Biogenesis of Oestrogens from [^{14}C]Testosterone in the Mitochondrial Fraction of the Human Placenta

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The biosynthesis *in vitro* of oestrogens from C_{19} androgens by human placental tissue was first described by Meyer (1955). Ryan (1959) demonstrated that the enzyme systems necessary for the aromatization of ring A of C_{19} steroids are located in the microsomal fraction of the human placenta, but he was unable to demonstrate their presence in the mitochondrial fraction. NADPH and molecular oxygen are essential for the reactions (Ryan, 1959). Among the numerous subsequent studies on the mechanism of oestrogen biosynthesis *in vitro* in the human placenta (Longchamp, Gual, Ehrenstein & Dorfman, 1960; Morato, Hayano, Dorfman & Axelrod, 1961; Gual, Morato, Hayano, Gut & Dorfman, 1962; Morato, Raab, Brodie, Hayano & Dorfman, 1962; Wilcox & Engel, 1965; Akhtar & Skinner, 1968; Townsley & Brodie, 1968) none has described oestrogen formation in the mitochondrial fraction. This communication reports the conversion of [^{14}C]testosterone into radioactive oestrone and oestradiol-17 β in high yield by carefully prepared mitochondrial fractions from human placenta.

Full-term placentae were dissected free from foetal membranes and cut into small pieces, which were washed repeatedly with 0.9% NaCl. This tissue, after disruption (Waring Blender) in 0.25 M-sucrose in 0.01 M-tris-HCl buffer, pH 7.7 (3 ml./g. of tissue), was homogenized either in a Potter-Elvehjem-type homogenizer or in a Sorvall Omni-Mixer. After filtration through gauze the homogenate was centrifuged at 700g for 10 min. and the supernatant was then centrifuged at 6000g for 10 min. to yield a crude mitochondrial fraction. The 6000g supernatant was centrifuged at 10000g for 45 min., the pellet discarded and the microsomal fraction sedimented by centrifugation at 60000g for 75 min.

The mitochondrial fraction was washed twice in 10 vol. of 0.20 M-mannitol and 0.07 M-sucrose in 0.01 M-tris-HCl buffer, pH 7.7, and sedimented by centrifugation at 10000g for 10 min.; the 'fluffy layer' of light mitochondria was discarded each time. This washing procedure was repeated three times with centrifugation at 10000g for 15 min. to yield the mitochondrial fraction that was used in the studies on oestrogen formation. Allmann, Bachmann, Orme-Johnson, Tan & Green (1968)

used similar techniques to obtain mitochondrial fractions from rat and bovine liver tissue. The microsomal fraction was washed twice in 20 vol. of 1.15% (w/v) KCl and sedimented by centrifugation at 60000g for 50 min. each time.

All the operations described above were done at 0–4°.

The washed mitochondrial fraction was suspended in 0.05 M-potassium phosphate buffer, pH 7.4, at a concentration equivalent to 9 mg. of mitochondrial protein/ml. Similarly the microsomal fraction was suspended at a concentration equivalent to 37 mg. of microsomal protein/ml. Measurement of mitochondrial and microsomal protein gave values of 1.8 mg. and 3.7 mg. respectively/g. wet wt. of placental tissue. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955), with bovine serum albumin as standard.

Incubations were carried out in a Dubnoff metabolic shaking incubator at 37° in an atmosphere of air. Each reaction flask contained [^{14}C]testosterone (200 $\mu\text{g.}$, 0.81 μC) dissolved in ethanol (0.5 ml.), NADP $^{+}$ (20 μmoles), glucose 6-phosphate dehydrogenase (10 units) and glucose 6-phosphate disodium salt (100 μmoles), each dissolved in 0.05 M-potassium phosphate buffer, pH 7.4, to give a final volume of 7.0 ml. NADPH was generated by incubation for 30 min. Mitochondria (68 mg. of protein) or microsomes (73 mg. of protein) were then added with buffer to give a final volume of 15.0 ml. The mixture, containing 725 μmoles of potassium phosphate, was incubated for a further 60 min. The mitochondrial incubations were carried out in duplicate.

At the end of the incubation period the reaction was stopped by the addition of ethyl acetate. The contents of the reaction flask were extracted with ethyl acetate (3 $\times$ 30 ml.) and the combined extract was evaporated to dryness. The organic residue was dissolved in toluene (20 ml.), which was extracted with a solution of M-NaOH (4 $\times$ 20 ml.). The combined alkali extract, after adjustment to pH 7.5–8.0, was extracted with ether (3 $\times$ 50 ml.), and the combined ether extract, after being washed with water (2 $\times$ 5 ml.), was evaporated to dryness to yield the phenolic fraction. This mixture was chromatographed on paper in the solvent system

toluene-light petroleum (b.p. 80–100°)–methanol–water (5:5:7:3, by vol.) described by Bush (1952). Two radioactive zones on the chromatogram were detected by scanning; the mobilities of these radioactive areas corresponded to those of oestrone and oestradiol-17 β . The two zones were eluted with methanol and each solution exhibited an absorption maximum at 281 nm. and a minimum at 249–250 nm.

To each of the dried eluates the corresponding unlabelled compound was added and a series of successive crystallizations from methanol was carried out to establish the radiochemical purity of the isolated compounds. After obtaining constant specific radioactivities in both the crystals and mother liquors, the compounds were acetylated and the derivatives were again successively crystallized to yield crystals and mother liquors exhibiting the same specific radioactivities, both unaltered from the previous crystallization step.

The chromatogram eluates of the oestrone and oestradiol-17 β zones obtained from the two mitochondrial incubations were shown by isotopic-dilution analyses to be 88 and 93% (oestrone) and 93 and 93% (oestradiol-17 β) radiochemically pure. For example, unlabelled oestrone (100.9 mg.) was added to one of the [^{14}C]oestrone eluates (507 200 d.p.m.) to give a pool specific radioactivity of 5027 d.p.m./mg. After the third crystallization the specific radioactivities of the crystals and of the mother liquor were 4765 and 4640 d.p.m./mg. respectively, and after the fourth crystallization were 4692 and 4772 d.p.m./mg. After chemical formation of oestrone 3-monoacetate the specific radioactivities (calculated for the free compound) after the third crystallization were 4730 and 4689 d.p.m./mg. respectively for the crystals and the mother liquor. The results indicate that the radioactivity associated with the [^{14}C]oestrone accounts for 93% of that originally in the pool.

The radioactivities of the phenolic fractions isolated from the two incubations with mitochondria and the one with microsomes were 88, 79 and 90% respectively of that of the substrate [^{14}C]testosterone. The sum of the radioactivities associated with the radiochemically pure oestrone and oestradiol-17 β accounted for 68 and 89% of the radioactivity present in the phenolic fractions obtained from the two incubations with mitochondria. The minimum radiochemical yields of the sum of oestrone and oestradiol-17 β from substrate [^{14}C]testosterone were 60 and 70%. The radiochemical yields of oestradiol-17 β were 153 and 166% of the corresponding oestrone in these mitochondrial incubations.

Since the placental metabolites of 19-norsteroids, 1 β -hydroxy-19-nortestosterone and 1 β -hydroxy-19-norandrost-4-ene-3,17-dione, are converted into oestradiol-17 β and oestrone by base but not by

placental microsomes (Townsend & Brodie, 1966), mitochondria were incubated with [^{14}C]testosterone in the usual manner but the base partition step of the isolation procedure was omitted. The organic extract of the incubation mixture was partitioned between hexane and aq. 70% (v/v) methanol. Paper chromatography of the aq.-methanol fraction revealed two zones of radioactivity corresponding in mobilities to oestrone and oestradiol-17 β , with no evidence for the presence of either 1 β -hydroxy-19-nortestosterone or 1 β -hydroxy-19-norandrost-4-ene-3,17-dione. Thus the radioactive oestrogens isolated from the incubation mixtures are enzymic products and are not artifacts formed in the isolation procedure.

A mitochondrial preparation that had been previously heated in a boiling-water bath for 15 min. was incubated with [^{14}C]testosterone under the conditions described above. Radioassay of the phenolic fraction isolated from this incubation mixture revealed that only 1% of the substrate radioactivity was present. This result confirms that the conversion of testosterone into oestrogens is enzymic.

Additional incubations of mitochondrial and microsomal preparations from three placentae were carried out in which the concentration of substrate [^{14}C]testosterone was not rate-limiting. In each instance the percentage of the radioactivity of the substrate [^{14}C]testosterone in the isolated phenolic fraction was used as an index of the yield of formed oestrogens. Thus the enzymic capacities of the two subcellular fractions to transform testosterone into oestrone and oestradiol-17 β were compared in each of the three placentae. It was found that, based on mg. of protein of the subcellular fractions, the formation of oestrogen by the mitochondrial system from each of the placentae studied was 28, 35 and 50% respectively of that observed in the microsomal system. In these experiments the mitochondrial fractions had been washed five times and the microsomal fractions twice. When one of these mitochondrial preparations was incubated after two washes only the yield of oestrogen/mg. of protein was equal to that of the corresponding twice-washed microsomal preparation. This finding suggests that the washing procedure is decreasing enzymic activity.

We submit that, at least *in vitro*, the human placenta transforms testosterone into oestrone and oestradiol-17 β in the mitochondrial as well as in the microsomal fraction, and that in the former these oestrogens are synthesized in relatively high yield.

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