7.4×10^{-5} M^{-1} min.$^{-1}$, while that for reaction at the aromatic ring is 2.3×10^{-5} M^{-1} min.$^{-1}$ (Table IV). Thus, the rate of reaction of methylamine at the aromatic ring is 27 times slower than the rate constant of 62 M^{-1} min.$^{-1}$, which was assigned to the attack of dimethylamine on the aromatic ring (Table VI). The absence of base catalysis of the methylamine reaction would reasonably be accounted for if the relatively slow rate of this reaction reflects a large free energy barrier for attack at the aromatic ring. It was concluded above that the attack of amine on the ring is not subject to appreciable base catalysis, and if this step is rate determining in the reaction with primary amines, base catalysis would not be significant for these reactions. The mechanism and rate constants for the second step of the methylamine reaction would be expected to be similar to those for the secondary amine reactions. (It is probable, by analogy with other base-catalyzed reactions, that water may act as a proton transfer catalyst for the second step. A mechanism which involves proton transfer from the amine to the leaving phosphate, either directly or through a water molecule, is possible and would be especially attractive for reactions of primary amines. The result of reaction paths of this kind would be to lower the energy barrier for the second step of the reaction at low base concentrations.) This analysis is consistent with the previous observation of Brady and Cropper that dimethylamine reacts 11 times faster than methylamine with 2,4-dinitrochlorobenzene in alcohol, in spite of the steric effect of the *o*-nitro group in this compound and the generally higher nucleophilic reactivity of secondary than of primary amines, if steric effects are not dominant.[24]

Thus, the disappearance of detectable general base catalysis in the reactions of amines with monosubstituted nitrobenzenes could occur through either an increase in the energy barrier of the uncatalyzed first step of the reaction or through a decrease in the energy barrier of the second step, as in the reaction of dimethylamine with *p*-chloronitrobenzene or with *p*-nitrophenyl phosphate at high concentrations of base catalyst. Analogous changes in sensitivity to base catalysis are seen in reactions of acyl compounds as the leaving group is varied.[25]

(24) H. K. Hall, Jr., *J. Org. Chem.*, **29**, 3539 (1964); G. Yagil and M. Anbar, *J. Am. Chem. Soc.*, **84**, 1797 (1962), and references therein.
(25) J. F. Kirsch and W. P. Jencks, *ibid.*, **86**, 833, 837 (1964).

The Migration and Elimination of Hydrogen during Biosynthesis of Cholesterol from Squalene[1]

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Contribution from Shell Research Limited,
Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent, England,
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Shrewsbury, Massachusetts. *Received February 19, 1965*

Lanosterol and cholesterol biosynthesized from 4R-4-H^3-mevalonic acid retain, respectively, five and three of the six labeled atoms present in the intermediate squalene. The cholesterol was degraded and, of the three H^3 atoms retained, one is at the 17α-position. The remaining two tritium atoms are most probably located at C-20 and C-24. This pattern of loss, retention, and distribution of H^3 is in complete harmony with the theoretical mechanism of squalene cyclization. The absence of tritium in the steroidal nucleus confirms the intermediate oxidation of the C-3 hydroxyl during biosynthesis and shows that when the double bond of cholesterol is formed the 5α-hydrogen atom is eliminated, not rearranged.

Eschenmoser, *et al.*,[3] reasoning from what was then known of the cyclization of squalene (II) to lanosterol (IV) and assuming that the stereochemistry of this enzymic process would be that observed for analogous chemical reactions, put forward a detailed mechanism for this cyclization. Briefly, the attack of an electron-deficient species, equivalent to an hydroxyl cation, initiates the cyclization of squalene to a cationic intermediate (III). Four rearrangements then follow: hydrogen from C-17 to C-20 and from C-13 to C-17, methyl from C-14 to C-13 and from C-8 to C-14. Finally a proton is lost from C-9 to give lanosterol. This mechanism has been strongly supported by the demonstration[4,5] that the rearrangements of methyl groups do occur as postulated. There has been no experimental support until now for the hydrogen migrations except the fact that enzymic cyclizations of squalene run in D_2O gave lanosterol free from stably bound deuterium.[6]

(1) (a) This work was initiated while one of us (E. C.) was a visiting scientist at the Shell Research, Ltd., Milstead Laboratory in Sittingbourne, Kent, England, during July–Aug. 1963, and was continued at the Worcester Foundation, Shrewsbury, Mass. (b) The work at the Worcester Foundation was supported by Grants CA-07137, A-5326, and CA-04663 from the U. S. Public Health Service.
(2) (a) Postdoctoral Fellow 1963–1964 on leave of absence from Hokkaido University, Sapporo, Japan; (b) Postdoctoral Fellow 1960–1964; (c) recipient of Research Career Program Award CA-K3-16614 from the National Cancer Institute.

(3) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).
(4) J. W. Cornforth, R. H. Cornforth, A. Pelter, M. G. Horning, and G. Popják, *Proc. Chem. Soc.*, 112 (1958); *Tetrahedron*, **5**, 311 (1959).
(5) R. K. Maudgal, T. T. Tchen, and K. Bloch, *J. Am. Chem. Soc.*, 80, 2589 (1958).
(6) T. T. Tchen and K. Bloch, *J. Biol. Chem.*, **226**, 931 (1957).

In the biosynthesis of squalene from mevalonate it has been shown[7] that the 4*S* proton of mevalonic acid (I) is eliminated stereospecifically and that the squalene retains all six 4*R* hydrogens of the contributing six molecules of mevalonic acid.[8] The fate of these retained hydrogens, when squalene is converted into cholesterol (V), is of unusual interest: only one of the six, that on C-24 of cholesterol, should have retained its original attachment. Of the other five, two should have migrated and three should have been lost: one in the final step of lanosterol (IV) formation, one when the 3β-hydroxyl is oxidized to the ketone before elimination of the 4-methyl groups,[9] and one when the 5,6-double bond of cholesterol (V) is formed.

Mevalonate labeled stereospecifically with H^3 at C-4, 4*R*-4-H^3-mevalonate, was synthesized[7] at Milstead and added to a specimen of 2-C^{14}-mevalonate to give a preparation with a specific activity of 6.11 μc. of H^3 and 0.83 μc. of C^{14} per μmole.[10] This doubly labeled mevalonate was then used for the biosynthesis of squalene, lanosterol, and cholesterol (see Experimental section) in a liver enzyme preparation.

The ratio of H^3 to C^{14} in the mevalonate was 7.37.[10] In squalene this ratio should be the same, since six labeled atoms of each element should be present (II). The cyclization of this squalene, according to the hypothesis of Eschenmoser, *et al.*,[3] should give lanosterol (IV) containing five positions labeled with H^3 and six with C^{14}, with a theoretical H^3/C^{14} ratio of $7.37 \times {}^5/_6 = 6.14$. In cholesterol (V), on the other hand, only three H^3-labeled and five C^{14}-labeled atoms should persist; the calculated H^3/C^{14} ratio is therefore $7.37 \times {}^3/_5 = 4.42$. The experimentally determined ratios (Table I), 7.36 for squalene, 6.20 for lanosterol, and 4.40 for cholesterol,[11] are in good agreement with the calculated values.

The cholesterol thus appeared to have the expected number of H^3-labeled atoms; it remained to be shown that these were attached to C-17, C-20, and C-24. A combined enzymic and chemical degradation was therefore carried out at the Worcester Foundation.

Enzymic cleavage of the cholesterol side chain by incubation with a corpus luteum preparation[12] yields isocaproic acid (VI), pregnenolone (VII), and progesterone (VIII). Should conversion of squalene to cholesterol proceed as postulated, the isocaproic acid (VI) derived from the side chain would contain one H^3-labeled atom and two C^{14}-labeled atoms. The expected H^3/C^{14} ratio in VI would then be $7.37 \times {}^1/_2 = 3.685$. Pregnenolone (VII) and progesterone (VIII) retain only the 17α-H^3 since the labeled atom at C-20 is necessarily lost in the enzymic oxidation. Thus both these ketones should have one H^3-labeled atom and three C^{14}-labeled atoms and a H^3/C^{14} ratio of $7.37 \times {}^1/_3 = 2.46$.

The enzymic conversion of the H^3-C^{14}-cholesterol biosynthesized from the 4*R*-4-H^3-2-C^{14}-mevalonate into isocaproic acid, pregnenolone, and progesterone is described in the Experimental section. As can be seen from Table I, all three products gave H^3/C^{14} ratios very close to or identical with the predicted values.

It remained to be shown that the H^3 in pregnenolone and in progesterone was attached to C-17 and to no other position. This was demonstrated by an unambiguous removal of the 17α-hydrogen from the pregnenolone and by the base-catalyzed exchange of the 17α-hydrogen in both pregnenolone and progesterone.

Pregnenolone (VII) was submitted to Baeyer–Villiger oxidation with peroxytrifluoroacetic acid to yield after saponification the known tetrol IX. The tetrol retained all the H^3 of pregnenolone as evidenced by the observed H^3/C^{14} ratio of 2.52 (Table I). Oxidation of the tetrol (IX) with chromic acid–acetic acid at room temperature gave the trione X devoid of H^3. Similarly, the equilibration of pregnenolone and of progesterone in boiling methanolic potassium hydroxide gave products virtually free of H^3 (Table I).

Table I. Ratios of H^3/C^{14} in Products Derived from 4*R*-4-H^3-2-C^{14}-Mevalonic Acid

Compound	Labeled positions, no. H^3	C^{14}	H^3/C^{14} ratio Calcd.	Found
Mevalonic acid (I)	1	1	..	7.37
Squalene (II)	6	6	7.37	7.36
Lanosterol (IV)	5	6	6.14	6.20
Cholesterol (V)	3	5	4.42	4.40
Pregnenolone (VII)	1	3	2.46	2.46
Progesterone (VIII)	1	3	2.46	2.48
$3\beta,5\alpha,6\beta,17\beta$-Tetrol (IX)	1	3	2.46	2.52
3,6,17-Trion-5α-ol (X)	0	3	0	0
Equilibrated VII	0	3	0	0.04
Equilibrated VIII	0	3	0	0.02
Isocaproic acid (VI)	1	2	3.68	3.64

The following conclusions can be drawn from this work: (1) Lanosterol and cholesterol biosynthesized from 4*R*-4-H^3-mevalonic acid retain, respectively, five and three of the six labeled atoms present in the intermediate squalene. (2) Of the three labeled atoms retained in cholesterol, one is at the 17α-position; the second is lost when cholesterol is converted enzymically to isocaproic acid and pregnenolone and is therefore attached to either C-20 or C-22; the third appears in the isocaproic acid and is therefore attached to C-23–27. (3) This pattern of loss, retention, and distribution of H^3 is in complete harmony with the theoretical mechanism of squalene cyclization[3]; thus it is probable that the two labeled positions in cholesterol not located precisely were at C-20 and C-24. (4) The absence of H^3 from the cholesterol nucleus (except at C-17) confirms the intermediate oxidation at C-3 during biosynthesis and shows that when the double bond in cholesterol is formed the 5α-hydrogen atom is eliminated, not rearranged.

(7) J. W. Cornforth, R. H. Cornforth, C. Donninger, and G. Popják, *Proc. Roy. Soc.* (London), to be published; *cf.* G. Popják, Abstracts 6th International Congress of Biochemistry, Volume VII, 1964, p. 545.

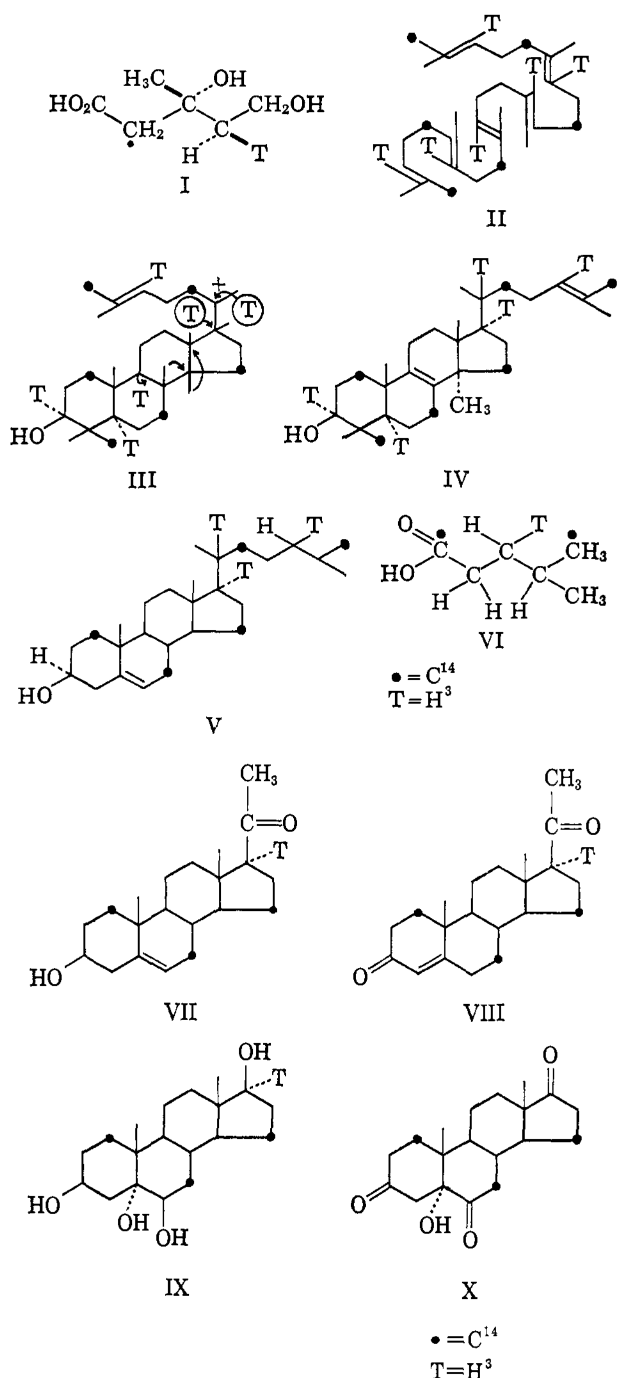
(8) The notation of absolute configuration, *R* and *S*, follows the convention proposed by R. S. Cahn, C. K. Ingold, and V. Prelog, *Experientia*, 12, 81 (1956).

(9) K. Bloch in "The Biosynthesis of Terpenes and Sterols," a Ciba Foundation Symposium, G. E. W. Wolstenholme and M. O'Connor, Ed., J and A. Churchill Ltd., London, England, 1959, p. 4.

(10) The specific radioactivities and H^3/C^{14} ratios are given in figures rounded off to the nearest second decimal place; this accounts for the small discrepancies that may be noted in the H^3/C^{14} ratios as shown and calculated from the specific activities.

(11) The H^3/C^{14} ratios for cholesterol, as measured on several samples, ranged from 4.37 to 4.45, with a mean value of 4.40.

(12) S. Ichii, E. Forchielli, and R. I. Dorfman, *Steroids*, 2, 631 (1963).



Experimental

Biosynthesis of Squalene and Sterols from 4R-4-H^3-2-C^{14}-Mevalonic Acid. The biosynthesis was carried out essentially as described before with the supernatant (S_{10}) of a rat liver homogenate centrifuged at 10,000*g* for 30 min.[4] A preparation of S_{10} (150 ml.), made from 122 g. of rat liver, was incubated for 3 hr. at 37° aerobically in three batches of 50 ml. each and supplemented at the start and at the end of the first and second hour with ATP (3 m*M*), DPNH (1 m*M*), TPN^+ (1 m*M*), and glucose 6-phosphate (3 m*M*).[13] The concentration of 4*R*-4-H^3-2-C^{14}-mevalonate (specific activity: 6.11 μc. of H^3 and 0.83 μc. of C^{14} per μmole) in the incubations was 1.5 m*M*.

The unsaponifiable matter extracted from the combined incubations was first divided into squalene and total sterols by chromatography on alumina[4]; the sterols were then precipitated as digitonides from which they were recovered (23.7 mg.) by treatment of the digitonides with pyridine and precipitation of the digitonin with diethyl ether. The sterols were then chromatographed on a silicic acid–Celite (2:1, w./w.) column 80 cm. long and 1.5 cm. in diameter, with pure benzene as eluting solvent.[14] Lanosterol and cholesterol (23.0 mg.) were obtained, among other sterols, as individual fractions. By collecting 5-ml. fractions, lanosterol was eluted sharply between fractions 45 and 55 and cholesterol between fractions 78 and 95; this pattern of elution was established previously also with authentic unlabeled substances. Another major radioactive component (4-methylstenol?) was eluted between fractions 59 and 75 and two others, after cholesterol, between fractions 96 and 120. For the identity of lanosterol and cholesterol we relied on the chromatographic evidence, and in the case of cholesterol on mass spectrometric analysis. The H^3/C^{14} ratios for lanosterol were measured on portions of fractions 48, 49, and 50, and for cholesterol on portions taken from the pooled cholesterol specimen.

The utilization of mevalonate for the biosynthesis of squalene plus sterols was approximately 35%; 10.7% of the C^{14} of mevalonate used was found in squalene and 89.3% in sterols, 46% of the C^{14} in the latter being associated with cholesterol, 4% with lanosterol, and the remainder with three not fully identified sterol fractions. The specific activity of cholesterol was 3.56 μc. of H^3 and 0.81 μc. of C^{14} per mg.

Conversion of H^3-C^{14}-Cholesterol into Pregnenolone, Progesterone, and Isocaproic Acid. Source of Enzymes and Incubation Procedure. A soluble enzyme preparation was made from an acetone powder of a bovine corpus luteum homogenate as described previously.[12] Acetone powder (1 g.) was extracted with 0.066 *M* phosphate buffer, pH 7.2, and the extract was centrifuged at 105,000*g* for 30 min.

Incubations were carried out in 30-ml. flasks as follows: 100 μg. of cholesterol in benzene solution was added to each flask, followed by the addition of 0.05-ml. of propylene glycol. The benzene was removed under a stream of nitrogen. To this was added 1.0 ml. of 0.066 *M* phosphate buffer at pH 7.2, 0.1 ml. of 0.1 *M* $MgCl_2$, 1.5 mg. of TPNH in 0.15 ml. of 0.066 *M* phosphate buffer, and 1.0 ml. of the enzyme preparation. The mixture was incubated at 37° in air for 90 min. in a Dubnoff metabolic shaker.

Extraction and Isolation of Pregnenolone and Progesterone. The reaction was terminated by addition of 20 ml. of 80% ethanol, the content of the flasks was combined, and the mixture was stored for 2 hr. at −20°. The precipitated proteins were separated by centrifugation and the aqueous alcoholic solution was collected. After the removal of most of the alcohol under reduced pressure, the aqueous residue was acidified with 3 *N* sulfuric acid and thoroughly ex-

(13) At each supplementation the amount of cofactors added was such that it would have resulted in the concentrations shown if there had been no cofactor present in the incubation mixture. Thus, *e.g.*, 150 μmoles of ATP were added to each 50-ml. incubation at the start and at the end of the first and second hour of incubation.

(14) I. D. Frantz, Jr., E. Dulit, and A. G. Davidson, *J. Biol. Chem.*, **226**, 139 (1957).

tracted with ether. The ethereal solution was washed several times with 0.1 M sodium hydrogen carbonate and the alkaline extracts, containing isocaproic acid, were saved. The ether solution was washed with water, dried, and concentrated to a steroidal residue.

The residue was chromatographed for 3 hr. on paper (Whatman No. 1) in a ligroin–propylene glycol system. Three major radioactive zones were detected. The zones corresponding to pregnenolone and progesterone were eluted and each was rechromatographed for 3 hr. on paper in the Bush A system (80% aqueous methanol–hexane) at 37°. Single radioactive spots were detected with mobilities corresponding to those of authentic samples. The steroids were eluted with ethyl acetate.

We needed an amount of the labeled pregnenolone and progesterone sufficient for dilution with inactive material so that the projected chemical manipulations could be carried out on recrystallized material. Since the specific activity of the biosynthetic cholesterol was 3.56 μc. of H^3 and 0.81 μc. of C^{14} per mg., it was calculated from the expected yields of the steroids that about 3 mg. of this cholesterol would provide the amounts of pregnenolone and progesterone needed. There was, however, a technical complication. While cleavage of the side chain of cholesterol with corpus luteum preparations proceeds well on the microgram level, an increase of the amount of substrate to 100 μg. per incubation did not result in a proportionately greater yield. Hence it was necessary to recover and reincubate the unchanged cholesterol several times until enough product was accumulated.

The zone corresponding to cholesterol on the initial ligroin–propylene glycol chromatograms of the steroids was eluted and chromatographed for 8 hr. in a heptane–Phenyl Cellosolve system. A single zone was detected which showed the mobility of cholesterol.

The product was eluted and submitted to a final chromatography in Bush A system (3 hr.) to give crystalline cholesterol. The recovered homogeneous cholesterol was reincubated. Three additional sets of incubation experiments were carried out and processed as above.

Isolation of 3-H^3-1,5-C^{14}-Isocaproic Acid. The sodium hydrogen carbonate washings of the ether extracts of steroids were acidified with 3 N sulfuric acid and extracted with ether. The ether solution was washed with a small amount of water and dried. The volume of ether was reduced and then a slight excess of alcoholic potassium hydroxide was added. The slightly alkaline mixture was evaporated to dryness; a portion of the residue was transferred to a scintillation counting vial and acidified with 0.04 ml. of acetic acid. Scintillation solution was then added and the mixture was counted. The identity of this acidic component with isocaproic acid had previously been established[12]; we did not characterize it further.

Processing of 17α-H^3-1,7,15-C^{14}-Pregnenolone and 17α-H^3-1,7,15-C^{14}-Progesterone. The radioactive pregnenolone (about 4.07×10^5 H^3; 1.5×10^5 C^{14} d.p.m.) was diluted with 100 mg. of nonradioactive material and recrystallized once from methanol and twice from n-hexane–methylene chloride. The H^3/C^{14} ratio remained constant after the first crystallization.

The progesterone (about 4.3×10^5 H^3; 1.57×10^5 C^{14} d.p.m.) was diluted with 100 mg. of unlabeled material and was recrystallized twice from n-hexane–methylene chloride. The H^3/C^{14} ratio remained unchanged.

To evaluate whether a high counting impurity was included in the diluted samples, 2 mg. each of pregnenolone and progesterone were diluted with 20 mg. of unlabeled cholesterol, and each mixture was chromatographed on thin layer plates (silica gel HG 254; benzene–ethyl acetate (9:1)). The recovered pregnenolone and progesterone were rechromatographed in the same system and then crystallized from n-hexane–methylene chloride. The H^3/C^{14} ratio remained unchanged.

17α-H^3-1,7,15-C^{14}-3β,5α,6β,17β-Tetrahydroxyandrostane (IX). To a stirred, ice-cooled mixture of 17α-H^3-1,7,15-C^{14}-pregnenolone (80 mg.), dry methylene chloride (7 ml.), and disodium phosphate (3 g.), a solution of trifluoroacetic anhydride (1.1 ml.) and hydrogen peroxide (90%, 1.2 ml.) in dry methylene chloride (7 ml.) was added. After 3 hr. the mixture was poured onto ice and the steroids were recovered with methylene chloride. The methylene chloride solution was washed with aqueous sodium hydrogen carbonate and water, then dried. Removal of the methylene chloride left a residue which was dissolved in methanol (10 ml.), and 2 N sodium hydroxide (5 ml.) was added. The alkaline mixture was refluxed for 1 hr. under nitrogen. Most of the methanol was removed under reduced pressure, water was added, and the steroids were extracted with ethyl acetate. The extract was washed with saline, dried, and concentrated to yield crystalline IX. The product was crystallized once from methanol–ethyl acetate and once from ethanol–water to m.p. 260° (reported[15] m.p. 261–263°). The infrared spectrum (in potassium bromide) showed no carbonyl absorption.

1,7,15-C^{14}-5α-Hydroxyandrosta-3,6,17-trione (X). To a solution of tetrol IX (25 mg.) in acetic acid (2 ml.) a mixture of chromium trioxide (23 mg.) in aqueous acetic acid (80%, 1.4 ml.) was added. After 16 hr. at room temperature water was added and the steroids were recovered with ethyl acetate. The extract was washed with a sodium hydrogen carbonate solution in water and dried. Removal of solvent left the solid X, which was recrystallized three times from ethanol to m.p. 240–242° (reported[15,16] m.p. 240–244°, 248°); ν_{max}^{KBr} 3540 (5α-OH), 1738 (17-ketone), and 1710 (3 and 6 ketones) $cm.^{-1}$.

Equilibration of 17α-H^3-1,7,15-C^{14}-Pregnenolone (VII) and of 17α-H^3-1,7,15-C^{14}-Progesterone (VI). A sample of the pregnenolone was dissolved in 8% methanolic potassium hydroxide (2 ml.), then aqueous methanol (50%, 2 ml.) was added. The solution was refluxed for 4 hr. in an atmosphere of nitrogen. Water (3 ml.) was then added, and most of the methanol was removed in a stream of nitrogen. The aqueous residue was extracted with ether. The extract was washed with saline, dried, and concentrated, yielding crystalline pregnenolone which was counted.

A sample of the progesterone was treated exactly as above to yield finally a noncrystalline residue. The sirup was counted as such.

(15) T. Rull and G. Ourisson, *Bull. Soc. Chim. France*, 1581 (1958).
(16) A. Butenandt and B. Riegel, *Ber.*, **69**, 1163 (1936).

Measurements of H^3 and C^{14}. All radioactive counting was done in Packard Tri-Carb scintillation spectrometers: at Milstead Laboratory in a Model 314EX, and at the Worcester Foundation in a Model 3002. Both laboratories used the same scintillator solution (4 g. of 2,5-diphenyloxazole and 100 mg. of 1,4-bis-2-(5-phenyloxazolyl)benzene per l. of toluene). Reference standard solutions of the benzhydrylamide of the H^3-C^{14}-mevalonate and of the squalene and cholesterol biosynthesized from it were counted at both laboratories: the H^3/C^{14} ratios found at the Worcester Foundation were consistently higher by a factor of 8.12/7.37 than those measured at Milstead. Since further experimental results obtained with the same specimen of H^3-C^{14}-mevalonate as used here await publication from Milstead Laboratory, the data of radioactive assays from the Worcester Foundation have been all multiplied by the factor 7.37/8.12.

Chemistry of Conjugate Anions and Enols. V. Stereochemistry, Kinetics, and Mechanism of the Acid- and Enzymatic-Catalyzed Isomerization of ^{5}-3-Keto Steroids[1,2]

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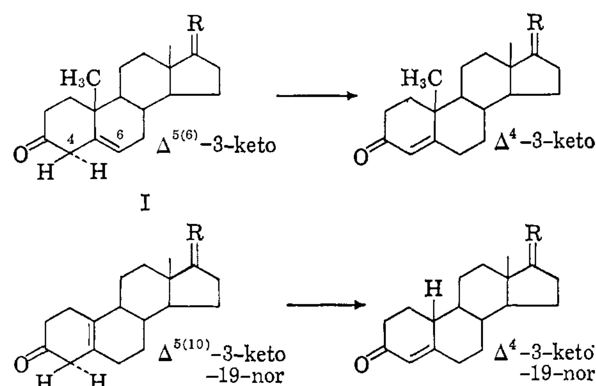
Contribution from the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts. *Received March 12, 1965*

The acid-catalyzed isomerization of Δ^5-3-keto steroids has been shown to proceed by formation of the $\Delta^{3,5}$-dienol via a rate-determining loss of a C-4 proton. The kinetic isotope effect ($k_H/k_D = 4.1$) and the solvent isotope effect ($k_{D_2O}/k_{H_2O} = 1.64$) rule out a mechanism involving C-6 protonation of the double bond followed by C-4 proton loss. Loss of the 4α-proton was shown to be slightly favored over 4β-proton loss in the enolization process. The enzymatic reaction with Δ^5-3-ketoisomerase was found to involve a stereospecific diaxial proton transfer from the 4β- to the 6β-position and was shown to be intramolecular by the simultaneous isomerization of deuterated and nondeuterated substrates. The loss of the 4β-proton was found to be rate determining in the enzymic reaction $V_{max_H}/V_{max_D} = 5.35$, $k_{m_H} = 3.1 \times 10^{-4}$, $K_{m_D} = 1.4 \times 10^{-4}$. It is proposed that the Δ^5-3-ketone undergoes conversion to the enol through carbonyl protonation by a donor group (AH) on the enzyme followed by 4β-proton loss to a basic group (B). In a fast step, reprotonation at the 6β-position by BH and proton removal from the carbonyl by A forms the Δ^4-3-ketone and regenerates the enzyme. The kinetics of the reaction and the failure of BH to undergo proton exchange with the medium are discussed.

Introduction

In 1955 Talalay and Wang[4] isolated an induced enzyme from *Pseudomonas testosteroni* that catalyzes the isomerization of a number of $\Delta^{5(6)}$-3-keto steroids and of $\Delta^{5(10)}$-19-nor steroids to the corresponding α,β-unsaturated ketone. Since the enzyme requires no cofactor and the over-all reaction is an extremely simple one, the isomerase reaction appeared particularly attractive for a study of stereochemistry, kinetics, and mechanism. Apart from its simplicity the enzymic reaction is characterized by two points of exceptional interest, the first being the extremely high turnover number of 17×10^6 min.$^{-1}$ (at maximum velocity one mole of enzyme isomerizes 17×10^6 moles of androst-5-ene-3,17-dione (I, R = ketone) per minute), which classifies this purified crystalline enzyme as the most active known.[5] Furthermore, it had been demonstrated[4,5] that enzymatic isomerization carried out in deuterium oxide led to the incorporation of only 0.12 atom of deuterium into the product, indicating that the net process involved a transfer of hydrogen from the C-4 position of the β,γ-unsaturated ketone to the C-6 position of the resulting α,β-unsaturated ketone product. In contrast, both the acid- and base-catalyzed isomerization led to the incorporation of one or more atoms of deuterium into the product.



In this paper we report on the mechanism and stereochemistry of both the acid-catalyzed and the enzymatic isomerization process. Apart from the obvious interest as a model for the enzymatic reaction, the chemical reaction constitutes a study which clarifies certain fundamental points concerned with the chemistry of

(1) This work was supported by the National Institutes of Health Research Grant No. AM-4044 and the American Cancer Society Grant T-185.

(2) Presented in part at the 6th International Congress of Biochemistry, New York, N. Y., July 1964, Abstracts IV, p. 139.

(3) To whom inquiries should be addressed.

(4) P. Talalay and V. S. Wang, *Biochim. Biophys. Acta*, **18**, 300 (1955).

(5) F. S. Kawahara and P. Talalay, *J. Biol. Chem.*, **235**, 1, 1960; F. S. Kawahara, S. F. Wang, and P. Talalay, *ibid.*, **237**, 1500 (1962); S. F. Wang, F. S. Kawahara, and P. Talalay, *ibid.*, **238**, 576 (1963).