THE BIOLOGICAL ACTIVITY OF THEELOL

BY ROLAND K. MEYER, LLOYD C. MILLER, AND GEORGE F. CARTLAND

(From the Research Laboratories, The Upjohn Company, Kalamazoo)

(Received for publication, September 11, 1935)

In 1930 Marrian (1) reported the isolation and the chemical and biological activity of an estrogenic substance obtained from human pregnancy urine. He found this substance had chemical and biological characteristics differing from theelin which had been isolated and characterized by Veler, Thayer, and Doisy (2) and Butenandt (3). Marrian called this new substance trihydroxyestrin. Later Doisy and Thayer (4) and Butenandt and Hildebrandt (5) also isolated theelol from human pregnancy urine, and the close agreement between the physical and chemical properties of the theelol preparations is presumptive evidence that those isolated in the various laboratories were identical. This statement is also applicable to the crystalline substance isolated from the ether-soluble fraction of human placenta by Collip and his coworkers (6).

However, as one reviews the work which has been published concerning the biological activity of theelol, it is apparent that there is great discrepancy among the reports of the various workers. Thus Curtis and Doisy (7) state that the biological activity of theelol is one-half that of theelin, i.e. 1,500,000 rat units per mg. Marrian and Cohen (8) also report an activity of the same order. However, Butenandt and Störmer (9) have found the biological activity of theelol to be approximately one-hundredth that of theelin. Butenandt and Browne (10) assayed a preparation of theelol (Doisy) and reported an activity of about 50 mouse units per mg., which agreed with a parallel assay on their own preparation which they call Follikel Hormonhydrat.

1 Doisy designates the same substance as theelol and Butenandt as Follikel Hormonhydrat.
Biological Activity of Thee101

In assaying the urine which we were using as the source of thee101 we found that it contained relatively small amounts of estrogenic activity. Therefore, we did not expect to obtain a good yield of thee101 from this urine, since Doisy and Thayer (4) have stated that approximately 80 per cent of the estrogenic activity of human pregnancy urine can be accounted for as theelol on the basis of yields of crystalline material. However, we were surprised to find that we obtained relatively good yields of a crystalline substance having the chemical and physical properties of theelol but very much less active biologically than theelin, and also less active than reported by Doisy for theelol. A specific example is afforded by the following experiment. A butanol extract from 110 gallons of human pregnancy urine assayed biologically as containing 125,000 rat units. From this butanol extract we were able to isolate a theelin fraction which contained approximately 75 per cent of the original biological activity, or 94,000 rat units. In addition, we obtained a total of 682 mg. of crystalline theelol which, when we assayed it, was not equivalent to more than a total of 50,000 rat units. This crystalline theelol was obtained as three fractions; the first two fractions, which comprised 90 per cent of the total, melted at 274–275° and 271–273° (uncorrected). \( [\alpha]_{D}^{25} = +75.2° \) in ethanol. The third fraction was not as pure and melted at 265–268° (uncorrected).

Thus we satisfactorily accounted for the biological activity of the original butanol extract. However, on the basis of the report of Doisy and Thayer (4) and Marrian (1) we should have had the equivalent of 1,200,000 rat units in the original butanol extract on the basis that 682 mg. of crystalline theelol should have possessed an activity of approximately 1,120,000 rat units, as contrasted with a found biological potency of 50,000 rat units.

These facts, together with the importance of knowing whether the biological activity of theelol is one-half or one-hundredth that of theelin, made it of interest to investigate further the activity of theelol.

Materials and Methods

Late pregnancy urine was acidified to Congo red and then extracted at room temperature with butanol. The theelol was then
prepared from these butanol extracts by the method of Doisy and Thayer (4) and that of Butenandt and Hildebrandt (11). These methods both yielded similar crystalline compounds; in Table I we have recorded the physical, chemical, and biological characteristics of three lots of theelin prepared by the two methods.

2 gm. of Theelin 76-LCM-2 (m.p. 275–276° uncorrected; \([\alpha]^{25}_{D} = +71^\circ\) in ethanol), which had been recrystallized three times, were recrystallized seven additional times, with the following solvents used in the order named: ethanol, acetone, ethyl acetate, ethanol, methanol, ethanol, ethanol. This procedure yielded 115 mg. of Theelin 128-GFC-15, having the properties described in Table I.

The biological activity of theelin was determined by the vaginal smear method based on that described by Kahnt and Doisy (12). This method makes use of the castrated adult rat which has been primed with an estrogenic substance 1 week prior to its use. The

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Method of preparation</th>
<th>No. of times recrystallized</th>
<th>M.p. (uncorrected)</th>
<th>([\alpha]^{10}_{D})</th>
<th>Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Mature spayed rat</td>
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<td>Oil Aqueous*</td>
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<td></td>
<td></td>
<td></td>
<td>Immature rat Aqueous*</td>
</tr>
<tr>
<td>Theelin 131-GFC-15</td>
<td>Doisy and Thayer</td>
<td>3</td>
<td>276–277</td>
<td>+70</td>
<td>11.1 5</td>
</tr>
<tr>
<td>Theelin 76-LCM-3</td>
<td>Butenandt and Hildebrandt</td>
<td>3</td>
<td>275–276</td>
<td>+71</td>
<td>10 5</td>
</tr>
<tr>
<td>Theelin 128-GFC-15</td>
<td>&quot; &quot;</td>
<td>10</td>
<td>275–276</td>
<td>+72</td>
<td>4000</td>
</tr>
<tr>
<td>Theelin, international standard</td>
<td>&quot; &quot;</td>
<td>252–253</td>
<td>909</td>
<td>1250</td>
<td>1000</td>
</tr>
</tbody>
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* Aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate.
unit is determined on the basis of the production of a full squa-
mous or predominantly squamous and epithelial vaginal smear in
75 per cent of animals. The injections are made in three equal
amounts in 8 hours. We have taken as our unit the amount of
hormone required to produce this type of smear in 50 per cent of
the animals. We have not classified as negative those animals
having smears which contain a very few leucocytes in addition
to the squamous and epithelial elements.

In addition to the above method we have used that described
by Curtis and Doisy (7). In this method the intact immature
female rat is employed and a unit is determined on the basis of
the amount of hormone required to produce canalization of the
vagina in 60 per cent of the animals within a period of 10 days of
the first injection. The hormone is injected twice daily for 3
days. Rats 18 or 19 days old were used by Curtis and Doisy,
but in our study we have used animals 19 to 21 days of age.

In both of the methods used in our investigation the estrogenic
substances assayed have been administered both in corn oil and
in aqueous 10 per cent alcohol containing 0.5 per cent sodium
carbonate.

We have experienced considerable variation in the same group
of animals as regards their sensitivity to theelin and theelol. This
is in agreement with the experience of Curtis, MacCorquodale,
Thayer, and Doisy (13). For this reason we have made all but
preliminary assays of theelol in parallel with the international
standard theelin and have adopted the policy of making repeated
assays with ten or more rats at intervals, in contrast to making
only one or two determinations on a group of twenty rats. The
biological activity given in Table I is the average of several
separate determinations. Especial effort has been made to keep
comparable all groups of rats used in parallel assays. By using
the international standard theelin we have a standard of refer-
ence which is available to other laboratories. Use of such a
standard in the assay of unknown substances makes it possible
and advantageous to report the biological activity of the un-
known in terms of the standard.

Results

In Table I are shown the results of the determination of the
biological activity of three preparations of theelol as compared
with that of the international standard theelin. These results demonstrate that theelol is relatively an inactive estrogenic substance, when injected in the adult spayed female rat. Our data also show that international standard theelin is approximately 90 times more active biologically than theelol when administered in oil and 250 times when given in 10 per cent alcohol containing 0.5 per cent sodium carbonate. In contrast, the biological activity of theelol when determined by the method in which the immature female rat is used is very great and is approximately 4 times greater than international standard theelin in this regard. Our results obtained with adult spayed rats are at great variance with those reported by Curtis and Doisy (7), Marrian (1), and Marrian and Cohen (8). These workers report the activity of theelol to be one-half that of theelin. The results we have obtained with immature rats are of the same order as those reported by Curtis and Doisy. As has been stated, Butenandt and Stöhrmer (9) have found the biological activity of theelol to be one-hundredth that of theelin when determined by the vaginal smear method, and our results obtained with oil solutions are in accord with their findings.

The recrystallization of Theelol 76-LCM-3\textsuperscript{3} seven additional times, which yielded Theelol 12G-FGC-15 (Table I) did not result in any change in the physical characteristics or biological activity.

The medium in which the hormone is administered is of importance in determining the number of units in a given amount of theelol. Thus an oil solution injected three times in 8 hours gave results which indicate that the number of rat units is about 10 per mg., as compared to 5 per mg. when theelol is administered in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate. If theelol is injected into adult spayed rats as a suspension in aqueous 10 per cent alcohol, the physiological effect is greatly enhanced. Thus, if the concentration of theelol is 0.166 mg. per cc., the biological activity is about 50 rat units per mg. However, if the concentration of theelol is decreased to the

\footnote{Browne (14) states that Doisy has reported that 0.006 mg. of theelol is required for a rat unit when a full squamous vaginal smear is required in 75 per cent of the animals.}

\footnote{This preparation is the one from which a sample of theelol was converted to theelin by use of potassium bisulfate at high temperature (Cartland, Meyer, Miller, and Rutz (15)).}
extent that it is in solution, the activity is of about the same order as when administered in water or in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate. These results indicate that the ease with which thee101 is absorbed determines to a large extent its biological activity. Thus aqueous solutions of thee101 are absorbed more rapidly and are biologically less efficacious than oily solutions or aqueous suspensions from which the rate of absorption is slower.

The activity of theelin is so great as compared with thee101 that it is more difficult to determine whether or not suspensions of it would give a greater physiological effect. However, in our hands administration of theelin in an oily solution (injection three times in 8 hours) has given results which indicate that the biological activity is perhaps less when it is administered in oil than in aqueous solution or aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate.

DISCUSSION

The cause of the great difference in the biological activity of thee101 as reported by us and that found by other investigators is not definitely known. However, the difference may depend on whether or not a partial or full estrus smear is taken after a positive response. Thayer and MacCorquodale (16) reported that the bioassay of thee101 showed an enormous difference, depending upon whether a partial or full estrus smear was considered in the determination of the biological unit. Butenandt and Störmer (9) have treated thee101 preparations, having relatively great biological activity, with ketone reagents and thus showed that thee101 was contaminated with theelin. They have suggested that the high activity of thee101 reported by some investigators is a result of its being contaminated with theelin. However, MacCorquodale, Thayer, and Doisy (17) have failed by use of the same methods employed by Butenandt and Störmer (9) to reveal the presence of theelin in their routine preparations of thee101. Butenandt and Störmer (9) have also suggested that there may be an active and in inactive form of thee101, thus explaining in another way the discrepancy in the reports on the biological activity of thee101. If there are two forms of thee101, we have not obtained any evidence of the second and more active form.
During the process of preparing theelol in our laboratory we have consistently noted that recrystallizations of the first crude theelol result in changes in melting point and specific rotation which indicate increase in purity. Concomitant with these recrystallizations we have observed decrease in biological activity. However, continued recrystallizations do not give indications of any further increase in purity and the biological activity remains constant. This small activity we believe represents a true activity of theelol itself and not that of a more highly active contaminant. This statement is difficult to prove definitely but the small amount of theelol necessary to produce canalization of the vagina of the immature rat in contrast to that possessed by theelin is evidence which tends to support the contention that theelol does have a biological activity—little as regards the adult spayed rat; great when determined in the immature rat.

Browne (14) and Butenandt and Browne (10) have also stated that they believe that theelol has a low grade of biological activity as determined by the vaginal smear method and that this activity is a result of the conversion of theelol to theelin by the tissues. Browne (14) has obtained evidence that less theelol is required to produce estrus in the intact immature rat than in the spayed rat. This, together with other data, has caused him to suggest that the ovary is particularly efficient in the conversion of theelol into theelin.

We have data which show that the amount of theelol, in terms of rat units, necessary to produce uterine bleeding in immature monkeys is much less than theelin (unpublished data). These data, together with certain other facts, indicate that the quantitative relationship between theelol and theelin differs with different methods of assay.

CONCLUSIONS

1. Theelol possesses relatively little biological activity when compared with international standard theelin and determined by the vaginal smear method. International standard theelin is approximately 90 times more active than theelol when administered in oil and 250 times when injected in aqueous 10 per cent alcohol containing 0.5 per cent sodium carbonate.

2. Theelol is 4 times as active as international standard theelin when determined by the opening of the vagina of the immature rat.
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