Methods in Hormone Research

VOLUME III

Steroidal Activity in Experimental Animals and Man Part A

METHODS IN HORMONE RESEARCH

Volume I: Chemical Determinations

Volume II: Bioassay

Volume III: Steroidal Activity in Experimental Animals and Man Part A

Volume IV: Steroidal Activity in Experimental Animals and Man Part B

Methods in Hormone Research

Edited by

RALPH I. DORFMAN

The Worcester Foundation for Experimental Biology
Shrewsbury, Massachusetts

VOLUME III

Steroidal Activity in Experimental Animals and Man
Part A

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PREFACE

Volumes I and II of this series have dealt with methods for the chemical and biological determination of hormones and related compounds. In considering the bioassay of hormones in Volume II, attention was focused on the more usual activities of steroid hormones. During the past years a considerable number of so-called "nonhormonal" activities of steroids have been discovered and this volume, the third in the series, presents discussions of these newer methods. Specifically, this volume presents reviews of the methods dealing with the following types of activities not considered previously: protection against irradiation damage, anti-mammary tumor activity, influence on body lipids, anti-aldosterone, central depressant action, and the copulatory reflex activity.

A second feature of the present work has been the documentation of the many steroids studied for these nonhormonal activities and for certain hormonal activites as well. This information is vital for many facets of hormone and steroid research, from the more practical details of fashioning more valuable therapeutic agents to supplying the biological data on the activities of steroids which form the very bases for an understanding of structure-activity relationships and of mechanisms of steroid hormone action.

The richness of bioassay information makes it impossible to have a presentation of all the available information in a single volume. Since a second volume is needed, Volume IV will bear the same subtitle as this volume—Steroidal Activity in Experimental Animals and Man, Part B.

Again I acknowledge with thanks the gracious cooperative effort of the many who made this volume possible. A hearty thanks to the many contributors who labored so long and so well; to Mrs. Iola Graton, Mrs. Elaine Joseph, and Mrs. Madeline Daley, who efficiently and cheerfully put all things right with the spirit "above and beyond the call of duty," and to the staff at Academic Press.

RALPH I. DORFMAN

Shrewsbury, Massachusetts
July 1964

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STEROIDAL ACTIVITY IN EXPERIMENTAL ANIMALS AND MAN, PART B

Chapter 1

Estrogens

C. W. EMMENS AND L. MARTIN

I. Types of Estrogen

A. Natural Estrogens

The three steroid estrogens about which much more is known than any others are estradiol-17β, estrone, and estriol. These substances, together with a series of more recently discovered estrogens such as 16β-hydroxyestrone (Layne and Marrian, 1958), are characteristic of the human. The biological activities of the newer human estrogens have, however, been poorly studied to date.

Estradiol-17β [Fig. 1 (1)] is the most potent natural estrogen according to practically all methods of testing and appears to be a precursor in at

least the human of the other steroidal estrogens. It seems likely that it is the only estrogen produced in quantity by the human ovary and probably by that of a number of other mammals. However, it is also produced by the adrenals, placenta, and, in the stallion, by the testes. Little appears in the urine of the nonpregnant human, but the quantity rises, perhaps 100-fold, in late pregnancy. At the ovulation peak of the menstrual cycle, estrogen excretion suddenly falls—the level of estradiol-17β falls together with that of estrone, but prior to that of estriol by about 24 hours (Merrill, 1958). In adrenal cortical tumors (Diczfalusy and Luft, 1952; Landau *et al.*, 1954) and uterine carcinoma (Belassi and Ricca, 1951), estradiol excretion increases, together with that of the other estrogens. Estradiol-17α is found in the urine of pregnant mares and is of much lower estrogenic potency than its stereoisomer.

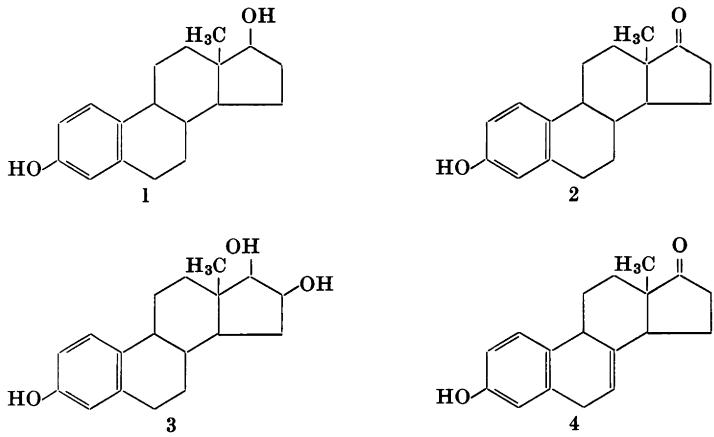

FIG. 1. Four important natural estrogens: (1) estradiol; (2) estrone; (3) estriol; (4) equilin. From Emmens (1959).

Estrone [Fig. 1 (2)] is found in the urine and in adrenal and placental extracts. The ratio of the potency of estrone to estradiol-17β varies a great deal with test methods; parenteral routes of administration in general give a decidedly lower potency to estrone. By intravaginal administration in the mouse, however, it is not much less potent than estradiol-17β; various test methods give ratios of about 0.67 (Emmens, 1962a). The slopes differ for the two compounds in such tests, so that the estimate is approximate. The excretion of estrone parallels that of estradiol-17β in most urine, but at a higher absolute level. This is at a ratio of about 3:1 in pregnancy (Brown, 1956), postmenopausal women (Brown, 1955a), and the male (Cameron, 1957). The ratio is somewhat lower in the various phases of the menstrual cycle (Bauld, 1956; Brown,

1955b) and is highly variable in abnormal states such as liver disease and adrenal tumors (Cameron, 1957; Landau *et al.*, 1954).

Estriol [Fig. 1 (3)] is an estrogen peculiar to primates and also, as far as is known, the pussy willow (Skarzynski, 1934). In nearly all reported instances it has been isolated from human sources, including urine and placentas. It is both less active than and different in action from estradiol-17β and usually from estrone. In vaginal smear tests by parenteral injection, the more water-soluble estriol is very much less potent than the above estrogens unless it is given by multiple dosage. In such tests by local administration, its potency is raised to that of estrone only under optimal conditions of testing (Biggers and Claringbold, 1954; Martin and Claringbold, 1960; Martin, 1960), but is still below that of estradiol-17β. In uterine weight tests, estriol is the prototype of the so-called "impeded" estrogens (Huggins and Jensen, 1955), with dose-response lines attaining only part of the total responses seen for estradiol-17β or estrone. Both estriol and estrone are formed in the body from estradiol-17β and are excreted in the urine mainly as sulfates and glucuronides (Marrian, 1948). Estriol is much increased in pregnancy, up to 1000-fold in the urine, when it is almost certainly manufactured by the placenta. At the end of pregnancy, urinary estriol glucuronide falls very abruptly but free estriol increases. It is not established whether parturition is affected by this change, which is observed only in the human.

Equine steroids, including *equilin* [Fig. 1 (4)] and *equilenin*, are peculiar as far as is known to the horse family and are found in the urine of pregnant mares. No very recent studies of their activity seem to be available.

B. Natural Proestrogens

There is every reason to believe that the estrogens mentioned above are themselves active without further change, except perhaps in the case of "impeded" estrogens in the uterus. Other so-called proestrogens, however, are almost certainly not estrogenic, but their apparent activity is due to metabolites (Emmens, 1941b). Yet other estrogens are of one class or the other only by inference. The weakly estrogenic androgens would seem to fall into the class of proestrogens, thus androst-5-ene-3β,17β-diol is proestrogenic (Emmens, 1941b) while testosterone itself gives rise to estrogenic metabolites (Callow *et al.*, 1939). The extent to which such an androgen exhibits biological estrogenicity is probably dependent on the ratio in particular tests of its androgenic potency to the estrogenic potency of the metabolites in question.

C. Synthetic Steroidal Estrogens

A considerable number of steroids with estrogenic properties have been synthesized. Usually such compounds have other activity as well; indeed, the great majority have been made with other activities in mind, and have turned out to have estrogenic action also. These compounds, such as ethynyltestosterone, the various derivatives of 19-nortestosterone including norethynodrel, are usually proestrogens or perhaps in the odd instance contaminated with a proestrogen or even an estrogen. In most cases little has been done in the way of comparative studies or accurate potency determinations, and potency is usually low.

D. Esters

The effects of esterification in prolonging the effects of steroids were studied extensively with androgens by Miescher *et al.* (1936). In a series of aliphatic esters of testosterone, the longer the ester chain, the more prolonged the effect, and the higher the median effective dose. Esters of estradiol-17β were investigated by Parkes (1937) using the breast feathers of the Brown Leghorn capon, which turn from black to fawn under estrogenic stimulation, and grow at about 2 mm. per day. The length of the feminized zone thus gives an index of prolongation of action. With free estradiol-17β, high doses gave no more than 2 days feminization, which was maximal at about 2 mg. A single injection of 1 mg. of estradiol -17β diacetate feminized the plumage for 6 days, that of the 3-benzoate-17-acetate for 19 days, and that of the monobenzoate for 12 days. Intravenous injection of the monobenzoate gives no prolongation of action (Deanesly and Parkes, 1937), since the prolongation depends on a delay in absorption from the site of injection. There is similarly no prolongation by oral administration in the mouse (Emmens, 1939b) although monoesters and diesters of various natural and synthetic substances are prolonged in action on the vaginal smear (Emmens, 1939b; Dodds *et al.*, 1938). By the intravaginal route in the mouse (Emmens, 1941b), esterification is again without effect, except that estriol glucuronide and estrone sulfate are probably inactive by that route (Emmens, 1962a); neither compound, however is strictly speaking an ester.

Considerable attention was paid to the biological assay of esters in the early days, and a special standard, estradiol-17β monobenzoate, was set up for comparison with them. It soon became apparent that its use was limited to comparison with other samples of the same compound. The problem has been solved by the production of pure characterized

substances for normal use, which do not need to be assayed biologically in ordinary circumstances.

Diczfalusy (1954) and Diczfalusy *et al.* (1953) have described long-chain water-soluble polymeric phosphoric esters of estradiol-17β. These compounds, although only weakly active in conventional Allen-Doisy tests, prolong the duration of vaginal cornification in spayed mice to an extent very much greater than that produced by the same dosage of estradiol-17β-3-monobenzoate or ethynylestradiol-17β. Duration of action, which may be up to 30 days or more, is proportional to the molecular weight, and presumably chain length of the compounds. They are not active orally.

II. Relative Potencies in Vaginal Smear Tests

A. Parenteral Tests

Most of the early testing of estrogenic activity employed the vaginal smear technique in ovariectomized rats or mice. It soon became apparent that relative potencies were very highly influenced by technique. Emmens (1939a) and Pedersen-Bjergaard (1939) published monographs in which the information to date was summarized and drew similar conclusions from it and from their own experiments. These inferences were, that it was at that time impossible to draw meaningful conclusions from attempts at assaying other steroids in comparison with the recently established international standard estrone, which could only be employed for the standardization of estrone itself, and similarly for the other new standard, estradiol monobenzoate, as mentioned above.

There were quite large discrepancies in the biological unit of international standard estrone. This, indeed, was why the standard had been set up. The differences are illustrated in Table I from Pedersen-Bjergaard (1939). It will be seen that even apparently very similar techniques in the hands of different investigators gave up to 12-fold differences in the biological unit, as with four aqueous injections in the spayed mouse.

When it came to actual assays, the situation was worse, particularly with estriol. Tables II and III, from Emmens (1939a), have been reprinted a number of times, but still serve to illustrate the state of affairs as well as any other data since collected. It is seen that according to the particular author and test method used, the ratio of potencies of estradiol-17β to estrone varied between 0.8 and 12 [omitting the so-called β-form of Whitman *et al.* (1937), which would correspond to the less potent present-day estradiol-17α]. With estriol, however, the variation

was over a 250-fold range, from 1 to 0.004. Even within one experimenter's results, as much variation is found. Thus, in Table IV, from Pedersen-Bjergaard (1939), we find the relative potency of estradiol

TABLE I

SOME BIOLOGICAL UNITS FOR ESTRONE, INTERNATIONAL STANDARD[a]

Spayed Mice	(μg.)
Whole dose given in one oily injection:	
Doisy[b]	0.05
Butenandt[b]	0.05–0.1
Dose divided into three or four oily injections:	
Schoeller *et al.* (1935)	0.5
Hain and Robson (1936)	0.09
Dose divided into four aqueous injections:	
Doisy[b]	0.02
Rowlands and Callow (1935)	0.047
Parkes[b]	0.061
Marrian[b]	0.063
Butenandt[b]	0.05–0.1
Hain and Robson (1936)	0.250
Spayed Rats	
Whole dose given in one oily injection:	
Burn[b]	0.96
D'Amour and Gustavson (1936)	1.30
Girard[b]	ca. 4.15
Dose divided into four oily injections:	
D'Amour and Gustavson (1936)	1.5
Hain and Robson (1936)	3.3
Dose divided into three or four aqueous injections:	
Doisy[b]	0.70
D'Amour and Gustavson (1936)	0.74
Meyer *et al.* (1936)	0.80
Girard[b]	ca. 1.84
Hain and Robson (1936)	2.5

[a] From Pedersen-Bjergaard (1939).

[b] Unpublished observations in 1939.

varying from 0.08 (mice, single injection) to 7.6 (rats, 5 aqueous injections) or even 8.0 if oral administration in guinea pigs is included for the moment. The relative potency of estriol varied from 0.0049 (mice, 5 aqueous injections) to 0.062 (rats, 5 aqueous injections) in the only two

TABLE II

THE RATIO OF POTENCIES OF ESTRIOL AND ESTRONE FOUND BY DIFFERENT INVESTIGATORS WORKING WITH OVARIECTOMIZED RATS IN THE ALLEN-DOISY TEST[a]

Number and nature of injections	Estrone: estriol ratio	Reference
3 Aqueous	250	Meyer *et al.* (1936)
3 Aqueous	2	Curtis and Doisy (1931)
4 Aqueous	2	Cohen and Marrian (1934)
4 Aqueous	1	Burn and Elphick (1932)
1 Oily	4.5	Burn and Elphick (1932)
1 Oily	100	Butenandt and Stormer (1932)
3 Oily	90	Meyer *et al.* (1936)
? Oily	2	Marrian (1930)

[a] From Emmens (1939a).

TABLE III

THE RATIO OF POTENCIES OF ESTRADIOL AND ESTRONE FOUND BY DIFFERENT INVESTIGATORS USING OVARIECTOMIZED RATS OR MICE IN THE ALLEN-DOISY TEST[a]

Animals	Number and nature of injections	Estradiol: estrone ratio	Reference
Rats	3 Oily	6	Schoeller *et al.* (1935)
Rats	6 Aqueous	7	Schoeller *et al.* (1935)
Mice	3 Oily	0.8	Schoeller *et al.* (1935)
Mice	6 Aqueous	3	Schoeller *et al.* (1935)
Rats	1 Oily	3	David *et al.* (1935)
Mice	1 Oily	2	David *et al.* (1935)
Mice	3 Oily	2	David *et al.* (1935)
Mice	6 Aqueous	2	David *et al.* (1935)
Mice	5 Oily	5–10	Dirscherl (1936)
Rats	3 Oily (β-form)[b]	12	Whitman *et al.* (1937)
Rats	3 Oily (α-form)	0.3	Whitman *et al.* (1937)

[a] From Emmens (1939a).

[b] Modern nomenclature.

examples of injection quoted. With oral administration in rats it rose to 8.8. Emmens (1939a) found that the relative potency of estriol in mice rose from 0.014 to 0.42 with a change from two to four oily injections.

A more recent repetition of this type of finding is available from Zondek and Sulman (1951), in a comparison of the estrogenic action of estriol in infantile intact, infantile castrate, and adult castrate mice and rats. The figures are shown in Table V and exhibit a 10-fold variation in potency within comparable animals according to the vehicle of administration. These investigators indicate that the ovary of the infantile rat

TABLE IV

EFFECTS OF ROUTE AND FREQUENCY OF ADMINISTRATION ON POTENCY IN DIFFERENT SPECIES[a]

Method of assay	MED (μg.)		
	Estradiol-17β	Estriol	Estrone
Mice, 5 aqueous injections	0.100	39	0.19
Mice, 1 oily injection	4.4	—	0.35
Mice, 5 oral administrations	7.5	26	11.7
Rats, 5 aqueous injections	0.145	18	1.11
Rats, 1 oily injection	5.2	—	2.76
Rats, 5 oral administrations	90	25	220
Guinea pigs, 5 aqueous injections	630	—	460
Guinea pigs, 1 oily injection	280	—	600
Guinea pigs, 5 oral administrations	1,120	—	8,900
Monkeys, 1 oily injection	1,000	—	1,000
Monkeys, 1 oral administration	20,000	—	5,000

[a] From Pedersen-Bjergaard (1939).

or mouse is not important in converting estriol to estrone or other active compound, but leave open the question of the adult ovary's possible activity.

The duration of action of various estrogens given by injection in ovariectomized mice was studied by Emmens (1939b), who showed that esterification of diethylstilbestrol resulted in increasing duration of effect of minimally effective doses as the ester chain increased in length, and of even greater prolongation of action if multiples of the lowest effective dose were given. Only a few steroid estrogens were studied and showed the same effect. Estrone methyl ether was also somewhat prolonged in action at low multiples of the lowest effective dose. The data

TABLE V

ESTROGENIC EFFECTS OF ESTRIOL IN SPAYED AND INTACT FEMALE MICE AND RATS (49)[a]

	Weight, gm.	Vehicle[b]	Estrogenic Action
Mice:			
Adult castrate	20–25	water	10
Infantile intact	12–15	water	10
Adult castrate	20–25	oil	1
Infantile intact	12–15	oil	1
Rats:			
Adult castrate	150–160	water	200
Infantile castrate	30–35	water	100
Infantile intact	30–35	water	100
Adult castrate	150–160	oil	15
Infantile castrate	30–35	oil	10
Infantile intact	30–35	oil	10

[a] From Zondek and Sulman (1951).

[b] Water vehicle contains 0.01 *N* NaOH + 10% ethyl alcohol; oil is olive oil.

TABLE VI

THE DURATION OF ACTION, IN DAYS, OF VARIOUS ESTROGENS WHEN GIVEN BY SUBCUTANEOUS INJECTION TO OVARIECTOMIZED MICE[a]

Substance	Lowest effective dose (μg.)	Dose (as multiple of lowest effective dose)									
		1	2.5	5	10	20	40	100	200	2000	8000
Diethylstilbestrol	0.1	2	1	2	—	—	—	5	7	—	14
Diethylstilbestrol dipropionate	0.25	6	5–6	6	6	9	17	—	—	—	—
Diethylstilbestrol dibenzoate	20.0	21	29	34	30	28	—	—	—	—	—
Diethylstilbestrol dipalmitate	50.0	18	30	38	—	—	—	—	—	—	—
Diethylstilbestrol dimethyl ether	5.0	1	6	11	12	—	—	—	—	—	—
Estrone	0.1	½	—	—	—	4	4	—	—	7–8	—
Estrone methyl ether	1.25	1	1	4	6	6	14	—	—	—	—
Ethynyldihydroequilin	0.05	½	½–1	1	2	2	2	3	—	—	—
Ethynylestradiol-17β-di-*n*-butyrate	2.0	10	—	—	—	—	—	—	—	—	—

[a] From Emmens (1939b). Doses are shown in terms of the lowest effective dose of each preparation.

are given in Table VI. The same compounds given by mouth showed no such effects (Table VII), except for a somewhat lengthened action of estrone methyl ether.

In the spayed ewe, Robinson and Reardon (1961) have studied the relative potencies of a number of estrogens, both natural and synthetic, in causing both vaginal and behavioral estrus. The ewe exhibits these reactions only if a period of about 12 days on progesterone is followed by an injection or injections of the estrogen. This resembles the

TABLE VII

THE DURATION OF ACTION, IN DAYS, OF VARIOUS ESTROGENS WHEN GIVEN BY MOUTH TO OVARIECTOMIZED MICE[a]

Substance	Lowest effective dose (μg.)	Dose (as multiple of lowest effective dose)							
		1	2.5	5	10	20	40	200	3200
Diethylstilbestrol	0.5	½	—	—	—	2	3	—	7–8
Diethylstilbestrol dipropionate	0.5	½	1	2	2	2	3	—	—
Diethylstilbestrol dibenzoate	1.0	½	2	1	2	3	—	—	—
Diethylstilbestrol dipalmitate	1.0	½	½	1–2	3	3	2	—	—
Diethylstilbestrol dimethyl ether	2.5	2	2	1–2	4	4	3–4	—	—
Estrone	2.0	½	—	—	—	—	—	5	—
Estrone methyl ether	2.5	½	1–2	1	—	2	2	—	—
Ethynyldihydroequilin	2.5	½	1	1	—	—	—	—	—
Ethynylestradiol-17β-di-*n*-butyrate	0.5	½	1	1	—	—	—	—	—

[a] From Emmens (1939b). Doses are given in terms of the lowest effective dose of each preparation.

natural course of events, in which progesterone from the corpus luteum of the previous estrous cycle primes the animal to respond when a peak of estrogen secretion occurs at the time of ovulation. Robinson and Moore (1956) describe the detection of vaginal estrus in the sheep. The data are listed in Table VIII, and include the three natural estrogens of the human group, esters of estrone and estradiol, and for comparison, three common synthetics. The priming dose of progesterone was 20 mg. per ewe every 2 days for six injections, and the estrogens were given either as a single injection or in three injections spread over 24 hours, commencing 48 hours after the last injection of progesterone. All injections were subcutaneous. The MED's (median effective doses) follow

the same pattern as most results with rodents; estradiol is the most potent of the steroid group, followed by estrone and then estriol. Behavioral estrus parallels vaginal estrus remarkably closely.

In most instances with steroids, there was no difference between the effects of one as against three injections of estrogen, except where noted for behavioral estrus. The narrow limits of error, particularly for behavioral

TABLE VIII

MEDIAN EFFECTIVE DOSES, WITH 95% FIDUCIAL LIMITS, OF A NUMBER OF ESTROGENS TESTED IN THE EWE[a]

Estrogen	Vaginal response MED (μg.)	Behavioral response MED (μg.)
Class 1—Natural estrogens (free):		
Estradiol-17β	9.2–11.7–14.6	9.7–11.2–12.8[b]
Estrone	39.5–54.1–74.0	71.1–81.7–93.7[c]
Estriol	754.0–961.0–1241.0	877.0–985.0–1108.0
Class 2—Natural estrogens (conjugated):		
Estradiol-17β benzoate	3.7–6.6–11.6	13.5–14.9–16.4
	(2.8–5.0–8.7)	(10.1–11.1–12.3)
Estrone benzoate	17.0–33.3–65.3	90.0–100.3–111.9
	(12.8–25.0–49.0)	(67.2–74.9–83.6)
Class 3—Synthetic estrogens:		
Stilbestrol	3.6–8.2–18.6	23.2–27.5–32.7[b]
Hexestrol	6.5–12.7–25.9	21.0–30.5–44.5
Dienestrol	22.4–40.1–68.8	80.4–95.3–112.7[b]

[a] From Robinson and Reardon (1961). Figures in parentheses are values for estrogen component of conjugated molecules.

[b] Calculated from data for divided injections (× 3) to give maximum potency.

[c] Calculated from data for single injection (× 1) to give maximum potency.

estrus, are illustrated in Fig. 2. These are characteristic of responses in the spayed ewe, which gives decidedly greater precision per observation in such tests than do laboratory rodents.

The potencies of various steroids other than those listed above have been reported in many different ways and with varying degrees of precision. Interest today attaches mainly to the estrogenic activity of some of the newer synthetic steroids, particularly the norsteroids,

manufactured because of their potential progestational, anabolic, or other properties rather than as estrogens per se. Drill and Riegel (1958) give some informative data about various such steroids gathered in the course of a comprehensive program of manufacture and testing, but most of the determinations of estrogenic activity were made in conjunction with the chick lipid test and will be reported below. In vaginal

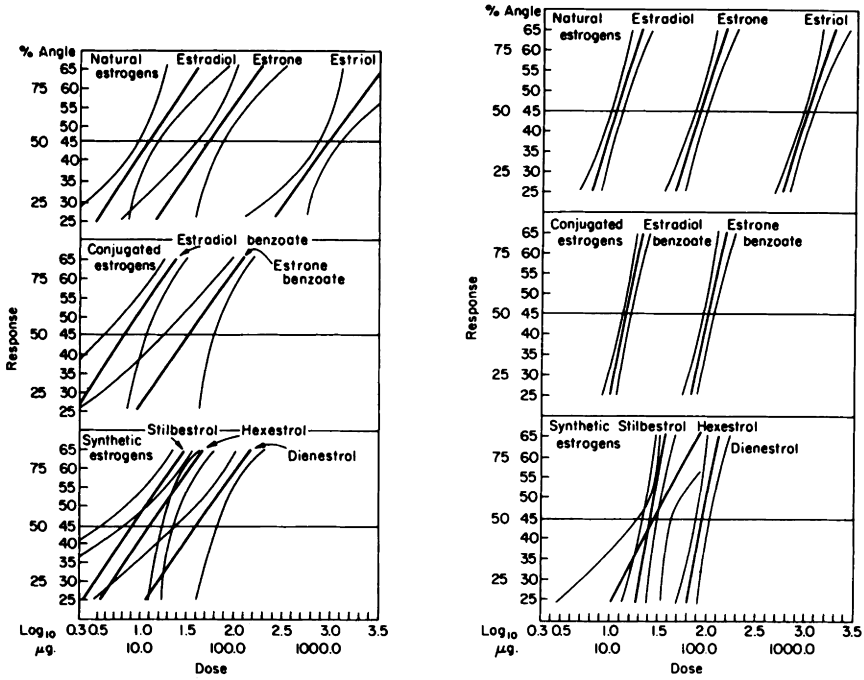

FIG. 2. Dose-response lines for vaginal estrus (on the left) and behavioral estrus (on the right) in the ewe, with 95% fiducial bands. From Robinson and Reardon (1961).

smear tests, 17-ethyl-19-nortestosterone and 17-ethynyl-19-nortestosterone were found to be without estrogenic activity, while 17α-ethynyl-17β-hydroxy-estr-5(10)en-3-one (norethynodrel) had 3–5% of the activity of estrone. Other reports are not entirely consistent with this. Emmens *et al.* (1960) found evidence of estrogenic activity in vaginal smear tests with 100 μg. intravaginal doses of 17-ethynyl-19-nortestosterone in the mouse, confirmed by other methods of testing, but not with corresponding doses of 17-ethyl-19-nortestosterone or 19-nortestosterone. The latter compound, however, gave positive estrogenic responses in both mitotic count and tetrazolium reduction tests (see below). A number of other workers have also reported 17-ethynyl-19-nortestosterone to be

weakly estrogenic (Saunders and Drill, 1956; McGinty and Djerassi, 1958; Pincus *et al.*, 1956; Martin and Cuningham, 1960).

It is of great interest that Katzman *et al.* (1960) report the activity of 17-epiestriol as greater than that of estradiol-17β in vaginal smear tests in rats and mice. Their results are shown in Table IX, which also

TABLE IX

COMPARATIVE ACTIVITIES OF SOME NATURAL ESTROGENS IN VAGINAL SMEAR TESTS[a]

Estrogen	Spayed mouse MED (μg.)	Immature rat MED (μg.)
Estradiol-17β	0.02	0.09
Estriol	0.10	0.13
16-Epiestriol	0.20	0.20
17-Epiestriol	0.044	0.054
16,17-Epiestriol	20.0	1.80

[a] From Katzman *et al.* (1960).

includes 16,17-epiestriol, added as a note to their reprints. Earlier reports have always indicated that 17-epiestriol is a relatively weak estrogen (cf. Huffman and Grollman, 1947).

B. Local Tests

Vaginal smear tests by local application have been reviewed by Emmens (1950, 1962a), whose group has particularly concentrated on the method. Freud (1939) and Mühlbock (1940) investigated some of the variables associable with these tests and showed that although intravaginal administration in the mouse was not successful with oily media, the use of 50% glycerol gave consistent results of very high sensitivity. The MED of estradiol-17β, estrone, and estriol was found by Mühlbock (1940) to be 500, 250, and 750 pg. (picograms or 10^{-12} gm.) respectively. The relative inaccuracy of such tests is now known to be such that these doses almost certainly do not differ significantly.

Local instillation of potent estrogens into the mouse vagina results in local utilization, without generalized action in the whole body, and thus the effective dose is a small fraction of that needed by other routes. Robson and Adler (1940) showed that if a separate vaginal pocket is

constructed in the spayed mouse from the lower vagina, doses of estrogen which are effective in stimulating characteristic changes in this pocket do not affect the upper vagina. Emmens (1941b) confirmed these results and extended them to show that estrogens may be classified into true estrogens, with which the effective intravaginal dose is typically one one-hundredth or less of the corresponding subcutaneous dose, and proestrogens, with which the two doses are identical, within experimental limits of error, or perhaps with the intravaginal dose somewhat higher than the subcutaneous one, rather than the reverse. In the case of the proestrogens, the separate vaginal pocket (Robson and Adler, 1940) is affected by the same dose which causes reactions in the pocket receiving the active substance (Emmens, 1942a).

Further experiments (Emmens, 1941a, b, 1943, 1947) showed that all of the natural estrogens examined are true estrogens, excepting the weakly estrogenic androgens, but that many synthetics are proestrogens. However, stilbestrol, dienestrol, and hexestrol are true estrogens. Proestrogens must be metabolized in the body, probably in the liver, before becoming estrogenic; hence it makes little difference where they are injected, even if intravaginally, since they must circulate before exerting an estrogenic effect. Esterification does not change the potency of estrogens when they are given locally, and many substances which interfere with subcutaneous and other assays do not affect potency ratios in intravaginal assays. Unfortunately, the slope of the dose-response line is poor, and intravaginal assays are not very precise unless large numbers of animals are used, when cornification is the criterion of response. Fortunately, however, newer response criteria (see below) give much better slopes.

Since relative potencies turned out to be so similar to those of the common estrogens, both natural and synthetic, a considerable amount of work was done to determine if these compounds were in fact different in potency, when they were given under optimal conditions so that each might exert its fullest potential. Using a single two-application technique, Emmens (1941b) found the results shown in Table X for various true estrogens. If methyl ethers and some of the synthetics are omitted, the most potent compounds are alike in activity, and such activity is confined to substances which may be presumed to have some common structural relationship. A corresponding table of proestrogens (Emmens, 1941b) contained a variety of synthetic compounds, but included androst-5-ene-3β,17β-diol and ethynyltestosterone. The structural formulas of the true estrogens listed in Table X are shown in Fig. 3. Biggers (1951, 1953a) found that, still using the two-injection technique, the potency of estrogens is enhanced if they are given in aqueous egg

albumin. Estrone in 1% egg albumin showed a 3-fold or more increase in potency (as compared with instillation in saline), whereas the potencies of estradiol-17β and estriol in 1% egg albumin were, respectively, 1.75 (1.15–2.66, $P = 0.05$) and 1.09 (0.70–1.70, $P = 0.05$) compared with

TABLE X

SUBSTANCES HAVING A HIGH S/L RATIO[a]

No.	Substance	Median effective dose when given by: Subcutaneous injection (μg.) (S)	Intravaginal application (μg.) (L)	S/L ratio
1	Estrone	0.075	0.00029	260
2	Estrone methyl ether	0.9	0.015	60
3	Estradiol-17β	0.025	0.0005	50
4	Ethynylestradiol-17β	0.03	0.00025	120
5	Estriol	2.0	0.001	2000
6	Ethynyldihydroequilin	0.045	0.0005	90
7	Diethylstilbestrol	0.12	0.00037	320
8	ψ-Diethylstilbestrol	0.45	0.001	450
9	Diethylstilbestrol dimethyl ether	8.0	0.02	400
10	Ethyl-propyl-stilbestrol	0.6	0.0035	170
11	Di-isopropyl-stilbestrol	4.7	0.015	310
12	Di-*n*-butyl-stilbestrol	50.0	0.16	310
13	Hexestrol (*meso*)	0.16	0.0009	180
14	Hexestrol (racemic)	8.9	0.025	360
15	4,4-Dihydroxy-γ,δ-diphenyl-β,δ-hexadiene	0.1	0.00058	170
16	Triphenylchloroethylene	65.0	1.0	65
17	3,3′,4,4′-Tetrahydroxy-γ,δ-diphenyl-*n*-hexane	12.5	0.2	63
18	1-Ethyl-2-(*p*-hydroxyphenyl)-6-hydroxy-1, 2, 3, 4-tetrahydronaphthalene	ca. 1.0[b] mg.	ca. 8.0	ca. 125

[a] From Emmens (1941b).

[b] In milligrams.

estrone. Estriol was thus brought up to the same activity as estrone, while estradiol-17β was more potent. Using bovine plasma albumin, 0.1% gave erratic responses, presumably because of too tight a binding, but 0.01% gave enhanced potencies as did 1% egg albumin, although the relative potencies of the three natural human estrogens were not determined by this method.