Eli Lilly Lecture: Estrogen Metabolism and Action[1]

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I SHOULD LIKE to consider two related and converging themes of research that have occupied the attention of our laboratory for a number of years. They were both stimulated by the clinical observations of the palliative effects of steroid hormones including estrogens upon certain tumors of the breast and prostate gland. These observations placed in question the previously held view that tumors are wholly autonomous and not subject to control by normal physiological mechanisms. These hormonal effects gave a great impetus to studies of the biochemistry of the steroid hormones, and especially of the estrogens.

Perhaps it would be appropriate to indicate the state of the art in those days. It is now difficult to conceive of a time when isotopically labeled steroids could not be purchased at the corner drug store, when paper, thin-layer, gas-liquid and other chromatographic and partition techniques for separation were not yet available and when the most reliable procedure for the detection and estimation of estrogens in biological samples was the bioassay of Allen and Doisy or other similar methods. The introduction of those techniques that we now take so much for granted provided us with new ways of inquiring into the mode of action of steroid hormones in influencing normal and tumor growth. However, the time was then not yet ripe for a direct assault on this question. Basic information was needed to understand the mechanisms whereby the levels of circulating hormones were regulated in the normal individual and in disease states. Broadly speaking, the circulating levels of hormones are controlled by the rates of synthesis, the rates of degradation, the efficiency of the transport processes and the efficiency with which the hormones are extracted from the blood stream by the cells of the tissues that they affect.

Thanks to the pioneering work of Pincus, Hechter, Dorfman, Heard and many others, a very substantial body of information relating to the biosynthesis of the corticosteroids, of progesterone, and of the androgens from cholesterol and smaller molecules had become available by the early 1950s. Progress in the estrogen field was retarded, partly because of conceptual difficulties. Arguing from the fact that phenylalanine and tryptophan, both aromatic compounds, are essential amino acids for many mammalian species, it was believed that mammals were incapable of synthesizing benzenoid compounds and that the biogenesis of estrogens proceeded along pathways separate from those of the other steroid hormones. Although several examples of aromatization in mammals had been recorded, it now appears that, in many cases in which this phenomenon has been observed *in vivo*, intestinal microorganisms were the responsible agents (1). There are, however, well-documented examples of the formation of aromatic compounds by animal tissues *in vitro* (2, 3). Nevertheless, it remains true that the estrogens are the only aromatic compounds that play a major role in mammalian economy that are synthesized by the mammal from small acyclic precursors. However, there

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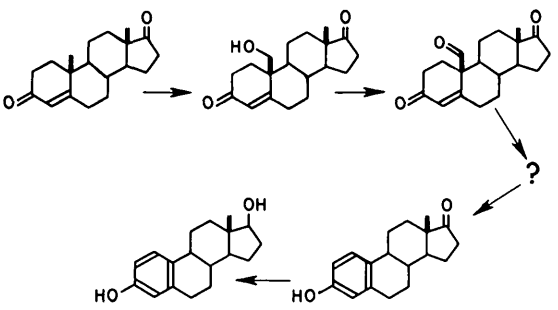

FIG. 1. Biosynthesis of 17β-estradiol.

was indirect biochemical evidence in humans for transformation of androgenic steroids to estrogens. The evidence consisted of the demonstration by either bioassay or chemical estimation of an increase in the excretion of estrogens after androgen administration (for review see ref. 4).

The direct proof had to await the development of refined separation methods and the availability of isotopically labeled steroids. The first such evidence for a biosynthetic link between estrogens and other steroids was that of Heard *et al.* (5), who administered labeled testosterone to a pregnant mare and isolated from her urine labeled estrone. These observations were followed in quick succession by the observations of Baggett *et al.* (6), who demonstrated the conversion of testosterone to 17β-estradiol by surviving slices of human ovary. Shortly before this, Meyer (7) isolated 19-hydroxyandrostenedione and demonstrated its conversion to estrone by a variety of endocrine tissues. It was soon found that placenta, testis, and certain adrenal cortical tumors could also carry out the transformation of androgens to estrogens (8). It remained, however, for the definitive work of Ryan and Smith (9–12) to establish securely that, in the ovary, estrogens are synthesized by the same pathways and utilize the same precursors and intermediates as the other steroid hormones.

The details of the transformation of androst-4-ene-3,17-dione to estradiol are still incompletely understood, but what progress has been made resulted from use

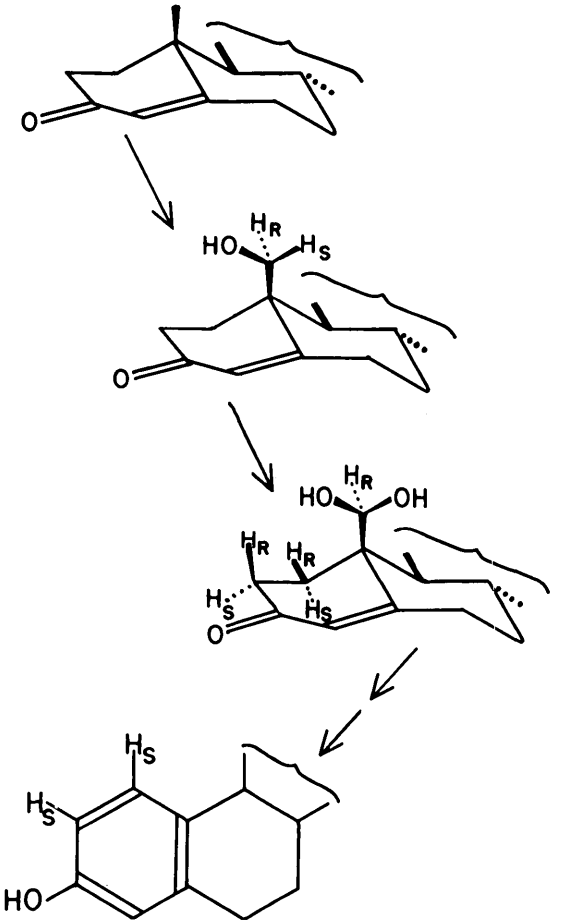

FIG. 2. Stereochemistry of the aromatization reactions.

of the placental microsome system developed by Ryan (13). It is now generally accepted that the first step is hydroxylation of carbon 19 (Fig. 1). This has been established both by trapping experiments and by a kinetic method (14). The next step, which also requires TPNH and molecular oxygen, is a second hydroxylation of C-19 leading to the formation of the 19-aldehyde. This has now been established by Akhtar and his colleagues (15). They showed further that it was the 19-pro-S hydrogen that was removed in the oxidation of 19-hydroxyandrost-4-ene-3,17-dione to 19-oxo-androst-4-ene-3,17-dione (16) (Fig. 2). We have continued our interest in the aromatization problem and recent results

obtained by Dr. Braselton confirm the formation of 19-oxo-compounds both from testosterone and androst-4-ene-3,17-dione and from their 19-hydroxy derivatives. Both 19-oxoandrost-4-ene-3,17-dione and 17β-hydroxy-19-oxoandrost-4-en-3-one have been identified by their mass spectra. There is still little evidence concerning the final stages of the aromatization reaction but it is known that there is at least one more step that requires TPNH and molecular oxygen. The examination of the final stages in aromatization is now under intensive investigation in our laboratory.

I wish now to turn to the other theme that I mentioned in my introductory remarks. In the earliest studies that Dr. Kenneth Ryan carried out in our laboratory, he made use of two techniques, then new. By joining together a fluorometric method for the estimation of estrone and 17β-estradiol and the countercurrent distribution procedure for their separation, he was able to examine the interconversion of these two compounds in a variety of human and animal tissues. Of the tissues studied, human placenta showed the highest activity in carrying out these transformations (17). Thus, it seemed appropriate to embark upon the fractionation of placental tissue in the hope of isolating the enzyme or enzymes that catalyze the reactions. Following the observations of Coppedge *et al.* (18, 19) and of others who demonstrated the requirement for a pyridine nucleotide in the oxidoreductions (Fig. 3) and those of Pearlman and DeMeio (20), who identified, by chemical means, estrone as an oxidation product of 17β-estradiol, Dr. Langer (21) developed a rapid, sensitive and precise spectrophotometric assay for 17β-estradiol dehydrogenase activity. Such a procedure is an indispensable prerequisite for any attempt to isolate and characterize an enzyme. She also discovered the remarkable stabilizing effect of glycerol upon the enzymatic activity; an observation that was crucial to its subsequent purification.

One of our primary objectives in studying 17β-estradiol dehydrogenase was to obtain information concerning the structural features of the steroid molecule that made it suitable to serve as substrate. In parallel with these studies were experiments on the interaction of steroids with the purine bases that are constituents of deoxyribo- and ribonucleic acids (22, 23). The complexes formed with nucleic acid constituents had such large dissociation constants and showed such low structural specificity that even then we did not consider direct interaction between steroid hormones and nucleic acids as an attractive possibility for a mode of action of hormones. Nevertheless, the structural specificity that was observed could be regarded as a preview of the kinds of specific interactions one could expect between steroids and other biologically important molecules. The experiments with purine bases, nucleosides and nucleotides offered the advantages of working with a precisely defined system with components of known structure and was an early example of what are now known as hydrophobic interactions.

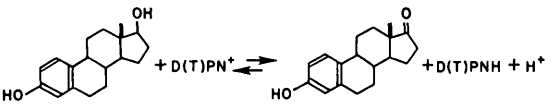

FIG. 3. 17β-Estradiol dehydrogenase.

In considering how steroid hormones might interact with other molecules to express their biological activity, we reasoned as follows: Because of their structural complexity, the steroid hormones are information-rich molecules. The information that they contain is expressed very precisely and very specifically in a three-dimensional form. One needs only to point to the profound effects of changing the configuration of one substituent on a steroid molecule upon its biological activity to realize this. Although polynucleotides contain large amounts of information, it is largely expressed in the linear sequence of the purine and pyrimidine bases and only secondarily in the three-dimensional structure of the

macromolecule. Although it might seem reasonable to postulate that steroid hormones act by interaction with complex lipids or complex polysaccharides, a more appealing notion and one more consistent with modern ideas concerning the nature and chemistry of growth is that the steroid hormones interact with proteins. The three-dimensional structures of proteins are such that the widest latitude and the greatest specificity are provided by the spatial disposition of polar and nonpolar amino acid residues. Proteins seemed then and still seem to provide the needed structural variation and specificity to be complementary to steroid hormones whose biological activities are in so many cases precisely determined by the nature and stereochemistry of substituents on the parent nucleus.

These ideas provided the impetus for our attempts to purify 17β-estradiol dehydrogenase from human placental extracts. At the time this work was undertaken it seemed only remotely possible to purify an enzyme that we now know comprises only about 0.03% of the soluble extract of human placenta; we therefore embarked upon substrate specificity studies with preparations purified 200-fold (21) that contained at most 5% of the protein of interest. Our experiments (24) were designed to investigate those structural features of the steroid molecule that influenced the fit of enzyme and substrate as measured by the affinity of the enzyme for its substrate and by the velocity of the enzymatic reaction. These and later studies (25, 26), in conjunction with investigations from other laboratories, led us to postulate that 17β-estradiol dehydrogenase interacts with the α-side of the steroid molecule. This is almost implicit in the reaction since it is the α-hydrogen at C-17 that is abstracted in the oxidation of 17β-estradiol. It was also found that substituents distant from the site of the reaction in ring D exerted a significant effect upon the fit of enzyme and substrate and upon the reaction rate. More profound were the effects of changes in configuration at the asymmetric centers at carbon atoms 8, 9, 13 and 14. While these studies afforded some view of the effects of structural variations of the steroid, they revealed essentially nothing about the structural characteristics of the protein partner in the interaction. This phase of the problem can only be studied with a pure enzyme. Our laboratory and that of Talalay and his associates embarked upon the purification of the 17β-estradiol dehydrogenase of human placenta, and in 1962 Jarabak *et al.* (27) reported a 2500-fold purification of the enzyme.

More recently (28) we have been able to achieve a 3000-fold purification of placental 17β-estradiol dehydrogenase to yield a protein that is homogeneous by polyacrylamide disc gel electrophoresis at two pH values and has a molecular weight of 48,000. Unlike earlier samples from our own and other laboratories, the pure enzyme does not attack non-aromatic steroids. Jarabak (29) has also isolated a homogeneous enzyme that has somewhat different properties from our preparation (30). The unusual properties of this protein, which include cold-inactivation (31) and serious instability in the absence of glycerol (21), make it an interesting subject for study. We are now in the process of stockpiling pure enzyme for physicochemical studies and for attempts to examine the nature of the amino acid residues in the active site. In this way we are proposing to probe the structure of that part of the enzyme molecule that is complementary to the steroid substrate.

Some years ago Dr. Weiert Velle, who had mastered the difficult technique of working with hydroxylapatite for the fractionation of protein mixtures (32), became interested in an unresolved disagreement concerning the mechanism of the stimulation by 17β-estradiol of the enzymatically catalyzed transfer of hydrogen from reduced triphosphopyridine nucleotide to diphosphopyridine nucleotide in human placental extracts. The phenomenon itself had

been discovered by Villee and Hagerman (33) and had been shown by Talalay and Williams-Ashman (34) to be a transhydrogenation. The disagreement arose in the interpretation of the data. The Talalay group (35) maintained that, in the enzymatic transfer of hydrogen from one pyridine nucleotide to the other, 17β-estradiol and estrone were oxidized and reduced, respectively, in a reaction catalyzed by the 17β-estradiol dehydrogenase of human placenta which appears to have dual pyridine nucleotide specificity (21). Thus, the 17β-estradiol plays a coenzymatic role in the hydrogen transfer (Fig. 4). Villee and Hagerman confirmed the experimental findings but maintained that there is, in addition, a transhydrogenase system that is not associated with estradiol dehydrogenase.

The resolution of this apparent conflict required several years of intensive work by Dr. Harry Karavolas. By means of hydroxylapatite chromatography, he was able to separate two transhydrogenase systems from placental extract (37) (Fig. 5). One of these we have designated the "17β-estradiol activated transhydrogenase" and the other, the "transhydrogenase function of estradiol dehydrogenase." The mechanisms of the two systems are fundamentally different; in the 17β-estradiol activated transhydrogenase, activation of the enzyme occurs without chemical change of the estradiol molecule. 17β-Estradiol and estrone are interconverted by the transhydrogenase associated with 17β-estradiol dehydrogenase activity (Table 2) and deuterium is lost from the 17α-position of the 17β-estradiol molecule. They differ in many other respects (Table 1). The thermal instability of the estradiol-activated transhydrogenase led to its destruction in the heat treatment employed by Jarabak *et al.* (27). The two systems differ markedly in their behavior toward high salt concentrations, an early clue to the existence of two transhydrogenase systems.

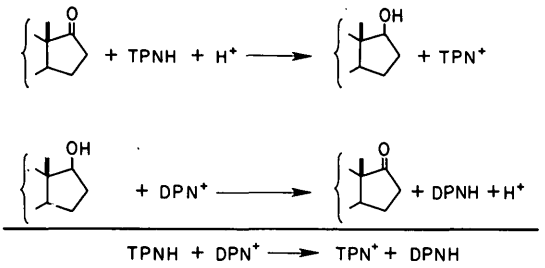

FIG. 4. Transhydrogenase reactions.

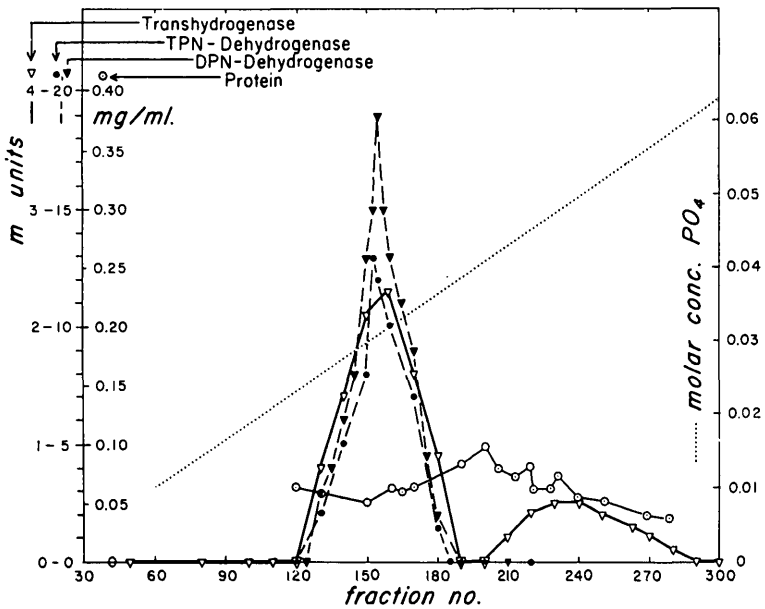

FIG. 5. Separation of 17β-estradiol activated transhydrogenase from the transhydrogenase function of 17β-estradiol dehydrogenase by hydroxylapatite chromatography. [From Karavolas, Orr and Engel (38).]

TABLE 1. Transhydrogenase function of estradiol dehydrogenase[a]

	20 min	2 hr
	nmoles	
Estradiol-17α-^{2}H[b] incubated	74	74
Estrone formed	9.5	10.5
DPNH formed	15.6	72.7
Estradiol recovered (% ^{2}H at C-17)	86	50

[a] From Karavolas, Orr and Engel (38).
[b] The estradiol-17α-^{2}H incubated contained 95.8% ^{2}H at C-17.

Moreover, the spectrum of activators for the two systems is distinctly different (Table 1). Both are activated by 17β-estradiol and neither by 17α-methylestradiol. The nonsteroidal estrogen, diethylstilbestrol, affected only the 17β-estradiol activated transhydrogenase; it cannot undergo the cyclic oxidoreduction that characterizes the transhydrogenase function of 17β-estradiol dehydrogenase. Finally, the fact that estrone does not affect the 17β-estradiol activated transhydrogenase confers upon the system an unexpectedly high degree of specificity. The concentration of 17β-estradiol required to activate the system is of the order of 10 nM (Fig. 6). The specificity for activation and the low concentration of 17β-estradiol required to elicit the effect seem to put this system in a special category. It is the only soluble enzyme thus far known that is specifically activated by a steroid hormone present in a concentration that could occur physiologically in the tissue from which it is derived.

The original observations that led Villee and Hagerman (33) to the discovery of the 17β-estradiol activated transhydrogenase and its close relative, the transhydrogenase function of 17β-estradiol dehydrogenase, were made on placenta and uterus and were at that time believed to constitute a possible mode of action for the estrogenic hormones. It was proposed that the transhydrogenase(s) could control the levels of oxidized and reduced pyridine nucleotides and thus determine the amount of DPNH oxidized with production of ATP, and the amount of TPNH available for biosynthetic processes. This concept has been overshadowed by recent attention to aspects of estrogen action at the level of nucleic acid and protein synthesis. These studies have led to a better understanding of the metabolic processes initiated and stimulated by these potent physiological agents. The studies of Jensen (39), of Shyamala and Gorski (40), and of many others have disclosed that in target tissues

TABLE 2. Properties of the two soluble estradiol-sensitive pyridine nucleotide-linked transhydrogenase activities of human placenta[a]

	Estradiol activated	Transhydrogenase function of estradiol dehydrogenase
Phosphate concentration (mM) at which activity is eluted from hydroxylapatite	45–55	20–30
Presence in extract after heating to 55 C	No	Yes
Effect of 1.67M NaCl	Increased activity	Inhibition
Association with DPN (and TPN)-linked estradiol dehydrogenase activity	No	Yes
Kinetic isotope effect with estradiol-17α-^{2}H	No	Yes
Formation of estrone during transhydrogenation	No	Yes
Loss of 17α-^{2}H during transhydrogenation	No	Yes
Structural specificity for activation		
Estradiol	+	+
Estradiol-17α-^{2}H	+	+
Estrone	−	+
17α-Methylestradiol	−	−
Diethylstilbestrol	+	−

[a] From Karavolas, Orr and Engel (38).

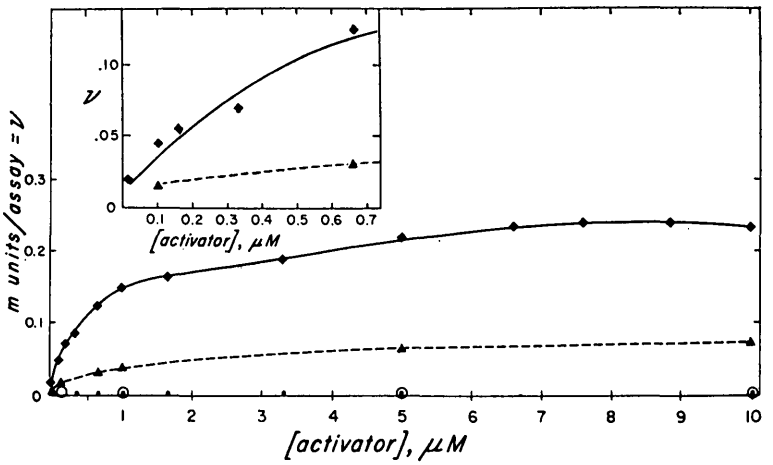

FIG. 6. The effects of estrone (⊙), 17α-methyl-17β-estradiol (○), diethylstilbestrol (▲) and 17β-estradiol (◆) on the activity of 17β-estradiol activated transhydrogenase. [From Karavolas, Orr and Engel (38).]

for estrogens there are proteins that have a very high affinity for 17β-estradiol and that appear to be related to estrogen action. Yet, it is fair to say that there is still a gap between the binding of 17β-estradiol to cytoplasmic or to nuclear proteins and the metabolic events that are initiated. The role of these macromolecules is still a mystery; they are present in extremely minute amounts, and have thus far resisted extensive purification. It is important that the affinities of these estrophilic macromolecules are sufficiently high so that they are capable of extracting 17β-estradiol from plasma and extracellular fluid. Clark and Gorski (41) report dissociation constants of the order of 1 nM for estrogen receptor proteins from rat uterus. A further important characteristic of the estrophilic proteins is that it has so far been impossible to dissociate the estrogen-protein complex without destroying the integrity of the protein structure. These observations are consistent with the views that have been expressed previously that, in the interaction of a specific protein with a steroid hormone, the protein reads all of the information that is present in the steroid molecule. In order to do this it must virtually engulf the steroid in a largely hydrophobic pocket that cannot be opened without damaging the protein molecule. This concept is also consistent with the very high affinity of the protein for 17β-estradiol. Finally, the structural specificity for retention of estrogenic substances by uterine and other target tissues is also noteworthy. In the uterus, 17β-estradiol and nonsteroidal estrogenic compounds are bound, but not estrone. This behavior is similar to that of the 17β-estradiol activated transhydrogenase discussed above and suggests that it plays a specific but, as yet, unknown role in cellular economy.

A possible role proposed by Dr. Karavolas is that the 17β-estradiol activated transhydrogenase serves to control the rate of 17β-estradiol biosynthesis in the placenta. *In vitro* experiments carried out with the placental microsome system of Ryan (13) indicated that there is an obligatory sequence for the transformation of androstenedione to 17β-estradiol (42) (Fig. 7). There are at least four steps in which TPNH and oxygen are required. The ev-

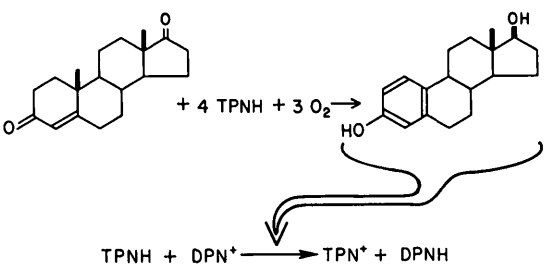

FIG. 7. Feedback control of 17β-estradiol biosynthesis in the placenta.

idence suggests that the final reaction is the reduction of estrone to 17β-estradiol. When its concentration rises, the 17β-estradiol activated transhydrogenase functions to reduce the concentration of TPNH, controlling the reaction at the rate limiting step, 19-hydroxylation. Thus, in placenta the level of 17β-estradiol in the tissue may serve to control its synthesis.

Whether or not our hypothesis has any significance, I wish once again to draw attention to this system, a soluble one, as a suitable model for the study of a protein whose activity is dependent upon interaction with a steroid hormone at a concentration that brings it into the range of physiological interest.

We have considered a somewhat meandering trail of research on the metabolism and action of the estrogens; sometimes the distinction becomes blurred. At the present time we can begin to discern some of the structural characteristics possessed by the estrogens that are involved in their binding to proteins. There are clearly several levels of both affinity and specificity. 17β-Estradiol dehydrogenase and its transhydrogenase function have a broad specificity for aromatic 17β-hydroxysteroids and the dissociation constants for the complexes are of the order of 1–10 μM. The 17β-estradiol activated transhydrogenase has a much sharper steroid specificity and a dissociation constant that has not been measured but is probably of the order of 10 nM. Finally, the 17β-estradiol receptors of target tissues show an extremely high steroid specificity and probably even smaller dissociation constants (1 nM).

Our next big task is to understand these observations in terms of the structures of the protein partners in the interactions. In this way, we will be able to gain new insights into hormone metabolism and action. The synthesis of new hormone analogues, synergists and inhibitors, as well as the synthesis of proteins that possess steroid binding properties could lead to the development of new therapeutic tools and new methods for the regulation of population. An understanding of the molecular mechanisms in hormone action on the reproductive system will eventually be translated into a clearer comprehension of the means by which the levels of circulating hormones influence the growth of neoplastic tissues and eventually enable us to control or even to prevent tumor growth.

Acknowledgment

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