# THE BIO-ASSAY OF β-ESTRADIOL*

S. A. THAYER, E. A. DOISY, JR., AND EDWARD A. DOISY

Occasionally, a scientist makes a discovery which stands out clearly as the contribution which has had a profound effect on the subsequent developments and advance in a particular field of science. That Edgar Allen's introduction of the vaginal smear method[1] for detection of the "ovarian follicular hormone" was such a discovery is evidenced by the tremendous advances in our knowledge of ovarian physiology and in the chemical study of the estrogens resulting from the application of his method. Allen's contribution was the base upon which many subsequent investigations in all parts of the world were founded.

Although Allen's original procedure has been modified in many respects, still the basic principle remains unchanged. Nearly every investigator in the estrogen field has developed his own modifications of assay technic. In this laboratory five different procedures have been used for many years in the study of the activity of estrogens. This report deals in part with the potencies of β-estradiol, β-estradiol-3-benzoate, estrone, and α-estradiol as determined by these technics.

β-Estradiol was first obtained by Schwenk and Hildebrandt[15] by catalytic reduction of estrone. Subsequently, Butenandt and Goergens[2] and Wintersteiner et al.[18] reported more details on its preparation, chemical and physical properties, and potency, and Wintersteiner and Hirschmann[17] reported its isolation from the urine of pregnant mares. More recently, Stroud,[16] Fish and Dorfman,[8] Heard et al.,[9] and Pearlman and Pearlman[13] have shown that β-estradiol is an important metabolite of α-estradiol and estrone in certain species of laboratory animals.

Our interest in the metabolism of estrogens has led us (Doisy, Thayer, and Van Bruggen[6]) to a study of the products of excretion following the administration of β-estradiol to a monkey. An increase

* From the Laboratory of Biological Chemistry, St. Louis University School of Medicine, St. Louis. We are indebted to the Theelin Fund administered by the Committee on Grants for Research of St. Louis University for financial support.

in the estriol, estrone, and estradiol fractions occurred. In an attempt to ascertain the nature of the estradiol fraction a differential assay which was based on the data submitted in this paper was employed; the results indicated that the estradiol fraction contained a non-ketonic estrogen which was more active than was β-estradiol. Pearlman and Pincus[14] have subsequently contributed another procedure for the characterization of the weak-phenolic non-ketonic fraction which is based on the alteration of potency caused by the oxidation of the 17-hydroxyl with aluminum *tert*-butoxide.

Since the bio-assay of β-estradiol showed this compound to have very low estrogenic activity, and metabolism studies as well as theoretical considerations indicated the possibility of the conversion of β-estradiol to estrone and α-estradiol, it seemed possible that β-estradiol might owe its estrogenic property to compounds produced by its metabolism, i.e., it might be a pro-estrogen. Consequently, it was subjected to the procedure used by Emmens[7] for differentiating estrogens and pro-estrogens. Data in the experimental section clearly indicate that β-estradiol conforms to Emmens's description of the behavior of a true estrogen in that vaginal cornification is produced by vaginal application of only a minute fraction of the amount required when administered subcutaneously.

## *Experimental*

*Preparation and Characterization of β-Estradiol*: The β-estradiol* used in our experiments was obtained by purification of the phenolic non-ketonic fraction of an extract of mare's urine by adsorption on a column of Merck's alumina. Petroleum ether containing ethyl alcohol was used for elution. Subsequent purification of the fractions having a specific rotation less than $+60°$ was effected by recrystallization from aqueous ethyl alcohol and from acetone until the melting-point remained constant.

Since the potency of α-estradiol is so much greater than is that of β-estradiol, contamination of the latter with even a small quantity

* The β-estradiol was prepared from a light-colored partially purified residue from the commercial production of estrone which was obtained from Eli Lilly and Co. We wish to express our appreciation to Mr. George B. Walden for this material.

of the former would lead to markedly erroneous values for bio-assay. Consequently, the β-estradiol was carefully purified and characterized.

β-Estradiol: M.P. 222-223° (corr.)—no depression of melting-point when mixed with β-estradiol prepared by reduction of estrone.

$[\alpha]_{25^\circ}^{D} = +52.2^\circ$ (dioxane)

| | | | *C* | *H* |
|---|---|---|---|---|
| Analysis: | $C_{18}H_{24}O_2$ | Calculated: | 79.35 | 8.89 |
| | | Found: | 79.10 | 8.63 |
| | | | 79.24 | 8.89 |

The benzoate and acetate were made for characterization of β-estradiol. The melting-point of the diacetate agrees with the values reported by Butenandt[2] and by Whitman et al.[18] Crystallized from aqueous acetone, our product did not contain solvent of crystallization.

β-Estradiol diacetate: M.P. 139.5–140° (corr.).

| | | | *C* | *H* |
|---|---|---|---|---|
| Analysis: | $C_{22}H_{28}O_4$ | Calculated: | 74.12 | 7.92 |
| | | Found: | 74.01 | 8.05 |
| | | | 74.13 | 8.19 |

Our experiments with the monobenzoate gave data which indicate that this compound may exist in different crystalline modifications having different melting-points and consequently the melting-point of the benzoate is not entirely satisfactory for identification purposes. (In this connection see Hirschmann and Wintersteiner.[17]) In addition to the platelet form observed by Butenandt[2] and by Whitman et al.,[18] we have frequently obtained the compound in long needles, and also in a mixture of needles and plates. Occasionally upon recrystallization of an analytically pure sample from purified solvents the melting-point decreased several degrees. Furthermore, although we have obtained the benzoate from aqueous methyl alcohol containing 0.5 mole of solvent of crystallization (the form reported by Butenandt), usually the compound did not contain solvent of crystallization.

β-Estradiol-3-benzoate: M.P. 153.5–154.5° (corr.).

| | | *C* | *H* |
|---|---|---|---|
| Analysis: $C_{25}H_{28}O_3$ | Calculated: | 79.74 | 7.50 |
| | Found: | 79.89 | 7.81 |
| | | 79.55 | 7.68 |

*Assay of β-Estradiol*: The assay of β-estradiol was conducted by the procedures which were used in the study of α-estradiol which had been isolated from liquor folliculi.[10] Although the methods were described briefly in our earlier publication, a slight degree of repetition is introduced in this paper to aid the reader.

*Modified Butenandt Procedure;*[3, 10] *A Single Injection of Estrogen in Oil; Mice*: Mice were ovariectomized as previously described by Allen and Doisy.[1] Vaginal smears were made daily for a period of two weeks following ovariectomy and animals that did not show a negative smear each day were discarded. Two weeks after ovariectomy, the mice were primed with an aqueous solution of estrone containing 0.1 γ or one international unit. Smears were taken at 9:00 A. M., 1:00 P. M., and 5:00 P. M. starting 48 hours after the injection.

One week after priming, the mice were divided into two groups of 20 animals. Each of one group of 20 animals was injected subcutaneously at 9:00 A. M. with the oily preparation being tested; each of a similar group was injected with the standard estrone preparation dissolved in oil, and the proportion of positive effects in each group was determined. After one or two trial experiments, the dosages could usually be so adjusted that approximately the same percentage responses to the unknown and standard were obtained. In accordance with the work of Coward and Burn[4] a 50 per cent positive response was regarded as one unit; the unknown was evaluated in terms of the standard response curve and the concurrent response to the standard.

Mice that failed to respond positively to an injection within 80 hours were immediately primed with an oily solution containing 0.05 γ of estrone. Owing to the slow rate of absorption mice were not used again until four weeks had elapsed following a positive response. The volume of oil injected was usually 0.2 cc. and in all experiments the unknown and standard were administered in the same volume.

*Mather Modification of the Marrian-Parkes Procedure;*[11, 12] *Four Injections of an Aqueous Solution; Mice*: Ovariectomized mice were given subcutaneous injections of four equal quantities of an aqueous solution at 8:00 A. M. and 5:00 P. M. on two consecutive days. Smears were taken at 9:00 A. M. and 5:00 P. M., starting 16 hours after the last injection, and the last smear was taken 48 hours after the first smear. In this method the same principles were followed as in the modified Butenandt method, using comparable animals and the same method of determining potency of a preparation. The estrogens and the standard preparation used in this assay procedure were dissolved in an aqueous medium. Animals that showed a negative response from previous injections were primed with an aqueous solution containing 0.1 γ of estrone before they were used for another assay. In this method the test animals were used every two weeks.

*Thayer-Doisy Procedure; Three Injections of an Aqueous Solution; Mice*: Injections were made at 9:00 A. M., 1:00 P. M., and 5:00 P. M. on the same day. The same principles regarding priming, estimation of activity, etc. which were used in the preceding method were followed in this procedure, with the exception that the mice were used for assay at intervals of seven days. We have used this method for many years as a routine procedure to determine the biological activity of estrogens.

*Allen-Doisy Procedure;*[1] *Three Injections of an Aqueous Solution; Rats*: Ovariectomized rats were given subcutaneous injections of three equal quantities of an aqueous solution at intervals of four and one-half hours. All rats used had given a positive response one week before use for assay due to priming with 1.2 γ of estrone or a previous assay. The same principles were followed as in the other procedures for determination of units. In this method the changes of the cells in the vagina may be regarded as positive if a few leukocytes are present along with the nucleated epithelial cells and squamous non-nucleated epithelial cells.

*Curtis-Doisy Procedure;*[5] *Six Injections of Aqueous Solution; Immature Rats*: The estrogen was injected at 9:00 A. M. and 5:00 P. M. on three successive days. Changes in the procedure from the original method as published by Curtis and Doisy were: the requirement of opening of the vagina with a positive smear, and application of the same procedure for determination of units as in the other assay methods.

The results obtained from using these five different assay procedures are given in table 1.

TABLE 1

POTENCIES OF β-ESTRADIOL, β-ESTRADIOL MONOBENZOATE, α-ESTRADIOL AND ESTRONE AS DETERMINED BY FIVE DIFFERENT METHODS OF BIO-ASSAY

| *Compounds* | *Butenandt modified units/mg.* | *Marrian-Parkes modified units/mg.* | *Thayer-Doisy units/mg.* | *Allen-Doisy modified units/mg.* | *Curtis-Doisy modified units/mg.* |
|---|---|---|---|---|---|
| β-Estradiol | 700 | 8,000 | 2,000 | 200 | 700 |
| α-Estradiol | 35,000 | 70,000 | 40,000 | 17,000 | 5,000 |
| Estrone | 20,000 | 35,000 | 20,000 | 1,700 | 1,000 |
| β-Estradiol benzoate | 1,000 | 2,400 | 450 | 200 | 200 |
| $R^1$ | 28 | 4.4 | 10 | 8.5 | 1.4 |
| $R^2$ | 1.4 | 0.3 | 0.22 | 1. | 0.3 |

$$R^1 = \frac{\text{Potency of estrone}}{\text{Potency of } \beta\text{-estradiol}}; \quad R^2 = \frac{\text{Potency of } \beta\text{-estradiol-3-benzoate}}{\text{Potency of } \beta\text{-estradiol}}$$

*Ratio of Estrogenic Potencies by Systemic and Local Administration*: In order that our data might be comparable to those published by Emmens,[7] the systemic mouse unit for β-estradiol was determined on a group of mice injected at 9:00 A. M. on each of two consecutive days with one-half of the total dosage of oily preparation. Smears were taken on the third and fourth days. The systemic mouse unit was 1.65 γ or 600 units per milligram.

Emmens's procedure was also used to determine the local unit, i.e., the amount required by local application to the vagina to produce cornification in that tissue. Sixty-two per cent of 29 mice responded to the administration of 0.033 γ with cornified smears. This gives a S/L ratio $\frac{1.65}{0.033} = 50$, the same value reported by Emmens for estradiol, presumably α-estradiol in view of the values of the systemic and local units given, respectively, 0.025 γ and 0.0005 γ.

## *Discussion*

Butenandt's data[2] indicate that estrone is approximately 37 times as potent as is β-estradiol, while our figures obtained by Butenandt's

assay procedure show the ratio of potencies to be 28. The comparison of potencies published by Hirschmann and Wintersteiner[17] was based on assays in which rats were injected with a solution of estrogen in oil and therefore is not directly comparable with our assay in mature rats. Pearlman and Pincus[14] have also used rats in an assay procedure differing somewhat from ours. Nevertheless, the ratios which are respectively 13, 12, and 8.5 show an acceptable agreement. In our work, in which various procedures were used, the ratios of potencies varied from 28 to 1.4. Apparently, the potency of β-estradiol with respect to estrone is improved by divided dosage and the greatest division of dosage gives the highest potency for β-estradiol in relation to estrone. Furthermore, the lowest relative potency was found with the method (Butenandt) using a single injection of mice with an oily preparation.

The comparison of potencies of β-estradiol and its benzoate yields some rather astonishing figures. The potency of the benzoate ranges from 1.4 times to 0.22 of the potency of β-estradiol, depending upon the method of assay.

Perhaps, the most apparent conclusion is that the potency of an estrogen depends to a great extent on the method used for assay and that it is necessary for the accurate comparison of potencies of two compounds that they be assayed by exactly the same procedure.

## *Summary*

1. β-Estradiol has been prepared from mare urine and characterized by its physical constants and the preparation of derivatives.

2. β-Estradiol and β-estradiol-3-benzoate have been assayed by five procedures in use in this laboratory. A comparison of the potencies of these compounds and of β-estradiol and estrone shows that the relative activities depend in part on the method of assay.

3. Determination of the potencies by subcutaneous administration and by local application to the vagina shows that β-estradiol is a true estrogen.

### References

1 Allen, E., and E. A. Doisy: J. Am. Med. Asso., 1923, *81*, 819.
2 Butenandt, A., and C. Goergens: Ztschr. f. physiol. Chem., 1937, *248*, 129.
3 Butenandt, A., and E. Ziegner: Ztschr. f. physiol. Chem., 1930, *188*, 1.
4 Coward, K. H., and J. H. Burn: J. Physiol., 1927, *63*, 270.

5 Curtis, J. M., and E. A. Doisy: J. Biol. Chem., 1931, *91*, 647.
6 Doisy, E. A., S. A. Thayer, and J. T. Van Bruggen: Federation Proc., 1942, *1*, 202.
7 Emmens, C. W.: J. Endocrinol., 1940-41, *2*, 444.
8 Fish, W. R., and R. I. Dorfman: J. Biol. Chem., 1940, *140*, xl; 1942, *143*, 15.
9 Heard, R. D. H., W. S. Bauld, and M. M. Hoffman: J. Biol. Chem., 1941, *141*, 709.
10 MacCorquodale, D. W., S. A. Thayer, and E. A. Doisy: J. Biol. Chem., 1936, *115*, 435.
11 Marrian, G. F., and A. S. Parkes: J. Physiol., 1929, *67*, 389.
12 Mather, A.: J. Biol. Chem., 1942, *144*, 617.
13 Pearlman, W. H., and M. R. J. Pearlman: Arch. Biochem., 1944, *4*, 97.
14 Pearlman, W. H., and G. Pincus: J. Biol. Chem., 1943, *147*, 379.
15 Schwenk, E., and F. Hildebrandt: Naturwissenschaften, 1933, *21*, 177.
16 Stroud, S. W.: J. Endocrinol., 1939, *1*, 201.
17 Wintersteiner, O., and H. Hirschmann: J. Biol. Chem., 1937, *119*, cvii.
Hirschmann, H., and O. Wintersteiner: J. Biol. Chem., 1937-38, *122*, 303.
18 Wintersteiner, O.: J. Am. Chem. Soc., 1937, *59*, 765.
Whitman, B., O. Wintersteiner, and E. Schwenk: J. Biol. Chem., 1937, *118*, 789.