COMPARATIVE ACTION OF VARIOUS OESTROGENIC COMPOUNDS ON MOUSE VAGINAL SIALIC ACIDS (II)

By

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ABSTRACT

Oestrogens administered in lower doses than necessary to induce full cornification of the mouse vagina induce mucification. It was shown previously that the degree of mucification could be estimated by quantitative determination of sialic acids. A suitable parameter for oestrogen assay was the measurement of vaginal sialic acid concentration which exhibited a clear cut dose response curve. Eleven assays of various oestrogens were performed with this method. Their estimated relative potencies were in good agreement with other routine oestrogen assays. A statistically sufficient degree of precision was found. The sensitivity was of the same order, or slightly higher, than the Allen-Doisy test.

In the previous study (Part I, Carlborg 1969) the action of diethylstilboestrol (DES) was studied with regard to the sialic acid (SA) content of the mouse vagina and concomitant histological changes. It was demonstrated that this oestrogen caused a dose dependent decrease in SA concentration. The present study used this characteristic mode of reaction of the mouse vagina as an assay for the relative potency in a number of oestrogenic compounds.

METHODS

Female mice (NMRI strain) of about 25 g bodyweight were spayed and then allowed to rest for ten days. All test compounds were dissolved in sesame oil (except oestriol which was dissolved in saline), the daily dose being contained in 0.1 ml. Subcutaneous injections were made into the interscapular region once daily for three days. Force feeding was instigated using a suitable plastic tube inserted into the stomach of the animal. About 24 h after the last dose the animals were sacrificed by a blow on the
head, the vaginae were then dissected out and immediately weighed wet on a torsion balance. The samples were ground in a tissue homogenizer in 1.25 ml of distilled water. The analysis of SA was performed using the Direct Ehrlich Method (Werner & Odin 1952; Carlborg 1966).

**STATISTICAL METHODS**

Dose intervals were logarithmic, and it was found suitable to increase successive dose levels by a factor of 1.56. Data from the two dose levels chosen for estimation of the relative potency of a particular compound were subjected to calculation of the slope (b) (designating the logarithm of the dose as the independent variable) and its standard deviation (sd). The group means were calculated with 95% confidence limits. The precision of the assay was indicated by lambda (λ) (Dorfman 1954; Bancroft 1957; Borth et al. 1957).

When using an assay for the comparison between activities of several compounds it is necessary to have a statistically acceptable degree of parallelism between the slopes. If not, a comparison cannot be made and a different kind of action must be suspected.

A suitable statistical procedure including an F-test has been described by Ostle (1960). This procedure tests the hypothesis of parallelism between multiple slopes and whether it should be rejected or not at various levels of risk. The use of this procedure is justified if three conditions are valid: 1) the population from which each experimental group is obtained should be normally distributed; 2) all observations within a group should be independent of each other and the experimental groups should be independent of each other; 3) the same sd should be present in all experimental groups.

Conditions 1) and 2) are considered valid as they have been taken into account during the design of the experiment. The same strain, weight and age of mice were used. Condition 3) is also considered valid as the differences in sd were not significant between the experimental groups.

**MATERIAL**

The following compounds were studied*: The 3 natural oestrogens, oestrone (OE₁), 17β-oestradiol (OE₂) and oestriol (OE₃); diethylstilboestrol (data included from Part I) and hexoestrol; compound F 6066 (Sexovid®, cyclofenil NFN, bis [p-acetoxyphenyl]-cyclohexylidene methane) and the research compound F 6103 (bis [p-acetoxyphenyl]-2-methyl cyclohexylidene methane); mestranol and ethinyloestradiol (EOE₂), the latter two compounds also being administered by force feeding.

In pilot experiments two dose levels were chosen to be used for assay of the relative potency of the compounds. These levels were on the descending slope for SA concentration as described in Part I. Forty-one animals (controls) were injected with oil only.

* The compounds were generously supplied by the following companies: OE₁, OE₂ and OE₃ from Sigma Chemical Co.; DES and EOE₂ from N. V. Organon; hexoestrol from Recip Pharmaceuticals; mestranol from Astra Pharmaceuticals; F 6066 and F 6103 from Ferrosan Pharmaceuticals.
RESULTS

For each compound a corresponding dose dependence for total SA content and vaginal weights was found as described for DES in Part I. Therefore, the concentration of vaginal SA was used as the dose dependent parameter.

The mean control value was 2.82 µg SA/mg ± 0.17 µg/mg 95% confidence limits. In all the pilot experiments an identical low level of SA concentration was recorded at comparatively high dose levels. Therefore, 72 measurements could be pooled into one group which constituted the opposite limit to the controls. The mean SA concentration in this group was 1.24 µg/mg ± 0.06 µg/mg 95% confidence limits. The suitable range for investigation was the difference between these extreme means (1.6 µg/mg). These data are shown in Fig. 1, where the two group means from the assay of a particular compound also have been connected by a dose response line.

Table 1 includes statistical data. The slope (b) varied from -1.89 to -4.94 with an average value of -2.97. This means that a ten fold increase in the dose would result in a theoretical decrease in SA concentrations of 2.97 µg/mg. The statistical procedure used for testing of parallelism between multiple slopes resulted in an F-value of 1.28. This figure should be compared with the corresponding table value of F distribution. There are 10 degrees of freedom with regard to groups and 135 degrees of freedom with regard to individual observations. At P < 0.05 the table value is 1.90 which exceeds the calculated value (1.28). The result is therefore not inconsistent with the hypothesis of parallelism between all slopes at the 5% level.

Assuming parallelism between slopes, an attempt was made to calculate the

![Dose response curves of various oestrogenic compounds.](image1)

*Fig. 1.*

Dose response curves of various oestrogenic compounds. E1 = oestrone; E2 = oestriol; E3 = oestradiol; EE2 = ethinyl oestradiol; S = diethylstilboestrol; H = hexoestrol and M = mestranol. The dotted curves for mestranol and ethinyl oestradiol represent responses when administered by force feeding. For oestradiol 4 dose levels are included. The upper shaded area represents the mean control level ± 95% confidence limits; the lower shaded area represents the mean SA concentration at maximal SA suppression ± 95% confidence limits.

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Table 1.
Statistical data of the action on mouse vaginal sialic acids by various oestrogens.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route</th>
<th>Dose pairs μg daily dose</th>
<th>n</th>
<th>b</th>
<th>s_b</th>
<th>λ</th>
<th>Potency</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MED</td>
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<tr>
<td>oestrone</td>
<td>sc</td>
<td>0.050:0.032</td>
<td>18</td>
<td>-1.96</td>
<td>0.30</td>
<td>-0.15</td>
<td>0.0289</td>
</tr>
<tr>
<td>oestradiol</td>
<td>sc</td>
<td>0.020:0.0125</td>
<td>18</td>
<td>-1.97</td>
<td>0.37</td>
<td>-0.19</td>
<td>0.0173</td>
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<tr>
<td>oestradiol</td>
<td>sc</td>
<td>5.0:3.2</td>
<td>17</td>
<td>-2.45</td>
<td>0.49</td>
<td>-0.20</td>
<td>1.20</td>
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<tr>
<td>DES</td>
<td>sc</td>
<td>0.040:0.025</td>
<td>14</td>
<td>-4.41</td>
<td>0.27</td>
<td>-0.061</td>
<td>0.0330</td>
</tr>
<tr>
<td>hexoestrol</td>
<td>sc</td>
<td>0.050:0.032</td>
<td>10</td>
<td>-2.62</td>
<td>0.47</td>
<td>-0.18</td>
<td>0.0382</td>
</tr>
<tr>
<td>F 6066</td>
<td>sc</td>
<td>40.0:25.0</td>
<td>16</td>
<td>-4.29</td>
<td>0.31</td>
<td>-0.072</td>
<td>34.0</td>
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<tr>
<td>F 6103</td>
<td>sc</td>
<td>2.0:1.3</td>
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<td>-1.98</td>
<td>0.39</td>
<td>-0.19</td>
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<tr>
<td>mestranol</td>
<td>sc</td>
<td>0.50:0.032</td>
<td>14</td>
<td>-2.95</td>
<td>0.26</td>
<td>-0.087</td>
<td>0.448</td>
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<tr>
<td>mestranol</td>
<td>or</td>
<td>1.6:1.0</td>
<td>16</td>
<td>-1.89</td>
<td>0.47</td>
<td>-0.25</td>
<td>2.04</td>
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<tr>
<td>EOE2</td>
<td>sc</td>
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<td>-3.23</td>
<td>0.33</td>
<td>-0.10</td>
<td>0.0148</td>
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<tr>
<td>EOE2</td>
<td>or</td>
<td>0.50:0.32</td>
<td>13</td>
<td>-4.94</td>
<td>0.34</td>
<td>-0.070</td>
<td>0.406</td>
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</table>

sc = subcutaneously; or = orally
n = number of animals
b = slope
s_b = standard deviation of the slope
λ = index of precision
MED = mean effective dose
mean effective dose (MED) of each compound. Using the b value the regression equation of y (SA concentration) on x (log dose) was determined. The MED for a compound was arbitrarily set at the dose needed to give an SA concentration of 1.90 μg/mg. This figure was chosen as being midway between the control value and the steady low level obtained at comparatively high oestrogen levels. The value of 1.90 was thus inserted into the equation and the corresponding MED was subsequently calculated.

The MED for each compound calculated in this way is listed in Table 1. In one case, oestrone, the range between the experimental groups does not include an SA concentration of 1.90 μg/mg and the corresponding dose value had to be extrapolated.

A second way of expressing the relative potency between compounds was used. DES was considered as a reference compound and the activity of the other compounds was calculated as percentage activity of DES.

In addition a 4-point statistical calculation of relative potency of two compounds was performed (Borth et al. 1957). Oestradiol (0.0125 and 0.02 μg) was compared with F 6066 (25 and 40 μg). The following data were obtained: average slope (b) = -3.40; sd = 0.27; t = -0.079; t (parallelism) = 1.88 (the corresponding t-value for 28 degrees of freedom and P < 0.05 = 2.05; the result is therefore not inconsistent with parallelism at the 5% level). These data indicated that statistically there was a sufficient degree of validity of the assay. Subsequent calculation of relative potency indicated OE2 to be 1966 times more potent than F 6066. In Table 1 it can be seen that the MED for OE2 was 0.017 μg and the MED for F 6066 was 34.0 μg. When these data were used for the determination of relative potency, a figure of 2000 was obtained. Thus, the two methods of calculation were in close agreement.

DISCUSSION

In a previous study (Part I) on DES, the principles for the assay were discussed. Each of the compounds tested in the present study (Part II) caused the same vaginal changes in SA concentration as did DES, but the dose levels at which these changes could be observed varied between the compounds. The possibility of using this characteristic vaginal reaction as an assay of oestrogen potency was evaluated in this study. Statistical treatment of the data showed that the index of precision (λ) varied from -0.061 to -0.25 with a mean value of -0.14. This figure was of the same magnitude as that found in other routine assays of oestrogen potency in laboratory animals (Dorfman & Dorfman 1954). The spread of results within an experimental group was narrow enough to conclude that 5 animals per group provided sufficient information. The statis-
tical evaluation of parallelism between all slopes indicated a similar action in the compounds tested.

The reproducibility of the method was tested by comparison with the data obtained for OE₂ in the author's previous paper (Carlberg 1966). In this study, the doses had not been selected according to the criteria in the present assay, but the data lent themselves to calculation of the slope and the estimation of MED. Control mice had a mean SA concentration of 2.45 μg/mg and the low concentration at comparatively high oestrogen levels was 0.95 μg/mg. The range for useful investigation was thus 1.50 μg/mg. The slope was calculated to be -1.38 which was a lower value than that found in the present study. With this exception the statistical data from the two investigations agreed closely as did the MED which was calculated to be 0.011 μg (c.f. 0.017 μg in the present assay).

Using spayed mice and Allen-Doisy technique, the activities of the three natural oestrogens following subcutaneous administration were determined (Emmens 1939a,c). The same order of activity was obtained as in the present study. OE₂ was the most potent compound followed by OE₁ and OE₃. The sensitivity for OE₂ was found to be 0.1 μg in total dose. As the present assay gives the daily dose, the MED should be multiplied by 3 for comparison and resulted in a total dose of 0.052 μg. This value was about half of that reported by Emmens (1939a,c). Considering the biological basis for the present assay it should provide a lower MED than the dose needed for complete vaginal cornification. There is thus a good agreement with the previous reports on the activity of OE₂. Oestriol was of remarkably low activity in the present type of assay, and did not appear to show any specificity for the vagina as compared to OE₁ and OE₂.

Most bioassays on DES and hexoestrol indicate that these compounds have an action equivalent to or higher than that of α-oestradiol. Sealey & Sondern (1941) found the activity of DES to be 4 times that of α-oestradiol using the Allen-Doisy test on immature rats following subcutaneous administration. In a similar assay Dodds et al. (1939) found hexoestrol and DES to be respectively 2.8 and 2.0 times more potent than OE₁. The present study indicates a rather similar activity of OE₁, hexoestrol and DES, OE₂ being the most active.

The oral effectiveness of EOE₂ is well known, although subcutaneous administration is more effective. Using the Allen-Doisy technique on spayed mice (Emmens 1939b) estimated the MED following oral administration to be 60 times higher than the subcutaneous MED. Inhoffen & Hohlweg (1938), using a similar test on rats, found EOE₂ to be equal in potency to α-oestradiol when given subcutaneously. The corresponding oral dose was found to be 30 times higher. Harmer & Broom (1950) also using the Allen-Doisy technique on spayed rats, found the oral dose to be about 13 times higher (author's calculation from Harmer & Broom's (1950) data). The data from the present
assay agree well with these reports, the ratio of the oral/subcutaneous MED being 28.

Dorfman & Kincl (1966), in a study using the increase in mouse uterine weight following the administration of an oestrogen, reported EOE₂ to be about twice as active as mestranol by gavage. They compared the activity with that of OE₁ but absolute doses were not reported. Subcutaneously, mestranol was estimated to have a relative potency of about 23 % of that of OE₁. The present assay gives a corresponding figure of 6.5 %.

As shown above, the oral MED for EOE₂ was 28 times higher than the subcutaneous MED. The corresponding figure for mestranol was 4.6. These data indicate a comparatively higher oral activity for mestranol than for EOE₂ if related to the subcutaneous MED.

The oestrogenic action of F 6066 and F 6103 has been tested by Einer-Jensen (1965, 1968). He used the mouse uterus test and determined the MED of these compounds and compared them with the corresponding MED for oestradiol benzoate. The data on absolute doses were not readily comparable with those in the present assay. He estimated oestradiol benzoate to be about 1000 times more potent than F 6066 and F 6103 to be 20 times more potent. The present study using the concentration of vaginal SA indicated a similar ratio of activity between F 6066 and F 6103, the latter being 18 times more active and OE₂ times more active. The difference may be explained by the fact that Einer-Jensen (1965, 1968) used oestradiol benzoate instead of OE₂ and used a different end point.

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