Table. 1. *Biosynthesis of cholesterol from squalene in the presence of tritiated water and the distribution of label at positions 7 and 8*

Samples (10 ml.) of a 500 g. cell-free supernatant of rat-liver homogenates (1.25 ml. of 0.1 M-sodium phosphate buffer, pH 7.3/g. of liver) were incubated with 0.02 ml. of squalene at 37° for 3 hr. under oxygen. Each flask contained 30 μ moles of glucose 6-phosphate, 17 μ moles of glutathione, 5 μ moles of NADH and 2.5 μ moles of NADPH, and 200 mc of tritiated water. The isolated cholesterol from each flask had 150000 counts/min. The radioactive samples were dissolved in NE213 or NE220 liquid-scintillation fluid (Nuclear Enterprises Ltd., Sighthill, Edinburgh) and counted in an LDL 2022 liquid-scintillation counter (Isotope Developments Ltd., Beenham, Berks.) at 15% efficiency. All samples were corrected for quenching by internal standardization.

	Cholesterol (III)	5 α -Hydro- peroxide (VII)	7-Oxocholes- terol acetate (IV)	3,7-Diben- zoate (V)	7-Dehydro- cholesterol benzoate (VI)	% of total radioactivity	
						At C-7	At C-8
Counts/min./m-mole	38170	32300	32570	32380	30470	15.0	5.2
Loss of activity (%)	0	15.3	14.7	15.2	20.2		

hydrogen atoms are removed from positions 7 and 8 are therefore 15.0 and 5.2% respectively. This corresponds to a radioactivity ratio loss 2.9:1 associated with the 7 α - and 8 β -C-H bonds; this is in close agreement with our expected ratio 3:1 (see above).

These experiments, in our view, conclusively establish the obligatory role of a cholesta-5,7-diene system (Scheme 1, reaction 3) in cholesterol biosynthesis.

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Akhtar, M. & Marsh, S. (1966). *Biochem. J.* (in the Press).
 Dempsey, M. E. (1965). *J. biol. Chem.* **240**, 4176.
 Dvornik, D., Kraml, M. & Bagli, J. F. (1966). *Biochemistry*, **5**, 1060.
 Fieser, L. F. (1953). *J. Amer. chem. Soc.* **75**, 4386.
 Fieser, L. F., Fieser, M. & Chakravarti, R. N. (1949). *J. Amer. chem. Soc.* **71**, 2226.
 Haslewood, G. A. D. (1938). *J. chem. Soc.*, p. 224.
 Nickon, A. & Bagli, J. F. (1959) *J. Amer. chem. Soc.* **81**, 6330.
 Popják, G., Goodman, de W. S., Cornforth, J. W., Cornforth, R. H. & Ryhage, R. (1961). *J. biol. Chem.* **236**, 1934.
 Wilton, D. C., Akhtar, M. & Munday, K. A. (1966). *Biochem. J.* **98**, 29c.

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A New Placental Metabolite of Oestr-4-ene-3,17-dione: a Possible Source of Error in Oestrogen Estimation

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During studies on the mechanism of aromatization of C₁₈ and C₁₉ steroids Townsley, Possanza & Brodie (1966) have isolated a placental metabolite of 19-norandrostenedione (oestr-4-ene-3,17-dione) that has been identified by chromatographic, microchemical, stereochemical and isotope-dilution techniques as 1 β -hydroxy-19-norandrostenedione (1 β -hydroxyoestr-4-ene-3,17-dione). The production of this metabolite is of especial interest for three reasons. (1) Treatment of the compound with either

acid or base results in the formation of oestrone (3-hydroxyoestra-1,3,5(10)-trien-17-one). Consequently, any such material present in extracts of tissues or body fluids used for the estimation of oestrogens by the commonly used procedures (e.g. Brown, Bulbrook & Greenwood, 1957) would be converted into oestrone in this way and result in artificially elevated values. (2) 1 β -Hydroxylation was suggested as a possible intermediate step in the transformation of androgens into oestrogens by

Morato, Raab, Brodie, Hayano & Dorfman (1962). (3) To our knowledge this is the first report of the isolation of a 1β -hydroxylated steroid of mammalian origin.

In the present communication we give evidence for the identity of the compound and discuss the implications of its occurrence.

[4- ^{14}C]19-Norandrostenedione (250 $\mu\text{g.}$; 2.42×10^5 disintegrations/min./ μmole) and a NADPH-generating system (consisting of 60 μmoles of NADP $^+$, 200 μmoles of glucose 6-phosphate and 20 units of glucose 6-phosphate dehydrogenase) were incubated, with gentle shaking in air at 37° for 1 hr., with 240 ml. of a 10000g supernatant fraction of human term placenta, prepared in water according to the procedure of Ryan (1959). After evaporation of acetone added to quench the reaction, 95% of the radioactivity was recovered from the incubation mixture by extraction with ethyl acetate. Analysis of the extract was effected by 'over-running' in the light petroleum-propylene glycol paper-chromatographic system to remove non-radioactive impurities and re-running the same chromatogram for 3 hr. in toluene-propylene glycol (Zaffaroni, 1953). Radioactive areas on the chromatogram were located with an 880 Chromatogram Scanner (Vanguard Instrument Co., La Grange, Ill., U.S.A.), and the ultraviolet-absorbing radioactive material of R_F 0.22 in the latter system was eluted with methanol to give 62000 disintegrations/min. (26% yield). Portions of this material were then subjected to a variety of procedures designed to determine its identity and properties.

(a) When chromatographed together with 3,17 β -dihydroxyoestra-1,3,5(10)-triene (E-17 β) and relevant reference compounds in the toluene-propylene glycol paper-chromatographic system for 24 hr. ($R_{E-17\beta}$ 1.6), in the B₃ paper-chromatographic system of Bush (1952) for 24 hr. ($R_{E-17\beta}$ 0.32) and in the thin-layer-chromatographic system acetone-benzene (7:13, v/v) on silica gel GF₂₅₄ (E. Merck A.-G., Darmstadt, Germany) (R_F 0.5), the mobility of the radioactive material agreed with that of authentic 1 β -hydroxy-19-norandrostenedione (Bolt *et al.* 1965) and was different from the 2 α -hydroxy isomer in the first system.

(b) Acetylation with pyridine and acetic anhydride at room temperature for 1 hr. yielded a product with the R_F value of authentic 1 β -acetoxyoestr-4-ene-3,17-dione in the A paper-chromatographic system of Bush (1952) and the thin-layer-chromatographic system ethyl acetate-benzene (3:7, v/v) (R_F 0.5 and 0.4).

(c) Sodium borohydride reduction at 0–5° for 10 min. in methanol yielded a product with the R_F of 1 β ,17 β -dihydroxyoestr-4-en-3-one in the B₅ paper-chromatographic system (R_F 0.5) of Bush (1952) and ethyl acetate-benzene (7:13, v/v) thin-layer-

chromatographic system (R_F 0.17). This was different from the R_F of the 10 β -hydroxy isomer.

(d) After treatment with 5 ml. of N-KOH for 1 hr. at room temperature, the mixture was neutralized and extracted with toluene. The dried extract was chromatographed in a modified Bush B₃ system [methanol-water-light petroleum (b.p. 60–90°)-benzene, 4:1:4:1, by vol.], together with appropriate standards. The radioactive area corresponded in R_F (0.5) to oestrone and yielded a blue colour with Turnbull's blue reagent [FeCl_3 - $\text{K}_3\text{Fe}(\text{CN})_6$].

(e) A sample of the unknown compound, containing approximately 20000 disintegrations/min. diluted with 18.7 mg. of authentic 1 β -hydroxy-19-norandrostenedione, was crystallized three times from dichloromethane-hexane. The specific activities of the material from successive crystallizations as determined by scintillation counting (model 314EX; Packard Instrument Co., La Grange, Ill., U.S.A.) were 356, 361 and 359 disintegrations/min./ μmole and of the mother liquors 345, 343 and 344 disintegrations/min./ μmole respectively. A further crystallization from benzene-hexane-methanol failed to reduce the specific activity (crystals 360; mother liquor 350 disintegrations/min./ μmole). The crystals remaining from the fourth crystallization (4.5 mg.) were acetylated as described above. The reaction mixture was analysed by thin-layer chromatography with ethyl acetate-benzene (7:13, v/v) as solvent, and elution of the ultraviolet-absorbing material corresponding to authentic 1 β -acetoxyoestr-4-ene-3,17-dione yielded 4.4 mg. Two recrystallizations from diethyl ether-light petroleum gave material of specific activity 328 and 329 disintegrations/min./ μmole respectively.

To provide further evidence of the position and stereochemical orientation of hydroxylation, [4- ^{14}C , 1- ^3H (83% β)]19-norandrostenedione (Brodie, 1966) with a $^3\text{H}/^{14}\text{C}$ ratio 59:1 was incubated with the 10000g placental supernatant fraction, and the incubation mixture analysed as described above. The 1 β -hydroxy-19-norandrostenedione isolated had a $^3\text{H}/^{14}\text{C}$ ratio 13:1 (78% loss of tritium), implicating the 1 β -position since hydroxylation reactions have been shown to occur by direct replacement of hydrogen (Hayano, 1962).

In incubations similar to those described above, under conditions where 82% of androst-4-ene-3,17-dione was converted into oestrone, 19-norandrostenedione was converted 4.5% into oestrone and 8% into 1 β -hydroxy-19-norandrostenedione. If a base partition has been incorporated into our procedure, the 1 β -hydroxy-19-norandrostenedione produced would have formed oestrone to give a total conversion of 12.5%. This suggests that previous values, obtained *in vitro*, of 12–20% conversion of 17 β -hydroxyoestr-4-en-3-one into oestrogens (Ryan,

1959; Gual, Morato, Hayano, Gut & Dorfman, 1962) probably include a contribution due to the formation of oestrogen as an artifact from the 1β -hydroxy derivative, since base partitions were used.

Whether a similar situation exists with studies *in vivo* is not known. Although *in vitro* the 19-nor-steroids are relatively poor precursors of oestrogens, *in vivo* they, and their 17α -substituted derivatives, appear to be converted into oestrogens to an extent similar to or greater than testosterone (17β -hydroxyandrost-4-en-3-one) (Breuer, 1964; Dimick, Heron, Baulieu & Jayle, 1961; Engel, Alexander & Wheeler, 1958). Since the isolation and estimation procedures used in such studies involve acid hydrolysis and partition between aqueous base and organic solvent, the presence of 1β -hydroxylated derivatives would artificially inflate the value for oestrogens. For example, such a derivative could be the precursor of the oestrogenic artifact of 17α -ethynyl- 17 -hydroxyoestr-4-en-3-one that Brown & Blair (1960) suggest may be produced during the analysis of urine.

Owing to the increasing use of 19-nor-steroids as progestational agents, there is an active interest in their metabolism. The present communication provides evidence for the possibility of producing 1β -hydroxy metabolites that, in investigations of oestrogen biosynthesis, make contributions of artifacts unless adequate precautions are taken.

Stereochemical studies and the cofactor requirements for aromatization of C_{18} and C_{19} steroids suggest the possibility of 1β -hydroxylated androgens' being intermediates in aromatization (Morato *et al.* 1962; Townsley *et al.* 1966). However, neither

the isolated radioactive 1β -hydroxy-19-norandrostenedione nor the authentic non-radioactive compound was converted into oestrogens by the placental system under conditions where appreciable amounts of oestrogens were formed from 19-norandrostenedione.

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- Bolt, C. C., Mijs, W. J., Zeelen, F. J., Szpilfogel, S. A., De Flines, J. & Van der Waard, W. F. (1965). *Rec. Trav. chim. Pays-Bas*, **84**, 626.
 Breuer, H. (1964). *Int. J. Fertil.* **9**, 181.
 Brodie, H. J. (1966). *Tetrahedron*, **22** (in the Press).
 Brown, J. B. & Blair, H. A. F. (1960). *Proc. Roy. Soc. Med.* **53**, 433.
 Brown, J. B., Bulbrook, R. D. & Greenwood, F. C. (1957). *J. Endocrin.* **16**, 49.
 Bush, I. E. (1952). *Biochem. J.* **50**, 370.
 Dimick, D. F., Heron, M., Baulieu, E. E. & Jayle, M.-F. (1961). *Clin. chim. Acta*, **6**, 63.
 Engel, L. L., Alexander, J. & Wheeler, M. (1958). *J. biol. Chem.* **231**, 159.
 Gual, C., Morato, T., Hayano, M., Gut, M. & Dorfman, R. I. (1962). *Endocrinology*, **71**, 920.
 Hayano, M. (1962). In *Oxygenases*, p. 181. Ed. by Hayaishi, O. New York: Academic Press Inc.
 Morato, T., Raab, K., Brodie, H. J., Hayano, M. & Dorfman, R. I. (1962). *J. Amer. chem. Soc.* **84**, 3764.
 Ryan, K. J. (1959). *J. biol. Chem.* **234**, 268.
 Townsley, J. D., Possanza, G. & Brodie, H. J. (1966). *Fed. Proc.* **25**, 282.
 Zaffaroni, A. (1953). *Recent Progr. Hormone Res.* **8**, 51.

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The Biotransformation of Gramine into Tryptophan in Barley Shoots

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The alkaloid gramine (VI) is formed in germinating barley from tryptophan (Bowden & Marion, 1951a,b). Wenkert (1962) suggested that a key intermediate in the above biotransformation is a protonated 3-methyleneindolenine species (IV) (Scheme 1), which could arise by a reverse Michael degradation of the Schiff base (III) formed by the

interaction of tryptophan (I) and pyridoxal phosphate (II). Attack of the intermediate (IV) by ammonia, followed by methylation, could lead to gramine (VI). Wenkert's (1962) hypothesis has been supported by experiments *in vivo* (O'Donovan & Leete, 1963; Gower & Leete, 1963) and by amine fragmentation *in vitro* (Whitlock & Digenis, 1964).