

THE CHEMISTRY OF ESTROGENS AND ANTIESTROGENS: RELATIONSHIPS BETWEEN STRUCTURE, RECEPTOR BINDING, AND BIOLOGICAL ACTIVITY

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INTRODUCTION

The biological activity of steroid hormones, such as estrogens, depends upon their interaction with certain high affinity binding proteins, called receptors, that are found in the cells of target tissues. The interaction between a steroid and its receptor is of high affinity (K_d = 0.1 - 1 nM) and is characterized by a high degree of stereospecificity. Therefore, it is not surprising that small alterations in the structure of certain estrogens can greatly affect the receptor binding affinity of these compounds and their biological activity.

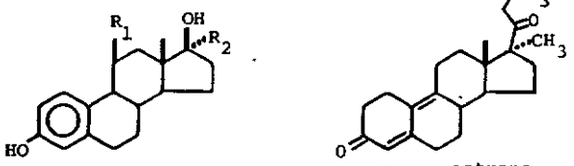
In this paper, we will examine several facets of the relationship between the chemical structure of estrogens and antiestrogens and their biological activity, both in terms of receptor interaction and physiological responses. We will also consider the variety of chemical structures that can embody estrogenic activity. It will become apparent that the biological consequences of chemical alteration have a dual origin - altered receptor affinity and altered pharmacodynamics; both factors are important in determining biological activity.

RESULTS AND DISCUSSION

Optimization of the Selectivity of Estrogen Interaction with Receptor. In addition to their desired interaction with the estrogen receptor, estrogens interact with other binding sites. This non-receptor binding can be to specific serum binding proteins such as rat alpha-fetoprotein and human sex steroid binding protein, to enzymes involved in estrogen metabolism such as dehydrogenases and hydroxylases, and to low affinity, non-saturable (i.e., non-specific) binding sites such as albumin and lipids. These interactions reduce the fraction of a dose of an estrogen that is "free" and thus available for interaction with the receptor (serum and non-specific binders), and they can shorten the serum half-time (metabolizing enzymes).

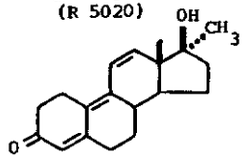
A considerable effort has been mounted by the pharmaceutical industry to develop estrogens that will have increased potency because they interact with the receptor more selectively. An example of this is found in a detailed study

by Raynaud.[1] Raynaud has shown that two structural modifications, a 17α-ethynyl group and an 11β-methoxy group, both enhance the selectivity with which an estrogen interacts with the receptor and increase its biological potency (Scheme 1). The 17α-ethynyl group reduces binding to serum binding proteins



	R_1	R_2	RBA[a]
estradiol	H	H	100
	OCH_3	H	16
	H	C≡CH	160
moxestrol (R 2858)	OCH_3	C≡CH	40

promegestrone (R 5020)



methyltrienolone (R 1881)

[a]Relative binding affinity

Scheme 1.

and blocks the action of the 17β-dehydrogenase, a major pathway of estradiol metabolic inactivation; the 11β-methoxy group blocks binding to serum proteins very effectively and also lowers the lipophilicity of the compound, reducing its non-specific binding. A particularly important feature of these two modifications is that, while they reduce non-receptor interactions dramatically, they cause relatively small changes in the binding affinity to the estrogen receptor. Thus, 11β-methoxy-17α-ethynylestradiol (R 2858 or "moxestrol") is an estrogen with increased potency due to the increased selectivity of its receptor interaction. Raynaud has also found that moxestrol is a preferable ligand to use in measuring the concentration of estrogen receptors in in vitro assays;[2] its increased selectivity of receptor interaction translates into decreased levels of background or "non-specific" binding. Along a similar vein, the Roussel Company has developed analogs of testosterone (R 1881) and progesterone (R 5020) that have increased selectivity of receptor interaction due to their decreased binding to serum proteins (Scheme 1).[3]

Another aspect of the selectivity of receptor binding concerns the extent to which modified steroid hormones of one class will bind to receptors for another class of hormones. Again, the most extensive work in this area has been done by the Roussel Company where the binding affinity of a large number of modified estrogens, androgens, progestins and corticosteroids has been measured to the

corresponding four receptor systems.[3] The "parent" ligands, estradiol, testosterone, progesterone, and cortisol, have high receptor selectivity, that is, they bind with highest affinity to their own receptors. Structural modifications can be made that will increase the binding selectivity somewhat, but other alterations result in compounds with roughly comparable binding affinities for receptors of two or more classes. These compounds can have interesting mixed activities.

Structural Modifications of Estrogen Derivatives Designed for Affinity Labeling or Breast Tumor Imaging. We have been interested in developing two types of estrogen "reagents", affinity labeling agents and tumor imaging agents. Compounds of the first class are estrogens that contain a functional group that is chemically or photochemically reactive, so that once the estrogen-receptor complex is formed, the two members can be covalently linked. The second type of agent embodies a gamma-emitting radionuclide, so that its in vivo distribution (and presumably its concentration in an estrogen receptor-containing breast tumor) can be detected externally by gamma imaging techniques. A feature required by both of these types of agents is high affinity for the estrogen receptor, and in developing these agents we have made a considerable study of how structural modifications affect receptor binding.

We have prepared derivatives in the two series based on the steroidal estrogen estradiol and on the non-steroidal estrogen hexestrol. The latter compound has the advantages that it does not bind to high affinity serum binding proteins, its affinity for the estrogen receptor is three-fold greater than that of estradiol, and its chemistry is simpler. In examining the receptor binding affinity of various estradiol and hexestrol derivatives (see Table 1), we have noted the following: For small structural modifications (entries 1 and 5), the binding affinity of the derivative relative to that of the parent is roughly equivalent in the two systems. However, with the introduction of larger substituents (entries 3, 6-9), the binding affinity of the hexestrol derivatives can exceed that of the estradiol derivatives by a wide margin.

We have suggested[4] that the greater tolerance of hexestrol towards these sorts of substitution derives from its symmetry and conformational flexibility (Scheme 2). For example, introduction of a substituent ortho to the phenolic hydroxyl group in *meso*-hexestrol produces a racemic mixture of enantiomers, one of which can bind with its substituent in a position congruent with either a C-2 or C-4 substituted steroidal ligand, and the other which can bind at sites roughly equivalent to steroid positions C-15 and C-17. Thus, while the corresponding substituent at the 2- or 4-position of the steroid is positioned unam-

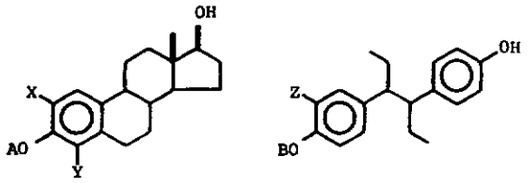


TABLE 1

RELATIVE EFFECTS OF SUBSTITUENTS ON RECEPTOR BINDING OF STEROIDAL VS NON-STEROIDAL ESTROGENS[a]

	Relative Binding Affinity[b]		
	Estradiol		Hexestrol
	A		B
1. H	100		100
2. CH_3	3.0		6.5
3. $EtOCOCH_2$	0.034		0.54
	X	Y	Z
4. H	100	100	100
5. F	86	128	68
6. NO_2	0.03	6.2	5
7. N_3	3	0.9	24
8. Br	1.2	10	5.3
9. I	0.03	0.03	4.6

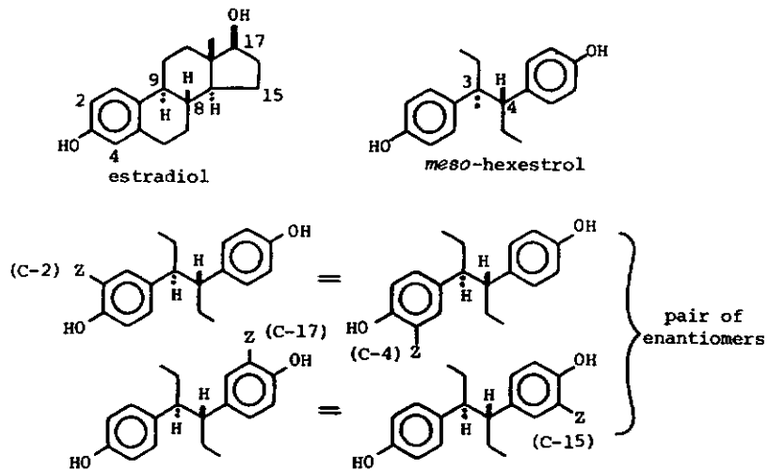
[a]Data from ref 4, 5.

[b]Binding affinities are measured in rat or lamb uterine cytosol by a competition assay with ^{3}H-estradiol (ref 4). Numbers are % K_a relative to that of the unfunctionalized ligand.

biguously within the receptor binding site, the symmetry and flexibility of hexestrol provide several alternative modes of binding for the group.

Stereochemical Ambiguities in the Mode of Binding of Hexestrol Derivatives. In the preceeding argument we have implied that the hexestrol derivatives are bound by the estrogen receptor in such a manner that the configuration at carbons 3 and 4 are congruent with carbon atoms 8 and 9 at the B-C ring junction of estradiol (Scheme 2). There are some binding data that indicate the importance of configuration at these centers (Scheme 3): Compared to *meso*-hexestrol, (+)- and (-)-hexestrol have a binding affinity of only a few percent;[6] these stereoisomers are epimeric at only one of these positions. A more extreme example is the enantiomer of estradiol, which is epimeric at all five chiral centers in the steroid and is bound with an affinity only 1% that of estradiol.[7]

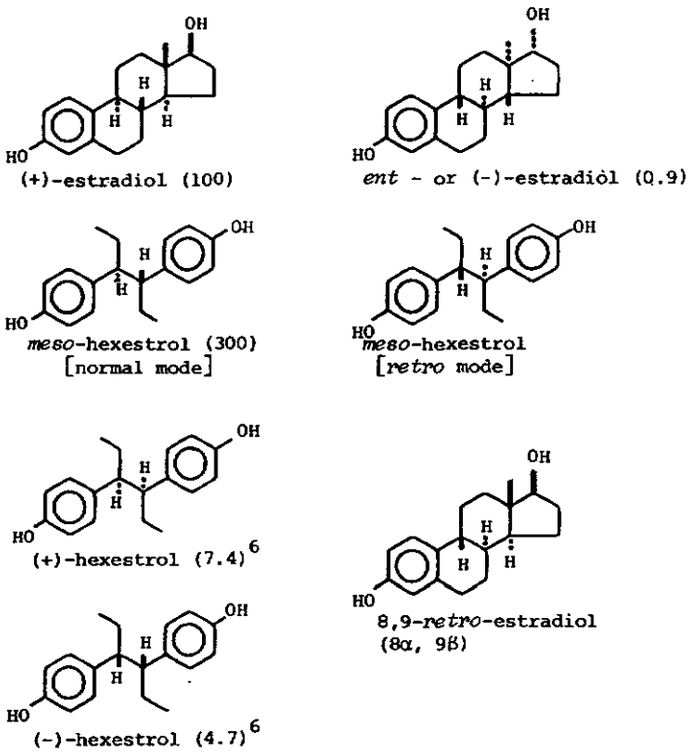
However, although these data are suggestive, one cannot say with certainty that the receptor does not bind *meso*-hexestrol in a *retro* mode (Scheme 3).



Scheme 2.

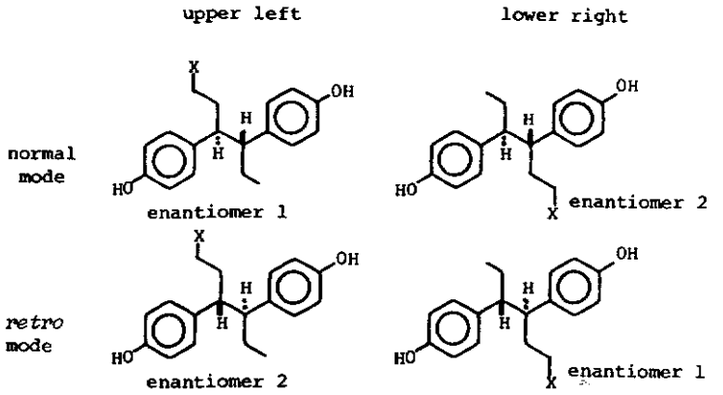
In the *retro* mode of binding, *meso*-hexestrol is rotated 180° from its presumed normal orientation in which it mimics (+)-estradiol; this makes the configurations of carbons 3 and 4 just the reverse of that found in (+)-estradiol. (The term *retro* is derived from steroid nomenclature to indicate a reversal of configuration at two adjacent centers; thus, 8α,9β-estradiol could be called 8,9-retroestradiol; Scheme 3). While a single inversion of configuration, such as is found in (+)- and (-)-hexestrol causes a considerable distortion in the conformation and steric contour of the molecule, the steric disposition of *meso*-hexestrol in the *retro* orientation appears quite similar to that in the normal orientation. Unfortunately, 8,9-retroestradiol, which would be the most appropriate steroid analog to use to judge the binding of *meso*-hexestrol in the *retro* orientation, is not available for binding measurements.

Recently, we have prepared a number of hexestrol derivatives that are functionalized at the end of the hexane chain.[8] These compounds have the same configuration at carbons 3 and 4 as *meso*-hexestrol, but because they are unsymmetrical, they are also mixtures of enantiomers. In this series, we have been able to resolve the enantiomeric derivatives by fractional crystallization of the quinine salt of the acid dimethyl ether, so that binding measurements can be made separately on the individual enantiomers. We had hoped to use the binding behavior of these enantiomers to probe the question of the normal vs *retro* mode of binding of *meso*-hexestrol.



Scheme 3.

The various modes of binding of the enantiomers are shown in Scheme 4. If the enantiomers are oriented in the normal fashion (with the configurations of carbons 3 and 4 congruent with the configurations of carbons 8 and 9 of estradiol), then one enantiomer projects its substituent into the upper left region of the receptor and the other into the lower right. However, if they bind in the *retro* fashion, then this is reversed: the first enantiomer projects its group into the lower right and the second into the upper left. Therefore, if the enantiomers show very different binding affinities, this indicates that the corresponding regions of the receptor (upper left vs lower right) have very different tolerances for the substituent *and* that the hexestrols are being bound unambiguously in one mode (either normal or *retro*). If the enantiomers have nearly the same binding affinity, then either the corresponding regions of the receptor have very similar tolerance to substitution, or one enantiomer is bound in the normal and the other in the *retro* fashion.



Scheme 4

Our preliminary binding data with two of these derivatives, the methyl and the pentyl ester, are shown in Table 2. It is clear that both enantiomers have

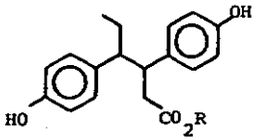


TABLE 2

ESTROGEN RECEPTOR BINDING AFFINITIES OF 1-HEXESTROL ESTERS[a]

R	Relative Binding Affinity[b]		
	racemic	(+)	(-)
$-CH_3$	8.8	7.0	8.8
$-CH_2CH_2CH_2CH_2CH_3$	3.8	4.0	4.2

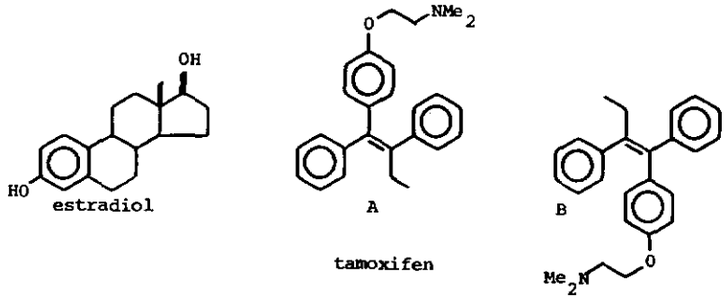
[a]Data are from S. W. Landvatter, K. E. Carlson, and J. A. Katzenellenbogen. Unpublished.

[b]Binding affinities are measured in rat uterine cytosol by a competition assay with ^{3}H-estradiol (ref 4). Numbers are % K_a relative to that of estradiol.

very similar binding affinities; so, at present, the question of the mode of binding of *meso*-hexestrol to the estrogen receptor is unanswered. We are planning to make other derivatives of these enantiomers, and should they prove to show larger differences in binding affinity, they will help to resolve this stereochemical ambiguity. While, as yet, we do not know the absolute configura-

tion of the enantiomers we have resolved, the configuration of the corresponding norhexestrol acids is known,[9] and we plan to interrelate the two systems.

We are hopeful that stereochemical studies of this type may permit us to resolve another long-standing ambiguity: the preferential mode of binding of the triarylethylene estrogens and antiestrogens. This stereochemical uncertainty can be exemplified by the antiestrogen tamoxifen (Scheme 5), where it is not clear whether the molecule is bound at the estrogen binding site in orien-



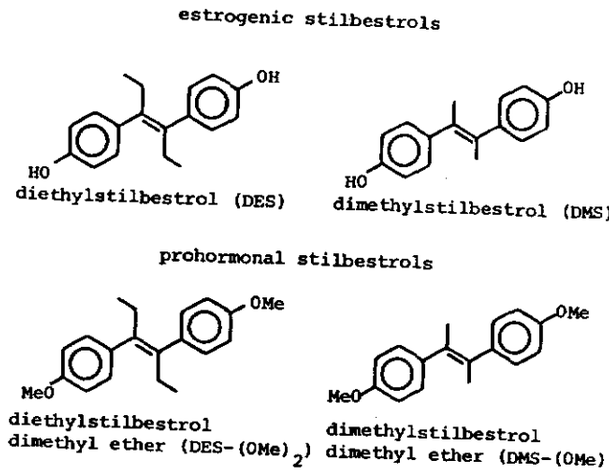
Scheme 5

tation A or B relative to estradiol. The stereochemical mapping of receptor bulk tolerance and mode of binding that we can do with the enantiomers of the chain substituted-hexestrol derivatives may go a long way towards resolving this ambiguity.

Prohormonal Estrogens: Chemical Modifications That Affect Both Receptor Binding and Pharmacokinetics. When estrogens interact with the uterus in vivo, they first bind to the unfilled receptor that is present in the cytoplasm. This complex then is translocated to the nucleus where it interacts with chromatin binding sites in a manner that is thought to initiate the biological response by differential alteration of gene expression. From studies that have been done by ourselves[10] and Clark,[11] it is apparent that stimulation of uterine growth in the rat requires estrogens that are capable of interacting with the estrogen receptor and translocating receptor to the nucleus. There is an additional requirement, however: For the uterine growth stimulation to be effective and long-term, it is essential that the compounds maintain elevated levels of the nuclear estrogen receptor for an extended period of time. Thus, estradiol, which binds tightly to the uterine estrogen receptor and maintains nuclear receptor levels elevated for 4-6 hours, causes a pronounced uterine weight increase. On the other hand, estriol, which is bound less tightly and is cleared more rapidly, causes only a temporary (1 hr) increase in nuclear estrogen receptor levels and consequently effects only minor

increases in uterine weight. The short duration of action of estriol contributes to the "impeded" nature of its response.

Chemical modification of short-acting estrogens to produce prohormones (i.e., inactive hormone derivatives that are metabolized to active hormones) can have a curious effect: the prohormonal estrogen may have a reduced receptor binding affinity, yet its biological activity can be increased. We have examined prohormonal derivatives in the stilbestrol series, where this phenomenon is particularly well illustrated.[12] The dose response curves for a 3-day uterine weight response in the immature rat are shown in Figure 1 for estradiol, diethylstilbestrol (DES), dimethylstilbestrol (DMS), and their corresponding dimethyl ethers (DES-$(OMe)_2$, DMS-$(OMe)_2$) (Scheme 6). DES is a potent estrogen,



Scheme 6.

giving a uterine weight response like that of estradiol, but DMS is a weak, impeded estrogen, active only at the highest doses. Conversion of the stilbestrols to the prohormonal form (dimethyl ethers) has an interesting effect: The receptor binding affinity of 330% for DES (relative to 100% for estradiol) drops to 1% for DES-$(OMe)_2$; the uterotrophic potency of DES-$(OMe)_2$ is also lower than DES. In contrast, while the receptor binding affinity of 21% for DMS drops to 0.1% for DMS-$(OMe)_2$, the uterotrophic potency increases markedly. While these results appear paradoxical when considered in terms of the receptor binding affinities of the compounds themselves, this phenomenon can be understood on the basis of differences in the pharmacokinetic properties of DES, DMS, and their dimethyl ethers.

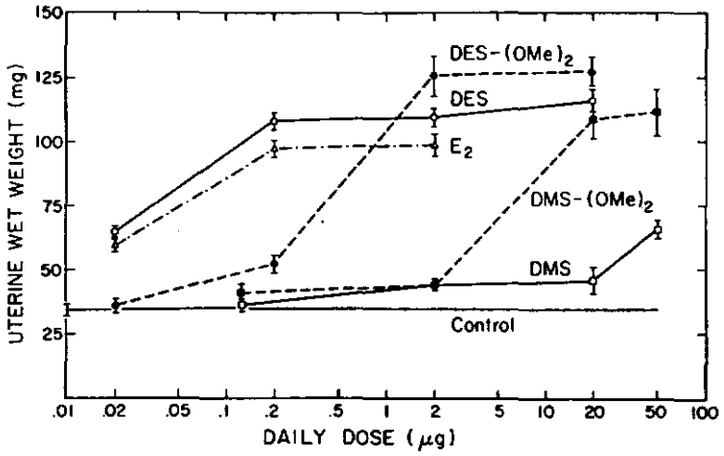


Figure 1. Dose-response curves showing the uterotrophic activities of DMS, DES, estradiol and stilbestrol methyl ethers. Rats (21 days old) were injected s.c. with the indicated daily dose of compound in 0.5 ml saline once daily at 24-h intervals on 3 successive days and uterine wet weights were determined at 24 h after the last injection. Control animals received saline alone. Each value is the mean of determinations from at least 5 individual animals ± SEM. (From ref 12)

As can be seen in Figure 2A, DES causes a rapid accumulation of estrogen receptor in the nucleus, with elevated levels being maintained for at least 12 hours; this temporal profile of nuclear receptor is very effective in stimulating a uterine weight increase. DMS, on the other hand, causes only a transient increase in nuclear receptor, with levels returning to control by 6 hours (Fig. 2B). This elevation is of insufficient duration to effectively stimulate uterine weight increase. Conversion of both DES and DMS to the prohormonal forms (dimethyl ethers) causes the elevation in nuclear receptor levels to persist for longer periods.

In terms of the uterotrophic dose-response relationships (Fig. 1), methylation has a differential effect, however: Methylation of DES, which is a potent uterotrophic agent itself, simply decreases the total amount of active compound that is made available through metabolic activation, reducing its effectiveness at low doses and shifting the dose response curve to the right. In contrast, methylation of DMS, which is a weak uterotrophic agent because of its very brief duration of action, makes it more potent because by extending its period of action it provides a profile of nuclear receptor levels that is much more

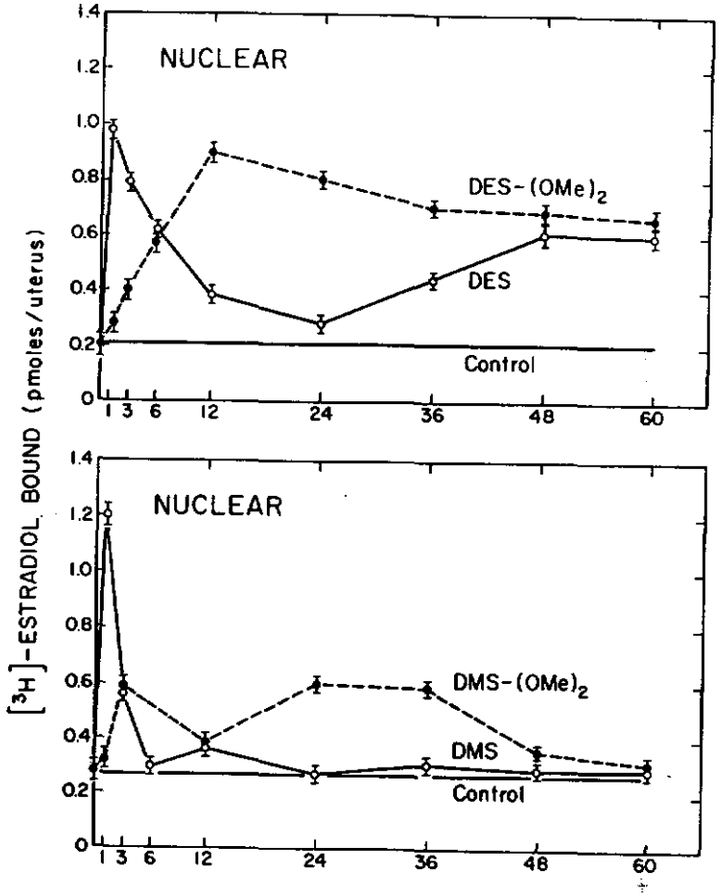


Figure 2. Content of specific estrogen binding sites present in nuclear fraction of the immature rat uterus as a function of time after a single injection of DES, DMS, DES(OMe)$_2$, or DMS(OMe)$_2$ (Scheme 6) or control vehicle saline. Rats (21 days old) were injected s.c. with 20 μg of compound in saline and at indicated times, high affinity binding sites in uterine nuclear fraction were determined by an exchange assay (ref 12). Each point represents the mean of 2-3 determinations with 3 uteri per determination, and is corrected for nonspecific binding. (From ref 12)

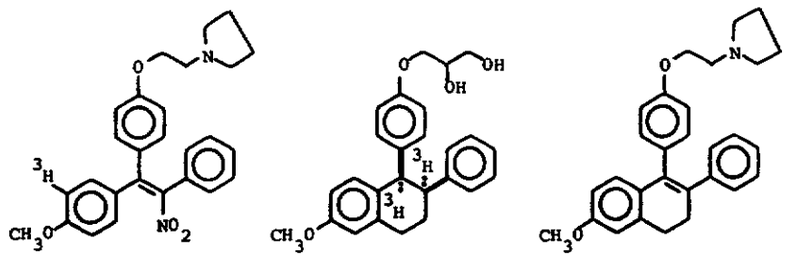
appropriate for the stimulation of uterine growth. Thus, methylation causes the dose response curve for DMS to shift to the left.

There are undoubtedly many other examples of chemical alterations of hormones (to prohormonal forms) that increase biological activity and potency by a mechanism that does not involve receptor binding directly, but rather by

causing appropriate alterations in pharmacokinetic properties of a metabolite of the derivative (the free hormone).

Antiestrogens: Prohormonal Estrogen Antagonists. Studies by Clark[13] indicated that the triarylethylene-type antiestrogen nafoxidine can occupy uterine estrogen receptor sites for greatly extended periods of time. The prolonged duration of action of this compound, as well as its relatively slow onset of action (in terms of nuclear estrogen receptor elevation), together with related studies we had done on the onset and duration of action of other antiestrogens (notably CI-680 and its demethylated analog 9411X27),[14] suggested to us that the antiestrogens commonly-administered may in fact be prohormonal forms that are slowly converted into the active compounds in vivo.[15]

In order to investigate this point directly, we have prepared two antiestrogens in high-specific activity, tritium-labeled form (Scheme 7).[16,17] One



Scheme 7.

of these compounds, CI-628, is an antiestrogen that was synthesized by Dr. Horace DeWald of the Parke-Davis Co. It is one of the more potent antiestrogens. The second compound U-23469 was prepared by Daniel Lednicer while at the Upjohn Company. This antagonist can be considered to be an analog of the better known Upjohn antiestrogen nafoxidine that was developed in order to eliminate the undesirable photosensitivity reaction that was experienced by individuals who used nafoxidine. The sites of tritium labeling in each compound are shown in Scheme 7.

We have investigated the interaction of both of these antiestrogens with the uterine estrogen receptor in vitro[14,16,17,18] and have found that the binding characteristics of the labeled compounds correspond closely to those that were previously measured indirectly on the unlabeled compounds, by competitive binding assays with tritium-labeled estradiol. These compounds were then administered to rats, and their in vivo receptor interactions were investigated. The most striking finding was that in each case a more polar metabolite of the antiestrogen appeared to accumulate selectively in the nuclear estrogen receptor. This was seen particularly clearly with ^{3}H-U-23469.[17,18] Figure 3 shows thin layer chromatograms of extracts of serum and of the uterine nuclear fraction

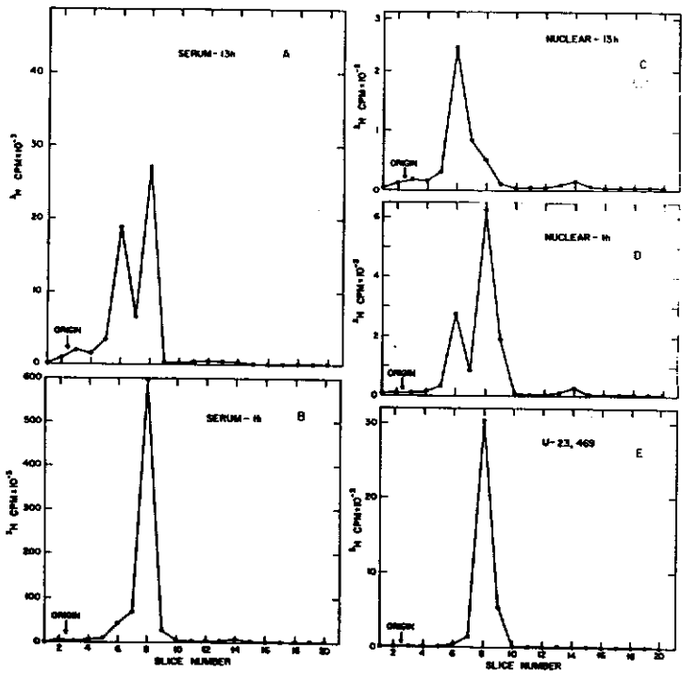


Figure 3. Thin-layer chromatographic profiles of authentic ^{3}H-U-23469 (panel E) and of serum (panels A and B) and of uterine nuclear radioactivity (panels C and D) after in vivo injection of ^{3}H-U-23469. Immature rats were injected with ^{3}H-U-23469 (25 µg sc/rat) and at 1 h and 13 h after injection, serum was prepared and extracted with ethylacetate; at the same times, uteri were excised and homogenized and the three-times washed nuclear pellet was then ethanol extracted. The extracts were concentrated and analyzed on thin-layer silica gel plates developed in anesthetic ether:ethanol (98:2 v/v). (From ref 17)

at various times after ^{3}H-U-23469 injection. The nuclear fraction has accumulated a substantial fraction of the polar metabolite by 1 hr (Fig. 3D), a time when it is barely detectable in the serum; by 13 hr, the radioactivity in the nuclear fraction is due almost entirely to the metabolite (Fig. 3C), while the majority of the extractable serum activity is still U-23469 (Fig. 3A). Similar

selective accumulation of a more polar metabolite in the uterine nuclear receptor fraction was observed after ^{3}H-CI-628 injection. These data suggest that the polar metabolites of both antiestrogens have a higher affinity for the estrogen receptor than do the parent compounds and are thus selectively accumulated in uterine nuclear fraction by virtue of their higher receptor binding.

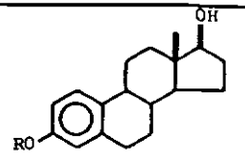
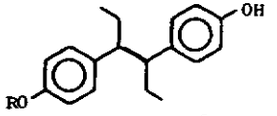
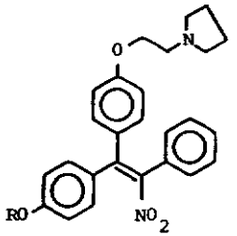
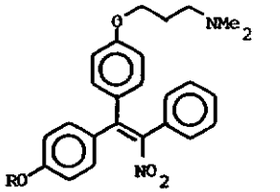
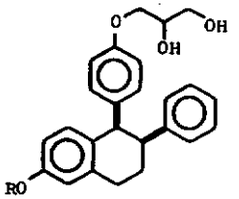
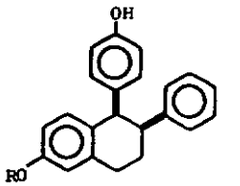
The lower chromatographic mobility of the antiestrogen metabolites suggested that a polar group was being unmasked or added, and it appeared most likely that the methyl ether function of each antiestrogen was being cleaved oxidatively. Both of the free phenols were synthesized, and by cochromatography in several solvent systems, they were shown to be identical to the polar compounds generated metabolically.

Table 3 shows the binding affinities of the antiestrogens, their polar metabolites and several related compounds for the uterine estrogen receptor as measured by competitive binding assays. With both the Parke-Davis antiestrogens, CI-628 and CI-680, the binding affinity increases by a factor of 7-15 upon removal of the methyl ether. A similar ratio is found between the binding affinities of the phenol and phenyl methyl ether forms of the estrogens estradiol and hexestrol. The antiestrogen U-23469, however, appears to be unique in that its binding affinity increases nearly 400-fold upon removal of the methyl ether function. Also, in this system, addition of the glyceryl ether group causes only a modest decrease in binding affinity (1 vs 3 and 2 vs 4), while it causes a 50-fold decrease in the binding affinity when added to hexestrol. These data again indicate that in dealing with a stereospecific binding site, analogous chemical modifications in different ligand systems can have very different outcomes in terms of receptor binding affinity.

The Estrogen Receptor - Stereospecific Yet Structurally Tolerant. The results presented in the preceeding sections support the fact that the binding of ligands to the estrogen receptor is stereospecific, and in numerous instances small structural or stereochemical alterations can cause large changes in binding affinity and in biological potency. The receptors for other steroid hormones demonstrate comparable stereospecificity. On the other hand, despite its stereospecificity, the estrogen receptor does have a remarkable capacity for binding (sometimes with high affinity) ligands with structures quite remote from that of a steroidal estrogen. We have already seen examples of the bisphenol (hexestrol) type of estrogen (Table 1) and of the triarylethylene antiestrogens (Table 3), but even more remarkable is the binding affinity of the estrogen receptor for the compounds shown in Table 4. These compounds include plant natural products such as the flavones genistein, mirestrol, and the fluor-

TABLE 3

BINDING AFFINITY OF ANTIESTROGENS, ANTIESTROGEN METABOLITES AND RELATED DERIVATIVES TO THE ESTROGEN RECEPTOR[a]

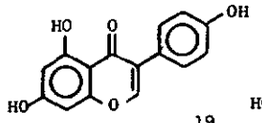
Compound	Substituent	RBA[b]
	estradiol R=H	100
	R=CH_3	3
	*meso*-hexestrol R=H	300
	R=CH_3	19
	R=CH_2CHCH_2OH (OH)	6.7
	"polar metabolite" CI-628 R=H	100
	R=CH_3	5
	9411X27 CI-680 R=H	222
	R=CH_3	34
	"polar metabolite" U 23,469 R=H 1	39
	R=CH_3 2	0.1
	R=H 3	30
	R=CH_3 4	0.24

[a]Data are from refs 14, 16, 17.
[b]Binding affinities are measured in rat uterine cytosol by a competition assay with ^{3}H-estradiol (ref 4). Numbers are % K_a relative to that of estradiol.

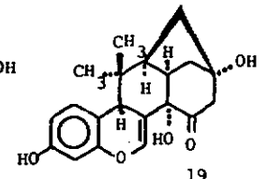
TABLE 4

STRUCTURAL VARIETY IN ESTROGENIC SUBSTANCES[a]

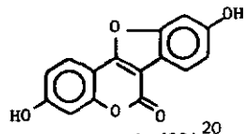
Natural Products:



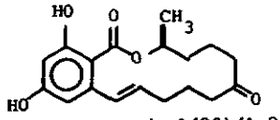
genistin (0.9)[19]



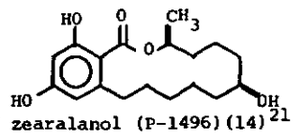
mirestrol (5)[19]



coumestrol (20)[20]

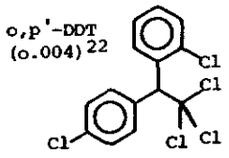


zearalenone (P-1492) (1.8)[21]

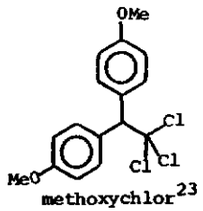


zearalanol (P-1496) (14)[21]

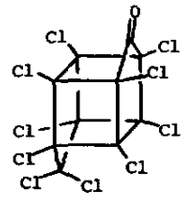
Xenobiotics:



o,p'-DDT (0.004)[22]

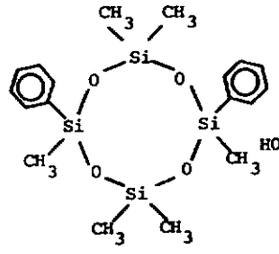


methoxychlor[23]

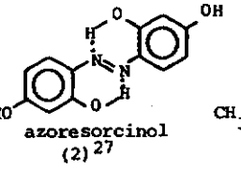


chlordecone (kepone) (0.04)[24,25]

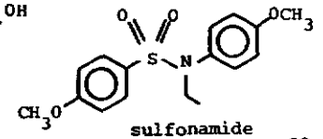
Other Synthetics:



cis-2,6-diphenylhexamethyl-cyclotetra siloxane[26]



azoresorcinol (2)[27]



sulfonamide (weakly estrogenic)[28]

[a] Relative binding affinities to the uterine estrogen receptor are given in parentheses. Reference numbers are given as superscripts.

escent coumestrol. The mold metabolite zearalenone (P-1492) and its more potent derivative zearalanol (P-1496) are bound with good affinities; we have studied in detail the estrogenic potencies and receptor binding of several compounds of this type (β-resorcylic acid lactones).[21] Xenobiotics such as o,p'-DDT bind weakly, but others such as methoxychlor can be metabolized (demethylated) to compounds with high affinity. Perhaps most remarkable is the estrogenic activity of chlordecone or kepone. We have investigated this compound and have found it to have high uterotrophic activity in the rat, although its potency (and receptor binding affinity) are low.[24] Other synthetic materials with unusual structures that are estrogenic are the cyclic tetrasiloxane, discovered as an estrogenic contaminant in a "bioinert" dimethyl-methylphenyl siloxane copolymer, azoresorcinol and other azobenzenes, which are also mutagenic (and thus possibly carcinogenic), and even some sulfonamides that also have antibacterial and antifungal activity.

CONCLUSION

The estrogen receptor binds estrogens and antiestrogens with high affinity and high stereospecificity, but it also binds a considerable variety of other ligand types, steroids, stilbestrols, plant and mold natural products, xenobiotics and other diverse synthetic chemicals. In many cases, structural changes affect binding affinity in a rational and predictable fashion, while in other instances they do not. Since the uterine growth-promoting effects of estrogens depend both on receptor occupancy and the duration of that occupancy, structural alterations that convert estrogenic agonists or antagonists into prohormones can have a complex effect on biological activity and potency; while the receptor binding of the prohormonal derivatives may be decreased, their potency and activity may be increased due to their more favorable pharmacokinetic properties. Thus, a full understanding of structure-activity relationships of estrogens and antiestrogens requires careful study of their receptor binding *in vitro* and an examination of the time course of their action *in vivo*. An analysis of their metabolism is also important, as it can lead to compounds with both decreased or increased biological activity. In the latter case, the receptor itself can be used as an agent for the selective extraction of metabolites with higher receptor binding affinity, ones that are presumed to be of biological importance in the action of estrogens and antiestrogens.

ACKNOWLEDGMENT

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DISCUSSION

KUPFER: Is the metabolite of CI-628 estrogenic or antiestrogenic?

KATZENELLENBOGEN: The metabolites of the antiestrogens CI-628 and U-23,469 have been synthesized and tested for uterotrophic and antiuterotrophic activity in immature rats. Both of the compounds have antiuterotrophic activity, but like the parent compounds, they are mixed agonists/antagonists in terms of this response.

KUPFER: Is nafoxidine demethylated in vivo? In vitro, we could not demonstrate demethylation of nafoxidine using liver microsomes, derived from rats which were induced to elevate monooxygenase activity, and fortified with NADPH.

KATZENELLENBOGEN: We have not studied nafoxidine, because we have not prepared it in radiolabeled form. However, I believe that by analogy with CI-628 and particularly U-23,469 (which is a nafoxidine analog that was prepared to reduce phototoxicity), we should expect that nafoxidine will be demethylated in vivo. Furthermore, this is supported by the available (though indirect) evidence (prolonged activity and slow onset of receptor translocation to the nucleus). We have found that liver microsomes can demethylate ^{3}H-U-23,469 in vitro. The incubations were fortified with an NADPH-generating system, and, at low concentrations of the antiestrogen, 50% of the demethylated metabolite was produced within 30 minutes. The capacity of the microsomal demethylase activity is low, however; so, at higher U-23,469 concentrations, the precent of demethylation is lower (this work is soon to appear - ref. 17, Katzenellenbogen et al., this volume). I doubt whether you could detect this demethylation indirectly (e.g., by formaldehyde analysis), because of the insensitivity of these methods. We were less successful in observing demethylation of ^{3}H-CI-628 by liver microsomes in vitro. We suspect that the nitro function of this compound may be shutting down the electron transport system needed for P-450 action.

McLachlan, ed. Estrogens in the Environment

ESTROGEN ACTION IN NORMAL AND ABNORMAL CELL GROWTH

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INTRODUCTION

Estrogens are hormones which stimulate growth and development of the secondary sex characteristics and organs in the female animal. Thus they constitute a normal and very important component of the physiology of the female. Yet it is well known that estrogens can be dangerous substances if not handled properly or under certain abnormal patho-physiological states. In this article it is our purpose to discuss both the normal and abnormal growth responses that are stimulated by estrogens. In order to understand these relationships it is necessary to clarify our current knowledge about estrogen action at the cellular and molecular level.

II ESTROGEN ACTION IN NORMAL CELLS

The interaction of cellular components with steroid hormones depends on the amount of free hormone that is available to the cell. This amount is determined by a complex interplay of mechanisms which involve binding components of the blood and intercellular spaces. Neither the interplay nor the mechanisms themselves are completely understood at this time. For a more extensive discussion see Clark and Peck.[1] However, the following are the minimal points which should be considered in deriving a model of this system: a) Steroids are bound with different affinities by specific and nonspecific blood binding components. These interactions determine the quantity of free steroid available for entry into cells. A steroid hormone that is not bound tightly to blood binding sites is physiologically more potent than one which is bound tightly, provided that all other variables remain constant. b) Steroids have different metabolic clearance rates and their physiologic effectiveness depends on this variable. Thus steroid receptors may have a low affinity for a given hormone and yet, if this hormone has a long half life in the body, its potency may be greater than anticipated. c) Steroids which are bound to blood components may enter intercellular spaces of some organs via protein permeable vascular beds, thereby creating a local elevation of total steroid concentration. Subsequent dissociation of

these complexes will allow the maintenance of free steroid levels within the intercellular space. d) Steroids can also enter the intercellular space in the free form. There they may bind to intercellular proteins which augment the ability of blood binding components to maintain elevated tissue levels of hormone.

Free estrogen readily enters all cells at physiological temperatures and is subsequently bound by many cellular components. The best known of these components is the cytoplasmic estrogen receptor which we will call the type I site. Type I sites are soluble protein macromolecules with a high affinity for estradiol, a dissociation constant (K_d) of 0.1-1.0 nM, and are present in target cells at approximately 10,000-20,000 sites per cell. We have recently described a second class of estradiol binding sites (Type II) which have a lower affinity for estradiol, K_d of approximately 50 nM.[2,3] These sites also are soluble protein macromolecules and their numbers per cell can be 5-10 times that of type I sites. Non-specific sites (NS) include a multiple array of cellular components which have a much lower affinity for estrogen than either of the above mentioned sites, K_d of 1 - 100 μM; however, their number is extremely large and hence they can be significant binding constituents.

The precise binding state of estrogens *in situ* is not known, however all of the above components undoubtedly contribute to the accumulation of estrogen by target cells. Type II and NS sites may act as buffer-like accumulators of estrogen that retain estrogen against a declining concentration gradient in the blood. Such a function could easily occur because of the extremely large number of these sites. Once such binding has occurred, reequilibration via dissociation of estrogen from these sites could elevate the cellular content of available estrogen for binding to type I sites. The binding of estrogen to such lower affinity sites may also facilitate the binding of estrogen to type I sites. This is possible because the large number of lower affinity sites increases the probability that estrogen binding of any kind will occur and hence the likelihood of binding to type I sites would be increased. This is especially true if there are localized interactions between type II and type I sites. Another possibility is that type II sites are precursors of type I sites. Type I sites are replenished to the cytoplasm by *de novo* synthesis and/or by recycling from the nucleus after estrogen administration.[4-6] Little is known about this phenomenon but it appears possible that type II sites may constitute a precursor form of such sites or are part of the nuclear mediated events which ultimately lead to receptor replenishment.

Once the type I estrogen complex has formed it undergoes translocation to the nucleus where it binds to a large number of sites on chromatin. Translocation of type I estrogen complexes results in the depletion of these sites from the cytoplasm. Chromatin binding sites can be classified as either acceptor or non-acceptor sites. Acceptor sites are generally visualized as a specific complex of chromosomal proteins, probably non-histone proteins, which the type I estrogen complex recognizes and binds to with a very high affinity. Non-acceptor sites are considered to be secondary sites on chromatin where the receptor-hormone complex can bind with a lower affinity. Although these non-acceptor sites have a lower binding affinity for the complex, they are present in such large numbers that they constitute a major component of the chromatin binding mechanism. These binding interactions between the receptor-steroid complex and non-acceptor sites may serve to maximize the number of receptor-hormone complexes which can be accumulated and thus maximize the chance that binding of some complexes to acceptor sites will occur.

The binding of type I estrogen complexes to acceptor sites is thought to make gene sites available for transcription by RNA polymerase which subsequently results in elevated cellular RNA and protein synthesis. These synthetic events may be very restricted such that the hormone appears to stimulate only a few cellular functions. This is the case with aldosterone which enhances sodium transport in kidney tubules but does not have a general metabolic or growth effect on renal cells. In contrast, hormones that cause growth, such as estrogens and androgens, stimulate many cellular events which ultimately lead to hypertrophy and hyperplasia of specific target tissues (For a review of the topics discussed above see Clark and Peck.[1])

A second class of estradiol binding sites are also present in the nuclei of uterine cells which we have called nuclear type II. These are not translocated from the cytoplasm, but are stimulated or activated by estrogen administration and may represent chromosomal proteins which are present in the nucleus of estrogen sensitive cells at all times.[2,7,8] The function of these sites is not known; however, they may be thought of as integral components of the mechanisms which control RNA and/or DNA synthesis, and thus may represent "nuclear acceptors" which are activated by the binding of type I complexes. Additional possible functions for nuclear type II sites include the following: (a) an amplification mechanism to amplify the nuclear events initiated by binding of the receptor to acceptor sites; (b) components of the nuclear processing machinery which interact with the receptor estradiol complex and bring about recycling or replenishment of the receptor; (c) components of the "off-reaction" involved either in turning off receptor stimulated events or in removing hormone and/or receptor from the nucleus. It is possible that all of the above functions

could work in concert, and therefore, nuclear type II sites may represent a combined gene activation-receptor processing unit.

III ROLE OF ESTROGENS IN CARCINOGENESIS

Chronic exposure of mice and rats to various estrogens results in preneoplastic and neoplastic changes in the vagina, uterus, pituitary and mammary gland.[9-15] Endometrial hyperplasia and cancer occur in women who have been exposed to either endogenous or exogenous estrogens for prolonged periods of time. These cases include women with ovarian tumors which produce estrogens,[16] women who fail to ovulate and as a result are exposed to estrogen without the normal intervention of the luteal phase of the cycle[17,18] and women who have taken estrogens for many years because they lack functional ovaries.[19-21] Exposure of the human fetus to diethylstilbestrol has been associated with the development of vaginal adenosis and clear cell adenocarcinoma in the female offspring.[22-24]

In the studies cited above, the quantities of estrogen administered were very high and/or the exposure was extended for long periods. Hence, the conclusion that estrogens play some role in carcinogenesis, whether causative or permissive, must be tempered by the realization that the hormone exposure was non-physiological. Under normal physiological circumstances estrogens may have no carcinogenic potential; however, as discussed below, it has been suggested that certain estrogens do function in this capacity.

Diethylstilbestrol (DES) and estrone are considered by many people to have specific cancer-causing "properties". In contrast, estriol has been proposed as a protective agent against the development of neoplasia.

Our viewpoint on these topics which differs from the above derives both from considerations discussed in Part II and from the following observations. A single injection of estradiol to a female rat stimulates a number of biochemical and metabolic events in the uterus as well as other target organs. Among these are glucose oxidation, amino acid and nucleotide uptake, water imbibition, histamine mobilization, eosinophil accumulation and stimulation of nuclear RNA polymerase activities. These activities are increased within the first six hours after hormone administration and are normally termed early uterotrophic events. Late events, such as DNA synthesis, sustained stimulation of RNA polymerase activities and cellular hypertrophy and hyperplasia occur between 12 and 36 hours after estradiol treatment. All of these responses can be stimulated maximally by a single injection of a low dose of estradiol (0.2 μg/100 g body weight). This level of hormone causes the nuclear accumulation and retention of approximately 10-20% of the total number of uterine type I sites (∿2000 sites/cell)

and the activation of maximal levels of nuclear type II sites. The retention or occupancy of nuclear acceptor sites by ∿2000 type I complexes/cell for six or more hours appears to be a requirement for the stimulation of nuclear type II sites and all of the above mentioned late uterotrophic events.[25-28]

The importance of long term nuclear occupancy by the estrogen receptor hormone complex has been shown by using short acting estrogens such as estriol and dimethylstilbesterol. These hormones do not stimulate significant uterine growth after a single injection; however, they do stimulate all early uterotrophic events. This failure to stimulate true uterine growth is correlated with a rapid loss of nuclear sites by the receptor-estriol complexes and a failure to stimulate elevated levels of nuclear type II sites.[7] These concepts are presented in Table 1. The rapid loss of receptor estriol complexes from the nucleus is probably due to the dissociation of estriol from the receptor or to a failure of the complex to bind tightly to nuclear acceptor sites. If type I sites are kept continually occupied by either serial injection of estriol or by estriol implants, true uterine growth occurs and nuclear type II sites are elevated. Estriol has been classified as an estradiol antagonist and it clearly is when the two hormones are administered as a single injection. However, when the two hormones are implanted no antagonism is detected. This apparent paradox is resolved by considering the fact that following injection, both receptor estradiol and receptor estriol complexes are in competition for nuclear binding sites. Since the receptor estriol complex dissociates rapidly from nuclear sites before it can fully stimulate uterotrophic responses, the net effect of receptor estradiol complexes is reduced. When the two hormones are implanted, receptor estriol and receptor estradiol complexes occupy nuclear retention sites equally well. Therefore, late uterotrophic events are maximally stimulated resulting in no antagonism.[29]

These results have several important implications. Estriol has been classified as a weak estrogen in the past, but from the above data it is clear that it acts as such only when it is administered as a single injection. In contrast, when it is present in a continuous fashion, as it is under a number of physiological circumstances, it is a highly effective estrogen. A protective role has been ascribed to estriol in breast cancer. This suggestion is based on the observation that Oriental women, who have a high (Estriol)/(Estradiol + Estrone) ratio in the blood, also have a low incidence of breast cancer.[30-32] This hypothesis was formulated on the assumption that estriol was a "weak" estrogen under all circumstances and that during each menstrual cycle estriol would act to reduce the "carcinogenic potential" of the more potent estradiol.

TABLE 1

EFFECTS OF ESTRADIOL AND ESTRIOL ON EARLY AND LATE UTEROTROPIC RESPONSES

Response	Comparison of Estradiol (E_2) and Estriol (E_3)
Initial nuclear accumulation of receptor hormone complex	$E_2 = E_3$
Long-term retention of receptor hormone complex by the nucleus after an injection	E_2, longer than 6 hrs E_3, shorter than 6 hrs
Early uterotropic events: RNA polymerase I and II activity, template activity, histamine mobilization, water imbibition	$E_2 = E_3$
Late uterotropic events: sustained and elevated RNA polymerase I and II activity, sustained-RNA polymerase initiation sites, RNA + DNA synthesis, cellular growth	$E_2 >> E_3$
True uterine growth after paraffin implant of hormone	$E_2 = E_3$
Receptor occupancy in the nucleus after paraffin implant of hormone	$E_2 = E_3$
Stimulation or activation of nuclear type II sites after a single injection	$E_2 >> E_3$
Stimulation or activation of nuclear type II sites after paraffin implant of hormone	$E_2 \cong E_3$

Our results and those of others[33] indicate that this theory is suspect and, in light of recent evidence which shows that estriol and estradiol are of equal potential in facilitating the onset of mammary tumors in mice,[34] we suggest that the estriol theory of mammary cancer protection is untenable.

Diethylstilbestrol (DES) is considered by some investigators and many lay persons as a "monster" drug with special cancer producing potential. This attitude results from the public's knowledge of the studies mentioned above in which DES was employed in an attempt to extend pregnancy and in which cervical and vaginal abnormalities eventually developed in some progeny.[22] Although DES may be linked with the production of abnormalities in these cases, this does not confer any special carcinogenic potential to this compound. Any estrogen which is not bound tightly in the blood such as DES or is given in sufficiently high concentrations probably would produce similar results. As mentioned earlier, it has been known for many years that estradiol and DES will cause vaginal cancer in mice and thus DES is really not different from the physiological estrogen.

Estrone has also been indicated as the estrogen which causes endometrial cancer in postmenopausal women.[35] Estrone is formed by the aromatization of adrenal androstenedione in adipose tissue and constitutes the major estrogen in postmenopausal women. Siiteri et al.,[35] suggest that estrone may cause cancer by acting as an unopposed estrogen. However, they also suggest that estrone may differ qualitatively from estradiol in the biochemical events that result from nuclear binding of receptor estrone complexes. The latter suggestion has led many investigators to assign special significance to estrone as an intrinsic cancer causing hormone. Our results suggest that no qualitative differences exist between the steroidal estrogens except in their abilities to promote receptor retention in the nucleus.[36] Ruh et al.,[37] examined the ability of estrone to stimulate induced-protein synthesis in the uterus in vitro and could show no qualitative differences between estradiol and estrone. Our interpretation of the role of estrone as a carcinogenic agent in postmenopausal women is that the unopposed action of any estrogen, when present in high steady state levels in the blood, will provide an environment wherein the predisposition to cancer can manifest itself. Hence we ascribe no special carcinogenic potential to estrone as opposed to other steroidal estrogens.

IV UNOPPOSED ESTROGEN ACTION AND HYPERESTROGENIZATION

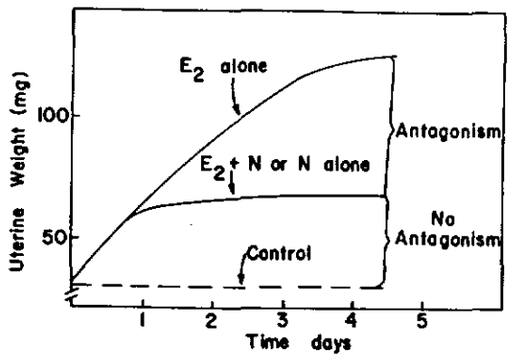
As discussed in Part III, continuous exposure to estrogens will result in neoplasia of the reproductive tract. This phenomenon probably results from the

continuous cellular activity which increases the probability that a "cancer causing" event can manifest itself. Under normal physiological circumstances the actions of estrogen are modified and decreased by the presence of progesterone. Progesterone decreases estrogen receptors in the cytoplasm of target cells and elevates the activity of enzymes which convert active estrogens to less active metabolities.[38,39] In addition, progesterone greatly reduces the amount of type II estrogen binding sites in the uterus.[8] All of these actions decrease the ability of estrogen to stimulate cell growth. Progesterone is present during the second half of the menstrual cycle in the human and transforms an estrogenized uterus into a secretory one which is capable of supporting pregnancy if conception occurs. This monthly exposure to progesterone may be a very important modulator of estrogen action and may act to protect against continuous exposure to estrogen. This protective effect of progesterone can be visualized as a monthly fluctuation in the effectiveness of estrogen which results in normal female function. Without this protective effect of progesterone, estrogen is free to act in an unopposed way which could lead to abnormal development and neoplasia of estrogen sensitive tissues.

We have examined the state of unopposed estrogen by exposing rats to triphenylethylene derivatives such as Clomid and Nafoxidine.[40-42] These compounds, which are usually called non-steroidal anti-estrogens, are used to induce ovulation in anovulatory women and for the treatment of breast cancer. These drugs are unusual estrogen agonists/antagonists that display estrogenic or anti-estrogenic properties depending on the type of target cell.

A single injection of Nafoxidine will stimulate a submaximal level of true uterine growth for longer periods of time than estradiol. This long-term stimulation of uterotrophic function is associated with long-term nuclear retention of the receptor-ligand complex and sustained stimulation of RNA polymerase activities and elevated chromatin RNA initiation sites. It appears that non-steroidal estrogen antagonists are slowly cleared from their nuclear binding sites and this long-term nuclear retention accounts for the prolonged stimulation of uterine growth.[26,40]

Nuclear binding of the estrogen receptor that is induced by triphenylethylene derivatives is accompanied by a depletion of cytoplasmic receptors that lasts for several days. This loss on cytoplasmic sites may be involved in the mechanism by which these compounds act as estrogen antagonists. That is, these compounds initially act as estrogens and undergo nuclear accumulation and retention, hence, they stimulate uterine growth. However, because they fail to stimulate replenishment of the cytoplasmic receptor they act as estrogen antagon-



Treatment	Nuclear Retention	Receptor Replenishment	Estrogen Sensitivity
E_2	long	+	+
N	very long	-	-
E_2 + N	very long	-	-

Figure 1. Effects of daily injections of estradiol (E_2) Nafoxidine (N) or E_2 + N on uterine growth.

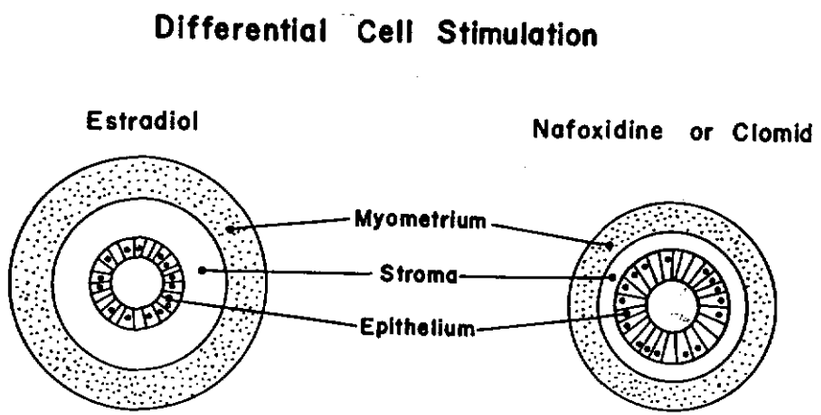


Figure 2. Differential cell stimulation of uterine luminal epithelium by Nafoxidine or Clomid.

ists upon subsequent estrogen injection. These varied interactions are undoubtedly involved in the complex mechanism by which triphenylethylene derivatives act; however, they do not explain how a compound can occupy a receptor for long periods of time (several days) with continued stimulation of the transcriptional machinery of the uterus and yet only result in limited uterine growth (Fig. 1). This paradox has been partially reconciled by the observation that triphenylethylene derivatives have a profound stimulatory effect on the epithelial lining of the uterus and very little effect on the stromal or myometrial elements.[43] (Fig. 2). Therefore, most of the uterine growth that is observed after Nafoxidine or Clomid treatment is due to growth of the epithelium. Since this component represents only a small percent of the total uterine tissue, the growth that is seen is slight when compared to that induced by estradiol which stimulates growth of all uterine tissues. These observations indicate that triphenylethylene derivatives have estrogenic properties in some cell types while acting as estrogen antagonists in others. These results make studies in which these compounds have been considered only as antagonists difficult to interpret. Such studies must be re-examined in light of these cell specific effects.

An important result of the above data with Clomid and Nafoxidine is the prediction that long acting estrogenic stimulation of epithelial components of the reproductive tract could lead to abnormalities and neoplasia. We have recently demonstrated that Clomid will cause reproductive tract abnormalities in adult animals when given as a single dose to one day old rats.[41,42] Similar results have been obtained by injecting Clomid into pregnant rats on day 5 of pregnancy and examining the off-spring after maturation. These results deomonstrate the potential danger which is inherent in the use of these drugs for the induction of ovulation in women. Women are treated for 5 days with Clomid in order to induce ovulation. If the treatment fails, it is repeated after 40 days. This exposure to Clomid every 40 days may continue for a year or more. Thus, some women may be exposed to hyperestrogenization for long periods of time which represents a potentially dangerous situation. Also, unless pregnancy is determined carefully, Clomid might be administered to a woman during early pregnancy which could have profound effects on the fetus.

It is predictable from the above discussion that triphenylethylene derivatives would be estrogenic in some estrogen dependent tumors, even though these drugs are routinely used to inhibit the growth of such tumors. We have observed that Nafoxidine stimulates growth and the production of progesterone receptors in a transplantable mouse mammary tumor line (MXT, Watson and Clark, in press). Nafoxidine causes estrogen receptor accumulation and long-term retention in tumor nuclei which closely resembles that previously described for the rat uterus. Thus it appears that tripehenylethylene derivatives can be either estrogenic or anti-estrogenic depending on the cell or tissue type.

V CONCLUSIONS

Steroidal estrogens probably act as a result of their ability to occupy estrogen receptors. This occupancy of specific sites results in the stimulation of transcriptional and translational events which cause appropriate responses in target tissues. These interactions are a normal and necessary component of the mechanisms which maintain the reproductive capacity of the female. Continuous occupancy of estrogen receptors, whether by physiological or synthetic estrogens, can cause tissue to grow abnormally (Fig. 3). This usually does not occur in the cycling female because progesterone interrupts the action of estrogen. This cyclic switch to a progestational state probably acts as a deterent to the inherent ability of estrogens to cause abnormal growth.

The concept of cell specific estrogen agonist/antagonists, as exemplified by the triphenylethylene derivatives, brings a new perspective to the question: What is an estrogen? Estrogens can no longer be classified as weak or strong depending on simple uterotropic assays. If this were done, as it has been in the past, Nafoxidine would be classified as a weak estrogen. If on the other hand, one examined the ability of Nafoxidine to stimulate epithelial cell growth it would be classified as a potent estrogen. The extent to which the cell specific estrogenicity of these drugs extends is not known at the present. However, it is possible that these effects can manifest themselves in other estrogen target tissues. It is possible that cell specific estrogenicity may reflect differential localization of receptor types. Cell specific localization of type II estrogen binding sites is also likely to be involved in the mechanism of action of these drugs.

Another complication is introduced by the concept of cell specific estrogen agonist/antagonists is that estrogen receptor binding cannot necessarily be equated with estrogen action. This has been a presumption in previous work on estrogen agonists that will require revision and modification in the future. The ability of Nafoxidine to act as an agonist in one cell and an antagonist in another implies that events subsequent to estrogen binding are dictated by the cell type. Control mechanisms must exist which determine whether the biosynthetic machinery of a specific cell will respond to a receptor ligand signal.

All of these complexities make proper evaluation of estrogenic substances difficult; however, before a clear picture of the pharmacology of estrogens will emerge, each of these problems must be addressed.

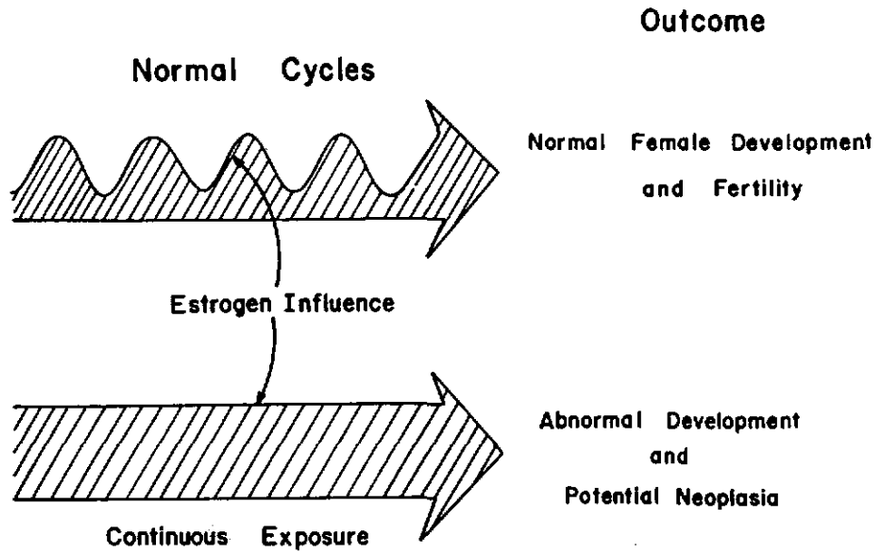


Figure 3. Effects of cyclic and continuous estrogen exposure.

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DISCUSSION

SMITH, R.: Can you tell us what organelle system(s) is observed by electron microscopy to be hypertrophied in the epithelial cells of the endometrium stimulated by nafoxidine?

CLARK: All cytoplasmic organelles are highly stimulated. In addition, the nuclei of these cells contain nuclear bodies which may be associated with hyperestrogenization.

LEROY: You mentioned that the aim of the saline injection of estradiol was to avoid, as much as possible, receptor replenishment. To what extent did you obtain that result compared with other types of administration?

CLARK: Since the replenishment of cytoplasmic receptors is not measurable before 4-6 hr after an injection of estradiol, we probably accomplished this goal. However, it is not possible to say how much recycling and reassociation of nuclear bound receptor takes place during this early period. Estradiol falls to extremely low levels in the blood after this time, and hence, it is not likely that any binding to replenished cytoplasmic receptor takes place during this period.

LEROY: In your nafoxidine and clomid experiments in which selective epithelial hypertrophy occurred, was there also hyperplasia, particularly in neonatal animals? As you know, the rat uterus does not respond to estradiol by increased DNA synthesis before 20 days of age according to Kaye et al.

CLARK: We do not know whether there is hyperplasia in the neonate.

SONNENSCHEIN: The slides you showed did not show an increase in cell number as measured by increased mitotic index when stimulated by clomid, but rather indicate a significant hypertrophy which must not be related to hyperplasia.

CLARK: That may be the case; however, it should be remembered that the animals were killed 96 hours after injection. Perhaps hyperplasia occurs before this time. It is well established that DNA synthesis occurs between 12-30 hr after estradiol administration in older animals. This time sequence may also exist in the neonate.

CUNHA: The hypertrophy of neonatal uterine epithelium caused by clomid may be mediated via the underlying stromal cells. One example of this can be found in tissue recombination experiments between epithelium and mesenchyme from wild-type and androgen-insensitive Tfm organs. In this case the epithelial response to androgen is strictly dependent upon the presence of wild-type (androgen-sensitive) mesenchyme.

CLARK: This is quite possible. However, the important point is, regardless of the mechanism, hyperestrogenization of the uterine epithelium takes place.

MARTIN: I would like to comment on Dr. Cunha's remarks. We have studied the effects of tamoxifen on adult rat uterus and find similar hypertrophy of luminal epithelium but no DNA synthesis. Yet tamoxifen appears to have the same effects as estradiol on stromal DNA synthesis. In view of this similarity we suspect that the tamoxifen effects on epithelium are not mediated by differential effects on stroma.

PALMITER: Tamoxifen appears to be a pure antiestrogen in chickens. It has no effect by itself on RNA induction in the chick oviduct and, when given with estradiol, it can, at the appropriate dose, completely suppress estradiol-induced RNA accumulation. This raises the question of whether these drugs (tamoxifen, nafoxidine, and chlomiphene) are species specific in their action, or whether they may be subject to metabolism into active estrogens in some species (e.g., mice and rats) but not others (e.g., birds).

CLARK: I believe that this may be the case. As Dr. Martin will show, there are species differences in the mouse and the rat. We have observed that nafoxidine is fully estrogenic in a transplantable mouse mammary tumor line (Watson and Clark, unpublished observations). Yet in other tumor systems it is antiestrogenic. Perhaps we should call these drugs "species/cell specific estrogen agonists/antagonists." It is difficult to believe that metabolism to active forms is the solution to the problem since nafoxidine is estrogenic in the epithelium and antiestrogenic in the myometrium of the rat uterus (assuming that metabolic activation is taking place in the liver and not in the uterus).

WEISZ: From an environmental and iatrogenic point of view, the obviously alarming aspect of your findings is the delayed effects of injection of neonates with the so-called "antiestrogens," specifically the uterine tumors and ovarian pathology found in adulthood. These findings suggest an effect on the process of differentiation during a critical period in development. Do you have any information on where the primary sites of damage might be? Is it the uterus, the ovary, or the CNS? For example, did you find any evidence of an effect on the ovary, such as depletion of oocytes shortly following the administration of nafoxidine? Alternatively, did you note any alterations in vaginal cyclicity during the period immediately after puberty -- alterations that might suggest changes in the differentiation of the CNS mechanisms regulating gonadotrophin release. The change in cyclicity need not necessarily be in the form of constant vaginal estrus since there may be more than one way of altering gonadotrophin regulation.

CLARK: Neonatal and fetal exposure to clomid and the resultant estrogenization of various target tissues, probably including the CNS, does occur during critical periods of development. In many ways the effects resemble the persistent vaginal estrus syndrome. A large proportion of the animals exhibit a high percent of estrus vaginal smears -- others are in persistent diestrus. The ovaries vary from cystic to atrophic. It is quite likely that oocyte damage has occurred in many animals. I cannot answer this question fully because the developmental studies are not complete.

McLachlan, ed. Estrogens in the Environment

ESTROGEN RECEPTOR IN THE MAMMALIAN LIVER

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INTRODUCTION

Estrogen effects on mammalian liver composition and function including synthesis of plasma proteins have been described over the past decade[1,2]. Estrogen receptors have only recently been demonstrated in mammalian liver. This chapter will describe some of the information available concerning the estrogen receptor system in the mammalian liver. In several respects, this liver system is of pertinence to the topic of estrogens in the environment. One major source of exposure is the use of estrogen containing birth control pills (and estrogens after menopause) by women. The estrogen related side effects of the oral contraceptives may be, at least in part, initiated by interaction of estrogens with this receptor in the liver. The liver is the site for extensive metabolism of estrogens and other chemicals. Metabolites with estrogen receptor binding capability might be formed in high concentration and attach to the estrogen receptor in liver with the possibility of undesirable effects.

CYTOPLASMIC ESTROGEN RECEPTOR

Female Rat Liver Cytosol Estrogen Receptor

Properties. Although putative estrogen receptors were easily demonstrated in the cytosol of target organs such as the rat uterus and pituitary, early attempts to demonstrate an estrogen receptor in mammalian liver cytosol were unsuccessful. In these early studies, liver preparations from immature female rats were used to avoid the possibility that endogenous estrogen, secreted by the mature ovary, might have occupied the estrogen receptors.[3-6] This laboratory found that estrogen binding is readily detectable when the liver cytosol is prepared from adult female rats.[7-10] The binding of tritiated estradiol (^{3}H-estradiol) was determined using gel filtration columns to separate macromolecular bound radioactivity from free radioactivity. An unusual feature of the liver is that the level of estradiol binding present in rat liver increases 5 to 10 fold at about the time of puberty[8,10] (Figure 1 top). The radio-

activity extracted from the macromolecular bound fraction has been identified as unchanged estradiol by thin-layer chromatography and by methylation to 3-methoxyestradiol.[8]

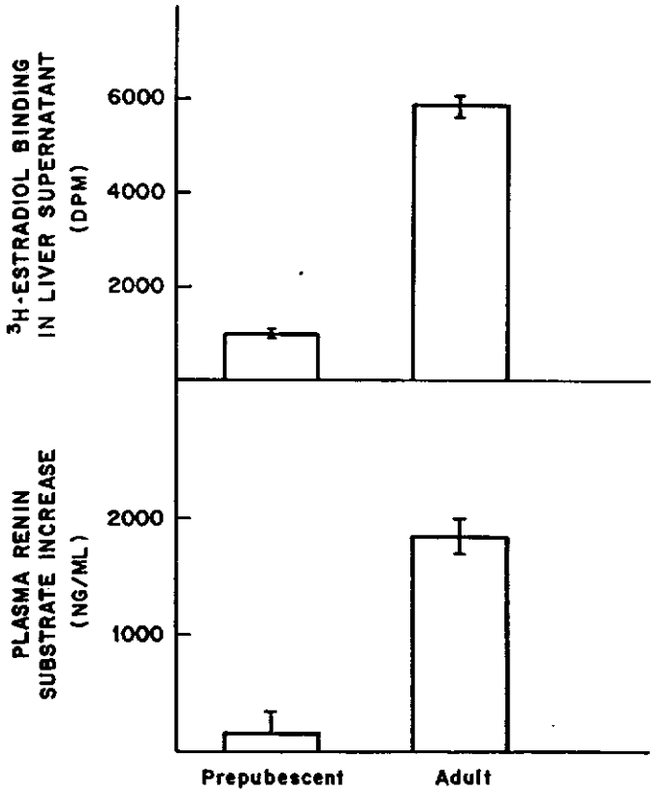

Fig. 1. Developmental correlation of estrogen binding in liver and estrogen induction of plasma renin substrate. Livers were homogenized in 6 volumes of buffer and cytosol prepared by ultracentrifugation. Macromolecular binding of 2×10^{-9}M [^{3}H]E_2 was measured by gel filtration after incubation in ice for 1 hour in 0.2 ml of liver cytosol from 27-day-old prepubescent rats and 200-g adult female rats of the control groups (top). Groups of five animals each of prepubescent and adult rats received subcutaneous injections of 100 µg of 17α-ethinyl estradiol or the vehicle alone (propylene glycol as control) at 0 and 24 hours. At 48 hours plasma renin substrate was measured by radioimmunoassay. The control levels of plasma renin substrate were 1040 ± 80 ng/ml for the prepubescent group and 1100 ± 80 ng/ml for the adult group. The graph indicates the increase above control in the estrogen-treated groups (bottom). The bars represent the standard error of the mean. Reproduced from Eisenfeld et al, Science 191, 862-865, 1976. Copyright 1976 by the American Association for the Advancement of Science.

The binding of ^{3}H-estradiol to female rat liver cytosol is highly specific for estrogens. Nonradioactive estrogens including ethinyl estradiol and diethylstilbestrol reduce the binding of ^{3}H-estradiol, while other steroids including testosterone, progesterone, corticosterone, cortisol and dexamethasone, even in 5000 fold molar excess, do not.[8,9]

Female rat liver cytosol binds ^{3}H-estradiol with a high affinity (0.7×10^{-10} M at 4 C). The capacity of the high affinity system is 4.7 fmole per milligram of tissue (58 fmole per milligram of supernatant protein).[8] The concentration of estrogen receptors in the adult female rat liver is approximately 1/3 of the concentration found in uterine cytosol.[9] Assuming that one estrogen molecule binds to each receptor, the number of estrogen receptors is estimated to be in the range of 10,000 per hepatocyte.

The ^{3}H-estradiol binding macromolecules appear to contain protein: the binding is diminished after incubations with papain, trypsin or chymotrypsin but is not diminished by incubations with ribonuclease. Sodium p-chloromercuriphenylsulfonate, a reagent which reacts with sulfhydryl groups, or heating the preformed complex to 50°C for 10 min, will also diminish the binding.[8,9]

The sedimentation coefficients for the estradiol binding macromolecules from female liver cytosol have been determined by ultracentrifugation in sucrose gradients. The supernatants were incubated with 5×10^{-9}M radioactive estradiol in the presence and absence of 1×10^{-7}M diethylstilbestrol for 1 hr in ice, gel filtered and then the macromolecular bound fraction analysed by linear sucrose gradients. For liver supernatant, the specific binding (the difference between the binding in the presence and absence of diethylstilbestrol) is distributed as 38 percent in the 8S region and 62 percent in the 4S region.[9] The properties of the estrogen binding sites of the adult female rat liver cytosol appear to be identical to those observed in uterine cytosol.

The liver estradiol binding protein has been partially purified by ammonium sulfate fractionation. The protein is precipitated by ammonium sulfate at 30 percent of saturation. There is a 20-fold increase in the binding per milligram of protein relative to cytosol.[10]

The properties of the putative estrogen receptor in rat liver cytosol have been studied by other laboratories using a variety of experimental techniques. All recent studies of the adult female rat liver cytosol have described a high affinity, estrogen specific binding component.

Beers and Rosner have studied binding of estradiol by adult female rat liver cytosol after partial purification with ammonium sulfate at 40% of saturation.[11] The binding specificity and the Kd determined with this 40% fraction are identical to that previously indicated for cytosol. Both 8S and 4S peaks of binding activity are observed when the 40% fraction was analysed in linear sucrose gradients containing low salt concentrations. Addition of nonradioactive estradiol moderately diminishes the 8S and 4S peaks.

Chamness et al homogenized liver from adult female rats (Holtzman strain) in thioglycerol and glycerol containing phosphate buffer and analysed the binding of the cytosol by sucrose gradients.[12] Only the binding in the 8S peak is diminished by addition of diethylstilbestrol. Other drugs have been studied for their ability to diminish the binding. One unusual finding in this study is that 17-alpha-estradiol is as effective a competitor as estrone and estradiol in reducing the 8S binding. A group of drugs have been described as anti-estrogens based upon their ability, in certain experimental conditions, to antagonize the effects of co-administered estradiol. Two anti-estrogens, nafoxidine and CI-628 in 25 fold molar excess, also partially decrease the radioactive estradiol binding in the 8S region of the sucrose gradients. These and other anti-estrogens have previously been shown to partially compete with estradiol for binding to the uterine receptor when added in sufficient concentration.

Estradiol binding has also been measured in protamine sulfate precipitates of adult female rat liver cytosol.[12] Protamine sulfate precipitates the 8S estradiol specific binder observed by sucrose gradients. High affinity binding (Kd = 0.9×10^{-10}M) is observed by incubating ^{3}H-estradiol with the protamine precipitate.

Estradiol binding by adult female rat (Sprague-Dawley strain) liver cytosol has also been observed by two additional laboratories.[13,14] Both prepared cytosols from livers homogenized in Tris-EDTA with dithiothreitol buffer. When estradiol binding is determined using charcoal assays and the results analysed by the method of Scatchard, Viladiu et al, observes a Kd of 0.9×10^{-10}M and a capacity of 13 fmoles per mg protein; Powell-Jones et al observes a Kd of 1×10^{-9}M and a capacity of 76 fmole per mg protein.[13,14] Both laboratories have also analysed the estradiol binding using linear sucrose gradients. Both observed both 8S and 4S peaks. Viladiu et al observes that the 8S and 4S peaks are reduced when a 100 fold excess of nonradioactive estradiol is included although the 8S peak is reduced to a greater extent.[13] Powell-Jones et al observed that the 8S peak alone is reduced by estradiol and diethylstilbestrol and that no reduction is observed with progesterone, dihydrotestosterone or dexamethasone.[14] Thus, all recent studies indicate a high affinity estrogen specific binding component in liver cytosol of adult female rats. Except for differences in 8S versus 4S distribution of receptor (which could be due to the various experimental conditions utilized), the results are in agreement.

Developmental Correlation With A Response. As previously indicated, the concentration of estradiol binding sites is several fold higher in the liver cytosol from adult female than from prepubescent female rats. The adult rat is known to respond to administration of ethinyl estradiol by an increase in the concentration of plasma renin substrate. This is thought to be a direct effect of estrogen on the liver; an estrogen added to the isolated perfused rat liver increases the synthesis of renin substrate.[15] The effect of administration of ethinyl estradiol on plasma renin substrate levels was examined in prepubescent and adult female rats.[8,10] Ethinyl estradiol (100 micrograms) was given to 25 day old and to adult female rats. The controls received the vehicle alone. The estrogen was injected at 0 and 24 hours and renin substrate was determined at 48 hours (by radioimmunoassay of angiotensin I generated by purified rat kidney renin in the presence of inhibitors of converting enzyme and of angiotensinases). Plasma renin substrate levels increase only 15% in the prepubescent rat while in the adult the increase is 167% above control (Figure 1 bottom). Plasma renin substrate is also increased by administration of glucocorticoids. In contrast to the ethinyl estradiol effect, the administration of dexamethasone markedly increases plasma renin substrate in both the prepubescent and the adult.[10] These observations are consistent with the possibility that the estradiol binding sites in liver are the receptor and that the prepubescent rat has less plasma renin substrate response to ethinyl estradiol administration because of an insufficient concentration of the estrogen receptor.

Physiologic Variation of Cytosol Receptor. The maintainance or synthesis of the estrogen receptor appears to require pituitary hormones. After hypophysectomy, the estrogen receptor level in adult female rat liver cytosol diminishes to 5% of control.[12] The level in the intact rats is 14 and in the hypophysectomized rats 0.7 fmole per mg protein. Beers and Rosner have also examined the level of estradiol binding by liver cytosol following hypophysectomy. 2 weeks after hypophysectomy the level is one fifth of that present in intact females. In contrast, the level of binding 2 weeks after ovariectomy is 2½ fold greater than that of intact females.[11]

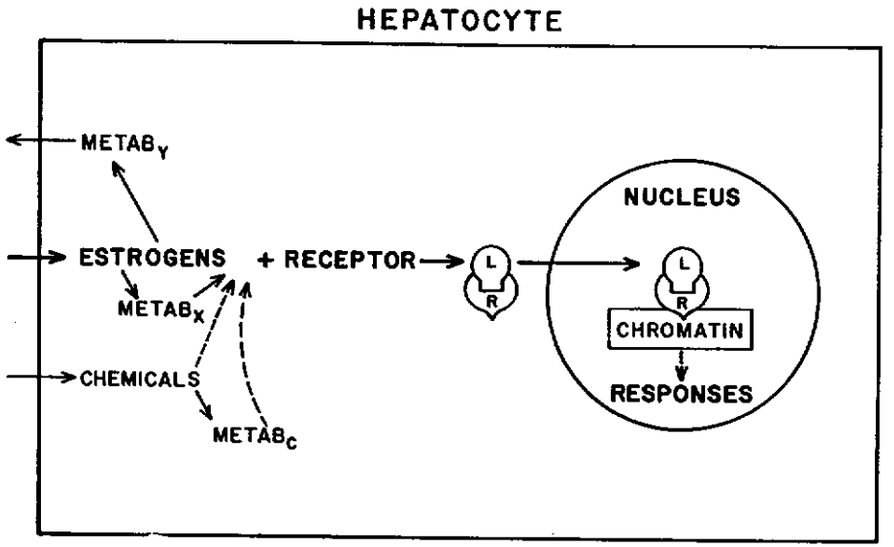

Fig. 7. Schematic Interaction of Estrogens and Other Chemicals with the Liver Estrogen Receptor.

Estrogens enter the liver parenchymal cell from the blood stream. These estrogens or a subset of metabolites formed in the liver ($METAB_X$) may attach to the estrogen receptor (R) in the cytoplasm. The complex may then translocate to the nucleus and attach to chromatin. Estrogens are also metabolized to derivatives not capable of attaching to the receptor ($METAB_Y$).

It is a theoretic possibility that some chemicals which are not usually considered as estrogens or certain metabolites formed in the liver from chemicals ($METAB_C$) might also be capable of attaching to the estrogen receptor and translocating as a complex to the nucleus.

(L) represents the ligand which can be an estrogen, estrogen metabolite, chemical or chemical metabolite attached to the receptor.

rate of receptor estrogen complex formation is second order, a low concentration of receptor and a low concentration of potent estrogen in cytoplasm may result in a lower rate, in liver relative to other target organs, in the formation of receptor-estrogen complexes. As was stated earlier, 60 min after subcutaneous administration of 5 micrograms ethinyl estradiol to adult female rats, the receptor in the uterus is depleted from the cytoplasm and is found in the nucleus while only a small fraction of the receptor in the liver is translocated. The number of receptor-estrogen complexes in the nucleus is then estimated to be 800/liver cell and 8000/uterine cell. Only at higher doses of ethinyl estradiol (e.g. 100 micrograms) is a substantial portion of the cytoplasmic receptor in liver translocated to the nucleus.[25]

A third and more speculative possibility is that there may be differences among cell types at steps subsequent to the translocation of the estrogen receptor complex to the nucleus. In the nucleus it is postulated that the complex interacts with specific acceptors (acidic non-histone proteins) on the chromatin. These multiple acceptors may regulate activity at different genes and lead to the variety of responses. The characteristics of the binding of the receptor-estrogen complex may vary at the chromatin acceptors among different cells and even for the variety of chromatin acceptors responsible for multiple effects in the same cell. It is proposed that the steroid-receptor complexes may change the attachment of RNA polymerase to initiation sites on the genes and may regulate the synthesis of selective messenger RNA molecules.[40] The quantitative and temporal relationships between binding of the receptor-estrogen complexes to the chromatin acceptors and the initial responses (e.g. mRNA synthesis) may also be highly variable. It is known that more cytoplasmic receptor-estrogen complexes translocate to the nucleus shortly after estrogen administration than are retained for several hours. The mechanisms for short and long-term disposal of the complexes in the nucleus are not known.

Another possible difference is that certain metabolites of estrogens formed in the liver might bind to the receptor, translocate to the nucleus and influence liver function. This is illustrated in Figure 7 by a subset of metabolites ($METAB_X$). It has previously been indicated that catechol estrogens are formed, bind and translocate the estrogen receptor in rat liver slices.[29] Catechol estrogens can compete with 3H-estradiol for receptor binding in

several experiments have been interpreted to suggest that administered catechol estrogens may exhibit both estrogenic and anti-estrogenic activities.[42] 2-hydroxyestradiol has also been shown to stimulate dry and wet weight gains of the uterus of the rat but was not as potent as estradiol.[43]

The study of estrogen receptor translocation in liver slices is the first demonstration of catechol estrogens bound to estrogen receptors in the nucleus of a target organ. The nature of the biologic activity of catechol estrogens in the liver is unknown but conceivably could encompass both forms of estrogenic activity. In addition, in liver, catechol estrogens may have other effects. They can be converted by hepatic microsomal enzymes into highly reactive, electrophilic compounds which will bind covalently to hepatic microsomal proteins.[44] Incubation with mushroom tyrosinase will also lead to the formation of reactive intermediates capable of binding to DNA.[45] These data suggest the speculative possibility that receptor-mediated localization of catechol estrogens in the nucleus might enhance covalent binding to DNA and contribute to hepatoma development.

Perhaps the most important difference is that some administered estrogens may vary markedly in their capability to attach to the liver receptor and produce liver effects. It is likely that certain estrogens, which may be potent in other target organs, will not bind to the receptor in the liver and initiate liver mediated responses because they are rapidly converted to inactive metabolites upon entering the liver parenchymal cell. Furthermore, while some estrogens may form metabolites which can bind ($METAB_x$) and which might be toxic, other estrogens might not form receptor binding and toxic metabolites. This is illustrated in Figure 7 as another subset of metabolites ($METAB_y$).

The metabolism of most estrogens in the liver is directed toward bioinactivation. However, mestranol, the other estrogen used in certain preparations of the combined oral contraceptives, is likely to require liver metabolism for activity. Mestranol is the 3 methyl ether of ethinyl estradiol. Mestranol binds poorly to estrogen receptors.[46] It has been shown that after administration of mestranol to rats, that it is o-dealkylated to ethinyl estradiol and it is ethinyl estradiol which is retained in the uterus.[47] O-dealkylation of mestranol to ethinyl estradiol is a reaction of the liver microsomal drug metabolizing system.[48]

This symposium will emphasize that other chemicals which are not usually classified as estrogens may also be capable of binding to the estrogen receptor and/or have some estrogenic activity (Fig. 7). Examples include the pesticide, kepone,[49] and alkyl phenols.[50] Since the liver is exposed to high levels of dietary substances via the portal vein from the intestinal tract, the liver may be exposed to higher levels of ingested environmental estrogens than other target organs for estrogens.

The liver might also convert compounds which are not capable of attaching to the estrogen receptor into metabolites which are. This is illustrated in Figure 7 by the subset of chemical metabolites ($METAB_c$). Examples of metabolites with enhanced estrogen receptor binding capability may include phenolic derivatives of DDT[51] and hydroxylated metabolites of dimethylbenzanthracene.[52] These metabolites might be generated in the liver. Some of the liver-generated metabolites with estrogen receptor binding capacity might be present only in the liver in sufficient concentration to attach and translocate to the nucleus. It is also conceivable that this receptor interaction may stabilize otherwise reactive compounds, translocate as a complex to the nucleus and thereby bring potentially toxic compounds in proximity to the chromatin and DNA.

PHYSIOLOGICAL IMPLICATIONS

Mammals

High concentrations of estrogens are likely to be required for liver effects. The liver estrogen receptor system may not have sufficient potent estrogens attached to be functional in the non-pregnant state. It might become operational during pregnancy only in those mammals that have substantial increases in the concentration of maternal plasma estrogens during pregnancy. High concentrations of plasma estrogens are observed during human pregnancy. Estrogen effects on the human liver during pregnancy may include changes in hepatic composition and secretion, including increased synthesis of certain plasma proteins (e.g. transcortin and thyroxine binding globulin) and decreased synthesis of other plasma preteins (e.g. haptoglobin).[1,2] The plasma concentration of transcortin begins to increase after the ninth week of human gestational age at a plasma threshold of 1,300 picograms of estradiol per ml of plasma (about ten times higher than in non-pregnant women). As the plasma estrogens increase with gestational age, the maternal transcortin levels are also further elevated.[53]

Non-mammalian vertebrates

A putative estrogen receptor has recently been demonstrated in chicken liver cytosol. The receptor was separated from other estrogen binders by precipitation with ammonium sulfate at 33% of saturation. After administration of estrogen in vivo, the apparent receptor levels diminished in cytosol and increased in the nucleus.[54] The receptor has previously been shown in the nucleus after non-radioactive estrogen administration in vivo and using radioactive estradiol exchange conditions with the nuclei.[55-57]

In amphibia, fish and birds estrogens dramatically change liver function. In response to estrogen an egg yolk protein precursor, vitellogenin, is secreted by the liver into the plasma. Vitellogenin accumulates in the ovarian follicles where it is split into the egg yolk proteins, which are phosvitin and lipovitellin.[58] In model systems, addition of estradiol to male amphibian (xenopus) liver maintained in culture can change the synthesis of secreted proteins to 80% vitellogenin and decrease the secretion of albumin from 30% to near zero.[53]

Estrogen administration has also been shown to increase chick liver synthesis of the triglyceride containing plasma proteins - very low density lipoproteins. A correlation has been observed between the high doses of estrogen required to increase nuclear receptors in the liver and to increase plasma triglycerides.[57]

IMPLICATIONS FOR CONTRACEPTIVES

The combined oral contraceptives contain an estrogen (either 17-alpha ethinyl estradiol or mestranol) and a progestin. This combined pill is the most effective method of birth control currently available.[59] Users of the combined oral contraceptive have a lower unwanted pregnancy rate and less breakthrough bleeding than users of the oral contraceptive containing only a progestin (minipill). The lower pregnancy rate of the combined pill is in part due to an estrogen action at the hypothalamic-pituitary axis. FSH secretion is reduced and the ovarian follicles do not mature.[60] The estrogen also acts at the endometrium to prevent irregular shedding of cells which leads to bleeding between periods. Accordingly, the inclusion of the estrogen provides substantial benefits: however, there are also deficits.

Rare but serious side effects associated with the combined oral contraceptives have been related to the presence of the estrogen. The estrogen may

in incidence of heart attacks is most marked in women older than 40 years of age who are still taking the birth control pill and who also smoke cigarettes.[62] The rare liver tumors are usually benign, but a few fatalities have been reported following hemorrhage into the liver and peritoneal cavity.[63] For women in their earlier reproductive years, the mortality from the pill is low. It is lower than deaths related to unwanted pregnancies if no contraceptives are used and is in the same range as the mortality due to other single, reversible methods of birth control (including deaths due to unwanted pregnancies if the method fails).[64]

Estrogens have been used alone in some other clinical situations. Estrogen replacement therapy during and after menopause has been associated with an increased risk of gallbladder disease,[65] hypertension[66] and the detection of endometrial cancer.[67,68] There has also been one report of a hepatoma found during estrogen therapy (which regressed after discontinuing the estrogen).[69] An increase in thromboembolism was detected in therapeutic trials when high doses of estrogens were administered to men either with prostatic cancer[70] or after a heart attack.[71]

The mechanisms by which estrogens increase the incidence of the side effects are not established. The side effects might be produced by the estrogens acting in multiple organs and by several separate mechanisms. At least some of the major side effects may be initiated by the interaction of an estrogen with the liver producing changes in liver function which may then contribute to a side effect in a susceptible person.

The liver is likely to be the estrogen target organ for the observed increase in the incidence of gallstones and of the hepatomas. It has been shown that women using the combined oral contraceptives have an increased concentration of cholesterol relative to bile acid in the bile secreted by the liver. The cholesterol, which is normally kept in colloidal solution by the bile acids, is supersaturated in concentration and precipitates as stones in the gallbladder.[72]

Although the blood vessels are ultimately involved in the cardiovascular complications, it is not known if this is a direct or indirect estrogen effect. One report has described vascular lesions thought to have some distinctive features in oral contraceptive users with fatal thromboses.[73] Indirect influences on blood vessels could be exerted by changes in clotting mechanisms, platelet function, atherogensis and hypertension. The levels of plasma

An increase in clotting factors VII and X[74] and a decrease in the clotting inhibitor antithrombin III[75] may contribute to the enhanced occurrence of thrombosis. Estrogens might contribute to heart attacks both by clotting abnormalities and by accelerating atherosclerosis via enhanced hepatic synthesis of plasma triglycerides and prebetalipoproteins.[76] Elevated levels of plasma renin substrate observed in oral contraceptive users might initiate the development of hypertension.[77] Renin substrate is cleaved by the enzyme renin from the kidney into angiotensin I which is rapidly converted to the potent vasoconstrictor, antigiotensin II. The metabolic changes mentioned and some of the side effects (e.g. many cases of hypertension) are reversible upon discontinuation of the oral contraceptive.

As a unifying concept, it is possible that most of the oral contraceptive side effects are due to an estrogen interaction in the liver. The estrogen may change liver function including the synthesis of critical plasma proteins that influence the cardiovascular system.

The newer combined oral contraceptives contain lower amounts of both the estrogen, ethinyl estradiol, and of the progestin. Decreasing the amount of the ethinyl estradiol to about 30 micrograms seems to retain contraceptive effectiveness (and to produce regular menstrual bleeding patterns in most women). Some estrogen related plasma protein changes are observed with 30 micrograms ethinyl estradiol in the combined pill, but the changes are less than with higher doses of the estrogen.[78] The only evidence to date that reducing the ethinyl estradiol dose diminishes a major side effect is that pills containing 50 micrograms increase the incidence of thrombosis less than do preparations containing more than 50 micrograms.[79]

It is not yet known whether decreasing the ethinyl estradiol dose below 50 micrograms will further reduce the risk of thrombosis or of the other side effects. An ongoing large-scale prospective study of oral contraceptive users and non-users has recently indicated that an increased risk of cardiovascular disease is still observed in oral contraceptive users, but insufficient information is available to assess the value of reducing the ethinyl estradiol dose below 50 micrograms.[80,81]

The doses of estrogens that elevate plasma transcortin (most likely a direct liver effect) are relatively high in the human. The doses of estrogens for half-maximal increase in transcortin (administered daily for 2 weeks to men) are 90 micrograms ethinyl estradiol, 1.3 milligrams diethylstilbestrol

Perhaps, a safer combined contraceptive might be obtained by changing the estrogen. If the major side effects are mediated by estrogen-receptor interactions in the liver, another estrogen could be selected or developed which preferentially minimized the liver-estrogen interaction or function while producing estrogen effects in the hypothalamic-pituitary axis and in the endometrium.

Routes of administration other than swallowing the estrogen containing contraceptives may aid in achieving desired systemic effects without liver effects. Parenteral routes of administration avoid the first pass effect of the estrogen being absorbed from the gut into the portal vein to the liver. By parenteral administration, systemic levels of certain estrogens may be sufficiently high to produce the desired effects in other target organs but upon entering the hepatocyte certain estrogens might be rapidly metabolized to inactive derivatives and not attach and translocate the liver estrogen receptor.

SUMMARY

An estrogen receptor has been demonstrated in the mammalian liver. It is a cytoplasmic protein with a high specificity and affinity for binding estrogens. The receptor-estrogen complex can translocate to the nucleus of the liver parenchymal cell. Theoretically, other chemicals or their metabolites formed in the liver could also interact with the hepatic estrogen receptor. The receptor-estrogen complex may modify hepatic function including changing the synthesis of some critical plasma proteins. At least some of the major side effects of the estrogens in oral contraceptives (and in preparations used after menopause) may be due to the estrogen directly acting at the liver. Estrogen receptor function in liver may differ from that in other targer organs in that the metabolism of the estrogen in the hepatocyte may regulate the amount and identity of the estrogen attached to the receptor. This difference might provide a molecular foundation for the possibility of diminishing side effects while maintaining the desired effects of estrogens. It might provide a basis for the possibility that the newer combined oral contraceptives with lower amounts of ethinyl estradiol will have less side effects. Alternatively, further elucidation of the function of the estrogen receptor in the mammalian liver might lead to the design or selection of a

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Estrogenic Responses *in vivo* and *in vitro*

McLachlan, ed. Estrogens in the Environment

ESTROGENIC RESPONSES IN VIVO AND IN VITRO

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The organizers of this symposium are attempting to address the questions of (1) What is an estrogen? and (2) What is the extent and significance of the estrogenic burden in the environment? In everyone's mind as we consider the "environmental burden of estrogens" is the possibility that estrogens cause human cancer. There is no clear evidence that estrogens cause human cancers. Indeed, the same studies in England that showed an increased incidence of thromboembolic disease among women using the steroidal contraceptive pill showed a decreased incidence of mammary tumors among the pill users. However, the question remains to trouble us.

An estrogen was originally defined operationally as a substance that would induce estrus in an experimental animal such as the rat. Both the natural estrogens, such as estradiol 17β and the synthetic nonsteroidal estrogens such as diethylstilbestrol are very powerful hormones which produce effects at concentrations about three orders of magnitude lower than those required for effects by other steroids such as glucocorticoids or progestins. A great many investigators have used the uterus of the immature rat or the adult castrate rat as the target organ of estrogens. These have shown that estrogens injected in vivo increase the blood supply, causing hyperemia, increase capillary permeability and the uptake of water, electrolytes and amino acids by the uterine cells. The rate of glycolysis and certain other aspects of carbohydrate metabolism are increased. Oxygen consumption is increased, the synthesis of three major types of RNA - messenger, ribosomal, and transfer - are all increased, estradiol increases the uptake of nucleotide precursors of RNA, the activity of RNA polymerases, and the template activity of the chromatin in the uterus. It increases the synthesis of phospholipids and proteins and the population of ribosomes in the cell. It increases uterine motility and the content of actin and myosin in the myometrium, although the latter response is delayed for several days. It increases the activity, in other words, of a host of enzymes within the uterus. The estrogen induced release of uterine histamine, which appears to be involved in the rapid onset of hyperemia and the water imbibition which occurs within the first hour or two of the treatment of a rat with estradiol, is difficult to correlate with our usual concept that estradiol reacts with a

receptor and is taken into the nucleus to activate the genome. Thus, this effect on histamine may represent an entirely separate hormonal action which serves to augment the other biological effects of estradiol.

The surprising finding that in many systems the metabolite, dihydrotestosterone, has a greater biological activity than the classic androgen testosterone has raised the possibility that the biologically active form of other hormonal steroids may be a metabolite. Specifically it has raised the question of whether some metabolite of estradiol or diethylstilbestrol may have a greater biological activity than estradiol-17β. Despite an intensive search no such metabolite of the estrogens has yet been found. The fact that responses to estradiol are demonstrable rapidly with in vitro systems makes it unlikely that some metabolite of estradiol must be produced before the estrogenic response can occur.

Not too long ago it was generally held that estradiol, and indeed all hormones, can have effects on their target cells only in vivo or only in a system with intact cells. With more modern experimental techniques it has been readily possible to show that estrogens and other hormones have clear-cut effects in cell-free systems. An early example was the estrogen-dependent pyridine nucleotide transhydrogenase of human placenta described by our laboratory in the 1950's.[1] The question of whether this stimulation reflected an estrogen activation of a transhydrogenase, as our laboratory reported, or whether it was due to estradiol acting as a substrate for the estradiol - 17β hydroxysteroid dehydrogenase as postulated by Talalay[2] was settled when Karavolas and Engel[3] confirmed our separation of the two enzymes and showed that the human placenta contains an estrogen-dependent pyridine nucleotide transhydrogenase in addition to the 17β hydroxysteroid dehydrogenase. In the last decade the classic studies of Schimke,[4] O'Malley[5] and their collaborators have clarified the molecular mechanisms by which estradiol or diethylstilbestrol stimulate the production of ovalbumin by the chick oviduct. Our understanding of the first steps in the process, the binding of estradiol to a specific cytosol receptor and the movement of the estrogen-receptor complex into the nucleus, began with the work of Elwood Jensen and his colleagues[6] in the rat uterus. It is now clear that under the proper circumstances cell free systems as well as intact cells and entire organs will respond to exogenous estrogens.

More than 30 years ago it was found that prolonged estrogen treatment of the Syrian hamster results in renal adenocarcinoma.[7] These renal tumors are malignant and estrogen-dependent and appear in essentially all male hamsters that have been treated continuously with estradiol or diethylstilbestrol for 250 days or more. These experiments showed further that progesterone has a protective effect in this system; animals can be protected against induction of the tumor by estrogens if they are treated simultaneously with progesterone. The tumors are not only [illegible]

can be transplanted into other hamsters only if the host has been estrogen treated. This seems to be an excellent model system to obtain further understanding of the molecular mechanisms involved in estrogen-induced tumorigenesis.

The question of whether the induction of a tumor by estrogen is similar at the molecular level to the hormonal effect of estrogen on its target tissue is an intriguing one. Estrogen receptors are present in small amounts in the normal hamster kidney and increase markedly in amount in response to long-term estrogen implants.[8] Receptors for progesterone have also been defined in the hamster kidney and increase in amount within two months after the estrogen pellet is implanted.[9] They are present in the renal adenocarcinoma and probably account for the anti-tumor effect of progesterone. It is known that the chick oviduct responds to exogenous estrogen with the production of a different spectrum of messenger RNA's, including a great deal of the mRNA that codes for ovalbumin.[10] An intriguing question at present is whether the kidney tumor produces a spectrum of messenger RNA's that is different from that produced by normal kidney cells. When the mRNA's from normal and tumorous kidneys are read out in a cell-free protein synthesizing system, is the spectrum of proteins produced by the two systems different? With the exception of one hybrid line of mice, in which the incidence of induced renal tumors is some two per cent,[11] the carcinogenic effect of estrogen on the kidney appears to be unique to the hamster.

These renal tumors grown in organ culture are estrogen dependent and will survive and grow when small amounts of estrogen are present in the medium. They undergo necrotic changes and fail to survive beyond the second week in the absence of estrogen in organ culture medium.[12] Work in our laboratory has shown that primary cell cultures of the renal tumor require the addition of estradiol to the medium for growth. The cultured cells contain an estrogen binding protein and the addition of estrogen increases the number of progesterone binding sites.[13] In contrast, the addition of progesterone to the medium inhibits estrogen induced growth. The cells growing in monolayers were primarily ones with an epithelial morphology, but some fibroblasts were present. About 20 percent of the cells contained tufts of motile cilia. These remained motile for three or four days in culture. Cilia are not observed on untransformed kidney cells, but are unique to the estrogen dependent adenocarcinoma and have been reported in other estrogen dependent tissues such as the oviduct. The tumor cells in culture had about 1,000 estrogen binding sites per cell, a value about one-tenth that found in fresh tumor tissue or in isolated rat uterus cells. A human renal carcinoma has some properties in common with the hamster renal carcinoma, but there is no evidence to suggest that the human renal carcinoma is induced by estrogen. Studies in our laboratory have shown that a specific estrogen binding

protein is present in the renal cytosols of intact, castrated, and estrogenized hamsters as well as in the primary renal tumors and its metastases.

An early event in the hormonal stimulation of the uterus by estrogens is an increased activity of ornithine decarboxylase.[14] Increased ornithine decarboxylase is a feature of most, if not all, processes involving increased cell growth such as the growth of the liver after partial hepatectomy and the growth of hepatomas. An increased ornithine decarboxylase activity in the kidney can first be observed about three months after the implantation of the estrogen pellet, at about the time when there is an increased synthesis of progesterone receptors. When kidney cells from hamsters that have not been previously treated with estrogens are prepared and grown in culture the cells respond when DES is placed in the culture medium with an increased ornithine decarboxylase activity that is evident within three hours. Estradiol - 17β also increased the activity of ornithine decarboxylase in kidney cells whereas estradiol - 17α inhibited it. That these responses represent de novo synthesis of the enzyme rather than activation was indicated by the fact that the increased activity was completely inhibited by the addition of cycloheximide.

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ESTROGENS, ANTI-ESTROGENS AND THE REGULATION OF CELL PROLIFERATION IN THE FEMALE REPRODUCTIVE TRACT IN VIVO

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INTRODUCTION

Estrogens are often described as mitogenic hormones, and there is much interest in how tney regulate cell proliferation, particularly in relation to their putative carcinogenicity. Current literature sometimes gives the impression that once the estrogen-receptor complex gains and holds the cell nucleus, DNA synthesis follows inevitably. However, their name reminds us of their many non-mitogenic actions, for 'estrogen' means something that generates estrus, i.e. female sexual receptivity or heat. In hamster and dog such effects on behaviour are spectacular, yet in women, estrogens fail to induce a 'vehement bodily appetite' (Oxford English Dictionary).

Comparable species differences in proliferative responses are common, and it is salutory to reflect how different our view of estrogens might be had the uteri of rabbit, dog, cat, ferret or spotted hyena been the popular test-beds, rather than those of the rat, mouse and guinea pig, for in the former progestins stimulate endometrial epithelial proliferation[1-5], even to the extent of inducing hyperplasias[6,7], whereas in the latter, endometrial epithelial proliferation is induced by estrogens and suppressed by progestins[8-11].

Current views of how estrogens regulate growth derive largely from homogenized-immature-rat-uterus, yet results from this system may not apply to the uterus and vagina of the adult rat or of a related species like the mouse. Whole organ homogenate studies also disregard the fact that the uterus is a collection of tissues, each responding differently to stimulation, even though the primary estrogen-receptor interaction may be the same. The relatively easy application of the techniques of molecular biology offered by homogenates and the quest for unifying hypotheses at the molecular level leads to neglect of the possibilities that specific tissue responses depend on the structural and physiological integrity of the whole organ; that estrogen-induced growth is regulated by cell and tissue interactions as well as modulation of receptors and that such regulation involves physical and structural factors as well as

It is pertinent that physical manipulations of target organs mimic the effects of estrogens. A suture at the tip of the rodent uterus increases cell proliferation along the length of the organ[12] and may induce squamous metaplasia[13]; dilation of the uterus with saline induces as much epithelial proliferation as estradiol[14]; mechanical irritation induces cornification of the vagina[15]. It is also pertinent that no one has yet reproducibly duplicated in vitro, the rapid and spectacular effects that estrogens have on target-cell proliferation in vivo. Although there are established cell lines which respond in culture to estrogens and anti-estrogens[16], primary monolayer cultures from mouse[17] and human endometrium[18] are insensitive to hormonal stimulation, rapidly become multinucleate and polyploid irrespective of hormonal environment and soon die out.

Organ culture has been as disappointing: claims that vaginal fragments maintained on plasma:embryo-extract clots responded to estrogen[19] were not confirmed by later workers who found that, even in synthetic media, the epithelia grew and cornified whether estrogens were present or not[20,21]. Grant[22] obtained limited effects on mouse uteri in organ culture with progesterone but not estrogen: in similar experiments Carter and McLaren[23] claimed to duplicate the in vivo effects of estrogen and progesterone on endometrial proliferation, but the responses were minute. Our own experiments[24] with uteri from intact immature, or ovariectomized mature mice (maintained for 24-48 h on floating lens-paper in Eagle's medium containing 1 μc [^{3}H]-thymidine/ml plus combinations of various dose levels of estradiol-17β, progesterone, insulin, prolactin, cortisol and fetal calf serum, with high and low oxygen tensions and monitored by autoradiography), uniformly failed to demonstrate any stimulation of DNA synthesis in the epithelia or connective tissue stroma.

Clearly the conditions required to obtain proliferative responses to estrogen in vitro are stringent and have not yet been met. In vivo the endometrium is well vascularized, with no cell far from a capillary; moreover cell proliferation whether epithelial or stromal, induced by estrogen or decidualization, is always preceded by increased endometrial hyperaemia and vascular permeability[25-28]. These may be the crucial deficits in vitro.

To explain why estrogen-sensitive tumors become insensitive in vitro, Sirbascu[29] postulates that in vivo estrogens induce the synthesis of essential circulating growth factors ('estromedins'). It seems unnecessary to invoke such extra-target-organ factors for normal tissues since complete responses can be

which are retained in the organ, the response being proportional to the amount of hormone retained[33-35].

SOME CONCEPTS OF CELL KINETICS

In vivo the cell populations of the uterus and vagina are continuously turning over, albeit at different rates. Turnover means that cells are continually being produced by mitosis, and lost by desquamation or death in situ[36], the relative magnitude of the rates of cell birth and cell death determining whether populations expand, decline or remain constant. In females undergoing sexual cycles, rates of cell turnover in the reproductive tract are amongst the highest in the body[37,38], as the populations swing abruptly from expansion to decline, rarely achieving a 'steady state'[37,38]. This is important to remember, as most classical methods of cell kinetics are valid only for populations in steady state growth or turnover[39].

Irrespective of whether populations fluctuate or remain steady, in the long-term normal cells achieve a necessary balance - one dead for each one born. Despite much speculation[40] there is no unequivocal evidence that this is because each division gives one stem cell and one cell destined to die, or because amongst dividing cells there is an equal probability of one, both or neither offspring surviving; operationally the alternatives are indistinguishable.

Overall effects of a hormone on proliferation may be monitored by counting the number of cells. By pinning rodent uteri to the same length before fixation, the number of cells/transverse section is then a measure of the size of the whole population[41]. Birth rates may be estimated from the proportion of cells in mitosis (Mitotic Index), if the duration of mitosis (T_M) is known; often it is not and is also assumed to remain constant over widely differing growth rates. On the other hand T_M is difficult to estimate with precision, particularly in non-steady state conditions[39].

After mitosis there is an interval (G_1) before the cell re-enters the period of DNA synthesis (S), with a further interval (G_2) between the end of S and the next mitosis. Clearly the frequency of division in a population depends on the average time taken by cells to complete the sequence.

The durations of S (T_S) and G_2 + M (T_{G_2+M}) can be estimated by the fraction of labelled mitoses (FLM) technique[42]. The population is exposed briefly to a radioactive DNA precursor, usually [^{3}H]-thymidine (pulse-labelling), and the

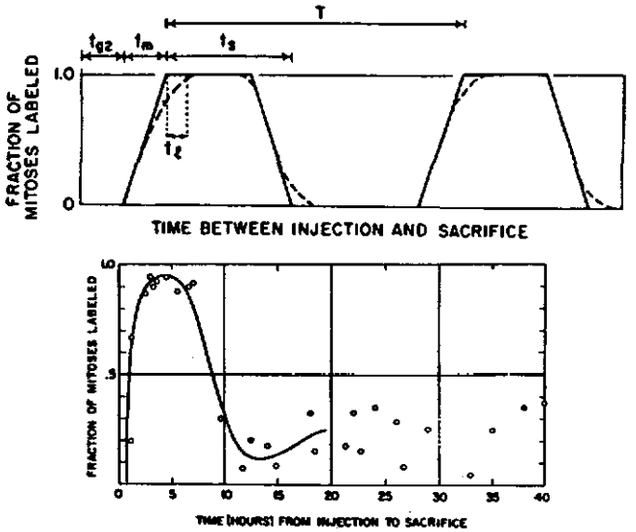

Fig. 1. Fraction of mitoses labeled vs time between injection and sacrifice. Upper, hypothetical case where each phase of cell cycle is of constant duration, t_{G2}, t_m and t_s are respectively the durations of G2, M and S, T is generation time and t_l, labeling time. Lower, actual case; lower ileum C57 brown mice: each point represents one animal, 100 mitoses scored in each case. From Quastler and Sherman, 1959, with permission.

that were in S appear and the proportion of labeled mitoses rises to 100%, then declines as cells that were originally in G1 enter mitosis. The interval between labeling and the rising limb of the FLM curve gives an estimate of $T_{G2 + M}$, and that between the rising and falling limbs of the first peak an estimate of T_S (Figure 1). Since cells in S normally proceed to mitosis, knowledge of T_S and the [^{3}H]-thymidine pulse-labeling index (L.I.) of the whole population gives an estimate of the cell birth rate[39].

It is accepted that proliferation rate is regulated in G_1, because its mean duration (T_{G_1}) varies widely between populations growing at different rates whereas T_S and T_{G_2} vary little, if at all[43]. G1 is often regarded as a deterministic sequence through which cells pass slowly, rapidly, or not at all[44]. If this were so, FLM curves would show a second peak whose position varied as G1 lengthened or shortened: this is how text books usually show FLM curves[39]. In fact most second peaks are broad[39,42]

Intermitotic times are known to be extremely variable, even in homogeneous populations in rapid steady state growth[39,43]. Tissue culture and FLM studies show that almost all this variability arises in G1 and increases with decreasing proliferation rate[50]. This led to the proposal[43] that after mitosis all cells enter an indeterminate state (A), located in G1, which they leave at random, to enter a deterministic replicative sequence (B) containing S, G2, M and part of G1, and that proliferation rates are regulated by altering the probability of undergoing the random transition from the indeterminate to the replicative phase, rather than the duration of the latter. There is now much evidence that this is true for cells in tissue culture[50-52]; in vivo data are compatible, but insufficiently precise to preclude models which account for variability in G1 by a normal distribution of proliferation rates[53].

In slowly proliferating tissues it is often envisaged that only a proportion of cells cycle continuously from mitosis to mitosis, the remainder being sequestered into a non-cycling compartment (G_0) from which they can be stimulated to resume cycling[39]. Such subpopulations have been postulated to account for discrepancies between intermitotic times estimated from T_S and L.I. and those estimated from the peaks of FLM curves[48,49] and for the sudden increases in L.I. seen after hormonal stimulation. Allegedly, G_0 populations can be distinguished from those with a long G_1 by techniques such as continuous [^{3}H]-thymidine labeling. In practice there are the problems of re-entry of G_0 cells into cycle, and of distinguishing putative G_0 cells from the dead, the moribund and the terminally differentiated[39]. One attempt to deal with these problems led to a model in which all cells enter G_0 and leave at random[54], and which is indistinguishable from the transition probability model[43,55].

Cell proliferation in the vaginal epithelium

Cornification of the vagina is the basis of the Allen-Doisy assay, the most specific bioassay for estrogenicity. Substances are given intravaginally or systemically to spayed rats or mice, and vaginal smears are taken 2-3 days later; a positive smear contains cornified cells and no leucocytes[56]. Uterine weight tests are less specific in that various non-estrogenic hormones are uterotrophic; on the other hand, some estrogenic substances fail to induce positive smears[57] or do so only transiently[58]. The cornification response depends on increased epithelial cell proliferation[59] and assays based on this[31]

Fig. 2. Transverse sections (5µ H & E) of vaginas from adult ovariectomised Sprague-Dawley rats (b.w. approx. 300g) left, and Q.S. mice (b.w. approx. 30 g) right: a, b, untreated; c, d, 4 weeks after 2 s.c. injections in arachis oil 24 h apart of 5 or 1.5 mg respectively of Tamoxifen; e, f, after 4 weeks with 800 or 100 ng/ml respectively of estradiol-17β in the drinking water.

In ovariectomized rodents the epithelium comprises one or two basal layers plus a superficial mucified layer of cells (Figure 2a, b). Proliferation is

rate of entry of the basal cells into S increases[62-64]. This lag varies with route of administration, the estrogen used and the interval between ovariectomy and experiment. Increases in mitotic index follow those in [^{3}H]-thymidine labeling index with no evidence of any cells being arrested in G_2 in the untreated animals[63]. After a single administration of estrogen the mitotic index rises to a peak 24-36 h later depending on dose and route of administration[31 63 65]: it then falls. With increased cell production rate, cell layers increase in number, and those on the surface stratify, then cornify and finally desquamate (Figure 2e, f). Usually cornification develops after the mitotic index has fallen[31,65].

The epithelium can be maintained in the cornified state indefinitely by continuous administration of estrogen by injection, inclusion in the drinking water[66] or use of long-acting polymer[67]. In such experiments basal-cell mitotic and [^{3}H]-thymidine pulse labeling indices first rise to high values, then decline, though always remaining well above control values (Figure 3). Continuous labeling with [^{3}H]-thymidine shows that all basal cells participate[67,68]. FLM data indicate that increases in proliferation rate arise mainly from shortening in the mean length of G_1, though there may also be a shortening of S phase[69,70].

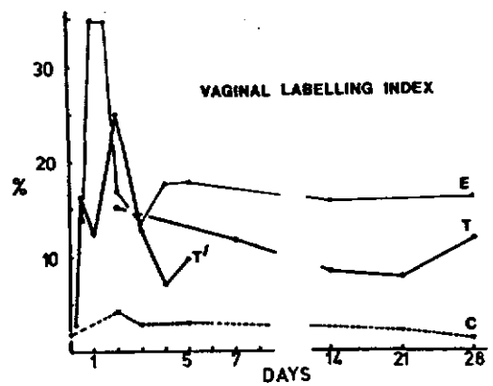

Fig. 3. Vaginal basal cell [^{3}H]-thymidine pulse labeling indices (%) from adult ovariectomized rats: 1 µc/g b.w. [^{3}H]-Tdr given 1 h before sacrifice; 2000 cells counted/organ: points are means from at least 4 rats, S.E.M.'s were usually < 10% of mean. C, untreated; T', 250 µg Tamoxifen daily s.c. in oil; T, 5 mg Tamoxifen s.c. in oil days 0 and 1 only; E, 0.5 µg estradiol-17β

The initial rise and fall in the indices of proliferation can be accounted for as follows: initially most cells are in G_1, estrogen abruptly increases the rate of transit from G_1 to S, producing a quasi-synchronous wave of cells in S and M; as cells divide again, synchrony decays because of the inherent variability of the cell cycle, and the indices of proliferation settle at new equilibrium values.

With continuous estrogenic stimulation, proliferation rates in the uterine epithelia also rise sharply but fall back to control values (see later). The difference between the two tissues may arise from the differing paths followed by new-born cells; vaginal cells rapidly move into the superficial layers exerting little constraint on the basal population, whereas uterine cells remain, to die *in situ*.

Using continuous estrogen administration, Peckham *et al*[68] showed that basal labeling indices and vaginal epithelial thickness measured in the same animals, varied with hormone dose but correlated almost perfectly with each other, suggesting that thickness is simply a reflection of proliferation rate. It therefore follows that cells have essentially the same life span after leaving the basal layer. The surface cells differentiate into mucified or cornified cells depending on the degree of estrogenic stimulation. If commitment to differentiate happened at a set age it would occur at different levels in the epithelium depending on the cell production rate, commitment to one or other pathway being determined by the particular environmental factors at that level.

Growth and cell proliferation in the uterus

In immature rat uteri, estrogen stimulates cell proliferation in all tissues[71]. This does not happen in the adult organ[9]. Figure 4 shows transverse sections of uteri from adult castrate rats and mice, to which this discussion is confined. In both there is an outer myometrium of two layers, one circular and one longitudinal. Both react to estrogenic stimulation by hypertrophy and changes in contractility, but in the short term show little cellular proliferation[8,9,41] which is usually a response to distension[72].

The myometrium encloses a complex connective tissue stroma, comprising a highly vascularized matrix of collagen, elastin, fibroblasts and mast cells plus an everchanging and often large population of wandering cells including large and small lymphocytes, plasma cells, macrophages, polymorphs and eosinophyls. The eosinophyl content of the rat uterus is influenced by estrogen[73],

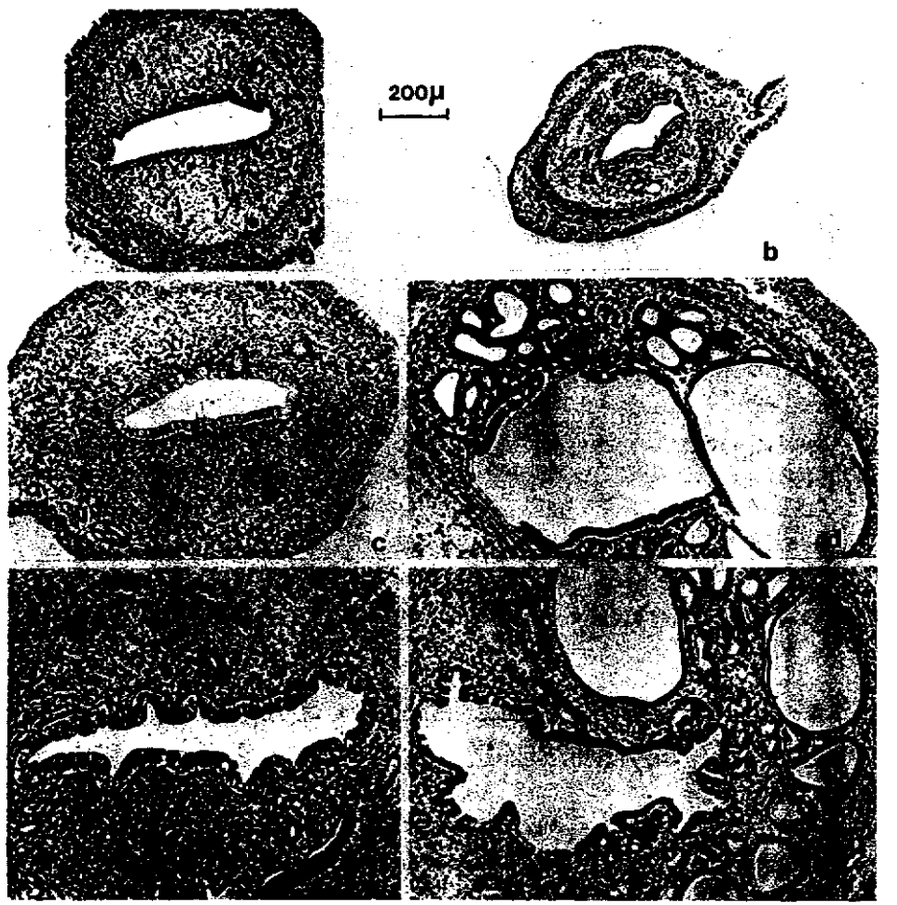

Fig. 4. Transverse sections (5µ H & E) from uteri of adult ovariectomised Sprague-Dawley rats (b.w. approx. 300 g) *left*, and Q.S. mice (b.w. approx. 30g) *right*: a, b, untreated; c, d, 4 weeks after 2 s.c. injections in arachis oil 24 h apart of 5 or 1.5 mg respectively of Tamoxifen; e, f, after 4 weeks with 800 or 100 ng/ml respectively of estradiol-17β in the drinking water.

estrogen and progesterone[12]. In general, there is little quantitative data concerning these populations though they may contribute substantially to total

in the ovariectomized animal following estrogenic stimulation, but labeling and mitotic indices remain low[41,76]. In ovariectomized mice such proliferation appears to be confined to the endothelial cells and occurs later than that in the epithelia[41]. With continuous estrogen treatment stromal cell proliferation remains elevated in the mouse uterus[76] but returns to control values in the rat (Figure 11). Extensive stromal proliferation occurs in the uteri of pregnant mice[77] and rats[78], immediately before ovum implantation, and can be duplicated in ovariectomized animals by progesterone pretreatment followed by estrogen[8,9]. In mice there is a lag of 6 h, the [^{3}H]-thymidine pulse labeling index then rises to a peak at 15 h, followed by a peak in mitosis[79]. The cell population concerned seems capable of only one division in response to estrogen and then becomes refractory to estrogenic stimulation[80,81]; this insensitivity also involves the stromal vasculature[28]. Thus prolonged treatment with estrogen and progesterone does not induce a stromal hyperplasia: instead the stroma becomes atrophic, and this may contribute to the long-term inhibition by progesterone of epithelial proliferation in the mouse uterus[82]. In the normal course of pregnancy, stromal cells differentiate into decidual cells[84]. It seems likely that the hormonal induction of one division followed by withdrawal from the cell cycle are prerequisites, serving to bring cells into a state of sensitivity to the transforming stimulus. Although a progesterone-induced failure in estrogen-receptor replenishment may account for the refractory state in rats, we find no diminution in the uptake, retention or metabolism of [^{3}H] estradiol administered <u>in vivo</u> by mouse uteri in the refractory state[83].

The uterine epithelia are of particular interest because in the human, hyperplasias of these tissues are considered to be precursors of endometrial cancers[85], most of which are epithelial in origin. In ovariectomized rodents the epithelia constitute only some 10% of the uterine cell population[41], but exhibit striking changes in proliferation rate with hormonal stimulation. The luminal and glandular epithelia, though similar in morphology, are distinct tissues showing striking differences in response to estrogens and anti-estrogens.

In intact female mice the luminal cell population appears to turn over completely each estrous cycle[36,38]. After ovariectomy the uterus atrophies and luminal cell numbers plummet. One or two weeks after ovariectomy or estrogen priming one finds 350-450 luminal cells/section and [^{3}H]-thymidine labeling indices ranging from 3-9%. If spayed animals are left for 2 months without estrogenic stimulation the number of cells/ [illegible]

cells (< 2%), and evidence of cell death - basophylic Feulgen positive droplets, singly or in clusters, sometimes labeling with [^{3}H] thymidine, which appear in large numbers during epithelial regression[36,41]. Regression after brief estrogenic stimulation does not involve shedding of cells into the lumen or their phagocytosis by macrophages or granulocytes. Nuclei do not become pycnotic. Cell death occurs by karyorrhexis; the products appearing between and within otherwise normal-looking cells and presumably phagocytosed by them[41]. We have found no intermediates between apparently normal cells and the debris, nor do we know how long debris survives; however, it is a useful indicator of the rate and timing of cell death.

Estimates of the average generation time in the luminal epithelium of untreated spayed mice vary between laboratories[41,81], with disagreement as to whether all or part of the population turns over[48,62]. Estimates of the duration of S also vary, but in our strain the FLM technique indicates 8-10 h[81].

Following systemic[41] or local[32] application of estradiol-17β to recently primed spayed mice there is a lag of 5-6 h before the rate of entry of luminal cells into S increases (Figures 5, 6). During the lag, cell debris disappears within 2 h; vascular flow and stromal edema increase from 2-3 h onwards[41], and protein synthetic rates peak at 4-6 h[87,88]. From 6 h on the [^{3}H]-thymidine pulse labeling index rises rapidly until 12-15 h then falls as cells leave S to enter G_2 and mitosis. The mitotic index increases from 12 h onwards, giving an estimate for $T_{(S + G_2)}$ in the stimulated epithelium of about 6 h[41]. This agrees well with estimates obtained from FLM curves[81]. Cell death rates remain low throughout this period. Continuous labeling with [^{3}H]-thymidine (Figure 6) shows that all cells enter S: pulse labeling[41] and FLM data[81] indicate that some go through a second round of proliferation. Certainly cell numbers double by 24 h (Figure 5).

In spayed mice left for 2 months without estrogenic stimulation, the response is generally slower; a longer lag and a lower rate of entry into S and division (Figure 5). As a result, cell numbers may not increase significantly by 24 h. There are also strain differences; long-term unstimulated spayed C57 mice may show no proliferation over the first day of stimulation[76]. If a second dose of estradiol is given 24 h after the first (Figure 5), the lag before [^{3}H]-thymidine labeling indices rise again is 6 h irrespective of the length of the first lag. If no further estrogen is given increasing numbers of dead cells appear and cell numbers fall precipitately[41]. Clearly, continuing estrogenic

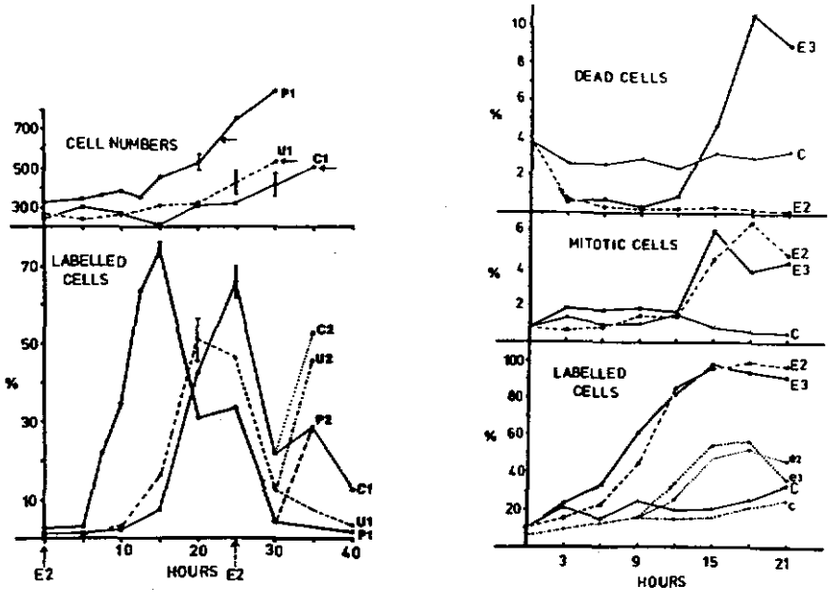

Fig. 5 (left). Uterine luminal cell numbers and [^{3}H]-thymidine pulse labeling (1 µc/g b.w. [^{3}H]-thymidine 1 h before autopsy) indices (%) in ovariectomized mice after a s.c. injection in oil of 50 ng estradiol-17β. Results are means from 5 mice with representative S.E.Ms. Arrows in top graph show where cell numbers double. P1; QS mice primed 1 wk before experiment with 3 x s.c. injections of 100 ng estradiol in oil. U1, C1; QS and C57 black mice respectively, ovariectomized and left untreated for 2 months before experiment. P2, U2, C2 are corresponding responses to second s.c. injection of 50 ng estradiol 24 h after the first.

Fig. 6 (right). Percentage of dead, mitotic and [^{3}H]-thymidine labeled cells in the luminal epithelium of ovariectomized primed QS mice: C, controls; E_2, E_3 after a 100 ng s.c. injection of estradiol-17β or estriol respectively at time 0. 1 µc/g b.w. [^{3}H]-thymidine was given every 3 h and the mice were killed 1 h after the last injection (continuous labeling). c, e_2, e_3 indicate labeling indices in the glands of these mice. Results are means for groups of 5 mice.

a lag of 6 h during which dead cells disappear; luminal cells then enter S at an increased rate. Continuous [^{3}H]-thymidine labeling shows that almost all do so (Figure 6). Yet cell numbers do not increase[89], for the cell death rate increases at the same time as the mitotic rate (Figure 6). In the whole uterus [^{3}H]-estriol is not retained for as long as [^{3}H]-estradiol[89]. Apparently it is retained for sufficient time to induce DNA synthesis, but not long enough to

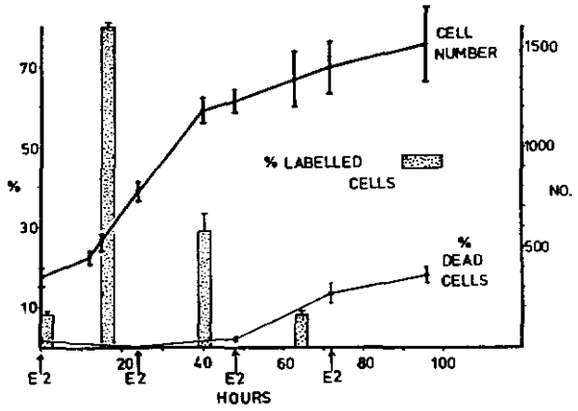

Fig. 7. Uterine luminal cell numbers, [^{3}H]-thymidine pulse-labeling and dead cell indices (%), in ovariectomized primed QS mice given s.c. injections of 100 ng estradiol-17β in oil at 24 h intervals. 1 µc/g b.w. [^{3}H]-thymidine was given 1 h before sacrifice, at time 0 h, or 15 h after the 1st, 2nd or 3rd estradiol injections. Dead cell counts (from a separate experiment) were made at time 0 h or 24 h after each estradiol injection. Results are means ± S.E.Ms for 5 mice.

cell death, and cell numbers double[89]. Increased DNA synthesis followed by premature cell death can be induced by minute doses of estriol applied directly to the uterine lumen, negligible amounts remaining in the epithelial nuclei six hours after instillation[32]. Such experiments suggest that increased transcription may be obligatory for doubling cell numbers but not entry into S.

Although initial stimulation of the mouse uterus induces most luminal cells to enter S, with continuing stimulation the proliferation rate drops until the pulse-labeling and mitotic indices barely rise above the untreated control level (Figure 7). This rise and fall has often been described[90-94], but the most extensive kinetic data are those of Lee[76] for C57 mice treated for 23 days with estrone in the drinking water. Following an initial rise and fall, the mitotic and labeling indices do not remain depressed but rise and fall again from days 7-13 (Figure 8) and again from days 16-23. These patterns are not explicable in terms of estrogenic toxicity, or a refractoriness developing from saturation of some estrogen dependent process - otherwise the indices would stay down. Stormshak et al.[94] find that whole-uterus levels of DNA polymerase first rise, then fall during continuous estrogen stimulation. However, cell culture

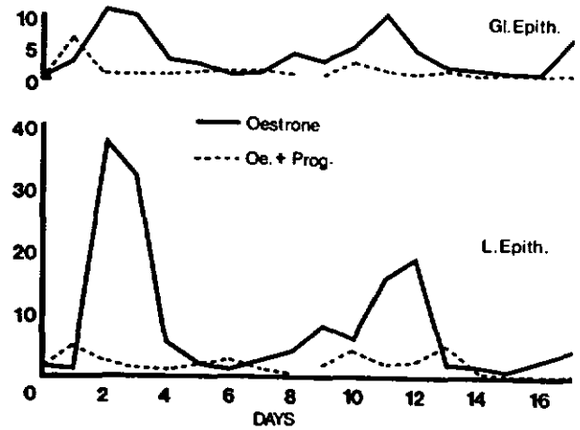

Fig. 8. Mean number of uterine epithelial mitoses/section for groups of 5 ovariectomized but unprimed C57 black mice given estrone (100 ng/ml) continuously in the drinking water with and without daily s.c. injections of 160 µg progesterone: 50 µg colchicine was given s.c. 2 h before sacrifice. Data of Lee[82] with permission.

of changing proliferation rate.

As in the vagina, initial synchrony and subsequent desynchronization would produce a peak mitotic index followed by a fall to a new equilibrium level, but this would not account for the successively smaller synchronous responses to estrogenic stimulations, as cell numbers increase (Figure 7).

Conceivably those cells which survive ovariectomy-induced uterine regression, are estrogen-sensitive stem cells, each capable of dividing into one stem cell and one infertile cell. There is some evidence for this:
a) a second injection of estradiol usually induces a peak [^{3}H]-thymidine labeling index of less than 50%[41], c) continuous administration of [^{3}H]-thymidine starting after cell numbers have doubled, labels only 25% of cells compared with 100% in spayed controls[62], c) if a pulse of [^{3}H]-thymidine is given when cell numbers have increased fivefold and the population allowed to regress by discontinuing estrogen administration, the same number of labeled cells as were present 1 h after labeling is found 7 days later, although total cell numbers have fallen by fivefold (Table 1).

Asymmetric division giving one stem cell and one infertile cell would halve

TABLE 1

PREFERENTIAL SURVIVAL OF [^{3}H]-THYMIDINE LABELED LUMINAL CELLS DURING UTERINE REGRESSION

1 hour			7 days		
No. cells	Labeled %	Labeled No.	No. cells	Labeled %	Labeled No.
1422 ± 145	13.6 ± 4.3	193 ± 61	406 ± 36	44.6 ± 3.5	181 ± 14
1341 ± 138	8.1 ± 1.0	109 ± 13	372 ± 34	36.0 ± 4.3	134 ± 16
1503 ± 112	2.7 ± 0.4	41 ± 6	314 ± 20	14.8 ± 4.3	46 ± 13

Mice given [^{3}H]-thymidine (1 µc/g b.w.) 15 h after the last of 3 s.c. injections in oil of 100 ng estradiol-17β were killed 1 h or 7 days later. See Martin *et al*[41] for technical details. Results are means ± S.E.Ms. for 5 mice.

within the proliferating population, and which involves a diminution in the probability of leaving G_1 but no increase in S, G_2 or M[45].

What mechanisms might be responsible? We have studied the *in vivo* uptake of [^{3}H]-estradiol-17β by the epithelium throughout the period of decreasing proliferation, and find only small decreases in total and nuclear content of hormone which are entirely compatible with the increasing numbers of dead cells which appear at this time[96] (see below). There is no fundamental reason why changes in proliferation rate should depend on changes in estrogen binding. All uterine tissues appear to bind estrogens equally well and translocate the hormone receptor complex to the nucleus[97] yet not all respond by proliferation.

Bullough[98] suggested that stem-cell proliferation is regulated by specific mitosuppressive substances (chalones) produced by the differentiated progeny - the concentration of chalones increasing with the size of the differentiated population - a simple negative feedback. Lee[99] finds no evidence for a uterine epithelial chalone arresting cells in G_1 (which one would predict from the FLM data), but some evidence of a factor which blocks cells transiently in G_2.

As discussed above, increases and decreases in uterine proliferation correlate with increases and decreases in vascular flow and permeability, while dilation of uteri induces as much epithelial proliferation as estrogen[14]. We have slit open *in vivo* the uteri of mice treated continuously with estrogen and several days later examined the epithelium. It did not spill over the cut edge, but

classes of protein in the uterine luminal epithelium[88] before there is any significant increase in incorporation of [^{3}H] uridine into RNA or in total RNA content[100]. Thus they probably result from increases in precursor pools as a consequence of increased availability of nutrients due to increased vascular flow and permeability. These various observations suggest that luminal proliferation is regulated by availability of space and nutrients as well as transcriptional events dependent on interactions of the estrogen receptor complex with the cell nucleus.

If the probability of a cell entering S is linked to the rate of protein synthesis[43], increased transcription and ribosome number may not become limiting until later stages of proliferation, a deficit leading to death only as cells attempt to divide, as in the estriol experiments[89]. Since total space and total nutrient flow are finite, as cell numbers increase, per capita space and per capita nutrients diminish, reducing the rates of protein synthesis, and of entry into S, despite continual estrogen stimulation. Conceivably the higher ribosome content of the stimulated cells allows the maintenance of a higher population density.

Autophagocytosis apart, it seems likely that a luminal cell will obtain nutrients primarily through its base: indeed the limits imposed by space may operate simply by restricting nutrient flow/cell by this route. Despite continuing estrogenic stimulation, dead cells appear in the lumen in increasing numbers from days 4-6 onwards and, as they do, another wave of mitosis develops. It appears that the first synchronized wave of mitosis produces a population of cells with a life span of 4-5 days: as they die, cell numbers fall transiently per capita space and nutrients increase and, with them, the rate of entry into S. The rat uterine luminal epithelium exhibits similar but less extreme fluctuations (Figure 9).

Whatever the regulatory factors, luminal cell numbers rise to a maximum within a few days and remain there or even decline slightly.

Gland cell proliferation

Uterine gland cells of rat and mouse resemble the luminal cells in being stimulated to proliferate by estrogens and prevented from doing so by progesterone. Epifanova[48] considered that luminal cells derive from gland cells but our observations indicate that in mice they probably don't - both

turn over; a few [^{3}H]-thymidine labeled, mitotic and dead cells are always visible. After initial estrogenic stimulation DNA synthesis and mitosis increase at the same time as in the lumen but peak indices are lower, cell death rates rise earlier; also, continuous [^{3}H]-thymidine labeling shows that only 50% of cells enter S (Figure 6). With continuous stimulation[76] gland proliferation rates rise and fall like those in the lumen (Figure 8). Nevertheless, cell numbers continue to rise long after the first fall in mitotic rate and all strains of mice tested rapidly develop adenomatous endometrial hyperplasias (Figure 4), which resemble those of the human endometrium[101], where they are also associated with prolonged unopposed estrogen action, and are viewed as likely precursors of endometrial cancer. Experimental approach to this question has been hampered by the rarity of spontaneous endometrial carcinoma in laboratory species. The mouse uterus may be a useful model since prolonged estrogen treatment does produce endometrial adenocarcinomas[102,103].

Although mice rapidly develop endometrial hyperplasias when stimulated continuously with estrogen, rats do not (Figure 4). For example, groups of 4 ovariectomized rats and of 5 mice were killed 5 or 8 weeks after being placed on drinking water containing estradiol-17β (rats, 800 ng/ml; mice, 100 ng/ml) or receiving s.c. injections in water of polyestradiol phosphate[67] (rats, 200 µg; mice, 40 µg) every 14 days. Vaginal smears were cornified throughout. At autopsy uteri were cleared and examined microscopically: 16/20 mouse uteri showed full length development of hyperplasias, and histological sections showed that 19/20 had developed the condition. None of the rat uteri showed any signs of hyperplasia.

There does not appear to be any fundamental difference in sensitivity to the mitogenic effects of estrogens, since uterine epithelial cell numbers plummet after spaying and are restored by estrogen in both species. What factors underlie such species differences? It is significant that the hyperplasias are restricted to the glands and develop after cell proliferation rates, which increased at the start of treatment, have fallen back towards control values in both lumen and glands. Estrogenic stimulation of excessive gland secretion would tend to distend the glands, increasing available space and thus cell numbers. Species differences might then result from differences in secretion rates or quirks of endometrial anatomy leading to retention of secretion. It is the latter which seems to be involved here. Histological examination shows

mouse become blocked, apparently by overgrowth of the luminal epithelium.

Several mechanisms can be envisaged whereby such hyperplasias might predispose towards neoplasia. Increased frequency of replication and size of replicating population would increase the chances of mutation. Breakdown products of the retained secretion may be carcinogenic, as might the products of cell death, which in the absence of any periodic sloughing of the epithelium, accumulate, and undergo autophagocytosis. Failure to undergo periodic sloughing might itself be important, by allowing the retention and accumulation of transformed cells.

The effects of anti-estrogens on target cell proliferation

Progesterone is usually regarded as an anti-estrogen. However, as the name suggests, its primary function is to support pregnancy. Since different species have different modes of ovum implantation, placentation etc., it is not surprising that its effects on the uterus vary with species[104]; antagonizing estrogens in one, synergizing with them in another. During early pregnancy in mice and rats[77,78] proliferation ceases in the endometrial epithelia. This is associated with rising levels of progesterone and can be duplicated in spayed animals with exogenous hormones. In mice 3 days progesterone treatment completely suppresses the acute proliferative responses of the uterine epithelia to estrogen[8], and even brief treatment partially inhibits the response. Cells are sensitive to progesterone early in G_1, and are blocked in this phase and not G_2; S is not prolonged[105]. Continuous progesterone suppresses epithelial cell turnover in otherwise untreated castrates[79], and suppresses the epithelial proliferation induced by continuous estrogen treatment[82]. Our own studies using homogenates of separated epithelium indicate that suppression does not involve qualitative or quantitative changes in epithelial estrogen binding or metabolism[87], though the pattern of epithelial protein synthesis is altered[88,106]. In the rat uterus, progesterone also suppresses epithelial proliferation, but autoradiographic evidence suggests that this involves inhibition of estrogen binding in the luminal, though (somewhat surprisingly) not the glandular, epithelium[107,108].

Progesterone is mitogenic in the uterine epithelia of rabbit[1] and bitch[7]. The spectacular proliferation of the glands of the rabbit uterus is the basis of highly specific assays for progestins[109,110]. In this species progesterone

stimulation[1,111]. Prolonged treatment induces endometrial hyperplasias in rabbit[6] and bitch[7]: in both cases they are associated with excessive secretion.

One can relate these varying effects of progesterone to different modes of ovum implantation. Thus it suppresses epithelial proliferation when pre-implantation embryos remain small and must attach to, or penetrate the luminal epithelium - processes which might easily be interrupted by division of the epithelial cells - whereas it is mitogenic when the embryo increases in size before implantation and requires increased glandular tissue to provide adequate nutrition. One wonders whether progesterone's mitogenic effects involve similar mechanisms to the mitogenic effects of estrogens.

Dimethylstilbestrol is one of the first competitive anti-estrogens to be described: given to spayed mice, intravaginally and simultaneously with estrogen, it abolishes the latter's mitogenic and keratogenic effects[112], acting competitively to prevent retention of estrogen[33,34]. However, it is only wholly effective in these limited conditions: intravaginally it is ineffective when given minutes after the estrogen[113], fails to displace estrogen already bound to receptors[35] and disappears rapidly from the target-organ itself. Given systemically it is only ever estrogenic despite many attempts to demonstrate antagonism. This was first attributed to its metabolism to estrogenic substances[112]. However, doses which intravaginally were anti-estrogenic in mitosis and smear tests were as effective as estradiol-17β in stimulating early nucleolar changes in the vaginal epithelium[114]. Thus DMS occupies receptors long enough to prevent the binding of estrogen, which is then lost, and to induce short-term responses, but not long enough to induce long-term responses. When its level in the vagina is maintained DMS is fully estrogenic: an intravaginal dose which is anti-estrogenic as a single application induces mitosis and cornification when it is divided and given as repeated applications over 5-10 h[115]. The estrogenicity of DMS and similar compounds, given systemically, thus reflects the depot effect of whole body administration, as much as metabolism[116].

Since all competitive anti-estrogens are weak estrogens it seemed that estrogenic potency simply reflected the time substances remained at receptor sites and that all anti- and impeded-estrogens would be fully effective agonists in conditions of continuous application[115]. This has not proved to be true for triphenylethylene-related antagonists but seems to hold for

dangers of compounds which appear to be only weakly estrogenic in conventional assays but which may be present continuously in the environment.

The classical 'impeded' estrogen[119] is estriol, equipotent with estradiol-17β in 6 h assays, but less potent in the long-term, and failing to induce maximal growth when given once daily[120]. Given with estradiol-17β, estriol reduces its effectiveness[119], apparently competing for receptors but dissociating rapidly from them[121]. Cole and McMahon[122] suggested that the protection afforded against breast cancer by an early pregnancy might result from high levels of estriol inhibiting the (putative) carcinogenicity of stronger estrogens. However, many groups have shown that estriol given continuously, is as effective as estradiol-17β in all respects including the genesis of mammary tumours[123]. Thus it would seem unlikely to be an effective antagonist in conditions of continuous secretion as in pregnancy.

The situation with non-steroidal anti-estrogens like Nafoxidine[124] and Tamoxifen[125] is far more complex. Emmens and I found insignificant antagonism of estrogens with Nafoxidine in mice or rats[126]. Harper and Walpole[125] in their original evaluation of Tamoxifen found it to be a weak but effective estrogen in mice. This was confirmed by Lee[82] who found that both compounds induced uterine cell proliferation in mice and failed to inhibit that induced by continuous estrogen treatment. Emmens[127], using vaginal smear assays, found subcutaneous injections of Tamoxifen had prolonged effects in mice which were initially estrogenic but later anti-estrogenic. Jordan[58] confirmed these findings. Using similar treatments we also showed the initial production of positive smears followed by negative ones[128], but found that both vaginal and uterine cell proliferation rates were raised far above control values for up to 60 days. The vaginal epithelium remained hyperplastic and multilayered throughout, though not cornified (Figure 2), while the uteri developed hyperplasias like those induced by estradiol (Figure 4). We argued that Tamoxifen is simply a weak estrogen in mice, and that its inhibitory effect on smears is an artefact of the criteria used to evaluate the smears. An alternative explanation is that Tamoxifen saturates the estrogen-receptor system in the vagina initially inducing maximal cell proliferation and thus sufficient cell layers for surface cornification. Thereafter proliferation rate drops because of desynchronization or non-receptor-mediated effects of the antagonist, the epithelium is no longer maintained at a thickness at which cornification occurs:

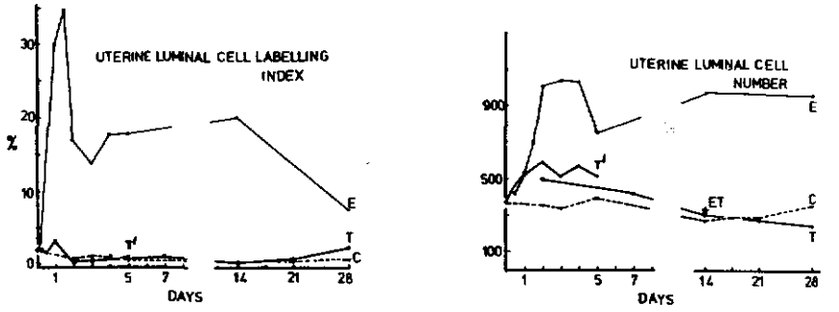

Fig. 9. [^{3}H]-Thymidine pulse-labeling indices (%) and number of cells/section in uterine luminal epithelium of adult ovariectomized rats. The results are means for at least 4 animals; S.E.Ms. were usually < 10% of the mean. 1 μc/g b.w. [^{3}H]-Tdr given 1 h before sacrifice, C, untreated; T', 250 μg Tamoxifen daily s.c. in oil; T, 5 mg Tamoxifen s.c. in oil, days 0 and 1 only; E, 0.5 μg estradiol-17β daily s.c. in oil (points up to day 5) or 800 ng/ml in drinking water (14 and 28 day points), ET had latter plus 2 x 5 mg Tamoxifen days 0 and 1.

It has always been apparent that neither Tamoxifen nor Nafoxidine are wholly effective estrogen antagonists in rat uterine weight tests[124,125], and that at dose levels which inhibit estrogen, both show significant though partial agonist activity. This does not apply to the individual uterine tissues. In adult castrate rats Tamoxifen induces prolonged hypertrophy of the uterine luminal cells (Figure 4) but no increase in [^{3}H]-thymidine labeling index, and cell numbers remain unchanged (Figure 9). Furthermore, it inhibits the acute increase in cell proliferation induced by estradiol-17β (data not shown) and the increased cell numbers induced by prolonged estrogen treatment (Figure 9).

In the glands Tamoxifen does not stimulate proliferation but increases cell death rate because, over 2-4 weeks the number of cells decreases six-fold below those of untreated castrates (Figure 10), even when estradiol is administered. This is the only case I know where an anti-estrogen reduces cell numbers below those in the castrate.

In contrast, Tamoxifen increases the number of stromal cells incorporating [^{3}H]-thymidine more than estradiol. Even more striking is the observation that the same doses of Tamoxifen in these animals significantly, consistently and persistently increase both [^{3}H]-thymidine labeling and mitotic indices in the

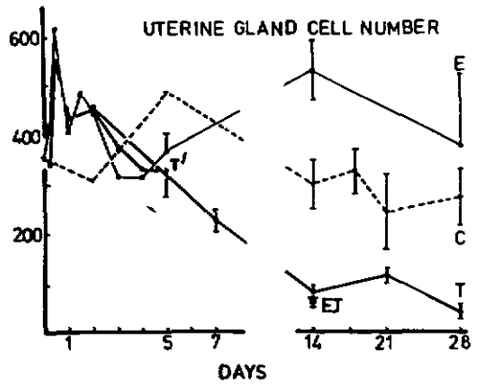

Fig.10. Number of cells/section in the uterine glandular epithelium of adult ovariectomized rats: symbols as in Figures 3 and 9.

The confusion is compounded by consideration of different species. Tamoxifen is an estrogen in mice, an agonist or antagonist in rats depending on the tissue, but a pure antagonist in chickens[129]. It was thought to be purely antagonistic in women, where it induces remission of mammary tumors without the side effects of high dose estrogen therapy, but recent reports indicate that these doses induce estrogenic changes in the vagina[130].

Difference in metabolism might account for some species differences in response, but would not easily accommodate similar actions in one organ in different species, and dissimilar actions in different organs of one species. To account for such diversity in terms of estrogen-receptors one must postulate that they vary, or that their ligand complexes act differently in different cell types, e.g. binding to other chromatin sites. From examples cited previously the disparities could involve tissue- and species-specific cell regulatory mechanisms that are not mediated by the occupation or replenishment of estrogen-receptors. One must also consider that while the estrogenic activity of compounds like Tamoxifen is mediated through the estrogen-receptor pathway its anti-hormone activity may not be, i.e. the inhibitory effects are non-specific but are enhanced in estrogen target tissues by virtue of an ability to occupy estrogen receptors. Tamoxifen may be a 'site-directed' cytotoxic agent.

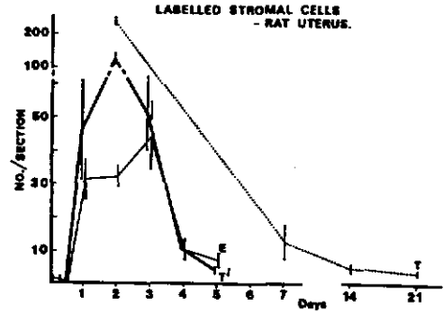

Fig. 11. Number of stromal cells/section in the uteri of adult ovariectomized rats, labeled by a pulse of [^{3}H]-thymidine. Experimental details as in Figures 3, 9 and 10.

cancer patients, therapeutically effective doses produce blood levels of 250 ng/ml or more[133], values far above those which saturate the estrogen-receptor pathway. In rats, Tamoxifen affects some glycolytic enzymes in liver and muscle[134].

As a further complication many responses stimulated by small doses of estrogen are inhibited by large ones. This is true of embryo implantation[135] and mammary growth in mice[136]. In vitro, high doses of estrogen inhibit various enzymes including the estrogen-sensitive human placental transhydrogenase[137]. Low doses of estrogen accelerate mammary tumor appearance in mice with pituitary isografts but high doses retard[138]. Quite low doses of estrogen inhibit growth of estrogen-sensitive rat mammary tumors[139,140]; massive doses induce regression of human mammary tumors[141], but have unpleasant side effects. Apparently the therapeutic usefulness of lower doses has not been investigated.

Where the same end-point can be achieved by agonism or antagonism, and where a compound is both partial agonist and partial antagonist, one must be cautious in attributing any result to one or other mode of action. Reversing the effect of a putative anti-estrogen by estrogen indicates an antagonist origin; failure to do so does not necessarily indicate an estrogen origin - the underlying antagonism may not involve competition. For compounds like Tamoxifen the questions remain. Do they inhibit breast cancer growth because they are estrogenic, because they are anti-estrogenic or because they possess

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DISCUSSION

CUNHA: Since the effects of steroidal and nonsteroidal estrogens on the developing reproductive tract have become appreciated in terms of teratogenic and possible carcinogenic actions, it is important to understand the normal development of the urogenital system. Tissue recombination studies in my laboratory have demonstrated that morphogenesis and differentiation of Mullerian epithelia within the female reproductive tract are induced by the subadjacent mesenchyme or stroma; that is, uterine epithelium differentiates as such because of the inductive properties of uterine stroma which induces and specifies this particular developmental course within the epithelium. This general concept applies to the development of both the male and female reproductive systems. One feature unique to reproductive organs is the expression of hormonal responsiveness mediated by highly specific hormone receptors for estrogens, androgens, and/or progestins. Utilizing tissue separation and recombination techniques, coupled with steroid autoradiography, we have been studying the development of nuclear androgen-binding sites. Tissue recombinations (UGM + BLE) composed of mesenchyme (UGM) of the embryonic urogenital sinus (a prostatic indicator) combined with epithelia (BLE) of embryonic urinary bladder differentiate as prostate-like glands. The presence of putative secretory products within the induced prostatic acini suggests that the inductive influence of urogenital sinus mesenchyme upon the bladder epithelia involves both morphological as well as biochemical alterations. This has been confirmed by autoradiographic localization of ^{3}H-dehydrotestosterone (^{3}H-DHT). Male hosts bearing UGM + BLE recombinants were grown for 30 days, castrated 24 hrs before injection of ^{3}H-DHT, and autoradiograms prepared by the methods of Stumpf and Sar. Epithelial cells of the host's prostate exhibited the characteristic nuclear uptake of label indicative of an androgen-target tissue. By contrast, epithelial cells of the host's urinary bladder did not concentrate ^{3}H-DHT over their nuclei. However, when embryonic bladder epithelia is induced by UGM to form prostate, the epithelial cells of the induced acini concentrate ^{3}H-DHT over their nuclei, a feature indicative of nuclear androgen binding sites. Therefore, the expression of nuclear

MARTIN: I can only agree with you about the importance of the mesenchyme in determining the type of epithelial response elicited by hormonal stimulation. We have long been aware that uterine epithelial proliferation may be regulated by changes in the state of the stroma, these in turn perhaps being regulated by changes in the vasculature. For example, we have some evidence from separated uterine tissues that the premature death of epithelial cells which follows estriol administration, results from a deficiency in stromal, rather than epithelial estriol levels. The changes in epithelial and stromal proliferation, induced in vivo by various estrogen-progesterone regimes, exhibit a striking reciprocity: as stromal mitosis increases, epithelial mitosis decreases, and vice versa. The death of epithelial cells in the antimesometrial lumen of the mouse uterus that occurs during decidualization is limited to the region where the underlying stromal fibroblasts differentiate into epithelioid decidual cells, and does not occur until the latter process is well advanced. All of these examples are suggestive. To prove stromal dependence is an entirely different kettle of fish, because none of these phenomena can be induced in vitro, even in the intact organ, let alone separated tissues. So one cannot, as yet, do anything resembling your own elegant crossover and recombination experiments.

LEROY: You said that glands and surface epithelia are totally different organs, but during the estrus cycle there is, in late estrus, a tremendous amount of luminal epithelial cells dying, while the glands are proliferating. How do you visualize the proliferative relationship between both compartments in this light? Do they evolve in complete independence or is there migration of cells from one compartment to the other? I would also like to know if you can obtain epithelial hyperplasia with chronic high doses of estriol although this latter is considered a weak estrogen?

MARTIN: I wanted to make the point that the two tissues behave as two distinct and different populations. I agree one cannot exclude the possibility of some flow from glands to lumen, particularly in the circumstances you describe. We have examined sections of mouse uteri over the first 3 days of pregnancy when gland cells are proliferating and luminal cells are dying; and while we lack precise data, it seems that the luminal population shrinks while the gland population expands. The tissues and the boundary between them remain distinct, the gland population later shrinking as cells die in situ. However, in circumstances where large chunks of endometrium are shed, as for example, after decidualization, or where the luminal cells are killed or removed en masse then, almost certainly, the surface epithelium could regenerate from the glandular epithelium as in the human endometrium. Regarding your second question, we can generate hyperplasia of the luminal epithelium with continuous estriol treatment, in the sense that cell numbers increase 4- to 5-fold over several days. We have not yet gone on long enough to see whether we can also generate the glandular hyperplasias, although experiments are planned. I would expect estriol to be effective in this regard also; there are now many groups who have shown that in conditions of continuous administration, estriol is capable of everything that estradiol does.

McLachlan, ed. Estrogens in the Environment

DISSOCIATION OF BIOLOGICAL ACTIVITIES IN METABOLITES OF ESTRADIOL

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The in vivo biological activity of any material represents the sum of the actions of the parent substance and of the products of its metabolism. The concept that the metabolism of an endogenous material represents solely a mechanism for its deactivation and disposition is now accepted as being largely invalid. Numerous examples exist where the biological potency of a substance is exceeded by one or more of its metabolites and in the case of hormones several have now been relegated to the status of prohormone.[1,2] While the notion that metabolic transformation can enhance the potency of an endogenous substance is a familiar one, the idea of metabolism modulating the qualitative nature of a biological response is more novel. In this contribution we wish to present evidence that the metabolism of estradiol controls the nature of the actions of this hormone and that therefore its biotransformation plays a critical role in its physiology.

The female sex hormone, estradiol, is distinguished by its exceptional potency relative to the other steroidal hormones and also by its very wide spectrum of biological activities which include profound responses in both central and peripheral target sites. The metabolism of estradiol in man and other mammals proceeds almost exclusively by oxidative pathways. An initial transformation is a massive oxidation to estrone which is a reversible reaction.[3] The equilibrium heavily favors the oxidized state, making estrone the central substance in the metabolic scheme of the female sex hormone.[3] The principal routes of metabolism subsequent to the oxidation to estrone are hydroxylations taking place at the terminal five carbon D ring or in the aromatic A ring (Figure 1). Hydroxylation in ring D is predominantly at the 16α position leading to 16α-hydroxyestrone which is then in part further transformed to estriol one of the major end products of estradiol metabolism. The epimeric product 16β-hydroxyestrone and the ensuing 16β-estriol are also formed, albeit in much lesser quantities.[4] A particularly intriguing product of ring D hydroxylation is 15α-hydroxyestriol or estetrol which is derived by successive 15α and 16α hydroxylations.[5, 6] This tetrol is the dominant metabolite of estradiol in the human fetus and neonate but it virtually dis-

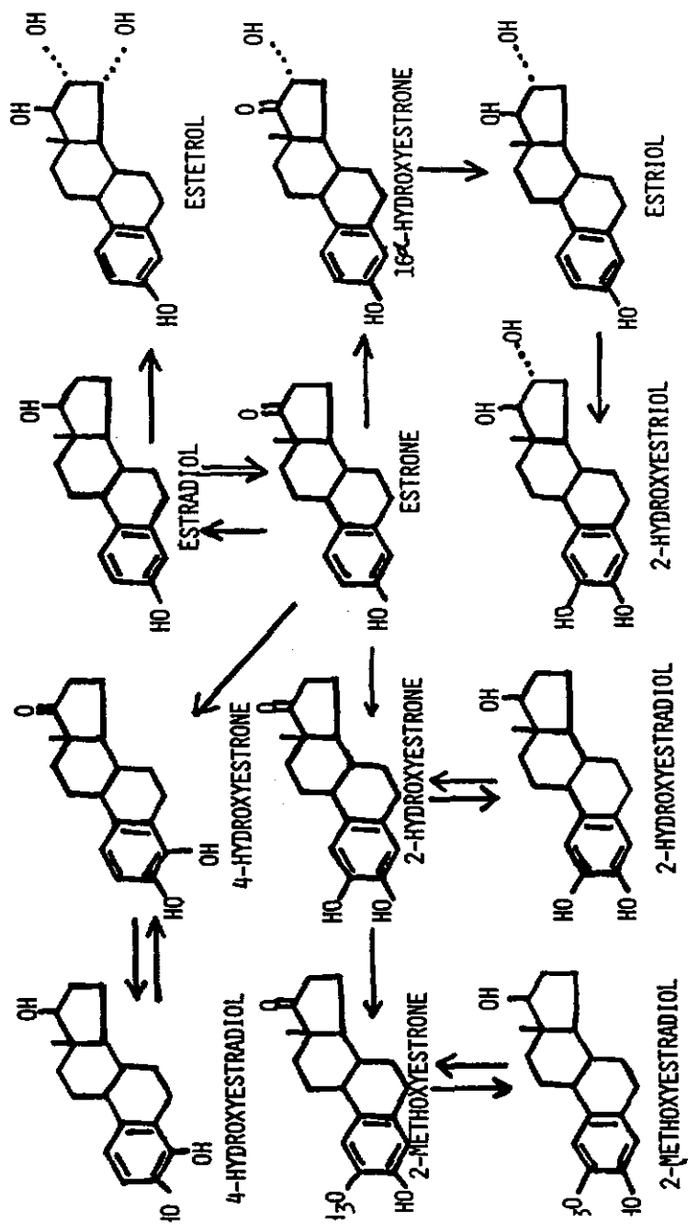

FIGURE 1

the C-2 position giving rise to the catechol estrogen, 2-hydroxyestrone.[7] Hydroxylation at the other possible ortho site gives rise to 4-hydroxyestrone,[8] a quantitatively lesser catechol estrogen with biological characteristics which are quite different from the preponderant 2-hydroxy isomer. The catechol estrogens are also found as the 17β-hydroxy derivatives, 2-hydroxyestradiol and 4-hydroxyestradiol[9, 10] which are mainly the products of the enzymatic reduction of the corresponding 17-keto catechol estrogens. In these cases also the equilibrium heavily favors the 17-keto derivatives. A product of both D and A ring hydroxylations, 2-hydroxyestriol is also present[11] but in a quantity such as to suggest that the two hydroxylating sites are largely mutually exclusive. The majority of the 2-hydroxyestrogens is excreted intact in the form of conjugates, but a considerable fraction is further metabolized to 2-methoxyestrogens.[12, 13] The O-methylation is accomplished by the enzyme catechol-O-methyl transferase[14] and is remarkably specific for the 2-position with very little of the 3-O-methylated product formed *in vivo*.

Even though a number of other metabolites of estradiol have been identified the products of the above metabolic pathways account for 90% or more of the total estradiol metabolism. For a long time studies of estradiol metabolism focused almost entirely on estrone, estradiol and estriol since these were considered to be the only quantitatively significant natural estrogens. The catechol estrogens because of their exceptional lability were largely destroyed during routine body fluid processing and their quantitative significance was not appreciated. The development of procedures such as highly specific radioimmunoassays requiring little separation[15, 16] and radioenzymatic methods which utilized stable intermediates[17, 18] as well as the elaboration of protective conditions for the preservation of the catechol estrogens during workup[16] finally permitted their quantitation and confirmed their role as the major metabolites of estradiol in the human. The content of the various metabolites of estradiol under discussion in human urine is presented in Table 1. The known plasma concentrations of the same estrogens as the free compounds are listed in Table 2. It is clear from inspection of the urinary data that the 2-hydroxylated estrogens exceed considerably the 16-hydroxylated derivatives and that the former is therefore the dominant metabolic fate of estradiol in man. In plasma the levels of 2-hydroxyestrone approximate those of estradiol and estrone and far exceed those of estriol.

Evaluation of estrogenic activity has been largely based on the induction of uterine weight gain in rodents deprived of endogenous estrogens by

TABLE 1

URINARY CONCENTRATION OF MAJOR ESTROGENS

	Women		Men
	Follicular	Luteal	
Estrone	9.8[a]	10.1[a]	3.1[c]
Estradiol	7.3[a]	6.7[a]	2.7[c]
Estriol	11.1[a]	13.0[a]	2.9[c]
2-Hydroxyestrone	18.2[b]	22.5[b]	9.7[b]
2-Methoxyestrone	14.2[b]	16.4[b]	12.9[b]

All values are expressed as mean µg/24 hours.
[a] See Reference 62.
[b] Ball, P., Reu, G., Schwab, J. and Knuppen, R. (1979) Steroids, 33, 563-576.
[c] Unpublished observations.

TABLE 2

PLASMA CONCENTRATION OF MAJOR ESTROGENS

	Women		Men
	Follicular	Luteal	
Estrone	76[a]	81[a]	43[c]
Estradiol	134[a]	136[a]	33[c]
Estriol	20[a]	25[a]	<10[c]
2-Hydroxyestrone	62[b]	71[b]	48[b]

All values are expressed as mean pg/ml.
[a] Fishman, J., Fukushima, D., O'Connor, J., Rosenfeld, R.S., Lynch, H.T., Lynch, J.F., Guirgis, H., and Maloney, K. (1978) Cancer Research, 38, 4006-4011.
[b] Ball, P., Emons, G., Haupt, O., Hoppen, H.-O., and Knuppen, R. (1978) Steroids, 31, 249-258.
[c] Unpublished observations.

castration or by immaturity. This test has served as the principal yardstick for establishing estrogenic potency in the extensive series of natural as well as synthetic estrogens.[19] In view of the large number of different biological responses induced by estrogens it is somewhat unfortunate that their physiologic relevance has been judged by this sole criterion. In addition, recently it has become apparent that even this pharmacological end

point can be incorrectly interpreted. The uterotropic evaluation of estradiol and its metabolites estrone and estriol indicated that estradiol was the most potent uterotropic natural estrogen, estrone was less potent but qualitatively identical to estradiol but estriol which induced only a fragmentary uterotropic response[20] was classified as an estrogen antagonist or an "impeded" estrogen.[21] Furthermore, because estriol failed to induce breast tumors in susceptible rodent strains and appeared to block such induction by estradiol and estrone it was considered to be a safe estrogen.[22] Indeed, its preeminence in pregnancy was then offered as the reason why an early pregnancy reduced the risk for breast cancer. Recent studies[23-25] showed, however, that these views of estriol were incorrect and that the anomalous pharmacological response to estriol was due to the short residence time of the receptor-estriol complex in the nucleus. When the substance was administered to the test animals not as a single injection but in a continuous mode its uterotropic response is indistinguishable from that elicited by estradiol.[26] Similarly administered estriol has now also been shown to be an inducer of breast tumors in rodents.[27] Since endogenous estrogens are secreted continuously the chronic administration mode approximates the in vivo situation far better than that of a single or even infrequently repeated injections. Endogenous estriol, therefore, must now be considered to be a potent estrogen agonist equivalent to estrone and estradiol.

The demonstration that the administration schedule can have profound effect on the uterotropic end point of a natural estrogen prompted us to reevaluate the uterotropic activity of the principal metabolites of estradiol under conditions of chronic administration.[28] The test substances were contained in subcutaneously implanted osmotic pumps which allowed for delivery at a constant rate of a selected amount of the estrogen to oophorectomized rats for a period of 3 days or longer. The increase in uterine weight gain was monitored at 24, 48 and 72 hour intervals. The result of these assays are presented in Table 3, where the wet uterine weights of the test animals are given as percent of the uterine weights of control animals. The relative dried uterine weights corresponded to the wet weights in each instance indicating that genuine tissue hyperplasia and not solely water inbibition was involved. It is clear that under these conditions of continuous administration the ring D hydroxylated metabolites, 16α-hydroxyestrone and estriol are highly active uterotropic agents and are equivalent

TABLE 3

UTEROTROPIC EFFECT OF ENDOGENOUS ESTROGEN ADMINISTERED CONTINUOUSLY

	Hours After Implantation		
	24	48	72
	(% of Control)		
Estrone	165	363	490
Estradiol	177	374	506
16α-Hydroxyestrone	155	365	474
Estriol	160	352	468
4-Hydroxyestrone	192	315	351
2-Hydroxyestriol	125	302	302
2-Hydroxyestradiol	233	288	285
4-Methoxyestradiol	139	194	260
4-Methoxyestrone	127	225	338
2-Hydroxyestrone	98	124	130
2-Methoxyestradiol	109	118	101
2-Methoxyestrone	108	105	105

Rats were implanted with osmotic pumps containing propylene glycol with 1 mg/ml ascorbic acid and 1 mg/ml of the various estrogens tested (1 µg/hr). Groups of animals were sacrificed at the indicated time intervals. Uteri were removed, blotted, and weighed. Each value is the mean of 4 animals per time point. The standard errors of the mean were 10% or less.

to estradiol at the dosage employed. In contrast, the major ring A hydroxylated compound, 2-hydroxyestrone, exhibits only negligible uterotropic activity and its metabolite 2-methoxyestrone is also similarly inert. The isomeric 4-hydroxyestrone as well as its 4-O-methyl derivative have considerable uterotropic activity, albeit very much lower than that of the 16α-hydroxy compounds. This difference in the uterotropic activity of the 2-hydroxy and 4-hydroxy derivatives emphasizes that the catechol estrogens cannot be considered to be biologically homogenous and that a distinction in the physiological role of the 2,3 and 3,4 ortho catechol estrogens must be made. Even though 2-hydroxyestrone exhibits only minimal activity, the 17β-hydroxy derivative, 2-hydroxyestradiol, did provoke a uterotropic response but it was of an anomalous nature. After inducing rapid uterine growth in the first 24 hours the metabolite did not elicit any further significant weight increases during the next 48 hours despite continuing administration. The other active compounds showed a cumulative uterine stimulation during

the entire 72 hour duration of the test. The reason and significance of the unusual uterine growth pattern induced by 2-hydroxyestradiol is presently unknown. Interestingly, 2-hydroxyestriol, the product of both 2- and 16-hydroxylations is of comparable estrogenic potency to 2-hydroxyestradiol but also exhibits an unusual time course in its response. The initial response to this metabolite is slower reaching a maximum at 48 hours but there is no further stimulation observed at 72 hours. Thus the 2-hydroxylated derivatives possess either no uterotropic activity or if they do it is of a nature different from that of estradiol and the other potent estrogens, estrone, estriol and 16α-hydroxyestrone.

The fetal and neonatal metabolite 15α-hydroxyestriol (estetrol) under the conditions of the study proved to be a rather ineffective estrogen inducing little uterine growth. As such, it is therefore a more suitable candidate than estriol for the role of the protective estrogen of pregnancy which could reduce the risk of subsequent breast cancer. It is possible to speculate that the presence of this metabolic pathway in the fetus reflects the need of the developing organism for the deactivation of the potent estrogens by either ring D or ring A hydroxylation, an aim which can be achieved only in the case of the former by double hydroxylation at 15 and 16.

In any *in vivo* pharmacological evaluation there is concern about the contribution of metabolism to the observed activity of the test substance. In the case of the compounds in Table 3 such considerations may apply to estrone whose activity may reflect its conversion *in vivo* to estradiol. Similarly 16α-hydroxyestrone activity may result from its transformation to estriol. The observed activity of the major metabolites estriol and 2-hydroxyestrone, however, is not subject to these considerations. Estriol is minimally metabolized except by conjugation[29,30] while 2-hydroxyestrone is only partially transformed to the equally inactive 2-methoxyestrone.[12,13] The very small transformation to 2-hydroxyestradiol may be responsible for the very minimal uterotropic effect of 2-hydroxyestrone.

The subcellular mechanisms of steroid hormone action requires binding to a specific cytosolic receptor in the target tissue as a prerequisite for the expression of estrogenic action. The affinity for such a receptor, however, does not ensure that the substance will exhibit biological activity. Table 4 contains the relative binding affinities of the metabolites of estradiol for the cytosolic estradiol receptor of the rat uterus. In general,

TABLE 4

RELATIVE BINDING AFFINITIES (RBA) OF ENDOGENOUS ESTROGENS FOR THE RAT UTERINE CYTOSOL RECEPTOR

Compound	RBA	S.D. or Range	n
Estradiol-17β	100		
4-Hydroxyestradiol-17β	45	±12	(5)
2-Hydroxyestradiol-17β	24	± 7	(7)
Estrone	11	± 8	(5)
4-Hydroxyestrone	11	± 4	(3)
Estriol	10	± 4	(5)
16α-Hydroxyestrone	2.8	± 1.0	(3)
2-Hydroxyestrone	1.9	± 0.8	(3)
4-Methoxyestradiol-17β	1.3	± 0.2	(2)
4-Hydroxyestradiol-3-Methyl ether	0.6	± 0.3	(2)
15α-Hydroxyestriol (Estetrol)	0.5	± 0.2	(3)
4-Methoxyestrone	0.13	± 0.04	(2)
2-Methoxyestradiol-17β	0.05	± 0.04	(2)
2-Methoxyestrone	0.01		(2)

the binding affinities correlate with the uterotropic potency of these estrogens with several notable exceptions. The receptor binding of 2-hydroxyestradiol exceeds that of estrone, estriol, and 16α-hydroxyestrone but the reverse is true of their uterotropic effects. Similarly, the significant binding of 2-hydroxyestrone is out of line with its inert nature insofar as uterine weight gain is concerned. This finding suggested that 2-hydroxyestrone may function as an endogenous anti-estrogen in that it occupies the receptor but fails to be processed for the expression of biological activity. In initial studies,[28] however, when 2-hydroxyestrone was coadministered with the estriol or estradiol the catechol estrogen failed to reduce the uterotropic impact of estradiol or estriol. Therefore, under conditions of equivalent concentration the substance does not play an anti-estrogenic role at the uterine target site.

Factors which contribute to the biological activity of an estrogen are not only its affinity for the receptor but also its availability for such binding. In the human, testosterone-estradiol binding globulin (TEBG) which is present in plasma[32] is thought to play a role in the control of

the biological availability of circulating gonadal hormone. We therefore measured the binding affinity of the various natural estrogens for this carrier protein. The results of these measurements are presented in Table 5 which lists the affinities of the different estrogens relative to estradiol.[33] An interesting feature of these results is the high affinity for TEBG of the 2-methoxy metabolites. They not only exceed that of their 2-hydroxy precursor but are even more effectively bound than estradiol. The physiological significance of this binding of the non-uterotropic natural estrogens to TEBG is at present elusive. In contrast the isomeric and more potent 4-methoxyestrogens are relatively poor ligands for the carrier protein. A more important result which emerges from these binding studies is the remarkable low binding affinity of the potent ring D hydroxylated estrogens, estriol and 16α-hydroxyestrone. This implies that in the human their activity can be expected to be expressed out of proportion to their relative concentration in plasma, since a much greater fraction of their circulating content will be free and available compared to the other active estrogens, estradiol and estrone.

TABLE 5

RELATIVE BINDING AFFINITIES (RBAs) OF TESTOSTERONE AND ENDOGENOUS ESTROGENS FOR HUMAN TESTOSTERONE-ESTRADIOL BINDING GLOBULIN (TEBG)

Competitor	RBA
Testosterone	460
2-Methoxyestradiol	130
2-Methoxyestrone	120
Estradiol-17β	100
4-Hydroxyestrone	35
2-Hydroxyestradiol	28
Estrone	20
4-Methoxyestrone	12
4-Methoxyestradiol	9
2-Hydroxyestrone	8
2-Methoxyestriol	4
Estriol	3
15α-Hydroxyestriol	1
16α-Hydroxyestrone	<0.5

The discussion up till now has focused on the peripheral activity of the estrogen as exemplified in their impact on the uterus. Estrogens, however, have also profound central activities ranging from regulation of pituitary hormone release[34] to the control of sexual[35] and feeding behavior.[36] Heretofore, it has been implicity assumed that the uterotropic potency reflected also the relative central activity of an estrogen. The demonstration, however, that the brain of both the rat and human is capable of 2-hydroxylation of estrogens,[37, 38] suggested that the product of this transformation, 2-hydroxyestrone, may have central actions even though it fails to exhibit uterotropic activity. The finding that the catechol estrogens, inhibited enzymes involved in the metabolism[39] and biosynthesis[40] of catecholamine neurotransmitters provided support for this concept. We therefore sought to measure the impact of continuously administered 2-hydroxyestrone on the plasma LH concentration of oophorectomized rats and compare it with the effects of the other natural estrogens.[41] The result of such tests are presented in Table 6. All of the potent uterotropic substances such as estradiol, estriol and 16α-hydroxyestrone produced a pronounced suppression of LH content with the latter being apparently the most effective. An ineffective uterotropic agent such as 2-methoxyestrone had no effect on the plasma LH concentration. The limited uterotropic activity of 2-hydroxyestradiol and also 4-hydroxyestrone was paralleled by the short term effects of these substances on plasma LH, with the suppression apparently ceasing after 48 hours. Most noteworthy, however, was the increase in plasma LH concentration induced by 2-hydroxyestrone. This material augmented the already high tonic levels of LH in the ovariectomized animals by a statistically significant 40% at 72 hours. This result provides evidence that the 2-hydroxyestrone which is inactive at the uterine target site has central effects and may be involved in the positive feedback of estradiol on pituitary gonadotropin secretion. This metabolite is therefore the first estrogen either natural or synthetic in which a separation of central and peripheral activity has been demonstrated and illustrates that estrogenicity should not be characterized solely in terms of uterotropic function.

An important consequence of the differential biological properties of estradiol metabolites is that the metabolism of the hormone in man has a major role in the modulation of its activity. Hydroxylation in ring D leads to compounds which retain or even exceed the uterotropic activity of

TABLE 6

EFFECT OF CONTINUOUS ESTROGEN ON SERUM LH IN OVARIECTOMIZED RATS

	Hours After Implantation		
	24	48	72
	(% of Control)		
2-Hydroxyestrone	110	134	142
4-Methoxyestrone	88	65	92
2-Hydroxyestradiol	35	31	61
4-Hydroxyestrone	21	28	50
16α-Hydroxyestrone	24	8	7
Estriol	10	8	18
Estradiol	12	12	19

estradiol and therefore this metabolic pathway serves to continue and extend this action of the female hormone. In contrast the competing A ring hydroxylation at C-2 leads to 2-hydroxyestrone and 2-methoxyestrone, both substances devoid of uterotropic activity and therefore this metabolic route terminates this particular biological role of the hormone.

The evidence that 2-hydroxyestrone possesses central activity renders the role of metabolism even more important in the expression of hormonal action. Not only does a metabolic pathway have the potential to alter the quantitative aspects of estradiol action but it also modulates its qualitative nature since 2-hydroxylation which terminates the peripheral activity of the hormone apparently initiates specific central estrogenic actions. It must be cautioned that at present it is uncertain whether the 2-hydroxyestrone formed peripherally and transported to the central system duplicates the function of the same material synthesized *in situ*.[42, 43]

The physiological significance of the different biological actions of the products of the two competing estradiol metabolic pathways can be gauged from changes observed in these pathways in human metabolic studies. A number of pathological situations have now been identified in which the metabolism of estradiol is predictably altered to increase either the 16-hydroxylation or the 2-hydroxylation pathway. Hyperthyroidism, whether endogenous or drug induced is invariably associated with a large increase in 2-hydroxylation of estradiol at the expense of 16α-hydroxylation which is correspondingly decreased.[44] The reverse situation obtains in hypothyroid

individuals who exhibit increased 16α-hydroxylation with a matched decrease in the transformation at C-2. The consequence of these changes is that in the presence of comparable secretion of the hormone, hyperthyroidism can be associated with hypoestrogenicity and hypothyroidism with hyperestrogenic consequences. The reproductive function deficits so frequently present in dysthyroid women can then possibly be related to these alterations in estradiol metabolism.

Another intriguing example of estradiol metabolic changes is the contrast between obese women and the undernourished females with anorexia nervosa. The latter exhibit estradiol metabolism distinguished by greatly increased 2-hydroxylation which is suppressed in the obese.[45,46] These changes in metabolism related to changes in body weight may also be responsible in part for the reproductive disturbances which so often accompany large changes in body weight in women.[47] More importantly, the hyperestrogenic direction of the metabolism of estradiol in the obese may contribute to the increased risk for endometrial cancer in these individuals.[48] Cirrhosis of the liver has been shown to result in a great elevation of 16α-hydroxyestrone formation[49] and the potent estrogenic character of this metabolite may be resonsible for the feminization so often present in men with cirrhosis.[50] A number of other examples of clinical syndromes associated with alterations of estradiol metabolism have been reported[51,52] and it is important to consider these as possible contributors to the symptomology of the disease.

Because of the relationship of estrogens to the etiology and progress of breast cancer much attention has been devoted to a possible role of estrogen metabolism in the epidemiology of this neoplasm. A dominant theme in these studies, derived from the presumed innocuous nature of estriol, was the estriol hypothesis.[53,54] This formulation proposed that a high content of estriol relative to estrone and estradiol was protective of the disease while a low ratio of estriol to estrone plus estradiol increased the risk. Some studies have indicated that women with breast cancer excreted less estriol than those without the disease[55,56] but other investigators failed to confirm this finding.[57,58] Blood production rates of estriol in breast cancer subjects were also found not to differ from controls.[59] Metabolic studies utilizing radiolabelled tracer hormone suggested that men with breast cancer[60] and to a lesser extent women with the disease[61] metabolize estradiol via 16α-hydroxylation to a greater extent than control subjects. This would appear to conform better with the finding of the fully estrogenic

character of estriol and its proximate precursor 16α-hydroxyestrone and the presumed relationship between uterotropic potency and carcinogenicity.

Prospective studies of individuals at risk for the disease failed to disclose a decrease in estriol excretion in either ethnic[61] or familial increased risk categories.[62] Young women who had an early pregnancy and who have therefore a decreased risk for breast cancer were recently reported to have increased urinary estriol excretion but it is uncertain whether this is related to the decreased risk.[63] In view of the accumulated biological and epidemiological evidence it seems prudent to consider estriol and 16α-hydroxyestrone as potent estrogens with all of the oncogenic properties of estradiol. The role of the catechol estrogens in the process is as yet unknown but based on their biological properties they could be expected to be non-participants in cancer induction and maintenance, if these are associated with the uterotropic character of the hormone.

The evidence that the consequences of the metabolism of estradiol include not only modulation of the intensity of the biological response to the hormone but also the very nature of that response adds another level of complexity to the eventual understanding of hormone action. The existence of such modulation needs to be considered in the study not only of the various essential physiological functions of the female sex hormone but also of its considerable real and presumed contributions to the etiology of several pathologies which are currently under investigation.

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DISCUSSION

VILLEE: I was interested in your observation that 2-hydroxyestradiol stimulates uterine growth but only for two days, then the organ weight plateaus. Do you think the 2-hydroxyestradiol induces the synthesis of a 17-hydroxysteroid dehydrogenase specific for 2-hydroxysteroids and that this enzyme converts the 2-hydroxyestradiol to 2-hydroxyestrone, which is inactive?

FISHMAN: Yes, it is possible.

ESTROGEN METABOLISM AND FUNCTION IN VIVO AND IN VITRO

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INTRODUCTION

It has become increasingly clear that steroid hormones are not simply synthesized by endocrine organs and secreted into the plasma in order to be concentrated in and act upon target tissues. Recent investigations have pointed to extensive and variable metabolic alterations in these steroids which occur in peripheral tissues. Extra-endocrine conversions often have a distinct influence on the plasma pattern of steroid hormone metabolites and ultimately on target tissue responses.

The metabolic fate of steroids depends on the age of the animal, its dietary and drug regimen as well as normal fluctuations in the total hormonal milieu. The net result of the interaction of peripheral tissues with steroids has been shown to include the conversion of precursors into active hormones and partial or complete deactivation of the ultimate hormone. An example of peripheral activation of systemic gonadal hormone precursors has been demonstrated by Longcope et al[1] and Schindler et al[2] in studies which established the aromatization of adrenal dehydroepiandrosterone (DHEA) or androstenedione by adipose and muscle tissue. The estrogen thus formed is active systemically and has been implicated in the growth of postmenopausal endometrial cancer[3]. In another example, plasma DHEA has been shown to be converted in situ to the active 5α-dihydrotestosterone by the skin's adnexa[4]. In this latter case the active androgen only stimulates the target tissue in which it has been formed and is apparently not active systemically.

The classical role of peripheral estrogen metabolism has been that of deactivation. Principally the liver has been shown to be active in the oxidation, reduction, hydroxylation and conjugation of estrogens[5,6]. Most tissues, in fact, may oxidize 17β-estradiol (E_2) to the less potent estrone (E_1).

While hepatically derived glucosiduronate conjugates of estrogens are rapidly removed from the plasma and excreted, sulfurylated estrogens persist for a considerable period in the plasma (7½ hrs.[7]). The estrogen sulfates are readily taken-up by target tissues via hydrolysis[8]. Although the ovary secretes an appreciable quantity of estrogens as sulfurylated hormones[9], it appears that the liver is responsible for the persistent level of plasma estrogen sulfates[10].

HEPATIC ESTROGEN METABOLISM IN VITRO AND IN VIVO

Utilizing a rat model it is possible to derive a clearer picture of the role of hepatic tissue in determining the plasma pattern of estrogen metabolites. The in vitro metabolic fate of estrogens in liver has been determined in incubations of minces[5,6]. In these experiments E_1 has been shown to be the dominant product of hepatic metabolism of physiological levels of E_2. Either of these estrogens or their metabolites may be conjugated to sulfuric acid or glucuronic acid[6]. Within the liver, glucosiduronidation is an end product of metabolism destined for excretion. This is not the case, however, for the sulfate esters which appear as dynamic metabolic intermediates and are also actively involved in target tissue uptake.

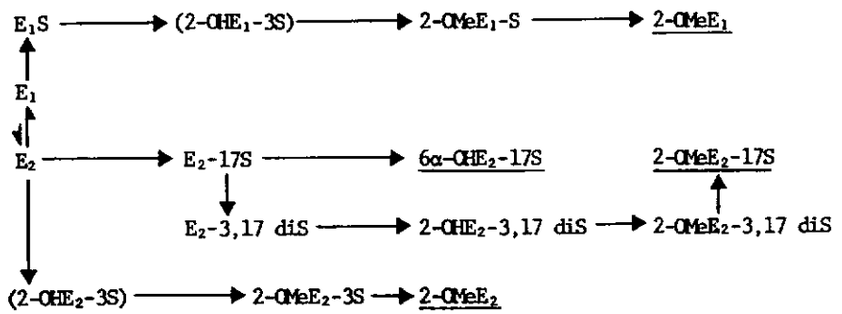

Fig. 1

An examination of the temporal relationships of the sulfurylated estrogen metabolites reveals certain precursor-product implications (Fig. 1 and ref. 5). In these experiments E_1 is sulfurylated at position-3 before hydroxylation (2-OHE_1) and methoxylation (2-OMeE_1) occur at position 2. After the relatively smaller amount of E_2 is sulfurylated at position-17β, a portion is hydroxylated at position-6. However, the majority of the monosulfate is disulfurylated and converted, presumably through the 2-hydroxy-3,17β-disulfate to 2-methoxy-estradiol-17β-sulfate. An even lessor amount of E_2 is monosulfurylated at position-3 and converted to free 2-methoxy-17β-estradiol. This pattern does not change in liver taken from rats in the various stages of the estrous cycle[5]. Hepatic sulfurylation of estrogens is not without influence by systemic conditions. For example, this metabolic process is increased by the available oxygen in vitro[6] and decreased by hypophysectomy in vivo[11].

Although the in vitro hepatic production of these metabolites in the rat has been demonstrated in several laboratories[5,12-14], the in vivo fate of this pattern of estrogens and their conjugates has not been well documented. In order to better understand the liver's effect on estrogen homeostasis, it is necessary to study factors other than the in vitro hepatic metabolism of estrogens. A more complete picture of the hepatic influence on the make-up of plasma estrogens requires that the fate of these compounds be studied in the enterohepatic circulation and answers be sought regarding the influence of the entire hepatic system on circulating estrogens and their conjugates.

Analysis of biliary estrogen metabolites. Following I.V. injection of 0.2 µg (7.7×10^7 dpm) of $^3HE_2$, 1.06% of the radioactivity was secreted into the cannulated bile duct within the first 3 hrs (Fig. 2). Cannulation of the duct in these experiments did not allow recycling of labeled estrogen metabolites through the enterohepatic circulation. Therefore, the decrease in biliary radioactivity over the initial 2 hr period represents, for the most part, rapid

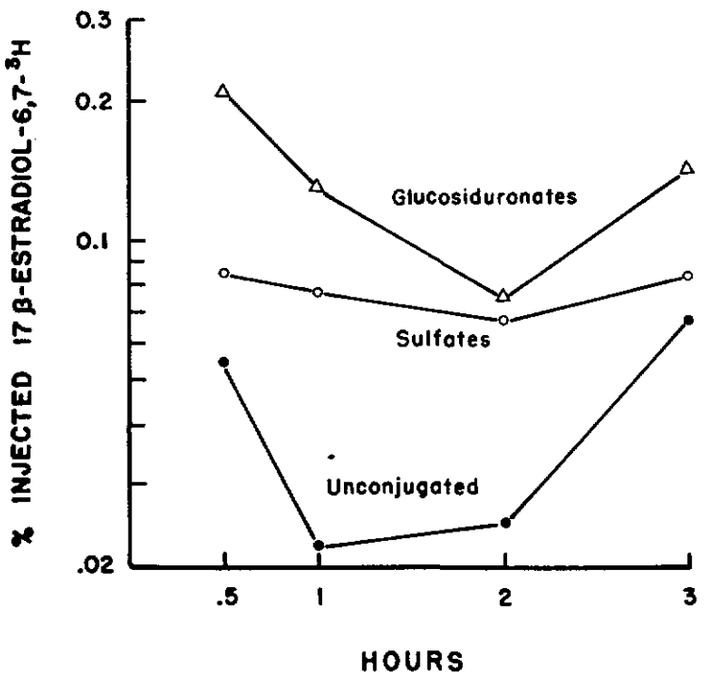

Fig 2. Secretion of labeled biliary estrogen metabolites following the injection of tritiated E_2 (0 hr). The bile flow was 1.2 ml/hr.

conjugation of circulating $^{3}HE_2$. Most of the biliary estrogens were isolated as glucosiduronates with a major portion being secreted as sulfates and a lesser amount as unconjugated estrogen metabolites. Noteworthy is the fact that during the 3rd hr following administration of $^{3}HE_2$ there was a marked increase in biliary radioactivity. This increase was particularly significant in the unconjugated and glucosiduronate fractions.

Analysis of the unconjugated estrogens on ITLC-SA and GLC[5] has shown this fraction to be made up of E_2, E_1 and 2-OMeE_1. The sequential appearance of these free estrogens in the bile over the three hour period is indicative of the established hepatic oxidation of E_2 to E_1 followed by conversion of E_1 to 2-OMeE_1.

Examination of the tritiated glucosiduronates recovered from bile showed 4 major peaks on DEAE-Sephadex chromatography[15] (Fig 3). Hydrolysis of the conjugates in each peak utilizing ketodase revealed the presence of E_1, 2-OMeE_1, 2-OMeE_2 and two very polar ^{3}H-compounds with characteristics of carboxylic acids

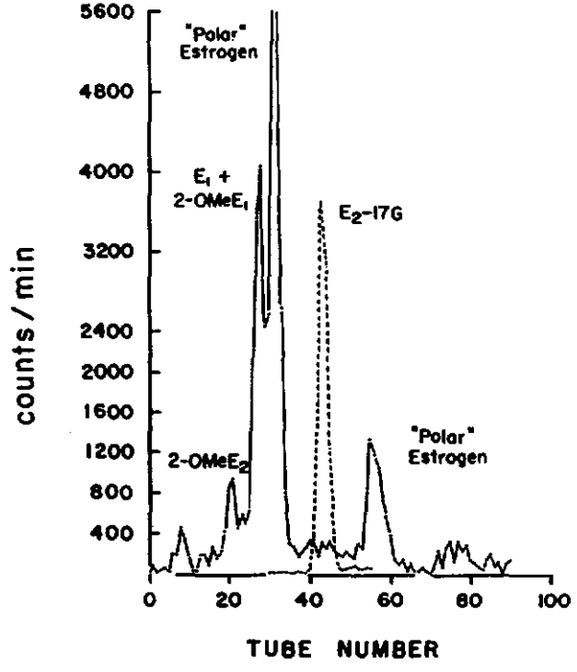

Fig 3. DEAE-Sephadex chromatography of labeled biliary estrogen glucosiduronates 2 hrs following injection of tritiated E_2 (——). Dashed line (----) depicts position of standard $^{3}HE_2$-17β-glucosiduronate. Elution was carried out with a linear NaCl gradient (0.05 - 2.0M).

after solution in 1.6% NaOH.* Again the increased biliary glucosiduronate fraction, 3 hrs after administration of $^{3}HE_2$, was the result of the higher levels of 2-OMeE_1 and 2-OMeE_2 conjugates. The major polar peak had the characteristics of a 2-hydroxy-estrogen with the A-ring ruptured following exposure to alkali. The carbon-14 methyl ester prepared from this peak with ^{14}C-diazomethane traveled on ITLC-SA with the methyl ester of authentic 2-OHE_1 synthesized following rupture with 1.6% NaOH (Fig 4).

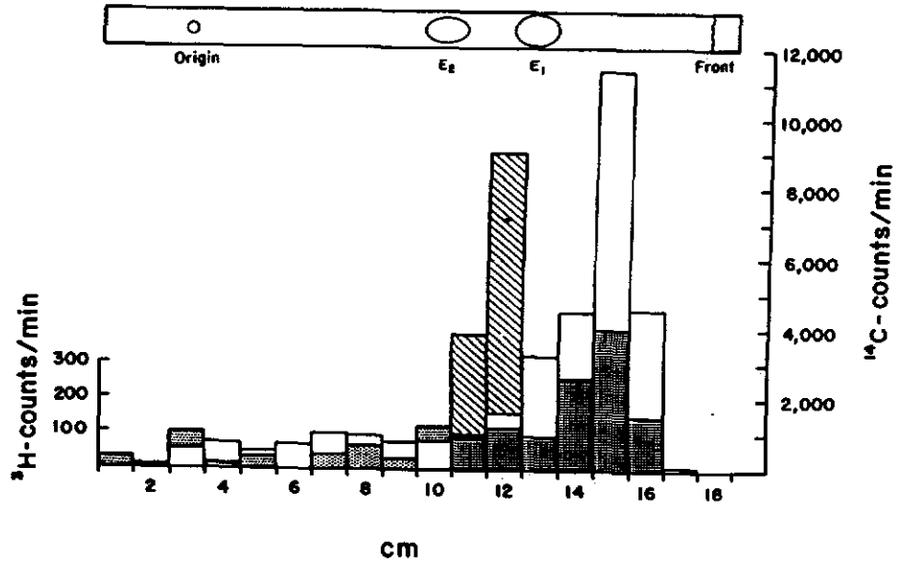

Fig 4. ITLC-SA of the major "polar" peak of tritiated estrogen biliary glucosiduronates. The compound has been exposed to alkali (1.6% NaOH) and extracted under acidic conditions before esterification with ^{14}C-diazomethane. (Peak B □ ^{3}H cpm; ▦ - ^{14}C cpm). Peak A is the chromatographed product of 2-OHE_2 treated similarily (▨ - ^{14}C cpm). The migration of E_2 and E_1 in this system ($CHCl_3$: MEOH, 97:3) is shown above.

The concentration of biliary estrogen sulfates was more consistent over the time period than were the levels of the glucosiduronate and unconjugated fractions. Analysis of the four major sulfate peaks from DEAE-Sephadex chroma-

*The hydrolyzed estrogen metabolites were routinely carried through a modified Brown procedure[5] from which the phenols were extracted from alkaline media (pH 9 bicarbonate buffered 1.6% NaOH) by ether. The carboxylic acids are then extracted following acidification.

tography showed that the sulfurylated biliary estrogens were also made up of at least 2 polar metabolites as well as estrone sulfate (E_1S, Fig 5). Significant peaks of diconjugated estrogens were not found during this time span. One of the polar peaks had the properties of a glutathione conjugate of 2-OHE_2 which had previously been isolated by Kuss[16] and Jellinck[17] from rat liver incubates (Fig 6).

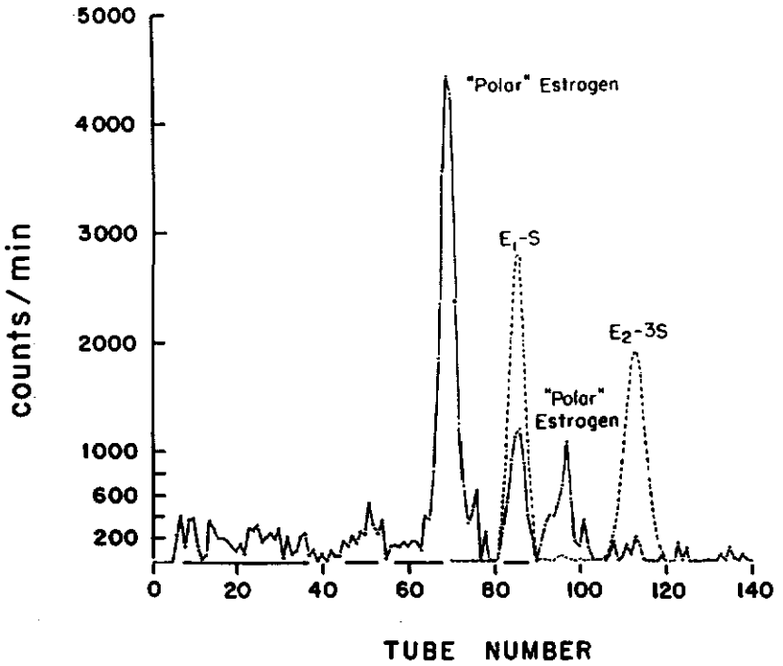

Fig 5. DEAE-Sephadex chromatography of labelled biliary estrogen sulfates ½ hr following injection of $^3HE_2$ (——). Dashed line (----) depicts position of standard E_1-S and E_2-3S. Elution was carried out with a linear NaCl gradient (0.05 - 2.0M).

It therefore appears that in vivo rat liver shares the capacity, demonstrated in in vitro experiments, to synthesize 2-methoxyestrogens and form the sulfate and glucosiduronate conjugates. The 2-hydroxy-estrogens, isolated herein as ruptured A-ring acids, are also formed and conjugated. Present in the biliary sulfates is a polar metabolite with characteristics of the glutathione conjugate seen by others in liver incubations. Curiously absent from the labeled biliary estrogens are the disulfurylated metabolites which were present in the incubation. Also evident from these experiments is the inability of rat liver to form detectable quantities of estriol in vivo and in vitro.

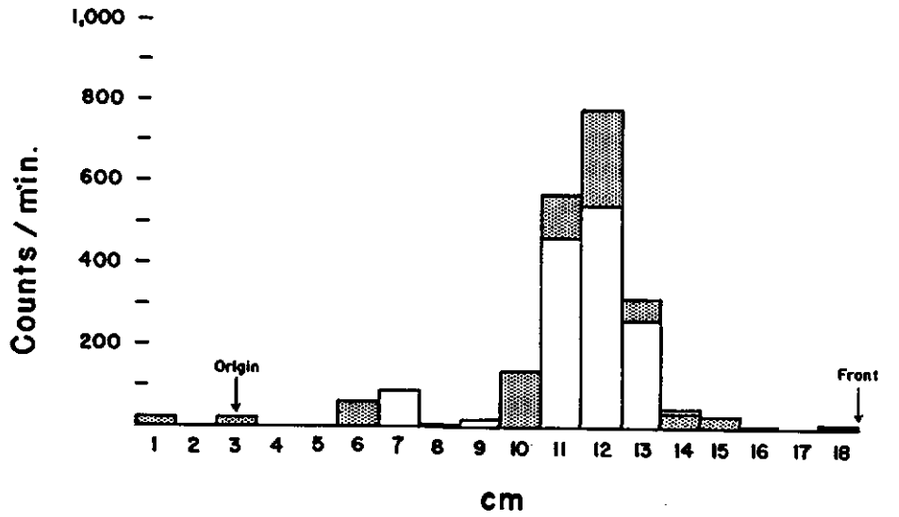

Fig 6. TLC (phenol-H_2O) of the major "polar" peak of tritiated biliary estrogen sulfates following hydrolysis with sulfatase[5] (□). Standard 2-OHE_2-1-S-glutathione-6,7^{3}H (▦) was a gift from Dr. J.S. Elce, Queens Univ., Kingston, Ontario.

Analysis of plasma estrogen metabolites. Germain to the point being discussed is which of these hepatic estrogen metabolites enter the circulation for possible influence on target tissues. The pooled plasma from 2 rats, each of which had been administered $^3HE_2$ (0.14 µg, 6.7 x 10^7 dpm), was assayed for the levels of labeled estrogens at various times (1/2, 1. 2, and 3 hrs) following the injection of labeled compound. E_2 and E_1 were identified by ITLC and GLC in the pooled plasma. Both these estrogens were found unconjugated, as esters of sulfuric acid and as glucosiduronates. Also present, but only in the conjugated fractions, were the polar estrogens identified above as the 2-hydroxy-metabolites. Detectable amounts of labeled 2-methoxyestrogens were not found in the plasma during this time span.

It is of interest to examine the disappearance of these metabolites from the plasma. In doing so we choose to compare the fate of the plasma estrogens in control rats to that of rats which had received (intubated) 5 mg of dimethylbenz-(α)-anthracene (DMBA) 2 months prior. At this level the carcinogen will bring about the appearance of hormone dependent mammary tumors in one-half the

treated Sprague-Dawley rats[18]. Tumors, however, had not yet appeared in these rats.

In the treated and control rats the plasma $^3H_{E_2}$ and $^3H_{E_1}$ displayed a similar rate of disappearance (Fig 7,8). Whereas the unconjugated estrogens and glucosiduronates were each present in like concentrations throughout the 3 hr period, the sulfate conjugates were initially lower in the plasma of the DMBA treated group (Fig 7). Sharp differences were found in the fate of the plasma estrogen sulfates in the carcinogen treated rats. E_1S and E_2-3S were present in much lower concentrations in the DMBA rats (Fig 9). More dramatically, the sulfated "polar" estrogens appeared in the plasma of the treated rats after 1 hr and persisted at appreciable levels until 3 hrs. These metabolites did not appear until 3 hrs in the control plasma. In treated rats the plasma gluco-

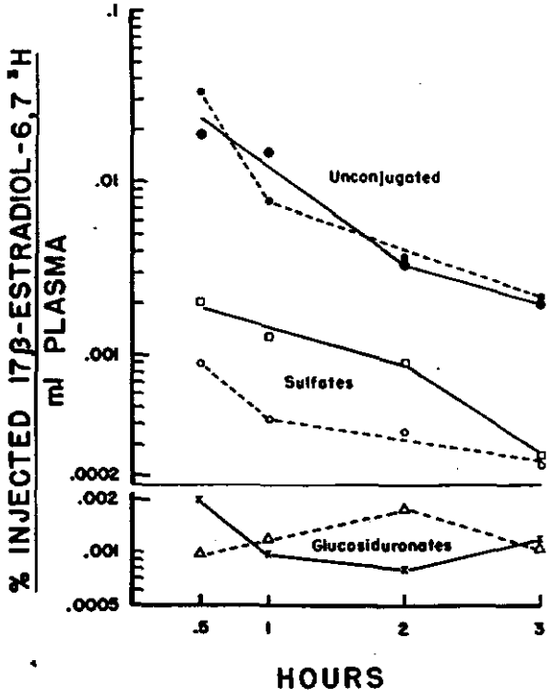

Fig 7. Conjugated state of labeled plasma estrogens following injection of $^3H_{E_2}$. The blood (approx. 12 ml/rat) was removed from each rat by heart puncture. Each point represents the estrogens in the pooled plasma from 2 rats. Solid lines depict estrogens from control rats. Dashed lines indicate estrogens from DMBA-treated rats.

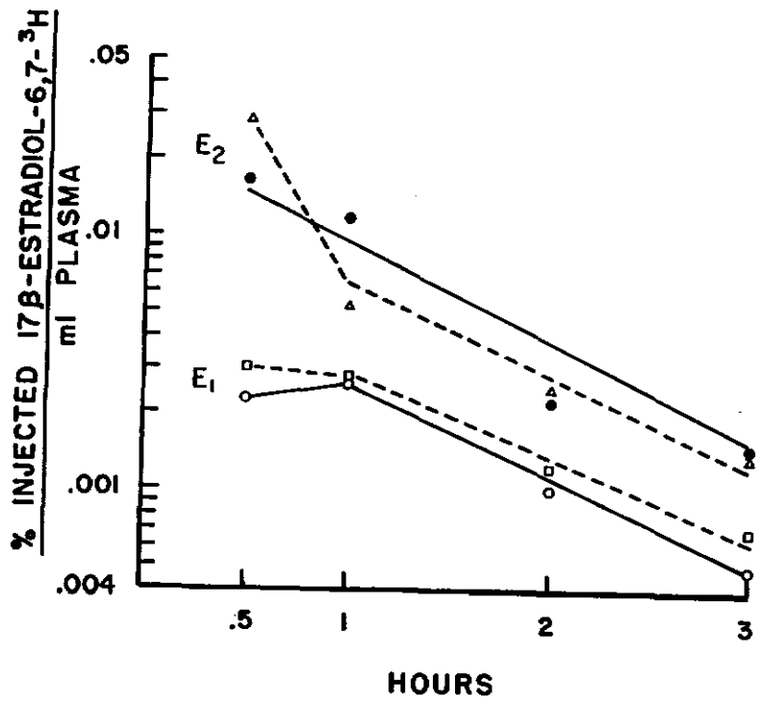

Fig 8. Disappearance of labeled unconjugated estrogens from the plasma of rats following the injection of $^3H_{E_2}$. Solid lines depict estrogens from control rats (2 per point). Dashed lines indicate estrogens from DMBA-treated rats (2 per point). (E_2 = ●, △; E_1 = ○, □).

siduronates contained less $^3H_{E_2}$ and more of the "polar" estrogens (Fig 10).

These experiments placed 140 ng of $^3H_{E_2}$ into the plasma pool of endogenous E_2. Normally the highest secretion rate of rat ovaries is approximately 10 ng/hr[19] or 30 ng over the duration of this experiment. Although above the physiological level, the quantity injected in these experiments would not be considered to be pharmacological, and it would be expected that the labeled E_2 would experience the same metabolic fate as the endogenous E_2. All of the metabolites observed in the bile and plasma are known products of <u>in vitro</u> liver estrogen metabolism. Furthermore, although other tissues have been shown to hydroxylate and/or conjugate estrogens (ovaries[20] adrenals[20], brain[21,22],

and uterus[23]) none but the liver would be expected to carry out the rapid conversions of such large quantities of estrogens as were observed in these experiments. The observed differences in plasma estrogens of DMBA treated rats are the result of metabolic changes of long duration brought about by the intubation of 5 mg of the carcinogen 60 days prior to analysis. The effect on target tissues of these plasma-borne sulfates of polar estrogens remains to be demonstrated.

It appears then that the pattern of plasma estrogen metabolites may be attributed to variations in hepatic estrogen metabolism. Interestingly many of these alterations in the estrogen molecule (principally hydroxylations) are known to be representative of the highly inducible (by carcinogens such as DMBA[24]) microsomal oxidase system (Cyt P_{450}).

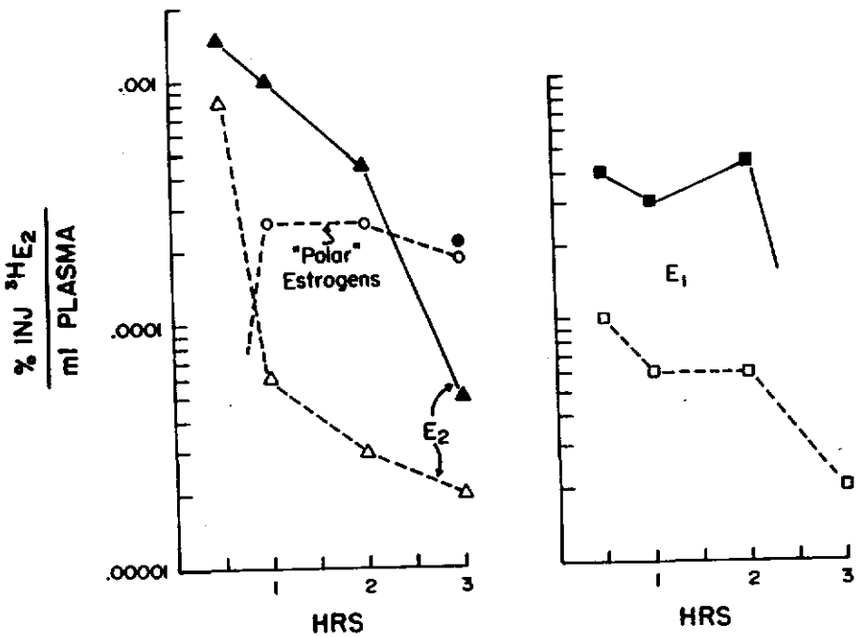

Fig 9. Disappearance of labeled sulfurylated estrogens from the plasma of rats following the injections of $^3H_{E_2}$. Solid lines depict estrogens from control rats. Dashed lines indicate estrogens from DMBA treated rats. The pooled plasma from 2 rats was analyzed for each point. (E_2 = ▲ ,△ ; E_1 = ■ ,□ ; polar estrogens= ● ,○).

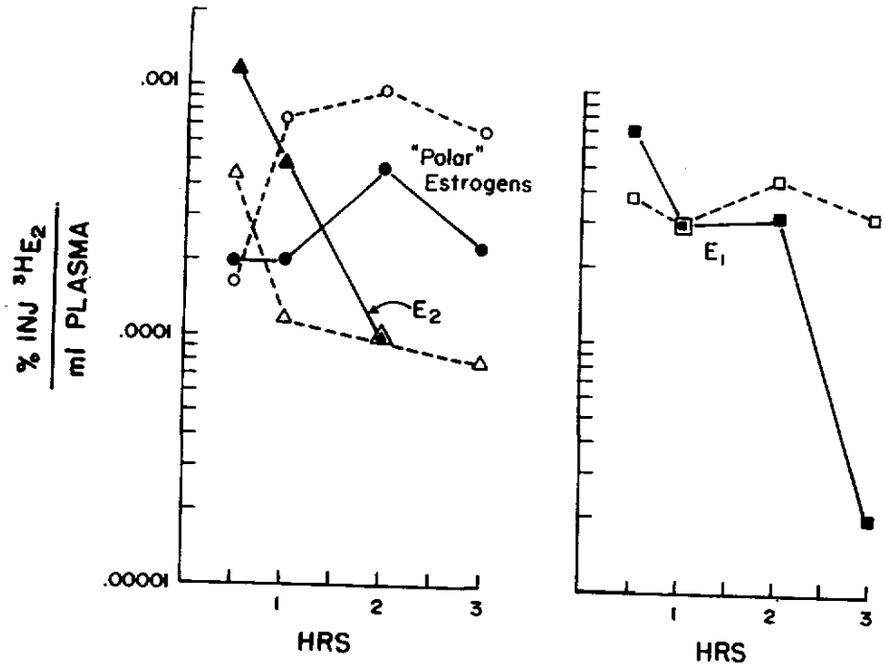

Fig 10. Disappearance of labeled estrogen glucosiduronates from the plasma of rats following the injection of $^3H_{E_2}$. Solid lines depict estrogens from control rats. Dashed lines indicate estrogens from DMBA-treated rats. The pooled plasma from 2 rats was analyzed for each point. (E_2 = ▲ ,△ ; E_1 = ■ ,□ ; polar estrogens = ● ,○).

THE FATE OF ESTROGENS IN TARGET TISSUES

Estrogens sulfurylated on the 3-hydroxyl are readily taken into target tissues such as the uterus[8]. Although hydrolyzed during uptake, these estrogens concentrate in the uterus at twice the rate of unconjugated estrogens. Once within the target cell E_2 shares a metabolic fate in addition to the well documented binding to and nuclear migration with its receptor.

In rat uterine cells the metabolic fate of E_2 is limited to the formation of estrone, and this metabolism is only present in mature uterus at estrus[8]. Higher mammals with longer estrous or menstrual cycles have displayed, in addition to this E_2 dehydrogenase activity, an estrogen sulfotransferase capacity[25-29]. In these tissues estrogen sulfotransferase appears to be induced by

progesterone, appearing only after the initial ovulation in porcine uterus[30] and only in the secretory endometrium of the mature female[25-29]. In the pig prolonged levels of plasma progesterone during implantation are reflected in a continuation of endometrial estrogen sulfurylation[27].

Therefore mature porcine and human uterine tissue have been demonstrated to contain an estrogen receptor[25,31] (E_2R) and, in addition, E_2 dehydrogenase activity[25-29] as well as the estrogen sulfotransferase[25-29]. The significance of these two enzymes in normal cycling uterus is unknown, but their dependence on the mature endocrine environment suggest that they may be important to the target tissue response mechanisms.

If one relates this metabolism of estrogens in target tissues to the nuclear uptake of E_2R complex some interesting correlations emerge. First, considering only the sulfotransferase activity, it becomes clear in both porcine[31] and human[26] endometrium that at the times when estrogens are maximally sulfurylated, there is a decreased level of nuclear receptor complex (Fig 11). In fact, if estrogen sulfurylation is maintained for a week or longer the nuclear E_2R disappears. Since at physiological levels E_2-3S does not bind to receptor[32], one could assume that during high sulfotransferase activity little or no E_2R is taken into the nucleus. The 3½ day half-life of nuclear E_2R thus observed agrees with an earlier report[33]. Significant levels of cytoplasmic E_2R can be demonstrated after sulfurylation begins. Soon thereafter the E_2R concentration decreases presumably since its nuclear induction by the chromatin interaction

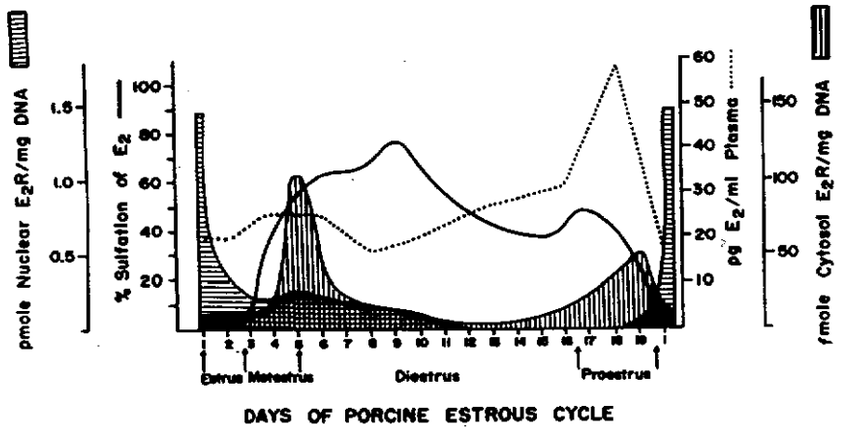

Fig 11. Composite plot showing relationship between gilt plasma E_2 levels (dotted line from ref. 34), uterine estrogen sulfurylation (solid line), cytoplasmic E_2 receptor (vertical striation) and nuclear E_2 receptor (horizontal striation). Taken from a previous publication from this laboratory[31].

of E_2R complex has ceased[31]. All of these changes occur in the presence of significant levels of plasma E_2[31] (Fig 11). This inverse relationship between endometrial estrogen sulfurylation and decreased nuclear uptake of E_2R has also been demonstrated in porcine uterus during and after implantation[27] and in human endometrial curettings[26].

It has been postulated that oxidation of E_2 to E_1 by uterine tissue served the purpose of deactivating the estrogen in situ particularly since E_1 binds receptor less tightly and is therefore less estrogenic[35]. This is unquestionably true; however, there is significant dehydrogenase activity in proliferative porcine and human uteri[25-29]. To this end the apparent increase in E_2 oxidation in secretory tissue is related to the decreased estrogen effects on this tissue. However, it may well be that estrogen sulfotransferase is more directly responsible for the retractiveness of secretory endometrium to E_2.

It has been established that the K_m of estrone in the estrogen sulfotransferase system is significantly lower that that of E_2[36]. The preference for E_1 over E_2 by the uterine sulfotransferase system is also apparent from the data in Table 1. If minces of porcine uteri are incubated for 2 hrs with a physiological level of $^3HE_1$ oxidation of labeled substrate to unconjugated $^3HE_2$ is

TABLE 1

UTERINE METABOLISM OF 17-β-ESTRADIOL[a]

	E_2-3S ← (c)	E_2 ⇄ (b)	E_1 → (c)	E_1-S	Total Oxidation[d]
Porcine Uterus	pmole	pmole	pmole	pmole	pmole
Immature	0	8.7	1.3	0	1.3
Proliferative	0	8.4	1.6	0	1.6
Secretory	1.7	3.3	1.7	3.3	5.0
Human Endometrium					
Proliferative	0	4.5	5.5	0	5.5
Secretory	1.4	1.5	4.2	3.9	8.1

[a] Incubations were carried out for 2 hrs in 2.5 ml Krebs-Ringer bicarbonate buffer containing 10 pmoles $^3HE_2$ and approximately 400 mg tissue.

[b] Estradiol dehydrogenase

[c] Estrogen sulfotransferase

[d] Total oxidation = E_1 + E_1-S

consistent in immature, proliferative and secretory tissue (Table 1). Although the degree of oxidation is greater in the human tissue, the amount of $^3H E_1$ produced is also similar in both the proliferative and secretory endometrium (Table 1). Upon the appearance of estrogen sulfotransferase activity in the secretory endometrium of either species there is preferential conjugation of E_1. It is in this sulfurylated E_1 fraction that the increased conversion of E_2 to E_1 resides. One could assume then, that the selective sulfurylation (removal) of the E_1 produced by the dehydrogenase equilibrium could result in the increased oxidation of E_2 seen in secretory endometrium (see total oxidation column in Table 1).

This concept is supported experimentally, since it has been demonstrated in secretory endometrium that the formation of E_1 reached a plateau within the first hour of incubation and the amount of E_1 formed was constant for the duration of the 2 hr period[25]. Furthermore the ratio of E_1S/E_2-3S was also constant from 60 min on. Total sulfurylation, on the other hand, increased linearly throughout this time period[25]. Therefore while the formation of E_1 in this tissue appears to reach a steady state, sulfurylation continues as long as there is substrate available.

A specific inhibitor of estrogen sulfotransferase would aid in acquiring information concerning the importance of these two enzymes in secretory uterus. Previous data from studies in our laboratory have elucidated an aromatic stacked system for the transition state comprised of E_1 and adenosine-3'-phosphate-5'-phosphosulfate (PAPS) on the sulfotransferase[36]. This knowledge brought about the design of an inhibitor which facilitated the stack through hydrogen bonding between the 6-amino group of adenine and a 4-nitro group on E_1[37]. A 3-methyl ether prevented this analogue from being sulfurylated while blocking the sulfurylation of estrogens.

Incubation of porcine uterine minces with 4-nitro-3-methoxyestrone in the presence of physiological levels of $^3H E_2$ resulted in inhibition of estrogen sulfotranferase (Table 2). Blockage was displayed by a 10^3-fold excess (over $^3H_{E_2}$) of inhibitor but was nearly complete (85% inhibition of sulfurylation) in the presence of a 10^4-fold excess of the analogue. Although a 10-fold excess was all that was required for 4-nitro-3-methoxyestrone to block the enzyme in solution[36], poor tissue permeability of this analogue necessitated higher levels in uterine incubations.

The data in Table 2 show that 4-nitro-3-methoxyestrone did not affect E_2 dehydrogenase activity since estrone made up the same fraction of unconjugated (32-39%) and sulfurylated (65-74%) estrogens whether the inhibitor was present or not. Furthermore neither was uterine steroid alcohol sulfotransferase

TABLE 2

INHIBITION OF PORCINE UTERINE ESTROGEN SULFOTRANSFERASE BY 4-NO_2-3-OMe-ESTRONE

Incubation[a]	Unconjugated Estrogen[c]			Steroid sulfates			
	$E_2 + E_1$	E_1		E_2-3S + E_1S	E_1-S		Total Steroid Sulfates[d]
	pmoles	pmoles	%	pmoles	pmoles	%	pmoles
Control	3.8	1.2	32	6.4	4.2	66	66.3
+ 4-NO_2-3-OMeE_1[b]							
10^3fold	4.5	1.5	34	5.7	4.3	75	75.4
10^4fold	9.2	3.7	40	0.96	0.67	70	68.5

[a] Incubations were carried out for 2 hrs at 37° with 400 mg whole porcine uterine tissue (day 5 of the estrous cycle) in 2.5 ml Krebs-Ringer bicarbonate buffer containing 4 x 10^{-9}M 6,7-^{3}H-estradiol-17β (sp. act. 44 Ci/mmole) and 1 x 10^{-4} $Na_2{}^{35}SO_4$ (sp. act. 554 mCi/mmole).

[b] 4-Nitro-3-methoxyestrone was added to the experimental incubations at two concentrations; one 10^3 times and the other 10^4 times the concentration of the tritiated 17β-estradiol.

[c] Following the incubation, the estrogens were extracted from the buffer with ethylacetate and from the tissue with $CHCl_3$: MEOH (3:1). The extracted estrogens were separated into unconjugated and sulfate esters by neutral alumina chromatography[24] (SA, Gilman).

[d] The total steroids conjugated to $^{35}SO_4$ included estrogens (product of estrogen sulfotransferase) and neutral steroids. The neutral steroid sulfates are the product of steroid alcohol sulfotransferase, an enzyme also found in porcine uteri[24].

blocked by this estrogen analogue, since the pmoles of total $^{35}SO_4$ conjugated to steroids remained unchanged (66-75 pmoles) in the presence of inhibitor. (It has previously been observed that porcine uterus conjugates neutral steroids to sulfate equally throughout the estrous cycle[25]).

Results of these studies utilizing a specific inhibitor of estrogen sulfotransferase with secretory uterine minces clearly demonstrated that in the absence of estrogen sulfurylation less E_2 was oxidized to E_1 (Table 2, $E_1 + E_1$-S = 5.4 pmoles in the control, or 4.4 pmoles in the presence of inhibitor). However, under these conditions of inhibited estrogen sulfotransferase the secretory endometrium contained a greater (3 fold, or 4.4 pmoles total E_1 from 10 pmoles E_2 added, Table 2) capacity to oxidize E_2 than did proliferative endometrium (1.6 pmoles total E_1 from 10 pmoles E_2, Table 1).

It appears then that both these enzymes display an increased activity in the secretory tissue. Yet there is a more dramatic increase in the sulfotrans-

ferase. Preparations of the 100,000g supernatant of porcine uterus which is free of microsomal sulfatase activity has shown that the soluble estrogen sulfotransferase is at least 10-times more active in secretory uterus than in the proliferative tissue. Presently kinetic studies are being carried out to establish further the extent of the variation of these enzyme activities in cycling endometrium.

A consistent amount of unconjugated estrone was formed from E_2 in incubates of uterine tissue removed from the host with any of the various hormonal milieu (immature, proliferative or secretory, Table 1). This value is near 15% in porcine uterus and 50% in human endometrium. Consequently the oxidation of E_2 to E_1 appears to be a basic property of this target tissue. Recently it has been

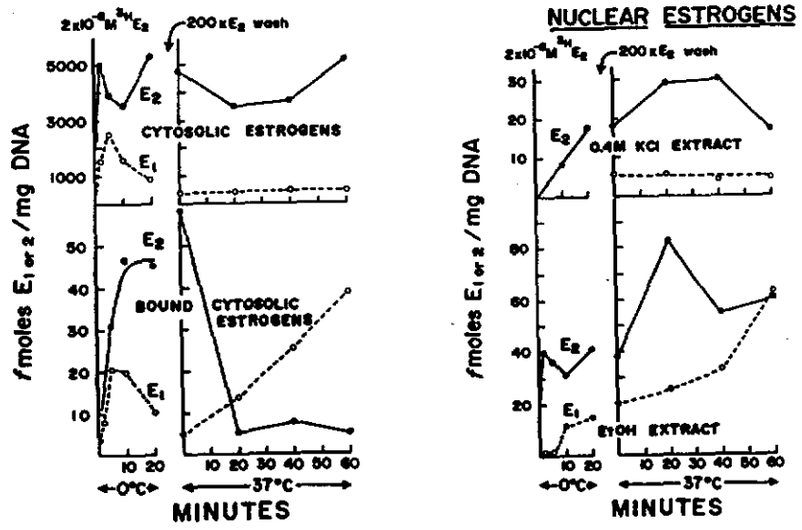

Fig 12. The fate of $^3H E_2$ in MCF-7 cells during cold and warm incubations. After reaching confluence in T_{75} flasks the cells were harvested with a rubber policeman and incubated with $^3H E_2$ as previously described[40]. Top left. Levels of $^3H E_2$ and $^3H E_1$ are shown in extracts of the total cytosol from cells incubated with 2 x 10^{-8}M tritiated E_2 for 20 min at 0° followed by a 1 hr incubation at 37° of these same cells in the absence of exogenous labeled E_2. Bottom left. The estrogens which were bound to high affinity, low capacity sites in these same cytosols are shown at various times of the incubations. Each point represents the cytosol from homogenates containing 0.231 to 0.336 mg DNA.

The nuclei were from the cells described above. The 0.4M KCl (top right) and ethanol (bottom right) extracts were obtained according to methods previously described[40]. The yield of nuclear DNA after centrifugation through 2.2M sucrose in TKM buffer was between 0.048 and 0.080 mg. Estrogens were identified as previously described[40]. (Chart from Brooks et al[40])

demonstrated by Pollow et al[38] that E_2 dehydrogenase activity can be found throughout the uterine cell; in the soluble fraction, microsomes, mitochondria and nuclei. The consistency of E_1 formation and the ubiquitous cellular distribution of E_2 dehydrogenase activity suggest that either E_1 itself or the oxidation of E_2 may have a role in uterine estrogenic response. In addition, a recent report by Bayard et al has indicated the presence of both E_2 and E_1 receptor in human uterine cytosol[39]. The ratio of these reported binding activities varied throughout the menstrual cycle.

TABLE 3

SPECIFIC ESTROGEN RECEPTORS IN THE NUCLEI FROM MCF-7 CELLS

Source of Nuclei	WHOLE NUCLEI[a] Specific E_2 Binding[b]		Specific E_1 Binding[b]	
	pmole/mg DNA	K_d x 10^{-9}	pmole/mg DNA	K_d x 10^{-9}
MCF-7 Cells[c]	0.31	0.73	0.15	3.3
	0.6M KCl EXTRACT[d]			
MCF-7 Cells[e]	0.45	0.78	0.07	1.8
+ 10^{-8}M E_2	1.37	0.78	0.61	1.9
	SALT RESISTANT[f]			
MCF-7 Cells	0.07	0.46	0.06	1.3

[a] Nuclei were isolated from cells homogenized in TKM buffer[39] pH 7.5 containing 0.1% saponin and washed twice with 0.25 M sucrose in TKM. Exchange assays[40] were carried out at 23° for 1 hr.

[b] Specific binding was determined by the Scatchard analysis utilizing 6 levels of $^3H E_2$ ± 200-fold unlabeled E_2 or $^3H E_1$ ± 200-fold unlabeled E_1.

[c] MCF-7 cells were grown past confluence in Eagles minimum essential medium supplemented with non-essential amino acids, insulin and antibiotics and made 10% with respect to calf serum.

[d] Nuclear proteins were extracted[39] by triturating nuclei at 0° for 1 hr in 0.6M KCl in TE buffer pH 8.5 and the binding assayed by the protamine sulfate technique of Horwitz and McGuire[41]. Under these conditions receptor which is bound to E_2 will not exchange with $^3H E_1$ at any temperature (0°, 23°, or 37°).

[e] After attaining confluence cells were exposed to the control medium plus 10^{-8}M E_2 for 1 hr at 37°, a procedure shown to saturate the nuclear receptor sites[41].

[f] The salt resistant receptors were assyaed by the exchange assay[40] carried out at 23° for 1 hr on the residual pellet from the 0.6M KCl extraction.

Utilizing the MCF-7 human breast cancer cell line, we have demonstrated that E_1 formed from E_2 *in situ* is bound to a cytoplasmic macromolecule with high affinity and low capacity[40]. Furthermore E_1 is formed within the nucleus from E_2, particularly in the salt-resistant nuclear fraction (Fig 12). This unexpected discovery would predict the presence of E_1 specific nuclear receptors in this neoplastic human cell line. We have carried out studies designed to gain information regarding the presence of a nuclear E_1 binding component. Whole nuclei, the salt extracted nuclear proteins and the residual nuclear pellet displayed high affinity low capacity binding for both E_2 and E_1 (Table 3). Furthermore the binding capacity for either of these estrogens increased upon exposure of the cells to 10^{-8}M E_2, a procedure shown to saturate nuclear receptors in the cells[42]. The observed E_1 binding in these experiments is displayed even in the presence of 5 μM unlabeled E_2.

The role of the E_1 receptor in estrogen activity is unknown at this time. However, its nuclear concentration has been seen to vary with the growth conditions of the cell culture (log growth *vs* stationary phase) and to vary in cultures before and after their exposure to exogenous E_2 (Table 3).

CONCLUSION

In vivo the liver is a major site of peripheral estrogen metabolism. A number of hepatic estrogen metabolites and their sulfate and glucosiduronate esters enter the plasma, some of which may interact with target tissues. This plasma pattern of metabolites can be demonstrated to have changed two months after a single administration of DMBA. These alterations are particularly evident in the increase in hydroxylated ("polar") estrogens which are products of the microsomal mixed oxidase system (Cyt_{450}).

Target tissues also have the capacity to metabolize estrogens to presumed less active forms. This metabolic activity is comprised of estrogen dehydrogenase and sulfotransferase, two enzymes which vary throughout the estrous and menstrual cycle, indicating their dependence on the hormonal milieu. Both enzymes increase in the secretory phase of the cycle. At present it appears that estrogen sulfotransferase is essential for the *in situ* deactivation of E_2 during the progesterone controlled uterine differentiation.

Uterine estrogen dehydrogenase, which is present throughout the cycle, may have a role in estrogen activity. Evidence is accumulating which indicates that the interconversion between E_2 and E_1 may play a role in the nuclear interaction of estrogen receptor complex.

ACKNOWLEDGEMENTS

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DISCUSSION

VILLEE: Do you think the variations in the relative amounts of estradiol and estrone in different tissues simply represent variations in the relative amounts of oxidized and reduced pyridine nucleotides?

BROOKS: The available ratios of NAD/NADH and NADP/NADPH in the different tissues would undoubtedly affect the amount of estrone formed from estradiol (e.g., porcine uterus vs. human endometrium). It is also possible that the reducing environment of the uterine cells would be different throughout the cycle in one species. However, we have shown in our experiments (and Pollow and Gurpide in their studies) that the estradiol dehydrogenase activity varies as indicated throughout the cycle in cell fractions supplemented with NAD (the preferred cofactor).

MCLACHLAN: Do you think the oxidation of estradiol to estrone occurs while the steroid is still on the receptor?

BROOKS: This we do not know. However, it is possible to speculate from the data available in Fig. 12 (Brooks et al., this volume) that the "bound" estrogen in the salt resistant fraction was oxidized from estradiol to estrone without mixing with the unlabeled estradiol in the cold chase. This figure shows the amount of ^{3}H-estradiol-associated radioactivity which is lost in the salt-resistant fraction during the 1 hr, 37° incubation appears as a like amount of radioactive ^{3}H-estrone.

KUPFER: Does the "nuclear" estradiol dehydrogenase utilize NADP or NAD or both?

BROOKS: Data reported by Pollow et al. (Acta Endocrinol. 79: 134, 1975) indicate that mitochondrial, microsomal and the soluble estradiol dehydrogenase prefer NAD over NADP (3-fold). This type of interaction is not

available for the nuclear enzyme. However, the nuclear experiments were carried out with NAD in the Pollow et al. investigation referred to herein (ref. 39, Brooks et al., this volume).

MULDOON: Since progesterone has been shown to induce uterine estrogen oxidoreductase, have you examined the effect of alterations in progesterone levels on your putative oxidation of undissociated estradiol-receptor complex to an estrone-receptor complex in the nucleus?

BROOKS: We have not yet examined the nuclear oxidation of "bound" estradiol to estrone in porcine or human endometrial nuclei. Pollow et al. (ref. 39, Brooks et al., this volume) have shown nuclear oxidation of estradiol to be greater in the nuclei from secretory endometrium (over proliferative) but these experiments did not indicate the bound state of the nuclear estrogens. Our experiments reported here were carried out utilizing the MCF-7 cell line which was cultured with only 10% calf serum as the hormone source. Although progesterone was undoubtedly present, the amount was not controlled.

DUAX: As I understand it, you propose that receptor bound estradiol might be metabolized to estrone in the nucleus. This appears to be consistent with our proposed model in which the A-ring of estradiol is the principal structural component responsible for receptor binding and the D-ring region is open to influence subsequent events such as the metabolism you propose.

BROOKS: Yes. I am aware of this. The support which your data gives to our investigations is greatly appreciated.

McLachlan, ed. Estrogens in the Environment

THE MECHANISM OF ESTROGEN ACTION: THE OLD AND A NEW PARADIGM

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This article deals with the mechanism of action of sex steroids with special emphasis on the effects of estrogens (E) on cell multiplication as opposed to hypertrophy and synthesis of specific proteins.

AN HISTORICAL PERSPECTIVE

The following is a brief review of the process which led researchers to postulate the currently accepted paradigm of the mechanism of estrogen action. In 1896, Beatson provided the earliest ideas about ovarian hormones action on certain secondary sex organs. Castration, he showed, improved the survival of women with breast cancer[1]. During the first half of the twentieth century, physiologists proved that castrated animals showed a significant atrophy of the uteri, vagina, and certain histological changes occurred in the pituitary, adrenals and other endocrine organs (For a comprehensive review, see 2). As a result of the injection of ovarian extracts first, and purified estrogens later on, cell multiplication and trophism of the secondary sex organs were reestablished. It is highly significant that no evidence of cell multiplication due to local effect of E administration on *bona fide* target cells has been reported to support the inference that E *per se* are able to affect the multiplication of such cells.

Organ culture of the uterus and vagina was used as a tool to study the effect of purified estrogens in an isolated system. This started in the '40s and still is being done today[3-7]. The results are as varied as the techniques, species, ages of the donors, and doses of estrogens used. Another variable is the lack of evidence for the presence of E receptors in these short-term culture systems[9].

The possibility of an indirect mechanism of E action was entertained by Clara Szego during the 40's. She reported that E did not significantly affect the uterus of eviscerated rats[10]. Despite this interesting finding the subject was not pursued further. During the 50's, Charles Huggins showed that either castration or large doses of E objectively induced the remission of experimental mammary tumors[11]. A number of models to explain the hyperplasia and hypertrophy due to E administration to castrated animals have been proposed [12-17]. They all center on the supposedly crucial role that E receptors play in the growth and development of E target organs. Receptors have been defined mainly as protein molecules that have the ability to bind their ligand with specificity and high affinity. Based on these features, Jensen has suggested that the term "estrophilins" instead of "E receptors" be adopted because it more accurately describes the properties of these molecules without prejudging their role in the measured response[14].

During the '70s, it became apparent that the hyperplastic effect seen in uteri and vagina of castrated animals subsequent to E parenteral injection could not be reproduced consistently in culture conditions of comparably designed experiments [18,19,20]. During the current decade several laboratories, including our own, investigated this specific area of research within the broad field of the mechanism of E action.

A SOMATIC CELL GENETIC APPROACH

We therefore must address the paradoxical finding that multiplication of E target cells in animals is stimulated by E administration whereas the proliferation of these same cells in culture conditions is not affected by E.

This analysis will focus on two main areas of research: a) the putative E-mediated growth promoting (cell multiplying) effect on certain target cells, and b) the induction of well characterized proteins as a result of E stimulation.

These two functions may overlap in some instances in animal experiments, but it is well accepted that they must not be linked. Indeed, no previous round of DNA synthesis is needed for E-sensitive specific proteins to be synthesized [21].

The application of cell culture techniques to the study of the mechanism of estrogen action has brought into focus several issues not well appreciated by means of animal studies alone. For example, the E "induction" of specific proteins seen in animal studies could be reproduced with relative ease in cell culture [21-28] but, the estrogen mediated cell multiplication of *bona fide* E target cells remained elusive. Again, both short-term and long-term systems have been used in attempts to demonstrate this latter effect of E. Short-term cultured cells, mentioned above, show a rapid fall of E receptors, bringing into question their property of being E targets along the duration of the experiment[9]. Moreover, short-term culture experiments that necessarily involve a mixture of several cell types (epithelial and mesenchymal) have provided conflicting evidence concerning the direct role of E in promoting cell multiplication[3-8].

We will also examine the evidence obtained using *bona fide* E_2 target cells in long-term culture, i.e., cells that have been established from E target organs, of both normal and malignant origin. These cloned cells carrying significant amounts of estrophilins grow indefinitely in culture conditions. We will briefly define the circumstances under which established clonal cell lines multiply in culture conditions.

CELL GROWTH IN CULTURE

Mammalian cells, as well as bacteria [29] and plant cells[30], growing in adequate culture media show an initial lag period of limited growth followed by a log or exponential period[31,32]. When the nutrients become exhausted, the growth of the cell population declines. In the log phase, the cell number increases exponentially with time, i.e., the logarithm of the cell number

increases linearly with time, or, $Ct = C_o e^{\alpha t}$ and $\ln \frac{Ct}{C_o} = \alpha t$, where C is cell mass, t is time and α is the instantaneous growth rate constant for the cultured cells. Hence, the multiplication of cells in culture does not represent a capricious, unpredictable or chaotic process, but follows principles valid for all cloned cells growing under adequate nutritional conditions found in the hosts from which they derive. One of the differences between in animal and in culture systems, however, is the limitless capacity for cells to multiply in culture conditions whereas cell multiplication is limited in the body of the host. Cells in both animals and in culture multiply in binary fashion. Figure 1 schematically summarizes the possible growth patterns cells may follow in culture and in live hosts.

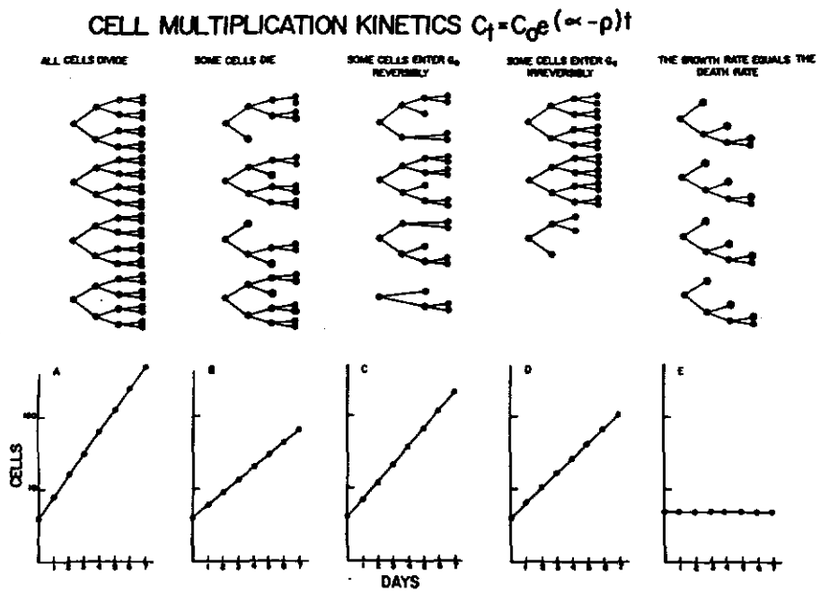

Fig. 1. Schematic representation of possible cell multiplication patterns of homogenous, discrete populations of cells growing in culture conditions and in live hosts. These slopes are representative of patterns of cell multiplication under steady state.

At this point we must call attention to the extensive literature on cell multiplication in general [31-32]. To the best of our knowledge, there is no evidence that normal cells and malignant cells differ significantly in the initiation of cell multiplication. As far as it is known, the difference between normal and malignant cells resides with their respective abilities to respond to signals to stop growing. Normal cells are able to recognize and process these signs, while malignant cells are not. Hence, in the study of cell multiplication, the specific origin and biological capacity for unlimited growth are not critical factors.

Growth of rat E-sensitive cells in animal - in culture systems. Our understanding of the mechanism of E action derives from a Somatic Cell Genetic approach in an 'in animal-in culture' system with cells carrying estrophilins [18, 33-36]. These cells show two well characterized growth patterns: a) E-sensitive-for-growth, i.e., cells grow more rapidly in animals in which the E concentration is higher (intact females and E-injected males and castrated rats); and b) autonomous or E-insensitive-for-growth, i.e., tumor cells that, despite carrying estrophilins, grow at the same rate regardless of the sex of the rats, the presence of gonads, or the sex hormone stimulation which the hosts may receive. Obviously, the transplantation of tumor cells in such hosts require that they should be isogenic, i.e., belonging to the same inbred strains from which the tumor originated.

The estrophilins of rat tumor cells in culture have physicochemical properties similar to those present in the original tumors. Their concentrations in the cytoplasm and the nucleus are similar to concentrations found in the tumors growing in animals. When the ability of both the E-sensitive-for-growth and the autonomous cells to react to a wide range of E_2 concentrations (10^{-7} to 10^{-11}M) is tested in culture, no increase in growth rate can be ascertained. Moreover, the cells grew at their maximal known growth rate in the estrogenless control medium (10-20% castrated and adrenalectomized calf and horse serum supplemented

media). These experiments were conducted in several laboratories with rat pituitary tumor cells, rat endometrial tumor cells and rat mammary cells [18,19, 33-36]. The growth rate of these cells was always exponential and followed a pattern predicted by the equation $C_t=C_o e^{\alpha t}$. That the rat pituitary cells are indeed responsive to E_2 stimulation is indicated by the reproducible induction of progesterone receptors or progestophilins (specific protein synthesis) both in culture as well as in animal experiments[28]. Once again, the induction of specific protein synthesis is not linked to a direct growth promoting effect.

One may ask, can the lack of E-dependent cell lines in culture be due to such cells being selected against during the establishment process? This is a verifiable possibility. By examining the behavior of these cells when they are challenged to grow in isogenic hosts, one can determine whether, in fact, these cells are E-sensitive-for-growth or not. An E-related pattern is considered a reliable criterion. This has been done with the rat cell lines mentioned above. For obvious reasons, human derived cell lines cannot be tested in this respect.

From the data briefly analyzed above we conclude that the growth rate of estrophilins-carrying cells, be they sensitive or autonomous when injected to animals, is not affected by the addition of E_2 to the media in culture conditions. This finding conflicts with the putative growth promoting activity of E over the uterus, vagina and other target organs in live animals.

The resolution of the paradox has been pursued in a few laboratories following two main avenues: a) that which postulates the existence of intermediary, synergistic growth promoting factors whose action is triggered by E administration and, b) that which acknowledges the data indicating that bona fide E-sensitive cells in culture grow exponentially regardless of the presence of E_2 in the culture media, and, hence, proposes a repressible model for cell multiplication. The first possibility postulates that E_2 acts on E_2-target cells by

inducing the synthesis and/or release of hormones or growth factors which in turn would affect E-sensitive-for-growth cells in the animal, or on cells in culture. The possibility that the pituitary was such an intermediary organ was investigated in the rat and in the quail (Coturnix coturnix Japonica) using as end points growth parameters in the uterus and the oviduct, respectively. The results indicate that the pituitary was responsible for 20-40% of the E-dependent weight and macromolecular synthesis increase of the castrated rat uterus [39,40], and a comparable percentage on similar parameters in the young quail oviduct[41]. Therefore, between 60-80% of the growth of these target organs after appropriate parenteral E_2 stimulation cannot be attributed to pituitary factors and hence, is due either to E_2 per se or other mechanisms. It should be remembered that in culture conditions, maximal growth of bona fide E-sensitive cells is obtained whether E_2 is added or not to the growth media. It seems unlikely, then, that E_2 per se is the growth promoting agent. The possibility that such growth factors, secreted by target organs, are transported in the serum and are responsible for the whole putative E_2 growth stimulation observed in animals has been explored in a preliminary fashion [42]. This approach awaits further assessment.

A second approach, the one which we favor, based on some of the above mentioned premises regarding cell multiplication, postulates that b) as growth appears to be a dominant, constitutive character in cells (normal and malignant), growth inhibition may be central to the understanding of the mechanism of estrogen action in this particular case, and to the subject of cell multiplication in general [20].

We will analyze next the rationale of our new assessment of the data available derived from our lab and that from others. It is of great importance to recognize at the outset that after a careful examination of the available

literature in the last 40 years we conclude that there is no evidence that the hyperplasia following the parenteral injection of E is unequivocably linked to direct, local effect of E at the level of the target cell. A critical interpretation of the available data indicates, however, that the injection or introduction of E_2 into castrated or immature females results in increased DNA, RNA and protein content of target cells, i.e., uterus, vagina, bird's oviduct or E-sensitive-for-growth tumors [43]. While it is true that there is conclusive evidence that labeled E_2 is indeed selectively taken up and retained by target cells because of the presence of the estrophilins mentioned above [12], there is no evidence that the interaction between the E_2 and any specific process will result in subsequent DNA synthesis. The relationship is inferential and, a reasonable inference at that. Nonetheless, it is important to emphasize that these events may be coincidental. As for the cause-effect relationship between E_2 stimulation to bona fide estrogen-sensitive-for-protein synthesis cells that results in specific induction of protein synthesis, this is probably an example of close correlation between incoming E_2 binding to estrophilins and then, at a yet to be defined site, the influence of either the E-receptor complex or any of its parts on the actual synthetic process or the triggering of the process. Although it is clear that the induction of specific protein synthesis by E is exerted both in animals and in culture conditions, the putative role played by estrophilins is still unclear, as is the location of such an interaction. This is so despite the elegant and thorough efforts of several laboratories [44,45].

AN ONTOGENETIC PERSPECTIVE OF THE MECHANISM OF E ACTION

1. Mechanism of estrogen action during the perinatal period.

This approach rests on the premise that growth inhibition (measured as inhibition of cell multiplication) is central to the understanding of the mechanism of estrogen action. The coexistence of high endogenous levels of E with atrophy of bona-fide E sensitive-for-growth cells in intact rats has been established [46-48].

The case in point is the perinatal stage in rats where levels of E are above those registered during the estrous cycle. At the same time, the levels of α-fetoprotein (AFP), a plasma protein that appears early in embryogenesis, are high during the perinatal stage diminishing to low levels toward the end of the 4th week of life [49-54]. AFP in murine species has the property of binding with high affinity to E[53]. To test whether the growth of the E-sensitive-for-growth tumor cells we developed was affected by the age of the hosts in which they were inoculated, we compared the latent period of E-sensitive-for-growth tumor cells inoculated in newborn and adult rats. The results of these experiments are presented in Figure 2. They indicate that the so called E sensitivity-for-growth is maintained regardless of the age of the hosts at the time of inoculation; equally important, there is a significant, equivalent delay in the appearance of the tumor when these cells were inoculated into 1 day-old male and female rats (10^6 or 10^7cells). This delay was not due to the trapping of all available circulating E because the latency period of the tumors was not significantly affected and never approached the growth rate seen in intact adult female rats when R_{2858}, a synthetic E that does not significantly bind to AFP, was injected for the first 20 days of postnatal life[54].

The inoculation of autonomously growing estrophilin-carrying cells, in this instance F_4C_1 rat pituitary tumor cells or AMNU rat mammary tumors, results in a latent period similar in duration regardless of the sex and age of the host [36, 54-56] (see Figures 2A and B).

The somatic cell genetic approach has been advantageously used in this situation because we could test whether the results obtained in the animals could be reproduced by exposing the same cells to an in culture environment in which the conditions prevalent in the host had been reconstituted. Thus, we demonstrated that increasing concentration of sera from fetal and newborn rats prevents the cell multiplication of the $C_2$9RAP cells in a typical dose-response pattern (Figure 3).

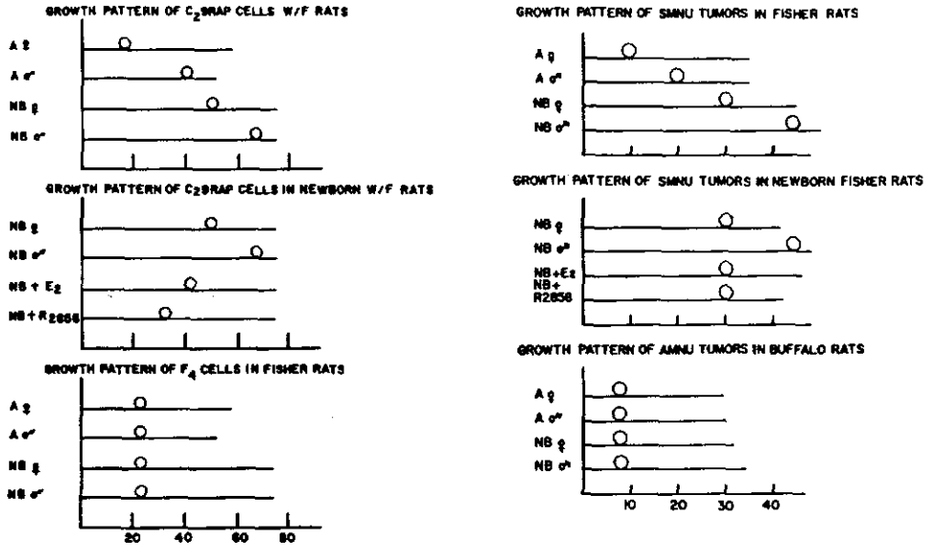

Fig. 2. Schematic representation of the growth pattern of rat pituitary tumor cells (A) of E-sensitive-for-growth (C_29RAP) and autonomous (F_4C_1) behavior. Rat mammary tumors (B) of E-sensitive-for-growth (SMNU) and autonomous (AMNU) behavior when injected into isogenic hosts is also shown for comparison. (see ref. 54 and 57).

This is indicative that sera contain an inhibitory substance which we suspected could be AFP. These experimental results suggested that AFP which is present in sera of fetal, newborn and Hepatoma 7777-bearing rats is heavily involved in the growth inhibitory responses seen in animals and in culture conditions[54].

Because it could be objected that the growth-inhibitory property of AFP might be a peculiarity of the E-sensitive-for-growth cells used here, i.e. pituitary tumor cells, we therefore demonstrated that indeed E-sensitive-for-growth rat mammary tumor cells also share the property of being inhibited during the perinatal period[57] (see Fig. 2). We have also confirmed this with rat endometrial cells that are E-sensitive-for-growth (unpublished results).

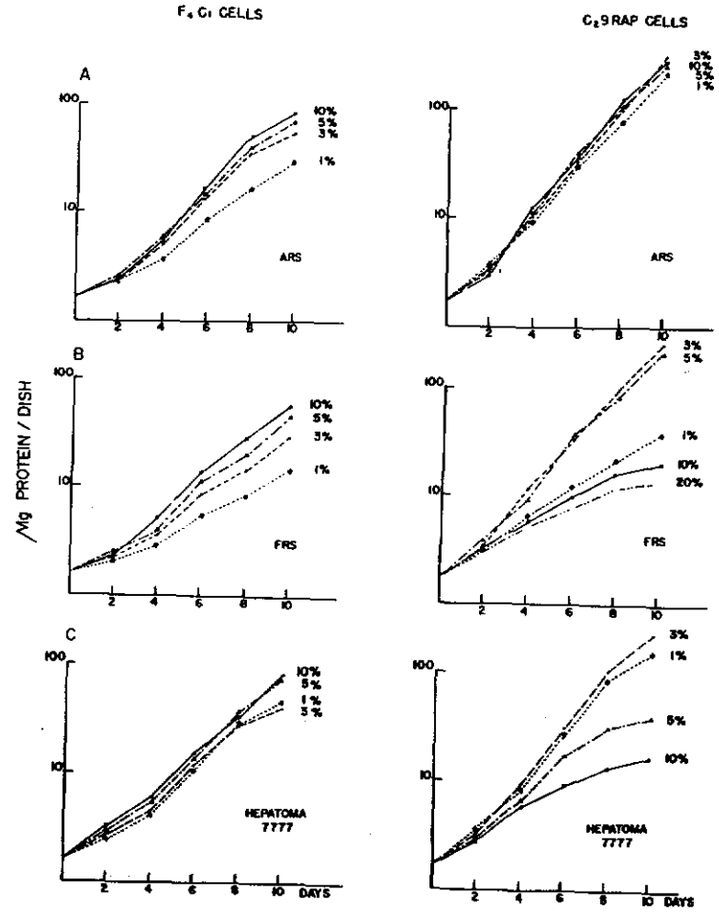

Fig. 3. Comparative growth rate of E-sensitive-for-growth (C_29RAP) and antonomous (F_4C_1) rat pituitary clonal cell lines challenged to grow in several concentrations of adult, fetal and Hepatoma 7777-bearing rat sera (see refs. 38 and 54).

The objection that this phenomenon, though related to high levels of AFP prevalent during the perinatal period, may be a reflection of the "general immaturity" of newborn rats was also considered. Again, using the somatic cell genetic approach we next used methylnitrosourea (MNU)-induced mammary tumors of

E-sensitive-for-growth and autonomous behavior now transplanted into adult hosts carrying high levels of AFP in their bloodstream. This was possible thanks to the availability of rat hepatomas in BUF rats that secrete AFP and others that do not secrete this protein in significant amounts. (These hepatomas were developed by H.P. Morris[53,58]). We demonstrated that rat mammary tumors are prevented from growing and are, in fact, induced to regress in the animals that carry Hepatomas 7777 which secrete high levels of AFP, whereas they grow undisturbed and prematurily kill the host in animals bearing Hepatomas 7787 and 7800 which do not secrete significant amounts of AFP[57, 59].

Recently we have purified AFP and by adding it to serum supplemented media at concentrations comparable to those existing in newborn rat serum we demonstrated that AFP inhibits the cell multiplying ability of $C_2$9RAP cells[60]. Furthermore, when AFP was washed off from cultures where it was apparently inhibiting cell multiplication, and replaced by a medium without AFP, the "inhibited" cells resumed cell multiplication at a rate similar to that of cells not exposed to AFP (Fig. 4). This is, in fact, the same fate these $C_2$9RAP follow when injected in 1 day-old W/Fu rats. F_4C_1 are insensitive to this treatment. Therefore, we may now assert that AFP plays the role of a specific inhibitor of E-sensitive-for-growth cells[54]. We have not yet determined how this inhibitory process is accomplished in molecular terms. This subject is currently being developed in our lab and a report on our findings is forthcoming.

That the acknowledged affinity of E to AFP which may result in "trapping" of available E does not impede E from entering cells and binding to E receptors with the same properties exhibited in adults, has been recently demonstrated in 5-day-old rats[61]. Moreover, it has also been shown that despite the 4 to 5 orders of magnitude differences registered between the molar concentrations of AFP and E in the bloodstream, bona fide E sensitive-for-protein synthesis cells

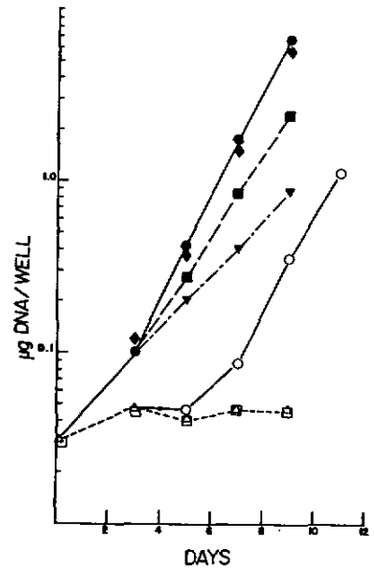

Fig. 4. Growth rates of E-sensitive-for-growth rat pituitary clonal cells ($C_2$9RAP) placed under different concentrations of rat purified AFP. ● Horse Serum (HS) 10%, ◆ HS 10% + 3mg BSA/ml, ■ HS 10% + 0.6 mg AFP/ml, ▽ HS 10% + 1.2mg AFP/ml, □ HS 10% + 3 mg AFP/ml, Δ HS 10% + 3 mg AFP/ml + 10^{-9}M R2858, and ○ cells grown for 7 days in HS 10% + 3mg AFP/ml and from then on in HS 10% devoid of AFP. The term E-sensitive-for-growth relates to the ability of these cells to multiply in animals with high levels of E.

such as rat pituitary cells in situ are able to respond to changing levels of circulating E during the perinatal period. Indeed, the negative feedback control mechanism for gonadotropins is operative during this period [46,62,63]. These results warrant reassessment of the available data regarding "free" and "bound" hormone and the degree of consequent "activity" [64]. In sum, we have been able to show that:

1) A discrete substance naturally occurring in normal and malignant states in rats is able to prevent the multiplication of both normal and malignant E-sensitive-for-growth-cells.

2) The presence of estrophilins in cells does not constitute a reliable marker for their sensitivity to E, when both cell multiplication [54] and induction of protein synthesis markers[28] are the parameter measured. Hence, though

the presence of estrophilins may be considered necessary, it is by no means sufficient to identify these cells as sensitive to E stimulation.

3) The effect of AFP on cell multiplication of E-sensitive-for-growth cells does not seem to be related to its ability to bind E with specificity [60],

4) We have reproducibly shown that E-sensitive-for-growth tumor cells of different origins (pituitary, mammary, endometrial) may be successfully and predictably prevented from multiplying and even induced to regress by strictly biological means [54,57,60,65].

The implications of these conclusions represent a significant departure from generally accepted notions about the etiology and pathophysiology of cancers.

We have recently found that AFP may affect the cell multiplication of androgen-sensitive-for-growth cells in rats. Hepatoma 7777-bearing adult male rats show atrophy and apparent hypoplasia in their prostate and seminal vesicles. This hepatoma secretes high amounts of AFP into the bloodstream (up to 16mg/ml serum). The effect of AFP is not reversed by dehydrotestosterone-filled silastic tubes implanted in castrated and adrenalectomized rats (Sonnenschein, Schatz and Soto, submitted). As AFP does not significantly bind androgens, we suggest that AFP is responsible for the growth-inhibition of sex accessories in male and female rats during the perinatal stage.

It is well known that AFP is normally present in most mammals during the pre- and/or perinatal stage. Different species have shown patterns that do not chronologically overlap with the one we analyzed for the rat. For example, mice have an AFP which binds with specificity to E as rat AFP does[53]. On the other hand, Syrian hamsters have an AFP which does not bind to E but follows a concentration pattern of temporal disappearance similar to that in mice [67]. Preliminary evidence indicates that the role of AFP in hamsters does not differ from the one we postulate above, namely, that of a cell-multiplication inhibitor of sex steroid-sensitive-for-growth cells (Schatz, Laugier, Soto and Sonnenschein, in preparation).

In humans, the pattern of AFP concentration during the pre-and perinatal stage is different from that of murine species and the Syrian hamsters and, in addition, human AFP does not bind E with high affinity [67,68].

To integrate these seemingly disparate pieces of evidence we can consider the mechanism of E action from an ontogenetic perspective. Two main periods can be identified within this approach: a) a pre- or perinatal period, reviewed above, and b) an adult period, to be reviewed next. We will first refer to the rat model and then extrapolate briefly to the human model.

2. Mechanism of estrogen action during adulthood.

The evidence reviewed above supports our contention for the existence of age-dependent, cell multiplication-inhibitory substances and explain available data related to the mechanism of E action centered around the effects of AFP on bona fide E-sensitive-for-growth rat cells of normal and malignant origin. It is well documented that AFP is present in all vertebrates studied during the perinatal stage [53]. Hence, the presence of AFP which is not under estrogenic control, or any known control for that matter, is unlikely to explain the available evidence in adult females. It is well accepted that AFP plasma levels become low beyond the first 4 weeks of postnatal life in rats. This, chronologically relates well with the beginning of puberty in males and females. As AFP levels are low in adult females, E_2 plasma concentration may be the agent apparently responsible for stimulating the cell multiplication of E_2-sensitive-for-growth cells of normal and malignant origin. As discussed above, this is consistent only with facts as seen in animal studies. Again, E-sensitive-for-growth-cells in culture grow at an exponential rate ($C_t=C_o e^{\alpha t}$) whether E_2 is present or not in the culture media [20]. To reconcile this paradox during adulthood we postulate the presence of (a) substance(s), circulating in blood that is (are) now under E_2 control for synthesis and/or release.

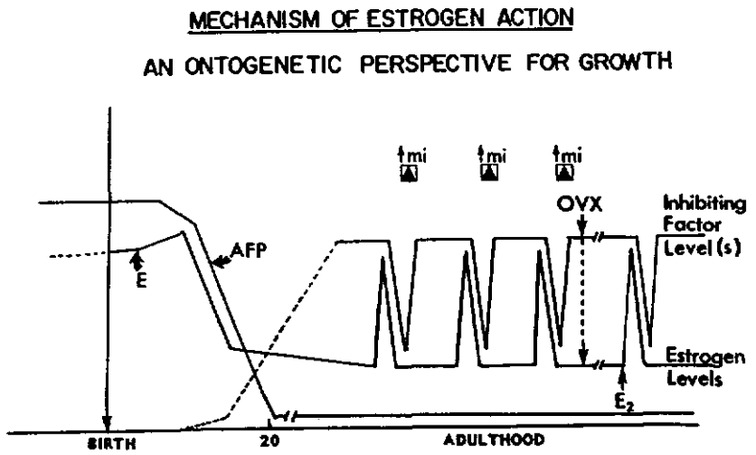

Fig. 5. Schematic representation of the ontogenetic perspective for understanding the mechanism of E action in the rat. A perinatal stage and an adult stage are suggested by available evidence (see Text). ↑ mi, indicates increased mitotic index; Ovx, ovariectomy; AFP, α-fetoprotein; E_2, 17β -estradiol. The broken line suggests the uncertainty of the level of the putative E_2-sensitive adult inhibitor during the prepubescent period.

Figure 5 schematizes the ontogenetic perspective of the mechanism of E action as seen in the rat. In it there are two clearly defined periods: 1) the perinatal period and 2) the adult period. The experimental data upon which we have based our interpretation of the results during the perinatal stage, has been discussed in the previous sections. For the adult period, we have reinterpreted data that suggest that E_2 *per se* was the element acting at the level of the E_2 target to elicit the cell multiplication effect considered central to the whole paradigm. For example, the cyclical pattern of hyperplasia that characterizes the estrous cycle in the rat uterus and vagina may now be interpreted as evidence for the

cyclical increase and decrease of the levels of the putative inhibitor(s) that are under E_2 control, namely, the rise in E_2 concentration in blood precedes the lowering of inhibitor(s) synthesis and/or release into the bloodstream. E_2, again, would affect the synthesis of a protein. This is consistent with all the evidence available regarding this item. It is well known that this is, in fact, the only consistent evidence between data obtained in animal studies as well as in culture studies where E_2 has shown to either increase or decrease the synthesis of specific, well defined proteins [21-28].

Moreover, the reproducible, unaltered exponential growth of *bona fide* E_2 sensitive-for-growth cells in culture conditions is compatible with the idea that the serum supplemented to the basal media in this type of system may have contained the inhibitors, but they were denatured during the process of shipping and storing (freezing) or preparing (heat inactivation). Another possibility in this context is that the inhibitor in question may be species-specific; that is, that the use of calf or horse sera supplemented even at concentrations up to 50-70% may not be effective to test the presence of a species-specific inhibitory substance we may be dealing with at this time. These are, of course, speculations over the meaning of negative data and it is justifiable to be somewhat skeptical on phenomenological approaches to such a fundamental topic.

We are currently exploring each of the problems listed above in order to get around the technical difficulties that have impeded the testing of our hypothesis. At this time we may report that in preliminary experiments, castrated female rat sera-supplemented media do not allow the growth of the E-sensitive-for-growth rat pituitary tumor cells in culture at the exponential rate we observe when we supplement the media with intact female rat sera (Soto and Sonnenschein, in preparation).

The literature-at-large has carried data that supports our contention for the existence of cell multiplication-inhibiting factors. One of the obvious implications of our model suggest that inhibitors are secreted by a cell type that may be at variable distance from the target of the inhibitory influence (Figure 6). The argument for the existence of such a mechanism is exemplified by the syndrome called gynecomastia present in men who suffer from alcoholic cirrhosis. Here, the increased cell proliferation of the breast tissue in males occurs regardless of the circulating levels of E[69]. We postulate that the lack of adequate amounts of healthy parenquimatous liver cells is responsible for low levels of an inhibitor for the proliferation of breast tissue that is tonically secreted by the liver under normal circumstances. Thus, the breast cells that no longer are prevented from proliferating will multiply accordingly.

The anomalous secretion of elevated amounts of E by ovarian or adrenal tumors in girls results in a syndrome of precocious puberty[70, and ref. therein]. The hyperplasia of female secondary sex organs ensues as a result of the lack of the E-sensitive cell-multiplication inhibitor. The removal of the tumor is followed by the atrophy of such organs. At the onset of puberty, the same proliferative process, but now under cyclical control, is established in these same girls (Figure 5).

Another example of the action of an E-sensitive cell-multiplication inhibitor is exemplified by the natural history of the vaginal and/or uterine dysplasia seen as a result of E stimulation of female human and mouse embryos of fetuses while <u>in uterus</u> [71,72]. To the best of our knowledge, these females do not develop this anomalous growth before puberty. We interpret this information as evidence for the presence of an inhibiting influence during infancy and childhood which prevents its expression. When the levels of E become high for prolonged periods (during pubertal cycles) the cells affected in uterus express their potential at a time the inhibitor is prevented from either being synthesized or secreted.

Once again, we would like to emphasize that there is ample evidence to indicate that E_2 is able to affect the synthesis of specific proteins even in the presence of AFP in rats[46,48,62,63] and perhaps guinea pigs[73]. This effect on specific protein synthesis takes place despite the presence of E-insensitive cell multiplication-inhibitors (AFP).

We predict that a good number of growth anomalies that include some forms of cancers may be better understood according to the schematic representation in Figure 6.

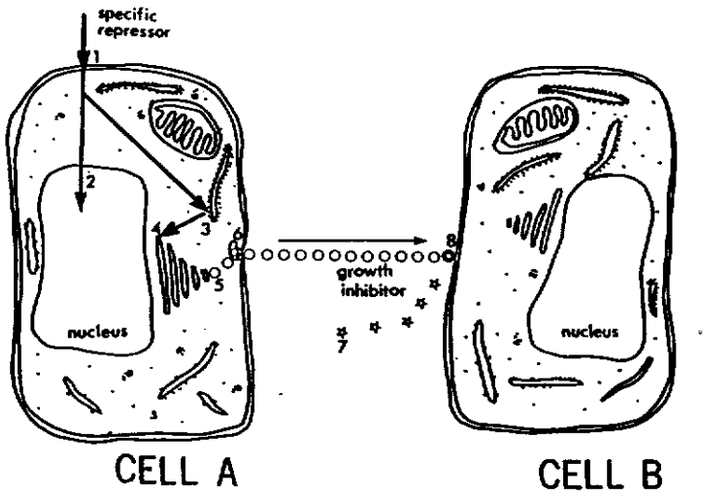

Fig. 6. Schematic representation of circumstances under which the target for inhibition of cell multiplication-inhibitory function (cell B) may be released from such inhibitory control. These circumstances include lack of sensitivity to the "specific repressor" at any of several locations where the influence is exerted within cell A (1,2,3,4,5,6). An anomalous processing of the synthesis or secretion of the "growth inhibitor" may also account for the release of cell B from the inhibition. The secretion of a "growth inhibitor" may be tonic (for example, AFP). It is conceivable that the specificity of the inhibitory recognition system (8) may require the interaction of the plasma membrane, the "growth inhibitor" and other specific ligands (7). The processing of the inhibitory message may also be affected and as result the target cell B is released from the inhibitory influence and sporadic or continued cell multiplication ensues. This model does not require a mutation in cell B to account for the transmission of the defect to the cell B progeny.

It illustrates a cell that secretes the cell multiplication-inhibitor and a cell that recognizes this molecule and reacts accordingly. The analysis of the evidence suggests, that:

1) The regulation of the synthesis and/or secretion of the inhibitor may be the most effective mechanism to regulate cell multiplication. Hyperplasia and/or hypoplasia may then be explained by an ephemeral, or continuous, spontaneous or experimental situation whereby the regulation of the secretion or action of the inhibitor is regulated or can be manipulated; 2) The normal function of the cell that secretes the inhibitor may be affected for prolonged periods of time. This results in the inordinate proliferation of target cells for such inhibitor. An example of this eventuality may be the gynecomastia syndrome briefly alluded to above[69]. 3) The target of the inhibitor may be impervious to the message carried by the inhibitor. This feature may develop spontaneously or may be induced experimentally (radiation carcinogenesis, chemical carcinogenesis or "spontaneous" loss of the recognition mechanism or the processing of the message within the target cell)[74]. Interestingly, the tumor that becomes autonomous does so by showing increased growth rate in the host in which it had been growing slower[75].

The secretion of the inhibitors postulated above is not done only by cells distant from the actual targets. This is exemplified by a strain of mice in which about 50% of the females develop gradually increasing anomalies in the cell multiplication control of oocytes. The case in point is the LT strain of mice where oocytes "spontaneously" divide and in some cases become implanted in the uterus and develop up to approximately the 7th-day-embryo stage or in cases develop as a teratocarcinoma[76]. A strong indication that the initiation of partenogenetic development may be due to the elimination of the cumulus cells from the oocyte has been presented[77]. This result added to that presented by

the characteristics of the ovary in LT strain of mice and similar observations in guinea pig ovarian tumors [78] again, strongly suggest that the initiation of cell multiplication, in this case of the oocyte, is triggered by the lack of responsiveness of cells to their specific inhibitors. In the present case analysis, the inhibitor is provided by a cell that is in close contact with its target.

CONCLUSIONS

In the preceding sections we have briefly covered many of the possibilities for the development of normal and anomalous cell multiplication patterns in in animal-in culture systems. We would like to stress that attempts to resolve the paradox of lack of reproducibility in culture conditions of the putative E_2- dependent growth promoting effects of E seen in animal experiments, must account for the lack of compelling evidence for an "inducible" mechanism of cell multiplication. Hence, we have explored the other alternatives, namely, that 1) cell multiplication is an inherent constitutive function of any living cell and 2) that cells will effectively divide whenever the agents that prevent them to do so are either eliminated, prevented from being secreted or else, when the target cells for the inhibitors become insensitive to their action. These generalizations cover most of the contingencies of the developmental aspects of living organisms, as well as those of malignant development. We are, in fact, suggesting again that the search for a character or marker in malignant cells, other than their inability to stop multiplying may be a fruitless enterprise. The lack of a comprehensive approach to the understanding of malignancy when the search for a circumstantial marker (chromosome aberrations, anomalous enzymatic pathways, carcinoembryonic antigens, viral infections, chemical carcinogenesis, etc., etc.) has been stressed, is now painfully clear[74]. In addition to the ontogenetic perspective proposed for the comprehension of the

mechanism of E action, we postulate that cell multiplication and ultimately DNA synthesis be considered "repressible" functions. Our "repressible" model, consistent with evidence we have been able to analyze above, closely relates to the question many investigators have posed themselves, namely, which is primum movens in the cell multiplication process[31,32]? It should be borne in mind that this is not just a semantic problem because we believe that the "repressible" model embodies the probable approach to the control of cell multiplication common to normal and malignant cells.

Fifteen years ago Charles Huggins wrote that... "With a single exception, all of the procedures found to be beneficial for human cancer of the breast were found directly at the bedside through clinical investigation. The vast amount of work in the laboratory on mammary cancer has yielded little that has been applied as therapy for women with cancer of the breast"[79]. We now believe that our experimental approach may contribute substantially to change such perspective for breast cancer and other forms of human cancers, as well. Needless to say, that we also believe that the new approach towards the understanding of the mechanism of E action and cell multiplication should experiment significant changes in priorities to be tested in the future.

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DISCUSSION

GOSKI: Extrapolations of studies of α fetoprotein (AFP) effects on tumor cell growth in vitro to in vivo effects on normal uterine growth are dangerous. We know that the rat uterus grows from the neonate to adult stages. We and other groups have observed that some nonsteroidal estrogens will induce uterine DNA synthesis in neonatal rats. Therefore, the young rat, in which AFP concentration is high, is capable of responding to estrogens. Estradiol is ineffective in the neonate, possibly due to its binding to AFP. Therefore, I would accept the data of Dr. Sonnenschein which indicate that AFP slows growth of cells in culture. I would not accept the extrapolation of these results to a model in which AFP is a primary regulator of uterine growth and cell proliferation in vivo.

SONNENSCHEIN: Obviously, we differ in our understanding of the subject of cell multiplication of the so-called estrogen-sensitive-for-growth cells. My understanding of the subject is adequately stated in my presentation.

SHEEHAN: We have obtained data showing that both DES and ethynylestradiol fail to bind effectively to AFP and that the estrogenic potency of these compounds is over 100-fold greater than estradiol in the neonatal rat in which AFP levels are high. Competent estrogenic responses are obtained in this system with 5 daily doses beginning on day 1. The correlation of ligand binding with estrogenic potency strongly suggests to us that AFP binding reduces the availability of estradiol to target tissue and prevents premature estrogenization in the neonate.

SONNENSCHEIN: Your results are at variance with those published by J. P. Raynaud, A. Kaye, E. R. DeSombre and others, which I referenced in my paper. Your interpretation of your results and those of others is at variance as well as with ours. I am confident that the assessment of all the available data, including that reported in this symposium, will eventually clarify whether the lack of cell multiplication of estrogen-sensitive-for-growth cells (of normal and malignant origin) during the perinatal stage is due to the

effect of AFP per se or by the property of rat AFP to bind estradiol with high affinity. We believe that we have shown sufficient data to support the first of the two conclusions mentioned above.

MARTIN: In vitro, AFP stopped the increase in cell numbers; did it do so by decreasing their birth rate or increasing their death rate? In other words, did it simply kill the cells?

SONNENSCHEIN: AFP stopped cells from dividing. Our preparation did not kill cells, as Figure 4 indicates (Sonnenschein et al., this volume). Once AFP was removed from the cell-containing wells, they restarted growth at an exponential rate.

MARTIN: You said there was no good evidence that estrogen increased proliferation by direct action on the actual target cells. However, there are many experiments with mouse vagina and mouse uterus which show that minute doses of estradiol applied directly into the organ induce cell proliferation. Moreover, experiments with ^{3}H-estradiol show that such minute doses are virtually 100% retained in the tissue. Furthermore, if retention in the target organ is prevented by competing out the estradiol with the antiestrogen, dimethylstilbestrol, the proliferation response is abolished. Have you any comment?

SONNENSCHEIN: Your evaluation of the data on this subject differs from ours and that of other investigators who showed estrogen-based ointments released estrogens which are absorbed into the bloodstream of treated human subjects even when small doses are applied. Our experimental data are consistent with this interpretation. We postulate that once estrogens enter the bloodstream, they affect cell multiplication of estrogen-sensitive-for-growth cells according to the scheme shown in Figure 6 (Sonnenschein et al., this volume). We do agree, I believe, that estrogens may affect specific protein synthesis in those target organs where they become bound to estrophilins, be they in the vagina, uterus, pituitary or liver, etc. This is valid to normal or malignant cells that carry these estrophilins.

LEROY: You stated that pituitary tumors injected into newborn animals treated with estradiol did not attain palpable size earlier than in controls. We did something similar with respect to the uterine response and found that by giving estradiol for 3 days starting at day 7 after birth, the mitotic response of the uterine epithelium could be advanced and was obtained on day 13 instead of day 20. So, there seems to be some discrepancy in this respect between your pituitary cells and the uterus.

SONNENSCHEIN: As I mentioned in my presentation, the study with pituitary cells was reproduced with mammary and endometrial tumor cells. Your observation is at variance with the results of a number of studies that I referred to in my original paper.

WEISZ: Maybe I could suggest one possible bridge between the two opposing views expressed so spiritedly during the discussion -- a suggestion at least with respect to the in vitro studies. We have heard a little from Dr. Cunha about the magic properties of the stroma and how it plays an essential controlling role in the characterization and the function of epithelial cells. Maybe the appropriate stromal cells need to be added to cultures of cloned cells to find out what estrogens can and cannot do.

SONNENSCHEIN: It is difficult to rule out the possibilities you propose without having the necessary data to properly evaluate the question and the answer. Of course, we realize that the epithelial cells get their nutrients and specific inhibitors through vessels located in the stroma. I may, however, speculate that it is not necessary to claim an interaction between the stroma and the epithelial cells in estrogen target cells to explain the evidence presented.

Environmental Estrogens: Exposures and Responses

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POTENTIAL FOR EXPOSURE TO ESTROGENS IN THE ENVIRONMENT

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Thousands of chemicals are introduced into our environment with little knowledge of their effects on two physiological processes which are central to our survival as a species -- reproduction and development. The overall human exposure to common chemicals, including drugs, is not known. However, the recent report that 8.5 kg/day of salicyclic acid was found in the waste water effluent of Kansas City[1] should raise concern. With millions of women taking oral contraceptives, some environmental contamination with estrogenic materials is a distinct possibility.

Some idea of the potential environmental burden of estrogenic materials can be illustrated by a few examples. The potent synthetic estrogen, diethylstilbestrol (DES), has been used extensively as a growth-promoting agent for poultry, cattle and sheep for over 20 years. In 1970 alone, over 27,000 kg of DES was used in the U. S. livestock industry[2]. The fate of this compound in a model ecosystem has been described in detail by Metcalf[3]. The well-known sequalea of prenatal exposure to pharmacologic doses of this chemical emphasizes the enhanced susceptibility of the developing organism to its effects[4].

o',-p DDT, an isomer of DDT which accounts for 15-20% of the commercial mixture of this formerly ubiquitous (estimated cumulative 200 million pounds used) pesticide, has been reported to be estrogenic. DDE, a metabolite of DDT, is commonly found in the body fat of humans and has been reported in human milk. This compound is very weakly estrogenic (see 5 for review).

Another series of compounds structurally related to DDT, the polychlorinated biphenyls (PCBs), have been reported to be uterotropic in rats. These compounds have been widely used as sealants and additives to paints, plastics, rubber, adhesives, printing ink and insecticides. These compounds represent important environmental contaminants and as such have been found in human milk. Gellert[6] has reported a correlation between the weak estrogenicity of some PCB mixtures (especially Aroclor 1221) and their ability to permanently alter the pattern of neuroendocrine reproductive function in rats exposed during development. The relevance of these studies to humans remains to be established.

However, PCBs have been reported to reach the fetus after maternal exposure in a number of species including humans[7].

Polycyclic aromatic hydrocarbons (PAH) are widespread environmental contaminants resulting from the combustion of fossil fuels. The report that a hydroxylated form of one of these compounds possesses weak estrogenic activity[8] raises the possibility that similar analogs of other PAHs may also be hormonally active. It is of interest that structural similarities between this class of chemicals and steroidal estrogens have been noted in the literature for many years.

The chemicals described previously were all derived synthetically or, in the case of PAH, were the result of a process such as combustion. It should be noted that some chemicals occurring naturally in the environment are also estrogenic. One, zearalenone, which has been described in detail by Mirocha's group[9], is a product of the fungus, Fusarium, which commonly infests corn in storage and has been implicated in hyperestrogenism in swine fed infested corn. Another class of naturally occurring environmental estrogens are the so-called plant or phytoestrogens. According to Lindner[10], more than 40 species of plants have been shown to contain substances that are active in biological assays for estrogenic activity. Two such estrogenic substances, coumestrol and genistein, have been extensively studied and interact with the cytoplasmic receptor for estradiol. As an example of exposure and response to environmental estrogens, the early report by Bennets and Underwood[11] of the large scale outbreak of infertility of sheep grazing on subterranean clover in Australia should be noted. Subsequently, genestein was isolated from this clover.

The literature on the hormonal activity of marijuana demonstrates a need for thorough mechanistic studies in this area and for a proper understanding of the appropriateness of the test system. Since the report of gynecomestia in men who smoked large amounts of marijuana[12], interest in the estrogenicity of the active ingredient of this material has continued. Reports that Δ^9-tetrahydrocannabinol (THC), the active component of marijuana, was uterotropic[13] were followed by reports that THC was not only uterotropic but also interacted with a high affinity with the uterine cytosolic estrogen receptor[14]. On the other hand, Okey and Bondy[15] have maintained that THC is not per se estrogenic either as a uterotropic agent or a receptor binder. These results were supported by the recent report that THC is not estrogenic in a primate uterine test system[16].

Such results raise the possibility that some so-called estrogenic effects of chemicals occurring as common environmental contaminants on a target organ may be an indirect effect of the agent on an organ peripheral to the supposed target. As an example, it has been reported (see Aulerich and Ringer for summary, 17) that administration of certain PCB mixtures to rats induces altered liver metabolism of endogenous estrogens and can result in a uterine weight response which is less than untreated controls. Careful attention to indirect peripheral effects of xenobiotics is crucial when evaluating them for hormonal activity.

Thus, compounds to which we may be exposed daily can possess varying degrees of estrogenic activity. Such exposures will obviously vary according to locale and occupation. Likewise, the responses of individuals to the same environmental agents may vary widely. Continued studies on the mechanism of action of estrogenic agents, defined broadly, as well as their distribution and potential for impact on human health should help provide some of the information necessary for better understanding our increasingly complex chemical environment.

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MODEL ECOSYSTEMS FOR ENVIRONMENTAL STUDIES OF ESTROGENS

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INTRODUCTION

Laboratory model ecosystems were developed to evaluate under carefully controlled conditions the disposition and fate of environmental contaminants in various substrates and within a variety of representative organisms of different phyla, class, and order (Metcalf 1977). The essence of model ecosystem technology is comparative, both (a) among ecological, behavioral, toxicological, and degradative processes in various organisms, and (b) between a wide range of contaminants where environmental effects can be related to physico-chemical properties. Thus if the relative model ecosystem behavior of a variety of organic compounds is compared to that of such widely studied pollutants as DDT, dieldrin and PCB's, it is possible to make meaningful judgments regarding the ultimate environmental fate of new organic compounds that have not yet been released into the environment or about older compounds whose real world fate has not yet been scrutinized.

METHODOLOGY

The essential methodology of laboratory model ecosystem evaluation of xenobiotics has been fully described (Metcalf et al 1971, Metcalf 1974, Metcalf et al 1975, Metcalf and Sanborn 1975, Lu et al 1975, 1977, 1978; Coats et al 1976, Metcalf 1977) and only a brief outline will be presented here. The essence of the investigation is the use of xenobiotic molecules radiolabeled with ^{14}C, ^{3}H, ^{32}P, ^{35}S or ^{36}Cl. The radiolabeled molecule can be followed both qualitatively and quantitatively throughout the substrates and organisms of the model ecosystem by liquid scintillation counting, thin-layer chromatography of extracts, and radioautography. Thus a wealth of information can be readily obtained about distribution, bioconcentration, food chain transfer, degradative pathways, comparative metabolism, and chemical nature of degradative products. The results can be quantified for comparison with other xenobiotics as bioconcentration or ecological magnification, i.e. amount of parent compound in organism/amount in water; as biodegradability index or amount of polar radioactivity/amount of non-polar radioactivity (from TLC plates); and as two parameters of total radioactivity in the organisms, the % parent compound, and the % unextractable - both measuring the overall degrada-

dative capacity of the organism. Gas-liquid chromatography can be used as an alternative to radiolabeling but is less informative.

The model ecosystem used in a large variety of experiments with pesticides (Metcalf et al 1971, Kapoor et al 1970, 1972, 1973, Metcalf et al 1973a, 1975a, Metcalf and Sanborn 1975, Lee et al 1976), industrial chemicals (Lu et al 1975, Metcalf et al 1973b, 1975b) energy conversion by-products (Lu et al 1978), carcinogens (Lu et al 1977), and veterinary drugs (Coats et al 1976), consists of a glass 10 gal. aquarium containing a sloping shelf of 15 kg of washed white quartz sand, bisected by a "lake" of 7 liters of standard reference water. On the terrestrial shelf are grown 50 Sorghum vulgare plants, comprising a "farm". The lake is stocked with a complement of microorganisms, plankton, 30 Daphnia magna, 10 Physa snails, and a clump of alga Oedogonium cardiacum. The aquarium is provided with a stream of air bubbles and Plexiglass top containing a small screenwire portion to retard evaporation and to permit gaseous exchange. The entire model ecosystem is housed in an environmental plant growth chamber with artificial "daylight" at 5000 ft. candles (54,000 lux) and kept at 26°C with a 12-hour daylight cycle. Contamination of the model ecosystem with the radiolabeled xenobiotic can be accomplished in several ways (a) by topical application to the Sorghum leaves from acetone using a micropipette, (b) to the sand or alternatively to a standard soil, (c) directly to the water, or (d) in a "model feed lot" modification (Coats et al 1976), by administration in feed of 3 white Swiss mice or 3 baby chicks housed in a 10 cm x 18 cm x 27 cm wire mesh cage located midway over the sloping shelf of sand so that their excretory products containing the radiolabeled parent drug and degradative products is distributed over the terrestrial "farm" and the "lake" of the model ecosystem. This "feed lot" model ecosystem is illustrated in Figure 1. More than 200 radiolabeled xenobiotics have been evaluated in these model ecosystems and the radiolabeled xenobiotic is typically applied to the Sorghum using 0.5 to 5.0 mg per chamber, that is equivalent to 0.1 to 1.0 lb. per A. (1.1 kg/hc) or applied to the 4 x 10 in. terrestrial shelf of the "farm". Typically, 10 5th instar salt marsh caterpillar larvae Estigmene acrea larvae are added to the system after treatment with the radiolabeled xenobiotic to consume the treated Sorghum leaves and to serve as the dispersing agent for the radiolabeled pollutant. After the model ecosystem has equilibrated for 26 days, 300 Culex pipiens larvae are added to the "lake" and after 30 days, three Gambusia affinis mosquito fish. These complete the food chain transfer of the xenobiotic and its degradation products. The experiment is concluded after 33 days when the water, sand, and organisms

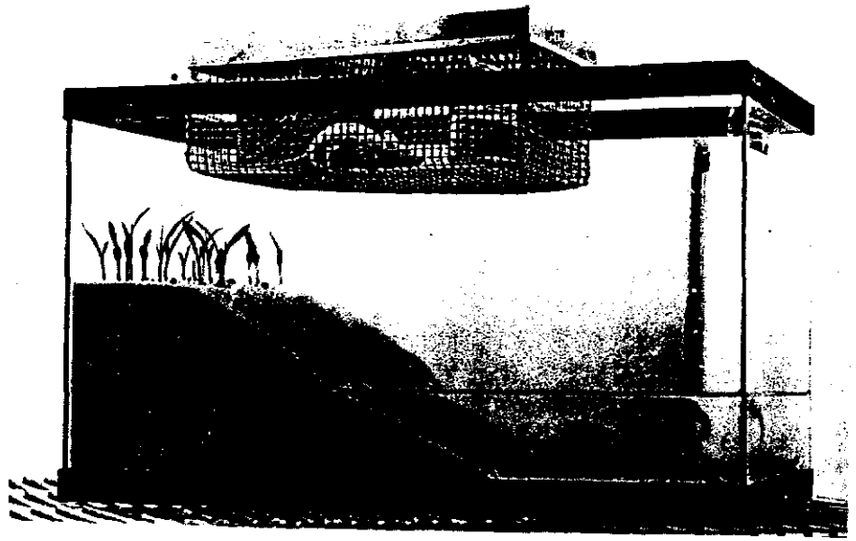

Fig. 1. Terrestrial aquatic model ecosystem with "feed lot" modification for evaluating environmental effects of veterinary drugs and feed additives (after Coats et al 1976).

are removed and weighed, total radioactivity determined, and aliquots extracted for TLC and radioassay.

RESULTS

Three xenobiotics with estrogenic properties of interest to this Symposium have been evaluated in the laboratory model ecosystem, i.e. diethyl stilbestrol (DES), methoxychlor, and chlordecone (Kepone^{R}). The results of the model ecostem evaluations will be reviewed briefly.

Diethylstilbestrol. This compound has been widely used as a feed additive to promote weight gain in cattle. DES is customarily administered at about 0.1-0.2 mg per kg per day and it is estimated that about 60,000 kg were used annually for this purpose in the United States. The environmental contamination from a cattle feed lot with ca 100,000 animals could approximate 1 to 2 kg per day. This situation was modeled in the "model feed lot" shown in Figure 1 (Coats et al 1976) by administering ^{14}C (monethyl-1-^{14}C),-DES, 52 mCi/m mole,

>98% radiopurity, orally in olive oil to 3 white mice at 0.5 mg/kg and allowing them to excrete into the model ecosystem for 3 days. Over this period, 95% of the administered drug was excreted, 61% as intact DES and 14% as polar conjugate (Coats et al 1976). At the conclusion of the experiment the distribution of ^{14}C DES and its degradation products was determined as summarized in Table 1.

The results indicate that DES is absorbed and stored in the organisms of the model ecosystem in appreciable quantities even after passage through the white mouse. The values for ecological magnification (E.M.) are low, snail Physa 36 and fish Gambusia 36. The biodegradability index (B.I.) values are correspondingly high snail 2.0 and fish 0.76. The comparative values for DDT were snail E.M. 34,500, B.I. 0.045, and fish E.M. 84,500, and B.I. 0.015 (Kapoor et al 1973). The model ecosystem behavior of DES reflects its H_2O solubility of 0.42 ppm and its relatively low octanol/H_2O partition coefficient 302 (Coats et al 1976). The phenolic groups of the parent molecule provide for ready conjugation and elimination of the molecule from the organisms.

Methoxychlor. This insecticide, 2,2-bis-(p-methoxyphenyl)-1,1,1-trichloroethane, is a biodegradable substitute for DDT. Methoxychlor is readily degraded in vivo by O-demethylation to produce 2-(p-hydroxyphenyl)-2-(p-methoxyphenyl)-1,1,1-trichloroethane, and 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (Kapoor et al 1970). This latter compound has obvious stereochemical similarities to diethylstilbestrol and to estradiol and is the probable source of the estrogenic properties of methoxychlor (Bittman and Cecil 1970). It is estimated that about 10 million lb. of methoxychlor is used annually as an insecticide for animal parasites and for home and garden. The model ecosystem fate of methoxychlor was investigated by Kapoor et al (1970) using ^{3}H ring-labeled compound, specific activity 4.52 mCi per m mole, radiopurity >99.9%. The radiolabeled methoxychlor was applied directly to the Sorghum plants of the model ecosystem at 1 lb. per A. The results are shown in Table 2.

Methoxychlor is substantially degradable in the fish Gambusia but is stored in relatively large amounts in the snail Physa. The E.M. values are fish 1545 and snail 120,000 and the B.I. values are fish 0.94 and snail 0.13 (Kapoor et al 1973). The phenolic in vivo O-demethylation products of methoxychlor are, like DES, subject to facile conjugation and elimination by the organisms of the model ecosystem (Table 2). Thus the parent molecule methoxychlor, H_2O solubility 0.62 ppm and octanol/H_2O partition coefficient 2,050 (Kapoor et al 1973) can be viewed as a lipophilic molecule that is readily absorbed from water by aquatic

TABLE 1

MODEL ECOSYSTEM FATE OF ^{14}C-DIETHYLSTILBESTROL[a/]

		DES equivalents - ppb				
		H_2O	Oedogonium (alga)	Culex (mosquito)	Physa (snail)	Gambusia (fish)
total extractable ^{14}C		0.115	9.2	20.8	11.5	12.6
unknown I	(R_f 0.90)[b/]	--	--	2.2	0.5	1.1
unknown II	(R_f 0.73)	--	1.8	1.6	1.4	2.4
unknown III	(R_f 0.63)	--	1.9	--	1.3	2.9
DES	(R_f 0.60)	0.0195	3.2	--	0.7	0.7
unknown VI	(R_f 0.43)	0.0124	--	5.7	1.8	0.9
unknown VII	(R_f 0.35)	0.0041	--	1.9	1.3	2.2
unknown VIII	(R_f 0.30)	0.0030	2.3	5.0	1.4	--
unknown XI	(R_f 0.10)	0.027	--	1.7	0.8	1.1
unknown XII	(R_f 0.05)	0.026	--	--	1.0	--
polar	(R_f 0.0)	0.022	--	2.7	1.4	1.2
unextractable		0.298				

[a/] Coats et al 1975

[b/] TLC with benzene:acetone (7:3)

TABLE 2

MODEL ECOSYSTEM FATE OF ^{3}H-METHOXYCHLOR [a/]

		methoxychlor equivalents - ppm			
		H_2O	Culex (mosquito)	Physa (snail)	Gambusia (fish)
total extractable ^{3}H		0.0016	0.48	15.7	0.33
$CH_3OC_6H_4C(=CCl_2)C_6H_4OCH_3$	(R_f 0.32)[b/]	--	--	0.7	--
$CH_3OC_6H_4HC(CCl_3)C_6H_4OCH_3$	(R_f 0.25)	0.00011	--	13.2	0.17
$CH_3OC_6H_4HC(CCl_3)C_6H_4OH$	(R_f 0.07)	0.00013	--	1.0	trace
$HOC_6H_4HC(CCl_3)C_6H_4OH$	(R_f 0.0)[c/]	0.00003	--	trace	trace
$HOC_6H_4HC(CCl_3)C_6H_4OH$	(R_f 0.0)[c/]	0.00003	--	--	--
unknowns		0.00009	--	trace	trace
polar	(R_f 0.0)	0.00125	--	0.8	0.16

[a/] Kapoor et al 1970

[b/] TLC with ether:hexane (1:1)

[c/] separated by TLC with hexane:chloroform:methanol)3:2:1)

organisms with subsequent in vivo conversion to estrogenic metabolites.

Chlordecone. This insecticide (Kepone ^{R}) is decachloro-octahydro-1,3,4-methano-2H-cyclo buta-(cd)-pentalene-2-one. It was widely used as a stomach poison bait for cockroaches and ants, and for thrips control on bananas. Production in 1975 was about 846,000 lb. Chlordecone has been shown to have estrogenic properties in laboratory animals (Eroschenko 1978) and in wildlife and humans exposed to effluents during manufacturing where an estimated 100,000 lb. contaminated the bed of the James River in Virginia. The model ecosystem fate of chlordecone was followed using gas-liquid chromatrography. The Sorghum plants were treated topically with 1.0 mg. of 99% pure chlordecone. After 33 days, the system was worked up in the standard way. The components contained the following concentrations of chlordecone: water 0.0206, alga 8.007, mosquito larva 55.458, snail 104.241, and fish 28.584 ppm. No degradation products were detected. The E.M. values for chlordecone were: snail 5066 and fish 1389, and the B.I. values were <0.001. Chlordecone is therefore, a highly persistent bioaccumulative pollutant than can be passed through aquatic food chains. Chlordecone has an octanol/H_2O partition of 4460 but has a relatively, high water solubility, >4000 ppm at 100°C, because of the facile hydration of the C=O group. This limits its bioconcentration to a moderate range. The model ecosystem results are in general agreement with the real world experience from chlordecone contamination of the James River and Chesapeake Bay where the compound has been shown to be highly persistent and bioaccumulative in a variety of organisms. These properties in combination with its estrogenic action, neurotoxicity and carcinogenicity make chlordecone a particularly dangerous environmental pollutant.

DISCUSSION

The laboratory model ecosystem provides a useful evaluation of the environmental fate of a very wide variety of organic xenobiotic compounds including estrogens. Model ecosystem studies provide reliable indicators for bioaccumulation, biodegradation, and degradative pathways in a variety of organisms. The data is useful quantitatively as well as qualitatively as shown in Figure 2 where log E.M. is plotted against log octanol/H_2O partition coefficient. There is clearly a highly precise relationship that has been confirmed for more than 200 xenobiotic compounds. The estrogenic substances chlordecone, methoxychlor, and DES conform closely to this relationship and it can be used to predict the propensity for bioaccumulation and food chain transfer of all estrogenic substances.

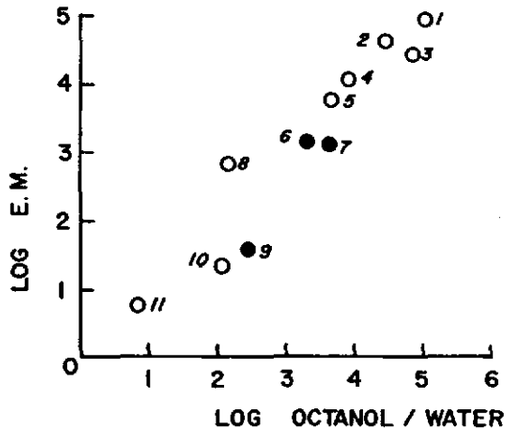

Fig. 2. Results of model ecosystem studies showing relation of log ecological magnification (E.M.) for the fish Gambusia vs log octanol/water partition coefficient. Estrogenic substances (solid circles) 6-methoxychlor, 7-chlordecone, 9-DES conform to general direct relationship as established with 1-DDT, 2-hexachlorobiphenyl, 3-DDE, 4-tetrachlorobiphenyl, 5-dieldrin, 8-chlorobenzene, 10-anisole, and 11-aniline.

ACKNOWLEDGMENTS

The writer gratefully acknowledges the assistance of Dr. Syed Ali and Dr. Po-Yung Lu in unpublished studies on the model ecosystem fate of chlordecone and in the determination of partition coefficients.

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DISCUSSION

GORSKI: Have you used the model ecosystem to study plant estrogens or other naturally occurring estrogens? These estrogens are likely to make up a larger environmental load or baseline than DES. The additional estrogen load due to DES should be compared with the baseline load.

METCALF: We have not made such studies of naturally occurring estrogens (e.g., Fusarium estrogens), but they would be very informative. A major difficulty is obtaining appropriate radiolabeled preparations of the estrogenic compounds.

RAO: In our environment, we have multiple chemicals simultaneously present at any one place. Have you considered studying the effect of multiple chemicals in your model ecosystem? Such experiments may provide a measure of the environmental load of chemicals and also may discern differential accumulation, if any, of one chemical over the other.

METCALF: We have begun to study effects of combinations of xenobiotics, (e.g., benzopyrene and piperonyl butoxide). There was a major change in the amount and nature of metabolites formed and stored in the animals of the model ecosystem. We have also studied effects of combinations of pesticides (e.g., the insecticide, carbaryl, and the herbicide, propanil). Major interactive effects were demonstrated. The laboratory model ecosystems are very well suited to measure and characterize such interactive effects.

SHIMODA: Is it possible to test the relative toxicities of certain compounds in your ecosystems?

METCALF: The laboratory model ecosystem is designed to produce information on the relative toxicities and ecological effects of xenobiotics. We have published a considerable amount of data on this subject.

KNIGHT: When you tested DES in your model ecosystem, did the sand layer contain any organic matter or any other source of enzymes such as polyphenolases? If not, what was the purpose of an inert "soil" layer which is unlike any soil in this country where DES is administered to livestock?

METCALF: After much thought, we standardized on sterilized sand as the most reproducible substrate for comparing a variety of xenobiotic compounds. The use of sand also provides the most severe test for evaluation of the test compound. If it is biodegradable under the standard test, this is a very positive piece of information. Supplementary information has been obtained by replacing the sand with two major soil types. The ultimate spectrum of characterization of xenobiotic compounds should perhaps involve both sand and soil. Nevertheless, the evaluation with the standard system permits us to state that any test compound has a relative environmental behavior compared with such widely evaluated xenobiotics as DDT, dieldrin, individual PCB isomers, DES, etc.

WEISZ: As I understand it, biodegradation is defined in terms of polar metabolite formation. This clearly diminishes the danger of these toxic substances becoming concentrated in lipids. However, as a reproductive biologist interested in development, I am concerned that these polar, water-soluble metabolites that are clearly accumulating in our environment might pose a special threat to the fetus. Fetuses are much more "watery" than the fully formed organism. Perhaps it is for this reason that steroids tend to be highly hydroxylated (i.e., polar), at least in the human fetus. Therefore, the polar derivatives of xenobiotics might pose a special threat to fetuses. It might be useful to introduce a pregnant mouse into your fascinating model ecosystem.

METCALF: This is a provocative suggestion. Clearly, highly polar metabolites such as 2,2-bis-(p-hydroxyphenyl) 1,1,1-trichloroethane produced from the insecticide, methoxychlor, represent exogenous estrogens that may be hazardous. Introduction of a pregnant mouse would be a logical way to evaluate this hazard. However, the "methodology" is not obvious.

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ESTROGENS AND ESTROGENIC ACTIONS IN FISH, AMPHIBIANS AND REPTILES

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INTRODUCTION

Estrogenic hormones appear to be ubiquitous in the vertebrate kingdom[1] and have been reported in invertebrate species and plants suggesting widespread occurrence of this molecular configuration in the biota.[2] Within the vertebrates, synthesis occurs in the gonads and brain of both male and female non-mammalian species.[3] In mammals, the placenta,[4] breast tissue, fat, adrenals, kidney, lung, bone, liver, thymus, muscle and skin[5] all have been found to synthesise estrogen. While the vertebrate ovary has been the major focus for studies of the regulated synthesis of these steroids, the importance of other sites of synthesis, particularly the brain, is difficult to deny on the basis of recent observations. Further, although the testicular synthesis of estrogenic steroids has been known for many years, it is only recently that studies on the importance of estrogens in male reproduction have been made.

The intent of this paper is to summarise the current status of knowledge of estrogen physiology in reptiles, amphibians and fish since the great bulk of information on estrogen physiology and biochemistry is derived from studies of relatively few mammalian species. It is hoped that this survey will aid in elucidating unifying principles applicable to all vertebrates and the identification of useful non-mammalian animal models for experimental study of specific problems.

SITES OF ESTROGEN SYNTHESIS

This subject has been thoroughly reviewed[6] and the following will serve only to summarise the major findings of the recent literature.

The Ovary. Estradiol 17β (E_2) and estrone (E_1) have been identified in tissue extracts of the most primitive vertebrates, the agnatha[7] and the biosynthesis of estrone and estradiol 17β have recently been demonstrated in ovarian homogenates of *Petromyzon marinus*.[8] In *Myxine glutinosa*, ultrastructural and steroid enzyme histochemical studies suggest that the follicle wall is the source of the steroids.[9] Similar studies of elasmobranchs, teleosts, and amphibians have been reviewed[6] and support the existence of estrogen synthesising ability in the ovary of these groups. Studies of reptiles are more

meagre but as in other vertebrates, biosynthetic studies have identified the ovarian follicle as the site of synthesis of estrogens.[10]

The Testis. Unlike the ovary, the testis has not been a frequently sought source of estrogens in non-mammalian species. However, recent studies[10] have demonstrated that testicular homogenates from the elasmobranch Squalus acanthias (spiny dogfish) and the urodele amphibian, Necturus maculosus (mudpuppy) convert androgen to estrone and estradiol 17β. In testicular homogenates from Necturus aromatase activity was much greater than in ovarian homogenates at the time of year at which the experiments were performed.

Central Nervous System. Since 1971, when aromatase activity was first demonstrated in the human fetal brain[11] this enzyme has been identified in several other mammals and in a variety of other species.[3] Thus, bird,[10] turtle,[12] snake,[10] bullfrog,[13] mudpuppy, sculpin, skate and dogfish,[10] all converted androgen to estrogen. Aromatase activity was extremely high in the teleost fish (Myoxocephalus octadecimspinosus) and amphibian (Necturus maculosus), both species in which gonadal aromatisation was low or negligible at the time of the experiment. The contribution which the central nervous system makes to peripheral estrogen levels remains to be determined.

PLASMA LEVELS OF ESTROGENS AND ESTROGEN BINDING PROTEINS

Plasma estrogen levels (Table 1)

The literature on blood estrogens in vertebrates other than mammals prior to 1972 has been reviewed.[6] At this time, the reports were 12 in number (5 in fish, 4 in amphibia). All identifications involved chromatographic techniques, but for the most part the identity of the estrogen was tentative in nature.

Since 1972, a number of reports have been added using radioimmunoassay and a reasonable assessment of the cyclic changes of plasma estrogens exists in one teleost fish, Pleuronectes;[19] an amphibian, Rana esculenta;[25] and two reptiles, the fresh-water turtle, Chrysemys picta;[27] and water snake, Natrix.[28] In the case of the fresh-water turtle, plasma levels of estradiol correlate well with levels of vitellogenin.

Plasma estrogen binding proteins (Table 2)

Sex hormone binding globulins (SHBG) have been identified in cyclostomes, elasmobranchs, teleosts and amphibians. Two high affinity binding proteins, which bind E_2 and progesterone (P) have been identified in the plasma of the lamprey Petromyzon marinus.[29] Among the elasmobranchs, a SHBG has been charterized in the skate Raja radiata[30] and in the dogfish Scyliorhinus canicula.[31] The elasmobranch SHBG binds estrogen, androgens and progesterone. The relative binding affinities are E_2 > progesterone > dihydrotestosterone (DHT) testoster-

Table 1. Plasma Estrogens in Non-Mammalian Vertebrates

Species	Method	Estradiol-17β	Estrone	Reference
FISH		Plasma Levels in ng/ml		
Scyliorhinus caniculus	CH	0.3 - 0.04	0.05 - 0.1	14
Torpedo marmarata	CH		0.8 - 2.96	15
Anguilla anguilla	CH	1.5 - 1.7	2.5 - 3.6	16
Conger Conger	CH	1.1 - 2.2	0.7 - 3.2	16
Murena helana	CH	7.2	11.7	16
Cyprinus carpio	CH	0.5 - 2.5	1.5 - 4.5	16
Salmo salar	CH	0.2 - 2.2	0 - 0.45	17
Ictalurus punctatus	CH	2 - 14	1.5 - 5.7	18
Pleuronectes platessa L.	CH	detected	detected	19
AMPHIBIANS		Plasma Levels in ng/ml		
Pleurodeles wahtlii	CH	0.6	0.6	21
Xenopus laevis	CH	Total estrogens = 40		22
Rana temporaria	CH	3 - 5	10.0	23
Rana esculenta	CH	110.0 - 230.0		24
Rana esculenta	IA	ND - 1.0	ND - 4.0	25
Necturus maculosus	(f) RIA	0.4 - 6.3	ND	26
	(m) RIA	1.9 - 9.8	ND - 1.87	
Rana catesbeiana	(f) RIA	0.2 - 0.8	0.1 - 0.2	3
	(m) RIA	0.2	0.1 - 0.2	
REPTILES		Plasma levels in pg/ml		
Chrysemys picta	RIA	ND - 1200	-	27
Natrix fasciata	RIA	10 - 540	-	28
Chelydra serpentina	RIA	13 - 284	-	20

ND - not detectable
D - detected
CH - Chromatographic
RIA - Radioimmunoassay
(f) - female
(m) - male

one (T) > corticosterone (B); diethylstibestrol binds poorly. The capacity of the SHBGs in elasmobranchs are on the order of 10^{-6}M, 100 times the capacity of SHBG in man. This elevated capacity is commensurate with the elevated levels (10^{-7}M) of circulating sex steroids in elasmobranchs. At present, the agnathans and elasmobranchs are the only vertebrates reported to have a single binding protein for the C_{18} and C_{19} sex steroids, and the C_{21} steroids.

Among the teleosts, SHBGs which bind E_2 and T with high affinity (K_A $10^8 M^{-1}$), have been demonstrated in the Atlantic salmon Salmo salar, the halibut Hippoglossus hippoglossus,[6] and the rainbow trout Salmo gairdnerrii.[32] The binding capacity of the teleost SHBG is in the range of 10^{-6}M, while circulating steroid levels are in the range of 10^{-7}M.[19, 20] Thus a large percentage of the total circulating steroid is still bound to a high affinity binding protein. In many teleost species, 11-ketotestosterone appears to be the major circulating androgen. In the Atlantic salmon, SHBG binds 11-ketotestosterone with a K_A = 1 x $10^7 M^{-1}$, but it is not clear whether testosterone and 11-ketotestosterone compete for the same binding site.[6] The large binding capacity of SHBG in teleosts is sufficient to bind a large percentage of the total circulating steroid.

In amphibians, high affinity binding proteins for E_2 and T have been demonstrated in the urodeles Pleurodeles waltlii and Salamandra salamandra, and in the anurans Discoglossus pictus and Rana temporaria.[33,34] Binding capacities are in the range of 3-15 x 10^{-7}M, commensurate with high circulating levels of sex steroid (1-10 x 10^{-7}M in urodeles, 1^{-8}M in anurans).[23]

Although a "medium" affinity testosterone-binding protein is present in the snake,[38] in the fresh-water turtle, Chrysemys picta, we have found an SBP which binds E_2, T, DHT and P (K_A = 2 x $10^8 M^{-1}$) sedimentation coefficient 6-7S.[39]

PHYSIOLOGIC EFFECTS OF ESTROGENS

Vitellogenesis

The subject of vitellogenesis in vertebrates has been extensively reviewed in the last 10 years.[40,41,42,43,44,45] In summary, vitellogenesis is the process of hepatic synthesis and secretion of yolk protein and its transport via the blood stream, and subsequent deposition in the oocyte. This occurs in all submammalian vertebrate classes. During this process, large amounts of protein and fat are mobilized and these changes are reflected in increased plasma levels of phospholipoprotein, lipid, and calcium during the breeding season of fish,[46,47] amphibia,[48,49] reptiles,[50,51] and birds.[52] Liver hypertrophy[50,53,54,55,56,57] is a corollary of vitellogenesis. In all submammalian vertebrates tested, estrogen treatment of either non-vitellogenic females or males cause changes similar to those observed during the natural breeding season as indicated above.[42]

The most detailed knowledge of this process and the role of estrogen comes from studies with the South African clawed toad, Xenopus laevis, and the domestic hen, Gallus domesticus. In the last two years, however, new information has appeared in the literature concerning vitellogenesis in elasmobranchs and reptiles. This new work will be summarized below and compared to the knowledge of amphibian and avian processes.

In elasmobranchs, the first demonstration of an estrogen effect was that of Woodhead[58] who described an increase in plasma calcium after estrogen treatment in Scyliorhinus canicula. Subsequently, Craik[59] has monitored the appearance of vitellogenin in blood and its subsequent disappearance and conversion into yolk granules in the same species using an isotopic method. Vitellogenin half-life times varied from animal to animal (132-303 hours, mean: 216 hours, at a water temperature of 7° ± 2°C) presumably due to differences in the stage of the reproductive cycle. Similar variation was found in the rate of vitellogenin synthesis. Estrogen-treatment (2.5 mg/ estradiol benzoate 13 days prior to P^{32} injection) of a male dogfish increased plasma vitellogenin to a value higher than in the normal mature females. Induction of vitellogenesis in nature appears to be temperature dependent since exposure to winter water temperatures in mid-summer (when ovarian weight is decreasing) stimulated vitellogenesis, but a winter photoperiod did not. Compared to similar parameters in Xenopus laevis, the rate of phosphoprotein synthesis in S. canicula is one tenth, plasma phosphoprotein levels about one fifth, and vitellogenin half-life approximately four times greater.

In a second study, Craik[60] has also measured plasma vitellogenin levels in female S. canicula by immunoassay. Vitellogenin is found in plasma throughout the year, the mean level of 0.4 mg/ml showing little seasonal variation except for a brief decline in plasma vitellogenin in October. This level of vitellogenin is one or two orders of magnitude lower than that typically found in other vertebrates during vitellogenesis. The lack of seasonal variation may be related to the fact that this species lays eggs continuously for 10-12 months. Craik[61] has demonstrated that the correlation between plasma estrogen level and "vitellogenin" is poor in Scyliorhinus, in contrast to the situation in Chrysemys (see below).

Our own data on Squalus acanthias (the spiny dogfish) are of interest. Using SDS-gel electrophoresis we have studied the level of vitellogenin in male and female dogfish in June and July. Plasma from male and early pregnancy (stage A; Hisaw and Albert, 1947[86]) female dogfish had no detectable vitellogenin but in late pregnancy females (stage C) with large (3-4 cm diameter) ovarian follicles, low levels of vitellogenin were detectable in some fish. Injection of estradiol

17β (4 x 1 mg) failed to induce a vitellogenin band in adult males and in stage A pregnant females injected with estradiol, levels of vitellogenin were undetectable or very low. By contrast, in stage C pregnant fish plasma vitellogenin increased progressively after estradiol injections and reached maximum on day 10 post injection (Fig. 1.) Since progesterone injections (4 x 2 mgs) reduced the effect of estrogen in stage C animals, it is possible that vitellogenesis in early pregnancy may be slowed by the presence of progesterone.

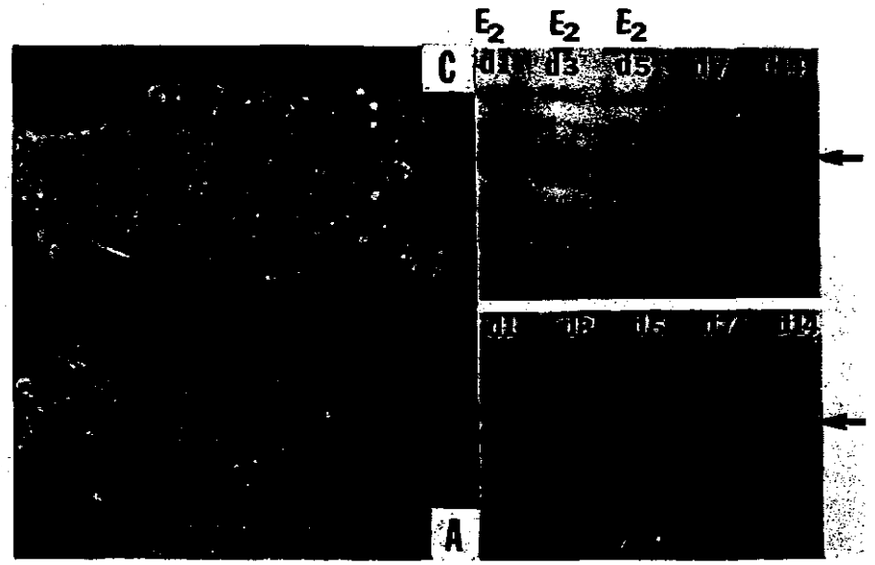

Fig. 1 Portion of the ovary and gel electrophoretic pattern of the plasma from different reproductive stages of the spiny dogfish Squalus acanthias. The left upper panel shows a portion of the ovary from a stage C pregnancy fish with growing follicles and left lower from a stage A pregnancy fish with small follicles. The right panel shows the electrophoretic pattern of the plasma from animals run on 0.1% sodium dodecyl sulphate, 7-15% polyacrylamide gels. Fish were injected with 3 x 1 mg of estradiol-17β in sesame oil and estrogen-induced vitellogenesis was observed only in stage C animal (above) and not in stage A animal (below).

In the reptiles, it appears that the endocrine control of vitellogenesis is similar to that of Xenopus and the domestic hen. Thus estrogen treatment induced liver hypertrophy and hepatic ultrastructural changes indicative of protein synthesis in snakes[50] and lizards.[62] Estradiol also stimulates RNA synthesis in the liver of squamates,[63,64] as in Xenopus laevis.[65]

The appearance of a unique plasma protein after estrogen stimulation has been demonstrated electrophoretically in snakes[52] and lizards.[66] Increased plasma protein and calcium levels have been observed in estrogen-injected, as well as naturally vitellogenic squamates.[50,66,67,57] In chelonians, Rao[69] noted an increase in the gamma globulin protein fraction of the serum after estrogen treatment in Testudo elegans. Hyperproteinemia and hypercalcemia also occur in female Chrysemys picta during the time of egg production and breeding, and in both male and female turtles during estrogen treatment.[51]

Recent studies in our laboratory have described the ovarian cycle of Chrysemys picta, which ovulates in late May or early June in Massachusetts.[70] From August to September most of the larger follicles in the ovary are atretic, but prior to hibernation in the fall, there is a 2 month period of vitellogenesis and ovarian growth, the largest follicles in the ovary reaching a size somewhat less than that of the mature preovulatory oocyte. On emergence from hibernation in spring, a second burst of vitellogenesis occurs, culminating in ovulation. Each of these period of vitellogenesis is associated with an increase in circulating estradiol 17β levels.[27] With the development of a radioimmunoassay for turtle plasma vitellogenin,[71] we have correlated high plasma levels of vitellogenin with estrogen peaks in spring and fall. Plasma vitellogenin levels drop precipitously in association with the periovulatory estrogen decline and progesterone surge.

Ovarian changes in squamates are more dramatic than in chelonians, with only a single, short period of intense yolk deposition prior to ovulation in the spring. Thus, in the snake, plasma calcium levels suggest a single narrow peak of vitellogenin production correlating with follicular growth;[50] this, in turn, correlates with increasing intensity of ovarian 3 beta hydroxysteroid dehydrogenase and an increase in oviduct mass[68] suggesting an increase in plasma estrogen level. Measurements of plasma estradiol 17β in the viviparous snake, Natrix, support these observations; snakes with the largest ovarian follicles having highest levels of estradiol, and snakes with follicles in the hydration stage the lowest.[28]

Few studies have investigated the possibility that hormones other than estrogen may be involved in the control of vitellogenesis. Follett, Nicholls and Redshaw[41] found that hypophysectomy did not alter the vitellogenic response of

Xenopus to estradiol 17β . In contrast, in the lizard Dipsosaurus dorsalis, Callard et al.,[64] were unable to elicit the usual estrogen related changes in liver weight, RNA/DNA ratio or plasma protein concentrations in hypophysectomized animals unless growth hormone was injected also. In keeping with the observations of Follett and Redshaw,[41] Wangh and Knowland,[72] and more recently, Green and Tata,[74] successfully cultured fragments of Xenopus liver and obtained in vitro synthesis of vitellogenin in response to estradiol 17β added at physiologic concentrations.

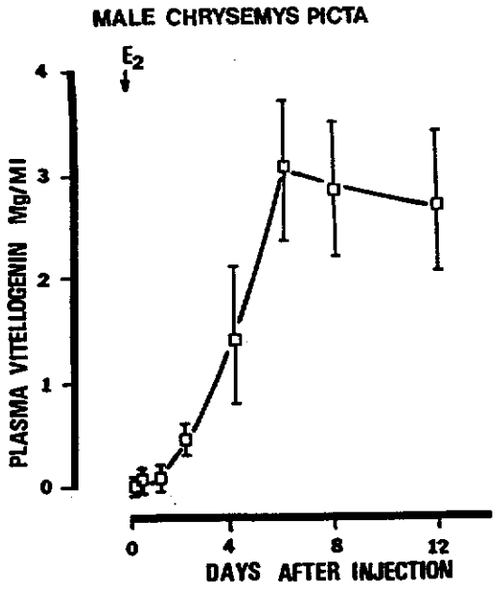

Fig. 2 Plasma vitellogenin level in male turtles as a function of time after receiving a single dose of estradiol-17β (100 ug per 100 g body weight). Plasma vitellogenin was measured by specific radioimmunoassay and results were expressed in mg vitellogenin per ml plasma as mean ± S.E.M. (n = 5).

Using the radioimmunoassay for vitellogenin in the fresh-water turtle, Chrysemys picta, we have documented the time course of induction of vitellogenin in male and female turtles. Vitellogenin was first detected in male turtle

plasma 8 hours after a single injection of estradiol 17β and reached a maximum (approximately 3 mg/ml for a dose of 1 mg/Kg body weight) at day 6 (Fig. 2.). Female turtles receiving the same dose demonstrated a ten times greater response and reached a maximum of approximately 30 mg/ml plasma at 10-14 days after estrogen administration. The level of vitellogenin in the plasma of estrogen-treated females was comparable to that found in female turtles during the peaks of vitellogenesis in the natural gonadal cycle. The estrogen-induced vitellogenic response is dose-dependent and estrogen specific (Fig. 3.). Non-estrogenic

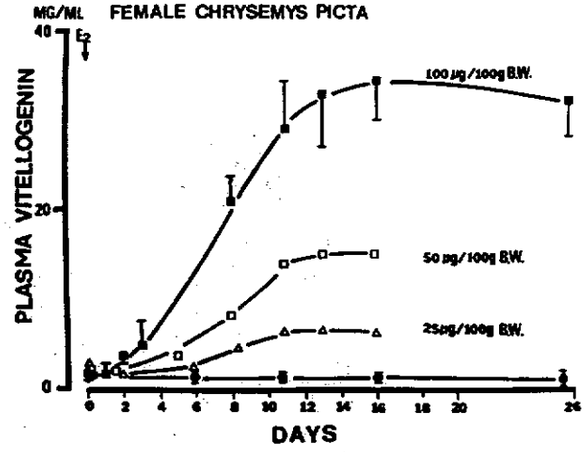

Fig. 3 Plasma vitellogenin level in female turtles, Chrysemys picta as a function of time after receiving a single dose of 100 ug (■—■), 50 ug (□—□), or 25 ug (△—△) estradiol-17β per 100 g body weight. ⊢●⊣ , mean ± S.E.M. (n =);

steroids, including high doses of androgens, failed to reproduce the estrogen action. When given multiple injections of estrogen, the level of vitellogenin in male turtles reached that of singly injected female turtles. Progesterone (5 mg/Kg body weight) delayed and suppressed estrogen-induced vitellogenesis, (Fig. 4.) but lower doses of progesterone (.75 - 2.5 mg/Kg body weight) were ineffective. In contrast to progesterone, testosterone inhibited estrogen action at the lowest dose, and was ineffective at the highest dose used (Fig. 5.). This may be explained by aromatization of the androgen to estrogen in this species.[3] When the rate of vitellogenin production in vivo was measured by P^{32} incorporation and secretion of labelled plasma protein, hypophysectomy

reduced the rate of appearance of P^{32}-labelled protein in the plasma of estrogen-treated male and female turtles. In addition, the maximal level of P^{32}-labelled protein reached in the plasma of hypophysectomized males was only half that in intact males. Similar, though less marked effects were noted when normal and hypophysectomized females were compared, possibly due to changes in ovarian uptake (Fig. 6.). In both sexes, growth hormone injections improved the estrogenic response in hypophysectomized animals. These data suggest a pituitary role in the regulation of vitellogenesis in turtles as has been suggested for lizards.[75,64]

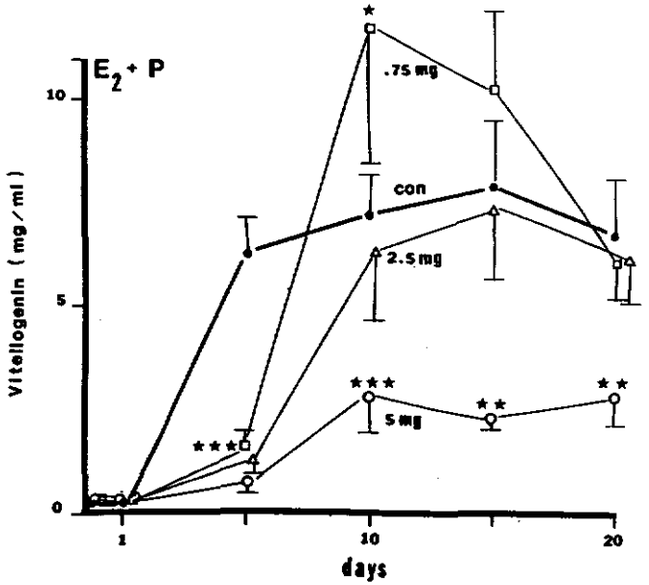

Fig. 4 Plasma vitellogenin level in male turtles, Chrysemys picta as a function of time after receiving a single injection of estradiol-17β (1 mg/Kg body weight) and various doses of progesterone. Control animals received estradiol-17β only, ●—● . Three doses of progesterone were given : □—□, 0.75 mg/Kg body weight; △—△ 2.5 mg/Kg body weight. Results expressed as mean ± S.E.M. (n = 5). Symbols **, and ***: statistically different from control values at the levels of $p<0.01$ and $p<0.001$.

More recently, we have investigated the in vitro synthesis of vitellogenin by turtle liver explant cultures. The rate of vitellogenin synthesis reached a maximum 5 days after estrogen 17β administration (minimal effective dose - 10 ug/100 g body weight). In animals injected with 5 mg/100 g body weight, hepatic vitellogenin production represented 80% of total protein secreted during the peak of vitellogenin synthesis. In in vitro studies over 15 days, using liver explants from castrated female turtles in completely defined media it was demonstrated that vitellogenesis can be readily induced by addition of estradiol 17β (10^{-6} - 10^{-8} M) to the medium. In contrast, in similar studies using male liver explants in complete medium with or without fetal calf serum and a variety of other additives, the synthesis of vitellogenin was not demonstrated.[76]

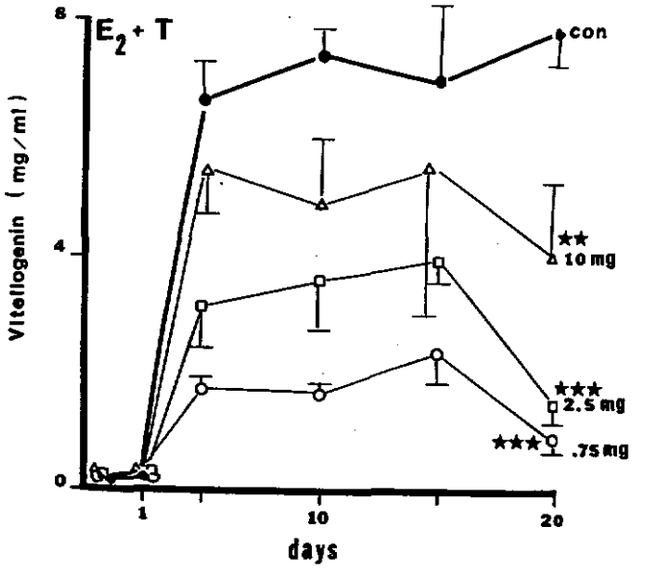

Fig. 5 Plasma vitellogenin level in male turtles, Chrysemys picta as a function of time after receiving a single injection of estradiol-17β (1 mg/Kg body weight) and various doses of testosterone. Control animals received estradiol-17β only, ●—● . Three doses of testosterone were administered: ○—○ , .75 mg/Kg body weight; □—□ , 2.5 mg/Kg body weight and △—△ , 10 mg/Kg body weight. Results expressed as mean ± S.E.M. (n = 5). Statistical differences indicated as in Fig. 4.

Effects of estrogens on the brain and expression of sex related behaviors

Estrogens have long been recognized to have important regulatory actions on the endocrine system through the central nervous system. In addition, behavioral patterns are sex and estrogen related in many vertebrate species.[77] As noted above, estrogens are also synthesised in the brain and probably have

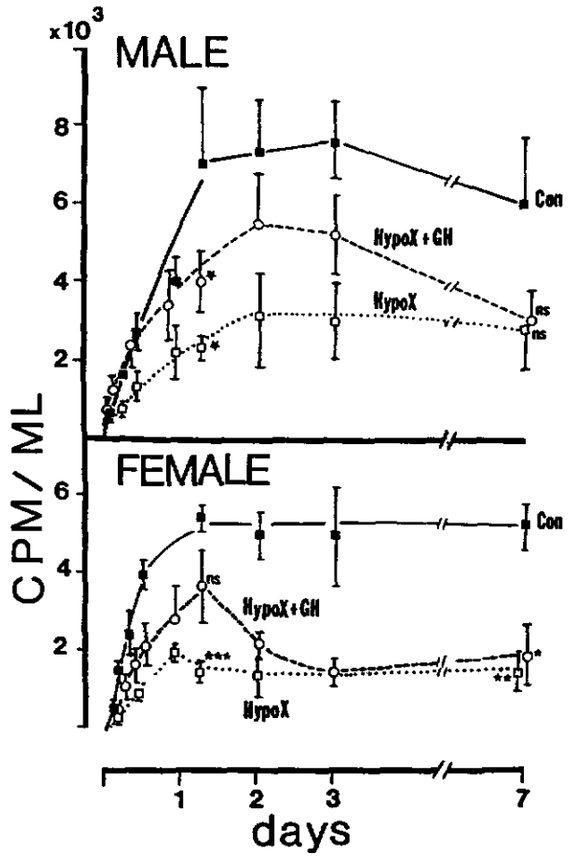

Fig. 6 Rate of p^{32} incorporation into plasma protein in estrogen-treated male and female turtles. All animals received six injections of estradiol -17β (1 mg/Kg/body weight) on alternate days. One group of hypophysectomized animales received daily injections of growth hormone (100 ug NIH GH-B_1). On day 12 after the first estrogen injection, 10 uCi p^{32} was injected i.m. to each turtle. Controls (E_2 only): ■——■; hypophysectomized and E_2: □——□ ; hypophysectomized and E_2 plus GH: ○——○ . Results are expressed as mean $\pm$ S.E.M. (n = 5); *, **, ***, indicates significantly different from control values (E_2 alone) at either p<.05, <.01 or <.001. NS - not significant.

important actions when introduced into the brain in a metabolic fashion.[3] A number of investigations, particularly in teleost fish (for review, see [6]) have demonstrated that estrogen injections prevent gonadal growth. Although a central nervous or pituitary site of action of estrogen was accepted as

possible, this was not directly demonstrated in a poikilothermic species until Lisk[78] observed that estradiol 17β implanted directly into the hypothalamus of male or female lizards (Dipsosaurus dorsalis) inhibited seasonal gonadal recrudescence. This observation was confirmed and extended in female Sceloporus cyanogenys. In this species, estradiol implants did not interfere with seasonal gonadal growth once begun, but prevented ovulation in lizards with mature oocytes.[79] Similar direct observations of an estrogenic negative feedback component in the regulation of ovarian function in amphibians and fish is currently lacking, although the involvement of the hypothalamus in gonadal regulation is quite clear.[80,81,82] Further, it is unlikely that the regulatory actions of estrogen are restricted to the hypothalamus since pituitary binding of labelled estradiol has been demonstrated in fish.[83]

That estrogen sensitive brain areas exist in non-mammalian species is supported by studies involving autoradiographic localization of sex steroid binding regions of the brain. Thus Morell and Pfaff[83] have demonstrated estradiol binding cells in the ventral telencephalon, preoptic area, and hypothalamus following administration of H^3 estradiol 17β in fish. Similar observations have been made in other fish,[84] two amphibians,[85] a lizard,[83,84] and a turtle.[84] Thus, in these species, as in mammals, areas of estradiol 17β concentration are predominantly in the anterior brain stem and basal forebrain. In general, no sex differences in the distribution of estradiol 17β binding neurons have been observed.

Although it is clear that labelled estradiol 17β can be taken up by neurons after peripheral injection, it is also clear that estrogens may be synthesized in situ from androgens by an active aromatising system found in representatives of all vertebrate classes (see above and Callard, G. V. et al.[3]) Although cytoplasmic and nuclear estrogen receptors have not been investigated in the brain of non-mammalian vertebrates, the autoradiographic studies described above, as well as correlated biochemical studies of Kelley et al.,[88] suggest a binding of steroids to cell nuclei, if not cytoplasmic receptors. The relative importance of estrogen formed in situ from androgenic precursors (aromatisation) and estrogen obtained directly from blood remains to be clearly demonstrated. However, in some non-mammalian species, at least, the in situ conversion of androgen to estrogen appears to be important for the expression of certain behavioral patterns.

In general, sex behaviors abolished by castration can be restored by androgen treatment in males and estrogen treatment in females.[77] However, evidence exists which suggests that the behavioral effects of testosterone in rodents may be due to the in situ conversion of androgen to estrogen and dihydrotestos-

terone. Aromatase and other androgen transforming enzymes are present in the brain of representative species of all vertebrate classes,[3] and there are physiological and behavioral studies that support the view that central target organ metabolism may be a phylogenetically ancient component of androgen action on sex behavior. A direct test of the role of androgen metabolites in the expression of male sex behavior in Xenopus laevis was made by Kelley and Pfaff.[89] These studies found that the restorative effects of testosterone and dihydrotestosterone on masculine type behavior in castrates were comparable; but that E_2 was ineffective. In contrast to these studies, in castrated Rana pipiens,[90] restoration of clasping appears dependent upon aromatization since the restorative effects of testosterone were blocked by a steroidal aromatase inhibitor; further, estradiol alone was an adequate substitute for testosterone in allowing expression of this behavior. Studies of this nature have also been performed in the lizard Anolis carolinensis.[91] In brief, although T is more effective than DHT or E_2 alone in restoring sex behavior in castrated lizards, there is some evidence for an interaction of the two metabolites and potentiating effects of DHT on estrogen-induced copulatory behavior have been clearly demonstrated. Thus, there may be species differences in the relative importance of T and its central metabolites E_2 and DHT in restoring male type sex behavior in castrates. Furthermore, circulating androgen and central androgen metabolism may also have a significant function for female receptive behavior in numerous animal species including Anolis.[3]

Secondary sexual characters

The comprehensive review of estrogenic effects on the secondary reproductive characteristics of fish, amphibia and reptiles by Ozon[6] leaves no doubt as to the importance of this class of steroids in regulation of the reproductive system in these vertebrate classes. The effects range from stimulation or maintenance of oviduct weight in intact and castrate animals (amphibia, fish and reptiles) and stimulation of oviduct alkaline phosphatase levels (amphibia) and differentiation of the Wolffian duct (elasmobranchs), to induction of gonopodial growth (teleost fish), cloacal swelling (cyclostomes) and inhibition of androgen-dependent nuptial coloration (teleost fish). More recently,[92] we demonstrated that estradiol 17β injections increases the amplitude of contractions and decreased the rest period between contractions in turtle oviduct segments in vitro.

The lowest limits of sensitivity of various parameters of estrogen action in fish, amphibians and reptiles appear to be similar (10-30 ng/g body weight) if the dose of estrogen administered is expressed on the basis of body weight.[93] However, the duration of exposure to hormone varies widely, and use of shorter exposure periods and lower doses is necessary particularly in sharks and teleosts. On the basis of available studies, it appears that the mammalian uterus is more sensitive to estradiol than currently known estrogen sensitive systems in other vertebrates. With expansion of our knowledge of non-mammalia receptor systems the reasons for the apparent differences may become clear. I is possible, for example, that important differences in the hormone delivery systems of non-mammalian and mammalian species exist. This is certainly suggested by differences in the amount of circulating estrogen, estrogen binding proteins in plasma in non-mammalian species, and problems encountered by inves tigators in the identification of estrogen receptors in at least one estrogen sensitive system of non-mammalian species, the liver (see above).

MECHANISMS OF ACTION

All of the physiologic actions of estrogen discussed above are presumed to occur in association with a specific interaction with receptors in steriod sen sitive cells. Classically, receptors are endoplasmic proteins which bind the steriod with high affinity, low capacity and strict stereospecificity. The steriod-receptor complex thus formed is translocated to the cell nucleus and there binds to chromatin. This interaction alters the pattern of gene expression in the target cell. Although there is a plethora of studies on mammalian and avian intracellular receptors (for review, see [94,95,96]) very little information exists for reptiles, amphibians and fish.

In the Reptilia, we have examined estradiol binding in cytosol prepared fro turtle oviduct.[97] Using the conventional techniques of charcoal-adsorption an glycerol density gradient centrifugation, no receptor binding of estradiol 17β was evident in turtle oviduct cytosol. However, a binding protein similar to that from plasma (see above Section IIb) in specificity, affinity, and sedimentation coefficient and dissociation kinetics, was found. In the presence of such a high affinity, high capacity binding globulin (Table 2.), it was necessary to use DNA cellulose affinity chromotography in order to demonstrate rece tor binding. Using this technique an estrogen-specific, high affinity, low capacity, estrogen binding protein was demonstrated in turtle oviduct tissue. In these parameters, the turtle material was comparable to the estrogen receptors of mammalian target tissues.

In addition to our own studies, Botte and his co-workers[98] reported high affinity binding of both E_2 and T in oviduct cytosol from the lizard, Lacerta sicula. There are several other reports of steroid binding in target organs o non-mammalian species. D'Istria and co-workers[99] have reported binding of E_2 and T in cytosol prepared from the skin and thumb pads of amphibians. Cytosol

Table 2. Binding characteristics of sex-hormone binding globulin throughout the vertebrates.

	Ligands Bound	K_A (M^{-1})	Capacity (M)	Reference
1. Agnatha				
Petromyzon marinus	E_2	-	-	29
	P	-	-	
2. Chrondrichthyes				
a. Rajiformes				
Raja radiata	T	$5\text{-}8\times10^{7}$	$1\text{-}2\times10^{-6}$	30
Raja eglanteria	E_2,P,C*	-	-	
b. Squaliformes				
Scyliorhinus canicula	T	7.5×10^{7}	1.6×10^{-6}	31
	E_2	3.2×10^{8}	2×10^{-6}	
	P	$3\text{-}4\times10^{8}$	$1\text{-}2\times10^{-6}$	
	C	$3\text{-}6\times10^{7}$	1×10^{-6}	
3. Osteichthyes				
Salmo gairdnerrii	T	3×10^{8}	2.4×10^{-6}	32
	E_2	2.8×10^{8}	2×10^{-6}	
4. Amphibia				
a. Urodela				
Pleurodeles waltlii	T	5×10^{8}	1.7×10^{-6}	34
	E_2	1×10^{9}	5×10^{-7}	
Salamandra salamandra	T	9×10^{8}	1.2×10^{-6}	
	E_2	1×10^{9}	6×10^{-7}	
b. Anura				
Discoglossus pictus	T	8×10^{8}	1×10^{-6}	34
	E_2	4×10^{8}	1.4×10^{-6}	
Rana temporaria	T	1×10^{9}	3×10^{-7}	
	E_2	5×10^{8}	3×10^{-7}	
5. Reptilia				
Chrysemys picta	E_2	2.4×10^{9}	$4\text{-}8\times10^{-7}$	39
6. Mammalia				
Homo sapiens	T	$1\text{-}2\times10^{9}$	$4\text{-}8\times10^{-8}$	*109;110
	E_2	$4\text{-}6\times10^{8}$		

prepared from the skin of the newt, Triturus cristatus, contained a high affinity binder for E_2 (K_A = 5-9 x $10^8 M^{-1}$).

An appropriate tissue for an investigation of estrogen receptors is the non-mammalian liver, since this organ is a model system for the investigation of estrogen action. Despite this, cytosolic estrogen receptors have not yet been confirmed in adult bird liver, although present in nuclei.[100,101,102] Nevertheless, Lazier[103] has confirmed the existence of a cytosolic receptor in embryonic chick liver. Similarly, an estrogen receptor has been reported in nuclear extracts of amphibian liver[101] and recently in cytosol.[104]

It is of interest that in both bird[105] and amphibian liver cytosol, other non-specific steroid binding proteins of variable affinity have been reported, and these do not appear to be present in extracts due to blood contamination.[106] Similarly, in turtle oviduct we have found a high affinity but non-specific (binds E_2 P and T) protein. The possibility exists that such binding proteins are ultimately of vascular origin since perfusion does not eliminate CBG - or SBP - like proteins from homogenates or enzyme-dispersed washed cells.[107] Thus, these binding proteins may serve to transport and concentrate steroids both inside and outside the cell and regulate the relative concentrations of free steroids in the cytoplasm. Watson et al.[108] have recently shown that other steroids can competitively inhibit formation of the estrogen-receptor complex. Thus, SBP, by maintaining high levels of non-receptor bound estrogen, may be a critical factor in the expression of steroid action. In non-mammalian species with high SBG and high steroid levels (See Tables 1, 2.) the function of SBG may be of even greater importance.[106]

SUMMARY AND CONCLUSIONS

Examination of the recent literature from studies of non-mammalian species reveals that the synthesis of estrogen occurs in both testis and ovary. Further, levels of estrogen found in the plasma of these species may be several orders of magnitude greater than in mammals, a fact probably related to higher levels of sex steroid binding protein in plasma. In addition, although extragonadal sources of estrogen have been reported in mammals, the brain of certain lower vertebrates appears more active in synthesising estrogen than either homologous gonad or the brain of mammalian species. Based on data from studies of direct estrogen feed-back effects on the nervous system, as well as estrogen binding studies, it is clear that estrogen exerts important regulatory effects on reproduction through the hypothalamic-pituitary unit. This is supported by behavioral studies, which are estrogen and possibly aromatisation-dependent. As might be expected, a variety of secondary sexual characteristics are dependent

upon estrogen for their expression. These include synthesis of vitellogenic protein, growth of the reproductive tract and gonopodial growth. Cytoplasmic and nuclear estrogen receptors have been demonstrated in amphibian and reptilian tissues, suggesting a mechanism of action in common with estrogens in mammalian species. However, the difficulties encountered in the identification of putative estrogen receptors in amphibian (and avian) liver, along with the demonstrated presence of other less specific, but high affinity 'binding proteins' in liver and oviduct, suggest that alternate mechanisms of estrogen action may be encountered in non-mammalian forms. Such findings may also have application for mammalian studies. Because of both similarities and differences, the use of non-mammalian forms as model systems in which to study specific hormonal problems encountered in mammalian endocrinology is of value; in addition, such studies will aid in the elucidation of unifying principles applicable to all vertebrates. Of particular value as a model for understanding estrogen effects is the non-mammalian liver. This organ is markedly influenced by estrogen, responding by synthesis of a number of proteins, of which vitellogenin is most readily quantifiable. Since it is likely that estrogenic effects on the mammalian liver are a product of the evolutionary history of the vertebrates, an understanding of estrogen action in the regulation of hepatic metabolism in non-mammalian species is of great importance.

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DISCUSSION

HERTZ: We found in our work on avidin induction in the avian oviduct that both estrogen and progesterone were required. We also found that the turtle egg contains avidin. This leads me to ask whether you have found a progesterone binding protein in turtle oviduct.

CALLARD: Recent work with Dr. W. Leavitt, at the Worcester Foundation, indicates that a progesterone receptor is present in the turtle oviduct.

McLachlan, ed. Estrogens in the Environment

ESTROGENIC PROPERTIES OF DDT AND ITS ANALOGS

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INTRODUCTION

Early studies in mammals and birds with chlorinated hydrocarbons (CH's) of the DDT class* demonstrated that these compounds exhibit effects which could be interpreted as causing alterations of the hormonal state of the animal. In turn these effects by CH's appeared somewhat conflicting and could be attributed to either estrogenic, antiestrogenic or antiandrogenic actions[1].

The probable reasons for the somewhat confusing observations were due to several factors: (a) Studies were conducted with technical grade DDT preparations containing several DDT isomers; the p,p' isomer is the major component of technical grade DDT, however the o,p' isomer is usually present as a major contaminant (up to 20%); (b) p,p'DDT is a potent inducer of the hepatic microsomal cytochrome P-450 monooxygenase[2,3] which catalyzes the metabolism of numerous xenobiotics and steroids, among these estrogens and androgens. Hence enhanced metabolism of an estrogen or androgen (endogenous or administered) by pretreatment with p,p'DDT would probably result in a decrease in estrogen and androgen activity and likely would be interpreted as an antiestrogenic or antiandrogenic action of DDT; and (c) o,p'DDT yields a "mixed bag" of effects, i.e., it can elicit both an induction of hepatic monooxygenase and exhibit estrogenic action[4-7]; these two effects of o,p'DDT might at times work in opposing directions and thus yield confusing observations.

In fact, an attempt to use technical grade DDT as an inducer of monooxygenase to diminish the uterotropic activity of administered estradiol 17β (E_2) led to the serendipidous discovery that o,p'DDT is estrogenic[5,6]. This observation and that of Bitman et al.[7] prompted numerous studies which demonstrated that o,p'DDT is estrogenic in several species[1] and could produce in neonatal rats the so called "early imprinting" (i.e., persistent vaginal estrus and anovulation)[8].

*Abbreviations and common names: p,p'DDT [2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane]; o,p'DDT [2,2-bis(p-chlorophenyl, o-chlorophenyl)-1,1,1-trichloroethane]; p,p'DDE [2,2-bis(p-chlorophenyl)-1,1-dichloroethylene]; methoxychlor [2,2-bis(p-methoxyphenyl)-1,1,1-trichloroethane]; HPTE [2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane]; E_2 (estradiol-17β); cytosolic estrogen receptor (R_c); nuclear estrogen receptor (R_n); ornithine decarboxylase (ODC).

Due to its relatively short half-life in the environment and mammals[9,10], methoxychlor has been proposed as a potential substitute for DDT, dieldrin and chlordane[11,12]. However, the estrogenic activity of methoxychlor[6,13] might be of some potential toxicological concern. It was previously observed that technical grade methoxychlor is more estrogenic than the purified preparation[13,14], raising the possibility that the estrogenic activity of methoxychlor is primarily or solely mediated by contaminants. Also early observations demonstrated that HPTE (a didemethylated analog of methoxychlor) is estrogenic, sustaining estrus in ovariectomized rats[15]. Since HPTE appears to be a major metabolite of methoxychlor in mice and rats[10,16], the possibility that methoxychlor acts via its metabolite(s) has been considered.

In the current studies we addressed ourselves to the following questions:

1. Do chlorinated hydrocarbons behave like typical estrogens in female and male rats?
2. Is o,p'DDT estrogenic in humans?
3. Is o,p'DDT per se estrogenic or are its metabolites estrogenic?
4. Does o,p'DDT exhibit antiestrogenic activity?
5. Is methoxychlor per se estrogenic or is the estrogenic action due to contaminants and/or metabolites of methoxychlor?

In this presentation we would like to share with you our findings on attempts to resolve the above questions and to expose some of our frustrations that in many incidences we have not been successful in designing experiments which would "end all experiments" and thus have been hitherto unable to unequivocally answer all of the posed questions.

In view of the multifaceted nature of the experiments performed in these studies, we described only briefly the methodology used and provided references where the interested reader could consult for more detailed procedures.

METHODS

A. Assays Involving the Estrogen Receptor:

1. Sucrose gradient sedimentation analysis of the in vitro binding of ^{3}H-E_2, in the presence or absence of chlorinated hydrocarbons, to the cytosolic estrogen receptor (R_c) derived from rat uteri or testes or from human tumors[17,18].

2. Dextran coated charcoal analysis for construction of a Scatchard plot and determination of total binding sites, affinity constants (K_D) and inhibition constants (K_I)[18,19].

3. Determination of the change in distribution of uterine cytosolic receptor (R_c) and nuclear receptor (R_n). Performed after administration of a compound of interest (e.g., E_2 or o,p'DDT) to rats or after incubation of isolated uteri with these compounds[20,21]. R_c and R_n are determined by an "exchange" involving incubation with ^{3}H-E_2 at 30° and 37°, respectively as previously described[22,23].

4. Determination of the effect of metabolism in vitro on the distribution of uterine R_c and R_n performed by simultaneous incubation of a given compound (e.g., methoxychlor) with rat liver microsomes in the presence of uteri from immature rats[24].

B. Assay of Uterine Ornithine Decarboxylase

After the administration of the potential estrogenic compound to immature or ovariectomized rats, the rate of decarboxylation of [1-^{14}C]-ornithine is determined[25-27].

C. Determination of Metabolism of o,p'DDT or Methoxychlor in vitro

1. Examination of the formation of acidic (phenolic) metabolites[16]. [^{14}C]-o,p'DDT or [^{14}C]-Methoxychlor is incubated with rat liver microsomes and NADPH. Reaction is terminated by addition of base and the residual substrate is extracted with an organic solvent. The radioactivity remaining in the aqueous phase, which upon acidification is soluble in organic solvents, represents acidic metabolites.

With methoxychlor, the rate of demethylation is also measured by assaying the formaldehyde formed[16].

2. Determination of products formed from methoxychlor[16]. The base-soluble products (see above) were isolated, separated by tlc and high pressure liquid chromatography (HPLC). The products which were more polar than methoxychlor, contained a monodemethylated methoxychlor (MDM) and a didemethylated methoxychlor (HPTE) and at least two more polar metabolites. The structure of MDM and HPTE was assigned based on chromatographic mobility in tlc and HPLC (coinjection with authentic compounds) and by GC/MS of derivatized metabolites (to be published elsewhere).

RESULTS AND DISCUSSION

Do chlorinated hydrocarbon pesticides behave as typical estrogens?

1. Interactions with the uterine estrogen receptor

The observations that among DDT derivatives o,p'DDT stands out as being by far the most estrogenic[6,27,28], prompted investigations on the mechanism of o,p'DDT-mediated estrogenic action. Thus, Nelson[14] demonstrated in

vitro that the addition of o,p'DDT to rat uterine cytosol inhibits ^3H-E_2 binding. This study used a charcoal procedure to remove unbound ^3H-E_2 and measured residual radioactivity, however, did not establish whether o,p'DDT did in fact inhibit the binding of ^3H-E_2 to the 8S cytosolic protein (estrogen receptor, R_c). Subsequent studies by us and others, using a sucrose gradient sedimentation analysis, have shown that o,p'DDT interferes with the binding of ^3H-E_2 to the 8S R_c[18,28,29]; also it was shown that the inhibition by o,p'DDT appears to be competitive without alteration of the number of total E_2-binding sites[14,18], indicating that o,p'DDT does not destroy the E_2-binding sites on the receptor.

To determine whether o,p'DDT acts in vivo like a classical estrogen we examined whether the administration of o,p'DDT to rats affects the distribution of the cytosolic (R_c) and the nuclear (R_n) estrogen receptors. As expected of an estrogen, o,p'DDT lowered R_c and increased R_n. However, by contrast to E_2, the duration of this effect was substantially longer with o,p'DDT than with E_2 (see section "Are DDT analogs estrogens or also antiestrogens?"). Also, we observed that the incubation of o,p'DDT with isolated rat uteri causes transolaction of R_c into R_n (see section below "Is o,p'DDT *per se* estrogenic?"). These findings suggested that o,p'DDT behaves like an estrogen.

2. Interactions with the testicular estrogen "receptor"

DDT given to cockerels inhibited testicular growth and development of secondary sex characteristics[30] and subnormal reproductive activity was observed in male dogs after DDT feeding[31]. Thus, the possibility that o,p'DDT interacts with the testicular high affinity estrogen-binding protein[33,34], was examined. In fact, we observed that o,p'DDT inhibited the binding of ^3H-E_2 to the rat testicular cytosolic 8S-"receptor", as observed in a sucrose gradient sedimentation and Scatchard plot analysis; the K_I for o,p'DDT was 2.4 $\pm$ 0.1 μM[35]. The lack of inhibition of ^3H-E_2 binding by methoxychlor or p,p'DDE, like with the uterine R_c, indicates that only estrogenic DDT analogs bind to the testicular estrogen receptor. Thus, it is tempting to speculate that the abnormalities produced in males by DDT are in some fashion related to the o,p'DDT, present in DDT, interacting with the testicular estrogen receptor.

3. Ornithine decarboxylase

To obtain additional information on the estrogenic action of o,p'DDT, we directed our studies to effects by o,p'DDT and E_2 on uterine ornithine decarboxylase (ODC). The extremely short half-life of ODC in certain tissues

and the observation that this enzyme catalyzes the key step in biosynthesis of polyamines which in turn are thought to be involved in various steps of protein synthesis and cell growth, has prompted speculations that ODC is involved in control of growth regulation[36,37].

We observed in the rat that o,p'DDT, like E_2, dramatically elevates uterine ODC levels (Table 1) and that this elevation most probably involves de

TABLE 1

EFFECT OF DDT ANALOGS ON UTERINE ORNITHINE DECARBOXYLASE IN THE IMMATURE INTACT AND IN THE OVARIECTOMIZED RAT

Treatment (10 mg/100 g BW)	ODC treated/control
	Intact
o,p'DDT	20[a]
o,p'DDD	16[a]
p,p'DDT	3[b]
p,p'DDE	2
p,p'DDD	1
	Ovariectomized
o,p'DDT	131[a]
o,p'DDD	36[a]
p,p'DDT	14[c]
p,p'DDE	1
p,p'DDD	2[b]

[a] $P \leq 0.001$; [b] $P \leq 0.025$; [c] $P \leq 0.005$
ODC activity (pmol CO_2/hr/mg protein) was 36.6 $\pm$ 5.3 and 4.6 $\pm$ 1.1 in intact and ovariectomized control rats, respectively. Results abstracted from Bulger and Kupfer.[27]

novo protein synthesis[27]. As expected o,p'DDT was found to be the most active DDT analog in elevating ODC levels. Whereas the peak of ODC activity was at 4 and 5 hours after E_2 administration in saline and corn oil, respectively[25,27], the peak of ODC after o,p'DDT injection in corn oil was at 6-7 hours[27]. This delay in o,p'DDT peak activity versus E_2 is also reflected in the time course of diminished R_c and increase in R_n produced after o,p'DDT administration (see below). The extent of induction of ODC by o,p'DDT and E_2 in ovariectomized-adrenalectomized rats[27] is similar indicating that o,p'DDT activity is not mediated via release of endogenous E_2. Lastly, we established that the induced uterine ODC by various compounds (E_2, DES, o,p'DDT, methoxychlor) exhibits similar kinetic constants; the K_m values for ornithine decarboxylation in

induced and control ODC were similar, ranging from about 2-3 x 10^{-5}M, also the K_I of inhibition by putrescine of induced and control ODC was similar[38]. These results suggest that induction of ODC by the various "estrogens", merely produced more of a similar, if not identical enzyme. Though, more work is necessary to establish whether induced ODC's by the various compounds are indentical, the above findings, nevertheless, support the treatise that the estrogenic activities of chlorinated hydrocarbons and of E_2 are mediated by the same mechanisms.

Is o,p'DDT estrogenic in humans?

Though o,p'DDT has been shown to exhibit estrogenic activity in several species[7,32], there is currently, to our knowledge, no direct evidence to indicate whether o,p'DDT is estrogenic in humans and whether the levels of o,p'DDT which have accumulated in human fat are sufficiently high so that estrogenic activity could be manifested.

To obtain evidence on whether human tissues might be expected to respond to the estrogenic action of o,p'DDT, we utilized human breast and uterine tumors as model tissues for normal human tissues[39]. The tumors selected were assumed to be hormone dependent, since they contained relatively high levels of a cytosolic high affinity estrogen binding protein (EBP*). The purpose of the study was to determine whether o,p'DDT binds to the same 8S fraction of EBP as does ^{3}H-E_2 and to determine whether the concentration of o,p'DDT required to interfere with ^{3}H-E_2 binding to EBP could be realistically achieved from exposure to environmental contamination.

Using a previously described procedure[18], we prepared a 105,000g cytosol from a mixture of several human breast tumors or uterine tumors. To aliquots of the cytosol were added o,p'DDT or the vehicle and subsequently ^{3}H-E_2 and the resulting solution was layered on top of a 10-30% sucrose gradient which was centrifuged for 15 1/2 hours at 250,000g. One-tenth ml fractions were collected from the bottom of the centrifuge tubes and the radioactivity representing ^{3}H-E_2 in each fraction was determined (for details see ref. 18). As can be seen in Table 2, o,p'DDT inhibited the binding of ^{3}H-E_2 to the 8S EBP in mammary and uterine tumors.

To determine whether o,p'DDT diminished the binding of ^{3}H-E_2 by interacting with the same site on EBP or by merely deactivating the binding site, the effect of o,p'DDT on the binding of nonsaturating concentrations of ^{3}H-E_2 to EBP was analyzed by a Scatchard plot. Figure 1 demonstrates that o,p'DDT competitively inhibited ^{3}H-E_2 binding without altering the total number of binding sites (see converging lines on the abscissa) indicating that there was no deactivation of the binding sites. Also as expected from a competitive inhibitor, the K_D for ^{3}H-E_2 binding increased with increased concentration of o,p'DDT (Table 3). In turn the K_I for o,p'DDT inhibition of ^{3}H-E_2 binding was

*This term has been used interchangibly with the terms estrogen receptor.

TABLE 2

THE EFFECT OF ADDITION OF o,p'DDT ON THE BINDING OF ^{3}H-E_2 TO THE CYTOSOLIC 8S ESTROGEN-BINDING PROTEIN (EBP) FROM HUMAN BREAST AND UTERINE TUMORS[a]

	Tissue	Addition (μM)	Binding of ^{3}H-E_2 (% of Control)
Exp. 1	Breast tumor	—	100
		o,p'DDT (48)	59.3
		o,p'DDT (96)	38.4
		E_2 (0.8)	0
Exp. 2	Uterine tumor	—	100
		o,p'DDT (96)	58
		E_2 (0.8)	0

[a]Calculated from a sucrose gradient sedimentation analysis (Kupfer and Bulger[39]).

TABLE 3

EFFECT OF o,p'DDT ON THE K_D VALUES OF ^{3}H-E_2 BINDING TO HUMAN MAMMARY TUMOR CYTOSOLIC EBP

Additions	K_D (M)
—	0.3 x 10^{-10}
o,p'DDT (0.33 μM)	0.5 x 10^{-10}
o,p'DDT (1.67 μM)	1.8 x 10^{-10}
o,p'DDT (6.67 mM)	3.3 x 10^{-10}

Determined by a Scatchard plot analysis, from Kupfer and Bulger.[39]

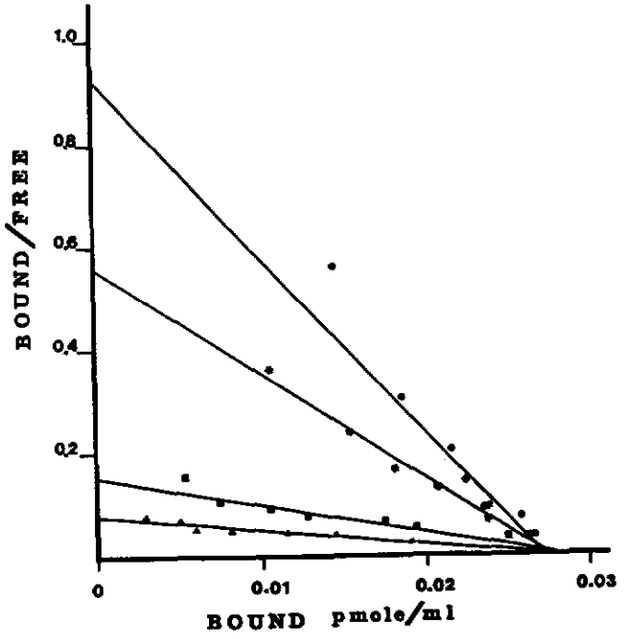

Figure 1. A Scatchard plot analysis of the inhibition of ^{3}H-E_2 binding to cytosolic EBP from human mammary tumors.

- ● No o,p'DDT
- * o,p'DDT (0.33 μM)
- ■ o,p'DDT (1.67 μM)
- ▲ o,p'DDT (6.67 μM)

For details see Kupfer and Bulger[39]. Reproduced with permission of PJD Publications, Ltd., Westbury, N.Y.

calculated using the equation for competitive inhibition: $K_I = \frac{K_D[I]}{K_D'-K_D}$ where K_D and K_D' represent affinity constants of ^{3}H-E_2 for the uninhibited and the inhibited EBP, respectively; [I] represents the molar concentration of the inhibitor (o,p'DDT). The mean value of three determinations gave a K_I = 0.5 ± 0.1 μM[18].

As to concentrations of o,p'DDT in human fat (μg/g fat), there is only limited amount of information[40]: 0.04-0.29 (Germany); 0.68 (Italy) and 0.12-2.16 (Luxemburg). Among workers in a DDT factory the mean of o,p'DDT was 24 ± 3 μg/g fat[41]. If one assumes that 1 g of fat is equal to 1 ml then the concentration of o,p'DDT in human fat in these European countries was 0.1-1.1 μM and in the U.S. in a DDT factory was about 67 μM. The K_I for o,p'DDT calculated by us (0.5 μM) is hence in the range of these concentrations of o,p'DDT in human fat. However, whether such concentrations of o,p'DDT do in fact accumulate in the human estrogen target organs and more importantly at the estrogen receptor sites in these organs is not known.

Is o,p'DDT *per se* estrogenic or are the metabolites estrogenic?

In their first study describing the observation that o,p'DDT is the estrogenic component of technical DDT, Welch et al.[6] addressed themselves to the question whether o,p'DDT is active *per se* or whether the metabolite(s) are responsible for the estrogenic action. Based on the *inhibition* of the uterotropic action of o,p'DDT and *potentiation* of this action of E_2 by the prior treatment of rats with CCl_4 [a hepatotoxin known to diminish markedly monooxygenase activity], Welch et al.[6] concluded that by contrast to E_2, o,p'DDT is not active *per se*, but requires metabolism for estrogenic action (see Table 4).

In view of the observations that there are stringent structural requirements for a DDT derivative to be estrogenic, e.g., p,p'DDT is only weakly estrogenic as compared with o,p'DDT and p,p'DDE and p,p'DDD are both inactive[6], it made sense to assume that the o-chloro substituted ring in o,p'DDT undergoes a monooxygenase-catalyzed hydroxylation at the para position; yielding a p-phenolic derivative which, by analogy to the classical nonsteroidal estrogen diethylstilbestrol (DES), is the estrogen. In fact, rats treated with o,p'DDT excreted phenolic metabolites of o,p'DDT[56]. Hence, the suggestion that o,p'DDT is not the estrogen *per se* but requires metabolic activation remained unchallenged for some time.

However, the observations that o,p'DDT inhibits competitively ^{3}H-E_2 binding to the uterine cytosolic high affinity estrogen binding protein (estrogen receptor, R_c)[14,18,29] demonstrated, at least in vitro, that o,p'DDT is active as an "estrogen" and suggested that o,p'DDT might have intrinsic

TABLE 4

THE EFFECT OF CARBON TETRACHLORIDE AND SKF 525A ON THE UTEROTROPIC ACTIVITY OF o,p'DDT AND E_2 IN IMMATURE RATS

Pretreatment	Treatment	Uterotropic Activity (% Control) Uterine wt.(±Δ%)	Uterine wt/BW (±Δ%)		Ref.
—	o,p'DDT	167			Welch et al.[6]
CCl_4	o,p'DDT	132 (-35)			
—	E_2	114			
CCl_4	E_2	138 (+24)			
—	o,p'DDT		125[a]		Nelson et al.[42]
CCl_4	o,p'DDT		137 (+12)[a]		
SKF525A	o,p'DDT		138 (+13)[a]		
—	o,p'DDT (13)	140	135		Current
CCl_4	o,p'DDT (13)	141 (+1)	149 (+14)		studies
—	E_2 (14)	136	154		
CCl_4	E_2 (13)	116 (-20)	118 (-36)		"

CCl_4 was given orally in corn oil (0.67 ml/kg); SKF 525A (25 mg/kg); o,p'DDT given i.p. (10 mg/kg) in dimethyl sulfoxide, E_2 given i.p. (0.6 µg/kg) in Welch et al.[6] and in current studies. Under treatment in parentheses number of animals used.

[a]These values were calculated from a bar graph, hence are only approximations.

activity in vivo as well. In fact, these findings prompted other investigators to reexamine the findings of Welch et al.[6]. Surprisingly, Nelson et al.[42] obtained diametrically opposing results, namely CCl_4 or SKF 525A treatment did not inhibit o,p'DDT action but in fact slightly potentiated the uterotropic action of o,p'DDT (Table 4). In view of these findings, these investigators proposed that o,p'DDT is active *per se*. To attempt to resolve the controversial observations by Welch et al.[6] on one hand and by Nelson et al.[42] on the other, we attempted to reproduce these experiments. In our hands, CCl_4 had no inhibitory effect on the o,p'DDT-mediated uterotropic activity; however, it appears that CCl_4 inhibited the E_2 effect (Table 4). Currently, we have no explanation for the contradictory observations in different laboratories.

To further explore the question whether o,p'DDT is active *per se*, we examined the effect of CCl_4 pretreatment on the induction of uterine ornithine decarboxylase (ODC) by E_2 and o,p'DDT[43]. To our surprise, CCl_4 inhibited both the E_2- and o,p'DDT-mediated induction of ODC without affecting the basal

levels (Table 5). The inhibition by CCl_4 of ODC induction was not accompanied

TABLE 5

EFFECT OF PRETREATMENT WITH CCl_4 ON THE ESTROGENIC ACTIVITY OF o,p'DDT AND E_2 IN OVARIECTOMIZED RATS

Pretreatment	Treatment	ODC[a] (Ratio treated to control)	R_c Total binding sites[b]	R_c $K_D(10^{-10}M)$	Cytochrome P-450[c]
—	—	1	2.2	0.7	0.4
CCl_4	—	1	2.3	0.8	0.2[d]
—	o,p'DDT	154			
CCl_4	o,p'DDT	59[d]			
—	E_2	74			
CCl_4	E_2	32[d]			

[a]ODC in pmol of CO_2/hr/mg protein; [b]in pmol ^{3}H-E_2 bound per mg protein.
[c]Cytochrome P-450 in nmol/mg of hepatic microsomal protein.
[d]$p \leq 0.025$; CCl_4 pretreated vs corresponding control.

Pretreatment: CCl_4 (0.67 ml/kg body weight) given orally in corn oil, 24 hours before treatment.

Treatment: o,p'DDT (5 mg) and E_2 (0.015 µg) per rat were given i.p. in 0.2 ml corn oil, 6 and 5 hours prior to sacrifice, respectively.

Results calculated from Bulger and Kupfer.[43]

by observable effects on the affinity for ^{3}H-E_2 nor on total binding sites for ^{3}H-E_2 in the cytosolic estrogen receptor (R_c); also there was no evidence for the presence of an inhibitor of ODC in the uterine cytosol from CCl_4-treated rats. These studies suggested that CCl_4 effects on ODC inductions were not related to effects on E_2 or o,p'DDT metabolism by the liver, though a decrease in both hepatic cytochrome P-450 and monooxygenase activity (not shown) was observed. We also observed that CCl_4 treatment 1 1/2 hours after the administration of E_2 had no effect on the induction of ODC by E_2, suggesting that after the "message" has been in progress, CCl_4 is no longer effective in inhibiting ODC induction.

Further evidence that o,p'DDT most probably possesses intrinsic estrogenic activity was recently obtained by us in vitro. As expected of an estrogen*, the incubation of o,p'DDT with rat uteri for one hour resulted in the translocation of R_c into the nucleus (R_n) without changing the total amount of receptor ($R_c + R_n$), (Table 6); a similar incubation with p,p'DDT and p,p'DDE,

TABLE 6

EFFECT OF INCUBATION OF o,p'DDT, p,p'DDT, p,p'DDE, HPTE OR E_2 WITH ISOLATED UTERI FROM IMMATURE RATS ON THE DISTRIBUTION OF THE CYTOSOLIC (R_c) AND NUCLEAR (R_n) RECEPTOR

	Compound added (μM)	R_c [a]	R_n [a]	Sum of $R_c + R_n$
Exp. 1	——	377	197	574
	o,p'DDT (10)	244[b]	303[c]	547
	——	462	135	597
	E_2 (0.02)	80	574	654
Exp. 2	——	1114	82	1196
	p,p'DDT (10)	967	76	1043
	p,p'DDE (10)	1123	68	1191
	HPTE (2)	311[c]	531[c]	842

[a] Mean values in fmol ^{3}H-E_2 bound to R_c or R_n per single uterus.

[b] $P \leq 0.05$; [c] $P \leq 0.001$

Exp. 1: n = 4 for o,p'DDT and its control (——)
n = 1 for E_2 and its control; E_2 was merely included as a positive control, invariably yielding translocation of R_c.

Exp. 2: n = 8, 3, 3 and 2 for control, p,p'DDT, p,p'DDE and HPTE respectively.

which exhibit little or no estrogenic activity, had no such effect. We believe that though possible, it is unlikely that in vitro uteri significantly convert o,p'DDT into a hydroxylated "estrogenic" metabolite(s). Preliminary experiments with [^{14}C]-o,p'DDT demonstrate that uteri could, at best, have formed 11 pmol of a base-soluble product of which only a portion appears to be associated with the uteri. Whether this represents sufficient amounts of a highly estrogenic compound remains to be established. Also uteri do not possess significant

*Under these conditions, we cannot distinguish an estrogen from an anti-estrogen.

monoxygenase-like activity, since uteri alone cannot demethylate methoxychlor into a phenolic product(s) which behave in vitro like active estrogens (see below).

The observations that phenolic derivatives of o,p'DDT, known to be formed in vivo[56], are active estrogens[57] may explain the reason for the confusion as to which are the active species in vivo. Most probably the estrogenic action of o,p'DDT in vivo represents the resultant activity of the parent compound as well as that of the metabolites. In fact, our findings in vitro support this conclusion; we observed that the incubation of o,p'DDT with liver microsomes and NADPH* (in the presence of uteri) did not alter the magnitude of "estrogenic" activity (translocation of R_c to R_n) of o,p'DDT as compared with a similar incubation of o,p'DDT with inactive (boiled) liver microsomes[24], suggesting that o,p'DDT and metabolite(s) are similarly active.

Lastly, our observation that o,p'DDT inhibits the binding of ^{3}H-E_2 to *mouse* uterine R_c[18], but nevertheless has little or no estrogenic activity in vivo in that species[44], points to the possibility that in that species metabolism of o,p'DDT yields inactive products; obviously other interpretations for such species peculiarity are possible**.

Is methoxychlor *per se* estrogenic or is the estrogenic activity due to contaminant(s)?

The observations that technical grade methoxychlor exhibits higher estrogenic activity than the more purified preparations of methoxychlor suggested that a contaminant(s) was responsible for the estrogenic activity of methoxychlor. Thus we addressed ourselves to the following questions: (a) Is methoxychlor estrogenic or only the contaminants are estrogenic?; and (b) If methoxychlor is not estrogenic are the metabolites of methoxychlor estrogenic?

To attempt to answer this question, we obtained laboratory grade methoxychlor (99% pure) and further purified this preparation by base extraction of the impurities and recrystallization of residual methoxychlor from hexane[47]. We observed that purified methoxychlor did not inhibit the binding of ^{3}H-E_2 to the rat uterine cytosolic R_c (Table 7); by contrast the laboratory grade

* Such conditions were shown by us to yield acidic (probably phenolic) o,p'DDT products.

** In the mouse p,p'DDT was initially thought to be inactive as inducer of hepatic monooxygenase and subsequently found to be a much weaker inducer than in the rat [45,46]. Hence, it is possible that a higher dose of o,p'DDT would be estrogenic in the mouse.

TABLE 7

EFFECT OF METHOXYCHLOR AND ITS DIDEMETHYLATED METABOLITE (HPTE) ON THE BINDING OF $^{3}H\text{-}E_2$ TO THE RAT UTERINE CYTOSOLIC ESTROGEN RECEPTOR

Compound added (μM)		% Inhibition of $^{3}H\text{-}E_2$ binding[a]
Purified Methoxychlor	(48)	0
Laboratory grade Methoxychlor	(48)	70
HPTE	(0.04)	27
HPTE	(0.40)	80
Diethylstilbestrol	(0.40)	94

[a]Determined from the radioactivity under the area of the 8S peak in a sucrose gradient sedimentation analysis from the data of Bulger et al.[47]

methoxychlor was inhibitory to $^{3}H\text{-}E_2$ binding. Furthermore, the isolated impurities were also inhibitory to $^{3}H\text{-}E_2$ binding to R_c. Also in vivo the purified methoxychlor was somewhat less active estrogen than the less pure preparation (Table 8). Lastly, we observed that incubation of purified methoxychlor with isolated rat uteri did not produce a translocation of R_c to R_n (Table 9). These observations demonstrated that methoxychlor does not bind significantly to R_c and most probably in vivo is not the active estrogen.

Is methoxychlor estrogenic or are the metabolites of methoxychlor estrogenic?

To examine this question we compared the in vivo activity of purified methoxychlor with that of the didemethylated metabolite of methoxychlor (HPTE). Our findings demonstrate that the metabolite is at least 50-fold more potent than methoxychlor, using induction of uterine ornithine decarboxylase (ODC) as a monitor (Table 8). These results strongly suggested that in vivo methoxychlor is not active *per se*, but did not rule out some intrinsic activity of methoxychlor. Thus we examined whether the incubation of methoxychlor with rat liver microsomes would yield metabolites which exhibit in vitro "estrogenic" action, i.e., interference with $^{3}H\text{-}E_2$ binding to R_c and whether the major metabolite (HPTE) would translocate in vitro R_c into the nucleus.

TABLE 8

EFFECT OF ADMINISTRATION OF ESTRADIOL, LABORATORY GRADE (LG), AND PURIFIED METHOXYCHLOR AND OF THE DIDEMETHYLATED METABOLITE OF METHOXYCHLOR (HPTE) ON UTERINE WEIGHT AND ORNITHINE DECARBOXYLASE (ODC) IN OVARIECTOMIZED RATS

Compound injected		ODC	Uterotropic action
(amount/rat)		(treated/control)[a]	
Purified Methoxychlor	(5 mg)	7	1.4
Methoxychlor (LG)	(5 mg)	14	1.6
HPTE	(0.1 mg)	17	1.2
HPTE	(0.5 mg)	55	1.3
E_2	(0.1 μg)	57	1.8

[a]All treated were significantly different from controls ($P \leq 0.05$); adapted from Bulger et al.[16]

We observed by tlc and HPLC that at least four metabolites were formed in vitro: the least polar is most probably the monodemethylated methoxychlor, the next in polarity is the didemethylated methoxychlor (HPTE)* and the most polar ones probably represent compounds with additional hydroxylations on the rings. All the metabolic products exhibited inhibition of $^{3}H\text{-}E_2$ binding to R_c, HPTE being the most potent inhibitor (Table 7). Scatchard plot analysis demonstrated that HPTE inhibited competitively $^{3}H\text{-}E_2$ binding to R_c (K_I = 6nM) without altering the number of binding sites[47]. In addition, incubation of HPTE with isolated rat uteri translocated the R_c into the nucleus (Table 6). Lastly we examined whether conditions which generate metabolites of methoxychlor in vitro in the presence of uteri would simultaneously cause the translocation of uterine R_c into the nucleus[24]. In that experiment, we incubated methoxychlor with rat liver microsomes (active vs. boiled) and NADPH in the presence of immature rat uteri. The incubation of methoxychlor with active, but not with boiled, microsomes caused a translocation of R_c (Tables 6,9). These findings support the hypothesis that in vivo also methoxychlor is not an estrogen, but requires metabolic transformation for estrogenic activity to be manifested. Surprisingly, the pretreatment of rats with SKF 525A to inhibit

*HPTE was characterized by tlc and HPLC (coinjection with an authentic compound), by GC/MS and by methylating with dimethylsulfate to yield a compound with chromatographic mobility identical to methoxychlor.

TABLE 9

EFFECT OF INCUBATION OF METHOXYCHLOR (M) OR THE DIDEMETHYLATED METABOLITE OF M (HPTE) WITH ISOLATED UTERI UNDER METABOLIZING CONDITIONS ON THE DISTRIBUTION OF THE ESTROGEN RECEPTOR IN UTERINE CYTOSOL (R_c) AND NUCLEI (R_n)

	Incubation[a]	R_c[b]	R_n[b]	$R_c + R_n$
Exp. 1				
	Complete (M + NADPH + liver microsomes)	110[c]	318[c]	428
	Complete minus NADPH	230	174	404
	Complete minus microsomes	271	177	448
	Complete minus M	241	169	410
Exp. 2[d]				
	Control	490	188	679
	Control + HPTE	187[c]	512[c]	699

[a] Incubations for 1 hour at 37° with 6 uterine horns (equivalent to 3 uteri); M (2 μM) or HPTE (2 μM) and liver microsomes (ca. 1.5 mg protein) as previously described by Kupfer and Bulger (values abstracted from that study).[24]

[b] R_c and R_n in fmol ^{3}H-E_2 per single uterus were assayed by an exchange with ^{3}H-E_2 as previously described.[22,23]

[c] $P<0.01$ against corresponding control (Exp. 2) or against incubations lacking an incubation constituent (Exp. 1).

[d] Exp. 2: These incubations did not contain NADPH and microsomes.

or with phenobarbital to stimulate the metabolism of methoxychlor, *did not* affect the uterotropic activity after administration of methoxychlor[42]. It is possible that the uterotropic action of methoxychlor in this study reflects the combined activity of methoxychlor and of the inherent impurities. Thus, since the impurities are more potent estrogens than methoxychlor and probably do not undergo further metabolic activation, SKF 525A and phenobarbital would not be expected to have an effect. Therefore, it is surprising, albeit interesting, that pretreatment with CCl_4 potentiated the activity of methoxychlor[42]. Currently no satisfactory explanation could be provided for this finding.

Are DDT analogs estrogens or also antiestrogens?

Attempts to answer this question *à priori* suffer from the lack of basic knowledge about the mechanism of action of antiestrogens on the molecular level. Hence, the question whether DDT derivatives are frank estrogens or also possess antiestrogenic activity has not been adequately explored. Also, the classical procedure to determine antiestrogenic activity which usually involves the demonstration that the administration of a given compound inhibits the uterotropic action of E_2, is not suitable for DDT analogs. These compounds possess two activities which might confuse the interpretation of results; i.e., they exhibit uterotropic activity and also they induce hepatic monooxygenase which in turn would result in increased metabolism of E_2 and lowered E_2 activity.

Several laboratories described differences in action between antiestrogens and estrogens. Thus, Clark et al.[48,49] observed that whereas estrogens permit a relatively rapid replenishment of R_c and disappearance of R_n, antiestrogens cause retention of R_n for a long duration and prevent the replenishment of R_c. Support for the findings that inhibition of estrogenic action by an antiestrogen is probably related to the duration of depletion of R_c by the latter was presented by Ferguson and Katzenellenbogen[50]. Also, studies from the same group[51] indicated that the above observations probably did not merely reflect the longer half-life ($t_{1/2}$) of the antiestrogen as compared with the $t_{1/2}$ of E_2. Based on widely different time span retention of nuclear receptor (R_n) with estrogens and antiestrogens and on the degree of salt extractability of these receptors, Ruh and Blaudendistel[52] proposed that there are different nuclear binding sites for estrogen- and antiestrogen-receptor complexes. Studies by Gardner et al.[53] with nafoxidine indicate that antiestrogens *do not* interfere with the early response to E_2 but do block the secondary (24 hours) response to E_2 and suggest that uterine nuclei contain different receptor acceptor sites for the regulation of the short and long term responses to estrogens. Horwitz et al.[54], using cancer cells in long term culture observed with the antiestrogen tamoxifen (T) a dual dose-dependent phenomena, i.e., at high concentrations (1μM), T acted like an antiestrogen by inhibiting cell growth and induction of the progesterone receptor (PR). At low concentrations (<0.1μM) T acted as an estrogen by enhancing PR formation. By contrast, nafoxidine acts as expected of an antiestrogen, by inhibiting cell growth and not inducing PR. Recently, Jordan

et al.[55] proposed that by contrast to estrogens, antiestrogens (tamoxifen) support only minimal cellular mitosis and DNA synthesis, yielding primarily hypertrophy; again no plausible mechanism for the differences is currently available.

As can be seen, knowledge on the mechanism of antiestrogen action at the molecular level is still inadequate. Despite these uncertainties as to the differences between estrogens and antiestrogens, we decided to examine whether o,p'DDT behaves more like an estrogen or like an antiestrogen. Hence, we examined the time course of depletion of the uterine cytosolic estrogen receptor (R_c) and the elevation of the nuclear receptor (R_n) after o,p'DDT administration to immature rats. As can be seen (Fig. 2), o,p'DDT appears

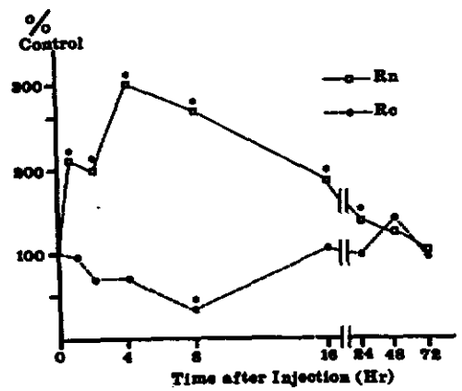

Figure 2. Time course of distribution of R_c and R_n after a single dose of o,p'DDT (100 mg/kg BW, ip). Values represent % of 0-time control (100%) R_c and R_n yielding 1975 and 119 fmol ^{3}H-E_2 bound per uterus, respectively. An asterisk indicates values different from 0-time at $P<0.05$.

to affect R_c and R_n essentially like E_2; also similarly to E_2, o,p'DDT produces higher levels of R_c at 48 hours than were initially present at 0-time; apparently antiestrogens do not cause this effect[48,49]. However, o,p'DDT also mimics somewhat the characteristics of antiestrogens in as far as both produce a longer retention of R_n and longer depletion of R_c than observed with E_2. The possibility that the differences in duration of action of o,p'DDT and E_2 is due to the different vehicles (corn oil vs. saline) used in the two studies has not been examined. Interestingly enough, however, the time course of the uterotropic activity of o,p'DDT closely resembles that of E_2, and both curves return to control levels at 72 hours.

The unlikely possibility that o,p'DDT effects are mediated through releasing endogenous estrogens in intact immature rats was examined by comparing with effects in ovariectomized rats (Table 10). Results demonstrate that

TABLE 10

THE EFFECT OF ADMINISTRATION OF o,p'DDT ON UTERINE WEIGHT AND ON THE DISTRIBUTION OF R_c AND R_n IN INTACT IMMATURE AND OVARIECTOMIZED RATS

Hours after o,p'DDT	Uteri (mg)/BW (100g)	R_c	R_n	$R_c + R_n$
	Intact			
0	73.4 ± 3.5	1504 ± 277	97 ± 13	1601 ± 289
8	110.5 ± 5.7[a]	762 ± 114[b]	262 ± 34[b]	1024 ± 102
	Ovariectomized[d]			
0	29.2 ± 1.0	936 ± 57	104 ± 15	1040 ± 62
8	56.6 ± 3.3[a]	637 ± 55[c]	179 ± 11[c]	816 ± 48

[a] $P \leq 0.001$; [b] $P \leq 0.05$; [c] $P \leq 0.025$
There was no difference between 0-time and 8 hours in DNA levels.
[d] Used 7 days after ovariectomy.

o,p'DDT is also estrogenic in ovariectomized rats, increasing uterine weight and translocating R_c into R_n and elevating ODC (Table 1), indicating that these activities of o,p'DDT are not mediated via ovarian steroids.

We decided to examine whether there are differences in the ability of antiestrogens (tamoxifen and nafoxidine) to induce uterine ODC versus that of E_2 and o,p'DDT and whether antiestrogens or o,p'DDT could block the induction of ODC by E_2 or o,p'DDT. The injection of tamoxifen (T) and nafoxidine

(N) to ovariectomized rats dramatically elevated ODC (Table 11). The duration of elevated ODC after T and N was markedly longer than after either E_2 or o,p'DDT. ODC essentially returned to control levels at about 18 hours after o,p'DDT or E_2, but not after T or N which had a much longer duration of activity. It is interesting that when the levels of ODC in animals pretreated with T or o,p'DDT, were allowed to return to control levels the subsequent induction of ODC by either E_2 or o,p'DDT was markedly suppressed (Table 12). A similar pretreatment with E_2 gave inconsistent results, i.e., only occasionally suppressed the induction of ODC by subsequent doses of E_2. The mechanism of

TABLE 11

TIME COURSE OF INDUCTION OF UTERINE ORNITHINE DECARBOXYLASE (ODC) AFTER A SINGLE INJECTION OF NAFOXIDINE (N) OR TAMOXIFEN (T) IN OVARIECTOMIZED RATS

Time after injection of N or T (hrs)[b]	ODC activity (Ratio of treated/control)[a]	
	NAFOXIDINE	TAMOXIFEN
Control[c]	1	1
2	2.8	3.9
4	24.6	26.8
6	155.5	138.3
24	220.0	71.1
48	17.7	ND

[a] Calculated from Bulger and Kupfer[59].
[b] Nafoxidine hydrochloride (50 µg) or tamoxifen citrate (1 mg) in H_2O were given i.p.
[c] 0-time controls for N and T were 3.6 ± 1.7 and 9.1 ± 1.2, pmol CO_2/hr/mg protein, respectively; ND = not determined.

the inhibition of ODC induction by antiestrogens and o,p'DDT is not understood. It is possible that inhibition of the "recycling" of R_c interferes with the action of E_2[49]. Several other possibilities were considered. The possibility that the inhibition of E_2 action merely reflects an increase in E_2 metabolism by the hepatic P-450 monooxygenase induced by T or o,p'DDT does not seem likely. We observed that p,p'DDE is a more potent inducer of the hepatic microsomal P-450 and monooxygenase activity than o,p'DDT, however, by contrast to o,p'DDT, p,p'DDE does not induce uterine ODC nor does it inhibit ODC induction by either E_2 or o,p'DDT. Also tamoxifen does not induce hepatic

TABLE 12

EFFECT OF PRETREATMENT WITH TAMOXIFEN, o,p'DDT OR p,p'DDE ON THE INDUCTION OF UTERINE ORNITHINE DECARBOXYLASE (ODC) BY E_2 OR o,p'DDT IN OVARIECTOMIZED RATS

	Pretreatment	Treatment	ODC activity[a] (Increase over control; control = 1)
Exp. 1	—	—	1
	—	E_2	37.3
	—	o,p'DDT	30.0
	o,p'DDT	—	0.1
	o,p'DDT	E_2	4.8
	o,p'DDT	o,p'DDT	1.2
Exp. 2	—	—	1
	—	E_2	129.8
	—	o,p'DDT	143.4
	Tamoxifen	—	1.9
	Tamoxifen	E_2	4.1
	Tamoxifen	o,p'DDT	3.6
Exp. 3	—	—	1
	—	E_2	50.3
	—	o,p'DDT	37.7
	p,p'DDE	—	1.7
	p,p'DDE	E_2	46.0
	p,p'DDE	o,p'DDT	40.8

[a] Calculated from Bulger and Kupfer.[4]

Pretreatment: o,p'DDT or p,p'DDE were injected i.p. (100 mg/kg BW) in 0.2 ml corn oil for 2 days. Tamoxifen citrate (1 mg/kg) was injected i.p. for 4 days.

Treatment: E_2 (0.5 µg/kg) or o,p'DDT (100 mg/kg in Exp. 1,3; 50 mg/kg in Exp. 2) were injected 2 days after pretreatment with o,p'DDT or p,p'DDE and 18-19 hours after Tamoxifen.

monooxygenase, but nevertheless induces ODC and inhibits ODC induction by E_2 or o,p'DDT.

Another possibility for inhibition of ODC induction is that a product is being formed which inhibits ODC induction. In fact, we observed that the administration of putrescine, a metabolic product of ornithine decarboxylation, inhibits the induction of ODC by o,p'DDT but had no effect on the basal enzyme activity.[38] Also we found that the addition of putrescine (1-4 mM) to uterine cytosol from controls, E_2- and o,p'DDT-treated rats competitively inhibited ODC. It is also possible that induction of ODC by T or o,p'DDT generates a specific protein which inhibits ODC activity by binding to ODC, a mechanism proposed by Heller, et al.[58] for the polyamine-mediated inhibition of ODC.

CONCLUSION

The results presented indicate that o,p'DDT acts as a typical estrogen on a variety of biological parameters. However, o,p'DDT also behaves possibly like an antiestrogen with respect to causing inhibition of the induction of uterine ornithine decarboxylase in response to subsequent treatments with E_2 or o,p'DDT.

With respect to whether o,p'DDT *per se* is the estrogen or whether the metabolites are the active species, our findings and those of others indicate that both o,p'DDT and its metabolites are estrogenic. Nevertheless, the question whether in vivo o,p'DDT is active *per se* has not been unequivically answered.

As to the estrogenic action of methoxychlor, evidence strongly indicates that methoxychlor is not active *per se*, but is metabolized by the hepatic monooxygenase into active phenolic metabolites. Also it appears that most preparations of methoxychlor contain base-soluble contaminants which are largely responsible for the estrogenic activity of the crude methoxychlor preparations.

Our newly developed in vitro assay which combines the "machinery" for metabolic activation of a proestrogen with the uterine detection system[24], should be helpful in the future in resolving questions whether a compound is active *per se* or is a proestrogen.

ACKNOWLEDGEMENTS

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DISCUSSION

SOTO: Your experiments show that both tamoxifen and nafoxidine induce ornithine decarboxylase while they are known not to induce hyperplasia of the rat uterus. Similarly, the uterus of newborn rats responds to estrogen by inducing ornithine decarboxylase in the absence of a growth response to estrogen. Would you or someone in the audience care to comment on the apparent lack of correlation between ornithine decarboxylase induction and growth?

LEROY: Since ornithine decarboxylase biosynthesis is generally considered to be related to tissue growth, I would like to ask if you have any idea how DDT derivatives affect uterine DNA synthesis and cell division?

KUPFER: We did observe that DNA levels increase following o,p-DDT treatment. We have no information on the mechanism of this increase and did not examine effects on cell division.

STANCEL: In response to Dr. Soto's and Dr. Leroy's questions, I would just like to say that o,p'-DDT administration to immature rats, similar to the ones Dr. Kupfer has been using, stimulates uterine DNA synthesis to a degree which is quantitatively very similar to that seen after estradiol administration.

LUCIER: In a normal individual receiving chronic low level DDT exposures, most of the DDT would be sequestered in adipose tissue and not create a direct estrogenic hazard. However, in dieting individuals, much of the DDT could be released thereby creating the possibility of estrogenic action. Are dieting individuals at special risks to the estrogenic actions of DDT and other estrogenically active organohalogens which are stored in adipose tissue?

KUPFER: There is currently no data to bear on this point. However, with respect to DDT stores in rats, German investigators have shown that following a single dose of DDT, the induction of hepatic monooxygenase lasts for about 90 days. However, if subsequently these animals were starved, the level of the monooxygenase "sky-rocketed." This was interpreted as being due to release of DDT from fat depots. Thus, if one is allowed to extrapolate these findings to the human and to o,p'-DDT, then one might answer your question affirmatively. Also loss of fat due to disease would most probably release the DDT from fat into the circulation.

MARTIN: Do you know if the ornithine decarboxylase is present in all of the uterine tissue or restricted to only one cell type?

KUPFER: We have not studied this and, to my knowledge, no one else has; but we feel that it is important that the localization of ornithine decarboxylase in specific cells of uteri be established.

McLachlan, ed. Estrogens in the Environment

MYCOTOXINS AS ESTROGENS

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INTRODUCTION

Mycotoxins are substances produced by fungi in feed and foodstuff which upon consumption cause deleterious effects in man and animals. Only one group of phytoestrogens cause mycotoxicoses in animals and these are called the zearalenones. They are produced by various species of Fusarium, but most frequently and most abundantly by F. roseum' Graminearum'.

Species of Fusarium are common and widespread in nature, occurring as saprophytes in the soil and in decaying plant material; they cause a variety of diseases such as wilts, blights and rots. Fusarium can cause serious economic losses in the major cereal crops such as rice, wheat, barley, rye, millet, sorghum and maize. Corn is most often infected by this pathogen in the corn belt of the USA and accounts for the majority of the cases of hyperestrogenicity found in swine.

Zearalenone and its derivatives are unique among the mycotoxins in that, according to the animal to which they are administered, they can either cause serious injury (zearalenone and zearalenol when consumed by swine), have little or no effect (zearalenone consumed by laying hens and broilers), promote growth (zearalanol as an ear implant in cattle and sheep) or serve as a beneficial drug (zearalanol when taken to alleviate post-menopausal distress in women). Swine are most frequently affected in nature. In the prepubertal gilt, the vulva become swollen and edematous and vaginal prolapse may result; the mammae increase in size. The uterus becomes enlarged and tortuous and the ovaries atrophied. Young males exhibit a feminizing effect, with atrophy of the testes and enlargement of the mammary glands. The most serious problem economically is infertility.

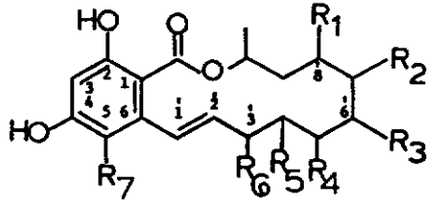
Recent and thorough reviews on the zearalenones can be found by Mirocha and Christensen[1] and Mirocha et al.[2]

ZEARALENONE AND ITS DERIVATIVES

Zearalenone is routinely produced in our laboratory by growing a high yielding isolate of Furarium roseum on autoclaved moist polished rice at a moisture content of about 60%. The rice cultures are incubated in 1 liter

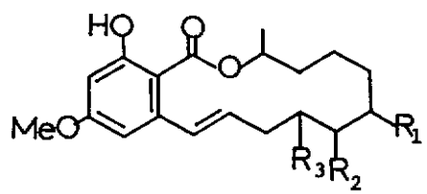
flasks at 24 - 27° C for 7 days and 10 - 12° C for 4 to 6 weeks to produce zearalenone between 1000 to 15000 mg per kg dry weight of cultures.[3] Some strains of F. roseum produce copious amounts of zearalenone when grown on rice at room temperature.[4]. International Minerals and Chemicals Corp. (Terre Haute, IN) was successful in obtaining a mutant of F. roseum (chemically induced) which produces large amounts of zearalenone in submerged fermentation tanks[5] as opposed to solid culture, the usual method of production.

Several derivatives of zearalenone have been isolated as minor metabolites of Fusarium roseum; they are listed in Figure 1. The hydroxylated derivatives of zearalenone are believed to be the products of metabolism by Fusarium. None of these derivatives except zearalenol has been found to be estrogenic. The metabolites, 3'-hydroxyzearalenone and 5-formylzearalenone, have not been tested for biological activity. α-Zearalenol is about 3 - 4 times more active in the rat uterotropic test than the parent compound.[6]

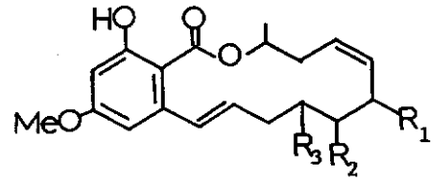


	R_1	R_2	R_3	R_4	R_5	R_6	R_7
ZEARALENONE[45]	H_2	H_2	=O	H_2	H_2	H_2	H
α-ZEARALENOL[6]	H_2	H_2	OH	H_2	H_2	H_2	H
8'-HYDROXYZEARALENONE[46,47]	OH	H_2	=O	H_2	H_2	H_2	H
3'-HYDROXYZEARALENONE[48]	H_2	H_2	=O	H_2	H_2	OH	H
6',8'-DIHYDROXYZEARALENE[49]	OH	H_2	OH	H_2	H_2	H_2	H
4',5'-DIHYDROXYZEARALENE[50]	H_2	H_2	H_2	OH	OH	H_2	H
7'-DEHYDROZEARALENONE[47]	H	H	=O	H_2	H_2	H_2	H
5-FORMYLZEARALENONE[47]	H_2	H_2	=O	H_2	H_2	H_2	CHO

Fig. 1. Derivatives of zearalenone produced by Fusarium roseum .



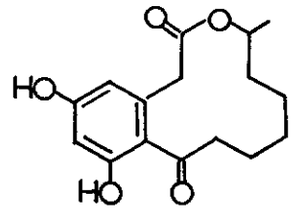
	R_1	R_2	R_3
Z1640-1	=O	OH	OH
Z1640-2	OH	OH	OH



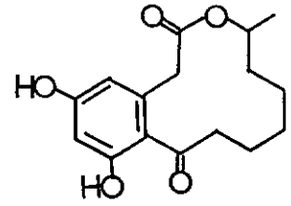
	R_1	R_2	R_3
Z1640-3	=O	OH	OH
Z1640-4	OH	OH	OH

Lederle culture Z1640
(Unidentified fungus)

d,l-Ethionine



Curvularin



Dehydrocurvularin

Fig. 2. Macrolides related to zearalenone produced by an unidentified fungus

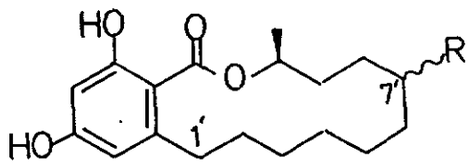
Recently Ellestad et al.[7] isolated four zearalenone-like macrolides (Fig. 2) from an unidentified fungus labeled Lederle Culture Z1640. All these derivatives were 4-methyl ethers, and did not exhibit any uterotropic or anabolic properties characteristic of zearalenone.[1] They attributed the lack of activity to the presence of phendic ethers. Their attempt to obtain the corresponding diphenolic compounds by incubating the cultures with D,L-ethionine resulted in the production of curvularin and dihydrocurvularin.[8] The latter metabolites, although related to zearalenone, are not estrogenic.

STRUCTURE AND UTEROTROPIC ACTIVITY

After zearalenone was patented as an anabolic agent by Andreas and Stob,[9,10] much attention was given to modify the molecule to enhance the anabolic activity. The estrogenic activity, as measured by the uterotropic response in either the rat or mouse, has been discussed by Pathre and Mirocha.[11]

TABLE 1.

ESTROGENIC ACTIVITY OF THE 7'-SUBSTITUTED DERIVATIVES OF ZEARALANE. THE ACTIVITY IS COMPARED WITH ZEARALENONE AND DIETHYLSTILBESTROL.

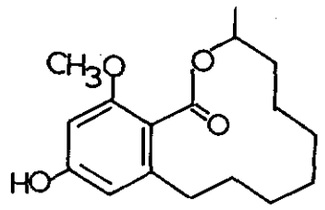


Compound	R	Uterotropic Activity Relative to Zearalenone	Uterotropic Activity Relative to Diethylstilbestrol
Zearalane	H	0.22 - 1.0	0.0001 - 0.0005
7'-Formylzearalane (diastereomeric Mixture)	CHO	50.1	0.025
7-Formylzearalane (Isomer A, 141 146 C)	CHO	18.0	0.009
7'-Formylzeaalane (Isomer B, 148 -152 C)	CHO	94.0	0.047
7'-Carboxyzearalane (Diastereomeric Mixture)	COOH	100.0	0.050
7'-Carboxyzearalane (Isomer A)	COOH	192.0	0.096
7'-Carboxyzearalane (Isomer B)	COOH	18.0	0.009

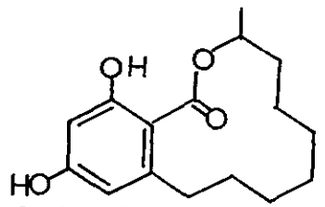
Zearalenone is approximately 0.005 times as active as diethylstilbestrol. Reduction of the double bond (1', 2') and/or the ketone (6') enhances its uterotropic activity. Deoxygenation of the 6' position does not impair the activity; however, substitution at the 6' position with a substituent other than an -OH group dimishes the uterotropic response. Introduction of any function in the aliphatic ring in addition to 6'-ketone diminishes its estrogenic activity.

Jensen et al.[12] modified the aliphatic ring of zearalenone to obtain several 7'-substituted derivatives of zearalane. Some of these derivatives are more active than zearalenone[13] (Table 1). A diastereomer of 7'-carboxyzearalane is almost one-tenth as active as diethylstilbestrol and 192 times more active than its parent compound. The formyl derivative is almost as active as the 7'-carboxy derivative. Although the uterotropic activity of each diastereomer is not similar, all the diastereomers of the 7'-substituted derivatives are significantly more active than zearalenone.

The ring size of the zearalenone molecule appears to be important since the natural macrolides such as lasiodiplodin and de-O-methyllasiodiplodin[14] which have a smaller ring size are devoid of estrogenic properties.



lasiodiplodin



de-O-methyllasiodiplodin

A marked reduction in the activity of zearalenone was noted when the C-4 position was modified.[11] In our tests 4-methoxyzearalenone and 2,4-dimethoxyzearalenone failed to show any significant uterotropic response in rats. Modification of C-2 yields the derivatives with varying degree of activity; nevertheless, they are less active than zearalenone.[15]

Configurational changes in the zearalenone molecule also affect the estrogenic activity. Zearalenol, as mentioned earlier, is 3 - 4 times more active than zearalenone where its diastereomer, β-zearalenol is equal to zearalenone in activity.[6] Peters and Hurd[16] demonstrated that the inversion of the configuration of zearalanone at C-10' results in loss of uterotropic activity. The isomerization[17] of the trans double bond (1, 2) in zearalenone gives the cis isomer which is about 3 - 4 times more active than trans-zearalenone in the rat uterotropic assay.[18]

BINDING CHARACTERISTICS OF ZEARALENONES TO ESTROGEN RECEPTORS

The interaction of zearalenone and its derivatives with specific estrogen binding sites has been recently described.[19] Boyd and Wittliff[20] examined the inhibition of estrogen binding sites in cytosol of lactating mammary gland by zearalenone and zearalanol. They found that: (i) both compounds inhibited [^{3}H]-17β-estradiol association with its binding site in a competitive fashion, and (ii) that zearalanol has a binding affinity five times greater than zearalenone. The dissociation constants determined in this binding study were 1.2×10^{-7} M and 2.2×10^{-8} M for zearalenone and zearalanol, respectively. The binding affinity of 17β-estradiol to cytosol receptor was 5.8×10^{-10} M.

Kiang et al.[19] studied the potency of some derivatives of zearalenone in competing with 17 β-estradiol for binding at the receptor sites. These derivatives were: cis-zearalenone, zearalenol (diastereomeric mixture), zearalanol, 8'-hydroxyzearalenone (F-5-3) and 6'-aminozearalenone. Their relative order of binding was found to be cis-zearalenone > trans-zearalenone > zearalenol > zearalanol. The other two derivatives, 8'-hydroxyzearalenone (F-5-3) and 6'-aminozearalene were inactive even when the concentration was increased by 100 fold. These two derivatives were also inactive in rat uterotropic assay.

Katzenellenbogen et al.[21] found that zearalanol (high melting point isomer) was more active in competing with estradiol for cytosol receptors than the low melting point diastereomer of zearalanol.

Zearalenone derivatives exhibiting binding to cytosol receptors have been shown to elicit an immediate translocation to the nucleus with prolonged nuclear retention.[20, 21]

Martin et al.[22] noted that zearalenone and zearalenols interact with the estrogen receptors of human breast cancer cells (MCF-7) in culture and that these derivatives markedly enhance tumor cell proliferation.

Zearalenone, like tamoxifen, has a nuclear retention greater than 17β -estradiol; however, unlike tamoxifen, zearalenone stimulates the replenishment of the depleted cytosol receptors.[19]

Despite the high affinity that zearalenone and its derivatives show for receptors, they are relatively weak uterotropic agents. From a dose-response curve, the uterotropic activity of zearalenone and α-zearalanol is approximately 1000 and 200 fold, respectively, less than that of estradiol.

Hobson et al.[23] examined the hormonal potency of zearalenone in non-human primates and used the estrogen-induced gonadotropin depression (LH depression) as a measure to estimate the estrogenic potency. Since the estrogen-induced gonadotropin depression occurs at lower levels of estrogen exposure, the assay system Hobson et al. developed was able to detect the injected estradiol, diethylstilbestrol or zearalenone at a level near that of the mouse or rat uterine-weight assays. They found that zearalenone is only slightly less potent than 17β-estradiol or diethylstilbestrol if given in a subcutaneous injection, but its potency when administered orally is significantly reduced.

ZEARALENONES IN ENVIRONMENT

Zearalenone is unique among mycotoxins insofar that it alone can be used as a commercial product.

A derivative of zearalenone enhances the growth rate in cattle and alleviates post menopause discomfort in women. The occurrence of the estrogenic syndrome in swine and of zearalenone in feedstuff has been documented by Mirocha and Christensen[1] and Mirocha.[2] Zearalenone has been found in corn, wheat, barley, oats, sorghum, sesame, hay, commercial animal rations and corn silage. Eppley et al.[24] reported a 17% incidence of zearalenone in 223 samples of marketable corn found in the corn belt ranging in concentration between 0.1 - 5.0 ppm. The occurrence of zearalenone has been summarized by Stoloff.[25] Suffice it to say that it has been found in most parts of the world where maize or other cereals are grown. The feedstuff submitted for analysis is usually associated with problems of infertility in swine and dairy cattle.

Not all cases of hyperestrogenism reported in farm animals are due to zearalenone as some feedstuff is contaminated with diethylstibestrol or both.[26] A more recent finding, perhaps of more significance, is that zearalenone has been found occurring together with trichothecene toxins in feedstuff, usually maize. Trichothecenes are a group of toxic compounds produced by fusarium and other fungi imperfecti.[27] As shown in Table 2, of six feed samples submitted to our laboratory for analysis because of possible implications in toxicosis to animals, five samples contained zearalenone together with deoxynivalenol and one together with T-2 toxin. The potential of synergism of the toxins often complicates diagnosis of the disease by a practicing veterinarian because of the different signs incited by the two toxins.

TABLE 2.

NATURAL OCCURRENCE OF FUSARIUM TOXINS IN FEEDSTUFF

Sample No.	Mycotoxins Found	Concentration (ppb)	Diagnosis	Feedstuff
FS-356	Deoxynivalenol[1]	1,800	Feed refused	Maize kernels
	Zearalenone	250	by swine	(Michigan)
FS-362	Deoxynivalenol	1,000	Feed refused	Maize kernels
	Zearalenone	175	by swine	(Indiana)
FS398A	Deoxynivalenol	100	Feed refused	Maize kernels
	Zearalenone	1,750	by swine	(Ohio)
FS-463	Deoxynivalenol	40-60	Feed refused	Commercial
	Zearalenone	3,600	by swine; and bloody stools	pelleted
FS-417	T-2 Toxin	76	Bloody stools;	Mixed feed
	Zearalenone	100	bovine	(Nebraska)
FS-483	Deoxynivalenol	1,000	Emesis in dogs	Mixed feed (Iowa)

[1] Also known as vomitoxin.

Table 3 lists additional samples of feeds in which zearalenone has been found. Notable of these is the presence of zearalenone in sesame seed used as a supplement for turkey feed.

TABLE 3.

NATURAL OCCURRENCE OF ZEARALENONE IN FEEDSTUFF ASSOCIATED WITH HYPERESTROGENISM IN SWINE

Sample	Concentration	Feedstuff
FS-435[1]	100-150	Maize kernels (Minnesota)
FS-449D[2]	150	Dry sow ration (Vancouver)
FS-453A[2]	66	Farrowing ration (Vancouver)
FS-453B	150	Dry sow ration (Vancouver)
FS-443B[2]	200	Corn kernels (Vancouver)
FS-443A[2]	250	Dry sow ration (Vancouver)
FS-447A	1,000	Lactation ration (Vancouver)
FS-447B	500	Gestation ration (Vancouver)
FS-475[3]	2,000-5,600	Milo (Minnesota)
FS-477[3]	1,500	Sesame meal (Univ. of Minn.)
FS-468A[2]	120	Corn kernels (Ohio)
FS-468B[2]	120	Mixed feed corn (Ohio)
FS-469	6,400	Corn kernels (Minnesota)
FS-470	6,800	Commercial pelletted Mixed feed (Minnesota)

[1] Rectal prolapse in gilts.
[2] Diethylstilbestrol was also present in these samples.
[3] Associated with hyperestrogenism in turkey poults.

Schroeder and Hein[28] reported the isolation of zearalenone from freshly harvested grain sorghum in Texas. They suggested that zearalenone contamination of sorghum in the field may be important when Fusarium head blight is severe in maturing grain sorghum during warm, highly humid weather. No mention of the concentration of zearalenone found was made. In addition, Stipanovic and Schroeder[29] reported zearalanol production by isolates of F. roseum obtained from milo. Refer to Figure 1 for a list of the derivatives of zearalenone produced by Fusarium in laboratory culture.

Sharaf and Negam[30] reported that corn oil used in their experiments exhibited estrogenic activity in rats and mice. This oil is used in animal and human diets in Egypt and they expressed concern. Although no tests were made for zearalenone or its presence implied, it is likely that the corn oil in question contained zearalenone and possibly some other Fusarium toxins.

Gross and Robb[31] attempted to determine whether zearalenone would be formed in field grown barley inoculated by an isolate of Fusarium culmorum, a known producer of zearalenone. The grain was analyzed for zearalenone at time of harvest but none was found; however, after storage for a 20 week period, zearalenone was detected. An unsprayed control plot was also handled in a similar manner; no zearalenone was found at time of harvest but did develop in storage. It is not surprising that zearalenone developed in both the experimental and control barley as it was stored at a moisture content varying between 34 and 42%. It appears that, at least in this study, the greatest potential for zearalenone production occurs in storage at moisture concentrations near optimum for development of Fusarium.

Caldwell and Tuite[31] conducted experiments to determine if dent corn inoculated with Fusarium roseum in the field would produce significant amounts of zearalenone. Small amounts of zearalenone were produced but never exceeding 5 ppm. They concluded that the biosynthesis of zearalenone is more important in storage than in field development. Zearalenone, however, can be found in freshly harvested corn.[33]

Sutton et al.[34] presented evidence for the translocation of zearalenone from inoculated stems into the ear of the corn but not vice versa. The highest concentration found in the ear was about 0.18 ppm whereas the inoculated stem averaged about 1.32 ppm. Inoculated ears contained an average of 12.4 ppm. Although, zearalenone was found in the ears of corn inoculated with Fusarium, it is not clear whether the metabolite was actually translocated or formed in situ by Fusarium spp. unaccounted for. Zearalenone is water insoluble and its translocation is difficult to explain unless a water soluble conjugate (e.g.,

a glucuronic acid adduct) was formed. The significance of this finding at this time is not clear.

The importance of α-zearalenol in nature was evidenced when Hagler et al.[6] demonstrated that α-zearalenol, which is three to four times more active than zearalenone, is the only diastereomer of zearalenol produced by Fusarium. The natural occurrence of α-zearalenol was recently reported[35] and is presented in Table 4. A sample of oats, received from Finland, suspected of being contaminated with mycotoxins and another sample of corn associated with procine hyperestrogenism and refusal of feed by swine in New York contained α-zearalenol together with zearalenone. Deoxynivalenol was also present in all these samples in significant amounts ranging from 0.15 to 4.0 ppm.

TABLE 4.

OCCURRENCE OF FUSARIUM MYCOTOXINS IN FEEDSTUFF

Sample	Zearalenone	α-Zearalenol	Deoxynivalenol
Oats	25.0	1.5	0.0
Oats	135.0	4.0	5.0
Corn	18.0	0.15	1.0

In 1978, stalk rot resulting from infection by Fusarium spp. was common in field corn in Minnesota. Because of wet weather early in the growing season, the roots of many corn plants did not penetrate deeply into the soil, and with the advent of dry weather in late August and September, many of these roots dried, providing an avenue of entrance for fungi into the basal portion of the stem. Subsequently, the interior tissues of the lower internodes were decayed. The decayed tissues of some of the stems were pink or reddish, characteristic of corn invaded by some species of Fusarium. Stems of such corn were found to be associated with significant amounts of Fusarium toxins namely zearalenone (2.8 ppm), T-2 toxin (0.11 ppm) and deoxynivalenol (1 - 5 ppm).[36] Corn stalks are often eaten by grazing cattle or fed to them as silage. The implication of corn stalks posing a hazard when fed to farm animals is apparent.

ZEARALENONE AND REPRODUCTIVE DISORDERS IN FARM ANIMALS

The effect of zearalenone on animals has been amply reviewed by Mirocha[2] and Mirocha and Christensen.[1]. Swine are the most sensitive of the large domestic animals and most commonly affected with hyperestrogenism on the farm although bovine hyperestrogenism due to zearalenone is also suspected. Other farm animals affected are dairy cattle, chickens and turkeys. Animals experimentally affected are rats, mice, guinea pigs, monkeys and lambs.

In swine, this estrogenic mycotoxin seems to cause more marked clinical signs in prepubertal animals than in mature swine. The prominent clinical signs consist of hyperemia and edematous swelling of the vulva along with a slightly turbid mucus discharge. Some gilts have vaginal prolapse and some also have rectal prolapse in severe zearalenone toxicoses.

Swine given feed amended with Fusarium infected cultures during gestation produced weaker piglets; stillbirth, weak piglets, mummifaction and spaylegs resulted in the offspring.[37] It is not clear what part zearalenone played because of the use of unpurified zearalenone.

Palyusik et al.[38] demonstrated that zearalenone-containing feedstuff decreased or completely inhibited the fertility of ganders and turkeycocks, depending on the concentration of toxin. The quantity of sperm and viability of spermatozoa were reduced. The epithelial cells of the testicles were seen in the convoluted tubules.

Vanyi and Szailer[39] reported that animal species whose spermatogenesis is inhibited by zearalenone in vivo are sensitive in the monolayer testicular cell cultures; testicular cells from resistant animals (chicken) were not affected. Swine testicle cultures were most sensitive followed by turkey and calf.

Zearalenone according to Vanyi et al.[40] causes sterility in sows by inciting a malfunction of the ovary. The oocyte dies in the Graafian follicles and despite signs of estrus, there is no ovulation. Zearalenone appears to initially cause maturation in gilts by stimulating primary and secondary follicles in the ovary and proliferation of uterine glands. Degenerative lesions develop, after one week of treatment (100 ppm in foodstuff) in the ovary and midpart of the uteral horn. After stopping toxin treatment, the clinically observed vulvar edema disappears, but the regressive lesions remain. Apparently degeneration of the uterine glands in the mucosa of the uterus contribute to the infertility by not supporting the settlement and nourishment of a ferilized ovum.

Chang et al.[40] studied the effect of pure zearalenone on the fertility of experienced sows with the objective of determining effects on conception, possible abortion or fetal resorption, litter size and condition of piglets at parturition. The animals were fed a ration amended with pure zearalenone at concentrations of 25, 50 and 100 parts per million. They were fed the ration from the time of breeding until parturition or end of the gestation period. The animals were bred 4 days after weaning their previous brood, a time most conducive for conception. Reproductive disorders observed in this experiment

included infertility, small litters, small pigs, a deformed pig, juvenile hyperestrogenism, and possibly fetal absorptions. These reproductive problems are similar to those associated with fusariotoxicosis. At 100 ppm of zearalenone, complete infertility was observed; clinical signs of infertility in all the treated infertile sows were nymphomania or pseudopregnancy. Besides the small size of pigs and one deformed pig, all the female suckling pigs in litters from sows treated with 25 or 50 ppm zearalenone developed hyperestrogenic signs within 7 days after birth.

EFFECT OF ZEARALENONE ON MICROORGANISMS

Investigations with Cochliobolus carbonum indicated that zearalenone at concentrations ranging from 50 to 200 µg inhibited the perithecia production[42] there was an increase in number of perithecia of certain isolates of this species. In F. roseum perithecial formation was enchanced as much as 100% after treatment with 0.1 to 10 g of zearalenone.[43] Amounts in excess of 10 µg inhibited perithecia formation. It has been now demonstrated that zearalenone acts as a hormone in Fusarium roseum where it regulates the production of the sexual stage; i.e., the formation of perithecia. In the fungus system, zearalenone acts in concert with cyclic 3'-5'-edenosine monophosphate to regulate perithecia production.[44]

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DISCUSSION

ULFELDER: Does estradiol produce the same clinical picture in swine as does zearalenone?

PATHRE: Yes.

REEL: Since various estrogenic materials are found at random to contaminate laboratory rodent feeds, it would appear that the newly instituted "certified chows" should be assayed by the vendor and guaranteed not to contain estrogenic contaminants at some stated level. If this is not provided, then the chow should not be a "certified chow." This requirement should be written into the FDA and EPA nonclinical guidelines for good laboratory practices.

PATHRE: I could not agree with you more on this point. Regardless of the FDA or EPA guidelines, it is a sound practice for laboratories to independently establish that the "certified chow" they are using is free of estrogenic contamination.

Xenobiotics as Estrogens

McLachlan, ed. Estrogens in the Environment

FUNCTIONAL MICROHETEROGENEITY AMONG ESTROGEN RECEPTORS: POSSIBLE RELEVANCE TO RECEPTOR ACTION.

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INTRODUCTION

More than 25 years have passed since it was first demonstrated that a protein exists in the cytoplasm of target cells (i.e., a receptor) that binds estradiol with high affinity[1]. Despite many direct correlations of the presence of this entity with the biological responsiveness of tissues to estrogens in vivo and numerous attempts to recapitulate aspects of these responses with receptor preparations in subcellular systems, the role of the receptors has remained an enigma. Most of the studies, however, have emanated from the view that estrogen receptors may act as all or none titrants in switching on or off the genetic expression mechanisms at specific gene sites. From the limited success of these studies it appears that this static view of receptor action may not apply so simply.

For this reason our laboratory has initiated studies in which the estrogen receptor is visualized as a working machine--transducing the conformational changes of the receptor, arising from a cyclic interaction with an entering estrogen, to a target molecule which is presumably another protein in most cases. To test the validity of this concept, we have looked first for evidence that receptors undergo some sequence of conformational changes during the binding of estradiol and second for evidence that endogenous and exogenous factors can modify this chain of events. Using simple alkyl phenols as analogs, we have found that the binding of estradiol is very likely an ordered process at the level of the receptor molecule and that the entry and exit of estradiol from the receptor can be influenced differentially by related alkyl phenol analogs, certain local anesthetic agents, low affinity steroids, and unidentified endogenous factors. The concentration-dependent effects which have been observed with certain of these agents have demonstrated a functional microheterogeneity of the receptors as they exist in fresh uterine cytosols. The possibility that this heterogeneity is related to their function is discussed. Finally, a hypothesis of receptor action is proposed in which the receptor functions as a mechanical transducer of conformational change to a range of target molecules.

INFLUENCE OF ALKYL PHENOLS ON THE BINDING AND RELEASE OF ESTRADIOL BY ESTROGEN RECEPTOR.

Estrogen receptors are noted for their high affinity (dissociation constant $K_D = 3 \times 10^{-9}$M at 0-4°C)[2]; at low temperatures, the displacement of bound estradiol by the free steroid is nearly negligible. Elevation of the temperature to 30-37°C facilitates the exchange process and has provided the basis for assay of occupied receptors[3]. One interpretation of this situation is that an entering estradiol molecule triggers a chain of conformational changes in the receptor molecule that end up capturing the steroid in some cage-like crevice or structure which effectively isolates it from the surrounding aqueous medium containing the high concentration of free estradiol. In such a case the interaction between the initial estradiol and the receptor need not be the same as that involved in the high affinity binding; it also follows that the exit of estradiol from the receptor may be by a different route than its entry.

While actual proof of this postulation will have to await a structural analysis of the isolated receptor molecule, support for the concept has been obtained by studying the ability of simple alkyl phenols to compete with or influence the binding of estradiol by receptors of the uterine cytosol[4]. From the list of compounds presented in Table 1 it is clear that alkyl phenols, containing a nonpolar mass that approximates the size of the B-ring of estradiol, are effective in preventing the binding of estradiol. The time course of such a competition reaction is shown in Figure 1 for tetrahydronaphthol-2 (THN), which was selected as a standard since it is equivalent to the A + B rings of estradiol. It is of interest that with each increase in the concentration of THN a greater fraction of the receptors was prevented from rapidly binding estradiol. This is particularly striking as it was found that THN was relatively ineffective in displacing estradiol from the receptors once it was bound[4].

p-secondary Amyl phenol (pSAP), a structural analog of the A + B rings of estradiol, presented quite a different picture. Not only did this compound inhibit the forward binding of estradiol by receptors of the uterine cytosol, it proved to be highly effective at 0°C in releasing or displacing estradiol which was already bound to the receptors (Fig. 2). The displacement appears to proceed with complex kinetics in which there is a fraction of receptors which rapidly release bound estradiol--followed by a much slower release of estradiol from the remaining receptors. The fraction of the receptor that was

TABLE 1

INHIBITION OF [^{3}H]ESTRADIOL BINDING BY ESTROGEN OF RAT UTERINE CYTOSOLS

	% THN Activity
Phenols with a fixed B ring	
5,6,7,8-Tetrahydronaphthol-2 (THN)	100
naphthol-2	105
naphthol-1	90
6-Bromo-2-naphthol	90
3-OH-naphthoic acid-2	0
3-NO_2-Tetrahydronaphthol-2	0
Assorted Phenols	
Phenol	0
p-Ethoxyphenol	0
2,4,6-Tribromophenol	0
3,4-Dimethyl-6-ethylphenol	0
α-Phenylcresol	65
4,4'-Dihydroxybiphenyl	100
Phenols with flexible 4-carbon side chains	
4-sec-Butyl phenol	50
2-sec-Butyl phenol	0
p-OH-cinnamic acid	0
Phenols with flexible 5-carbon side chain	
p-sec Amyl phenol (pSAP)	105
p-Isoamyl phenol	100
p-tert-Amyl phenol	65
o-sec-Amyl phenol	50
p-(α-OH-isoamyl) phenol	0

Cytosols were incubated for 1.5-3.0 h at 0°C with 1×10^{-3} µg/ml [^{3}H]estradiol in the presence of 50 µg/ml of the indicated alkyl phenol. The level of receptor-bound [^{3}H]estradiol was measured assaying 0.1 ml samples of the cytosol using the hydroxyapatite assay. The data are compiled from a series of experiments in which each test compound is compared to the activity of 50 µg/ml of THN in that experiment. The data are expressed as percentage of activity obtained for an equal weight of THN. Source: Mueller, G.C. and Kim, U.H. (1978) Endocrinol. 102, p. 1431.

caused to rapidly release estradiol increased linearly with the concentration of pSAP (Fig. 3), whereas the rate of the slow release was not affected greatly by varying the level of pSAP in the absence of a diluting source of unlabeled estradiol.

These results show clearly that pSAP in contrast to THN is an effective agent for rapidly displacing receptor-bound estradiol at 0°C and that the fraction of the receptors affected is directly proportional to the concentration of the pSAP which is used. The observation that this fraction is not representative of an equilibrium process is taken as evidence that not all the receptors of the cytosol are functionally equivalent. It appears that a microheterogeneity exists among them with respect to their susceptability to pSAP.

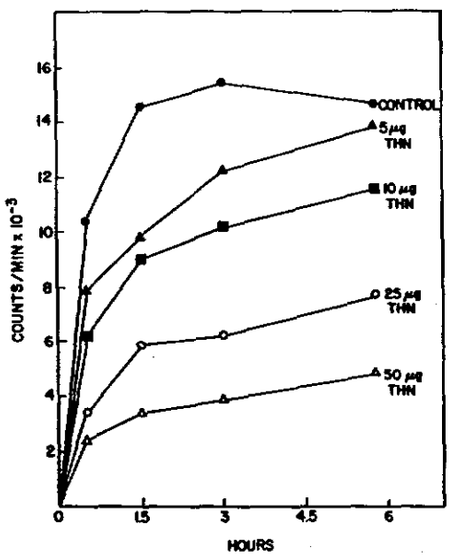

Fig. 1. Effect of THN on the binding of [^{3}H]estradiol. A cytosol from mature rabbit uteri was pretreated with 5-50 μg of THN/ml for 1 hr at 0°C and then exposed to [^{3}H]estradiol (1×10^{-3} μg/ml) at 0°C. At the indicated times, the level of [^{3}H]estradiol binding was determined by adsorption on hydroxyapatite columns. Data are expressed as counts per minute per 0.1 ml of cytosol. Source: Mueller, G.C. and Kim, U.H. (1978) Endocrinol. 102, p. 1430.

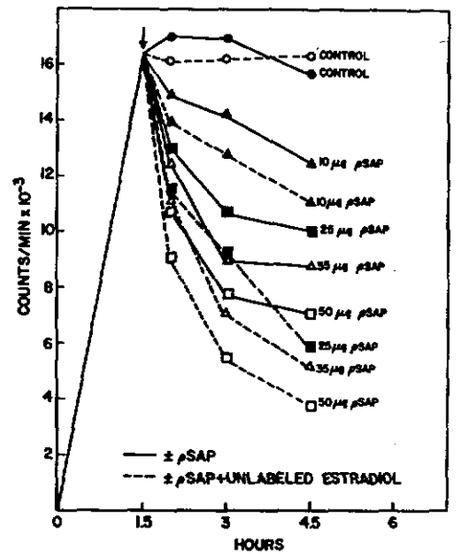

Fig. 2. Displacement of [^{3}H]estradiol from estrogen receptors by pSAP in the presence and absence of unlabeled estradiol. A cytosol from mature rabbit uteri was prelabeled with [^{3}H]estradiol (1×10^{-3} μg/ml) for 1.5 hr at 0°C. At this time, levels of pSAP (10, 25, 35, or 50 μg/ml), alone, (solid lines) or in the presence of 1 μg/ml unlabeled estradiol (dashed lines) as indicated were added to the incubation mixture. Control incubations were run in the absence of pSAP, with (○—○) or without (●—●) added unlabeled estradiol. The level of receptor-bound [^{3}H]estradiol was assayed over the next 3 hr at 0°C using the hydroxyapatite assay. Data are expressed as counts per min per 0.1 ml aliquot of cytosol. Source: Mueller, G.C. and Kim, U.H. (1978) Endocrinol. 102, p. 1432.

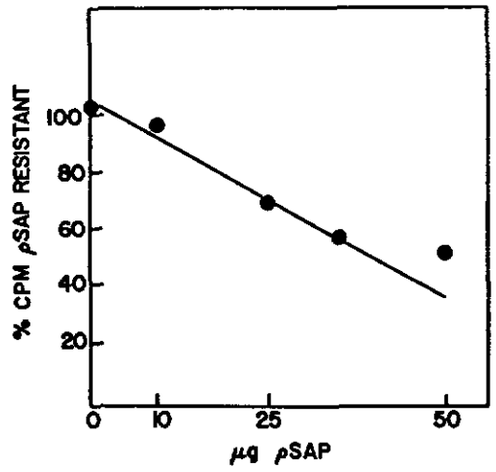

Fig. 3. Effect of pSAP concentration on the rapid release of receptor-bound [^{3}H]estradiol. The fraction of receptors which are caused to release [^{3}H]-estradiol in response to increasing concentrations of pSAP versus that fraction which is resistant has been determined by subtracting the amount due to the slow release in Fig. 2 (i.e., the pSAP independent rate determined in the interval from 3 to 4.5 hr) from the total released. The data are plotted as % of the receptor-bound [^{3}H]estradiol which is resistant to a given concentration of pSAP.

The finding that increasing concentrations of THN can act competively in the forward binding process to prevent increasing fractions of the receptor from binding estradiol, is taken as further evidence of this functional microheterogeneity. However, the fact that THN is relatively ineffective in displacing receptor-bound estradiol at 0°C argues that THN and pSAP affect the receptors in different ways. It is important to note that the effects of both THN and pSAP are mediated through low affinity interactions with the receptors, since gel filtration, sedimentation through a sucrose gradient, or treatment of the receptor preparation with dextran-charcoal is sufficient to completely free the receptors of either agent.

INFLUENCE OF OTHER ESTROGEN ANALOGS ON THE BINDING OF ESTRADIOL.

In the course of these experiments, a series of D-ring derivatives of estradiol were synthesized in the attempt to introduce a ligand which might be used to retrieve native receptors from cytosols by adsorption on an affinity column. When tested for their ability to bind to the receptors, it was found that estradiol derivatives with a chain of 3 or more carbons at the 17α position failed to bind to the receptor with high affinity. An example of such a compound is 17α-(2',3'-dihydroxypropyl)-17β-estradiol. While this compound only shows a low affinity interaction with estrogen receptors (at graded high concentrations; 100 to 1000X the level of estradiol), it acts like THN in blocking fractions of the receptors from binding radioactive estradiol at 0°C. Like THN, this agent is unable to displace receptor-bound estradiol even when the temperature is elevated to 30°C. In fact, elevating the temperature overcomes the inhibiting action of 17α-(2',3'-dihydroxypropyl)-17β-estradiol and results in a binding of the [^{3}H]estradiol. Similar to the situation with THN the fraction of receptors which is affected by this steroid increases with the concentration of the agent.

In this same series of studies, it was found that the so-called anti-estrogens, tamoxifen and nafoxidine, affect the estradiol receptors of rabbit uteri in a similar manner. Neither compound binds with high affinity, however, they prevent the binding of estradiol at 0°C in a manner which is overcome by elevating the temperature to 30°C. Again the fraction of receptors which is affected is linearly proportional to the concentration of the agent.

EFFECT OF TETRACAINE ON THE BINDING OF ESTRADIOL BY RECEPTORS

Additional evidence for the functional microheterogeneity of estrogen receptors comes from studies of the effects of local anesthetic agents on estrogen receptors. As shown in Fig. 4, the addition of increasing concentrations of tetracaine (TET) causes increasing fractions of receptor bound [^{3}H]estradiol to be rapidly exchanged or displaced by a diluting source of unlabeled estradiol at 10°C. The fraction of rapidly exchanging receptors is linearly proportional to the concentration of tetracaine in a manner which is similar to that cited above for pSAP. The fraction of receptors exhibiting rapid exchange with a given concentration of tetracaine is also linearly proportional to the temperature within the range of 0-20°C. In effect, increasing concentrations of tetracaine lowers the temperature at which a finite fraction of receptors will

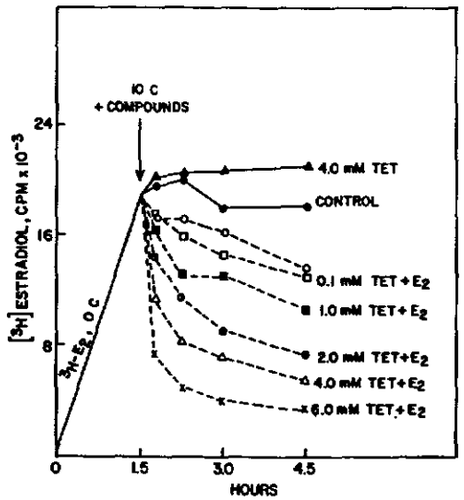

Fig. 4. Effect of tetracaine concentration on the exchange of receptor bound [^{3}H]estradiol. Rabbit uterine cytosol was pretreated for 1.5 hr at 0°C with [^{3}H]estradiol. After this interval the indicated levels of tetracaine were added ± a diluting pool of unlabeled estradiol and the temperature of the system was elevated to 10°C. At the indicated times aliquots were assayed by the hydroxyapatite method for receptor-bound [^{3}H]estradiol. The data are expressed as cpm x 10^{-3} of receptor bound [^{3}H]estradiol. Mueller, G.C. and Kim, U.H., unpublished observations.

exchange their bound estradiol. As will be shown elsewhere, tetracaine functions by relaxing the specificity of estrogen receptors for estradiol analogs as well as their affinity for estradiol. Presumably, it does this by reacting as a large hydrophobic anion at the surface of the receptor--possibly at sites which bind calcium, such as regions containing phosphatidyl serine. It does not mimic estradiol in anyway or compete directly at the estradiol binding site. The observation that the receptors respond fractionally to increasing concentrations of tetracaine suggests that the receptors differ from each other at a number of such sites. This situation may be responsible as well for the differences in the behavior of the receptors in the experiments with pSAP, THN, and estrogen analogs.

ESTROGEN RECEPTORS AS MACROMOLECULAR TRANSDUCERS - A DYNAMIC CONCEPT OF RECEPTOR ACTION.

These new observations on the properties of estrogen receptors have been integrated into a concept (Fig. 5) in which estrogen receptors are visualized as functioning cyclically as macromolecular transducers. It is proposed that the estrogen or analog interacts initially with the estrogen receptor at site A--a site that is distinct from the region of the receptor that finally binds estradiol with high affinity (site B). The interaction with site A is visualized as setting into motion a wave of conformational changes which spread through the structure of the receptor and ultimately causes the receptor to close on the steroid to capture it at site B. The observation that 17α analogs of estradiol, as well as analogs with extra mass or charge at position 16 fail to bind with high affinity, suggests that the binding may concern primarily the C-D region of the estradiol. At the very minimum, it suggests that the steric constraints are severe with respect to the receptor's ability to encompass the D-ring end of the steroid molecule.

To explain the ability of pSAP to displace estradiol at 0°C it is suggested that site A is unoccupied and exposed even though an estradiol molecule may already be sequestered at site B. pSAP, like any other entering estrogen analog, is visualized as directing a wave of conformational changes in the receptor which momentarily opens site B and releases the bound estradiol molecule. It is proposed that pSAP can do this at 0°C in contrast to THN or estradiol because the flexible secondary amyl side chain (i.e., bulk equivalent to the B-ring) directs the flexing of the receptor in quite a different manner than does the fixed B-ring structure of THN or estradiol. In this view it is proposed that the character of the structural change resulting from an attacking steroid is significantly directed by the structure of the entering molecule. It follows that the apparent difficulty in exchanging receptor-bound estradiol may arise in part from secondary estradiol molecules directing the conformational changes in a manner that reenforces the binding of the estradiol molecule that already occupies the high affinity site B. The observed interference of 17α-(2',3'-dihydroxypropyl)-17β-estradiol, nafoxidine, and tamoxifen in the competitive binding of estradiol may be explained in a similar manner if one assumes that the induced changes in the structure of the receptor are unfavorable to the capture of estradiol at site B. In this connection, it is important to keep in mind that the various peptide chain vibrations underlying the conformational changes would all display individual time

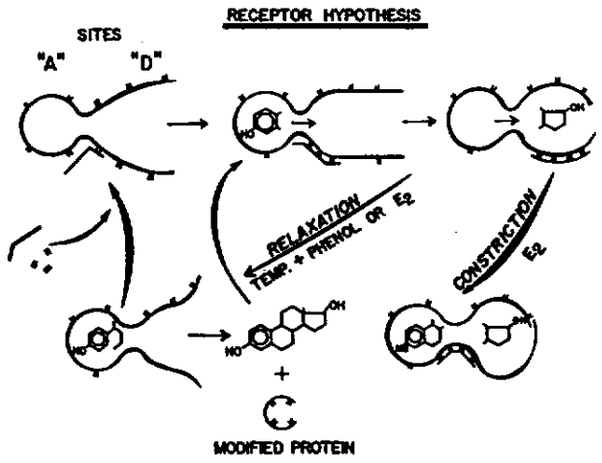

Fig. 5. A concept of estrogen receptor action. Estrogen receptors are depicted as flexible proteins which interact conformationally to form complexes with a range of proteins and/or nucleic acid-protein associations which exhibit some common recognition group or peptide character. It is proposed that the entering estrogen or analog attacks at site A to set in motion a wave of conformational changes amid the peptide chains of the receptor whose character is determined by the structure of the entering molecule. With estradiol and closely related estrogens, this wave of changes ends up with the capture of estradiol at site B (i.e., high affinity binding). With certain alkyl phenols and the estrogen antagonists, the induced conformational changes are contrary to those required for high affinity binding of estradiol. In all cases, however, it is proposed that the conformational changes of the receptor are transduced to the associated macromolecules of the receptor complexes. When the induced change in the target molecule makes the latter more fit for participating in a genetic expression process or a multienzyme catalytic process, a hormone response is achieved. The functional microheterogeneity of estrogen receptors which has been observed in binding studies is attributed to adsorbed small molecules which may play a role in the adaptive recognition and interaction of the receptors with their target molecules. Receptor action is visualized as operating cyclically.

constants--yet the high affinity binding would most likely be the result of a timely combination of interactions between the receptor chains and the steroid molecules. Accordingly, a single, wrongly-directed movement of the peptide chains making up the receptor, might strikingly reduce the probability of having a combinatorial arrangement of chains that is favorable to high affinity binding of estradiol.

While the postulated steroid-induced changes in receptor structure can be easily seen as playing a part in the high affinity binding of estradiol, it is proposed that they may play an even more important role through the conformational changes they inflict on associated macromolecules. In this connection, it should be noted that estrogen receptors hardly ever exist in a free state. In a low ionic environment they centrifuge as a broad set of receptor complexes with other proteins and are loosely referred to as the 8-9s receptors. The latter are dissociated to the 4s receptor state by 0.4 M KCl treatment, but reassociate again to give a new sedimentation pattern which reflects both the type and amount of other proteins that are present in the receptor preparations during this process[5,6]. Recent studies from our laboratory have also shown that the original 8-9s receptors of rabbit uterine cytosols are divisable into distinct subsets of receptor complexes according to the level of KCl that is required to dissociate them to the 4s state. In addition, it has been found that the dissociation of certain fractions of these receptor complexes can be influenced by the low affinity estradiol analogs and the estrogen antagonist, tamoxifen. Finally, we have found that both estradiol and dexamethasone influences the uptake of specific proteins from the cytosol into nucleochromatin[7].

These observations lead to the hypothesis that estrogen receptors, and very likely other steroid receptors, function by transducing steroid-induced conformational changes of the receptors to adjacent macromolecular targets. The altered target molecules, which are probably proteins in most instances, then fit with greater or lesser efficiency into the structural assemblies affecting the genetic expression processes or into the multienzyme complexes that are involved in certain catalytic mechanisms. In this way receptors are proposed to mediate selectively both positive and negative changes in gene expression or existing metabolic systems.

Inherent in this concept is the operation of a recognition mechanism between the receptor and its target molecules. In view of the heterogeneity of the receptor complexes in fresh cytosols and the range of biological responses that are mediated by a single hormone in target cells, it appears most probable that

the recognition is mediated by the complementarity of the receptor to some small peptide sequence, carbohydrate chain, lipid association, or other prosthetic group which is carried commonly by a specific range of proteins. This situation would be equivalent to a class-like recognition of certain targets and could account for the domain-like nature of the genetic responses which has been observed for at least one steroid[8]. The functional, microheterogeneity of estrogen receptors which has been observed in our present experiments would be best explain by lipids, peptides, carbohydrates, or other small molecules which are picked up or become associated with the surface of a common receptor protein during the course of the receptor-target molecule interactions. These components, whose association appears to be subject to considerable revision during fractionation and treatment of estrogen receptors with salts or elevated temperatures, may also play a role during the recognition process by contributing to the stability of the receptor-target intermediates or the release of receptors from such complexes as they function cyclically.

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McLachlan, ed. Estrogens in the Environment

OXIDATIVE METABOLISM OF STILBENE ESTROGENS

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INTRODUCTION

More than 40 years ago, the synthesis of diethylstilbestrol (DES, Figure 1) and the discovery of its estrogenicity started a new area in therapy with hormones.[1] For the first time in medicine, a synthetic compound mimicing the effect of a hormone was available. Aside from being inexpensive, the synthetic estrogen even had certain therapeutic advantages over the natural estrogens, viz. a longer lasting effect and efficacy after oral administration. Dienestrol and hexestrol (DIES and HES, Figure 1), other synthetic estrogens discovered at the same time, also shared these properties. Although DIES and HES do not really contain a stilbene moiety, they are generally considered together with DES as "stilbene estrogens".

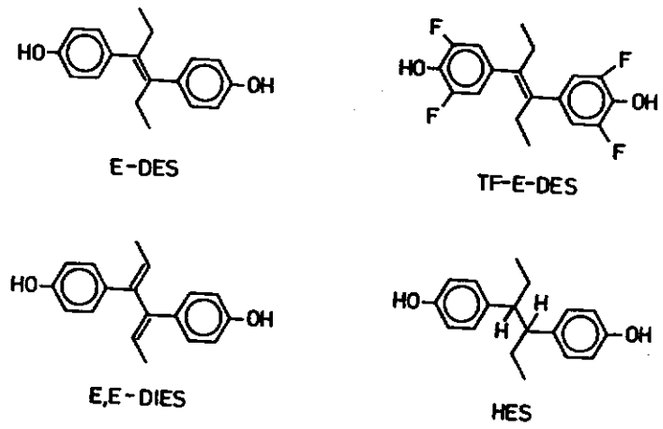

Fig. 1. Chemical structures of several stilbene estrogens.

Interest in the metabolism of stilbene estrogens arose soon after their synthesis, but faded away quickly after the identification of the glucuronides as the major metabolites. For several reasons, the belief became widely accepted that glucuronidation was the only metabolic transformation undergone by the stilbene estrogens.

Several years ago, however, interest in the metabolic fate of these compounds was revived by the association of *in utero* exposure to synthetic estrogens with genital tract abnormalities, including tumors, in young women (for review, see [2]). Since the early sixties, enormous progress has been made in the field of chemical carcinogenesis and teratogenesis. It is now generally accepted that electrophilic reactivity is one prerequisite for an organic compound to be carcinogenic; most compounds gain this reactivity through biotransformation in the affected organism, a process called metabolic activation. This raised the possibility that metabolism was important for the carcinogenic effects of DES.[3] Subsequent *in vivo* and *in vitro* studies have led to the identification of numerous oxidative metabolites of DES, and evidence is beginning to emerge that metabolic activation may, indeed, play a role in the carcinogenicity and fetotoxicity of this synthetic estrogen. These aspects are briefly reviewed in the present paper, together with new data on the metabolism of other stilbene estrogens.

METABOLISM OF DIETHYLSTILBESTROL

Structure of metabolites and intermediates

Oxidative metabolites of DES have been identified in the excretory products, mainly urine and bile, of humans, chimpanzees, Rhesus monkeys, mice, rats, and hamsters, as well as in *in vitro* experiments with microsomes and peroxidase (for review, see [4]). The structures of eleven metabolites have been elucidated so far, and these compounds are arranged in a metabolic scheme in Figure 2, together with their probable intermediates. The structures of 3'-hydroxy-DES, Z,Z-DIES, indenestrol A, 1-hydroxy-Z,Z-DIES, and 4'-hydroxypropiophenone (HPP) were confirmed through comparison with authentic reference compounds.

Figure 2 shows that at least four oxidative pathways are operative in DES metabolism:

- aromatic hydroxylation leading to the catechol, 3'-hydroxy-DES, and possibly proceeding through an arene oxide intermediate. The catechol is then methylated, probably at the 3'-oxygen, to yield 3'-methoxy-DES. At least in the rat, this reaction may occur at both aromatic rings, thus giving rise to 3',3''-dimethoxy-DES.

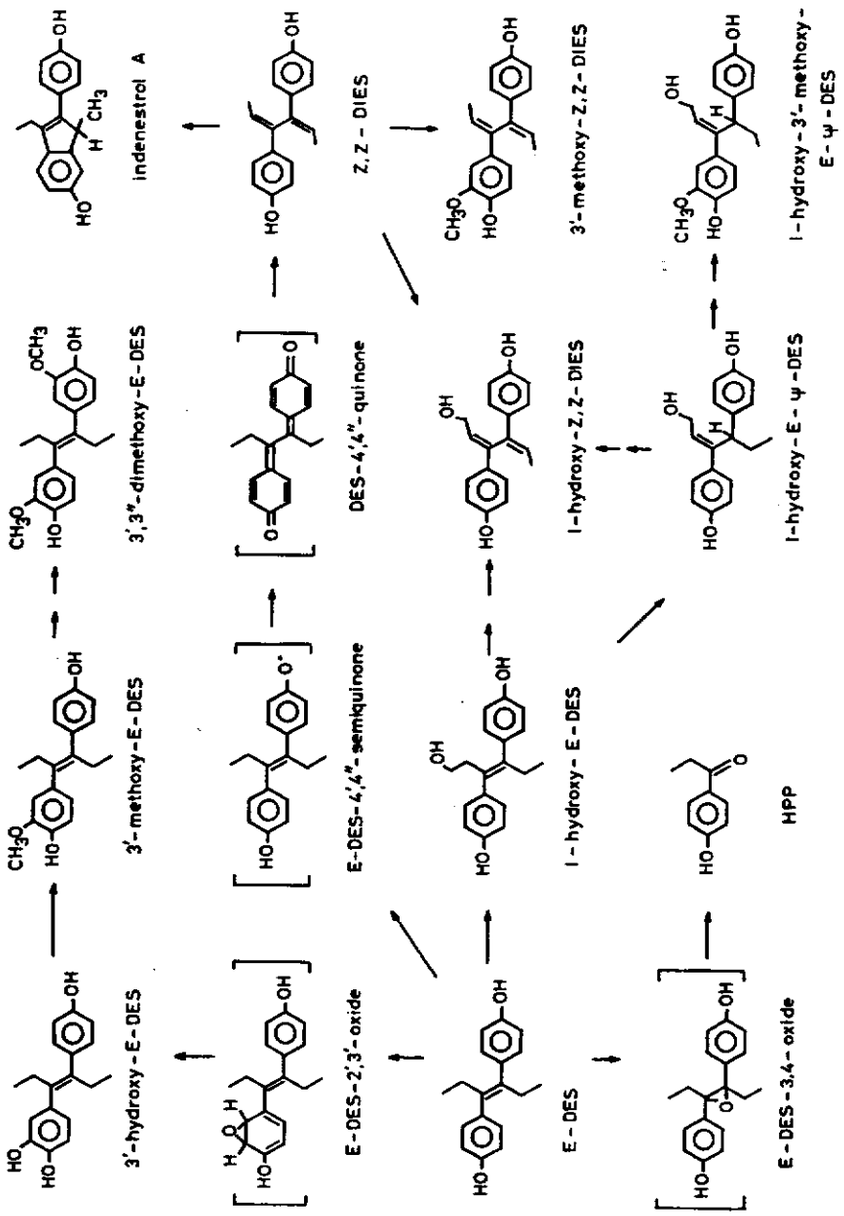

Fig. 2. Metabolic pathways of diethylstilbestrol. For nomenclature see [5].

- oxidation of the stilbenediol structure, finally yielding Z,Z-DIES. This reaction has been shown to be mediated by peroxidases,[6] and probably proceeds through a semiquinone and quinone. The resulting Z,Z-DIES may undergo further reactions, e.g. methoxylation, cyclization to indenestrol A, or hydroxylation to 1-hydroxy-Z,Z-DIES.
- aliphatic oxidation of DES leading to 1-hydroxy-DES. Whether this metabolite is the precursor of 1-hydroxy-ψ-DES is still unknown.
- cleavage of the DES molecule resulting in the formation of HPP. There is some evidence that this reaction proceeds through an olefinic epoxide.[7]

Species differences in DES metabolism

The pattern of oxidative DES metabolites excreted as urinary glucuronides by different species (Figure 3) reflects considerable species differences,

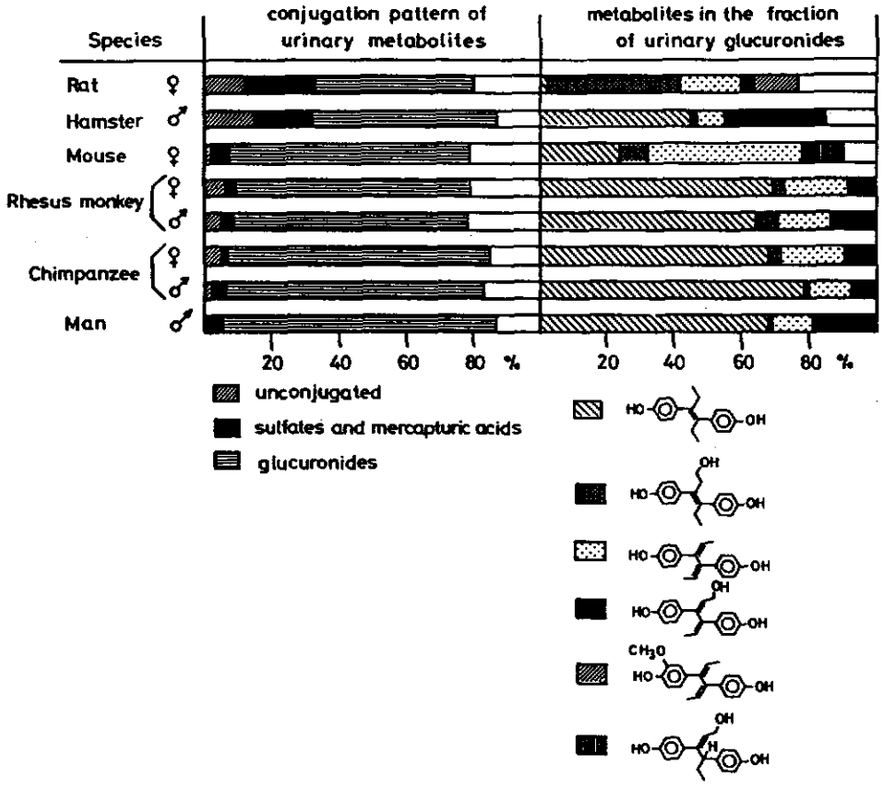

Fig. 3. Species differences in conjugative and oxidative metabolism of DES.

particularly between certain rodent species and primates. For example, the rat does not excrete significant amounts of DES as urinary glucuronide,[8] whereas the parent compound constitutes the major glucuronide in the urine of Rhesus monkey,[9] chimpanzee,[9] and man.[8] Some of the minor urinary DES metabolites, such as indenestrol A and 1-hydroxy-3'-methoxy-ψ-DES, are not included in Figure 3. Others, such as 3'-hydroxy-DES and its methylated derivatives are predominantly found in the bile.[8] Differences in conjugation reactions are much less pronounced, since glucuronidation predominates in all species (Figure 3).

Metabolism in fetal and newborn animals

Oxidative metabolites of DES have also been detected in the whole body extract of several rodent species during perinatal life, a susceptible period for DES carcinogenicity and teratogenicity. Thus, by using GLC-mass fragmentography, Z,Z-DIES, 1-hydroxy-Z,Z-DIES, and 1-hydroxy-ψ-DES were identified in the glucuronide fraction from 1, 4, and 8 day old mice injected i.p. with ^{14}C-DES.[7] More recently, eight oxidative metabolites have been identified in the whole body extracts from 15 day old hamster fetuses and placentas after administration of ^{14}C-DES to the mother or after intrafetal injection.[10] Z,Z-DIES, indenestrol A, 1-hydroxy-DES, 1-hydroxy-ψ-DES, 1-hydroxy-Z,Z-DIES, 3'-methoxy-DES, 3'-methoxy-DIES, and HPP were identified in the extracts through GLC-mass fragmentography after HPLC separation. It is likely that most of these metabolites were formed in the fetus rather than in the maternal organism, since the fetal concentration of metabolites was greater when DES was injected directly into the fetus than when the same dose was given to the mother. One to six percent of the tissue radioactivity could not be extracted from the fetus or placenta.

DES metabolites in human plasma

GLC-mass fragmentography was also used for the identification of DES metabolites in the plasma of two postmenopausal women treated with DES for remissions of breast cancer (Metzler, unpublished data). In the glucuronide fraction of plasma obtained 3 hours after an oral dose of DES (0.5 mg/kg), 3'-methoxy-DES, Z,Z-DIES, 1-hydroxy-Z,Z-DIES, and traces of indenestrol A were found.

GENOTOXICITY OF DES AND ITS METABOLITES

The studies reported have amply demonstrated that DES is metabolized by oxidative pathways in mammalian species including those which are susceptible to the carcinogenic and fetotoxic effect of DES. Several of the oxidative

routes are likely to involve reactive intermediates, such as the aromatic and olefinic epoxide of DES or the DES semiquinone and quinone (Figure 2). Indeed, it has been frequently observed in metabolic studies with radio-labelled DES that some non-extractable binding to tissue macromolecules occurs. Some of the reported findings are listed in Table 1.

TABLE 1

NONEXTRACTABLE BINDING OF DIETHYLSTILBESTROL METABOLITES TO TISSUE MACROMOLECULES IN VIVO AND IN VITRO.

Metabolic condition	Tissue macromolecules	Reference
In vivo	Rat hepatic tissue	11
	Rat plasma proteins	12
	Neonatal mouse tissue	7
	Hamster fetal tissue	10
Rat hepatic microsomes	Microsomal proteins	13,14
	DNA	15
Mouse hepatic or uterine microsomes	DNA	16
Mouse uterine peroxidase preparation	DNA and protein	6

Although these findings lend support to the formation of reactive metabolites, their importance to the toxic effects of DES remains to be established. The genotoxic activity of DES is still an open question. No mutagenicity of DES or its metabolites could be detected in the Ames Salmonella test system. Lately, however, DES has been reported to be active in other systems which assay genetic damage (Table 2). This was particularly pronounced in the sister chromatid exchange assay (SCE), where the potency of DES exceeded that of benz(a)pyren.[23] Moreover, this study showed that DES gains genotoxicity through metabolic activation, since its efficacy was suppressed by α-naphtoflavone, an inhibitor of drug metabolizing enzymes. The DES metabolites E-DES-3,4-oxide and Z,Z-DIES (see Figure 2) were more active than DES in this test by a factor of 10 and 70, respectively.

TABLE 2

REPORTED GENOTOXICITY OF DIETHYLSTILBESTROL

Assay System for Genetic Damage	Result	Reference
Salmonella test under activating conditions	Questionable	17
	Negative	18
Mammalian cell transformation in vitro	Negative	19
Induction of unscheduled DNA synthesis in HeLa cells in the presence of microsomes	Positive	20
Skin test in hairless mice	Positive	21
Mutations in a mouse lymphoma cell line	Positive	22
Sister chromatid exchange in human fibroblasts	Positive	23

METABOLISM OF TETRAFLUORO-DES AND HEXESTROL

For a future evaluation of the role of metabolites in estrogen toxicity it appeared to be of interest to study the biotransformation of derivatives of DES, in which certain metabolic pathways are blocked through structural alteration of the molecule. Examples for this are 3',5',3'',5''-tetrafluoro-DES (TF-DES, Figure 1) and meso-hexestrol (HES), both of which are potent estrogens.[24]

In vitro studies with peroxidase have shown that TF-DES could be oxidized to the respective dienestrol.[24] However, the aromatic hydroxylation resulting in catechol formation should be precluded by the four fluorine atoms in the molecule. In accordance with this assumption, no indication of a ring-hydroxylated or -methoxylated metabolite was found when the glucuronide fraction from rat bile was analyzed by GLC-mass spectrometry (Metzler, unpublished data). Unchanged TF-DES and a small amount of TF-DIES (Figure 4) appeared to be the only metabolites present in the bile. This is in contrast to the biliary metabolites found after DES administration, in which the monomethoxy- and dimethoxy-DES were major metabolites.[8]

When analogous studies were performed with hexestrol, no oxidation to dienestrol by peroxidase in vitro could be observed (Metzler, unpublished data). The biliary glucuronides from HES-dosed rats, on the other hand, contained a

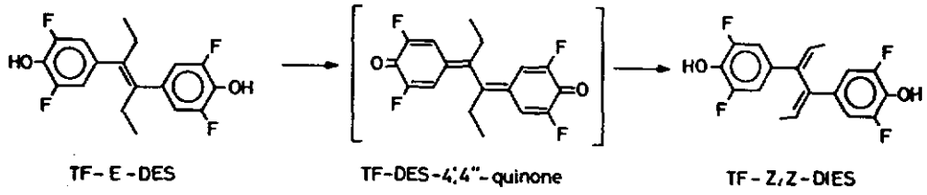

Fig. 4. Oxidation of TF-DES to its respective dienestrol derivative.

variety of metabolites (Figure 5), chiefly ring-hydroxylated and methoxylated compounds. Thus, HES metabolism proceeds via the aromatic hydroxylation pathway and not the peroxidase-mediated oxidation pathway. It will now be of considerable interest to see whether the toxic effects of TF-DES and HES differ from that of DES and, if so, whether they can be correlated with different metabolism.

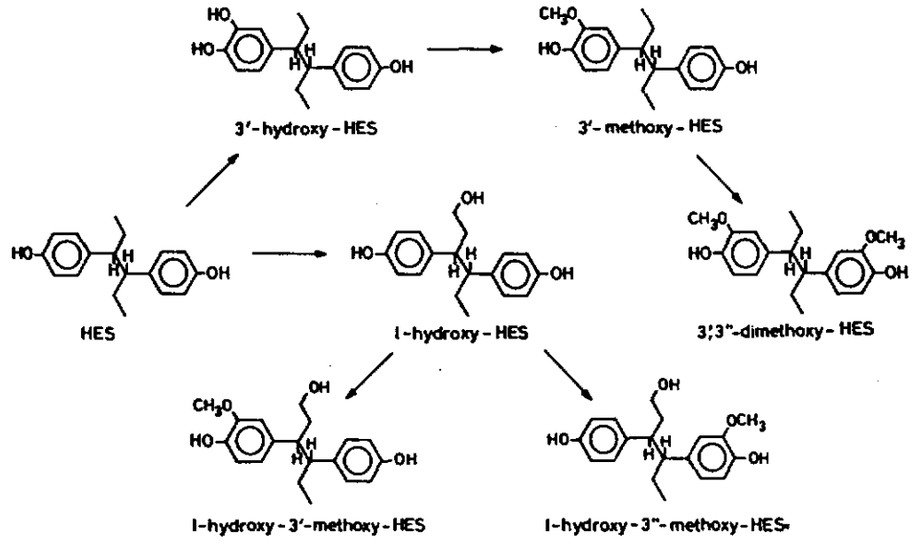

Fig. 5. Oxidative metabolism of meso-hexestrol (HES).

CONCLUSION

The extensive oxidative metabolism of DES to reactive and genotoxic compounds raises the possibility that metabolic activation of DES may be involved in the carcinogenicity and fetotoxicity of this synthetic estrogen. Several mechanisms may be considered for the role of reactive metabolites in the toxic effects of DES in estrogen target organs. For example, some of the metabolites may retain their estrogenicity and utilize the estrogen receptor for accumulation in the target tissue and for facilitated access to the nuclear DNA for covalent binding. The estrogenicity of some of the metabolites of DES has recently been investigated,[25] and E-DES-3,4-oxide was found to be highly estrogenic in vivo and in vitro. The organotropic effect of DES could also be explained by the formation of reactive DES metabolites within the target tissues. This is exemplified by the oxidation of DES to DIES by estrogen-induced uterine peroxidase.[6]

Comparative studies of DES metabolism and DNA binding in susceptible and nonsusceptible organs and in responsive and nonresponsive assay systems, as well as the effects of structural alterations of the DES molecule on its toxicity, should help further clarify the role of metabolic activation by distinguishing critical and noncritical pathways of metabolism.

ACKNOWLEDGEMENTS

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DISCUSSION

KUPFER: Since you indicated that DES-3,4-oxide is chemically stable, have you determined with this compound whether the cleavage product is actually formed in vivo?

METZLER: Yes. After administration of radiolabeled DES-3,4-oxide to mice, 4-hydroxypropiophenone (HPP) was the major in vivo metabolite identified in urine. However, the site of formation of HPP is still unknown. It may be the liver, but it could also be the intestinal bacteria due to enterohepatic circulation.

KUPFER: I hope you would not be surprised if hydroxylation of the fluorinated DES does, in fact, occur with displacement of fluorine atoms.

METZLER: We would not expect displacement of fluorine atoms. Nevertheless, we have screened the biliary metabolites of tetrafluoro-DES for both tetra-fluorinated and trifluorinated hydroxy and methoxy-DES, but did not find any of them.

OFNER: Do you have evidence for bis-catechol formation in the hepatic microsomal metabolism of DES?

METZLER: We have indeed found a bis-catechol of DES among the major products of microsomal DES metabolism, when microsomes from rat liver were used. Interestingly, it was not found in significant amounts with microsomes from hamster and mouse liver. This nicely fits the findings that the respective dimethoxy-DES is only found in the bile of rats but not other species.

KATZENELLENBOGEN: Quantitatively, how much DNA binding is seen with DES as compared with that seen with classical carcinogens?

METZLER: As far as I recall, the data for the in vivo and microsomal in vitro binding of DES to DNA, it is one to two orders of magnitude below that of benzo(a)pyrene.

KATZENELLENBOGEN: Since DES is now showing up as positive in some short term tests, it should be possible to address the very important question -- is the genetic toxicity of estrogens related to their estrogenic potency? It would be important to test estradiol, ethylnylestrogens, zearalenone, and phyto-estrogens as well as DES.

METZLER: I agree with you that other estrogens should also be tested for genotoxicity. The only comparative study I am aware of is that of Forsberg, who, by using a skin test in hairless mice, found that DES is positive in that assay, whereas dienestrol (presumably the estrogenic isomer, E,E-dienestrol) and ethynylestradiol are not.

KATZENELLENBOGEN: Is there any conversion of hexestrol into DES?

METZLER: In our preliminary study, using unlabeled hexestrol and looking at the biliary metabolites in the rat, no evidence for the conversion of hexestrol into DES has been found.

REEL: Does naphthoflavone also prevent covalent binding of DES (or its metabolites) to DNA or proteins? Such an experiment would indicate whether oxidized reactive intermediates (e.g., epoxides) are involved in covalent binding to macromolecules. Covalent binding of reactive intermediates might explain the positive activity of DES in the sister chromatid exchange assay as well as the teratogenicity of DES.

METZLER: The effect of α-naphthoflavone on the covalent binding of DES to DNA and proteins has not yet been investigated in this system.

SONNENSCHEIN: Could you let us know what is the concentration of the metabolites in plasma that are particularly genotoxic in cells in culture, and what is the concentration at which these metabolites are tested in culture conditions?

METZLER: Very little is known about the chemical nature of DES metabolites in plasma; those identified in human plasma by GLC-Mass fragmentography have not been quantitated. Only two metabolites of DES have been tested for genotoxicity so far; one of them, Z,Z-dienestrol has been demonstrated in the plasma of humans and mice; the other one, E-DES-3,4-oxide does not appear in plasma. In the culture system used for sister chromatid exchange, positive results were seen at nM concentrations of chemical.

McLachlan, ed. Estrogens in the Environment

ESTROGENICITY OF KEPONE IN BIRDS AND MAMMALS

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INTRODUCTION

Estrogens are essential for the normal development and function of the female reproductive system of vertebrates. However, excessive amounts of these compounds can have adverse effects on the function of reproductive organs in both sexes. All of the physiologically important estrogens are 17-carbon steroids, e.g. 17β-estradiol, estrone and estriol, that are synthesized in the ovary in response to pituitary hormones. A balance between synthesis, binding to serum proteins, metabolism to inactive compounds, and clearance keeps the effective concentration of these naturally occuring estrogens within a normal range during reproductive cycles. In addition to these naturally occuring steroidal estrogens, an increasing number of nonsteroidal compounds have been shown to have estrogenic activity. Some of these compounds are as potent as 17β-estradiol, e.g. diethylstilbestrol and hexestrol. Moreover, many of these escape the normal mechanisms involved in modulating estrogenic activity by not binding to serum steroid-binding proteins and by resisting metabolic degradation. The introduction and widespread use of insecticides has revealed that a number of chlorinated hydrocarbons, e.g. o,p' DDT and Kepone, also have weak estrogenic activity in birds and mammals. Because they are concentrated in the food chain, the estrogenicity of these compounds can become a serious problem.

Kepone, also known as chlordecone, is a chlorinated cage compound with the structure shown in Fig. 1. In the past, relatively small amounts of Kepone have been used to control insect pests. However, a related insecticide, Mirex (Fig. 1), has been applied to vast areas of the Southeastern United States to help control fire ants, and recent investigations have shown that Mirex can be converted to Kepone in the environment[1]. While Mirex has been deliberately applied in the field, Kepone was discharged into the air and water at its site of manufacture in Hopewell, Virginia where it contaminated large areas of the surrounding environment[2,3]. In addition to being an acutely toxic chemical to a wide range of animals, including man, Kepone also has adverse effects on vertebrate reproduction. In this paper we summarize our data relating to the

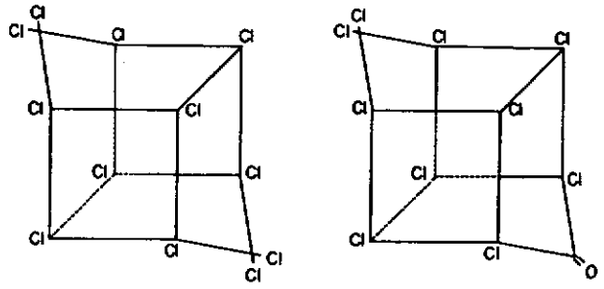

Fig. 1

estrogenic effects of Kepone on the reproductive organs of birds and mammals.

RESULTS

Effects of Kepone on quail oviduct and liver. Four to six-week-old, female Japanese quail were reared under reduced photoperiod of 6 hr light. Feed mixed with 10 to 200 ppm of Kepone was available *ad libitum*; another group was injected daily with 17β-estradiol (40 µg/day). After 3-4 weeks of this treatment, estradiol increased the wet weight of the oviduct 60-fold (Table 1). Kepone produced a dose-dependent increase in oviduct weight; 160 ppm was as effective as estradiol[4,5] (Table 1). When the same concentrations of Kepone were fed to mature, egg-laying quail, no significant changes in oviduct weight were noted. Kepone also doubled the weight of the liver in both females and males[4], and the

Table 1. Effects of Kepone on Quail Oviduct and Liver Wet Weight

Chemical	Dose	Duration	Oviduct Weight	Liver Weight
		days	*g*	*g*
none	-	26	0.02	2.84
Kepone	10 ppm	26	0.03	-
Kepone	40 ppm	26	0.10	-
Kepone	80 ppm	26	0.36	-
Kepone	160 ppm	26	1.12	-
Kepone	200 ppm	21	1.60	6.41
Estradiol	40 ug/day	26	1.13	-

Kepone was dissolved in acetone and then mixed in the feed at the concentrations indicated. 17β-estradiol was dissolved in sesame oil (400 µg/ml) and injected intraperitoneally.

livers were yellow as is characteristic of estrogen stimulation of egg yolk production in this organ (Tables 1 and 2).

Effects of Kepone on quail testes. Feeding maturing male quail a diet containing 200 ppm Kepone for 3 weeks produced a significant increase in testes weight; all testes appeared large and edematous. When the same diet was fed for 6 weeks, some of the testes were still twice as large as controls, while others were smaller and atrophied; the average weight was still significantly greater than controls[6] (Table 2). The effects of Kepone on gross weight and appearance of the testes were not reversible within 60 days, whereas the effects on the liver were completely reversible.

Table 2. Testicular Weight Change due to Kepone Ingestion

Treatment	Concentration	Bird age	Duration of treatment	Testes weight	Liver weight
	ppm	*weeks*	*weeks*	*g*	*g*
Control	-	6	0	1.59	2.63
Kepone	200	6	3	2.72	7.75
Control	-	12	0	2.76	2.52
Kepone	200	12	6	4.47	7.65

Kepone was dissolved in acetone and applied to the feed at 200 ppm

Effects of 17β-estradiol and Kepone on the morphology of immature quail oviduct. Daily injection of 17β-estradiol (40 µg/day) stimulates dramatic morphological changes in the immature quail[7,8]. Within a few days, tubular gland cells evaginate from the original, pseudostratified epithelium into the underlying stroma to form long tubular glands in both magnum and shell gland regions of the oviduct. Furthermore, the epithelial cells lining the main lumen of the oviduct become heavily ciliated. The cilia exhibit a tall, uniform appearance with gentle bending. Secretory granules begin to appear in the luminal epithelial cells as well as in the tubular gland cells. During the next weeks, the accumulation of secretory granules continues and this process is associated with a highly developed endoplasmic reticulum and Golgi apparatus[9].

Ingestion of Kepone produces similar changes in the immature oviduct[5,7]. The most rapid developmental changes were observed with the highest doses of Kepone. The lowest dose (10 ppm for 26 days) increased the number of microvilli, initiated cilia growth and promoted tubular gland formation in both magnum and shell gland. Feeding Kepone at 40 ppm for 26 days produced further

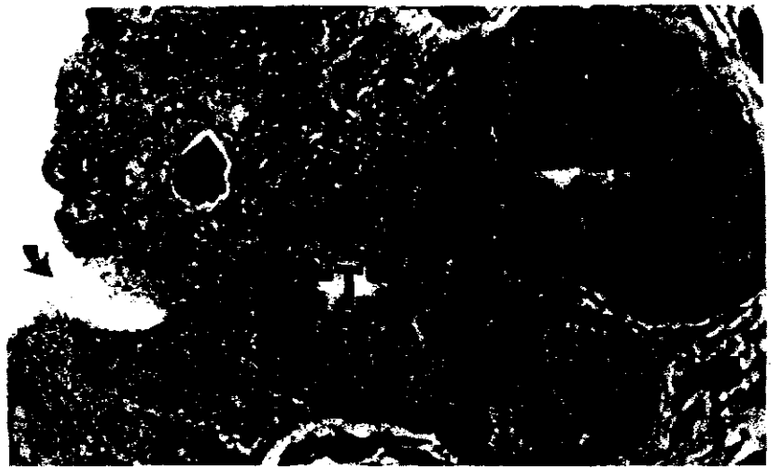

Fig. 2. Tubular gland formation (T) and invagination of luminal epithelium (arrow) in quail magnum after ingestion of 40 ppm Kepone for 26 days. 1500X.

Fig. 3. Fully ciliated magnum epithelium and tubular gland cell secretory granules (arrows) after ingestion of 160 ppm Kepone for 12 days. 1500X.

increases in ciliation and accelerated tubular gland formation in the entire oviduct (Fig. 2). Secretory granules were not observed in the tubular gland cells until 80 or 160 ppm of Kepone was fed for 18 or 12 days, respectively (Fig. 3). A large number of secretory granules were observed in both the magnum and the shell gland after 26 days of Kepone diet at 160 ppm. This secretory activity was correlated with abundant polyribosomes, well-developed endoplasmic reticulum, and dilated Golgi complex as shown in Fig. 4. The surface epithelial cells had a profuse covering of cilia (Fig. 5).

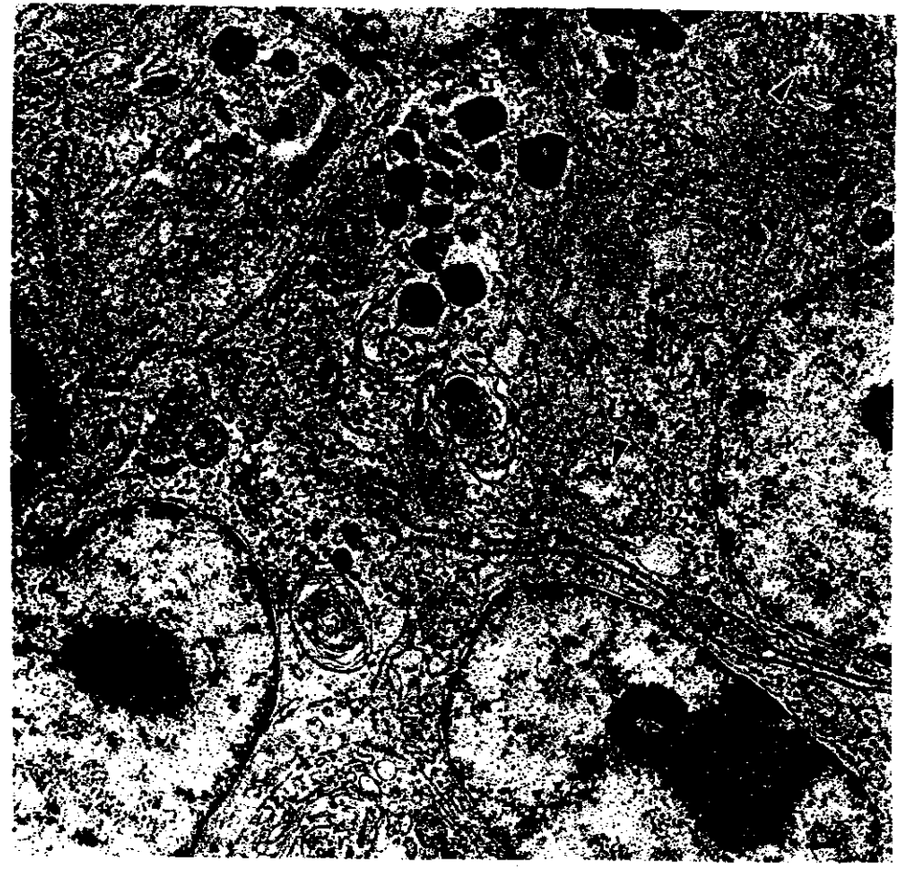

Fig. 4. Tubular gland cells in quail magnum after ingestion of 160 ppm Kepone for 26 days. Note the granules, swollen mitochondria (arrows) and loop-like configuration of the Golgi. 14,000X.

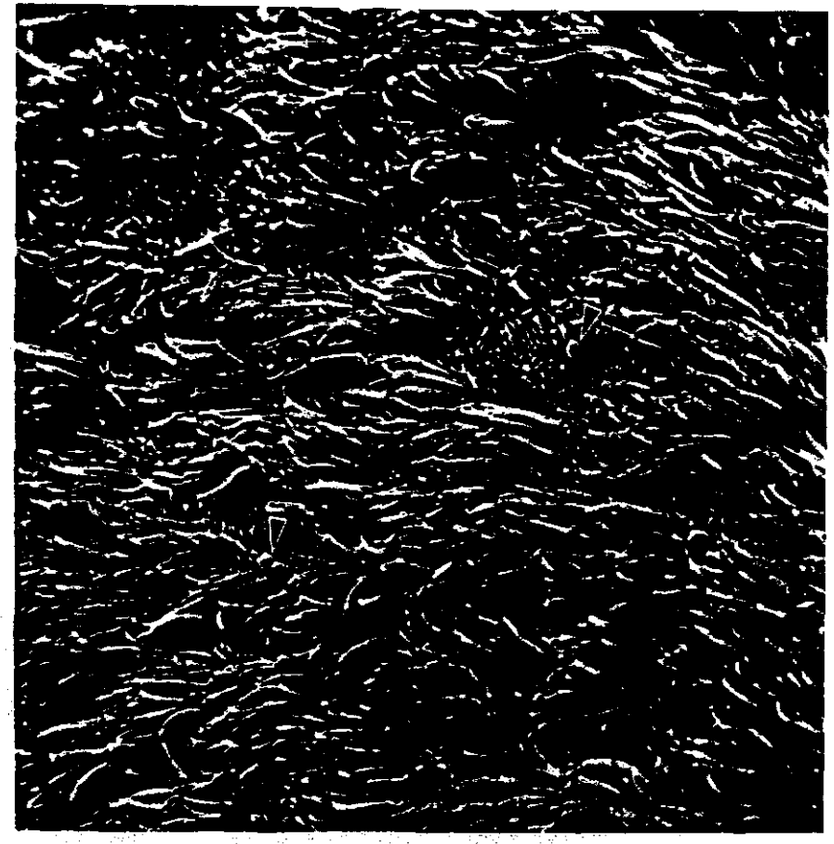

Fig. 5. Ciliation of quail shell gland epithelium with occasional granules (arrows) after ingestion of 160 ppm Kepone for 12 days. 3,800X.

Although the morphology of oviduct cells from birds treated with Kepone resembled that of estradiol-treated birds or laying quail in most respects, some features appeared abnormal and these are probably due to toxic effects of Kepone. For example, the luminal epithelial cells exhibited extreme swelling and the cilia frequently appeared more twisted and bent than in birds treated with estradiol (Fig. 6). Some mitochondria appeared swollen or vacuolated with a disrupted matrix and cristae (Fig. 4). Other mitochondria

Fig. 6. Distorted cilia and extreme apical protrusion of epithelial cells of the quail shell gland after ingestion of 80 ppm Kepone for 12 days. 12,750X.

exhibited myelin-figure formation. The Golgi complex formed loop-like structures around condensing vacuoles, other cisternae or multivesicular bodies (Fig. 4). The secretory granules in the tubular gland cells of Kepone-treated quail were somewhat smaller and less abundant than in the estradiol group. Most of the granules were either entirely electron dense or semiopaque, presumably representing different stages of condensation. However, some granules were not uniform in appearance, but rather seemed to contain material of two or three different densities (or composition) within the same granule. Similar granules have been observed in the chick oviduct after combined estrogen and progesterone treatment [9]. Since we have shown that Kepone can elevate

serum progesterone levels[10], this effect of Kepone may explain the appearance of these multi-component granules.

Reproductive performance of quail during chronic Kepone ingestion. Administration of 200 ppm Kepone to adult quail over an 8-month period significantly and irreversibly affected egg-laying performance. The total number of eggs laid per bird was only 30% of that of controls and less than half of these eggs were intact[11] (Table 3). Although the size of intact eggs was normal, they were significantly thinner and weaker in both dimensions[12] (Table 3).

Egg-laying became an important factor in quail longevity. Those quail that ceased laying soon showed signs of toxicity and died within an average of 17 days after laying the last egg. Post-mortem examination revealed that the ovaries were either totally regressed or contained mature follicles whose ovulation was apparently inhibited. These observations suggest that Kepone interferes with the secretion of gonadotrophins from the pituitary, perhaps

Table 3. Effects of Kepone on Reproductive Capacity of Adult Quail

Parameter	Group	Duration of Kepone Ingestion*	
		4 months	*8 months*
Total number of eggs/bird/month	Control	21.2	17.9
	Kepone	14.5	5.4
Number of intact eggs/bird/month	Control	14.3	16.3
	Kepone	7.7	2.3
Mean number of eggs/clutch	Control	5.7	4.1
	Kepone	3.8	2.6
Mean number of days between laying	Control	1.5	2.6
	Kepone	3.1	3.2
Mean egg weight *(g)*	Control	9.9	9.4
	Kepone	10.2	10.0
Eggshell weight *(g)*	Control	0.70	0.75
	Kepone	0.70	0.68
Eggshell thickness *(mm)*	Control	0.21	0.21
	Kepone	0.20	0.185
Mortality *(%)*	Control	0.0	0.0
	Kepone	54.8	90.5

**200 ppm*

due to either the estrogenic or toxic effects of Kepone. Since egg-laying helps eliminate Kepone from the body, the reduced reproductive function is self-perpetuating and leads to greater and greater toxicity.

Effects of Kepone on the histology of quail testes. Ingestion of Kepone (200 ppm) by male quail for 42 days resulted in 35% mortality[6] and had pronounced effects on the testes (Table 2). Microscopic examination of the testes of Kepone-treated birds showed that the seminiferous tubules were dilated and the germinal epithelium was eroded compared to controls (compare Figs. 7 and 8). Some of the testes were atrophied and in these spermatogenesis was severely reduced. In both the swollen and the atrophic testes, abnormal sperm with bulbous heads and coiled or bent tails were observed. The vas deferens of some testes contained phagocytic cells and cellular debris. Testicular atrophy is often associated with estrogenic influence on males.

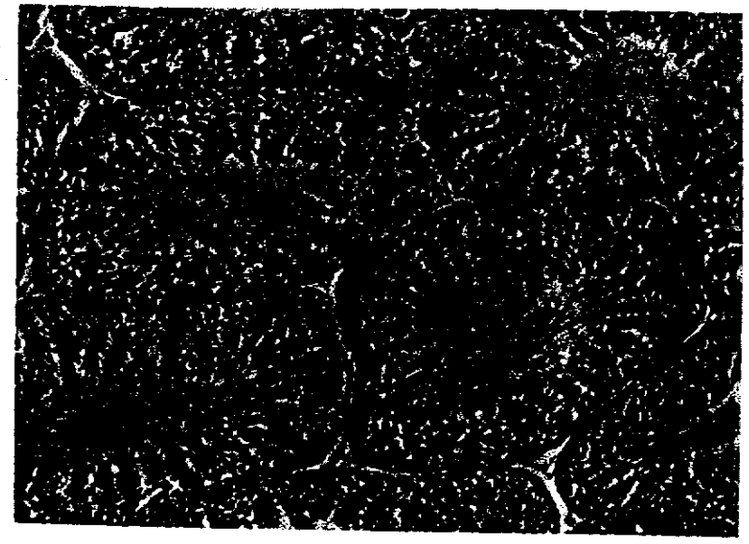

Fig. 7. Testis from adult control quail. 350X.

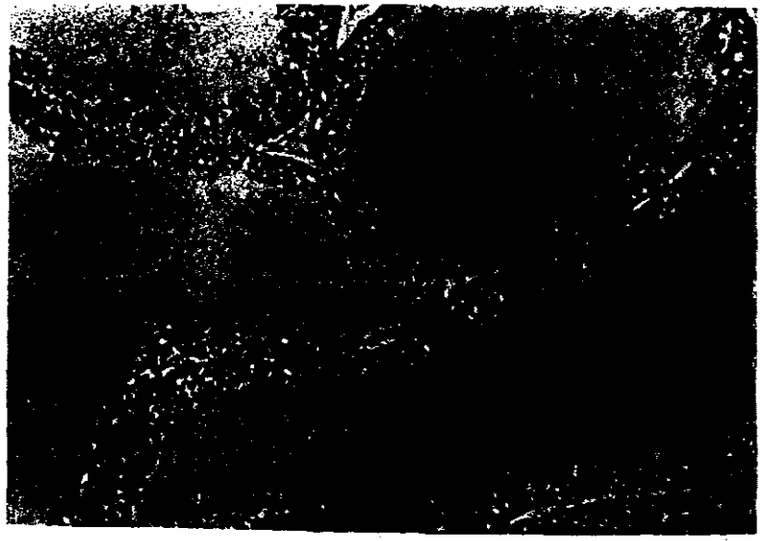

Fig. 8. Testis from quail fed 200 ppm Kepone for 42 days. Note severe dilation of seminiferous tubules and disruption of germinal epithelium. 350X.

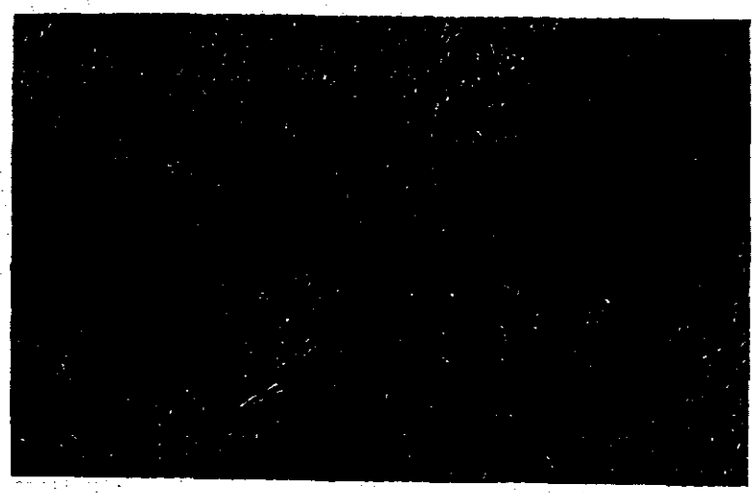

Fig. 9. Testis from a quail 60 days after Kepone (200 ppm for 42 days) showing some reversible changes in seminiferous tubules, germinal epithelium and spermatogenesis. 1000X.

Removal of Kepone from the diet for 60 days occasionally reversed the morphological appearance of the testes. The germinal epithelium became taller and more uniform and there was a substantial increase in the number of developing and mature sperm (Fig. 9). In other quail the testes became discolored and necrotic (Fig. 10).

Fig. 10. Necrosis and degeneration in the testis of a quail 60 days after Kepone (200 ppm for 42 days). 350X.

Effects of Kepone on the reproductive tract of neonatal female mice. One-day-old female mice received 10 daily injections of 17β-estradiol or varying amounts of Kepone. At least 5 neonates were terminated every 2 days for histological examination. Cellular proliferation and hypertrophy were obvious in the entire reproductive tract after estradiol injections and keratinization of the vagina was observed within 4 days in all animals. Microvilli increased in size and number in the non-ciliated cells of the uterus and Fallopian tube. The wet weight of the reproductive tract more than doubled (Table 4).

Table 4. Effects of Kepone on Mouse Reproductive Tract Weight and Vaginal Keratinization

Treatment	Reproductive tract weight	First appearance of keratinization	Mortality
	mg	*days*	*%*
Control	12.0	none	0
Kepone (30 ug/day)	29.0	10	0
Kepone (60 ug/day)	-	6	14
Kepone (125 ug/day)	45.3	4	17
Kepone (250 ug/day)	-	4	31
Estradiol (10 ug/day)	27.0	4	0

17β-Estradiol and Kepone were dissolved in sesame oil and injected intraperitoneally.

Fig. 11. Vagina of neonatal mouse after 4 injections of Kepone (125 ug/day). Note the fully developed and keratinized vaginal epithelium. 1000X.

Kepone also stimulated reproductive tract growth in a dose-dependent manner (Table 4). At concentrations greater than 125 µg/day , complete vaginal keratinization was evident within 4 days in over half of the neonates[13](Table 4 and Fig. 11). Scanning electron microscopy also revealed that ciliation and microvilli elaboration in the presence of Kepone closely resemble that obtained with estradiol. However, in areas of the Fallopian tubes and uterus, Kepone produced apical protrusions similar to those seen in quail oviduct after Kepone ingestion.

Histology of mouse testes after neonatal injection of Kepone. Neonatal male mice received the same daily injections of estradiol or Kepone as the females shown in Table 4 and they were terminated 10, 30 and 60 days after the last injection. The most dramatic changes in all of the testes were seen at the longer times. Estradiol effectively suppressed spermatogenesis; numerous seminiferous tubules were empty of developing and mature sperm. Neonatal injection of Kepone was less effective at all concentrations than estradiol at surpressing spermatogenesis. Even at the highest concentrations, most of the seminiferous tubules showed signs of active spermatogenesis. However, a few tubules were totally denuded of germinal epithelium and were lined instead by a single layer of lipid-containing cells. The severe swelling, discoloration and atrophy of the testes that was observed in quail reared on a Kepone diet was not seen in either neonatal or adult mice injected with Kepone.

Induction of egg white protein synthesis in chick oviduct by Kepone. The effects of Kepone on quail oviduct morphology, especially the proliferation of tubular gland cells and the accumulation of secretory granules, strongly suggest that Kepone is estrogenic. We assume that the secretory granules in the magnum tubular gland cells reflect the induction of egg white proteins: ovalbumin, conalbumin, ovomucoid and lysozyme[14]. To investigate this aspect of Kepone action we have relied on the chick oviduct system where the effects of estradiol and other classes of steroid hormones on the induction of egg white protein synthesis is the subject of intense study[15]. Figure 12 shows the effect of Kepone on the induction of conalbumin synthesis. Kepone was either added to the food or injected intraperitoneally and at various times the magnum portion of the oviduct was removed and pulse-labeled with ^{3}H-leucine. The percentage of total isotope incorporated into conalbumin was then determined immunologically[14]. With intraperitoneal injections of 12.5 mg/day, the rate of conalbumin synthesis rose from 1% to 12% of total protein synthesis

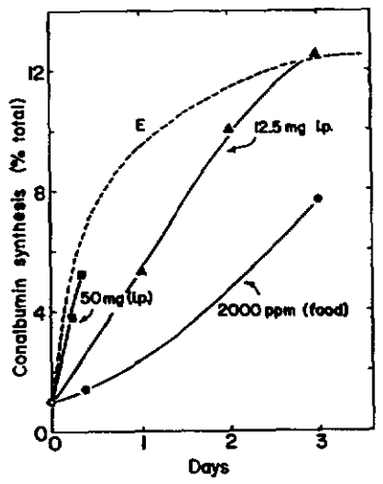

Fig. 12. Induction of conalbumin synthesis in chick oviduct by intraperitoneal injection of Kepone or ingestion of Kepone. Dotted line shows maximum rate of induction obtained with 17β-estradiol.

within 3 days. This is the same extent of induction observed with an optimal dose of 17β-estradiol-benzoate[14]. The initial rate of accumulation of conalbumin synthetic capactiy (mRNA) is equivalent to that obtained with subcutaneous injection of 10 μg of 17β-estradiol[16]. With 50 mg of Kepone, the induction of conalbumin synthesis was nearly maximal (equivalent to that observed with 40 μg of 17β-estradiol[16]) but this amount of Kepone is lethal within 8 hr. Fig. 12 also shows that 2000 ppm of Kepone in the feed induced conalbumin synthesis, but at a lower rate than by injection. With 500 ppm, conalbumin synthesis was 5.5% of total protein synthesis after a week[10] and never rose much higher. These results clearly show that Kepone induces egg white protein synthesis *in vivo*, but they do not prove that Kepone itself is estrogenic. Kepone might be acting indirectly by increasing the concentration of one or more of the naturally occurring active steroids, perhaps in a manner similar to that observed with other toxic compounds, e.g. ethionine, actinomycin D, and thioacetamide[17,18].

Kepone acts directly on the chick oviduct. To ascertain whether Kepone can act directly on the oviduct, we took advantage of the fact that oviduct minces will respond to steroids in culture in a manner that is qualitatively similar to that observed *in vivo*[19]. Table 5 shows that both conalbumin and ovalbumin synthesis are induced in culture by estradiol, progesterone and dexamethasone (a potent glucocorticoid). Other studies (not shown) reveal that each of these hormones acts via its own receptors. Kepone is as effective in this culture system as any of the steroids. To ascertain which of these receptors Kepone might be interacting with, we included the anti-estrogen, tamoxifen, in a parallel set of cultures. Tamoxifen binds to estrogen receptors but does not elicit estrogenic activity[20]; hence, it competes with compounds that normally bind to estrogen receptors. Table 5 shows that tamoxifen blocked the estradiol and Kepone-mediated induction of ovalbumin and conalbumin synthesis but had little effect on progesterone or glucocorticoid-mediated induction of these same proteins. This experiment shows that tamoxifen and Kepone are specific for estrogen receptors and that tamoxifen is not toxic at the concentration used.

Table 5. Induction of Ovalbumin and Conalbumin Synthesis in Chick Oviduct Cultures by Steroid Hormones and Kepone: Antagonism of Estradiol and Kepone Action by Tamoxifen

Chemical addition	Tamoxifen	Ovalbumin synthesis	Conalbumin synthesis
		% of total protein synthesis	
none	-	0.03	1.36
17β-Estradiol	-	0.61	3.32
17β-Estradiol	+	0.01	0.84
Kepone	-	0.59	3.01
Kepone	+	0.00	0.98
Progesterone	-	0.99	2.31
Progesterone	+	0.75	2.70
Dexamethasone	-	0.81	2.97
Dexamethasone	+	0.82	2.61

Oviduct tissue from estrogen-withdrawn chicks was incubated at 41° in Ham's F-10 media supplemented with 12% fetal calf serum (dextran-charcoal extracted) and the indicated hormones (10 nM) or Kepone (2.5 μM). Tamoxifen was used at 10 μM. After 8 hr, tissue was removed and labeled for 30 min in Hanks' salts containing 12 μCi/ml of ^{3}H-leucine. The incorporation of leucine into ovalbumin, conalbumin and total protein was determined as described by Palmiter[12].

Kepone binds to nuclear estrogen receptors. To pursue the observation that Kepone may be interacting directly with estrogen receptors, we measured the ability of Kepone to compete with labeled estradiol for nuclear estrogen receptors. Exchange assays have been devised in which unlabeled estrogen bound to nuclear receptors can be exchanged for labeled estradiol of known specific activity[16,21]; these assays are useful both for quantitating the number of nuclear receptors and for measuring the relative affinity of different compounds for these receptors[16]. Fig. 13 shows that Kepone competes with ^{3}H-estradiol in an exchange assay with nuclei from estrogen-treated chicks but it is about 0.02% as effective as estradiol; hence its affinity for the estrogen receptor is about 5000-fold lower than that of estradiol. Kepone does not compete with ^{3}H-progesterone for nuclear progesterone receptors, demonstrating the specificity of these exchange assays[10,16]. The relative affinity of Kepone for the nuclear estrogen receptor shown in Fig. 13 is about an order of magnitude lower than that originally published[10]. We believe that the values shown here are correct since they were obtained with three different preparations of Kepone.

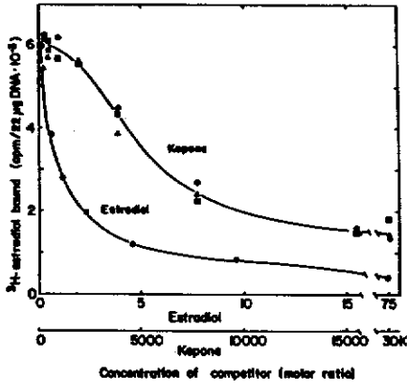

Fig. 13. Competition of Kepone with ^{3}H-estradiol binding to nuclear estrogen receptors. Nuclei from laying hens were incubated with ^{3}H-17β-estradiol (13 nM; 97 Ci/mmol) and the indicated molar excess of unlabeled 17β-estradiol (o) or Kepone (three different preparations ●, ■, ▲) as described by Mulvihill and Palmiter [16].

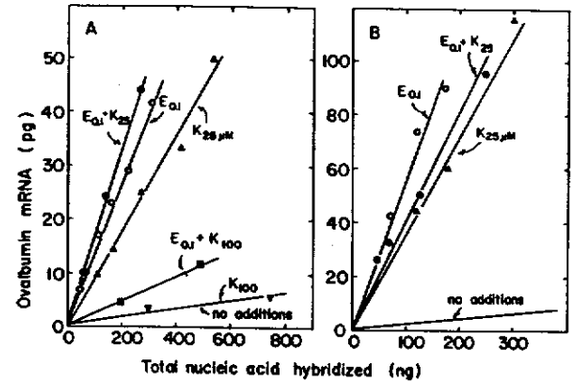

Fig. 14. Induction of ovalbumin mRNA by Kepone (K) and 17β-estradiol (E). Induction was for 11 hr (A) or 16.5 hr(B) as described in Table 5; subscripts indicate micromolar concentrations. Samples of total nucleic acid were hybridized with cDNAand the % hybridization was used to calculate mRNA content [19].

Induction of ovalbumin mRNA in culture by Kepone. More recent biological assays are also consistent with a lower potency of Kepone than reported[10]. For these assays, we monitored the induction of ovalbumin mRNA by hybridizing radioactive complementary DNA (cDNA) to samples of total nucleic acid isolated from oviduct cultures after 10–16 hr of induction. In these assays, 1 pg of ovalbumin mRNA per microgram of total nucleic acid is equivalent to about 5 molecules of ovalbumin mRNA per cell[19]. Fig. 14 shows that 25 μM Kepone induces ovalbumin mRNA in oviduct cultures almost as effectively as 100 nM 17β-estradiol (20 nM estradiol is the minimum concentration that gives maximal ovalbumin mRNA induction[19]). Other studies (not shown) indicated that 1.6 μM Kepone gave only marginal induction of ovalbumin mRNA; thus, there is about a 1000-fold difference in biological activity of Kepone and estradiol in culture and *in vivo* (see above). The differences in biological potency of Kepone and estradiol compared to their relative affinities for receptors (Fig. 13) are small and may relate to differences in uptake of these two compounds by oviduct cells. Fig. 14 shows that 100 μM Kepone is less effective in culture

than 25 μM. This is probably due to toxic effects since this concentration of Kepone also inhibits the action of estradiol (Fig. 14).

DISCUSSION AND CONCLUSIONS

A large number of vertebrates have been, and will continue to be, exposed to Kepone. Our experiments have shown that when Kepone is administered to birds or mice in large amounts, it is quite toxic and the animals quickly succumb. When Kepone is administered in sub-toxic amounts, its estrogenicity becomes evident. In the environment, insecticides generally accumulate gradually and persist for long times in the bodies of exposed species. Thus, the presence of small amounts of Kepone in the environment may not pose an immediate hazard to animal life, but may instead pose an 'estrogenic hazard' in which reproduction of animals is threatened.

The undeveloped reproductive organs of mice, quail and chickens serve as convenient target organs for studying the estrogenic activity of insecticides. We have shown that Kepone induces the growth and development of the entire reproductive tract in females of these species. In mature animals, Kepone also has deleterious effects on reproductive function in both males and females. Until recently, the mechanism of Kepone action on the vertebrate reproductive system was not understood. We have shown that Kepone mimics estrogens by promoting cell proliferation and stimulating the accumulation of a complex secretory apparatus including polyribosomes, endoplasmic reticulum, and Golgi. Kepone also induces egg white protein synthesis and the accumulation of secretory granules in the avian oviduct. It does so by inducing the mRNAs for these proteins as is typical of steroid hormones[22]. Since Kepone acts in culture, we conclude that it is acting directly on oviduct cells. The observations that Kepone competes with 17β-estradiol for binding to nuclear estrogen receptors and that its action is blocked by the anti-estrogen, tamoxifen, strongly suggest that Kepone acts in the oviduct by binding to estrogen receptors. Presumably Kepone allows the translocation of cytoplasmic receptors into the nucleus where these receptors mediate mRNA transcription in the same manner as naturally occurring estrogens[23]. The mechanisms of transcriptional regulation by estrogens are currently under investigation. Kepone also has estrogenic effects in mice and rats[24], and Bulger et al.[25] have recently shown that it competes with estradiol for rat uterine estrogen receptors; thus it is likely that its mechanism of Kepone action in mammals is similar to that proposed for birds. In both the avian and the mammalian[25] species Kepone acts as a weak estrogen with a potency about 1000-fold lower than 17β-estradiol.

The estrogenicity of Kepone is surprising considering its structure (Fig. 1). This raises the question of whether it is Kepone itself, a contaminant of Kepone, or a metabolite that has estrogenic activity. Although it is possible that Kepone is metabolized *in vivo*, it is unlikely that these metabolites are responsible for the estrogenic activity since Kepone is also estrogenic in culture and in cell-free receptor binding experiments where the potential for metabolic conversion to other compounds is minimal. The possibility of an estrogenic contaminant in Kepone preparations is more difficult to eliminate; however, we have noted that three different preparations of Kepone have essentially the same activity (Fig. 13). Two of these preparations were highly purified and were obtained from different laboratories. It seems unlikely that a trace contaminant would be present in all three preparations at the same relative concentration. Also, other chlorinated hydrocarbons have been shown to have similar estrogenic activity[26]. The best studied examples are the analogs of DDT[27,28] which have recently been shown to compete with 17β-estradiol for estrogen receptors[29].

Although Kepone appears to be acting as a pure estrogen in culture, it is likely that its action *in vivo* is modulated by other hormones. For example, we have noted that Kepone leads to adrenal hypertrophy[4,7] and potentially to the secretion of active steroids such as glucocorticoids and progesterone. Indeed, we have shown that progesterone levels in the serum are significantly increased by Kepone[10], and we know that combinations of estrogen and progesterone have different effects on the oviduct than estrogen alone[9]. In addition, Kepone has obvious toxic effects *in vivo* and in culture that undoubtedly modify its estrogenic activity.

From the available data it appears that the estrogenicity of certain chlorinated hydrocarbons is mediated by direct binding of these compounds to estrogen receptors which then elicit pleiotrophic effects on target cells by mechanisms that are not yet fully understood but probably involve the regulation of gene transcription. As long as these estrogenic pesticides are present in the environment, their effect on the reproductive functions of animals will remain of significant importance.

ACKNOWLEDGMENTS

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DISCUSSION

SONNENSCHEIN: We have demonstrated that the 20-40% increase in wet weight and macromolecule synthesis in the quail oviduct that has been attributed to the role played by the pituitary is, in fact, mediated by the estrogen-dependent stimulation of ACTH secretion; this, in turn, would increase the level of corticosterone, which acts on the oviduct (Laugier, Sonnenschein, and Brard). Also, it is very comforting to see the reproducibility of the induction of specific proteins in quails by Kepone carried out in a culture system.

McLachlan, ed. Estrogens in the Environment

A MODEL FOR THE MECHANISM OF ACTION OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN[+]

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A consideration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)[*] in this symposium is not because this compound or its congeners possess any known estrogenic activity, but rather because these halogenated aromatic hydrocarbons produce their biochemical and toxical actions by binding reversibly to a cytosolic protein which mediates the ensuing gene expression in a manner analogous to that of 17β-estradiol and its receptor. I want to stress the 'biology' of the TCDD induced effects and not detail the properties of the receptor, and compare the system to that of sterold hormones.

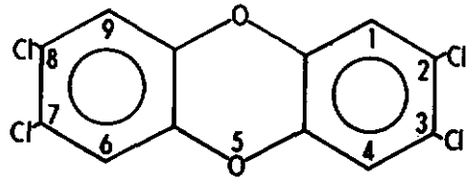

FIGURE 1 - 2,3,7,8 TETRACHLORODIBENZO-P-DIOXIN

Toxicity

TCDD is the prototype of a large series of halogenated aromatic hydrocarbons which includes other chlorinated dibenzo-p-dioxins, dibenzofurans, azo- and azoxybenzenes, and biphenyls, all of which produce a similar and characteristic pattern of toxic responses[1-9]. TCDD (and congeners) produces a

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[‡]Recipient of Research Career Development Award K04-ES-0017.

[*]Abbreviations: TCDD - 2,3,7,8-tetrachlorodibenzo-p-dixoin; AHH - aryl hydrocarbon hydroxylase; MC - 3-methylcholanthrene; ALAS - δ-aminolevulinic acid synthetase; ED_{50} - dose which produces one half the maximal response, in this case induction of hepatic AHH activity; K_D - equilibrium dissociation constant for binding.

wasting syndrome with a latent period to death of days to weeks which is characteristic of the species. Species differ in their sensitivity to TCDD: acute-oral LD_{50} 1 µg/kg guinea pigs, 20 µg/kg rats, 100 µg/kg mice, and $>$ 1000 µg/kg hamsters and bullfrogs[10-13]. This histopathic damage produced is also species specific and consists of: 1) lymphoid involution especially the thymus, accompanied by supression of cellular immunity occurs in most species; 2) embryotoxicity and teratogenesis also is observed in mose species; 3) chloracne and hyperkeratosis has been observed in humans, rabbits, monkeys and hairless mice; 4) an edematous syndrome is characteristic in chickens and to a lesser degree in mice; 5) hepatocellular damage is observed in rats, mice and rabbits and a disturbance in hepatic porphyrin synthesis (hepatic porphyria) has been reported in humans, and mice); and 6) bone marrow depression has been reported in monkeys. All of the congeners which have been tested have been reported to produce all of these symptoms.

The ultimate target organ, that tissue whose functional disruption leads to death, is unknown. TCDD produces histopathology in a variety of tissues, but cellular necrosis has rarely been described, except in the liver where parenchymal cell necrosis is mild to moderate, and as infrequent focal necrosis seen in the thymus cortex.

Pharmacokinetics and drug metabolism

The pharmacokinetics of TCDD has been most thoroughly studied in the rat, where it is primarily concentrated in the liver and to a lesser degree in fat, with a whole body half-life of about 3 weeks[14]. There is evidence that TCDD is slowly and partially metabolized to as yet unidentified metabolites appearing in the bile[14,15]. However, there is no evidence of appreciable covalent binding of tritium-labeled TCDD to cellular macromolecules in vivo, and no evidence to suggest that TCDD metabolites are involved in toxicity.

Enzyme Induction

The report by Bleiberg et al[16] that workers in a factory producing the herbicide 2,4,5-trichlorophenoxyacetic acid, and exposed to the contaminant TCDD, developed chloracne and porphyria cutanea tarda, a disturbance of hepatic porphyrin synthesis, suggested a specific biochemical lesion that could be studied in laboratory animals. At the time, it was believed that porphyrigenic chemicals act by inducing δ-aminolevulinic acid synthetase (ALAS), the initial and rate-limiting enzyme in the heme pathway. We found that the administration of TCDD to chicken embryos produced a dose-related induction of ALAS activity and that for 14 other halogenated dibenzo-p-dioxin congeners, there was a well defined relationship for induction of enzyme activity[17].

In rats and mice, TCDD does not significantly induce ALAS[18], but it does produce porphyria in mice[19], apparently by reducing uroporphyrinogen decarboxylase activity[20]. Since a major fraction of the heme synthesized in liver is incorporated into cytochrome P-450, compounds which disturb heme synthesis often produce some alteration in the concentration of cytochrome P-450 mediated microsomal monooxygenase activity. The microsomal monooxygenase system, is responsible for the metabolism of lipophilic compounds to more polar derivatives. Several distinct species of cytochrome P-450 have been purified from rat and rabbit liver[21,22], and shown to differ in their primary structure, and the spectrum of substrates they catalyze. These cytochrome P-450 species are differentially induced by the administration of certain compounds. Aryl hydrocarbon hydroxylase (AHH) activity, assayed as the in vitro rate of metabolism of benzo(a)pyrene to its 3-hydroxy metabolite, can be considered a measure of one or two of these cytochrome P-450 species (cytochrome P_1-450) which are selectively induced by polycyclic aromatic hydrocarbons and by TCDD.

Induction of hepatic AHH activity in the chicken embryo

Following the administration of TCDD to 18 day old chicken embryos there is a dose-related increase in hepatic AHH activity; one half of the maximal induction (ED_{50}) is produced by 3 ng/egg or 0.3 nmoles/kg. A large number of halogenated dibenzo-p-dioxins have been tested for their potency to induce AHH activity and the following structure-activity relationship has emerged: 1) halogenated must occupy at least 3, and for maximal potency, four of the lateral ring positions, - positions 2, 3, 7 and 8 (see Figure 1); 2) the order of substitution is Br$>$Cl$>$F, NO_2; and 3) at least one ring position must be unsubstituted. (The octachloro-congener is inactive, but both hepatochloro-analogues are inducers.) The same structure-activity relationship was observed for the induction of ALAS activity (18).

The most important observation has been that for chlorinated dibenzo-p-dioxins there is an excellent correlation between their potency to induce AHH activity (and ALAS activity) and their toxic potency.

Genetic Expression of AHH Activity in Inbred Strains of Mice

The classical inducers of AHH activity and cytochrome P_1-450 are the polycyclic aromatic hydrocarbons, such as 3-methylcholanthrene (MC). In a comparison of MC and TCDD for their capacity to induce hepatic AHH activity in the rat, we found MC and TCDD produce parallel dose-response curves, both compounds produce the same maximum response, simultaneous administration of maximally inducing doses of MC and TCDD produces no greater response than that

evoked by either drug alone. However, TCDD is 30,000 times as potent as MC, and a single administration produces sustained enzyme induction which lasts over 35 days[23], a reflection of the long biological half-life of the compound.

When randomly bred or certain inbred strains of mice are administered MC, they respond with the induction of hepatic AHH activity; certain other inbred strains when challenged with MC fail to respond[24,25]. The prototypical strain responsive to MC is C57BL/6J and prototypical non-responsive strain is DBA/2J. In crosses and backcrosses between C57BL/6J and DBA/2J mice, the trait of responsiveness to aromatic hydrocarbons is inherited in a simple autosomal dominant mode and the genetic locus controlling this trait is called the Ah locus.

Cell cultures derived from DBA/2J fetuses are moderately inducible in culture by benz[a]anthracene and MC, and application of MC topically to the trachea and skin of nonresponsive strains induces AHH activity. These observations suggest that the mutation in nonresponsive mice is not due to a defective structural gene for cytochrome P-450, but rather a regulatory mutation controlling the expression of the cytochrome.

The extraordinary potency of TCDD relative to MC prompted us to examine its effects in inbred strains which are responsive and non-responsive to MC. TCDD induced AHH activity in all strains tested regardless of their response to MC[26]. In C57BL/6J mice, the enzyme activity and the spectral properties of the cytochrome P_1-450 that were induced by MC or TCDD, or both compounds administered together, were found to be similar to those induced in DBA/2J mice by TCDD. These results suggest that the same gene product(s) is induced in C57BL/6J mice by both compounds, and in DBA/2J mice by TCDD.

Thus, mice which are nonresponsive to MC, do respond to a more potent stimulus, TCDD, and therefore these mice do have the structural and regulatory genes necessary for the expression of AHH activity. The failure of these mice to respond to MC suggests that they fail to recognize MC as a signal for induction. We postulated that the mutation in nonresponsive mice results in a defective recognition or receptor site for AHH induction, a receptor which has a diminished affinity for MC. Consider the hypothesis that TCDD acts as the same receptor as MC, but because of its greater potency, and hence its presumed greater affinity, it is able to saturate this receptor even in nonresponsive mice, and initiate the induction of AHH activity. This hypothesis predicts inbred mice which are nonresponsive to MC, should respond to TCDD, but should be uniformly less sensitive to TCDD, than strains which are responsive to MC.

We administered various doses of TCDD to six inbred strains of mice and 24 hours later measured their hepatic AHH activity[27]. The data are presented in Figure 2 as the log of the dose versus the fractional response (control activity = 0, maximal activity = 1.0). The three inbred strains which are nonresponsive to MC (DBA/2J, AKR/J and SJL/J) are less sensitive to TCDD, than are the responsive strains (C57BL/6J, BALB/cJ and A/J), i.e. the log-dose response curves for the nonresponsive strains are shifted to the right. The ED_{50} for AHH induction in responsive strains is approximately 1 nmole/kg, and for nonresponsive strains the ED_{50} is $\geq$ 10 nmoles/kg. The data support the hypothesis that TCDD and MC act on the same receptor, and that the mutation in nonresponsive strains is due to a receptor with a diminished affinity for inducing compounds.

Cytosolic Binding Species

We next wished to search for a macromolecular species which had the in vitro binding properties one would predict for the hypothesized receptor based on the in vivo biology. Specifically, one would expect such a moiety to have the following in vitro binding properties: 1) reversibly bind TCDD with a high affinity and the binding affinity (K_D) should correspond to the in vivo

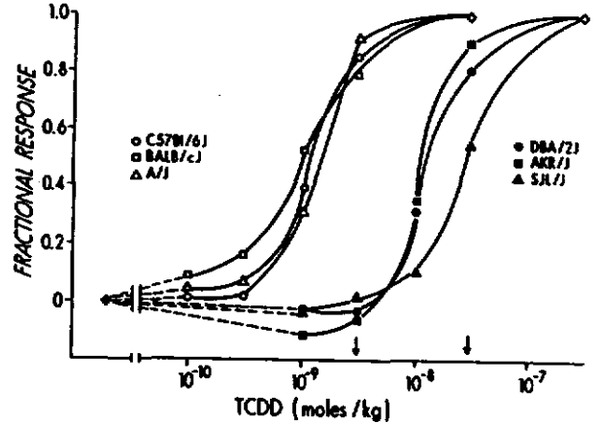


Fig. 2. Log dose-response curves for the induction of hepatic AHH activity by TCDD in six inbred strains of mice. The mice were injected intraperitoneally with varying doses of TCDD dissolved in p-dioxane; 24 hours later the animals were killed and their livers were assayed for AHH activity. The data are expressed as fractional responses to eliminate strain differences in basal and maximally induced enzyme activities (see text). Each point represents the average of four or five animals.

potency (ED_{50}) for AHH induction; 2) the binding species from mice which are nonresponsive should have a lower affinity for TCDD than the binding species from responsive mice; 3) the rank order of binding affinities of other halogenated dibenzo-o-dioxins, and other chlorinated aromatic hydrocarbons should correspond to their rank order of potencies to induce AHH activity, and 4) other compounds, such as the polycyclic aromatic hydrocarbons, which induce AHH activity, should bind to this moiety.

[1,6-(^{3}H)]-TCDD of high specific activity (52.5Ci/mmole) was prepared. An initial series of experiments suggested that there was a moiety which bound ^{3}H-TCDD in the liver cytosol (105,000xg supernatant fraction) of C57BL/6J mice.

The binding of ^{3}H-TCDD to liver cytosol was measured by the charcoal-dextran binding assay. When hepatic cytosol from C57Bl/6J mice was incubated with varying concentrations of ^{3}H-TCDD and ^{3}H-TCDD plus a 200-fold excess of unlabeled 2,3,7,8-tetrachlorodibenzofuran, we observed a small pool of high affinity sites (Fig. 3A)[28]. In Fig. 3B the same data for specific binding (displaceable, high affinity binding) is presented in a Scatchard plot. The binding affinity for TCDD, K_D, is 0.27 nM which compares quite favorably with the in vivo potency for AHH induction in C57BL/6J mice (ED_{50} = 1 nmole/kg).

The specific binding of ^{3}H-TCDD to liver cytosol from C57BL/6J mice was greater at all concentrations of the radioligand, than was the specific binding to liver cytosol from DBA/2J mice. Unfortunately, the limited aqueous solubility of ^{3}H-TCDD prevents us from achieving a sufficient concentration to saturate the cytosol binding species from nonresponsive mice and estimating the K_D.

Another expectation of the cytosol binding species if it is the receptor, is that the in vitro binding affinity of halogenated dibenzo-p-dioxin congeners should correspond to their in vivo potency to induce AHH activity. The binding affinities of these unlabeled congeners are measured by their capacity to compete with the specific binding of ^{3}H-TCDD to mouse liver cytosol, and the results in Fig. 4 are expressed relative to the binding affinity of TCDD (TCDD = 100). The biological potencies of these congeners to induce hepatic AHH in the chicken embryo (ED_{50}) are also expressed relative to TCDD. As seen in Figure 4, there is a very good correspondence between the rank order of binding affinity and biological potency for these compounds.

Other compounds which induce AHH activity and cytochrome P_1-450 such as the polycyclic aromatic compounds (MC, benzo[a]pyrene and benz[a]anthracene) and β-naphthoflavone compete for specific cytosol binding sites; but other compounds which induce different types of microsomal monooxygenase activities

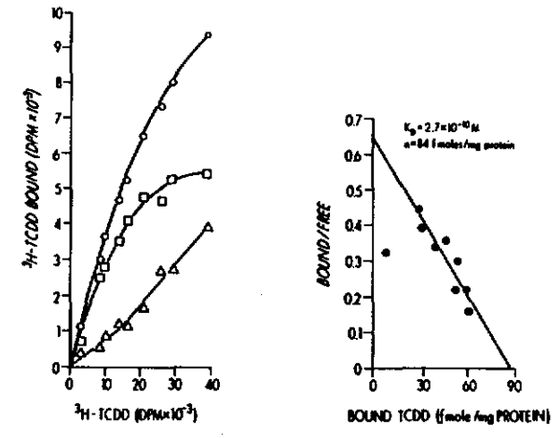


Fig. 3. A) Total, nonspecific and specific binding of (^{3}H)-TCDD to hepatic cytosol from C57BL/6J mice. The cytosolic fraction of liver from C57BL/6J mice at 2 mg protein/ml in buffer was incubated with varying concentrations of (^{3}H)-TCDD and (^{3}H)-TCDD plus a 200 fold excess of 2,3,7,8-tetrachlorodibenzofuran for 2 hours at 0°. Samples were treated with a suspension of charcoal-dextran to remove unbound ligand, and the radioactivity quantified. Total binding - (^{3}H)-TCDD alone (o); nonspecific or nondisplaceable binding - [^{3}H]-TCDD plus 2,3,7,8-tetrachlorodibenzofuran (△); specific or displaceable binding (□). B) Scatchard plot of the specific binding in A.

(e.g. phenobarbital) fail to compete with ^{3}H-TCDD for specific binding in the cytosol. 17β-Estradiol, testosterone, progesterone, hydrocortisone, and thyroxine (all at a 10^4 or 3×10^4 fold molar excess) fail to compete with ^{3}H-TCDD specific binding.

The cytosolic binding species has all the properties predicted for the induction receptor, most notably, stereospecific recognition of inducing compounds. Recent experiments both in vitro and in vivo, indicate the cytosol binding species mediates the specific uptake and binding of ^{3}H-TCDD to hepatic nuclei, and the experiments suggest that the ligand-receptor complex in the cytosol translocates to the nucleus[29]. The Ah locus, which determines the trait of responsiveness or nonresponsiveness to polycyclic aromatic hydrocarbons or greater or lesser sensitivity to TCDD, appears to be structural gene locus for the cytosolic receptor protein. (The biochemical

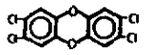
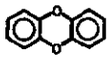
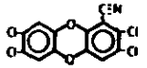
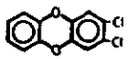
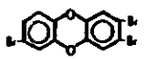
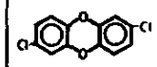
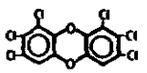
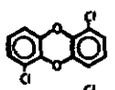
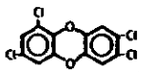
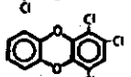
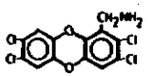
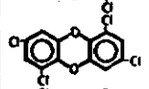
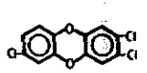
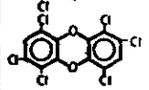
	RELATIVE BINDING AFFINITY	RELATIVE BIOLOGICAL POTENCY		RELATIVE BINDING AFFINITY	RELATIVE BIOLOGICAL POTENCY
	100[†]	100[††]		inactive (5.4×10^{-7})*	inactive (9.4×10^{-6})**
	167	100		inactive (2.7×10^{-6})	inactive (9.4×10^{-8})
	43	43		inactive (5.4×10^{-6})	inactive (9.4×10^{-8})
	20	22		inactive (5.4×10^{-6})	inactive (4.7×10^{-7})
	16	8		inactive (5.4×10^{-6})	inactive (9.4×10^{-8})
	13	3		inactive (2.7×10^{-6})	inactive (9.4×10^{-8})
	14	0.06		inactive (1.1×10^{-6})	inactive (9.4×10^{-6})

Fig. 4. The cytosol binding affinities and biological potencies of dibenzo-p-dioxin congeners relative to TCDD. The binding affinities of the dibenzo-p-dioxin congeners for hepatic cytosol were estimated by the capacity of these compounds to compete with (^{3}H)-TCDD for specific binding sites in C57BL/6J liver cytosol. The binding affinities are expressed relative to TCDD which is assigned a value of 100. For inactive analogues, the highest concentration tested that was judged to be soluble is given in parentheses.

The biological potency (ED_{50}) of each congener was the dose that produced one half the maximal induction of hepatic AHH activity in the chicken embryo, and potency was expressed relative to TCDD (TCDD=100). For inactive compounds, the highest dose tested (in moles/kg) is given in parentheses. This assumes the weight of an average chicken egg is approximately 50 g.

a - The absolute value of the K_D for TCDD is 0.27 nM.
b - The absolute value of the ED_{50} for TCDD induction of hepatic AHH activity in the chicken embryo is 0.31 nmoles/kg.
c - The highest concentration tested that was judged soluble in moles/liter.
d - The highest concentration tested in moles/kg.

and genetic evidence all suggest, but does not prove this). The mutation in nonresponsive mice results in an altered receptor with a diminished affinity for the inducing compound.

Carlstedt-Duke[30] et al have further characterized the properties of the TCDD-binding protein in liver cytosol. They found the ligand-receptor complex, but not the unbound receptor, bound to a DNA-cellulose column. The receptor-^{3}H-TCDD complex migrated in a glycerol gradient with a sedimentation coefficient of 6S. These investigators found that if ^{3}H-TCDD was added to cytosol, diffuse bands were formed on isoelectric focusing presumably due to aggregation of the receptor[31]. If the cytosol was treated briefly with trypsin, the ^{3}H-TCDD-receptor focused as a sharp peak at pI 5.2.

Coordinate Gene Expression

The administration of MC or TCDD to laboratory animals stimulates not only AHH activity, but a number of other hepatic enzyme activities. There is evidence for several of these enzymes, that this increase in activity represents true induction, i.e. de novo protein synthesis. MC and TCDD have been shown to induce the following hepatic enzymes: δ-aminolevulinic acid synthetase, UDP-glucuronosyl transferase, glutathione-S-transferase B, aldehyde dehydrogenase, DT-diaphorase, and ornithine decarboxylase. The induction of several of these enzyme activities have been shown to segregate with the Ah locus in inbred strains of mice, (for a more detailed discussion see ref. 32). It would appear that the Ah locus controls not only the expression of AHH activity, but also the coordinate expression (and perhaps repression) of a number of other enzymes (Fig. 5).

Structure-activity relationship

Other halogenated aromatic compounds, the chlorinated dibenzofurans, azo- and azoxybenzenes, and biphenyls and the brominated biphenyls produce similar toxic responses to that of TCDD[33]. Each of the congeners of these compounds which has been tested, induce AHH activity and compete with ^{3}H-TCDD for cytosol specific binding.

We can generalize about the structure-activity relationship of each of these classes of halogenated aromatic hydrocarbon. Consider a prototype of each class - TCDD, 2,3,7,8-tetrachlorodibenzofuran, 3,4,3',4'-tetrachloroazoxybenzene, and 3,4,3',4'-tetrachlorobiphenyl - depicted in Fig. 6. These compounds are all approximate stereoisomers and their molecular structures can be thought of as roughly fitting into a rectangle 3 x 10Å with halogen atoms in the four corners. Planarity or near planarity seems to be essential. In Figure 6 the in vitro binding affinity in the chick for each of these com-

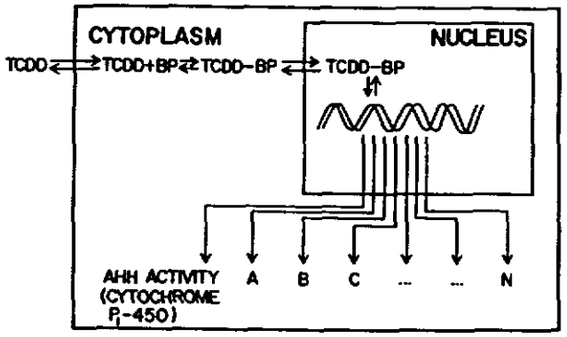


Fig. 5. Schematic representation of the model proposed for the pleiotropic response mediated by the TCDD-receptor complex. BP is the TCDD-binding protein or receptor.

pounds, and for a number of other tetrahalogenated aromatic compound which are approximate stereoisomers, are compared. The toxicity of these latter compounds has not been tested.

A Model for the Mechanism of Toxicity

For the halogenated dibenzo-p-dioxins and the other halogenated aromatic hydrocarbon congeners, there is a correlation between their potency to induce AHH activity and their toxic potency. It is difficult to envision how the induction of AHH activity, per se, is involved in specific organ toxicities such as chloracne, thymic involution or teratogenesis. We suggest that the toxicity of these chlorinated aromatic hydrocarbons is mediated through the receptor, that is, the initial event in their toxic action is the stereo-specific recognition and binding to the cytosolic binding species. It is postulated that the ligand-receptor complex controls a battery of genes, and the sustained expression (or repression) of one or more of these genes which are coordinately expressed results in the observed toxic syndrome.

The model has an obvious prediction. Since the Ah locus determines the receptor, its binding affinity, and sensitivity to induction of AHH activity, toxicity should segregate with the locus. That is, inbred strains of mice with a high affinity receptor, and sensitivity to induction of AHH toxicity, should be sensitivity to toxicity from TCDD, and mice with a low affinity receptor should be less sensitive to the toxicity of TCDD.

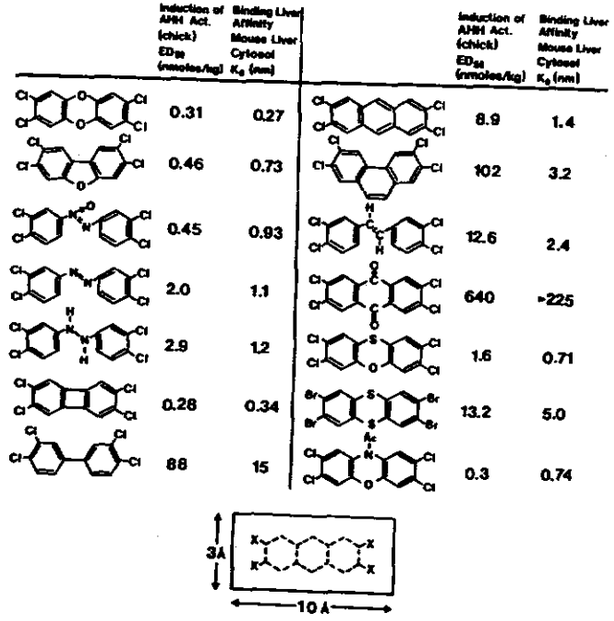


Fig. 6. Isosteric tetrahalogenated aromatic hydrocarbons: Comparison of their potencies to induce AHH activity and their cytosolic binding affinities. The biological potency of each compound was determined from the log dose-response curve for induction of hepatic AHH activity in the chicken embryo. The binding affinity of each congener was determined by its capacity to compete with (^{3}H)-TCDD for specific binding sites in liver cytosol from C57Bl/6J mice.

We have found that C57BL/6J mice are more sensitive to the dose-related thymic involution produced by TCDD, than are DBA/2J mice, and the hybrid B6D2F$_1$/J mice show intermediate sensitivity. In the backcross of B6D2F$_1$/J and DBA/2J mice, thymic involution segregated with the locus. In 10 inbred strains of mice, sensitivity to teratogenicity, measured as the incidence of cleft palate formation, followed the strain distribution of Ah locus, (with one exception, a responsive strain CBA/J which failed to develop cleft palates)[34].

In summary, 1) the correlation between the structure activity relationship of congeners to bind to the receptor and their toxic potency and 2) the segregation of toxicity produced by TCDD with the Ah locus, suggest that the toxicity of halogenated aromatic hydrocarbons is mediated through their binding to the receptor and the gene expression that ensures.

We do not understand why nonhalogenated agonists for the receptor, e.g. polycyclic aromatic hydrocarbons and β-naphthoflavone do not produce the characteristic spectrum of toxic responses produced by TCDD and its congeners. There are two obvious possibilities. The nonhalogenated agonists some how bind differently to the receptor or the receptor-ligand complex binds differently to the nuclear sites and doesn't fully express the same battery of genes that the halogenated aromatic hydrocarbons do. Alternatively it is possible that it is the sustained occupation of the receptor that is essential, and the long biological half-lives of halogenated aromatic compounds relatively to the rather rapidly metabolized polycyclic aromatic hydrocarbons accounts for this difference. In support of this latter possibility we have found that MC and β-naphthoflavone will produce thymic involution, a response characteristic of TCDD toxicity.

The data suggest that while the cytosol receptor may be essential for toxicity it may not be sufficient. We recently examined over 20 cell lines in culture, primary cell cultures, established cell lines, and transformed cells, for toxicity to TCDD. While a number of the cells showed AHH induction in response to TCDD, indicating the presence of the receptor, none show any signs of toxicity such as inhibited growth rate, cell death, or reduced plating efficiency (Knutson, J. and Poland A. - manuscript in preparation).

At present, we do not know the size of the gene battery controlled by the receptor, not its physiologic functions other than increase in the enzymes which metabolize foreign chemicals, nor if there is a endogenous modulator of the receptor.

Mutagenicity and Carcinogenicity

Recently, several groups have found TCDD to be a potent carcinogen in chronic feeding studies[35,36]. Kociba et al.[35] in a very thorough study maintained rats on diets supplying a daily dose of 0, 1, 10 and 100 ng/kg for two years. Female rats feed the highest concentration of TCDD developed a significant increase in hepatocellular carcinoma, stratified squamous cell carcinoma of the hard palate and nasal turbinate and keratinizing squamous cell carcinomas of the lung. At a dose of 100 ng/kg/day nearly one half the female rats developed one of these neoplasms, suggesting TCDD has a carcinogenic potency comparable to that of aflatoxin B_1, which at lifetime dose of 1 μg/kg/day has been estimated to produce a 50% incidence of hepatocellular carcinoma in rats[37].

The carcinogenic potency of TCDD is surprising in light of 1) the lack of convincing evidence that TCDD is mutagenic in any in vitro bacterial test systems[38-40], and 2) failure to demonstrate significant covalent binding in vivo. We have recently examined the in vivo binding of ^{3}H-TCDD to rat liver macromolecular fractions[41]. If one assumes unextracted radioactivity is equatable with covalently bound ^{3}H-TCDD, the maximum binding to DNA is 6 pmole of TCDD per mole of nucleotide, 4 to 6 orders of magnitude lower than that of most other chemical carcinogens[42], and equivalent to one molecule of TCDD bound to the DNA in every 35 diploid cells. These results suggest that it is unlikely that the mechanism of oncogenesis is through covalent binding and somatic mutation.

Recently Pitot et al.[+] have shown that partially hepatectomized rats which received a single dose of diethylnitrosamine and then administered a bimonthly dose of TCDD equivalent to 100 ng/kg/day, developed a large increase in altered hepatocellular foci and hepatocellular carcinoma. This suggests TCDD acts as a tumor promoter, in the liver and is approximately a million times as potent as phenobarbital in this system.

The mechanism(s) by which TCDD produces toxicity and carcinogenicity are unknown. Understanding these processes is important because 1) TCDD is the prototype of a large series of the halogenated aromatic hydrocarbons, many of which are environmental contaminants, 2) the studies to date suggest this series of compounds acts by rather a unique mechanism (s).

ACKNOWLEDGEMENTS

We would like to thank Dr. Andrew Kende and his postdoctoral fellows. Dr. James Wade, Mark DeCamp and John Airey who synthesized all the compounds employed in this work. We are also grateful to acknowledge the contributions of Drs. Bill Greenlee and Joyce Knutson on the biological aspect of this work.

[+]Pitot, H., Goldsworthy, T. and Poland, A. (Manuscript in preparation).

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DISCUSSION

DUAX: For your tricyclic compound, the corner pocket theory would appear to be a safe one. However, in some of your compounds, like TCAB and TCAOB, it isn't clear to me that the rings must be coplanar. Three halogens (the number you show to be necessary) could sit in the corner pockets without having the

rings be coplanar.

POLAND: I agree with you. The idea of planarity came from a study of halogenated biphenyls. We are not sure whether the 3 or 4 halogen atoms have to have the same planar touchdown points or whether the intervening rings must also be in the same place. Ideally, a hexachlorinated tryptycene would answer this question, but we haven't synthesized it.

KUPFER: Is methylcholanthrene or BNF more toxic to responsive mice than to nonresponsive ones?

POLAND: As you know, Dr. Nebert and his colleagues at NIH, have looked extensively at the question of whether the toxicity and carcinogenicity of polycyclic aromatic hydrocarbons segregate with the Ah locus. The evidence suggests that it depends on the particular polycyclic, the route of administration and the particular toxic response. I have suggested that methylcholanthrene can produce two kinds of toxicity: one arises from its metabolism to reactive metabolites which can cause cell damage by covalent binding to macromolecules; the second mechanism, less recognized, is that methylcholanthrene, BNF and the halogenated aromatic compounds elicit certain toxicities by binding to the cytosol receptor. This requires a tissue where the monooxygenase activity is low, so the polycyclic aromatic hydrocarbons persist long enough, and where the response from reactive metabolite formation and toxicity, does not mask the toxicity arising from the gene expression.

STANCEL: You mentioned that TCDD will cause atrophy of the thymus. Glucocorticoids will also produce this effect. Have you determined whether this response to TCDD and glucocoritcoids is additive or synergistic?

POLAND: TCDD will produce thymic atrophy in adrenalectomized rats. Dr. Joyce Knutson has found that the mouse lymphoma cell line S49G3, which was killed by dexamethasone, does not respond to TCDD. Glucocorticoids do not compete with ^{3}H-TCDD for receptor binding.

LUCIER: If the Ah locus correlates with the toxicity for TCDD, then why is the guinea pig, which is nonresponsive to the inductive actions of TCDD, the most susceptible species to TCDD toxicity?

POLAND: George, I wish I understood the difference in species sensitivity. Let me point out that among all the halogenated aromatic compounds that comprise this group, the potency of a congener to induce aryl hydrocarbon hydroxylase (AHH) activity (measured in chick liver or a rat hepatoma cell culture) corresponds to its toxic potency in a variety of animals. The chicken embryo and mouse are about equally sensitive to TCDD induction of hepatic AHH activity (ED_{50}, 0.3 to 1.0 x 10^{-9} moles/kg), but differ over 100-fold in their sensitivity to the LD_{50} of TCDD. Dr. Knutson has looked at over 20 cell lines in culture, and while many are inducible with TCDD, none show toxicity. I suggest that the receptor is essential for toxicity, but not sufficient.

SHIVERICK: Does TCDD induce benzo(a)pyrene hydroxylase activity in extrahepatic tissues, particularly adrenals and lung?

POLAND: TCDD is an excellent inducer of AHH activity in nonhepatic tissues including lung, bowel, kidney, and skin. I do not have data on the adrenal gland.

MARTIN: Would you care to speculate why God gave the cell a specific receptor system for TCDD.

POLAND: You have abviously asked the major question. My working hypothesis is that TCDD and other xenobiotics which bind to the receptor are mimetics of a physiologic regulator (an endogenous inducer). We have looked for the presence of a small molecular weight (dialyzable) factor which would compete with ^{3}H-TCDD for receptor binding and not found anything as yet. One corallary of your question is that it is not certain that the "purpose" of this regulation is only the induction of the drug-metabolizing enzymes. So both the overall function of the response, and a physiologic inducer remain speculative.

Environmental Impact of Estrogens

McLachlan, ed. Estrogens in the Environment

PERSONAL EXPERIENCES EMPHASIZING THE ENVIRONMENTAL IMPACT OF ESTROGENS

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Research Professor of Pharmacology, The George Washington University Medical Center, 2300 Eye Street, N.W., Washington, D.C. 20037

In these few minutes given me for our orientation to this morning's session it seemed of interest to briefly describe for you three past personal encounters with environmental estrogens.

About 20 years ago the Director of the N.I.H. laboratory at Helena, Montana, complained that his previously fertile mouse breeding colony had become virtually non-productive during the preceeding two months. He also noted that his male mice had developed a high frequency of inguinal hernias so that they would be dragging a scrotally placed mass of intestine behind them as they walked across the floor of the cage.

Gardner et al.[1] had described such hernias in male mice chronically treated with estrogens. Also infertility in sheep ingesting a form of clover producing effective amounts of an estrogenic flavone had been described in New Zealand[2].

These features of this laboratory's problem suggested that the cause of the trouble might be a dietary source of estrogen. The affected colony had been maintained for years on a widely used brand of dried pellets. We found that an extract of these pellets contained a uterotrophic substance in sufficient concentration to provide a fully effective dose in a few days ration.

During these investigations some of the untreated control mice in our own studies on other aspects of estrogen metabolism began to show enlarged uteri. After severely reprimanding our animal care-taker for mixing up our animals we cleaned house and soon again encountered the same phenomenon in untreated ovariectomized mice.

The culpable substance in both our own and in the Montana laboratory was found to be diethylstilbestrol which had inadvertently contaminated large lots of the pelleted feed. The feed had been milled on the same equipment used by the manufacturer to prepare highly potent concentrates of stilbestrol for admixture to cattle feed.

A year or so later a distinguished pediatrician referred to me a five year old girl and her seven year old brother who presented at about the same time with bi-lateral modular breast enlargement (Figures 1 through 4). This simultaneous occurrence within a family suggested an exogenous source of estrogen, particularly since Zondek in Israel had just written me of an outbreak of

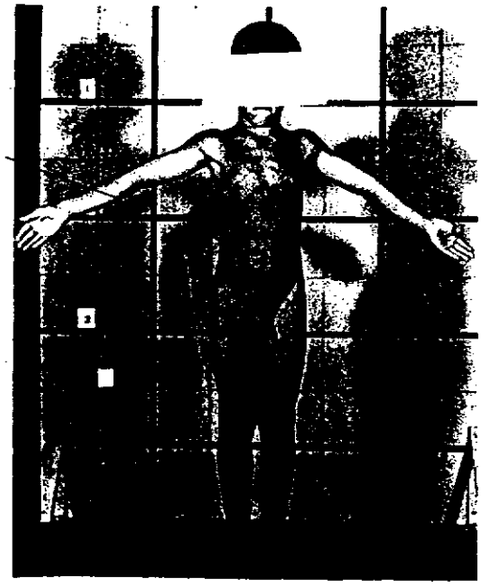

Figure 1. 7 year old boy with breast enlargement.

Figure 2. 5 year old girl with breast enlargement.

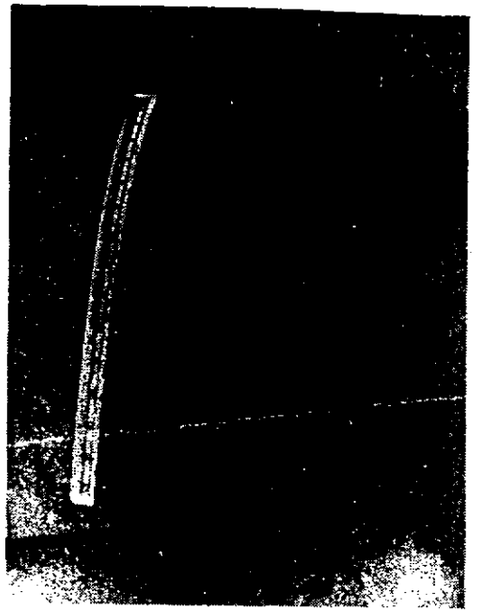

Figure 3. Breasts of girl in Figure 2.

Figure 4. Breasts of boy in Figure 1.

gynecomastia in a kibbutz attributable to the ingestion of chicken necks containing residual stilbestrol pellets. Exhaustive study in our patients' home one evening with emphasis on the medicine chest revealed no probable estrogen source. However, as I was about to leave the home I noticed that the mother had set the table for the following morning's breakfast and had provided each child with a large vitamin capsule. The mother and father were fortunately obliged to omit this dubious health practice for budgetary reasons.

The remaining vitamin capsules were found in our laboratory to be heavily contaminated with diethylstilbestrol, each containing the equivalent of 150 gamma of estrone. They had been manufactured in a garage in Philadelphia by a marginal firm which also prepared stilbestrol tablets, on the same uncleaned equipment. The F.D.A., which moved rapidly in those days, soon terminated this practice.

Six months later a physician referred to me her 8 year old and 10 year old sons who had simultaneously presented with an abrupt nodular gynecomastia (Figures 5 and 6). She had been giving these boys daily doses of isoniazid because of prior household exposure to tuberculosis. This medication had been obtained from a firm of dubious standing and proved to be heavily contaminated with stilbestrol[3].

Two years later our untreated control rats began showing a highly sporadic occurrence of uterine enlargement. The spotty character of this effect in the laboratory this time exculpated the diet and the animal care-taker, and the technicians. However, this feature suggested an environmental effect in our laboratory. Then one day we found the same scattered effect among a group of rats freshly received from our central animal production facility. Careful review of the practices in that facility revealed that the rats were being dusted at various times with an insecticidal powder in order to keep their infestation with ectoparasites to a minimum. It had been determined previously that the insecticide DDT had some estrogenic potency and so we undertook a study of the dusting powder in use. The active estrogenic ingredient proved to be technical grade Methoxychlor, which the animals had ingested by grooming themselves and their cage partners[5].

I hope that this account of these experiences will prove instructive for you and will serve as an appropriate introduction to this morning's discussion of ambient estrogens.

Figure 5. Breasts of 8 year old boy.

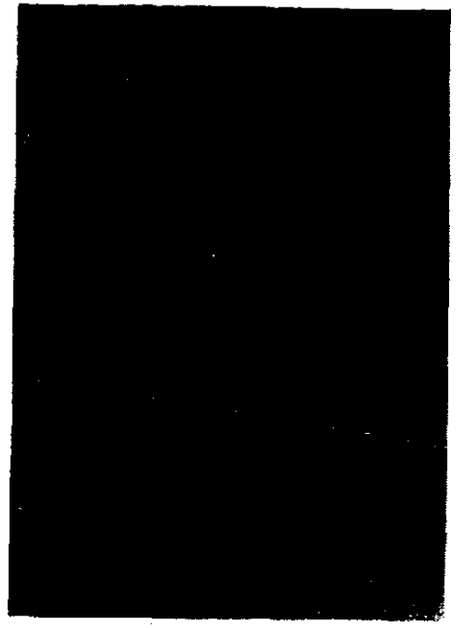

Figure 6. Breasts of 10 year old boy.

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ASSOCIATION OF EXOGENOUS ESTROGENS AND CANCER IN HUMANS

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INTRODUCTION

When an epidemiologist is invited to discuss a specific carcinogen or class of carcinogens at a meeting composed primarily of laboratory scientists, the relevant human observations frequently are of great biologic interest in offering insights into basic mechanisms of carcinogenesis but of minor public health significance because of the relatively few humans exposed to the substance in question. This is often the case for therapeutic drugs which exhibit carcinogenic potential. In these circumstances the public health signficance of exposure is even further diminished because the individuals exposed are often quite ill and willing to accept substantial risk of serious side effects in order to obtain the potential benefits of the therapeutic drugs in question.

Estrogenic drugs, however, certainly do not fit this description. These agents, for which there is abundant laboratory evidence of carcinogenicity, have been, and continue to be widely used at high doses for long periods of time by large numbers of healthy women. It is estimated that between 4 and 6 million Americans (mothers, daughters, sons) have been exposed to diethylstilbestrol (DES) during pregnancy.[1] A recent survey indicated that as many as 50% of all recently menopausal women in one area of the U.S. have taken estrogens for climacteric symptoms for a median duration of 10 years.[2] Current estimates also indicate that approximately 80 million women in the reproductive ranges throughout the world use oral contraceptives.[3] Therefore, not only is the human species currently participating in a massive natural experiment to evaluate the potential carcinogenicity of these compounds, but the public health significance of even small alterations in carcinogenic risk due to these drugs is substantial.

As evidence of the level of concern over the carcinogenicity of these drugs, in the last two years I have participated in two comprehensive reviews of various aspects of this subject for two international agencies.[3,4] However, new information is appearing at such a rapid rate, that such reviews need to be updated at least annually. In attempting to review the human

evidence with respect to potential carcinogenicity of a wide variety of estrogenic drugs, I will rely heavily on these two recent reviews, updated with subsequently published studies and the results of several studies completed but not yet published.

EARLY STUDIES

Until the mid 1960's, only a small proportion of the general population used estrogens, and only a very small proportion had used them for extended periods of time. Thus, the few early case-control studies to assess this exposure were inadequate to detect any alteration in cancer risk.[5-7] Six follow-up (cohort) studies had been done of women intensively exposed to estrogen preparations either for symptoms of the climacteric or for treatment of osteoporosis.[8-14] As recently as 1971 several of these studies were cited as indicating that estrogen replacement therapy was associated with "protection" against virtually all forms of cancer.[15] If correct, the biologic and public health implications of these observations would have been impressive. However, little attention had been paid to these observations of apparent protection by either cancer researchers or the general public, a fact lamented by the author summarizing these observations in 1971.[15] Perhaps part of the reason for this lack of attention arose from at least an implicit understanding of some of the deficiencies of these studies. In each of these studies, rather than identifying a group of exposed persons and then following them for cancer, information was simply extracted from existing medical records. Often a group was composed of only those patients who were followed up to a certain date; in others patients were included only up to the date they were last seen, with no additional follow-up efforts made. These methods could have resulted in underestimates of the cases of cancer occurring in these groups.

A few other comments concerning these early studies are in order since they are often resurrected in current debates over these medications. In several of the studies, only a small number of patients and/or a short follow-up period were involved. Most carcinogenic effects manifest themselves only after long latent periods, so the relevant follow-up period may occur only many years after initial exposure. Indeed, endocrine phenomena associated with changes in breast cancer risk (e.g., oophorectomy and an early age at natural menopause) do not exert their effect until about 10 years following the event.[4] In addition, small cohorts and short follow-up make it likely that a relatively small or moderately increased risk will be

overlooked. With this in mind, it is important to note that in the 6 studies cited above: a) In two no women were followed more than 10 years[8,14]; b) in one only 86 women were followed for more than 10 years[11]; c) in one the number followed for 10 years could not be determined, but the longest follow-up was only 15 years[10]; d) in one although the longest follow-up was 25 years, the average for all 292 patients was 5 years, indicating that very few women were followed up for more than 10 years[9]; e) in one the entire study group consisted of those who had used the medication for 10 years, but the longest follow-up was only 14 years after starting use[13]. Many of these studies also suffered from flaws in their analyses. Such flaws included calculating expected numbers of cancers for periods of time during which the group was not under observation for malignancy, calculating expected numbers of cancers for organs that had been removed from these populations (e.g., the uterus and ovaries among women having undergone total hysterectomies), and making no adjustments for the protective effect against breast cancer of an oophorectomy or an early age at natural menopause.

In summary, prior to the early 1970's, the numbers of persons exposed to estrogens, particularly long term users, were too few for an adequate evaluation to be made by case-controls studies. In addition, each of the 6 early cohort studies was so deficient in either conduct or analysis that the results were uninterpretable.

DES DURING PREGNANCY

In 1971, the same year that the protective effect of estrogens was being promoted, an epidemiologic study was published which indicated that an unusual cluster of a rare form of vaginal cancer in females aged 14 to 22 years, noted at one hospital, was related to intrauterine exposure to DES by these young women.[16] Later studies confirmed these results and indicated that the in-utero exposure related to an excess risk of clear cell adenocarcinoma both of the vagina and the cervix.[1] Shortly thereafter a registry of this disease in young women was established. Currently, this registry contains reports on over 350 cases of clear cell adenocarcinoma of the vagina or cervix.[17] Among those cases with an available maternal pregnancy history, approximately 2/3 indicated in-utero exposure to DES or similar estrogens, such as hexestrol and dienestrol. While the relative rarity of this tumor, along with a lack of accurate estimates of exposure, have made an estimation of the actual risk of this malignancy among the exposed difficult,

a reasonable range for this estimate has been established. It appears that the incidence of clear cell adenocarcinoma is somewhere between 1 per 1,000 to 1 per 10,000 through the age of 24, among the exposed daughters.[17] Since the use of DES during pregnancy was not common prior to the early 1950's, accurate estimates for what occurs beyond the age of 24 are not yet available. An analysis of the age-incidence pattern for this disease is quite interesting.[17] The rates rise very sharply at age 14, peak at age 19, and then decline rapidly. The steepness of the ascending limb of this curve is noteworthy, considering that the relevant exposure occurred 15 to 20 years prior. Usually, if the latent period for a disease is long, it tends to have a wide range. In this circumstance, while the average latent period is 19 years, the range is quite constrained. It appears that something associated with puberty (perhaps the concomitant surge of endogenous estrogens) is acting as a powerful promoting agent, leading to the manifestation of this disease.

With the enthusiasm for describing various features of clear cell adenocarcinoma associated with in-utero DES exposure, it is easy to forget that the influence of this exposure on cancers of other hormonally sensitive sites has not yet been evaluated adequately. Recent reports have suggested, and denied, that cervical and vaginal intraepithelial neoplasia (squamous cell dysplasia and carcinoma in-situ) might be more common in DES exposed women.[18,19] The women exposed to DES in-utero are just now entering the age range where cancers of the cervix and breast begin to appear, and it will be a number of years before they reach the high risk ages for cancers of the endometrium and ovary. Therefore, the need for continued evaluation of this exposure seems to be obvious.

Male Offspring

It has been noted recently that males exposed in-utero to DES demonstrate a number of teratogenic effects.[20] There is a clear excess of abnormalities of the external genital tract among exposed males. These consist primarily of a history of cryptorchidism and the finding of an increased number of hypoplastic testes and epididymal cysts. In addition, single semen determinations suggest an increase in sperm abnormalities, such as low sperm counts, decreased sperm motility, and possibly an increased number of abnormal sperm forms. The implications of these findings for potential carcinogenic effects are as yet unknown. However, two small case-control studies of testicular cancer have raised the suspicion of an increased risk of this tumor associated with in-utero exposure to DES.[1,21]

Mothers

It is often overlooked that the mothers who took DES during the pregnancy had a substantial, albeit short-term, exposure to exogenous estrogens. The dose regimen most popular in the late 1940's and early 1950's called for between 10 and 12 grams of DES to be administered during the pregnancy. Currently the only information we have concerning the potential risks to the mother associated with this exposure comes from the follow-up of women who participated in a randomized clinical trial conducted at the University of Chicago in the early 1950's.[22] Among this group, more patients in the DES-exposed group developed cancer in reproductive organs than among women not treated with DES. Thirty-two cases of breast cancer were observed in the 693 women exposed to DES compared to 21 cases in the 668 women in the comparison group. More cases of ovarian cancer (4 versus 1) and cancers of the uterine cervix (7 versus 3) occurred in the DES-exposed group than the comparison group. However, fewer cases of endometrial cancer (3 versus 5) occurred in the exposed in comparison to the unexposed. Thirty-eight of the exposed women have died compared to 28 in the comparison group. All of this difference was seemingly attributable to deaths due to cancers of the breast and gynecologic organs (e.g., 12 deaths were attributed to breast cancer among the exposed versus 4 among the comparison group). These observations provide some cause for serious concern about the carcinogenic potential of a large dose of DES for the breast and gynecologic organs of the mothers taking the medication. However, there is need for caution since the differences observed in this study were based on a relatively small number of cancers and could be due to chance alone. Further studies will be needed to confirm or deny the implications of these observations.

MENOPAUSAL ESTROGENS

As noted, early studies of menopausal estrogen use failed to identify any excess risk, and indeed had implied a substantial amount of "protection" against virtually all malignancy. Since 1975 there has been a dramatic reversal in the weight of the evidence concerning the carcinogenic consequences of the use of these substances.

Endometrial Cancer

Late in 1975, two case control studies were published which indicated that the use of conjugated estrogens for symptoms of the climacteric was associated with a relatively high risk of endometrial cancer.[23,24] The first study, conducted in a cancer clinic, indicated a 4 to 8-fold increased

risk of endometrial cancer among estrogen users compared to nonusers. The second study, from a large prepaid health plan, indicated an 8-fold excess risk overall for users of estrogen, and a dose-response relationship with duration of use, rising to 14-fold among those who had used estrogens for 7 years or more. Both of these studies were based on record reviews. A subsequent study in a large retirement community utilized both health plan records and personal interviews and obtained results similar to the first two.[25] In addition, this study indicated a comparable relative risk associated with the use of nonconjugated estrogens, and a dose-response relationship with the dose of the tablet usually used. Since these first reports, eight independent investigations have found similar results using a wide variety of study designs.[26-33] Three were case-control interview studies,[26-28] two were case-control studies involving a review of medical records,[29,30] two were case-control record-linkage studies in large group practices,[31,32] and one was a cohort study of the frequency of subsequent primary malignancies of the endometrium among breast cancer patients treated with non-steroidal estrogens.[33] Since the first reports, the methods employed in a number of these studies have been criticized and questioned in a variety of ways.[30,34] Targets for criticism have included the following: the use of control women who had diseases with risk factors different from those of endometrial cancer; the possibility of inadequate control for endometrial cancer risk factors in the analyses; an interval between first exposure and diagnosis that was too short to be consistent with current concepts of carcinogenesis; the exclusion of women who had had a hysterectomy from control groups; the accuracy of the endometrial cancer diagnoses; and the possibility of a surveillance bias (those using estrogens being more likely to have endometrial cancer diagnosed or diagnosed earlier than those not using estrogens). In the variety of studies that have been reported since the initial papers, and in several commentaries,[4,34,35] each of these criticisms has been addressed adequately, without altering any conclusions concerning the association.

In addition, the conclusions of these analytic studies have been supported by evidence of rising incidence rates of endometrial cancer following the dramatic increase in use of estrogens for symptoms of the climacteric in the United States.[36,37]

In summary, a number of recent studies utilizing a variety of designs have found a consistent, strongly positive association between a number of estrogenic substances and the risk of endometrial cancer, with positive dose-response relationships both with the strength of the medication and

with the duration of use. These observations have been supported by a dramatic rise in the incidence rates of endometrial cancer in concert with the dramatic increase in the use of these medications.

While most of the important issues have been addressed adequately by the current studies, there are at least two remaining issues that need to be addressed. First, no adequate evaluation has been made of the influence on endometrial cancer risk associated of the addition of progestational agents to estrogenic compounds used for hormonal replacement therapy. It has been suggested that this addition might at least partially diminish the risk of endometrial cancer in women undergoing estrogen therapy.[38,39] Until such time as this has been evaluated, however, it should be noted that the sequential cyclic use of estrogen and progestins in oral contraceptives has been related to an increased risk of endometrial cancer.[40] The other major issue requiring further data concerns the risk among women who have stopped using estrogens. Very recent evidence seems to indicate a plateauing of the incidence rates of endometrial cancer, and perhaps even a slight downturn in the rates, following quite closely on the dramatic reduction in use of menopausal estrogens after the initial reports in 1975.[37] In fact, a recent study of individuals in a large group practice indicated that the decline in incidence rate in this group practice following the reduction in estrogen use was due to the decrease in use, since the incidence rates among those using estrogens remained at the same high level.[32] Very recently, the first study to attempt to address the risk among former users has been reported.[28] While the numbers of relevant observations are small, two features are noteworthy. First of all, even among former users who stopped some time ago, a substantial elevation in risk remains. However, after standardization for amount of estrogen received, there is evidence of a meaningful reduction in the excess risk of endometrial cancer very soon after the women stopped using the medications. These observations are particularly exciting for their immediate relevance to cancer prevention, and for their biologic implications with respect to understanding cancer initiation and promotion.

Breast Cancer

A number of reports from a cohort study carried out in Nashville, Tennessee have appeared in the literature since the early 1970's.[41] Although this study had some of the same faults as those described under "early studies", it was better designed and analyzed and was the first that did not describe "protection" against breast cancer. Among the 735 women who were followed for an average of 15 years, 21 cases of breast cancer were observed versus

18 expected. A criticism of the development of the expected value in the study has indicated that it may be too high. In addition, although half of the total group had undergone bilateral oophorectomy, the anticipated protection against breast cancer due to this procedure did not occur.

In 1976, another, and much larger, cohort study was reported in which 1891 women given conjugated estrogens for symptoms of the climacteric were followed for an average of 12 years.[42] Breast cancer was observed in 49, whereas 39 were expected on the basis of rates in the general population. The relative risk of breast cancer increased with duration of follow-up, progressing to about two-fold after 15 years. In addition, after ten years of follow-up observation, two factors related to lower risk of breast cancer, nulliparity and oophorectomy, were no longer so related. In this study, estrogen use was also related to an increased risk of breast cancer among women in whom benign disease developed after they had started the drug.

A number of recent case-control studies of breast cancer have shown no significant association with estrogen use; however, none of these have been able to address the question of long-term use.[43-48]

Since the cohort studies had raised the question of excess risk in long-term users, a number of case-control studies to evaluate this association have been initiated. As yet the results from these studies have not been published. However, I am aware of the preliminary results of at least three of these studies, all three of which seem to lend some support to the estimate of a two- to three-fold excess breast cancer risk among long term users of conjugated estrogens.[49-51]

Ovarian Cancer

In a recent study, a statistically significant excess risk of ovarian cancer was reported among a small group of women who had been treated both with DES and conjugated estrogens for symptoms of the climacteric.[52] In this study, there was no significant elevation of risk for those women who had received only conjugated estrogens. As noted previously, in a follow-up study of women exposed to DES, four women treated with DES during pregnancy subsequently developed ovarian cancer, compared with 1 in a control group of comparable size.[22] On the other hand, one recent record linkage case-control study, and one interview case-control study have not found an association between conjugated estrogen use and the risk of ovarian cancer.[53,54] Taken in the aggregate, the human observations, together with a suggestion of an association between DES and the development of ovarian cancers in laboratory animals,[55,56] indicate the need for further investigation. Several studies

of ovarian cancer designed to address this issue are currently reaching their analysis phase.

Other Cancers

Two cohort studies since 1971 have reported lower than expected numbers of cancers other than those of the breast and reproductive system, and especially of colon cancer.[41,42] While the lack of any evidence of a dose-response relationship for this association weakens arguments in favor of a protective effect, additional studies need to be done to explain these observations.

Benign Breast Disease

The only benign neoplasm that has been extensively evaluated for its relationship with the use of estrogens for symptoms of the climacteric is benign breast disease. However, the results of these evaluations have been conflicting. Three case-control studies have failed to find an association between estrogen use and the risk of surgically confirmed benign breast disease,[43,47,57] while one case-control study has found a two and one half-fold excess risk.[58]

ORAL CONTRACEPTIVES

The situation for oral contraceptives is somewhat different than that for DES and other estrogens used in treatment. For these other hormones, adequate human evaluations lagged behind the use of these medications for an unfortunately long period of time. The human exposure circumstances surrounding oral contraceptive use is truly a story unique in the annals of therapeutic drug history. Prior to 1960 essentially no one had used these agents outside of the clinical trial context. In less than ten years of their introduction in 1960, fully 60% of young women in this country had had significant exposure to these potent combinations of estrogenic and progestational agents.[59] As noted previously, current estimates are that 80 million women worldwide are using these medications for contraception.[3] Because of this abrupt widespread use by a healthy population of potent physiologic agents for which there was laboratory evidence of carcinogenicity, a number of people started calling quite early for appropriate evaluations to be done in women.[60] Because of this concern, the literature on the subject is quite extensive.

Benign Breast Disease

With one exception, a number of case-control and cohort studies have been consistent in finding a deficit of benign breast disease in current oral contraceptive users. (Tables 1 and 2) This deficit is consistently observed

Table 1. Case-Control Studies of Oral Contraceptive Use: Benign Breast Neoplasia

Investigator and Year	Age Range (Years)	Disease*	Number of Cases	Relative Risk By Duration of Oral Contraceptive Use (Years) 0	<1	1-	2-	3-	4-
Vessey (61) 1972	16-39	FC	117	1.0	1.0			0.3	
		FA	86						
Sartwell et al. (57) 1973	20-70	FC and Misc, FA	306	1.0	1.3			0.5	
			71	1.0	1.0			1.8	
Boston Collaborative Drug Surveillance Program (43) 1973	20-44	FC	62	1.0			0.5		
		FA	30						
Kelsey et al. (62) 1978	20-44	FC	209	1.0	0.9	0.7	1.2	1.1	0.4
		FA	123	1.0	0.9	1.4	0.8	0.8	0.4
Fasal & Paffenbarger (63) 1975	15-49	FC, FA And Misc.	446	1.0	1.4		0.4		0.5
Nomura & Comstock (58) 1977	20-49	FC	275	1.0			1.0		
		FA	45						
Hoover et al. (64) 1978	26-50	FC, FA and Misc.	342	1.0	1.2	1.1	0.6	0.6	
Ravnibar et al. (47) 1979	15-49	FC	266	1.0	0.9			0.4	
		FA	106	1.0	1.2			0.6	

* FC = Fibrocystic Disease
* FA = Fibroadenoma
* Misc = Miscellaneous

Table 2. Cobort Studies of Oral Contraceptive Use: Benign Breast Neoplasia

Investigator, Year and Period of Enrollment	Age Ranges of Coborts at Enrollment (yrs.)	Disease*	Number of Cases	Relative Risk by Duration of Oral Contraceptive Use (Years) 0	<1	1-	2-	3-	4-
Royal College of General Practioners (65) 1974 (Subjects enrolled during 1968-1969)	15-49	FC,FA,and Misc (clinical diagnosis)	859	1.0	0.9	0.9	0.8	0.6	0.5
Ory et al. (66) 1976 (Subjects enrolled during 1970)	25-49	FC (histological diagnosis)	499	1.0	0.9	0.7		0.4	
		FA (histological diagnosis)	83	1.0	1.2			0.5	
Vessey et al. (67) 1976 (Subjects enrolled during 1968-1974)	25-39	FC,FA,and Misc. (clinical diagnosis)	263	1.0			0.5		

*FC = Fibiocystic Disease
* FA = Fibioadenoma
* Misc = Miscellaneous

with respect to fibrocystic disease but has only been inconsistently linked to fibroadenoma. The apparent protective effect is related to duration of use and may persist for some time after cessation, however the association among former users has yet to be investigated adequately. For current users of oral contraceptives with a total exposure of longer than two years, the risk of being hospitalized for benign breast disease is only about 25% of those who have never used oral contraceptives.[61] The cohort study from the Royal College of General Practitioners was able to take advantage of the popularity of two varieties of a particular brand of contraceptive.[68] The only difference between the two varieties was in the dose of progestin involved. This study seemed to indicate that the apparent protective effect was directly related to the strength of the progestational component.

Obviously, the important question is whether this apparent protective effect against benign breast disease will be relevant to breast cancer. Since benign breast disease identifies a group at high risk for breast cancer, these findings with respect to diminished risk of benign breast disease have been somewhat encouraging. However, a recent study indicates caution.[69] In this study, the cases of benign breast disease were reviewed and scored according to an index of ductal atypia. The marked protective effect associated with oral contraceptives seemed to apply primarily to the form of the disease associated with the least atypia (the form that may not be a risk factor for breast cancer). In fact, for the type of benign disease associated with the highest subsequent risk of breast cancer (the one with the most severe atypia), oral contraceptives were associated with an actual increased risk.

Breast Cancer

To date, studies on the relationship of oral contraceptive use to breast cancer have yielded inconclusive results.

Cohort studies have provided only limited information, due to the small numbers of incident cases observed thus far (Table 3). In one study, 16 cases have been reported and the lowest rate was among those using oral contraceptives, however, the differences were not significant.[67] In another cohort study, 31 cancers were reported, and the standardized rates were no different in users, ex-users, and nonusers.[65] It should be noted that in this study only 5% of women had used hormones for more than five years. In another cohort evaluation, hospitalization rates for breast cancers in users and nonusers based on 137 cases of cancer observed over a 30 month period yielded no significant differences between the rates in users and nonusers.[66]

Table 3. Cohort Studies of Oral Contraceptive Use: Breast Cancer

Investigator and Year	Age Range (Years)	Number of Cases	Relative Risk by Duration (Years) Of Oral Contraceptive Use			
			Never Used	<1	1-	2-
Royal College of General Practioners (65) 1974	15-49	31	1.0		1.1	
Ory et al. (66) 1976	25-49	137	1.0	0.6		0.7
Vessey et al. (67) 1976	25-39	16	1.0		0.4	

An increasing number of case-control evaluations of oral contraceptive use have been reported (Table 4). The most recent report from the ongoing case-control study at Oxford has the largest numbers of cases reported to date.[71] Among the 621 total cases and their matched controls, there was no evidence of excess risk associated with ever use of oral contraceptives and no evidence of a dose-response relationship with number of years of use. When analyzed by age there was some evidence of excess risk in the oldest age group under study (ages 46-50). However, the trends in the next oldest age group (ages 41-45) were for the most part in the opposite direction and the authors interpreted this as evidence that the positive association was likely a result of chance, since they had investigated the risk in a number of subgroups. Among the 487 patients for whom clinical stage information was analyzed, those who had never used oral contraceptives had more advanced tumors at presentation than those using the pill in the year prior to diagnosis. The former pill users occupied an intermediate position with respect to clinical stage. These differences in clinical stage were reflected in differential survival patterns also. Since there was evidence that these differences were not due to a diagnostic (surveillance) bias, the authors suggested that these results may indicate that oral contraceptives may have had a beneficial effect on tumor growth and spread. Even in a study of this size, because of the recency of introduction of oral contraceptives only 3.5% of the control group had used oral contraceptives for more than 8 years (the longest duration of use category evaluated).

Additional studies have produced similarly negative results, with the same reservation as that of the Oxford study, that of a paucity of long-term users.

Two recent case-control studies may be worthy of separate note. In one, no significant differences were found in the risk of breast cancer between cases and controls who had ever used contraceptives.[63,72] However, a positive association was noted for long-term contraceptive users who also had a history of surgically treated benign breast disease, and among a small group of women who had used oral contraceptives prior to their first childbirth. In the other study, no overall association between contraceptive use and breast cancer was noted.[70] However, among women with a natural menopause there was a consistent finding of excess risk among oral contraceptive users with evidence of a dose-response relationship for those women who had another breast cancer risk indicator (those who had a history of surgically treated benign breast disease, those who had a late age at first birth, those with a

Table 4. Case-Control Studies of Oral Contraceptive Use: Breast Cancer (100 or More Cases)

Investigator and Year	Age Range (Years)*	Number of Cases	Relative Risk by Duration (Years) of Oral Contraceptive Use		
			Never Used	<2	≥2
Arthes et al. (5) 1971	15-75	119	1.0	0.8	0.6
Ravnihar et al. (47) 1979	20-49	190	1.0	0.9	1.0
Brinton et al. (70) 1979	PRE	126	1.0	—— 0.8 ——	
	NAT	160	1.0	—— 1.7 ——	
Vessey et al. (71) 1979	16-50	621	1.0	0.9	1.0
Paffenbarger et al. (72) 1977	15-49	452	1.0	1.1	1.3

* PRE = Premenopausal
* NAT = Natural Menopause

family history of breast cancer, and those with a late age at natural menopause). There are a variety of difficulties involved in interpreting studies in which a number of subgroups have been evaluated. Interpretation of the studies reported here is hampered by these difficulties. However, the observations of excess risk associated with long term use of oral contraceptives by women who are already at high risk because of the presence of another breast cancer risk indicator should be cause for concern, and should stimulate more intensive evaluations for possible synergistic effects. In addition, the suggestion of increased risk among oral contraceptive users who used the pills at a young age needs attention also. This time in a woman's life appears to be one when she is particularly sensitive to hormonal and other events that influence breast cancer risk.[73,74]

Endometrial Cancer

Since 1975, numerous case reports have appeared concerning the development of endometrial carcinoma in young women with a history of use of sequential oral contraceptives. A report in 1977 concerning a series of 30 women under age 40 who both developed endometrial carcinoma and had a history of oral contraceptive use found that the proportion of users of sequential oral contraceptives among this group was much higher than expected from national rates of use of sequential versus combination agents.[75] This association became even stronger when women with other known risk factors for the disease, short durations of contraceptive use, or use for reasons other than contraception were removed from the analysis. In addition, women who developed endometrial carcinoma in association with sequential contraceptive use had fewer of the previously established risk factors for the disease than did a similar series of young endometrial cancer patients diagnosed prior to the introduction of oral contraceptives. The proportional exposure method used in this analysis is open to criticism. However, taken in the aggregate, there appears to be an increased, although not quantified at this time, risk of endometrial cancer among users of sequential oral contraceptives.

Cancer of the Uterine Cervix

Few data are available concerning the risk of invasive carcinoma of the cervix associated with use of oral contraceptives. The data available relate primarily to the risk of development of dysplasia and/or carcinoma in-situ of the uterine cervix. Thus, much of the evidence is made more difficult to interpret because of the various controversies in pathology and epidemiology concerning these entities.

A number of studies concerning the possible carcinogenic effects of oral contraceptive use on the uterine cervix have utilized data abstracted from programs for the cytological detection of cervical neoplasia by comparing the prevalence of cervical neoplasia in users and nonusers of oral contraceptives.[76-85] These studies therefore have been based on data that were not collected with a view to research on the effects of oral contraceptives and they have yielded conflicting results and are difficult to interpret.

Four case-control studies have been conducted.[86-89] Three have found no association, while the fourth[89] conducted among black women attending a screening program in Atlanta, Georgia, found a positive association with some evidence of a dose-response relationship (the risk rising to five-fold over that of nonusers for contraceptive users of three years or greater). While the results of this study were standardized for a number of factors, no information was available on a number of confounding factors directly related to sexual activity. Another problem was the substantial disagreement with the original histologic diagnosis of cancer in-situ on the part of one of the two pathologists who reviewed the slides.

Four cohort studies have thus far been reported.[90-93] In two, no significant differences in cancer or in-situ precancerous lesions were found between contraceptive users and users of methods other than the diaphragm.[90,91] Diaphragm users have been noted on a number of occasions to have a substantially reduced risk of cervical neoplasia. One cohort study concerning 17,942 women enrolled pre-paid health plan detected a significantly increased relative risk of cancer in-situ among oral contraceptive users, a risk which increased with duration of exposure.[92]

A number of risk factors were taken into account into the analysis of this study, but information on risk factors related to sexual activity were not available. A subsequent investigation of these variables in this group indicated that when these factors were taken into account, the association between duration of oral contraceptive use and carcinoma in-situ remained, but was less marked.[94] This finding again illustrates the importance of sexual activity as a major confounding variable with regard to the study of cervical neoplasia and contraception.

In 1977, the results of a 7 year follow-up of a group of contraceptive users and nonusers was reported.[93] This study was a follow-up of patients with cervical dysplasia. Rates of progression of cervical dysplasia to carcinoma in-situ were compared for users of oral contraceptives and nonusers. Over 90% of the nonusers used intrauterine devices. The results of this

study suggested that extended oral contraceptive use (for 6 years or greater) appeared to increase by several times the rate of conversion of cervical dysplasia to carcinoma in-situ among women with dysplasia at the time they began to use oral contraceptives.

As this brief review indicates, the studies addressing the issue of cervical neoplasia and contraceptive use have been numerous, conflicting, and difficult to interpret. Detailed discussions of the methodologic issues involved could occupy a number of pages, and have been summarized elsewhere.[3,4] The general conclusion that can be achieved at this time based on the available data would be that there is a suggestion of an increased risk of cervical dysplasia and carcinoma in-situ among long-term oral contraceptive users who also have other factors predisposing them to these conditions. However, to date, all of the potential sources of bias and confounding in these studies have not been controlled adequately, so this conclusion must remain a tentative one at this time.

Ovarian Cancer

Three recent studies have suggested that patients with ovarian cancer have a less frequent history of use of oral contraceptives than controls.[54,95,96] It has also been noted that this apparent "protective" effect is biologically consistent with the other risk factors for ovarian cancer, which indicate that patients with "incessant" ovulatory activity tend to be a higher risk than those who have had less ovulatory activity.[96] It should also be emphasized that this apparent protective effect may be a relatively acute effect, with the long-term consequences of contraceptive use on ovarian cancer yet to be evaluated.

Liver Neoplasms

Increasing numbers of reports of hepatocellular adenomas in young women have appeared in the literature since 1973.[97,98] These neoplasms, although benign, are highly vascular and often present as emergencies because of intrahepatic or abdominal hemorrhage with shock.

Two case-control studies have linked these tumors to the use of oral contraceptives.[99-100] The relative risk associated with oral contraception is quite high (100 times that of nonusers for those who have used contraceptives for 3 to 5 years and over 500 times that of nonusers for those who have used for 7 years or more). The relative risk also appears to be higher for contraceptive users over age 30, and appears to be higher among contraceptive users who took pills with higher doses of estrogen and progestin. While the relative risk is high, the absolute risk does not appear to be large for this rare tumor. Preliminary calculations suggest that the amount of hepatocellular adenoma among women under age 30 is no more than 3 per 100,000 contraceptive users per year.[3] Over age 30 the absolute risk is greater, but not yet estimated.

Several malignant hepatomas of the liver have been reported among women using oral contraceptives. In one instance, such malignant tissue was found in an hepatic adenoma in a contraceptive user.[101] Whether these reports indicate any excess risk or not is impossible to determine, since no controlled study has been conducted to date.

Malignant Melanoma

A possible association between oral contraceptive use and malignant melanoma of the skin was based on analysis of incidence data from a cohort of 17,942 women.[102] A total of 22 cases were found during the period of observation and the age adjusted rate per 100,000 persons per year was 17.6 for those who had never used contraceptives, 24.1 for users of less than 4 years, and 29.5 for those using 4 years or longer. These differences were not statistically significant. As an adjunct to this study, an additional case-control study of 37 melanoma cases in the tumor registry of the same health plan, but not among women in the identified cohort, was conducted.[102] The estimated relative risk for who had ever used oral contraceptives was 1.8, but again this excess was not statistically significant. The excess risk among users of contraceptives appeared to be localized to the lower limbs. In neither study was any information ascertained about exposure to sunlight, the most important known risk factor for malignant melanoma. If users of contraceptives are more likely to spend more time out doors than nonusers, this could have biased the results of these studies. Evaluations are underway to test this hypothesis after control for sunlight exposure.

Other Tumors

Several series of cases of adenoma of the pituitary have been reported in young women, a high proportion of whom had recently stopped using oral contraceptives.[103,104] To date an adequate test of whether these tumors are related to contraceptive use has not been conducted.

In a review of 611 women who had been followed after the removal of a benign hydatidiform mole, approximately 10% subsequently developed an invasive mole.[105] Twenty-five percent of those who had taken oral contraceptives prior to the return of human choriogonadotropin levels to normal underwent this malignant transformation in comparison to about 9% of those who had not taken oral contraceptives. This suggests that increased development of invasive trophoblastic disease may be due to the use of oral contraceptives.

CONCLUSIONS

Diethylstilbestrol was first produced in 1938. That same year the occurrence of cancer was reported in animals exposed to DES.[106] Similar timely laboratory observations followed upon the introduction of conjugated estrogens and the various synthetic components of oral contraceptives. Unfortunately, appropriate human evaluations could not be carried out on the same agents until the proscribed latent periods associated with human tumors had elapsed. Unfortunately also, even when these latent periods had elapsed appropriate human evaluations were often not undertaken. The last eight years has seen an aggressive attempt by a number of investigators to rectify this lack of appropriate evaluations. This has led to the cascade of reports in the literature which this review has attempted to summarize. As indicated, many questions remain unanswered, new questions have been raised, and the appropriate latent periods for a number of tumors have not yet elapsed. However, a substantial leap in our understanding of the neoplastic effects of estrogenic medications in humans has occurred in this time. Unfortunately, most of the news is not good. In-utero exposure to DES has been firmly linked to vaginal and cervical clear cell adenocarcinoma. In addition, an association of this exposure with congenital malformations of the external genitalia in males has been established, and a suspicion of increased risk of testicular cancer has been raised. Similarly, suspicion of an excess risk of cancers of the breast and gynecologic organs among the mothers taking this medication has been raised. The influence of this in-utero exposure to daughters on other tumors (cervix, breast, etc.) must await the aging of the exposed cohort into the ages at high risk of these tumors.

Marketedly elevated risks of endometrial cancer have been clearly linked to use of menopausal estrogens and recent observations have also raised a distinct suspicion of increased breast cancer risk among long-term users of these medications.

The use of sequential oral contraceptives has been related to an increased risk of endometrial cancer in young women and the prolonged use of oral contraceptives have been firmly linked to benign, though definitely neoplastic, liver tumors. Suspicions have also been raised with respect to oral contraceptive use and increased risk of cancers of the breast and cervix, at least among specific groups of women (particularly high risk women). These suspicions are currently being aggressively evaluated. In addition, evidence linking these agents with the development of malignant melanoma, pituitary adenoma, and choriocarcinoma have appeared.

Even initial optimism over notations of diminished risk of benign breast disease associated with oral contraceptive use has recently been moderated with the observation that this may not apply to the premalignant form of benign breast disease. It is hoped that the initial observations of diminished risk of ovarian cancer among oral contraceptive users are also not subsequently reversed when long-term effects are evaluated adequately.

The public health consequences of the use of any medication are ultimately judged on a risk versus benefit basis. Adequate assessment of risks and benefits of estrogenic drugs will take some time to determine. In the interim, these evaluations of the numerous natural experiments underway in human beings should be utilized to their fullest to elucidate biologic mechanisms of hormonally related neoplasia. Perhaps in this way we will be able to link this material with laboratory results in order to identify those laboratory observations which are particularly relevant. Hopefully, in this way we can establish a scientific basis for evaluating the wisdom of allowing human exposure to a substance without having to wait 20 to 30 years.

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OCCUPATIONAL EXPOSURE TO ESTROGENS - PROBLEMS AND APPROACHES

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INTRODUCTION

The Industry-wide Studies Branch (IWSB) of the National Institute for Occupational Safety and Health is currently engaged in an assessment of the extent and nature of occupational exposure to estrogenic compounds, and the possible resultant effects of that exposure.

This report will discuss briefly some of the available literature concerning reports of adverse effects of occupational exposure to estrogens, and the background and impetus for the current study. The report will conclude with an overview and discussion of the activities of IWSB and some of the current findings.

HISTORY OF OCCUPATIONAL EXPOSURES

Although considerable literature exists regarding acute and chronic effects of estrogens in women (primarily as a result of widespread contraceptive and other medical uses of estrogenic pharmaceuticals), reports of occupational exposures and the effects on males have been relatively few.

To date, a review of the literature has revealed approximately 15 reports of occupational etiology and perhaps half a dozen or so reports of effects of non-occupational etiology in women, men and children. These reports began approximately in 1940, and continue to 1978. Although space does not permit the discussion of details from all of these reports, it would be germane to summarize some of the circumstances described in these reports, and some of the pertinent clinical findings.

The hyperestrogenic syndrome (involving primarily acute or subacute toxicity) has been associated in various reports with exposures to diethylstilbestrol (DES) and its derivatives, natural or conjugated estrogens, hexestrol and its derivatives, and steroidal synthetics such as ethynyl estradiol and mestranol. There have also been a few reports of toxicity syndromes associated with some progestogenic compounds, including acetoxyprogesterone, and vinylestrenolone in combination with ethynyl estradiol. No studies recording long term followup of men and women occupationally exposed to estrogens or progestogens have been found.

Since DES was the first estrogen synthesized on a large scale, most of the earlier literature is concerned with subjective observations and objective clinical findings resulting from exposure in both occupational and non-occupational situations to DES. These findings were reported in association with the synthesis of DES (Scarff and Smith, 1942; Fitzsimons, 1944; Watrous, 1947; Klavis, 1953; Pagani, 1953; Watrous and Olsen, 1959; Pacynski, 1971; Burton and Shumnes, 1973; and Meyer et.al. 1978),[1-9] and with the manufacture and use of diethylstilbestrol pellets for caponizing cockerels (Katzenellenbogen, 1956).[10] Two of these papers (Pacynski, Katzenellenbogen) also reported signs of hyperestrogenism in the worker's children as a result of carryover of contamination to the worker's homes.

Two additional interesting reports of non-occupational exposure to DES involved the treatment with DES of a male criminal with a history of repeated sexual offenses (Dunn, 1940)[11] and the inadvertent exposure of two boys who apparently used hair lotion containing DES (Stoppleman et.al., 1955).[12]

The principal features of hyperestrogenism in males reported in these studies have included nipple sensitivity (manifested as tingling or tenderness of the nipple), or a feeling of pressure in the breast area, progressing in some cases to breast hyperplasia and outright gynecomastia. Subjective manifestations reported by some of the workers also included decreased libido and/or sexual potency. Findings in females included irregular menstruation, nausea, headaches, breast pain, leukorrhea, and ankle edema. Symptoms in exposed male and female children have included areolar pigmentation and enlargement of the nipples or breasts.

Goldzieher and Goldzieher (1949)[13] and Fisk (1950)[14] reported a total of six cases of gynecomastia, with elevated urinary excretion of estrogens, in male employees involved in the extraction processes of natural or conjugated estrogens. The case reported by Goldzieher was apparently due to skin absorption of estrogen after prolonged contact with concentrated alcoholic solutions (most estrogens are highly soluble in alcohol). Principal findings included tenderness and increased size of the breast and nipple pigmentation. Weight loss, headache, reduced libido and reduced size of the genitalia were also noted.

Pagani (1953)[5] reported menstrual disorders in two female pharmaceutical workers after pulverizing and selving blocks in hexestrol intermittently for eight weeks. Also noted were headaches, lumbar pain, mastodynia, and leukorrhea. Breast tenderness and enlargement were also reported in a number of male employees working with hexestrol dipropionate. Alterations in sexual functioning (decreased libido and potence) were also reported by the workers, although

no further details are given. One worker working with and exposed to hexestrol powder, apparently wearing contaminated clothing home, inadvertently exposed his daughter, who later developed symtoms of pressure in the breast area, increased areolar pigmentation, and leukorrhea.

Gambini et.al. (1976)[15] reported a clinical case of a male worker weighing and wet mixing ethynyl estradiol and vinylestrenolone in acetone. Principal findings in this case included gynecomastia and dysspermia (significant drop in sperm number and motility without signs of cell degeneration). After removal from work with the estrogens, both symptoms returned to normal within six months. The author theorized that the effect on spermatogenesis may have been due to the combined effect of the estrogen and progestogen. Briggs and Briggs (1974)[16] reported the production of azoospermia in five male volunteers given 20 ug of ethynyl estradiol and 10 mg of methyltestosterone twice per day for 12-18 weeks in the course of research in developing a male contraceptive. The effect on spermatogenesis proved to be reversible, and no other signs of hyperestrogenism were apparent, possibly as a result of the methyltestosterone present in the "pill" used.

Suciu et.al. (1973)[17] reported abnormal spermatogenesis, coincident with occupational exposure to a progestogen alone. The author reported observations in Poland of 22 workers employed in the preparation of acetoxyprogesterone. Seven of the workers were hospitalized for detailed examinations. Three of the seven had reduced number and motility of spermatozoa. Two cases also showed signs of sperm cell degeneration.

Finally, Harrington et.al. (1978)[18] reported a study of a worker population manufacturing oral contraceptive pills in which the principal active raw materials were mestranol and norethindrone. Five of the 25 male workers had clinical gynecomastia or a history of such, with or without decreased libido. Among 30 female workers, 12 reported at least one episode of intermenstrual bleeding in the preceeding 12 months, while none of the 60 non-factory matched controls reported such effects. Elevated serum levels of the ethynyl estradiol (active metabolite of mestranol) in workers were more frequent in those with high potential exposure, but the difference was not statistically significant ($p = 0.08$). However, blood sampling was not necessarily timed coincident with time of exposure. Industrial hygiene sampling data indicated wide variation in personal sampler values ranging from below the analytical limits of detection to 8.6 ug of mestranol per cubic meter of air, and up to 59.5 ug of norethindrone per cubic meter of air. It is interesting to note that despite subjective impressions that exposure potential was higher in the granulating and compression areas than in the packaging area, personal sampler results from

the packaging area averaged substantially higher than did similar samples obtained during the compression phase. However, the sampling and analytical methodology were at that time not perfected, and some caution should be used in interpretation of the sampling results.

IMPETUS FOR CURRENT NIOSH SURVEY

Based on the available literature, and in particular the recent study by Harrington et.al. as well as the suspicion that other pharmaceutical companies in the United States may well have had similar experiences with occupationally induced hyperestrogenism, an industry wide assessment of the extent and nature of the problem was begun by NIOSH in late 1977. The purposes of the study have been to focus on pharmaceutical companies synthesizing or using synthetic and natural estrogens, to delineate the extent of the hyperestrogenic syndrome in employees of the various firms involved, to evaluate the environmental situations at these sites, and if feasible, to conduct epidemiological studies designed to evaluate chronic or delayed effects.

INDUSTRY-WIDE STUDIES - OVERVIEW OF APPROACH

Before discussing the activities of NIOSH in the last year on this project, a brief overview of the research design used by the IWSB for this and many other occupational studies would be useful.

Basically, the approach consists of five major phases:

(1) Literature search
(2) Site selection
(3) Walk through surveys
(4) Protocol development and review
(5) Detailed studies

The literature review needs little discussion, but these are typically accomplished through manual searches as well as extensive use of available computerized data bases such as NIOSHTIC, Medline, Toxline, and others.

The site selection process utilizes information available from a variety of sources including trade associations, industrial directories, other government agencies, and direct contact with potential study sites. In the present study, for example, data from F.D.A. and industrial directories were utilized in developing a list of estrogenic products and manufacturing sites. These were followed up by direct contact with a number of manufacturing sites.

Once manufacturing sites of interest have been identified, site visits are arranged for the purpose of conducting walk through surveys. These surveys are essentially fact finding visits designed to provide a detailed description of each site in terms of the facility itself, the workforce, personnel and medical

records, and environmental conditions. These surveys include a tour of the pertinent operations to learn more about the manufacturing process and to observe first hand environmental conditions at each site.

Having completed the walk through surveys, an analysis of the situation in terms of need and feasibility for in-depth studies is made, and if indicated, a detailed study protocol is prepared in writing and subjected to peer, human subjects, and statistical reviews.

Finally, in-depth studies are conducted at those sites selected as representative of the industry. Depending on the study, in-depth industrial hygiene, epidemiological or cross-sectional medical studies may be conducted at each of the selected sites.

RESULTS TO DATE

To date, the literature search, site selection processes, and the walk through survey phase have been completed. A detailed study protocol for in-depth studies is now being developed.

Thirteen walk through surveys have been conducted to date involving 10 separate companies. These have included nine sites primarily manufacturing oral contraceptives containing various synthetic estrogens and progestogens (several of which also manufacture other products containing synthetic or natural estrogens or progestogens), one firm manufacturing estrogen replacement pharmaceuticals from synthetic estrogens, two firms manufacturing estrogen replacement pharmaceuticals from natural conjugated estrogens, and one firm manufacturing pharmaceuticals from DES, and which had in earlier years synthesized DES.

In general, many of these manufacturing sites (or facilities) are new and relatively small, although many of the established pharmaceutical companies are involved. In most of these companies, the workers involved with estrogens are rotated to work with other pharmaceutical products, introducing the possibility of confounding exposures. In general, estrogens are not synthesized in the United States at present, although it would have been desirable to evaluate such sites. Sites using DES in various products are numerous, but are usually very small operations involving sporadic production and few workers, and were thus not included (with the one exception noted) among the final sites selected for walk through surveys. Although these sites might be amenable to industrial hygiene evaluation, they were not considered to be good potential sites for medical or epidemiological studies.

Before discussing some of the findings from these surveys, it would be desirable to summarize (for orientation purposes) a typical tablet manufacturing

process, and an overall description of the workforce involved in manufacturing estrogen containing tablets.

Process Description. As with most dry product drugs, most estrogenic preparations are manufactured stepwise in batch operations. As shown in the block diagram, the basic steps include assembly and weighing of active ingredients, batch mixing of ingredients (usually wet mixing with subsequent drying and milling of the mix) followed by compression of the finished "granulation" into tablets by means of a tablet press. Tablets are then usually blister packaged (sealed between laminates of plastic and aluminum foil) or bottled, coated or uncoated, depending on the product. Drying of the wet granulation may be done "in-situ", or separately with a fluid bed dryer, tray-rack drier, or a spray drier, in which a slurry of the mix is pressure sprayed into a heated chamber. Subjectively, the greatest potential for operator exposures occurs during material transfers or loading of equipment, drying, milling, and tablet compression operations.

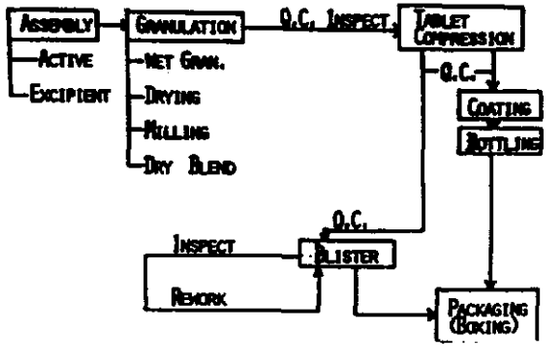

Fig. 1. Block flow diagram, tablet manufacturing.

However, some exposure via inhalation or skin contamination is also possible in (1) the packaging area (particularly in the areas in which uncoated bulk tablets are handled, or during tablet inspection and rework), (2) during the sampling and quality control testing of raw materials, granulation, and finished tablets, and (3) during maintenance and cleanup of production equipment and air handling systems.

Description of Workforce. Among the plants visited, job categories are fairly similar, although a variety of job titles are used. The general categories of jobs involved in "estrogen" areas, the approximate total number of persons employed in these areas at the plants visited are:

(1) Processing technicians: these workers assemble and prepare the batch mix (granulation) and then conduct and monitor the tableting process. Depending on the specific site, those people preparing the granulation and conducting tableting processes may be the same or different employees (300 employees).

(2) Packaging area operators: these employees load and package tablets, conduct product package inspection and re-work, and operate a variety of manual and automatic packaging equipment (1200 employees).

(3) Quality assurance and laboratory technicians: these employees collect samples of and inspect raw materials, granulations, and tablets for physical attributes and composition to verify quality (150 employees).

(4) Maintenance personnel: these employees are responsible for repairing and maintaining production equipment, air systems, and other facilities (200 employees).

Persons in a variety of auxiliary job categories may also be exposed to estrogens from time to time, such as area supervisors, materials handlers, janitors, etc. A rough estimate of the total number of employees in these jobs is 120.

Summary of Plant Inspections. One of the principal objectives of the present study is to delineate the extent of adverse clinical effects among workers occupationally in contact with estrogenic hormones. Although it is not possible to provide a definite answer to this question without detailed on-site studies, it may be valuable to present a brief overview of the history of the hyperestrogenic syndrome reported by the various drug companies visited during the previous phase of this project. A total of approximately 180 cases of symptomatology in employees were recorded by the thirteen companies visited, covering a time span from ca. 1940 to 1978. These cases have included both males and females, and have represented symptoms ranging from those purely subjective (e.g. nipple sensitivity) to overt gynecomastia or irregular menstruation. By far the largest percentage of the reported incidents were in males. Although details were not available from all plants, it was apparent that (in the companies from which a breakdown was available) the majority of symptoms were associated with the processing technicians, although occasionally other job categories were involved.

It should be noted that among the companies involved, the criteria for diagnosis of hyperestrogenism vary considerably. A summary of the history of cases reported by company physicians is presented in the following table (cases include some "repeats", i.e. more than one incident per individual):

TABLE 1.
HYPERESTROGENISM RECORDED IN THE THIRTEEN PLANTS BY COMPANY PHYSICIANS; CA. 1940-1978.

Date	Cases (male & female)	Recorded in (X) plants	No. of plants (of 13) Mfg. estrogen pharm.
ca. 1940-1972*	58 (DES)	1	----
1955-1978*	ca. 20	1	----
1966	1	1	7
1967	2	1	8
1968	0	1	9
1969	1	1	10
1970	18	3	10
1971	2	2	11
1972	5	2	11
1973	13	4	11
1974	14	4	12
1975	16	5	13
1976	15	5	13
1977	8	5	13
1978	8?	4?	13
TOTAL	181		

*The first two lines refer to cases recorded by two companies for which a breakdown by year was not available.

Although it may appear from this table that the incidence may have increased somewhat in recent years, the data must be interpreted with caution. First of all, it is obvious that the number of manufacturing sites had been increasing (until 1975), as well as the population at risk. Second, case finding by company physicians may have become more aggressive, reporting of symptoms by employees may have been more frequent in recent years, and record keeping may have improved. It should also be noted that case finding activities have varied considerably from plant to plant, ranging from annual or biannual physicals to frequent and aggressive interviews and examinations; in at least one plant, the latter has been done daily. Thus, the fact that a plant has reported a larger number of cases over the years may reflect more active case finding and more careful record keeping.

It is clear that definite statements regarding the past and present extent of hyperestrogenism cannot be made in a meaningful way from this data. However, although considerable progress has been made in environmental conditons at the various manufacturing sites, it is apparent that the industry has not totally solved the problem.

In the plants surveyed, the overall impression was that substantial effort has been made to improve environmental conditions and to protect employees at risk. A number of general approaches have been taken by various companies to accomplish this. Representative of these are:

(1) Isolation of the potentially most hazardous operations by such means as dedicated "estrogen" production facilities with separate air supply and exhaust systems, often with integral multiple filtration systems, and frequently with "double doors" or airlocks.

(2) Improvements in local exhaust systems and materials handling methods (e.g. closed system transfers of dry powders, i.e., pneumatic or gravity flow through closed pipes and conduits).

(3) Process changes to reduce the necessity for open handling or transfers of materials (e.g. use of fluid bed driers, "in-situ" drying instead of tray racks and ovens, and elimination of dusty procedures such as milling).

(4) Institution of strict work practices including procedures for entry and exit from isolated areas, and worker rotations to and from work with estrogens.

(5) Personal protective equipment such as disposable clothing, air supplied air hoods or helmets, and full air supplied suits of impervious material.

The above list of measures is a "composite" list of representative improvements made at various manufacturing sites. Not all of these have been instituted at all sites, and the above is certainly not an exhaustive list.

The above also does not mean there is no room for improvement. In spite of the near "ultimate" personal protective equipment used at many sites, it is apparent that exposures to estrogens are still occurring. Even the best personal protective equipment is not likely to totally eliminate exposure where it is used in areas with a high potential for environmental contamination, or where the personal protective equipment is regularly used for long periods of

time. This is not to advocate the phasing down or elimination of personal protective equipment, but it is likely that substantial improvement in reducing inadvertent exposures may necessitate the development and institution of better engineering controls including the use of totally enclosed processing methods.

Paradoxically, it should be noted that it is not easy for pharmaceutical companies to rapidly institute process changes or other environmental controls since each such change has to be approved by F.D.A.

CONCLUSIONS

The following conclusions have been drawn based on the walk through surveys conducted from May, 1978 to June, 1979.

(1) Subjective evaluation of the plants visited in the last year indicated considerable effort by the companies to reduce or eliminate exposures of employees to estrogens, and prescriptions for work practices and personal protective equipment appeared in general to be appropriate. In spite of this, the infrequent but continuing appearance in many of the plants of symptoms in employees attributable to exposure to exogenuous estrogens indicates that exposures are (at least occasionally) still occurring. It is not clear in many cases how this is happening. Periodic breakdown in the use of safety procedures by employees, or hypersusceptibility of some employees, cannot be ruled out. Further in-depth industrial hygiene studies would be necessary to precisely define hazardous operations and the most significant sources of exposure and route(s) of entry.

(2) Medical screening studies would be desirable in a representative sample of manufacturing sites to further define the nature and extent of hyperestrogenism in employees, and in particular, to better define the prevalence of "lesser" or less obvious clinical effects which might go unreported by employees, or which might not be so easily recognized with a routine physical examination.

(3) Although epidemiological studies (e.g. cohort mortality or reproductive studies) would be desirable to focus on possible chronic effects of exposure to estrogens, it appears that (with one possible exception) this would not be feasible. A cohort of sufficient size and/or latency for mortality studies has not been located, and reproductive studies would have to be conducted mainly with women working in the packaging areas, where exposure is very likely limited.
However, in one plant which has manufactured products from natural estrogens since approximately 1940, it may be possible to observe the effects of long term exposure to exogenuous natural estrogens, although the total cohort size may not be large enough to detect relatively rare conditions. A final decision on this possibility is pending at this time.

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DISCUSSION

MURRAY: You indicated in one of your slides that, at least in the last couple of years, the majority of the plants which you studied reported no cases of hyperestrogenism. You also suggested that varying approaches have been used by these plants to control estrogen exposure. During your recent walk-through visits, did you find a correlation between better control measures and a lack of reported hyperestrogenism? In other words, were the plants which reported recents cases of hyperentrogenism,those which had less comprehensive programs to prevent estrogen overexposure?

ZAEBST: An obvious correlation such as the one suggested was not evident from the findings of our walk-through survey. This is not surprising, however, in view of the number of variables which must be considered in looking for such a relationship. We consider the data presented in the table on the number of cases of hyperestrogenism (Zaebst et al., this volume) a rather crude measure of the actual extent of adverse effects resulting from either acute or chronic exposure to estrogens. This is true for several reasons. First, these numbers represent case finding and record keeping activities at various plants ranging from very good to dismal or even nonexistent. Second, reporting of symptoms by employees between plants and populations may vary considerably, depending on such factors as the background and beliefs of the employee population, as well as the quality of education and training given by the plant to its employees. Third, the numbers given in the table include only acute or subacute effects of a rather distinct nature, and do not necessarily include less obvious symptoms which an employee may not associate with exposure to estrogens, and thus will not report to the plant physician (e.g., slight nipple sensitivity, hypertension, or reduced libido). The presence of other variables also may explain the lack of an apparent correlation between extent of health effects and effectiveness or type of control measures. For example, frequently a plant with less comprehensive hygiene programs also had poorer record keeping, or may have manufactured estrogens less frequently, or used a less potent estrogen. We do, however, believe that improved hygiene measures are essential for minimizing exposures and prevention of hyperestrogenism. We have actually documented such a successful program in at least one plant.

GREEN: I wonder whether more data could be obtained by adopting some of the techniques used by manufacturers to recall products. By spot radio or newspaper advertising one could ask persons who might have had occupational exposure to come forward. They might then be asked the appropriate questions. Exposures could have occurred in laboratories other than those mentioned, i.e., universities and hospitals.

ZAEBST: Although the question is very relevant to case finding efforts, or to the possible establishment of a case registry of estrogen exposed workers, I do not feel that this approach would be practical or warranted for the purposes of the current project. However, I do feel that the suggestion has considerable merit for certain purposes; for example, in identifying more completely the the types of work places in which exposure to estrogens may occur, or as an adjunct to other means in establishing a case registry of occupationally exposed workers. The approach suggested should certainly be considered along with other approaches if our studies of occupational exposures to estrogens be expanded or redirected to other areas.

ARCOS: Hormones are used as growth promotants, as well as for feed efficiency and estrus control, in farm animals. Do you know of, or have you considered investigation, of human exposure during feed handling, preparation or administration?

ZAEBST: We are aware of the potential for human exposure to diethylstilbestrol and other estrogens in industrial plants preparing feed and in feedlot employees administering pellets or handling feed mixed with DES. To date, however, our efforts in this study have focused primarily on occupational exposure in pharmaceutical companies which manufacture estrogenic drugs for human use. Available resources have thus far limited our investigations to this aspect of occupational exposure to estrogens. In addition, the recent ban by the FDA on manufacture and transport of DES for use as a farm animal growth promoter (effective July, 1979), may limit or negate studies in this area. However, should FDA lift this ban in the future, we would consider expanding the scope of our study to include feedlot workers and other workers who may be exposed to estrogens.

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ESTROGENS ADMINISTERED TO FOOD-PRODUCING ANIMALS: ENVIRONMENTAL CONSIDERATIONS

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The use of estrogens to alter animal production started in the 1940's with diethylstilbestrol being used to caponize poultry. Currently slaughter cattle, sheep and poultry may be fed or implanted with estrogens (Table 1). Estrogenic materials may not be administered to food producing animals without specific permission from the Food and Drug Administration. The approval and withdrawal of approval of estrogenic products for livestock and poultry production has been a very active area for the past 30 years for this regulatory agency. This report will concern only the estrogenic products that were available during the last decade since this is when the use of such chemicals appears to have peaked. The contribution of plant estrogens to the environmental load will not be discussed here.

Estrogens in animal agriculture come from one of two sources: those that are administered exogenously and those that are produced endogenously. The exogenous estrogens include diethylstilbestrol (DES), estradiol benzoate, estradiol monopalmitate and zeranol. Estradiol benzoate[1] and estradiol monopalmitate are broken down in vivo to estradiol 17B. The endogenous estrogens include estradiol, estrone and estriol and they are produced by all species, sexes and classes of farm animals. Because estrogens are one of the most effective chemicals available for increasing the rate of weight gains and the efficiency of feed utilization in healthy animals they have become widely used in food animal production. Extremely small quantities of estrogens are effective in producing these effects in cattle[2, 3] and sheep[4]. Because of inefficiency of absorption, more drug is required to produce a similar effect orally than when the drug is implanted or injected.

Table 1

Estrogens Approved in the United States for Food-Producing Animals[a]

Compound	Animal	Route and Quantity of Administration	Use Restriction
Estradiol Monopalmitate[b]	Roasters	10 mg - implantation	Implant after 5 weeks of age & at least 6 weeks before slaughter
Diethystilbestrol[c]	Slaughter Cattle	Oral - 5-20 mg/day Implantation - 30 or 36 mg	Cattle may be slaughtered after 120 days & orally treated cattle after 7 days
Diethylstilbestrol[c]	Slaughter sheep	Implantation - 3 mg oral - 2 mg/day	Sheep may be slaughtered 70 days after implantation & orally dosed animals after 7 days
Estradiol Benzoate[b] and Progestrone (Synovex-S)	Steers	Implantation - 20 mg Estradiol	Steers may be slaughtered 60 days after implantation
Estradiol Benzoate[b] and Testosterone (Synovex-H)	Slaughter heifers	Implantation - 20 mg Estradiol	Heifers may be slaughtered 60 days after implantation
Zeranol	Slaughter cattle and suckling beef calves	Implantation - 36 mg	Cattle may be slaughtered 65 days after implantation
Zeranol	Slaughter lambs	Implantation - 12 mg	Lambs may be slaughtered 40 days after implantation

[a]Title 21 Code of Federal Regulations, Part 500-599, 1978

[b]Currently the subject of regulatory action intended to withdrawal approval of its use in the United States[36]

[c]Withdrawal of approval has been ordered[6]

DIETHYLSTILBESTROL (DES)

The most widely used estrogen in food animal production is DES. During the past 30 years it has been used in poultry (caponizing agent), cattle and sheep. Its use in poultry began in 1947 and lasted until 1966[5]. At that time FDA withdrew its approval because DES residues were found in edible portions of treated birds. Even during peak use periods, the use of DES never exceeded 1.0% of the marketed chickens.

The use of DES in cattle and sheep began in 1954 and 1958, respectively. Even though the use of DES has been ordered discontinued[6] a stay of the order is anticipated. Too the quantity (Table 2) of DES that has been used and may now still be in the environment makes a discussion of DES very pertinent if one is to evaluate the environmental load of estrogens due to animal agricultural uses. DES was approved prior to current environmental assessment requirements so the sponsors have never conducted studies to evaluate its impact on the environment. Hence, the movement of DES has never been followed in a single study, through the animal to the soil, to the streams and to the aquatic life.

Callantine, et al[7], Hinds, et al[8] Melampy et al[9] and Mitchell et al[10], using varied analytical sophistication, showed that most of the DES administered to ruminants is excreted in the feces and urine regardless of the route of administration. More recently Aschbacher[11], Aschbacher and Thacker[12], Aschbacher et al[13], and Rumsey et al[14, 15] used 14C-DES in sheep and cattle and showed within experimental error (Table 3) that all orally administered DES can be accounted for in the feces and urine within seven days after dosing. Within the times tested all the DES absorbed from an implant can also be accounted for in the urine and feces (Table 3).

Table 2

Estimate of DES Used in Cattle and Sheep[a]

Year	Amount of DES (kg)
1970	27,600
1971	27,600
1972	26,100
1973	400[b]
1974	8,900
1975	5,700
1976	4,500
1977	29,200
1978	3,500

[a]Federal Register[40]

[b]Approvals for oral DES withdrawn from Jan. 1, 1973 to Jan. 24, 1974; approvals for implant DES were withdrawn from April 27, 1973 to Jan. 24, 1974.

Table 3

^{14}C-DES Excretion in Urine and Feces

Route of Administration	Species	Recovery of Dose (%)	Reference
Implantation	Steers	86.8	41
Oral	Steers	95.5	14
Implantation	Steers	80.8	15
Oral	Sheep	94.9	11
Oral	Steers	97.0	12
Injection	Chickens	90.8	42

Since the animal waste is not routinely separated in practical animal production situations, it is not important whether the urinary or fecal route of excretion dominates for environmental considerations. Except, however that the metabolites in the urine and feces are different. The fecal excretory material is dominated by the free, unconjugated DES, whereas the urine contains mostly conjugated materials which are water soluble. In both cattle and sheep DES is excreted mainly in the feces[16].

Several laboratories have studied the stability of DES in soil. Zondek and Sulman[17] reported that when a saturated water solution (about 12 ppm) of DES is permitted to migrate through "soil" over 97% of the DES "adheres" to the soil. Gregers-Hanson[18] showed that the soil effect on DES is independent of soil microbes. He sterilized soil with γ-irradiation (215 megarads - a level that ensures complete sterilization) and showed sterile soil to be capable of degrading DES. When 100 mg ^{14}C-DES (labeled in the ethyl side chains) is stored in 90 g "loamy soil" for three months, 70-80% of the DES radioactivity is not extractable with benzene. After 10 months over 95% of the radioactivity is not extractable with benzene. Since the CO_2 production could not account for the loss in DES, the author concluded that a change in the DES moiety takes place that does not involve a degradation of the ethyl groups.

The instability of aqueous solutions of DES has been reported. Aqueous neutral solutions of DES rapidly lose their estrogenic activity whether they are held at room temperature[17] in the cold[19], in Alkali[20] or under weak acidic conditions. The stability of DES in solution is influenced by its concentration with more dilute DES solutions being less stable than saturated solutions[19].

The importance of soil microorganisms in the degradation of DES is not clear. Zondek and Sulman[17] showed some bacteria to be very sensitive to DES. In fact Hanka and Lockhart[21] proposed an antimicrobial assay for DES using Staphylococcus aureus Strain II. Thayer et al[22] showed the Bacillus and Cellulomonai species to be very sensitive to DES whereas the Pseudomonas and Escherichia species are rather insensitive to DES. Yotis and Banman[23] determined that DES exerts its antimicrobial effect by causing cell leakage and they also concluded that gram negative organisms are less sensitive to DES than gram positive microbes. After studying the microbial isolates from stockpile cattle manure, Thayer et al[22] found no evidence of apparent bacterial selection in either bacterial species or numbers.

Hacker et al[24] used waste from a ^{14}C-DES implanted steer to study the fate of DES in acidic (pH6.5) and alkaline (pH7.5) sandy loam soils and to study the ability of certain plants to take up such radioactivity. During a 5-week growing period onions, wheat, lettuce, radish and bean parts (roots, leaves, bulbs, stems or fruit) were capable of accumulating significant levels of radioactivity. The plants grown in the acidic soil tended to have higher levels of radioactivity (except for lettuce). However, only radish leaves and lettuce roots grown in the alkaline soil showed estrogenic activity based on the mouse uterine assay and only about 50% of the radioactivity present corresponded with the biological activity. Cored sampling of the soil showed that the radioactivity did not migrate. Extracting of the soil with ethanol removed less than 15% of the radioactivity after 8 weeks.

Rumsey, et al[25] fed steers 20 mg DES per animal per day for 180 days and found 3.1 g of the 3.6 g of DES fed in fresh animal waste (urine and feces). When a portion of this waste was stored for 12 weeks the DES concentration dropped from 0.83 ppm to 0.64 ppm. Some of the waste was spread on a pasture for six months after which no DES could be found in the waste (assay sensitivity of 20 ppb) or in the top 20 cm of soil (assay sensitivity of 1 ppb).

Gregers-Hanson[26] showed that the radioactivity incorporated into rye grass or red clover grown in the presence of 20 mg DES (5 microcuries) per 1.3 kg "sandy soil", the plants after 8 months of plant growth were too small to be identified as DES. However, DES-Glucuronide was more rapidly taken up by corn plants than is free DES when the plants are grown in aqueous solution.

Thayer[27] reported that DES in stockpile manure in Texas is approximately 1/2 the feed concentration. Thus in arid areas DES does not appear to be degraded as rapidly as in moist[25] areas. Some reputable feedlot consultants have speculated that some of the animals with DES residues who had not been fed DES for long periods before slaughter may have been re-exposed by the DES in feedlot waste.

In a 33-day terrestrial-aquatic model ecosystem Metcalf[28] showed ^{14}C-DES to be taken up by certain aquatic species (snail, alga, water flea, mosquito larva and mosquito fish) and concentrated in their tissues to a greater concentration than was in the water. In this system free DES persisted throughout the 33-day study. The source of the DES was excreta from mice and day old chicks injected with ^{14}C-DES.

ZERANOL

Resorcyclic acid lactone (Zeranol) is a weakly estrogenic material isolated from the fungus, Gibberella zeae. It is administered by ear implantation (Table 1) to cattle and sheep for the purpose of making them grow more rapidly and more efficiently[29]. This material was first isolated from moldy corn so there is a natural source in the environment. Sharp and Dyer[30] implanted a steer with ^{14}C-Zeranol (72 mg) and found the ratio of radioactivity in urine to feces to be about 1:5. No attempt was made to characterize the nature of the radioactivity; however it may be fair to assume that the material in the feces was mostly free zeranol and the material in the urine consisted of zeranol conjugates[31]. No data was found in the literature on the stability of

zeranol in soil or water. Craig[32] reported that when fresh cow manure is spiked with zeranol to create an 1100 ppm zeranol mixture and stored at room temperature, it degrades at the rate of 60 ppm per week. This product enjoys a substantial portion of the potential slaughter cattle and sheep markets.

ESTRADIOL BENZOATE AND ESTRADIOL MONOPALMITATE

Estradiol benzoate and estradiol monopalmitate are used in slaughter cattle and poultry production, respectively (Table 1). These compounds are broken down *in vivo* to yield free estradiol 17-B. Cattle metabolize estradiol to estrone, estradiol 17-α and conjugates of estrone and estradiol 17β. Unlike other animals the major metabolite of estradiol 17B in ruminants is estradiol-17[33]. Estradiol 17α has very little estrogenic activity and is thought to be analogous to estriol in man. About 20% of the slaughter cattle are treated with estradiol.

The major metabolite of estradiol 17-B in poultry is estrone and estradiol 17α[34,35]. Less than 1.0% of the chickens slaughtered in this country are treated with estradiol monopalmitate. Both estradiol monopalmitate and estradiol benzoate are the subject of proposed regulatory action by the Food and Drug Administration that could lead to their withdrawal from the market[36]. Considering the quantity of estradiol currently given exogenously to either cattle or poultry, the quantity of endogenous estrogen produced *in vivo* and excreted into the environment far exceeds the quantity given exogenously. Adult cows excrete about 30 mg estrogens per day[37], cycling heifers excrete about 2.2 mg estrogen per day[38] and hens excrete 1.6 mg of estradiol per gram of dry excreta[39]. No reference was found on the excretion of estrogens by steers. Other farm animals (swine, horses and goats) also excrete large quantities of estrogens into the environment[33]. However no exogenous estrogens are approved for swine, horses and goats intended for slaughter.

No research report was found on the stability of estradiol, estrone or estriol in soil or fecal material. In water solutions natural estrogens appear quite stable. Wilder Smith and Williams[19] found an estrone-water solution (1 mg estrone/ml) to retain 94% of its estrogenic activity after 27 weeks at room temperature. A similar solution of estradiol retained 69% of its estrogenic activity after 28 weeks. Plant tyrosinase has the capacity to inactivate estrone[17]. Beet roots, potato juice, and mushroom extracts also have this capacity to inactivate estrone. Certain bacterial species[17] can degrade estrone. However no association of these microbes with soil or fecal species has been made.

CONCLUSION

Large quantities of estrogens are entering the environment via animal production. Several thousand kilograms of diethylstilbestrol, estradiol benzoate, estradiol monopalmitate and zeranol are intentially added to cattle, sheep and poultry annually. The fate of these chemicals in animal excreta, in the soil and in water (streams, rivers, etc.) is not well known. Much of the data available on the degradation of estrogens is old and was collected using analytical methodologies which do not meet today's standards of sophistication. No one knows the effect of 116 million cattle, 3 billion chickens, 58 million swine and 12 million sheep excreting estrogens into the environment. All available data indicate these estrogens just disappear with no carbon balance studies being available to show exactly where they do. One can take comfort from the fact that animals have been excreting estrogens into the soil and water for hundreds of years with no apparent accumulation or toxicity to plant or animal life.

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DESCRIPTION OF THE ESTROGEN-TREATED POPULATION OF YOUNG WOMEN IN THE UNITED STATES

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INTRODUCTION

In addition to the exogenous estrogens which most of us are exposed to indirectly from our environments, work places, and food, many Americans, mostly women, use estrogens directly and on purpose, as treatments intended to achieve desired effects. This paper describes the extent to which teenaged American women use treatments based on estrogen. It is an updated version of a paper which was originally prepared in December 1977 for a Conference on Estrogen Treatment of the Young.[1] The purpose of that conference, sponsored jointly by the Kroc Foundation, the Lawson Wilkins Pediatric Endocrine Society, and The National Institute for Child Health and Human Development, was to examine the risks and the benefits of the estrogenic treatments used by young American women, and to examine the balance between those benefits and risks, especially in regard to estrogen used to limit the growth of unusually tall girls.

MATERIALS AND METHODS

There is no single source of data by which one can measure or even estimate how many American teenagers are treated with estrogenic drugs. It is possible to estimate the extent of their estrogen treatment, however, by putting together data from several different sources. The information in this paper is of that nature -- estimates inferred from census data, natality data, survey data

*The opinions or assertions contained herein are the author's and are not to be construed as official policy of the Department of HEW.

and special studies. The oldest data used as a basis for the estimates is the percent distribution of 15-19 year-old women by marital status and race, which is from the 1970 census. The most recent data used in the estimates is the number and rates of births to 10- to 19-year old women, by race, in 1977. All of the other data used in the estimates are from special studies and national surveys which were conducted between 1973 and 1976. The outcomes are estimates of the proportions of American teenagers treated with estrogen during the mid-1970's. The last section of the paper is a discussion of recent trends which indicate that estrogen use by this age-group of women may have decreased slightly during the late 1970's.

Most of the data in this paper relate to 15-19 year-old females, although some incidental information on estrogen treatment of even younger children is included. Throughout the paper the term "teenager" refers specifically to 15-19 year old girls.

RESULTS

An estimate of the proportion of American teenagers treated with estrogen during any 12-month period during the mid-1970's is presented in Table 1. Approximately 20% (1 in 5), used some form of estrogen treatment each year. The treated proportion was a little lower, about 18%, for white teenagers. Black teenagers were twice as likely as white teenagers to be treated. Approximately 1 of every 3 black American teenagers was treated with estrogen during 1975. Only 14% of this estrogen use was for treatment of medical conditions. Most of it was estrogen use by healthy young women for non-medical purposes -- mainly for contraception and, to a lesser extent, for suppression of lactation following childbirth. The basis for each component of the estimate presented in table 1 is explained as each category of estrogen use is more fully described later in this report.

TABLE 1

ESTIMATED PERCENT OF 15-TO 19-YEAR OLD WOMEN IN THE UNITED STATES USING ESTROGEN DURING ANY 12-MONTH PERIOD IN MID-1970's, BY RACE

Purpose of Estrogen Use	All	White	Black
Oral Contraceptives for the Purpose of Contraception	15.8	14.0	29.7
Oral Contraceptives for Non-Contraceptive Reasons	0.7	0.7	0.7
Suppression of Lactation	2.3	1.9	4.5
All Other Purposes	1.0	1.0	1.0
ALL	19.8	17.6	35.9

Oral contraceptives (OCs)

Oral contraceptives are the major source of estrogen taken by teenagers. As shown in the top part of table 2, nearly 16% of teenage girls (1 of every 6) used oral contraceptives in 1976. This proportion, however, varies widely with marital status, race, and single-year-of-age within the age group. Table 2 is organized in 3 parts by race. The data for all 15-19 year-olds, regardless of race, are shown at the top of the table; data for white 15-19 year-olds are shown in the middle of the table; data for black 15-19 year-olds are at the bottom of the table. For each racial grouping, the data on oral contraceptive (OC) use by marital status is shown in two ways: In the first column the point prevalence of OC use within marital status groupings is shown as found in two national surveys conducted in 1973 and 1976. The 1973 National Survey of Family Growth measured oral contraceptive use by married 15-19 year-old women.[2] A 1976 survey by Kantner and Zelnick measured oral contraceptive use by never-married teenagers.[3] Since previously-married teenagers were excluded from both of these studies and no other information was available to describe their use of oral contraception, previously-married teenagers were arbitrarily ascribed an oral contraceptive prevalence halfway between that of married and of unmarried teenagers of the same race. Because the data in table 2 were developed from 3 different sets of data, each collected at a different time, the percentages presented are only estimates. Most of the OC use is among never-married women and the information on never-married women was collected in 1976; therefore, 1976 is the best reference date for the estimate. Although most teenagers who take oral contraceptives do so for their contraceptive effect, OCs are prescribed for some teenagers as treatments for medical conditions. The data in table 2 reflect all OC-use for married teenagers but only contraceptive use of OCs by never-married women in this age group.

TABLE 2

PERCENT OF 15-19 YEAR-OLD WOMEN USING ORAL CONTRACEPTION (OC) BY MARITAL STATUS AND RACE, UNITED STATES, 1976

Marital Status	% Using OC	Distribution of 15-19 Year-Old Women by Marital Status and OC Use		
		Using OC	Not Using OC	Total[a]
ALL RACES				
Never Married	12.2[b]	10.8	77.4	88.2
Currently-married	43.9[c]	4.7	6.0	10.7
Previously-married	28.1[d]	0.3	0.8	1.1
TOTAL	15.8	15.8	84.2	100.0
WHITE				
Never Married	10.2[b]	9.0	79.0	88.0
Currently-married	43.6[c]	4.8	6.2	11.0
Previously-married	26.9[d]	0.3	0.7	1.0
TOTAL	14.1	14.1	85.9	100.0
BLACK				
Never Married	28.1[b]	24.9	63.7	88.6
Currently-married	42.7[c]	4.1	5.5	9.6
Previously-married	35.4[d]	0.6	1.2	1.8
TOTAL	29.6	29.6	70.4	100.0

a. Distribution of 15-19 year-old women by marital status and race is based on 1970 Bureau of Census data.[4]
b. Based on data from Zelnik M. and Kantner J.F.: Sexual and Contraceptive Experience of Young Unmarried Women in the United States, 1976 and 1971. Family Planning Perspectives 9: 55-71, 1977. Includes only oral contraception used for the purpose of preventing pregnancy.
c. Based on data from the 1976 National Survey of Family Growth, National Center for Health Statistics.[2] Includes all OC use, including that for purposes other than contraception.
d. The percentage of previously-married women using oral contraception was arbitrarily set midway between the percentages for never-married and for currently-married women.

As of the mid-1970s, approximately 12% of all never-married and 44% of all currently-married teenagers were using oral contraception. Although OC-use was more common among married than among unmarried teenagers, relatively few teenagers were married and 2 of every 3 teenagers using oral contraception had never been married.

In 1976 the proportion of black teenagers using oral contraception was more than twice the proportion of white teenagers using oral contraception. This difference was due entirely to greater use of OCs by never-married black as compared to never-married white teenagers. White and black teenagers were extremely similar in the percent who were married and in the prevalence of OC use by married teenagers. Five of 6 black teenaged OC-users were single.

Since many young women discontinue oral contraception after only a relatively short period of use, the number of teenagers who have ever-used OCs is always greater than the number using OCs at any given point in time. Table 3 shows the percent of never-married teenagers who indicated that they had ever-used oral contraception and the percent who said that they were currently using oral contraception when they were interviewed for the 1976 Kantner and Zelnick study of teenage sexual and contraceptive practices. Slightly more than 15% of the total group had used OCs at some time. For every 15 girls who had ever used them, almost 11 were still using them at the time of their interviews.

With each year of additional age within the age group, the proportion of girls who have ever-used OCs increases, the proportion of girls currently using OCs increases, and the difference between the proportion who have ever used and the proportion currently using widens. By age 15, 5% of never-married teenage girls have tried oral contraceptives, and 4 of the 5 (80%) who have tried them are still using them. By age 19, more than a third of all never-married teenagers have used OCs, but only 56% of those who have tried them are still using them.

TABLE 3

PERCENT OF 15- TO 19-YEAR-OLD NEVER-MARRIED WOMEN WHO HAVE EVER USED OR CURRENTLY ARE USING ORAL CONTRACEPTIVES (OCs), BY RACE, UNITED STATES, 1976

Age (Years)	Have Ever Used OCs			Currently Using OCs		
	All	White	Black	All	White	Black
15	5.0	3.8	12.9	4.0	3.1	10.5
16	7.9	6.2	26.3	6.2	4.9	21.0
17	13.0	11.8	29.3	9.6	8.8	22.3
18	26.1	23.2	49.0	17.3	14.6	33.5
19	34.2	29.7	56.5	19.3	16.0	33.5
15-19	15.3	13.0	34.0	10.8	9.0	24.9

Based on data from: Zelnik M, Kantner JF: Sexual and Contraceptive Experience of Young Unmarried Women in the United States, 1976 and 1971. Family Planning Perspectives 9:55-71, 1977.

For the entire age group, both ever- and current-use of oral contraception is 2 to 3 times higher among black than among white teenagers. The racial difference in OC use is greatest between white and black 15- and 16-year-olds, and diminishes with increasing age. The prevalence of both ever- and current-OC-use is more than 4 times greater for black than for white 16-year-olds, but is only 2 times greater for black than for white 19-year-olds. This difference is associated with Zelnik and Kantner's finding of a 2- to 3-fold greater prevalence of sexual experience in black as compared to white 15- and 16-year-olds, with less than a 2-fold difference between the races for ages 17,18, and 19. The difference between ever- and current-use is smaller for black than for white teenagers in each year-of-age group. This means that, once they start using oral contraception, black teenagers are less likely than white teenagers to discontinue its use.

Medical (non-contraceptive) use of oral contraceptives

The overall estimate of the percentage of teenagers using estrogen, which was presented in table 1, included an estimated 0.7% of 15- to 19-year-old women using oral contraceptives for non-contraceptive (medical) reasons. Those medical reasons include dysmenorrhea, menorrhagia, functional uterine bleeding, amenorrhea, oligomenorrhea, intermenstrual bleeding, oily skin, and acne. [5]

The estimate of 0.7% is based on data from the National Disease and Therapeutic Index (NDTI), which projects data on physician prescribing practices based on information supplied by a representative, rotating sample of physicians in private practice. During the 12 months from April 1974 through March 1975, NDTI estimated that approximately 3 1/2 million prescriptions for oral contraceptives were written by private physicians for U.S. females between the ages of 10 and 19. [5] During the same time period, NDTI physicians reported that 12% of the OCs they prescribed for women of all ages were prescribed for purposes other than contraception. The non-contraceptive reasons they listed most frequently were regulation of menstruation (5% of all OC prescriptions), control of bleeding (2%), correction of hormone imbalance (1%), "cyclic therapy" (1%), reduction of discomfort (1%), and other reasons including treatment of acne and oily skin.

In table 2 the estimated proportion of 15- to 19-year-old women using OCs is based on data which included any use of OCs by married women, but only contraceptive use of OCs by never-married women. Since the proportion shown as never-married and using OCs does not include never-married women using OCs for non-contraceptive reasons, the estimate for that group is too low. As shown in the top section of table 2, approximately 11% of all women in this age group are never-married women using OCs for the purpose of contraception. Approximately 54% of that 11% received their OCs from private

physicians. [3] Thus, about 6% (54% of 10.8 = 5.9%) of the total age group are never-married women using OCs prescribed for their contraceptive effect by private physicians. Assuming that 12% of the OCs prescribed by private physicians for women in this age group were prescribed for non-contraceptive purposes, then the total percentage of unmarried 15- to 19-year-olds receiving OCs from private physicians is about 12% higher (6.6% instead of 5.9%). This adjustment adds 0.7% to the total proportion of teenage women estimated to be using OCs, an adjustment necessary to include teenagers using OCs for non-contraceptive purposes in the total.

Type and dosage of estrogen contained in oral contraceptives used by teenagers

Although the daily dose of estrogen in most OC formulations is relatively low, they are taken regularly over an extended period of time. The total dose of estrogen received by the young women who use them depends on how long they remain on oral contraception and on the type and amount of hormone contained in the specific OC formulations they use. Except for the progestin only "mini-pill," all OCs contain one of two synthetic estrogens -- mestranol (ME) or ethinyl estradiol (EE). EE is 50-100% more effective than an equal weight of ME in producing some of the biologic actions of estrogen. [6] Since 1973, about 52% of the OC market have been formulations containing ME; about 48% are formulations containing EE. [6] A trend towards decreasing use of high estrogen dose OCs began in 1969. Sartwell et al found that OC formulations containing 100 or more micrograms of either type of estrogen accounted for approximately 57% of OC sales in 1969, 47% in 1970, 36% in 1971, 31% in 1972, 27% in 1973, and 26% in 1974. [7] OC formulations containing 50 or fewer micrograms of estrogen were introduced in 1972 and accounted for 51% of the U.S. market that year. By 1977 only 17% of OCs sold in the United States contained 100 or more micrograms of estrogen and 70% of them contained 50 or fewer micrograms. [8]

Teenagers who use oral contraception may be even less likely than older women to use the higher dosage formulations. National Disease and Therapeutic Index data indicate that of the OCs prescribed by private physicians during the period from April 1974 through March 1975, less than 19% of those prescribed for use by 10- to 19-year-old women contained 100 or more micrograms of estrogen, as compared to more than 25% of the OCs they prescribed for use by older women.[5] The fact that younger women received a greater proportion of lower dose formulations could be due either to purposeful prescribing of lower doses to younger women, or to a tendency of women to stay on the same OC formulation once they have started on it, so that trends in prescribing practices affect new (younger) OC users more than older users. This tendency was documented during a recent study in which careful oral contraceptive histories of more than 200 women were taken and verified by medical records. [9]

Lactation suppression

The second most frequent purpose for which estrogen was prescribed for young women during the mid-1970's was to suppress lactation in newly-delivered mothers who were not planning to breast feed their infants. Unlike OCs, which provide a low dose of synthetic estrogen taken over extended periods of time, estrogen given to suppress lactation is administered as a large dose given as a single injection which is absorbed over several days or as tablets taken during the first several days postpartum. The supposed effect of the estrogen, which is given either alone or in combination with testosterone, is to relieve the discomfort and shorten the process of "drying up the milk." Although national data on this form of estrogen use are not available, it has been an extremely common practice. In the late fall of 1977, I asked nurses working in the labor and delivery suites of all hospitals with

obstetrics departments in the vicinity of Atlanta, Georgia, about the use of estrogen for lactation suppression in their hospitals. They reported that in each hospital at least 95% of obstetric patients who were not planning to breast feed received this treatment, and that in several hospitals prescription of estrogen was part of the "standing orders" for postpartum nursing care. Nurses in those hospitals asked all new mothers if they were planning to nurse their infants and, unless their physicians wrote specific orders to withhold the medication, administered estrogen to any not planning to breast feed.

The estimated percentage of teenagers treated with estrogenic drugs each year during the mid-1970s (table 1), includes 2.3% of all teenage girls (and 4.5% of black teenage girls) treated with estrogen for lactation suppression. The basis for these estimates is laid out in table 4, which also shows the number of births and the birthrate per thousand teenagers in 1977. The estimates on use of estrogen for lactation suppression are based on 2 assumptions: (1) At least 42% of new teenage mothers do not attempt breast feeding, and (2) every mother who does not attempt breast feeding receives the treatment. The first assumption is based on data from 1,303 expectant mothers who were questioned about their infant feeding plans during a 1976 study conducted for the American Baby Magazine.[10]

Of 571 expectant mothers less than 25 years old, 57.6% of those expecting their first baby and 51.5% of those expecting a second or higher-order child planned to breast feed the baby. The study also found that of women pregnant with their first child, the percentage planning to breast feed decreased with age (77% of women over 30, 69% of 25- to 29-year-old women, and 57.6% of women less than 25). The data in table 4 are based on the assumption that 57.6% of women who deliver babies before the age

TABLE 4

BIRTHS TO WOMEN LESS THAN 20 YEARS OLD AND ESTIMATED INCIDENCE OF TREATMENT WITH ESTROGEN FOR SUPPRESSION OF LACTATION, BY RACE, UNITED STATES, 1977

Race and Age (Years)	Births [a]		Treatment for Lactation Suppression	
	No.	Per 1000	No. [b]	Per 1000 [c]
All Races [d]				
12-14	11,455	1.2	4,857	0.9
15-17	213,788	34.5	90,646	14.7
18-19	345,366	81.9	146,435	34.8
15-19	559,154	53.7	237,081	22.9
White				
12-14	4,671	0.6	1,981	0.4
15-17	138,223	26.5	58,607	11.3
18-19	253,960	71.1	107,679	30.2
15-19	392,183	44.6	166,286	19.0
Black				
12-14	6,582	4.7	2,791	3.3
15-17	71,182	81.2	30,181	34.4
18-19	84,008	147.6	35,619	61.6
15-19	155,190	107.3	65,800	45.3

a. Source: National Center for Health Statistics: Final Natality Statistics, 1977, Monthly Vital Statistics Report, Vol. 27, No. 11, Supplement, 1979.
b. Assumes that 42.4% of mothers were treated.
c. Rates based on population projections from U.S. Bureau of the Census, Current Population Reports, Series P-25, No. 601, "Projections of the Population of the United States: 1975 to 2050," U.S. Government Printing Office, Washington, D.C., 1975.
d. Totals for all races is greater than the sum of white and black because of births to teenagers of other races.

of 20 attempt to breast feed, and that the other 42.4% receive estrogen for lactation suppression. This assumption probably underestimates the proportion of teenagers who receive lactation suppression medication because of the following 2 reasons: (1) Approximately 20% of births to 15- 19-year-old white women and 29% of births to 15- to 19-year-old non-white women are second and higher-ordered births, [11] which were found to be associated with a lower expectation of breast feeding. (2) Because the expectation of breast feeding decreases with age, it is likely that the percentage of teenage mothers who plan to nurse their infants is less than the percent planning to breast feed among the entire group less than 25 years old.

I conservatively estimated that approximately 23 of every 1,000 15- to 19-year-old women, (2.3%), received estrogen for lactation suppression in 1977. As with OCs, the percentage of the population treated increases steadily with age and is more than twice as high for black as for white women in every age group, a difference due entirely to the higher rate of young childbearing by blacks as compared to whites. Lacking better data, the same assumption of 42.4% not breast feeding has been applied to both races. This may have resulted in a further underestimate of the use of estrogen to suppress lactation in black teenagers.

Other uses of estrogens

Aside from contraception and suppression of lactation, other reasons for which estrogen products are prescribed for teenagers include a variety of ovarian and menstrual disorders; hirsuitism; vaginitis; certain skin problems common to adolescents; failure to stop growing tall; a number of rare but serious endocrine abnormalities such as Turner's Syndrome; and for postcoital contraception. [5,12] NDTI projections indicate that of all

estrogenic products prescribed by private physicians for 10- to 19-year-old women from April 1974 through March 1975, 91.1% were OCs and 3.2% were products usually used to suppress lactation. Less than 6% were products with other purposes -- 2.5% (about 95,000 prescriptions) were topical preparations or suppositories used mainly to treat vaginitis, 1.8% (about 69,000 prescriptions) were products usually used to treat ovarian or menstrual disorders, and 0.6% (about 25,000 prescriptions) were for oral forms of diethylstilbestrol (DES), for which postcoital contraception is probably the main use in this age group. The data for that same period also showed 37,000 prescriptions for topical application of estrogen to females less than 10 years old, 57% of which was for girls less than 3 years old.

Unless the prescribing patterns of physicians practicing in prepaid health plans, medical centers, public health agencies, or elsewhere in the public sector of medicine are extremely divergent from those of private physicians, it is unlikely that more than about 1% of 15- to 19-year-old women annually receive estrogen products other than OCs or estrogen to suppress lactation.

Prenatal exposure

The youngest group of Americans receiving estrogen are those exposed prenatally. A study of 920 mothers who delivered babies with birth defects in Atlanta, Georgia, hospitals between 1970 and 1975, found that 15% had received exogenous hormones during their pregnancies.[13] That 15% included 2.7% who took OCs while pregnant, 8.5% who had been given hormonal pregnancy tests, and 5.7% treated with hormones for threatened abortion. In May 1975 the Food and Drug Administration published a recommendation that estrogen not be used as a hormonal pregnancy test,

and there are indications that this use has declined since that time. The majority of the 5.7% treated for threatened abortion received progestational rather than estrogenic agents.

Trends

Several recent trends suggest that estrogen use by American teenagers may have decreased slightly in the past year or two. Although "the pill" is still the most popular method of contraception in the United States, the proportion of married couples using OCs decreased from 25.1% in 1973 to 22.3% in 1976.[14] This decrease is thought to be related to increased publicity about health risks associated with oral contraception during the past several years.[15] Although the popularity of oral contraception has declined most among older women, teenagers may also be effected to some degree. In 1972 81% of the teenaged patients of federally-subsidized family planning clinics were using oral contraception. The percentage choosing pills has declined slowly since then and was 78% in 1976.[16] The total percentage of teenagers using contraception of some type is increasing,[5] however, so that this slight decrease, 3% in a 5-year period of time, may not mean that a smaller percentage of teenagers are using OCs. The trend towards increasing use of the lowest dose pills and less frequent prescription of higher dose formulations is probably continuing.

The number of teenagers being treated with estrogen for suppression of lactation is probably also decreasing. Birth rates for 16-19 year olds decreased steadily during the 1970s.[17] Although birth rates for even younger teenagers increased until 1973, fertility in this age group has also begun to decline. With a smaller proportion of teenagers giving birth, fewer are available to be treated with estrogen. The percentage

of American mothers who attempt to breast feed their infants is increasing. In 1978 the American Academy of Pediatrics formally endorsed breast-feeding and recommended that physicians encourage all mothers to nurse their infants. Although teenage mothers are less likely than older mothers to breast-feed, this trend probably effects them too.

A third factor that may eventually have a large impact on decreasing the number of women given estrogen for lactation suppression is that the effectiveness of the treatment has been challenged. Much data is now available which demonstrates that the lactation suppression effect of estrogen is only temporary. A rebound of milk production occurs when the therapy is stopped. [18,19,20] Evidence to that effect was presented on January 31, 1978, to the Food and Drug Administration (FDA), which may have a public hearing on it. In the meantime, FDA has removed lactation suppression as an approved use for estrogenic formulations.

Most of the data on which the estimates in this paper are based were collected during the mid-1970s. Since then there has been increasing concern among women, health professionals, and the FDA regarding the safety of estrogen treatment. Ever since July 1977, FDA has required that literature to inform patients of the contraindications to use of estrogen and of the risks associated with its use be made available to every woman before she receives an estrogenic preparation to insure that she has given informed consent.[21] The concern which this kind of policy reflects, coupled with declining teenage fertility and increasing interest in breast-feeding have probably reduced, to some extent, the proportion of young American women who are using estrogen today. Nevertheless, the downward trend, if any, is slight.

SUMMARY

It is estimated that each year approximately 20% of all 15- to 19-year old American women receive estrogens in the form of a pharmaceutical agent. The proportion of black teenagers who receive some form of estrogen therapy is estimated at 36%, twice the proportion of treated white teenagers. The majority of this estrogen is used for the purpose of contraception. Less than 2% of teenagers receive estrogen for treatment of medical conditions.

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