THE PHARMACOLOGY OF THE ESTROGENS 6505

A. E. KELLIE
The Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, England

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An annual review may be considered to have achieved some measure of success if it assembles in cogent form apparently unrelated facts and reveals in them a common theme. This has proved to be most difficult at the present time in relation to the pharmacology of estrogens as the two most important lines of development; the study of the metabolism of the compounds and the mechanism of their action at a cellular level have little common ground, and appear to be following widely divergent courses. Impressive technical developments now permit a most detailed study of biosynthetic and catabolic pathways in vivo and in vitro. The availability of tritium-labeled estrogens and estrogen precursors (1) at high specific activity permits in vivo study in human subjects without risk, while the development of subcellular techniques has made it possible to follow these changes in vitro almost down to molecular levels (2). The combination of gas-liquid chromatography in conventional and capillary columns with the mass spectrograph (3) used as an analytical tool, now permits isolation and characterization of products at a level never dreamt of by the classical biochemist of a few decades ago. Yet in spite of these advances, the proliferation of minor metabolites, the recognition of conjugated forms, and the characterization of the principal enzymes systems, there is no evidence that any of these metabolic transformations has any direct connection with the action of estrogens at the cellular level. On the contrary, the considerable progress which has been made in the study of the action of estrogens on hormone-dependent tissues continues to emphasize the uniqueness of molecular structure in compounds, natural and synthetic, which possess biological activity (4).

METABOLISM OF ESTROGENS

Biogenesis.—The biogenesis of estrogens in the mammalian species during the menstrual cycle and in early pregnancy is centered in the ovary and is probably controlled via the hypothalamus by gonadotropic factors of pituitary origin. With the decay of the corpus luteum, the contribution of the ovary to the supply of estrogens and progestogens declines and the substantial increase in steroidogenesis which characterizes the latter stages of

pregnancy is achieved by the placenta and fetus acting as a co-ordinated endocrine organ—the feto-placental unit (5, 6). There is little doubt that at this stage of gestation too, gonadotropins play a significant role in balancing the production of estrogens and progestogens, but the precise nature and function of these factors remain unknown. Certainly the major proportion of the urinary gonadotropins that appear in late pregnancy are of chorionic rather than pituitary origin (7).

The small but significant amounts of the classical estrogens, estrone, estradiol, and estriol—which males excrete (8)—are probably of adrenal origin, for there is evidence that the adrenal cortex in vivo and in vitro (9) is capable of forming C_{18}-phenolic steroids from conventional precursors. The possibility that some estrone is formed from 4-androstenedione in peripheral tissues cannot be excluded (10).

The main steps of the biogenesis of the C_{18}-phenolic estrogens have been known for some time (11, 12). The organism prefers to build up C_2 units to the large C_{27} cholesterol molecule and to form the biologically active estrogens by a series of stepwise attacks by desmolase enzymes (C_{27}- C_{21}- C_{19}) (13) completing the process by an aromatase enzyme (C_{19}- C_{18}) found in the placentae of many species (14–16). Aromatization of the neutral C_{19} steroids proceeds by a series of oxidative attacks on the C_{19} angular methyl group, producing in turn the 19-hydroxy steroid, the 19-oxosteroid, and finally with the loss of formaldehyde (17) or formic acid (18) the C_{18}-phenolic estrogen. The final step which can be achieved in vitro using a microsomal fraction from human placenta has been studied in detail by several groups (19, 20). The enzyme that carries out this aromatization is very widely distributed, having been found in human, bovine, and equine placentae; human, dog, rat, mouse, and frog ovaries; in cow adrenal and stallion testis, and in human fetal liver (21). It is located in the placental microsomal fraction and requires both NADPH and molecular oxygen (14). The enzyme appears to act specifically on C_{19} steroids and 19-nor-C_{18}-steroids and is not inhibited by oxygen substituents at C–1β, C–2β, C–6β, C–9α, or C–11α (22). The presence of a 11β-hydroxyl substituent appears to inhibit aromatization. A similar, and possibly identical enzyme, which has been isolated from the microsomal fraction of equine and human placentae, is capable of aromatizing ring-B and thus producing the ring-B unsaturated estrogens equilin and equilenin (23). The immediate precursor of these compounds is believed to be $\Delta^{4,7}$-androstadiene-3,17-dione. The same enzyme converts 3β-hydroxy-$\Delta^{5,7}$-androstadien-17-one into the nonphenolic ring-B aromatic C_{18}-steroid, 3β-hydroxy-$\Delta^{5,7,9}$ estratrien-17-one (Heard's ketone) (24).

Catabolic pathways.—If the biosynthesis and secretion of estradiol represents the main purpose of phenolic steroidgenesis, it is difficult to understand the diverse ways in which excess hormone is squandered by deactivation and excretion. Known metabolites of the classical estrogens already exceed thirty (21) and the number grows annually as techniques improve and

the scale of operation expands, yet none have significant estrogenic activity. The principal sources of new compounds have been late pregnancy urine or the urine of normal subjects after the injection of isotope-labeled estrogenic compounds. In vitro studies, carried out with tissue slices, homogenates, or sub-cellular fractions, complement in vivo results, and in both techniques the use of radioactive precursors has greatly simplified problems of detection and isolation. Minor metabolites that have recently been characterized include 2-hydroxyestrone, 2-methoxyestrone, 2-methoxyestradiol, 2-methoxyestriol, 6α-hydroxyestrone, 6-hydroxyestriol, 11-dehydro-17α-estradiol (25), 14α-hydroxyestrone (26), 15α-hydroxyestrone, 15β-hydroxyestrone, 15α-hydroxyestradiol, 15β-hydroxyestradiol, 16α-hydroxyestrone, 16β-hydroxyestrone, 16-oxoestrone, 16-oxoestradiol, 16-epiestriol, 17-epiestriol, 16-17-epiestriol, and 18-hydroxyestrone (21). These compounds and other metabolites arise mainly as a result of enzymic hydroxylation at C-2, C-6, C-7, C-11, C-14, C-15, C-16, and C-18 followed in some cases by reversible oxidation to the corresponding oxo-steriod (27, 28). In general, steroid hydroxylase enzymes require NADPH and molecular oxygen and there is increasing evidence that cytochrome P450 is the oxygen activating catalyst common to many steroid hydroxylations (29). The enzymes are difficult to solubilize and are found in the microsomal and mitochondrial fractions of liver and adrenal tissue. Enzymes capable of introducing a hydroxyl substituent into aromatic steroids at C-6α, C-6β, C-16α, C-16β (microsomal), and at C-7α, C-7β, C-14β, C-15α (mitochondrial), have been characterized (21). Cytoplasmic oxido-reductase enzymes active at C-16α-OH (30), C-16β-OH (31), C-17α-OH (32), and C-17β-OH groups have been identified in many species; the latter enzyme, soluble 17β-hydroxysteroid dehydrogenase, has been extensively purified (27).

Hydroxylation at C-2 in the aromatic A-ring which is achieved by microsomal hydroxylases of the liver has quantitative significance in estrogen metabolism. The three compounds 2-hydroxyestrone, 2-hydroxyestradiol, and 2-hydroxyestriol are formed in vivo and in vitro (33, 34); they have been isolated from hydrolysed human urine and are formed in vivo when the corresponding estrogen is incubated with a microsomal fraction prepared from rat liver (35). The yields in vitro are surprizingly high (30%) and in the case of oestrone trace amounts of 4-hydroxyestrone (1%) have also been identified. Hydroxylation at C-10 has also been observed in vitro using rat liver microsomes with estrone as substrate; the product has a *p*-quininoid structure in the A-ring, and being unstable has so far only been isolated as the 2,4-dinitrophenylhydrazine derivative (36). The kinetics of the reaction of this enzyme suggest that it is different from the liver microsomal enzymes which cause hydroxylation at C-2 and C-4.

Transmethylation of 2-hydroxyestrogens occurs in man and can readily be demonstrated in vitro by incubating 2-hydroxyestrone, 2-hydroxyestradiol, or 2-hydroxyestriol with a cellular supernatant fraction prepared from rat liver (37). The enzyme S-adenosylmethionine:O-diphenol-O-methyl transferase (EC 2.1.1.6) which requires S-adenosylmethionine as a co-

factor is widely distributed in rat and human liver, human myometrium and endometrium, human placenta, and in the kidney and spleen of man, rat, and guinea pig. The enzyme and cofactor show low specificity; thus with 2-hydroxyestradiol as substrate the products include both 2-methoxyestradiol and 2-hydroxyestradiol-3-methyl ether (38). It is probable that the same enzyme is responsible for the transfer of the methyl group of S-adenosylmethionine to the 3 hydroxy group of epinephrine and other catecholamines. Cross effects between estrogen metabolism and that of the catecholamines have been reported (39).

Conjugation of estrogen metabolites.—In pregnancy and throughout the menstrual cycle women excrete estrogen and estrogen metabolites principally as monoglucuronides (40). Estrone is excreted as the 3-monoglucuronide (41), estradiol in small amounts as the 3- and 17β-monoglucuronide (42), and estriol as the 3- and 16α-monoglucuronides (41). The latter compound, estriol-16α-glucuronide, is by far the largest single conjugate in late pregnancy urine, although in some urines 16α-hydroxyestrone-16α-glucuronide is a substantial component (40). Estrogen sulphates do occur in late pregnancy urine but only as a small fraction of the total conjugates (43). The presence of estrone, estradiol, estriol, and 16-oxoestradiol as the 3-sulphates has been indicated by indirect evidence but these compounds have not been isolated in pure form. Somewhat surprisingly, estrogen bis-conjugates containing both sulphate and glucuronosidic residues make up a significant proportion of the total estrogenic conjugates of late pregnancy urine (44). The principal components of this fraction have been identified as estriol-3-sulphate-16α-glucuronide and 16α-hydroxyestrone-3-sulphate-16α-glucuronide (40).

At least two different types of glucuronyltransferase enzymes are involved in the formation of estrogen glucuronides, for conjugate formation at C-3 involves the formation of phenolic glucuronides, whereas conjugation at all other positions results in the formation of an alcoholic ether type of linkage.

An enzyme present in the microsomal fraction of the liver of many species, UDP-glucuronate glucuronyltransferase (EC 2.4.1.17) catalyses the transfer of the glucuronyl residue from uridine-5′-diphosphoglucuronic acid to a wide variety of phenolic hydroxyl groups (45). The enzyme, prepared from rat livers, will form the 3-monoglucuronide of estrone (46), estradiol, and estriol (47) in vitro but there are some species differences in that the enzyme from other sources shows some structural specificity (21). The phenolic glucuronyl transfer is not confined to the steroid field (45). A second glucuronyl transferase isolated from the human intestine (46, 47) forms the 3-monoglucuronide of estradiol and estriol but also conjugates nonphenolic steroids forming the 17β-monoglucuronide of testosterone and the 3α-monoglucuronide of androsterone (48). Enzyme preparations in this group have not been extensively purified and may contain mixed glucuronyl transferases.

The formation of ring-D glucuronides, of which estriol-16α-glucuronide is quantitatively the most important, can be achieved using a microsomal enzyme preparation from human liver (49, 50) and intestines (48, 51). The enzyme from human liver shows remarkable specificity towards the 16α-hydroxyl group of estriol and does not form either the 3-monoglucuronide or the 17β-monoglucuronide. With 16α-hydroxyestrone as substrate the 16-α-monoglucuronide is formed. A soluble enzyme has also been prepared from human liver (51) and intestine (52) which shows the same high degree of specificity, forming only the 16α-monoglucuronide of estriol and 16α-hydroxyestrone.

Somewhat unexpectedly, although estriol-17β-glucuronide does not appear to be present in late pregnancy urine (40), the human intestine contains a glucuronyl transferase which catalyses the formation of the 17β-monoglucuronides of estradiol and estriol in vitro but displays no activity towards the C-3 phenolic or 16α-alcoholic groups (52).

Conjugation of estrogens as sulfate, whether as monosulfates or bis-mixed conjugates, appears to be restricted to the phenolic C-3 position. Two such enzymes have been described. One capable of transferring the sulfate radical from the cofactor 3′-phosphoadenosine 5′-phosphosulfate to phenolic hydroxyl groups has been prepared from rabbit liver. The crude preparation will also transfer the sulfate radical to dehydroepiandrosterone, but in recent studies complete separation of the phenolic transferase has been achieved (53). Related sulfonyl transferase systems are widely distributed in endocrine tissues, being found in the liver of rat, rabbit, lamb, ox, and man, in the adrenal of the ox and man, as well as in ovarian, luteal, and testicular tissue (21).

A second estrogen sulfotransferase (3′-phosphoadenylsulfate-estrone sulfotransferase; EC 2.8.2.4,) isolated from bovine adrenals requires the same cofactor but appears to have high specificity for the natural estrogens (54). The classical estrogens, including equilenin, are conjugated at the C-3 position. In vitro low yields of stilbestrol and hexestrol sulfate are formed.

The rabbit so far appears to be unique in excreting estrogens as the double conjugate of 17α-estradiol and the 17α-epimers of estriol, with a glucuronic acid residue at the C-3 position and an N-acetylglucosamine residue at the 17α-position (55). In vivo and in vitro studies have confirmed that rabbit liver preparations transfer N-acetylglucosamine from UDP-N-acetylglucosamine to the hydroxyl group of 17α-estradiol-3-sulfate and to the 15α-hydroxyl group of 15α-hydroxyestrone-3-sulfate but not to the corresponding free steroids (56).

Some interesting water soluble products formed by incubating rat liver with 2-hydroxy [4-^{14}C] estrone or with the corresponding estradiol derivative have now been identified as conjugates with glutathione joined by thioether linkage to the A-ring at C-1 and C-4 (57, 58). The structure of these novel conjugates has been confirmed by partial synthesis. (2-Hydroxyestradiol-1-yl) [^{35}S] glutathione was unaffected by incubation with male cat liver, but with kidney tissue from the same source glutamic acid and glycine

residues were removed from the conjugate (59). The search for estrogen mercapturic acids in rat urine has, so far, given negative results.

In spite of the large numbers of estrogen metabolites and the many ways in which these may be conjugated, there is no evidence that any compound other than estrone, estradiol, and estriol either induces a significant response at cellular level or a physiological reaction in the intact animal. Nevertheless, the existence of water-soluble conjugates has important consequences, especially in the kidney and the feto-placental unit. The renal clearance of steroid glucoronides is very high, and metabolites that appear in this conjugated form are likely to have a relatively short biological half-life (60); in contrast, there is no doubt that the steroid sulfates play an extremely important role in secretion and transport. They are conserved by the kidney and may represent a substantial store of steroid hormones in an inactive form. The special role of steroid sulfates within the feto-placental unit has been revealed largely as a result of the work of Diczfalusy and coworkers (61, 62).

The Feto-Placental Unit

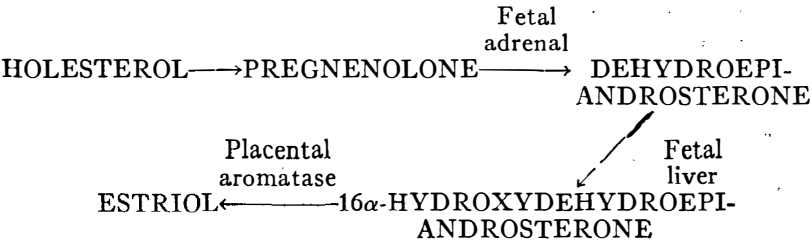
A striking feature of gestation is the progressive increase in the production of estrogens and progestogens. At full-term the output of estrogens is at least 1,000 times that in the nonpregnant state; the increased production of progesterone and related biologically active compounds is substantial but not of the same order. Immediately after ovulation in women, the site of formation of these compounds lies in the ovary and corpus luteum but after the first trimester these functions

ine embedding site and to the placenta (65). Because of the close relationship between the placenta and fetus, the former cannot be considered in isolation but as part of the feto-placental unit.

The need to consider the placenta and fetus together arises mainly because the enzymes and steroid substrates are distributed in complementary manner between the two tissues (66). There can be little doubt, in view of the scale of biogenesis, that the ultimate source of both estrogens and progestogens produced within the feto-placental unit is the cholesterol of the maternal circulation (67, 68). Although fetal liver, adrenal, and testis can synthesize cholesterol from acetate and the placenta cannot, yet cholesterol levels are high, relative to other steroids, on both sides of the placenta. The fetus, unlike the placenta, contains no 3β-hydroxy-Δ^5-steroid dehydrogenase and is unable to convert cholesterol or the C_{21} and C_{19} metabolites formed from it into Δ^4-3-one structures (69). For this reason the principal steroids produced by the fetus are pregnenolone (67) and dehydroepiandrosterone (69) which are excreted partly in the free form but predominately as the C-3 sulfates (70). The placenta, unlike the fetus, has little capacity to form sulfates; it can, however, hydrolyse neutral and phenolic sulfates and contains specific dehydrogenases which convert 3β-hydroxy-Δ^5-steroids to the corresponding Δ^4-3-one compounds (71, 72). Thus it can hydrolyse steroid sulfates secreted by the fetus, it can convert dehydroepiandrosterone into

androstenedione, the precursor of the estrogens (73, 74), and pregnenolone into progesterone, the key compound for the formation of the mineralo- and gluco-corticoids. It is clear that under these conditions the fetus lacking the necessary dehydrogenase is unable to convert the available pregnenolone to progesterone and it is accepted that the very considerable amounts of progesterone produced in pregnancy are formed almost exclusively by the placenta (67). Pregnanediol is considered to be a unique metabolite of progesterone and these facts are the justification for using the daily maternal pregnanediol excretion as an indication of placental function.

Reduction of circulating progesterone and androstenedione takes place in the fetus as well as in the mother and it has been shown that in the fetal liver both substrates are reduced to the corresponding 3α-hydroxy-5β-compounds. A different enzyme system must exist in the fetal lung, as with this tissue progesterone is reduced to the 3β, 5α structure while androstenedione is converted to 3α,5α compounds (75). In the feto-placental unit, as in the ovarian follicle, androstenedione is converted to estrone and estradiol but not to estriol. The human placenta at mid-term lacks an enzyme system capable of introducing a hydroxyl group at the C-16α position of phenolic steroids such as estrone and estradiol and there is accumulating evidence to suggest that estriol is largely formed from neutral steroids that have already been hydroxylated at C-16α (69, 76, 77). Such precursors include 16α-hydroxytestosterone, 16α-hydroxyandrostenedione, and 16-oxotestosterone, which are aromatized to estriol, 16α-hydroxyestrone, and 16-oxoestradiol respectively. The latter compounds, on reduction, would form estriol. According to this concept, estriol is formed by the feto-placental unit as follows,



As the feto-placental 16α-hydroxylase is mainly in the fetal liver and adrenal, the excretion of estriol in the maternal urine is currently used as an indication of fetal welfare in cases of threatened abortion (78). As the administration of estrone or estradiol to nonpregnant women causes a small increase in estriol excretion, the formation of this compound in the mother is probably by the phenolic route (79, 80); this metabolic pathway is, however, small in comparison with the output of estriol by the feto-placental unit. The excretion of urinary estriol by the pregnant mother falls rapidly after fetal death (78).

In general terms, the fetus lacks 3β-hydroxysteroid dehydrogenase and

depends on the placenta to furnish Δ^4-3-oxosteroids, while the placenta is deficient in the desmolase activity which converts C_{21} steroids to C_{19} steroids and is dependent on fetal and maternal sources for important C_{19} compounds. The 16α-steroid hydroxylase is abundant in fetal liver and adrenals but absent from the placenta; thus the adrenal supplies dehydroepiandrosterone sulfate which is converted to 16α-hydroxydehydroepiandrosterone in the fetal liver and then to estriol in the placenta. Clearly, analyses of conjugated steroid metabolites in the maternal urine are too remote from the feto-placental unit to provide a prompt and reliable indication of fetal welfare, and methods that measure individual estrogen and estrogen conjugate concentrations in both maternal and fetal blood are urgently needed.

Secretion of Estrogens

Labeled estrogens administered orally or by injection are poorly recovered in the urine of humans, rabbits, cats, and rats (81). At least half of the dose enters the enteroheptatic circulation in conjugate form and more than 90% of the radioactivity can be recovered from the feces. In women endogenous estrone, estradiol, and estriol are excreted mainly as glucuronides in the urine with a menstrual cyclic rhythm. The excretion of each estrogen rises during the follicular phase and reaches a maximum at, or about, ovulation and then declines towards the next period of menstruation (22). In some women there is a transient rise as the corpus luteum reaches maturity. Methods of measuring plasma levels of estrogens recently developed (10) show a similar cycle of estrogen concentration in peripheral blood. Throughout the follicular phase, estradiol levels lie in the 2–5 ng/100 ml range but the concentration rises steeply at ovulation (15–30 ng/100 ml range) and falls during the luteal phase. The same transient rise in estrogen plasma levels during the late luteal phase has been noted in some cycles examined (63).

The secretion (plasma levels) (82) and the excretion (64) of estrogens rise continuously throughout normal pregnancy and at full term may exceed 50 mg/day largely as a result of the production of estriol by the feto-placental unit. Estriol-16α-glucuronide is the major urinary conjugate, although substantial amounts of estrone and 16α-hydroxyestrone are also excreted in conjugate form (40).

Effect of estrogens in the intact animal.—The amount of estrogen that induces the development of secondary sex characteristics in females is minute in relation to that metabolized and excreted (83). The principal effect of estrogens on the female reproductive tract is exercised during the preovulatory phase on the epithelial lining of the uterus, cervix, and vagina, and leads to an increase in the thickness and vascularization of the endometrial mucosa. Some hypertrophy of the myometrium occurs, resulting in an increase in the uterine water-content and weight. At estrus there is proliferation of the vaginal epithelium accompanied by cornification and shedding of superficial layers of cells. Both increase in uterine weight and the ap-

pearance of estrus are used as the basis of estrogen detection and assay.

In contrast to other classes of steroids, estrogenic activity is not confined to members of the steroid group. The D-ring of conventional estrogens can be opened without loss of activity as in dl-cis-doisynolic and l-cis-bisdehydrodoisynolic acids (84), and further degradation resulting in the opening of the C-ring leads to methallenestril and to allenoic acid (85) which no longer retain the cyclopentanophenanthrene nucleus yet have high estrogen potency in the rat. Many synthetic compounds formed from stilbene analogues show remarkable estrogenic activity when injected or given orally. Thus trans diethyl stilbestrol is as active as estrone, and although it bears only a superficial structural resemblance to this compound, the physiological effects produced by these two compounds are indistinguishable (86). Many compounds structurally related to stilbestrol, e.g., hexestrol, dienestrol, and benzestrol, have high estrogenic activity and are effective by injection and by mouth. Somewhat less active synthetic compounds related to triphenylethane appear to have a more prolonged estrogenic effect by virtue of their solubility in body fat (87). Finally, a group of compounds that show structural resemblance to stilbestrol and triphenylethane, e.g., MER-25, U-11, 100A and CN-55, 945-27, antagonize the action of estrogenic compounds and are believed to act in this way by competitive blocking of estrogen receptor sites (88).

Several estrogenic compounds isolated from plant sources have a flavonoid structure. Mirestrol isolated from *pueraria merifica* is as active as estrone subcutaneously (89). Genestein, which occurs in subterranean clover grass, is a weak estrogen but has caused widespread infertility in Australian sheep (90); coumestrol, a related isoflavone found in clover, alfalfa, and some pulse seeds, is more potent than genestein (91).

Effects of estrogens on uterine tissue.—The obvious fact that estrogens induce physiological changes in the uterus led early workers to seek evidence of biochemical change in this organ. Szego & Roberts in 1953 ascribed the marked up-take of water by the uterus to a change in tissue permeability; they associated the growth and vascularization of the mucosa with protein synthesis (92). Mueller and colleagues observed pronounced rise in the rate of uptake of RNA-precursors some 6 hours after injection of estradiol into ovariectomized rats and were able to demonstrate increased protein synthesis after 40 hours (93). Both groups recognized three stages of response to estradiol which persist until the present time albeit with a shorter time schedule; the first stage involves the interaction of estrogen with a tissue component, the second stage involves primary biochemical events caused by this action, and the final stage amplifies these events into gross tissue changes. Mueller showed that the effect of estradiol on the rate of RNA synthesis was suppressed by simultaneous administration of puromycin, and advanced the hypothesis that estrogens might indirectly promote the formation of a protein inducer (94).

Insight into the nature of the primary cellular interaction came with the

synthesis of tritium-labeled estrogens of high-specific radioactivity (1). Jensen & Jacobson, in a classical study, demonstrated that the tissues that were the principal sites of estrogen activity, namely the uterus, ovary, and hypothalamus, had the ability to retain tritium-labeled estradiol selectively after a physiological dose of radioactive hormone. Retention by the nonresponsive tissues followed closely the decline in radioactivity in blood while the hormone-dependent tissues retained radioactivity for at least 6 hours (83). In liver, the principal site of metabolism, rapid transformation and conjugation of estradiol occurred but only radioactive estradiol was found in the uterus. Of a dose of 0.1μg of estradiol injected not more than 1% accumulated in uterine tissue. This type of tissue distribution experiment has been repeated with other potent oestrogens including hexestrol (95), diethylstilbestrol, and 17α-ethynylestradiol and has given basically similar distribution patterns (96). When [1,2-^{3}H] estrone was administered in this way, both radioactive estrone and estradiol were found in the uterus, and this observation raises the question as to whether estrone is an active compound only insofar as it is converted to estradiol. The selective retention of radioactive estrogens is inhibited by a preceding massive dose of estradiol and by anti-estrogenic compounds such as Upjohn 11,100, Parke-Davis C1-628, methallenestril, clomiphene, and ferocyclin, which are considered to act by occupying tissue estrogen receptors. Puromycin inhibits the uterotropic action of estradiol but does not influence binding by tissues (97).

The binding of estradiol by uterine tissue also occurs in vitro when intact surviving uteri or uterine homogenates are incubated with radioactive estradiol (98). If homogenates thus prepared are subjected to differential centrifugation in 0.44 M-sucrose or in a density gradient, approximately 30% of the unmetabolized radioactive estradiol appears in the highspeed supernatant fraction. The estradiol in this fraction is not free but is bound to a macromolecule variously cited as 9S or 8S (99). Very little radioactivity is found in the mitchondrial and microsomal fractions and the majority of the remaining radioactivity (60–65%) is associated with the nuclear pellet. Whereas the estradiol associated with the cytosol can be readily recovered by solvent extraction, that associated with the chromatin and acidic proteins of the nuclear pellet is more tenaciously held (100). Homogenization of the nuclear fraction with 0.3MKCl releases about half of the radioactive estradiol in the form of a complex with a different macromolecule, the 5S receptor (101). The remainder of the radioactive estradiol in the nuclear material can be released by treatment with deoxycholate solution but is no longer bound.

It is difficult to reconcile these finding with the results obtained by autoradiography of uterine slices after the administration of radioactive estradiol; they show heavy deposition around the nuclear membrane within the nucleus but very little radioactivity in the cell cytoplasm (102). Jensen, DeSombre & Jungblut suggest that the appearance of radioactive estradiol in the supernatant fraction is related to that seen in the nucleus and may arise

from the disruption of a fairly constant amount of the nuclear envelope during tissue homogenization (103).

Study of the effect of degradative enzymes and pH on the binding of estradiol by the macromolecules 8S and 5S led Toft et al (104) to conclude that both receptors were predominately protein in nature. Several attempts have been made to purify cytosol and nuclear receptors by standard procedures of gel-filtration, electrophoresis, and ion exchange chromatography and also by selective chromatographic separation on columns of cellulose co-valently linked to estradiol, but the purest material available at present is less than 4% pure (105). In spite of the impurity, this kind of material has been used as an antigen in the rabbit in an attempt to obtain antibodies specific to the receptor sites. Soloff & Szego (106) have prepared an anti-8S receptor γ-globulin fraction which abolishes estradiol binding to the 8S receptor. According to Jensen et al, the antigen-antibody complex is precipitated with the radioactive estradiol still attached (107).

The precise relation between the cytosol receptor (8S) and the nuclear receptor (5S) has been studied by several groups (108, 109). The latter complex is not formed by treatment of the nuclear fraction or isolated nuclei with estradiol, unless the cytosol receptor is also present, and this has raised the question of whether the 8S receptor represents a transport mechanism carrying estradiol to some pre-existing component in the nucleus, where the earliest biochemical responses are observed, or whether the 8S macromolecular-estradiol complex contributes to the formation of the 5S nuclear receptor. Two groups, independently, have shown that the formation of the nuclear 5S complex is a temperature dependent reaction and that it is associated with a decrease in the 8S binding capacity of the cytosol (108, 110). The 8S estradiol receptor complex shows a tendency to form larger aggregates and the molecular weight has been reported at values ranging from 50,000–200,000; it forms a well-defined complex with ribonuclease. In 0.3M KCl, and possibly also in vivo, the 8S complex dissociates reversibly into 4S sub-units which are stabilized by the presence of Ca^{++} even when the chloride has been removed (108). This stable form of the cytosol receptor has been purified some 5,000-fold by precipitation, gel-filtration, and ion-exchange chromatography (105).

Without reference to molecular-size, methods that have been developed to measure the concentration of high-affinity estradiol receptors in hormone-dependent tissue (111) have shown that the concentration of these sites varies in a cyclic manner throughout the estrous cycle in rats, being minimal at estrus and maximal at pro-estrus. The highest concentrations were found in the uteri of 10-day old rats and these values decreased rapidly to the range of values found during the estrous cycle. Systematic changes in the concentration of binding sites have also been observed in pregnancy and pseudo-pregnancy (112). The biological half-life of these receptors is short and at least one group maintains that estradiol is essential for their continued existence.

Effect of estrogens on uterine cell biochemistry.—Nothing is known of the way in which the nuclear receptor initiates biochemical changes in the cell. A current hypothesis is that the 8S estradiol complex or its 4S sub-unit in some way potentiates the nuclear protein-complex to remove inhibitors of gene expression. According to a recent review by Hamilton (113) the earliest biochemical changes that occur in the uterus of ovariectomized rats treated with estradiol are confined to the nuclei of the cells. Within minutes of the administration of the hormone it is possible to show a marked increase (+500%) in the rate of incorporation of labeled uridine and a corresponding increase in the specific radioactivity of nuclear RNA. Parallel with these changes, increased RNA-polymerase activity of isolated uterine nuclei has been observed and the increased polymerase activity is associated with the formation of two different types of RNA. That associated with the formation of ribosomal RNA reached a maximum at 12 hours (+125%) while polymerase activity producing a DNA-like RNA (base pair ratio 1) rose from 12–24 hours (+60%). Increased template activity of uterine chromatin, assayed in vitro, has also been observed after estradiol stimulation in vivo.

The incorporation of tritium-labeled uridine into nuclear RNA has also been used as a means of following the turnover of rapidly synthesized RNA from the nucleus to the cytoplasm with time. Estradiol injected into ovariectomized rats not only increased the rate of synthesis of nuclear RNA but accelerated the entry of the newly formed RNA into the cytoplasm and restored synthesis and transport of RNA to that found in the normal mature rat. The effect of estradiol on the synthesis of nuclear, mitochondrial, microsomal, and cytosol protein cannot be causally related to the synthesis and distribution of nuclear RNA but follows it in logical sequence. Study of the rate of incorporation of isotopically labeled amino acids into subcellular fractions following estradiol administration has shown that after a reduced incorporation lasting from 0.5–1.5 hours, there is a marked stimulation of protein synthesis in both nuclear and microsomal fractions. The increased rate of synthesis in the nuclei precedes that in the microsomes but the effect in the cytoplasm is substantial and prolonged; at 24 hours protein synthesis in this component is still raised while that in the nuclei has returned to normal values.

It has not been possible to establish the character of the RNA and protein formed as a result of estradiol stimulation and hence it is not known whether these represent new cellular components or an increased rate of synthesis of normal metabolites. Although the details of the precise mechanism of action of the hormone on the uterus is unknown, the following sequence of events within the cell has been recognized; the binding of estradiol to the nuclear receptor, the accelerated synthesis of nuclear and ribosomal RNA, the accelerated transport of RNA principally to the polyribosomes of the cytoplasm, and the increased synthesis of protein. These contemporary ideas still conform to the earlier concept of a preliminary stage

involving interaction of the hormone with a specific cellular receptor, the initiation of new biochemical events, and the amplification of these events, possibly by the release of genetic information, to gross physiological change.

The Determination of Estrogens in the Picogram Range

The high specificity of the binding of the estrogens to the 8S cytoplasmic receptor in vitro provides excellent conditions for the assay of estrogens by competitive binding and has made possible the determination of estrogens in the picogram range (114, 115). The dissociation constants for the equilibrium between this receptor and the natural estrogens are so low that for many purposes rabbit uterine supernatant fluid can be used in assay procedures without purification. The affinity of binding of stilbestrol is greater than that of the natural estrogens and this compound can also be determined at very low concentration (114). All estrogens bind to the same site and preliminary separation is necessary before assay but the sensitivity is so high that plasma estrogen levels throughout menstrual and estrous cycles can be measured (114, 115). Such methods open up new possibilities in the field of obstetrics and gynecology.

Anti-estrogens.—The low molecular weight estrogens are not antigenic, but when they are covalently linked to macromolecules, the complex may be used to form antibodies which show some specificity towards the estrogen so linked (116). Estradiol has been joined at C-17β via a succinyl link to bovine serum albumin (BSA) and the resulting compound when injected into sheep produced antibodies to BSA and to the complex (117). From the sheep serum it has been possible to prepare a γ-globulin fraction which forms complexes with estradiol and cross reacts with related estrogens. Vande Wiele (118) has shown that such preparations may be used in rats as an antiestrogen, combining with and removing from circulation endogenous estrogens. When administered up to 15 hours before the ovulatory period, this preparation inhibited the release of luteinizing hormone and suppressed ovulation. The administration of stilbestrol, which did not cross react with the anti-estrogenic preparation, restored normal ovulation (118).

In a second application, the antibodies to estrogens have been successfully adsorbed onto the wall of polypropylene tubes which have thus been converted into an insoluble but specific binding surface for estrogens (119). These tubes have been used as a medium for the determination of picograms of the natural estrogens by radio-immune assay. The antibody to the estradiol complex cross reacts with other natural estrogens which can be determined after preliminary separations. Measurement of plasma estradiol levels, whether carried out by competitive binding or by radio-immune assay, show peak concentration at ovulation more or less coinciding with the maximum concentration of luteinizing hormone. A smaller peak of estradiol concentration occasionally observed later in the cycle has been variously as-

cribed to estrogen production by the mature corpus luteum or to peripheral conversion of androstenedione to estrone.

A satisfactory theory of the action of estrogenic compounds must be able to explain why so many molecular structures display this activity. There is now evidence that the ability to bind to the cytosol receptor is a preliminary requirement for cell response and that the presence of this receptor differentiates hormone-dependent and nonresponsive tissue. It is possible that active compounds induce some allosteric change in either the cytoplasmic or nuclear receptor and that this modified receptor is a common factor in the process which leads to estrogenic change.

LITERATURE CITED

1. Uskoković, M., Gut, M. 1957. *J. Org. Chem.* 22:996–98
2. Dorfman, R. I., Ungar, F. 1965. *Metabolism of Steroid Hormones* N.Y.: Academic
3. Sjövall, J., Vihko, R. 1966. *Proc. 2nd. Int. Congr. Hormonal Steroids.* Milan. 210–15
4. Jensen, E. V. 1964. Proc. 2nd. Int. *Congr. Endocrinol.* Amsterdam: Excerpta Med. Found. 420 pp.
5. Diczfalusy, E. 1962. *Acta. Obstet. Gynec. Scand.* 41: Suppl. 1. 1–143
6. Diczfalusy, E. 1964. *Fed. Proc.* 23: 791–98
7. Loraine, J. A., Bell, E. T. 1966. *Human Chorionic Gonadotrophin in Hormone Assays and their Clinical Application.* 84–113. Edinburgh: Livingstone
8. Brown, J. B. 1955. The Technique and Significance of Oestrogen Determination. *Mem. Soc. Endocrinol.* 3:1–10
9. Meyer, A. 1955. *Experientia,* 11:99–102
10. Mikhail, G., Wu, C. H., Ferin, M., Vande Wiele, R. L. 1970. *Steroids* 15:333–52
11. Breuer, H. 1962. *Vitam. Horm.* 20: 285–335
12. Meyer, A. 1955. *Biochim. Biophys Acta* 17:441–42
13. Goodwin, T. W. 1960. 'Biosynthesis of Terpenes' and Related Compounds' *Recent Advan. Biochem.* 228–77
14. Ryan, K. J. 1959. *J. Biol. Chem.* 234:268–72
15. Starka, L., Breuer, J., Breuer, H. 1965. *Naturwissenschaften* 52: 540–1
16. Stitch, S. R., Oakey, R. E., Eccles, S. S. 1963. *Biochem. J.* 88:70–6
17. Breuer, H., Grill, P. 1961. *Z. Physiol. Chem.* 324:254–61
18. Morato, T., Hayano, M., Dorfman, R. I., Axelrod, L. R. 1961. *Biochem. Biophys. Res. Comm.* 6: 334–8
19. Talalay, P. 1957. *Physiol. Rev.* 37–362–89
20. Bolte, E., Mancuso, S., Eriksson, G., Wiqvist, N., Diczfalusy, E. 1964. *Acta Endocrinol.* (Khb.) 45:535–59
21. Breuer, H., Knuppen, R. 1969. Enzymes in Estrogen Metabolism. *Methods Enzymol.* 691–735
22. Gual, C., Morato, T., Hayano, M., Gut, M., Dorfman, R. I. 1962. *Endocrinology* 71:920–25
23. Starka, L., Breuer, H., Cedard, L. 1966. *J. Endocrinol.* 34:447–56
24. Starka, L., Breuer, H. 1966. *Biochim. Biophys. Acta* 115:306–11
25. Luukkainen, T., Adlercreutz, H. 1965. *Biochim. Biophys. Acta,* 107:579–92
26. Knuppen, R., Haupt, O., Breuer, H. 1967. *Biochem. J.* 105:971–78
27. Jarabak, J., Adams, J. A., Williams-Ashman, H. G., Talalay, P. 1962. *J. Biol. Chem.* 237:345–57
28. Meigs, R. A., Ryan, K. J., Petro, Z., Morrison, G. 1963. *Science* 142: 243–44
29. Cooper, D. Y., Narasimbulu, S., Rosenthal, O., Estabrook, R. W. 1968. *J. Biol. Chem.* 241:4011–15
30. Meigs, R. A., Ryan, K. J. 1966. *Function of the Adrenal Cortex* 2:897. N.Y.: Appleton
31. Breuer, H., Ozon, R., Mittermayer, C. 1963. *Z. Physiol. Chem.* 333: 272–81
32. Döllefeld, E., Breuer, H. 1966. *Biochim. Biophys. Acta* 124:187–89.
33. Hecker, E., Marks, F. 1965. *Bio-*

chem. Z. 343 :211–26
34. Marks, F., Süss, R. Morris, H. P. Hecker, E. 1967. *Krebsforschung* 69 :361
35. Fishman, J., Hellman, L., Zumoff, B, Gallagher, T. F. 1965. *J. Clin. Endocrinol.* 25 :365–68
36. Hecker, E., Walk, E. 1960. *Chem. Ber.* 93 :2928–37
37. Breuer, H., Vogel, W., Knuppen, R. 1962. *Z. Physiol. Chem.* 327 :217–24
38. Knuppen, R., Breuer, H. 1966. *Z. Physiol. Chem.* 346 :114–22
39. Breuer, H., Knuppen, R., Haupt, O. 1970. *Biochem. J.* 118 :9P
40. Smith, E. R., Kellie, A. E. 1967. *Biochem. J.* 104 :83–94
41. Beling, C. G. 1963. *Acta. Endocrinol.* (Kbh) 43 : Suppl. 79 :1–98
42. Noramly bin Muslim, 1970. *The Structure of Oestradiol Conjugates and Related Compounds of Human Pregnancy Urine.* Ph.D. Thesis, Univ. Western Australia.
43. Hähnel, R., Ghazali, M. 1967. *Biochem. J.* 105 :1047–53
44. Wilson, R., Eriksson, G., Diczfalusy, E. 1964. *Acta Endocrinol.* (Khb) 46 :525–43
45. Dutton, G. J., Storey, I. D. E. 1954. *Biochem. J.* 57 :275–83
46. Dahm, K., Lindlau, M., Breuer, H. 1967. *Acta Endocrinol.* (Kbh) 56 : 403–12
47. Dahm, K., Breuer, H. 1966. *Z. Klin. Chem.* 4 :153
48. Dahm, K., Breuer, H., Lindlau, M. 1966. *Z. Physiol. Chem.* 345 :139–49
49. Slaunwhite, W. R., Lichtman, M. A., Sandberg, A. A. 1964. *J. Clin. Endocrinol.* 24 :638–43
50. Diczfalusy, E., Franksson, C., Martinsen, B. 1961. *Acta Endocrinol.* (Kbh) 38 :59–72
51. Dahm, K., Breuer, H. 1966. *Acta Endocrinol.* (Khb) 52 :43–53
52. Dahm, K., Breuer, H. 1966. *Biochim. Biophys. Acta* 128 :30–36
53. Nose, Y., Lipmann, F. 1958. *J. Biol. Chem.* 233 :1348–51
54. Adams, J. B., Chulavatnatol, M. 1967. *Biochim. Biophys. Acta* 146 :509
55. Layne, D. S., Sheth, N. A., Kirdani, R. Y. 1964. *J. Biol. Chem.* 239 : 3221–25
56. Adams, J. B., Poulos, A. 1967. *Biochim. Biophys. Acta* 146 :493–508
57. Jellinck, P. H., Lewis, J., Boston, F. 1967. *Steroids* 10 :329–46
58. Kuss, E. 1967. *Z. Physiol. Chem.* 348 :1707–8
59. Elce, J. S. 1970. *Biochem. J.* 116 : 913–17
60. Kellie, A. E. 1961. *J. Endocrinol.* 22 :i
61. Diczfalusy, E. 1964. *Fed. Proc.* 23 : 791–98
62. Diczfalusy, E. 1969. *Acta Endocrinol.* (Kbh) 61 :649–64
63. Brown, J. B. 1960. *Advan. Clin. Chem.* 3 :151–233
64. Klopper, A., Michie, E. A., Brown, J. B. 1955. *J. Endocrinol.* 12 :209–19
65. Diczfalusy, E., Lauritzen, C. 1961. *Estrogens in the Human.* Berlin : Springer.
66. Diczfalusy, E. 1969. *Acta Endocrinol.* (Kbh) 61 :649–64
67. Solomon, S., Bird, C. E., Ling, W., Iwamiya, M., Young, P. C. 1967. *Recent Progr. Hormone Res.* 23 : 297–347
68. Hellig, H., Lefevre, Y., Gattereau, D., Bolte, E. 1968. *Int. Congr. Ser.* 170 :78 Amsterdam : Excerpta Med. Found.
69. Bolte, E., Wiqvist, N., Diczfalusy, E. 1969. *Acta Endocrinol.* (Kbh) 52 :583–97
70. Pion, R., Jaffe, R., Wiqvist, N., Diczfalusy, E. 1967. *Biochim. Biophys. Acta* (Amsterdam) 137 :584–87
71. Palmer, R., Eriksson, G., Wiqvist, N., Diczfalusy, E. 1966. *Acta Endocrinol.* (Kbh) 52 :598–606
72. Lamb, E., Mancuso, S., Dell'Acqua, S., Wiqvist, N., Diczfalusy, E. 1967. *Acta Endocrinol.* (Kbh) 55 :263–77
73. Mikhail, G., Wiqvist, N., Diczfalusy, E. 1967. *Acta Endocrinol.* (Kbh) 42 :519–32
74. Levitz, M., Condon, G. P., Dancis, J., Goebelmann, U., Eriksson, G., Diczfalusy, E. 1967. *J. Clin. Endocrinol.* 27 :1723–29
75. Benagiana, G., Mancuso, F. P., Mancuso, S., Wiqvist, N. Diczfalusy, E. 1968. *Acta Endocrinol.* (Kbh) 57 :187–207
76. Schwers, J., Govaerts-Videtsky, M., Wiqvist, N. Diczfalusy, E. 1965. *Acta Endocrinol.* (Kbh) 50 :597–610
77. Jackanicz, T. M., Diczfalusy, E. 1968. *Steroids* 11 :877–84
78. Kirschner, M. A., Wiqvist, N., Diczfalusy, E. 1966. *Acta Endocrinol.* (Kbh) 53 :584–97
79. Reynolds, J. W., Mancuso, S., Wi-

qvist, N., Diczfalusy, E. 1968. *Acta Endocrinol.* (Kbh) 58:377–95
80. Brown, J. B. 1962. *Recent Progr. Hormone Res.* 18:337–85
81. Karim, M. F., Taylor, W. 1970. *Biochem. J.* 117:267–70
82. Ferin, M., Zimmering, P. E., Vande Wiele, R. L. 1969. *Endocrinology* 84:893
83. Jensen, E. V., Jacobson, H. I. 1962. *Recent Progr. Hormone Res.* 18: 387–414
84. Miescher, K. 1944. *Helv. Chim. Acta* 27:1727–35
85. Horeau, A., Jacques, J. 1947. *C. R. Acad. Sci.* (Paris) 224:862–64
86. Dodds, E. C. 1955. Hormones in Reproduction, *Brit. Med. Bull.* 11:131–34
87. Robson, J. M., Schönberg, A. 1937. *Nature* (London) 140:196
88. Callantine, M. R., Clemens, L. E., Shih Yu-hsin, 1968. *Proc. Soc. Exp. Biol. Med.* 128:382–86
89. Terenius, L. 1968. *Acta Pharmacol. Toxicol.* 26:15–21
90. Bradbury, R. B., White, D. E. 1951. *J. Chem. Soc.* 3447–49
91. Leavitt, W. W., Meismer, D. M. 1968. *Nature* (London) 218:181–82
92. Szego, C. M., Roberts, S. 1953. *Recent Progr. Hormone Res.* 8: 419–69
93. Mueller, G. C., Herranen, A. M., Jervell, K. F. 1958. *Recent Progr. Hormone Res.* 14:95–139
94. Mueller, G. C., Gorski, J., Aizawa, Y. 1961. *Proc. Nat. Acad. Sci. U.S.* 47:164–69
95. Glasscock, R. F., Hoekstra, W. G. 1959. *Biochem. J.* 72:673–82
96. Jensen, E. V., Jacobson, H. I., Flesher, J. W., Saha, N. N. Gupta, G. N. et al. 1966. 'Estrogen Receptors in Target Tissues.' In *Steroid Dynamics,* Ed. T. Nakao, G. Pincus, J. Tait, N.Y.: Academic
97. Jensen, E. V. 1965. *Proc. 2nd Int. Congr. Endocrinol.* Lond., Amsterdam: Excerpta Med. Found. 420 pp.
98. Gorski, J., Toft, D., Shyamala, G., Smith, D., Notides, A. 1968. *Recent Progr. Hormone Res.* 24: 45–80
99. Toft, D., Gorski, J. 1966. *Proc. Nat. Acad. Sci. U.S.* 55:1574–80
100. King, R. J. B., Gordon, J., Cowan, D. M., Inman, D. R. 1966. *J. Endocrinol.* 36:139–50
101. Jensen, E. V., DeSombre, E. R., Jungblut, P. W., Stumpf, W. E., Roth, L. J. 1969. *Autoradiography of Diffusable Substances.* El. L. J. Roth, W. E. Stumpf, Academic 81 pp.
102. Stumpf, W. E., Roth, L. J. 1966. *J. Histochem. Cytochem.* 14:274–87
103. Jensen, E. V., DeSombre, E. R., Jungblut, P. W. 1966. *Proc. 2nd Steroid. Congr.* Milan; Excerpta Med. Int. Congr. Ser. III
104. Toft, D., Shyamala, G., Gorski, J. 1967. *Proc. Nat. Acad. Sci. U.S.* 57:1740–43
105. DeSombre, E. R., Puca, G. A., Jensen, E. V. 1969. *Proc. Nat. Acad. Sci. U.S.* 64:148–54
106. Soloff, M. S., Szego, C. M. 1969. *Biochem. Biophys. Res. Comm.* 34:141–47
107. Jensen, E. V., DeSombre, E. R. 1969. *Biochem. J.* 115:28P
108. Shyamala, G., Gorski, J. 1969. *J. Biol. Chem.* 244:1097–1103
109. Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. et al. 1968. *Proc. Nat. Acad. Sci. U.S.* 59:632–38
110. Rochefort, H., Baulieu, E-E. 1969. *Endocrinology* 84:108–16
111. Meśter, J., Robertson, D. M., Feherty, P., Kellie, A. E. 1971. *Biochem. J.* (in press)
112. Feherty, P., Robertson, D. M., Waynforth, H. B., Kellie, A. E. 1971. *Biochem. J.* (in press)
113. Hamilton, T. H. 1968. *Science* 161: 649–61
114. Korenman, S. G. 1968. *J. Clin. Endocrinol.* 28:127–30
115. Corker, C. S., Exley, D. 1970. *Steroids* 15:469–83
116. Erlanger, B., Borek, F., Beiser, S. M., Lieberman, S. 1959. *J. Biol. Chem.* 234:1090–94
117. Lieberman, S., Erlanger, B. F., Beiser, S., Agate, F. 1959. *Recent Progr. Hormone Res.* 15:165–200
118. Vande Wiele, R., Ferin, M., Raziano, J., Dyrenfurth, I. 1970. International Study Group for Steroid Hormones, *Research on Steroids* 4:(in press)
119. Abraham, G. E. 1969. *J. Clin. Endocrinol.* 29:866–70