THE ISOLATION OF THEELIN FROM HUMAN PLACENTA

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The occurrence of large amounts of estrogenic activity in human placenta has been known since the investigations of Fellner (1). The estrogens in this tissue had been studied from time to time without the attainment of success in the identification of any constituent until Browne (2) succeeded in isolating theelol. The identification of this substance with the theelol obtained from pregnancy urine was made by Butenandt and Browne (3).

In a study of the estrogens in this tissue Collip (4) observed that an estrogenic constituent was soluble in water and insoluble in ether, thus differing in these properties from the known estrogenic substances. This constituent was not identified, but the isolation of theelol from placenta and the later demonstration by Cohen and Marrian (5) that theelol occurs in human pregnancy urine as the glucuronide have given rise to the belief that this unknown water-soluble estrogen in placenta is probably theelol glucuronide. To distinguish between theelol and the water-soluble estrogen in placenta, Collip and coworkers (6) reserved the use of the name "emmenin" for the latter substance.

The occurrence of theelin as the major constituent of the ketonic activity in human placenta was established by the methods previously used for the identification of the ketonic estrogen in sow ovaries. The subsequent isolation of theelin in good yield from placenta confirms the reliability of these methods used in the study of the placental estrogens and in the identification of theelin in sow ovaries.

This study has been concerned only with the identification of the ketonic estrogen, and does not exclude the possibility that...
there may be estrogens other than theelin and thee101 in this tissue. From 422 kilos of human placenta we obtained 120,000 rat units of non-ketonic and 30,000 rat units of ketonic estrogen. This is a yield of 355 rat units per kilo, of which 20 per cent is ketonic.

Assuming that the 30,000 rat units of ketonic activity was due entirely to theelin, a total of 15 mg. of theelin should have been present in this extract; of this we have isolated in a pure crystalline form 12 mg., a yield of 80 per cent.

EXPERIMENTAL

The identification of theelin in placenta was carried out on an extract of ten human placentas. The phenolic fraction was prepared and separated into ketonic and non-ketonic fractions by Girard's Reagent T. We thus obtained 200 rat units of ketones and 600 rat units of non-ketones per kilo. Confirmation of the ketonic nature of the estrogen separated into the ketonic fraction was obtained through inactivation of this fraction by the semicarbazide treatment.

The identification of the ketonic estrogen as theelin was based on the methods utilized in characterizing the ketonic estrogen in sow ovaries. These included (1) reduction of the ketonic fraction with hydrogen in the presence of a platinum catalyst, or with sodium and alcohol; (2) determination of the partition ratios between 70 per cent alcohol and benzene for the ketone and for its reduction product; and (3) determination of the ratio of rat and mouse units for the ketone and for its reduction product. In every test the ketonic fraction from the placental extract behaved like pure theelin.

35 rat units of the ketonic fraction were successfully reduced by the catalytic method with the 8-fold increase in activity that is typical of theelin. Almost as good results were obtained by the reduction with sodium and alcohol, the preparation increasing in activity from 35 to 225 rat units.

For the ketonic fraction the ratio of rat-mouse units was found to be 420:35 or 12:1, for crystalline theelin 13:1. For the estrogen produced by the reduction of this ketone, the ratio was 800:280 or 2.85:1, for crystalline dihydrotheelin 3:1.

When partitioned between 70 per cent alcohol and benzene,
the activity in the ketonic fraction was divided in a ratio of 1:2.8; i.e., 25 rat units in the alcohol and 70 rat units in the benzene. After reduction, the activity partitioned in a ratio of 1.1:1 (30 rat units in the alcohol to 27 rat units in the benzene). These values are in good agreement with the ratios of 1:3 for theelin and 1:1 for dihydrotheelin.

Isolation of Theelin

Preparation of Extracts of Placenta—The isolation of theelin was carried out on an extract of 702 full term human placentas, the total weight of which was 422 kilos. These placentas were obtained from the delivery room, and were kept frozen until a sufficient number had been collected for convenient processing. They were then hashed, and the pulp was stirred with 2 volumes of 95 per cent alcohol. After standing for several days with occasional stirring, the tissue was filtered off and extracted in a continuous extractor for 24 hours with hot 95 per cent ethyl alcohol.

Both alcoholic extracts were combined, and the alcohol was removed by distillation. The aqueous sludge was then carried to dryness in a continuous still. The dry residue was leached several times with hot 95 per cent alcohol, and the phospholipids were precipitated from this extract by the addition of 2 volumes of acetone. The alcohol-acetone filtrate was distilled, and the resulting residue was redissolved in 95 per cent alcohol. Sufficient water was added to give a concentration of 70 per cent alcohol, and the cholesterol fraction was removed by extraction with low boiling petroleum ether. The alcohol was distilled from the aqueous phase, the resulting sludge diluted with water, and the estrogens extracted with ether.

The phenolic and acidic fractions were removed from this ether extract by thorough washing with 0.5 N NaOH. They were recovered by acidification and extraction with ether, and were then separated by washing out the acids with aqueous sodium carbonate.

The phenolic residue was further purified by dissolving in 50 per cent alcohol acidified with HCl and extracting with low boiling petroleum ether. The major portion of the estrogenic activity
remained in the 50 per cent alcohol, and was recovered therefrom by dilution and extraction with ether.

This residue was then fractionated with Girard’s reagent, separating it into ketonic and non-ketonic fractions. In this manner, 30,000 rat units of ketones were separated from 120,000 rat units of non-ketonic estrogens. About twice this amount of estrogenic activity was obtained from human placenta when the extraction was carried out on a much smaller scale; the low recovery in this case was probably due to an incomplete extraction in the initial stage. Two additional treatments of the non-ketonic fraction with Girard’s reagent did not appreciably increase the yield of ketones, the second treatment removing about 1000 and the third treatment removing less than 500 rat units to the ketonic fraction.

The residue of ketones was leached with hot 0.1 \text{ N} \text{ NaOH}; salt was added to flocculate the colloid and, after chilling, the solution was filtered through a sintered glass filter. The insoluble portion was dissolved in 95 per cent alcohol, and after distillation of the alcohol, the residue was again leached with hot 0.1 \text{ N} \text{ NaOH}. Several such leachings dissolved all the estrogens.

The concentration of the alkaline solution was increased to 0.2 \text{ N} by the addition of strong alkali, and the estrogens were removed from the aqueous phase by repeated extractions with ether. The ether was distilled, and the entire process of leaching and extraction, which effected a substantial purification, was repeated on the residue.

**Isolation and Identification of Ketonic Estrogen**—The semicrystalline product obtained by distillation of the final ether extract was dissolved in alcohol, and the theelin converted to the semicarbazone. The solution was evaporated, and the residue washed with water and then recrystallized from 95 per cent alcohol. The semicarbazone was hydrolyzed with 0.5 \text{ N} \text{ HCl} in 50 per cent alcohol; upon neutralization of the acid and distillation of the excess alcohol, the free theelin precipitated from solution. This was recrystallized several times from aqueous alcohol by use of a little norit for decolorization. The final product had the identical crystalline form and appearance of the theelin prepared from urine.

The weight of the theelin obtained was 7.0 mg. Simultaneous melting points (uncorrected) taken with standard theelin gave
251.5-253° for the placental theelin and 253-254.5° for the urinary theelin, while an equal mixture of the two melted at 252.5-254°. An equal mixture of equilenin and theelin melted at 222-240°, and an equal mixture of equilin and theelin at 212-235°.

The bioassay indicated identity with crystalline theelin; i.e., an activity of 10,000 international units per mg. For a 1 per cent solution in dioxane $[\alpha]_2^27 = +163°$.

Microcombustion Analysis—

C$_{18}$H$_{22}$O$_2$. Calculated. C 79.95, H 8.21

Found. " 80.09, " 8.30

The semicarbazone had the typical crystalline form of theelin semicarbazone. It was analyzed for nitrogen by the Pregl-Dumas method. Found, N 12.3; calculated for C$_{12}$H$_{23}$O$_2$N$_2$·$\frac{1}{2}$H$_2$O, N 12.49.

The estrogens lost to the petroleum ether in the purification process of partition between 50 per cent alcohol and petroleum ether were recovered, treated with Girard's reagent, and the ketonic fraction was purified as previously described. The theelin obtained upon hydrolysis of the semicarbazone was combined with the filtrates resulting from the recrystallization of the theelin previously isolated, and the entire fraction was treated with α-naphthoyl chloride in the presence of pyridine (7). In this way an additional quantity of 5 mg. of theelin was isolated as the naphthoate. The melting point was 208° (uncorrected) and the melting point of an authentic specimen of theelin α-naphthoate was 210°. The mixed melting point was not depressed.

Microcombustion Analysis—

C$_{29}$H$_{29}$O$_3$. Calculated. C 82.03, H 6.65

Found. " 81.71, " 6.88

SUMMARY

The ketonic estrogen of human placenta was shown by characteristic reactions to be theelin.

Theelin was isolated in crystalline form from placental extracts. The characterization clearly indicates that theelin is the principal ketonic estrogen in human placenta.
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