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Studies on the Metabolism of 16α-Hydroxyprogesterone in Humans; Conversion to Urinary 17-Isopregnanolone*

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Received March 23, 1962

The metabolic conversion of 16α-hydroxyprogesterone to 17-isopregnanolone has been demonstrated. The results also indicate that a C_{21}-Δ^{16}-steroid may serve as an intermediate and that its reduction occurs stereospecifically. Evidence has been obtained that 16α-hydroxyprogesterone may be secreted by the normal subject in amounts of 1–2 mg per day.

The isolation of Δ^{16}-androsten-3α-ol from hog testes (Prelog and Ruzicka, 1944) and from the urine of normal humans (Brooksbank and Haslewood, 1949, 1950, 1952) and, more recently, its quantitative estimation in human urine (Brooksbank and Haslewood, 1961) have stimulated interest in its biosynthetic origin. Burstein and Dorfman (1960) showed that this metabolite was formed from both cholesterol and pregnenolone in a woman with a virilizing benign adrenal tumor. They pointed out that such a result is consistent with two general mechanisms for the formation of the 16-olefin. In one of these, it would arise by direct 2-carbon elimination from a C_{21} steroid. Alternatively it could be formed by the dehydration of a 17β-hydroxy-C_{19} steroid such as testosterone. Although it has been demonstrated that rat testes and human liver are apparently both able to convert testosterone to $\Delta^{4,16}$-androstadien-3-one *in vitro* (Stylianou *et al.*, 1961a,b), no *in vivo* evidence exists that testosterone or any other C_{19} steroid can serve as precursor of a Δ^{16}-metabolite. Furthermore, Savard (personal communication, 1962) has recently shown that 50 mg of testosterone, when injected into adult males, does not re-

* Supported in part by U. S. Public Health Service Grant No. A-110. A preliminary report of this work has appeared (Calvin and Lieberman, 1962).

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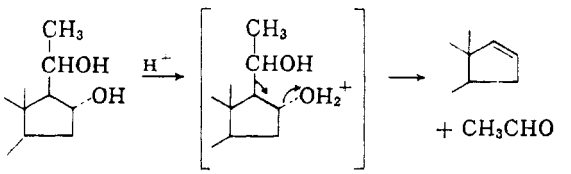

FIG. 1.—Suggested scheme for the formation of C_{19}-Δ^{16}-steroids by the degradation of C_{21}-16,20-glycols.

sult in an elevated excretion of Δ^{16}-androstenol. We have considered the possibility that the Δ^{16}-grouping could arise from a C_{21}-16,20-dihydroxy-steroid by a biochemical reaction which is analogous to the already well-known behavior of 1,3-glycols. Under acid catalysis, 1,3-diols undergo C-C bond cleavage to yield an olefin and a carbonyl compound (Wasserman, 1956). In Figure 1, the analogous reaction which would result in the degradation of a C_{21}-16,20-dihydroxy-steroid to a C_{19}-olefin is illustrated. In particular, the reaction sequence would produce Δ^{16}-androsten-3α-ol from a suitable C_{21}-3,16,20-triol, examples of which have on numerous occasions been isolated from the urine of both normal and abnormal subjects (Hirschmann *et al.*, 1961; Fukushima *et al.*, 1961; Fotherby, 1959). It is not unreasonable to suppose that these 16,20-glycols are derived from the Δ^4-3-keto steroid, 16α-hydroxyprogesterone, particularly since it has recently been demonstrated that the latter compound, which has been isolated in significant quantities from hog adrenals (Wettstein *et al.*, 1959), is also a product of the *in vitro* incubation of progesterone with hyperplastic human adrenals (Villee *et al.*, 1962) and normal human ovaries (Warren and Salhanick, 1961).

Therefore, in order to determine whether 16α-hydroxyprogesterone (I) may be converted to Δ^{16}-androsten-3α-ol via a triol intermediate, randomly tritiated I was injected into a normal male subject, with simultaneous ingestion of 200 mg of unlabeled I. Δ^{16}-Androsten-3α-ol was in fact isolated from the urine of this individual, but it was found to be free of tritium and therefore not a product of the peripheral catabolism of I. However, another unusual compound, 17-isopregnanolone (3α - hydroxy - 17α - pregnan-20-one) (III), was also isolated from the urine of this subject, and it contained a significant amount of tritium label.

This finding was particularly noteworthy in view of the fact that the side-chain attached to carbon-17 of the steroid nucleus is normally in the β-configuration, cis to the C_{18} methyl group. Steroids such as III, whose side-chain is in the alternate α-configuration, have rarely been isolated from biological systems. Examination of the literature reveals two such instances prior to the present study. 17-Isopregnanolone (III) was isolated from human pregnancy urine which had been heated at reflux temperature in 5% H_2SO_4 to hydrolyze water-soluble conjugates

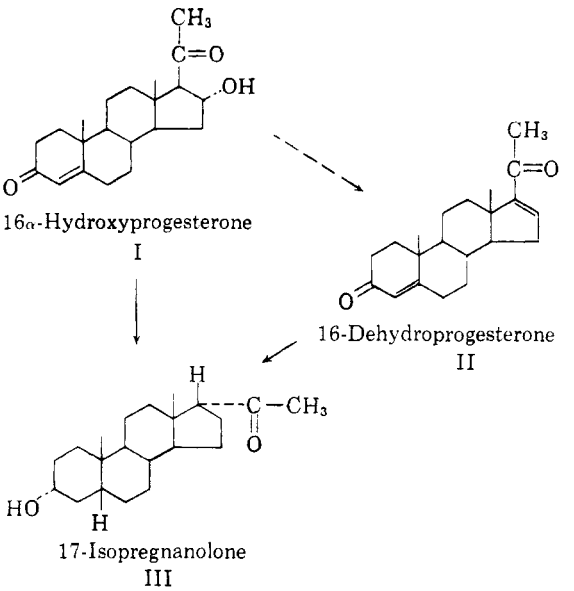

FIG. 2.—17-Isopregnanolone and its metabolic precursors.

(Lieberman *et al.*, 1948). Its normally occurring 17β-epimer, pregnanolone, was found also to be present in this sample of urine. Unfortunately, the techniques employed in this experiment did not exclude the possibility that the 17α-isomer was an artifact of isolation, since it may be prepared from pregnanolone by acid-catalyzed epimerization (Moffet and Hoehn, 1944). More recently, it has been shown that the mold, *Rhizopus nigricans*, can convert 16-dehydroprogesterone (II) to 11α-hydroxy-17α-progesterone (Meister *et al.*, 1953). However, until the present there has been no conclusive evidence for the natural occurrence of steroids whose side-chain is in the α-configuration. In view of our findings and the growing evidence that 16α-hydroxyprogesterone (I) may be a normal secretory product in humans, it is quite possible that III is endogenously excreted by normal individuals. In support of these conclusions, data will be presented indicating that the normal male studied in our experiments may produce as much as 1.5 mg of I per day.

Significantly, the above conversion probably proceeds through a 16-dehydro intermediate, such as II (see Fig. 2), since it was also possible to show in these investigations that II, when orally administered, can be metabolized to 17-isopregnanolone. This suggests that olefin-forming dehydrations may occur during the *in vivo* catabolism of steroids. Moreover, the apparent formation of a 17-iso metabolite from II is clearly analogous to the already cited microbiological conversion reported by Meister *et al.* (1953).

EXPERIMENTAL

Hydrocarbon solvents were purchased from Phillips Petroleum Co. and were distilled before

use. Dimethylformamide was a spectro grade solvent purchased from Eastman Chemicals. All other solvents were Mallinckrodt analytical grade reagents. Polar solvents packaged in metal drums were distilled before use.

Melting points were determined on a Kofler block and were corrected. Infrared spectra were determined on a Model 221 Perkin-Elmer double-beam spectrophotometer. Measurements of absorption in the ultraviolet were made with a Beckman Model DU spectrophotometer.

Radioactive samples were counted in the Packard Tri-Carb liquid scintillation spectrometer, Model 314-DC, with the counting chamber set at −2°. Dried samples in 5-dram vials (Wheaton Glass Co.) were dissolved in 5 ml of toluene containing 0.3% of 2,5-diphenyl-oxazole and 0.01% of 1,4-bis-(5-phenyl-oxazolyl)-benzene (Pilot Chemicals). The data referred to in this paper were obtained at high voltage tap 8 (1190 v), with pulse height discriminator settings of 10–50 v for tritium and 100 to infinity v for C^{14}. The current efficiency of the counter under these conditions was 17% for tritium and 48% for C^{14}, as determined with sealed standards of known dpm. All data have been standardized by correction for variations from these efficiencies. The ratio of two radioactive isotopes in a doubly labeled sample was determined by the simultaneous counting technique of Okita *et al.* (1957).

Column partition chromatography was performed on celite (Johns Manville No. 545). The techniques involved in this procedure are widely used in this laboratory and have been described in detail previously (Kelly *et al.*, 1962). Absorption chromatography was performed on Woelm neutral alumina (activity I), unless otherwise specified.

16α-Hydroxyprogesterone-H³.—16α-Hydroxyprogesterone (I) was randomly tritiated by the procedure of Wilzbach (1957) in the laboratories of the New England Nuclear Corp. When returned to this laboratory, the crude tritiated residue was dissolved in a saturated solution of $KHCO_3$ in methanol. It was allowed to remain in this solution for 24 hours at room temperature in order to exchange the labile tritium for hydrogen. The equilibrated product was then chromatographed for 5½ hours on paper in the Bush B_2 system (333 ml toluene, 167 ml ligroin, 300 ml methanol, 200 ml water) (Bush, 1952). The only detectable ultraviolet-absorbing zone occurred at a position about midway between the origin and the bottom of the chromatogram. Its contents were eluted with methanol and divided into two approximately equal fractions, A and B.

Fraction A was further purified as follows. It was chromatographed on paper for 5 hours in the E_2B system of Eberlein and Bongiovanni (1955) (500 ml iso-octane, 250 ml tertiary butanol, 450 ml water), the only detectable ultraviolet absorbing zone occurring at 0.8 of the distance between the origin and the solvent front. The contents of this zone were eluted and rechromatographed for 4 days on paper in the Bush A system (500 ml ligroin, 400 ml methanol, 100 ml water) (Bush, 1952). One ultraviolet absorbing band at about 0.15 of the distance between the origin and the bottom of the chromatogram resulted. After the addition of 5 mg of carrier I to this material, the mixture was rechromatographed on celite in the E_4 system of Eberlein and Bongiovanni (1955) (500 ml iso-octane, 225 ml tertiary butanol, 225 ml methanol, 50 ml water). Only one peak of radioactivity was detected, and this appeared in the seventh hold-back volume, coinciding with a peak of ultraviolet absorption (λ_{max} = 240 mμ). The radioactive fractions were pooled and chromatographed on alumina to remove colored impurities. The eluted I was then crystallized successively from methanol-ether and acetone, yielding products of respective specific activities of 4.9 × 10^6 cpm/mg (fraction A_1) and 4.6 × 10^6 cpm/mg (fraction A_2), as determined by counting measured aliquots of solutions prepared from weighed samples. Fraction A_2 was used in experiments 1 and 2 of the present study. To insure the absence of contaminants such as 16-dehydroprogesterone in fraction A_2, its homogeneity was further verified 9 months after the above purification. An aliquot of A_2 was diluted with carrier and the mixture rechromatographed on celite in the E_4 system. Only one radioactive peak was present, and it occurred in the expected hold-back volume. Aliquots of nine consecutive fractions from this peak were evaporated to dryness and the specific activities of the residues were determined by counting and spectrophotometric estimation, with $\epsilon_{240m\mu}$ = 16,500. The specific activities so estimated agreed within 5% of the mean value.

For experiment 3 of the present study, tritiated I derived from fraction B above was used after further purification as follows. Five mg of carrier was added to this labeled material and the mixture was chromatographed on celite in the E_4 system. The presence of several radioactive peaks confirmed the inhomogeneity of this sample. Therefore the fractions containing labeled I were pooled and rechromatographed on celite in a Bush-type system (250 ml hexane, 250 ml benzene, 400 ml methanol, 100 ml water) and then again in a modified Eberlein-Bongiovanni system (300 ml hexane, 200 ml tertiary butanol, 150 ml methanol, 50 ml water). One symmetrical peak was observed in both chromatograms. The specific activities in five consecutive fractions of the peak from the second chromatogram, as determined by relating cpm to absorbance at 240 mμ, showed a deviation from the mean of not more than 8%. These fractions (average specific activity 4.2 × 10^6 cpm/mg) were therefore pooled for use in experiment 3.

Administration of 16α-Hydroxyprogesterone-H³(I) and Isolation of 17-Isopregnanolone(III) from Urine

EXPERIMENT 1.—16α-Hydroxyprogesterone-H^3

(I) (1.6×10^6 cpm) was injected intravenously into a normal 25-year-old male subject. Simultaneously 200 mg of synthetic, unlabeled I was ingested, and urine was collected for 3 days. The pooled urine (2.5 liters) was adjusted to *p*H 4.5 and treated with 7×10^5 units of mammalian β-glucuronidase (Ketodase, Warner-Chilcott Co.) at 37° for 3 days. It was then extracted with an equal volume of ethyl acetate. After the organic extract was washed with a 5% NaOH solution and water, it was evaporated to dryness under vacuum. The resulting residue was chromatographed on Woelm neutral alumina containing 6% water; the gradient elution technique of Lakshmanan and Lieberman (1954) was used. A single peak of radioactive material containing more than 2×10^5 cpm was found not in those early fractions of the chromatogram where Δ^{16}-androsten-3α-ol would be expected to occur, but instead in those later fractions in which dehydroisoandrosterone ordinarily is found. Evaporation of the solvent from the early nonradioactive fractions revealed a crystalline residue whose distinct musk-like odor was unmistakably similar to that which has been attributed to Δ^{16}-androsten-3α-ol (Prelog and Ruzicka, 1944). This residue was rechromatographed on dry alumina. Elution with an ether-benzene (4:6) solution yielded a substance which, upon recrystallization from ligroin A, gave a sample weighing 0.6 mg and melting at 142–145°. The melting point of this product was not depressed by admixture with authentic Δ^{16}-androsten-3α-ol which had been synthesized by the procedure of Brooksbank and Haslewood (1961). Infrared spectrum determinations in CS_2 confirmed that the isolated and synthetic compounds were identical. It was thus concluded that the subject studied had produced Δ^{16}-androsten-3α-ol which was free of radioactivity and therefore not derived from I via peripheral metabolism.

As mentioned earlier, a significant peak of relatively nonpolar radioactive material was observed in the initial chromatogram. The fractions constituting this peak were pooled and rechromatographed on celite in heptane-methylcellosolve (system A). A single radioactive product was found in the third hold-back volume. From a solution of the latter in ligroin A, 10 mg of crystalline material deposited. This melted at 134–140°. Upon recrystallization from the same solvent, 5 mg, melting at 142–145°, was obtained. This product had a specific activity of 1.40×10^4 cpm/mg. The infrared spectrum of this substance and that of its acetate, both determined in CS_2, agreed with those recorded in the literature for 3α - hydroxy - 17α - pregnan - 20 - one (17-isopregnanolone (III) (Roberts *et al.*, 1958) and its acetate (Dobriner *et al.*, 1953). An authentic sample of III was generously made available to us by Dr. D. K. Fukushima. Its infrared spectrum, determined upon a KBr micropellet, was identical to that of the isolated material determined in a similar manner. Furthermore, the melting point of a mixture of the two samples was not depressed.

Additional crystalline material was obtained from the pooled mother liquor of the above samples by chromatography on alumina (eluted with ether-benzene [4:1]). This material and the previously crystallized product were then combined and rechromatographed on celite in isooctane–methylcellosolve (system B). The eluted compound was recrystallized from ligroin and melted at 143–146°. It exhibited no depression of melting point when mixed with authentic III, and its specific activity (1.30×10^4 cpm/mg) was in reasonable agreement with that of the initially isolated product.

To establish further that the radioactivity which was isolated was truly associated with III, the residue from the filtrate of the last crystallization was acetylated in pyridine with C^{14}-acetic anhydride in order to determine its specific activity (Ulick *et al.*, 1958). Upon chromatography of the resulting product on celite in heptane-dimethylformamide (system C), a single peak containing both isotopes was observed in the second hold-back volume. This material was rechromatographed in this system to insure the removal of acetylated impurities. It was observed that the peak fraction having the most tritium in both chromatograms also contained the most C^{14}. In the final chromatogram the ratios of H^3 to C^{14} in four consecutive fractions of the peak did not differ from the average by more than 8%. From the H^3/C^{14} ratio in this final product (5.80) and the specific activity of the C^{14} acetic anhydride (6.78×10^5 cpm/meq), the specific activity of the acetylated residue with respect to tritium was calculated to be 1.24×10^4 cpm/mg (3.94×10^6 cpm/meq). This procedure for determination of specific activity was employed without modification in experiments 2 and 3.

EXPERIMENT 2.—The same subject used for experiment 1 was injected with 1.0×10^6 cpm of 16α-hydroxyprogesterone-H^3. No carrier was fed, so that the total product isolated would be derived only from the trace dose of injected material and the endogenous production during the 3-day period of urine collection. The urine was worked up as in experiment 1, except that the organic extract was washed with dilute Na_2CO_3 (0.5 M) and with water. The milder alkali wash was used to insure that no artifacts were being formed during isolation.

To the crude neutralized organic extract, 5 mg of 3α-hydroxypregnan-20-one (pregnanolone), the 17β-epimer of III, was added in an attempt to isolate radioactivity associated with this compound. Chromatography of the mixture on celite in system A yielded one peak of radioactivity (1.3×10^5 cpm), with the approximate mobility of III. This was eluted earlier than the carrier, pregnanolone, which was devoid of radioactivity. The latter was identified by its infrared spectrum

TABLE I

SPECIFIC ACTIVITIES OF 17-ISOPREGNANOLONE ISOLATED FROM URINE AFTER ADMINISTRATION OF 16α-HYDROXYPROGESTERONE-H^3

Expt. No.	Injected (cpm)	Ingested	Sample	Specific Activity of 17-Isopregnanolone (cpm/mg)
1	1.6×10^6	200 mg 16α-hydroxyprogesterone	1st crystallization	1.40×10^4 [a]
			2nd crystallization	1.30×10^4 [a]
			Filtrate residue from 2nd crystallization	1.24×10^4 [b]
2	1.0×10^6	None	Noncrystalline	2.09×10^5 [b]
3	0.7×10^6	200 mg 16-dehydroprogesterone	1st crystallization	1.09×10^4 [a]
			2nd crystallization	1.03×10^4 [a]
			Filtrate residue from 2nd crystallization	1.08×10^4 [b]

[a] Determined by counting an aliquot of weighed sample (see text). [b] Determined by acetylation with C^{14}-acetic anhydride (see text).

and a mixed melting point determination.

The radioactive substance which appeared to be III was rechromatographed on system A and recovered as a single peak in the expected hold-back volume. Because of the limited amount of material, it could not be crystallized for direct identification. Its specific activity was shown to be 2.09×10^5 cpm/mg (6.66×10^7 cpm/meq) by acetylation with C^{14}-acetic anhydride. The specific activity of the acetic anhydride was 5.69×10^6 cpm/meq and the H^3/C^{14} ratio in the product was 11.7. That the substance which had been acetylated was in fact III was demonstrated by combining the acetate with a known amount of the doubly labeled acetate from experiment 1, and chromatographing the mixture on celite in system C. The ratio of H^3 to C^{14} in four consecutive fractions containing 85% of the counts chromatographed showed a deviation from the mean of not more than 3%. Furthermore, the mean of these four values was in perfect agreement with the H^3 to C^{14} ratio of the mixture before chromatography.

EXPERIMENT 3.—The same subject was injected with radioactive I (0.7×10^6 cpm), and simultaneously he ingested 200 mg of 16-dehydroprogesterone (II), unlabeled, in order to establish the possible role of the latter as an intermediate in the conversion of I to III. His 3-day urine specimen was worked up in a manner identical to that used in experiment 2, and the resulting extract was chromatographed in system A. One peak of radioactivity (0.8×10^5 cpm) occurred in the fractions where III was expected. The radioactive material was rechromatographed in system A and then on alumina. In this way, a radioactive, crystalline substance was eluted with ether-benzene (4:1) and ether. Upon recrystallization from ligroin, it melted at 143–146°. The infrared spectrum of this product in KBr (micropellet) was in excellent agreement with that of III, and its melting point was unchanged by admixture with authentic III. Its specific activity (1.09×10^4 cpm/mg) was considerably lower than that of the metabolite isolated in experiment 2, and therefore the ingestion of II in the present experiment appeared to have resulted in an increased excretion of III. The mother liquor of this initial product, after chromatography on celite in system B, yielded further crystalline III (melting point 143–146°) (specific activity 1.03×10^4 cpm/mg). The filtrate residue from the final crystallization had a specific activity of 1.08×10^4 cpm/mg as determined by acetylation wth C^{14}-acetic anhydride. The results of all three isolation experiments are summarized in Table I.

DISCUSSION

These studies have demonstrated that a normal male subject is able to convert 16α-hydroxyprogesterone (I) to 17-isopregnanolone (III). Moreover, the fact that the specific activity of urinary III after injection of radioactive I was observed to be considerably diminished by administration of unlabeled 16-dehydroprogesterone (II) suggests that the transformation of I to III proceeds via a Δ^{16}-intermediate. Such a pathway is analogous to the well-known interconversion of malate and succinate via fumarate in the classical Krebs cycle. Furthermore the known susceptibility of β-hydroxyketones to dehydration provides ample chemical precedent for the present observations.

The most unusual feature of these results is the stereochemical configuration at C_{17} of the final product. Of the two alternative possibilities, the epimer whose side-chain is in the β-configuration is virtually the only one encountered in naturally occurring steroids. In these experiments only the 17α-epimer was isolated. Attempts to demonstrate by reverse isotope dilution the occurrence of pregnanolone, the normal 17β-isomer, were unsuccessful; this fact implies that the reduction of the postulated Δ^{16}-intermediate was completely stereospecific and moreover confirms the conclusion that the isolated metabolite was not formed artifactually. It is of course conceivable that pregnanolone is a metabolite of the injected compound, but that it has been almost completely converted to more polar products such as pregnane-

diol, which were not examined in our experiments.

A value for the secretory rate of 16α-hydroxyprogesterone in the individual used in this study was calculated from the specific activity of the urinary 17-isopregnanolone derived from experiment 2, according to the isotope dilution technique previously described (Ulick *et al.*, 1958). The equation used for the calculation of the secretory rate was: $SR = [R \times M] / [sa \times t]$, where R represents the injected cpm, M the ratio of the molecular weight of the precursor to that of the metabolite, sa the cumulative specific activity of the isolated 17-isopregnanolone, and t the number of days of urine collection. The average secretion of 16α-hydroxyprogesterone during the 3-day period of urine collection was thus estimated to be about 1.5 mg per day. Obviously the precision of this value is subject to the previously discussed (Ulick *et al.*, 1958; Laragh *et al.*, 1960; Gurpide *et al.*, 1962) limitations of the method by which it was obtained. Specifically in this instance its validity would be impaired if any tritium had been introduced at C_{17} during the random tritiation of I and if this tritium were not removed during purification of the tracer by treatment with $KHCO_3$. This label would certainly have been lost during the conversion of I to III and would result in a falsely elevated value for the secretory rate of I as determined by the above method.

Incomplete separation of the doubly labeled acetate obtained in experiment 2 from impurities labeled only with C^{14} would also result in an estimation of a high value for the secretory rate of I. However, in the absence of evidence establishing the reality of either of these possibilities, it would appear that 16α-hydroxyprogesterone is produced in the normal male in amounts of about 1–2 mg per day, a level which makes it a quantitatively significant secretory product. Even with this secretion rate, however, it is unlikely that 17-isopregnanolone would be isolated from normal urine regularly since the conversion of the radioactive precursor to its urinary metabolite is approximately 10%.

The demonstration that a steroid β-hydroxy ketone has probably been dehydrated *in vivo* may have further significance in that it suggests a mechanism for the formation of estrogen from C_{19} precursors such as testosterone or Δ^4-androstenedione. A recent study of the steps involved in the biological aromatization of Δ^4-androstenedione indicated that oxygenation of C_{19} occurs with the formation of a C_{19}-hydroxy compound. This may be followed by dehydrogenation to yield a C_{19}-oxo compound as an intermediate (Morato *et al.*, 1961). Moreover, it also has been demonstrated in the same series of experiments that conversion of 19 - oxo - Δ^4 - androstenedione to estrone requires both TPNH and oxygen, suggesting an additional hydroxylation step. These observations can be rationalized by assuming that 19-oxo-Δ^4-androstenedione is enzymatically hydroxylated at C_1 and that the resulting β-hydroxy carbonyl compound suffers elimination of the elements of water and of the C_{19}-aldehyde group (possibly but not necessarily in a concerted mechanism) yielding an aromatic estrogen.

Addendum

Since this paper was submitted a new crystalline radioactive metabolite (m.p. 194–198°), has been isolated from the pooled urine of two female subjects after administration of 16α-hydroxyprogesterone both intravenously as tritiated tracer and orally as unlabeled carrier. The infrared spectrum of this compound was shown to be identical to that of authentic 3α-hydroxy-Δ^{16}-pregnen-20-one (16-dehydropregnanolone). The comparison of the two spectra was performed through the courtesy of Dr. T. F. and Mrs. B. Gallagher of the Sloan-Kettering Institute. The isolation of this metabolite has been previously reported (Fukushima *et al.*, 1954). Its apparent formation *in vivo* from 16α-hydroxyprogesterone supports the mechanism proposed in this paper for the formation of 17-isopregnanolone.

Acknowledgment

The authors are grateful to Dr. S. Bernstein of Lederle Laboratories for generously providing us with 16α-hydroxyprogesterone and 16-dehydroprogesterone and to Dr. D. K. Fukushima of the Sloan-Kettering Institute, who kindly furnished us with samples of pregnanolone and of 17-isopregnanolone and its acetate.

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The *in vitro* Conversion of Dehydroepiandrosterone-4-C^{14} to Estrogens by Ovarian Tissue*

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Received February 28, 1962

After the incubation of equine ovarian follicular tissue with dehydroepiandrosterone-4-C^{14}, radioactive estradiol, estrone, androstenedione, and testosterone were isolated and identified by the criteria of paper chromatography and recrystallization to constant specific activity. These results provide additional evidence for the existence of an alternate pathway for estrogen biosynthesis from pregnenolone through dehydroepiandrosterone with androstenedione and testosterone as intermediates.

Considerable evidence has accumulated to indicate that the same precursors are utilized in the biosynthesis of estrogens as for adrenal and testicular steroids (Ryan, 1959; Ryan and Smith, 1961; Solomon *et al.*, 1956; Sweat *et al.*, 1960). On the basis of this evidence the following partial biosynthetic pathway for estrogens has been proposed: Pregnenolone → progesterone → 17-hydroxyprogesterone → Δ^4-androstenedione → esterogens.[1] With the isolation of dehydroepiandrosterone from gonadal tissue, Neher and Wettstein (1960) proposed an alternate pathway from pregnenolone → 17-hydroxypregnenolone → dehydroepiandrosterone → androstenedione. The recent demonstration by Ryan and Smith (1961) that acetate-1-C^{14} can be converted to 17-hydroxypregnenolone and dehydroepiandrosterone by ovarian tissue, and the isolation of dehydroepiandrosterone from ovarian follicular fluid by Short (1961), suggest that this latter route might play a more important role in estrogen synthesis than was previously thought to be the case.

In the present study the conversion of dehydroepiandrosterone-C^{14} to estradiol and estrone in excellent yields by equine ovarian follicular tissue *in vitro* has been demonstrated. Testosterone and androstenedione were also isolated and identified as radioactive metabolites.

* Supported in part by USPHS grant no. C-3588.
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[1] The following trivial names for steroids are used in this manuscript: androstenedione = 4-androsten-3,17-dione; 19-hydroxyandrostenedione = 4-androsten-19-ol-3,17-dione; dehydroepiandrosterone = 5-androsten-3β-ol-17-one; estradiol (E_2) = 1,3,5-estratrien-3,17β-diol; estriol (E_3) = 1,3,5-estratrien-3,16α,17β-triol; estrone (E_1) = 1,3,5-estratrien-3-ol-17-one; progesterone = 4-pregnen-3,20-dione; 17-hydroxyprogesterone = 4-pregnen-17α-ol-3,20-dione; pregnenolone = 5-pregnen-3β-ol-20-one; 17-hydroxypregnenolone = 5-pregnen-3β,17α-ol-20-one; testosterone = 4-androsten-17β-ol-3-one.