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ENZYMATIC TRANSFORMATION OF dl-ESTRADIOL INTO d-ESTRONE AND l-ESTRADIOL

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In the last few years, various groups of workers [1–3] have developed simple methods for the complete synthesis of racemic estrone. The latter has been separated into its optical isomers by both chemical [4] and microbiological methods.

At the present time, methods are known for the microbiological separation of racemic steroids (dl-estrone [5], dl-estradiol and dl-androsterone [6], dl-corticoids [7], and dl-testosterone [8]) which, although they are fairly effective, are not always convenient to put into practice. In view of certain experimental difficulties and sometimes the lack of the required strains of microorganisms, we considered the possibility of using for this purpose β-hydroxysteroid dehydrogenase (E.S. 1.1.1.51) isolated from animal tissues.

The most convenient source of a highly active enzyme preparation proved to be human placenta. In the presence of coenzymes [nicotinamide adenine dinucleotide, oxidized (NAD^+) or reduced (NAD-H) forms] a partially purified enzyme preparation obtained from placental tissue effectively transformed d-estradiol and d-estrone. However, the results of these experiments could not be used for the isolation of adequate amounts of optically active estrogens because of the high cost of the NAD^+ and NAD-H.

Consequently, we have studied the conditions for using a homogenate of placental tissue already containing the required cofactor NAD^+. The first experiments showed that the homogenate mentioned transformed dl-estradiol almost completely in an alkaline medium (pH 10). Alkaline conditions also ensured the complete dissolution of the estrogen in the aqueous medium in the form of the corresponding phenoxide. Conversely, at pH 5.5 under the conditions most suitable for the transformation of estrone into estradiol in the presence of NAD-H we observed no high degree of transformation of d-estrone on the preparative scale. This fact can be explained both by the inadequate solubility of estrone in the aqueous buffer solution and by the necessity for carrying out a longer incubation, which affects the retention of the activity of the enzymes.

After having used the results of these experiments for the transformation of dl-estradiol, we showed that the l-form of estradiol present remained completely unchanged, while the d-entantiomer was converted completely into d-estrone. The preparative separation of the mixture of l-estradiol and d-estrone on a thin layer of alumina enabled us to isolate the components mentioned in the pure state.

As a result, we separated the racemate of estradiol in practically quantitative yield. Nevertheless, a fundamental disadvantage of this method continued to be the necessity for the use of fairly large amounts of enzyme preparation (homogenate) which sometimes complicated both the performance of the experiment and the isolation of the transformation products.

This defect could be eliminated by the addition of allyl alcohol as an activator of the enzymatic transformation. Thus, by adding 1.4 vol. % allyl alcohol to the homogenate we obtained the same results with considerably smaller (ten- to elevenfold) amounts of homogenate. Since it is known [9] that the natural androgens are substrates for 17β-hydroxysteroid dehydrogenase, it is very possible that the conditions for the enzymatic transformation of estrogens that we have developed may prove suitable also for the separation of racemic androgens into their antipodes.

The allyl alcohol did not play the part of a cofactor, since in the transformation of estrone into estradiol its conversion into acrolein might have been expected. As specially performed experiments showed, only the use of allyl alcohol causes the activation effect (and the transformation of estradiol) while the use of acrolein does not lead to the desired results. Consequently, the role of allyl alcohol can be limited to its functions only as an enzyme activator or, which is more probable, as a stabilizer for the latter.

Prolonged incubation of an enzyme sample in the presence of allyl alcohol (4–5 days at 35° C) under the conditions for the transformation of estradiol and a subsequent test of its activity showed that 17β-hydroxysteroid dehydrogenase retained its activity almost completely. Conversely, in the absence of allyl alcohol there was a considerable loss of activity (by a factor of 1.5–2) in the same time. Similar results were obtained on the prolonged storage (up to 2 months) of an enzyme preparation in the refrigerator at 2–5° C. Thus, allyl alcohol can be used successfully together with glycerol as an effective stabilizer for hydroxysteroid dehydrogenase.

Experimental

The analysis of the transformation products was carried out by thin-layer chromatography on microplates (7.5 × 2.5 cm) with a fixed layer of silica gel in the chloroform system and also by gas-liquid chromatography (Chrom-1

chromatograph, Czechoslovakia) in a column 80 cm long filled with Chromosorb W, 80–100 mesh, impregnated with 2.5% of SE-30 silicone elastomer at 221° C with a carrier gas (nitrogen) flow rate of 120 ml/min. The mixture of estrone and estradiol studied was acetylated with acetic anhydride in the presence of pyridine for 12 hr at room temperature, and after the usual working up it was transferred to the evaporator of the chromatograph at 270° C. Under these conditions the retention time for estrone acetate was 17.5 min and that for estradiol diacetate was 33.5 min.

The preparative separation of the estrone and estradiol was carried out by chromatography [benzene–ether (9:1) system] in a thin layer of deactivated alumina prepared from 18 ml of water and 90 g of alumina (activity grade II); the R_f value of the estradiol was 0.33 and that of the estrone was 0.60.

The homogenate and the sample of β-hydroxysteroid dehydrogenase were prepared by a previously described method [10].

dl-Estradiol. At 0° C, a solution of 10.5 mg of sodium borohydride in 0.26 ml of 5 N aqueous caustic potash was added to a solution of 70 mg of dl-estrone in 0.48 ml of 50% caustic potash. The mixture was left at this temperature for 2 hr and was then acidified with acetic acid to pH 6.0 and treated with 0.77 ml of water. The crystals that deposited were filtered off, washed with water, and dried. This gave 66.6 mg of racemic estradiol with mp 158°–159° C.

Incubation of d-estradiol with the homogenate. Two grams of polyvinylpyrrolidone, a solution of 25 mg of d-estradiol in 0.7 ml of 2.4% ethanolic caustic potash, and 550 ml of placental homogenate were added to 100 ml of a sodium carbonate buffer with pH 10.0. The mixture was incubated for 4 days in a thermostatted room at 35° C. After incubation, the solution was neutralized with hydrochloric acid and the transformation products were extracted with chloroform. The extract was evaporated in vacuum, the residue was treated with 1 N aqueous caustic potash solution, and the neutral products were extracted with ether. The aqueous layer was neutralized with hydrochloric acid and was again extracted with chloroform. The chloroform extract was dried over calcined sodium sulfate and evaporated to dryness in vacuum.

The chromatography of a sample of the residue in a thin layer of silica gel showed the complete absence of the initial estradiol. Preparative chromatography of the product on deactivated alumina in the benzene–ether (9:1) system yielded 18 mg of d-estrone with mp 256°–257° C giving no depression with an authentic sample. The IR spectrum of the sample of d-estrone obtained proved to be completely identical with that of the natural compound.

Incubation of racemic estradiol with the homogenate. 600 ml of glycerol, 4 g of polyvinylpyrrolidone, a solution of 66.6 mg of estradiol in 2 ml of 2.4% ethanolic caustic potash, and 1100 ml of placental homogenate were added to 2000 ml of a sodium carbonate buffer solution with pH 10.0. The mixture was incubated for 4 days at 35° C and was treated and extracted subsequently in the way described above. Chromatography in a thin layer of silica gel showed the presence in the transformation product of approximately equal amounts of estradiol and estrone. Gas-liquid chromatography showed the presence of estradiol and estrone in the mixture in a ratio of 1:1.

Preparative chromatography on the activated alumina in the system given above yielded 29 mg of d-estrone with mp 257°–257.5° C and 25 mg of l-estradiol with mp 173°–174° C, $[\alpha]_D^{20}$ −72° (c 2.0; chloroform). The IR spectra of the two compounds were completely identical with the IR spectrum of natural samples.

Incubation of d-estradiol with the homogenate in the presence of allyl alcohol. A solution of 30 mg of d-estradiol in 1 ml of 2.4% alcoholic caustic potash, 200 mg of polyvinylpyrrolidone, 70 ml of glycerol, 60 mg of placental homogenate, and 1 ml of allyl alcohol were added to 100 ml of a sodium carbonate buffer solution at pH 10.0. The mixture was incubated for 4 days at 35° C and was treated in the usual way. From the results of thin-layer and gas-liquid chromatography, the transformation product contained no traces of estradiol whatever. Preparative chromatography under the conditions described yielded 24 mg of estrone with mp 256°–256.5° C.

Summary

An enzymatic preparation of human placenta effectively transforms dl-estradiol into a mixture of d-estrone and l-estradiol, which can easily be separated by chromatography into the individual components. Allyl alcohol is a stabilizer for 17β-hydroxysteroid dehydrogenase.

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