fied product with the system isobutyric acid–water–concentrated ammonia (66:33:1)[39] showed only one spot, with R_f 0.50, *vs.* R_f's of 0.32 for 5'-thymidylic acid, 0.79 for the thymidine isostere I, and 0.69 for thymidine.

1-(*cis*-4-Carboxy-*trans*-3-hydroxycyclopentyl)-thymine (XIX).—A solution (*p*H 9) of 0.361 g. (1.5 mmoles) of 1-(*trans*-3-hydroxy-*cis*-4-hydroxymethyl)-thymine (I), 0.318 g. (3 mmoles) of sodium carbonate and 0.758 g. (9 mmoles) of sodium bicarbonate in 15 ml. of water was sealed with platinum catalyst (pre-reduced, from 0.038 g. of platinum oxide) and oxygen at 35 p.s.i. into a 183-ml., stainless steel bomb. This was rocked and heated at 85–90° for 24 hours, cooled, opened, charged with oxygen as before, sealed and rocked at 88–92° for another 47 hours. The cooled mixture was acidified and de-ionized by the careful, portionwise addition of 4.0 g. of Dowex-50 polysulfonic acid resin, and then filtered. The dark filtrate and washes were evaporated to a volume of *ca.* 5 ml., adjusted to *p*H 7 with concentrated aqueous ammonia, allowed to stand for 2 hours, then filtered from 0.048 g. of a solid which had separated, m.p. 317–319°. This material gave an infrared spectrum identical with that of thymine. The filtrate was adjusted to *p*H 8.5–9.0 with more ammonia, then subjected to chromatography[40] on a 1.5 × 27-cm. column of Dowex-1 formate. Eluate peaks were located qualitatively by evaporating a droplet from each cut (10–12 ml.) on filter paper; thymine derivatives at concentrations down to about 0.3 μmole/ml. were detected as dark spots when viewed under an ultraviolet lamp with a filter giving maximum transmission at 254 mμ. Quantitative assessments of peak homogeneities were made after appropriate dilutions, using a Beckman DU ultraviolet spectrophotometer, then plotting cut numbers *vs.* optical densities on graph paper. Water (cuts 1–15) eluted a peak centered at cut 7 with a shoulder at cut 10. Evaporation of cuts 6–8 left a glass which crystallized overnight. It was then washed with ethanol, leaving 0.011 g. (3% recovery) of crystals, m.p. 210–212°. These gave an infrared spectrum identical with that of the starting diol I. Dilute formic acid (0.05 *N*, cuts 16–29) gave a peak centered at cut 24. Evaporation of cuts 22–26 left 0.021 g. of a solid, m.p. 315–318° dec., which gave an infrared spectrum identical with that of thymine. Stronger formic acid (0.55 *N*, cuts 30–46) gave a peak centered at cut 40. Cuts 39–45 were evaporated and residual formic acid was removed by evaporation to dryness with 3 × 1 ml. of water. The residue was heated with 6 drops of water, allowed to stand and then collected by filtration; yield 0.018 g. (5%), m.p. 250–254°; $\lambda_{max}^{H_2O}$ 273 mμ (ε 9,000), min. 238 mμ (ε 1,680); λ_{max}^{KBr} 2.90 (–OH), 3.19 sh (–NH), 3.9, 8.20 and 8.30 (–COOH). When the potassium bromide disk was ground with the calculated amount of 0.01 *N* methanolic potassium hydroxide, then dried and repressed, it gave a spectrum in which the 3.9, 8.20 and 8.30 bands were gone and a new band at 6.35 μ (–COOK) was present.

Anal. Calcd. for $C_{11}H_{14}O_5N_2$: C, 51.96; H, 5.55; N, 11.02. Found: C, 51.42; H, 4.95; N, 11.02.

In a similar experiment with a heating period of 24 hours, the yield of hydroxy acid XIX was 1.2%, and 67% of the starting diol I was recovered.

(39) R. Zetterström and M. Ljunggren, *Acta Chem. Scand.*, **5**, 291 (1951).

(40) In an earlier run this ion exchange chromatography was done by a gradient elution method patterned after a procedure of H. Busch, R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **196**, 717 (1952). The concentrations of formic acid in peak eluate fractions were determined by potentiometric titration. These concentrations were then used in the modified chromatography which is described.

COMMUNICATIONS TO THE EDITOR

THE MECHANISM OF ESTROGEN BIOSYNTHESIS

Sir:

One of the more important biochemical problems related to steroid metabolism concerns the biosynthetic sequence and mechanism involved in the transformation of androgens to estrogens. It has been shown that 19-hydroxyandrost-4-ene-3,17-dione and 19-oxoandrost-4-ene-3,17-dione are excellent substrates for conversion to estrogens using human placental microsome preparations and are considered to be on the pathway going from androst-4-ene-3,17-dione to estrogen.[1] It also has been found that the 19-hydroxy compound forms both formaldehyde and formic acid in its conversion to estrogen while the 19-oxo structure yields mostly formic acid.[2] In attempting to fit these facts into a mechanism for estrogen formation, information concerning the stereochemistry of the hydrogen eliminated at C-1 was necessary. This communication is concerned with this point.

Two samples of androst-4-ene-3,17-dione specifically labeled with tritium at C-1 were prepared. The distribution of tritium in compound I was 25% 1α and 75% 1β and in compound II 93% 1α and 7% 1β. The proof for the distribution of tritium is outlined in the preceding publication.[3] These two compounds were then incubated with a placental aromatizing system.

Experimental.—Two sets of incubations were carried out with human placental microsomes supplemented with a reduced triphosphopyridine-nucleotide (TPNH) regenerating system as described earlier.[1] In the first experiment, the incubations, run with a total of 16.7 mg. of I, were extracted with methylene chloride. The combined crude extracts were partitioned between 50% aqueous methanol (mobile upper phase) and carbon tetrachloride (lower phase) in a Craig countercurrent distribution apparatus (99 transfers) and estrone was obtained from the appropriate tubes.[4] In analyzing for radioactivity, sample weights were determined by direct weighing of crystals (method A) and by spectrophotometric analyses (method B) using the absorption peak at 242 mμ for androst-4-ene-3,17-dione and the Kober reaction chromogen[5] for estrone. In experiment 2, the incubations containing a total of 200 μg. of II were extracted with methylene chlo-

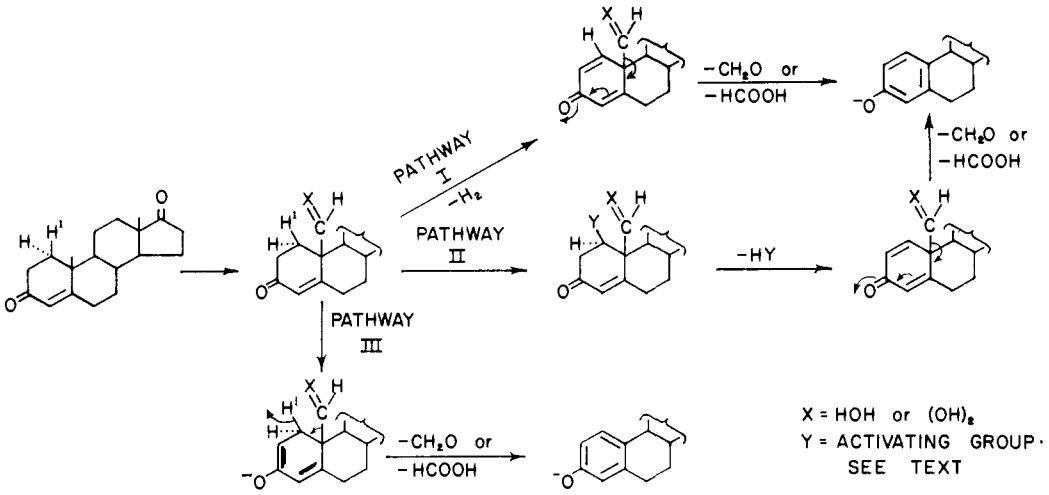
(1) T. Morato, M. Hayano, R. I. Dorfman and L. R. Axelrod, *Biochem. Biophys. Research Commun.*, **6**, 334 (1961).

(2) R. I. Dorfman, C. Gual, T. Morato, M. Hayano and M. Gut, Abstracts, International Congress on Hormonal Steroids, Milano, Italy, May, 1962, p. 270.

(3) H. J. Brodie, M. Hayano and M. Gut, *J. Am. Chem. Soc.*, **84**, 3766 (1962).

(4) This method of isolating pure estrone from tissue incubations of androstenedione has been well documented (B. Baggett, L. L. Engel, K. Savard and R. I. Dorfman, *J. Biol. Chem.*, **221**, 931 (1956); K. Ryan, *ibid.*, **234**, 268 (1959); see also ref. 7).

(5) J. B. Brown, *J. Endocrinol.*, **8**, 196 (1952).



ride. The crude dried extract was chromatographed on paper in the ligroin–propylene glycol system to effect a preliminary purification. The area near the starting line containing estrogens was eluted and chromatographed again in the toluene–propylene glycol system. Estrone was eluted and after being partitioned in aqueous sodium hydroxide, was rechromatographed in the Bush B_3 system. Aliquots of the estrone eluted from this chromatogram were analyzed for radioactivity along with starting material, concentrations being established by method B. Crystalline starting material was chromatographed on paper in the ligroin–propylene glycol and Bush A systems before counting. All determinations of radioactivities were carried out in a Packard Tri-Carb liquid scintillation spectrometer.

Results.—As illustrated in the table, when androstenedione had tritium predominantly in the 1β position (75%), most of it was lost in going to estrone (82% H^3 lost) whereas, when the tritium was essentially in the 1α position (93%), most of the label was retained (15% H^3 lost). This shows that the 1β hydrogen is eliminated preferentially in the aromatization process. This is contrary to conclusions reached in a recent study on the conversion of androstenedione to estrone in incubations with baboon and human ovaries. The authors claimed that the 1α-H is eliminated, a conclusion based on equivocal data and some unfortunate assumptions concerning the distribution and stability of the label in their steroid substrate.[6]

Discussion. The transformation of androstenedione to estrogen involves the elimination of the C-19 group and hydrogen from C-1 and C-2. The loss of the C-2 hydrogen is conveniently explained by a simple enolization of the C-3 oxygen to form a Δ^2 double bond since this structure would also favor the removal of a C-1 group in a later step. The problem of C-1 hydrogen removal involves the questions of when in the reaction sequence it is eliminated and how it is eliminated. With regard to the first point, the hydrogen is undoubtedly removed after the oxidation of the C-19 methyl group to the hydroxy or oxo compound and before or during the elimination of this group since androsta-1,4-diene-3,17-dione, 19-norandrost-4-ene-3,17-dione[7] and 19-norandrost-5-(10)-ene-3,17-dione do not appear to be good substrates for conversion to estrogen.

After the oxidation of the C-19 group, there are three general ways in which removal of the C-1 hydrogen can take place. These are illustrated in the figure. Modifications of the general types shown to involve different reaction sequences and enolization forms can be visualized. Pathway I shows a 1,2-dehydrogenation followed by the spontaneous elimination of the C-19 group. This pathway is not favored because a 1,2-dehydrogenation does not now appear likely, at least by the mechanism operating in microörganisms. With the Δ^1-dehydrogenase from *Bacillus sphaericus* (ATCC 7055) the C-1α-hydrogen is eliminated[8]; further there is no requirement for TPNH and oxygen which are essential in the placental aromatization process.[1]

TABLE I

CONVERSION OF ANDROSTENEDIONE TO ESTROGEN

Substrate	Product	% Loss of activity
Experiment 1		
Androstenedione (25%-1α-H^3, 75%-1β-H^3)	Estrone	
	d.p.m./μmole	d.p.m./μmole	
(1)	9.45×10^{4a}	1.74×10^{4a}	81.6
(2)	11.6×10^{4b}	2.07×10^{4b}	82.2
Experiment 2		
Androstenedione (93%-1α-H^3, 7%-1β-H^3)		
	d.p.m./μmole		
	2.23×10^{7b}	1.89×10^{7b}	15.3

[a] Weights for analysis determined by method A (see text); estrone m.p. 253°. [b] Weights for analysis determined by method B (see text).

(6) L. R. Axelrod and J. W. Goldzieher, *J. Clin. Endocrinol. and Metabolism*, **22**, 537 (1962). The authors assumed that their androstenedione substrate had tritium predominantly at 1α and 2α. From their method of synthesis, the reduction of a $\Delta^{1,4}$-dione, the label was, in all probability, predominantly beta (*cf.* ref. 3).

(7) C. Gual, T. Morato, M. Hayano, M. Gut and R. I. Dorfman, *Endocrinol.*, in press.

(8) H. J. Ringold, M. Gut, M. Hayano and A. Turner, *Tetrahedron Letters*, in press.

The more likely pathway for C-1 hydrogen elimination would involve either displacing it by an activated group "Y" (for example hydroxyl) which can later be eliminated[9] (pathway II) or eliminating it as a hydride ion concerted with the removal of the C-19 oxygenated group (pathway III). This latter pathway could take place either through the mechanism shown[10] or by a four-center type (cyclic) elimination involving an activated C-19 oxonium ion intermediate (not shown.) From present knowledge, these types of reactions could reasonably be expected to involve TPNH and oxygen and the 1β-hydrogen. Experiments are now in progress to define the more likely pathway.[11]

Acknowledgment.—We wish to thank Drs. H. J. Ringold and M. Gut for their active interest in various aspects of this problem.

(9) The group "Y" also could be eliminated in concert with the C-10 group to form a C-1(10) double bond.

(10) This is similar to a mechanism previously proposed (M. Hayano, H. J. Ringold, V. Stefanovic, M. Gut and R. I. Dorfman, *Biochem. Biophys. Research Commun.*, **4**, 454 (1961)) except that it involves the 1β-hydrogen.

(11) This work was supported in part by U. S. Public Health Service grants A-2672, FF-258, and training grant CRTY-5001.

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THE STEREOCHEMICAL COURSE OF THE CATALYTIC HYDROGENATION OF RING A UNSATURATED STEROIDS

Sir:

In the reactions of double bonds in ring A of steroids, attack usually occurs predominantly on the alpha face since this is often less hindered due to the presence of the 10β-methyl group.[1] Indeed, some workers have made "alpha face attack" a general rule for, in a recent publication,[2] it was assumed, based on the type of work cited in reference 1, that in the reduction of $\Delta^{1,4}$ steroids with tritium on palladium catalyst, the label goes mostly into the 1α and 2α positions.

For the study of the mechanism of estrogen biosynthesis, we wished to prepare a C-1 tritiated androst-4-ene-3,17-dione of known configuration. To do this 17β-hydroxy-androsta-1,4-dien-3-one was reduced with tritium gas over palladium.[3] We can report now that under these conditions the attack at C-1 is predominantly *beta* in a ratio of about 3:1. In contrast, however, when androst-1-ene-3,17-dione is reduced in the same manner the introduction at C-1 is over 90% alpha as expected.

(1) For examples of alpha attack during reduction, epoxidation and glycol formation, see L. F. Fieser and Mary Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, pp. 254, 266, 271–274. The less common beta attack has been shown in hydrogenations of Δ^4 and Δ^7 compounds where a ring junction is involved (pp. 272, 274).

(2) L. R. Axelrod and J. W. Goodzieher, *J. Clin. Endocrinol. and Metabolism*, **22**, 537 (1962).

(3) Compare P. Osinski and H. Vanderhaeghe, *Rec. trav. chim.*, **79**, 216 (1960).

The proof for the distribution of tritium in the two reduced products is outlined in the accompanying flow sheet. Before determining the distribution of tritium at C-1, the tritium at C-2 and other labile positions was removed by refluxing with KOH in aqueous methanol. Experiments have shown that the percentage of tritium lost from these positions ranges from 40 to 60%.[4] For clarity, tritium is shown at the positions of highest concentration in the reduced compounds III, IV, and V. All reductions were carried out with tritium gas over 5% palladium–charcoal in dioxane solvent. The structures of all compounds were proven from mixture melting points and by comparing their infrared spectra with authentic samples. The radiochemical purity was established by purifying to constant specific activity using paper chromatography and recrystallization.

Androst-1-ene-3,17-dione (I) was reduced and then refluxed with potassium hydroxide in aqueous methanol to give compound III. Compound III then was treated with dichlorodicyanoquinone (DDQ)[5] and incubated with *Bacillus sphaericus* (ATCC 7055)[6] to give compound IIIA and IIIB with losses of 89 and 93% of the tritium, respectively. This showed that the reduction and subsequent dehydrogenation were quite stereospecific for addition and removal of tritium at C_1. That the removal of tritium was from the alpha position was indicated from the literature on these reactions[5,6] and was proven conclusively in a recent study by Ringold, Gut, Hayano and Turner,[7] who showed that with 5α-androstane-3,17-dione-1α-H^2 the 1α-hydrogen (deuterium) is lost exclusively. They also showed that the reduction of the androst-1-ene-3,17-dione with deuterium proceeds almost exclusively alpha at C-1.

To determine whether the enzymatic and DDQ reactions were as specific for the elimination of the 1α-hydrogen with a C-4 double bond present III was oxidized to compound IV using the standard bromination–dehydrobromination technique.[8] Compound IV then was oxidized to compound IVA with DDQ. Here 76% of the tritium was lost, indicating that some of the 1β-hydrogen was eliminated with the C-4 double bond present. Dehydrogenation with *B. sphaericus* gave the same result as with the saturated compound, *i.e.*, loss of 93% of the tritium (IVB), showing that in this reaction the 1α-hydrogen is lost preferentially with the Δ^4-compound to the same extent as with 5α-androstane-3,17-dione.

Finally, 17β - hydroxy - androsta - 1,4 - dien - 3-one (II) was reduced catalytically to give a mixture of products.[3] The Δ^4-compound was isolated

(4) M. Gut and M. Hayano in "Advances in Tracer Methodology," Vol. I, S. Rothchild, ed., Plenum Press, New York 11, N. Y., in press. Osinski and Vanderhaeghe have reported (ref. 3) that after the reduction of the Δ^1 bond of the bismethylene dioxide of prednisone no loss of tritium was noted on treatment with potassium hydroxide. As can be seen, this is contrary to results obtained with C-1,2-tritiated testosterone and androstenedione.

(5) H. J. Ringold and A. Turner, *Chem. and Ind.*, 211 (1962).

(6) M. Hayano, H. J. Ringold, V. Stefanovic, M. Gut and R. I. Dorfman, *Biochem. Biophys. Res. Comm.*, **4**, 454 (1961).

(7) H. J. Ringold, M. Gut, M. Hayano and A. Turner, *Tetrahedron Letters*, in press.

(8) G. Rosenkranz, O. Mancera, V. Gatica and C. Djerassi, *J. Am. Chem. Soc.*, **72**, 4077 (1950).