Tetrahedron, 1963, Vol. 19, pp. 299 to 307. Pergamon Press Ltd. Printed in Northern Ireland

THE ISOMERIZATION OF β,γ-TO α,β-UNSATURATED STEROIDAL KETONES

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(*Received* 27 *July*; *in revised form* 20 *September* 1962)

Abstract—The isomerization of 12 steroidal $\Delta^{5(6)}$- and $\Delta^{5(10)}$-3-ketones to the corresponding Δ^4-3-ketones has been studied quantitatively under acid and enzymatic catalysis. Under both kinds of catalysis the $\Delta^{5(10)}$-series reacted more slowly. At pH 1 the difference in half-time periods for the two series was a factor of 10–38, but under enzymatic catalysis the difference was magnified to a factor varying from 29 to a few hundred, the exact values being dependent on precise structural features. Recently Talalay *et al.*[1] reported the rates of enzymatic isomerization of several of the $\Delta^{5(10)}$-compounds which we have measured, and our values are in agreement with theirs. The mechanisms of the two types of isomerization are discussed, and two new compounds, 5-estrene-3,17-dione and the diacetate of 17α-methyl-3,5-estradiene-3,17β-diol, are reported.

IT IS known qualitatively that steroidal $\Delta^{5(6)}$-3-ketones will isomerize faster to the Δ^4-3-ketones than will the $\Delta^{5(10)}$-ketones. Thus, Fieser[2] reported that 5-cholesten-3-one is isomerized to the Δ^4-isomer in 0·27M oxalic acid in warm 95% ethanol during 10 minutes, while Wilds and Nelson[3] observed that 5(10)-estren-17β-ol-3-one is stable to 1M oxalic acid in methanol at 40° for 25 minutes. On the other hand, no quantitative studies of the rates of these isomerizations have been reported. The importance of the reaction lies, among other things, in Talalay's recent work[1,4,5] which has shown not only that the isomerization can be enzymatically catalyzed but that the rate of the reaction depends on whether the double bond is in the 5(6)-position or the 5(10)-position.

Three different groups (Table 1) of steroidal β, γ-unsaturated ketones have been obtained by gift or by synthesis, *viz.*, (a) $\Delta^{5(6)}$-3-ketones, (b) $\Delta^{5(10)}$-3-ketones, and (c) 19-nor-$\Delta^{5(6)}$-3-ketones. Each group comprises a series in which C-17 is either a carbonyl group or C(OH)R where R is H, CH_3, C_2H_5, C≡C, or C=CH_2.

The synthetic route to these compounds followed previously established methods which we have modified to greater or lesser extents depending on the individual case. The details of the modified preparations are described in the experimental section. In general, the $\Delta^{5(6)}$-3-ketones were prepared through the corresponding 3β-alcohols which were oxidized with the Jones CrO_3-reagent by a modification of the procedure used previously by others.[6,7]

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[1] F. S. Kawahara, S-F. Wang, and P. Talalay, *J. Biol. Chem.* **237**, 1500 (1962).
[2] L. F. Fieser, *J. Amer. Chem. Soc.* **75**, 5421 (1953).
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[5] F. S. Kawahara and P. Talalay, *J. Biol. Chem.* **235**, PC 1 (1960).
[6] C. Djerassi, R. R. Engle, and A. Bowers, *J. Org. Chem.* **21**, 1547 (1956).
[7] K. Bowden, I. M. Heilbron, E. R. H. Jones, and B. C. L. Weedon, *J. Chem. Soc.* 39 (1946).

TABLE 1. RATES OF ISOMERIZATION

Compound	At pH 1·0[a]		Under enzymatic catalysis[b]			Origin of compound[c,d]
	$t_{1/2}$	$Rt_{1/2}$	$t_{1/2}$	$St_{1/2}$	$Rt_{1/2}$	
5-Androstene-3,17-dione (I)	5·2	1·0	21[e]	—	1·0	Syn.
			9·0[f]	—	1·0	
17α-Methyl-5-androsten-17β-ol-3-one	10	1·9	18	8·3	2·2	Syn.
17α-Ethyl-5-androsten-17β-ol-3-one	11	2·1	28	8·3	3·4	Syn.
5-Estrene-3,17-dione	6·9	1·3	2·0	3·4	0·59	Syn.
17α-Methyl-5-estren-17β-ol-3-one	4·2	0·81	7·8	3·2	2·4	Syn.
17α-Ethynyl-5-estern-17β-ol-3-one	4·2	0·81	9·2	3·2	2·9	Syn.
5(10)-Estrene-3,17-dione	198	38	720[g]	3·2	230	Syn.
			300[h]	1·3[i]	230	
5(10)-Estren-17β-ol-3-one	156	30	720	2·0	360	Sch.
17α-Methyl-5(10)-estren-17β-ol-3-one	132	25	348	2·0	174	GDS
17α-Ethyl-5(10)-estren-17β-ol-3-one	108	21	198	2·0	99	GDS
17α-Ethynyl-5(10)-estren-17β-ol-3-one	138	27	288	2·0	144	GDS
17α-Vinyl-5(10)-estren-17β-ol-3-one	150	29	354	2·5	142	GDS

[a] $t_{1/2}$ = half-time period in min of a solution which contained sufficient hydrochloric acid to make pH 1 and which was 5×10^{-5}M in steroid; $Rt_{1/2}$ = half-time value relative to the standard (5-androstene-3,17-dione, I) under the same conditions.

[b] $t_{1/2}$ = half-time period in min of a solution which was 5×10^{-5}M in steroid, which had pH 7·0, was 0·10M in phosphate buffer, and to which a small portion of similarly buffered cell-free extract (see Experimental section) of *Pseudomonas testosteroni* had been added; $St_{1/2}$ = half-time period of the standard (5-androstene-3,17-dione, I) measured under precisely the same conditions, i.e., with the same amount of the same enzyme preparation, and at approximately the same time as for the compound listed; $Rt_{1/2}$ = half-time value relative to the standard ($Rt_{1/2} = t_{1/2}/St_{1/2}$).

[c] Syn. = obtained by synthesis (see Experimental section).

[d] GDS = obtained through the courtesy of Dr. Frank Colton of G. D. Searle and Co., Chicago, Illinois; Sch = Schering A. G., Berlin.

[e] Enzyme dilution: 1/6250.

[f] Enzyme dilution: 1/2500.

[g] Enzyme dilution: 1/312.

[h] Enzyme dilution: 1/125.

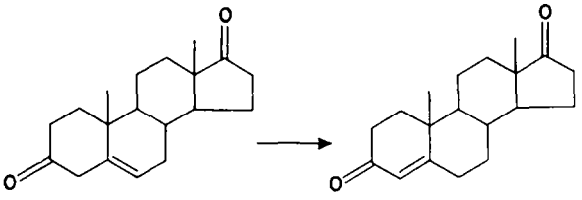
[i] On the assumption that the reaction is first order in enzyme, $St_{1/2}$ was calculated from $St_{1/2}$ (3·2 min) at the dilution of 1/312.

In the course of preparing the 19-nor-$\Delta^{5(6)}$-3β-alcohols, we were unable to duplicate the report[8] that the reduction of 17α-ethynyl-5-estrene-3β,17β-diol 17-acetate with lithium aluminum hydride in refluxing tetrahydrofuran yields 17α-ethynyl-5-estrene-3β,17β-diol. When the procedure was carefully followed several times, for reasons which are not clear, consistent results were not obtained, and a mixture of products was formed which did not contain significant amounts of the reported compound. However, it was obtained without difficulty by reduction with lithium aluminum hydride in diethyl ether at room temperature.

[8] J. Iriarte, C. Djerassi, and H. J. Ringold, *J. Amer. Chem. Soc.* **81**, 436 (1959).

Acid-catalyzed isomerizations.

The rate of isomerization of 5-androstene-3,17-dione (I) to 4-androstene-3,17-dione (II) in an aqueous medium was measured at different concentrations of hydrogen ion (hydrochloric acid) by observing the increase in absorption at 248 mμ as a function of time. That the reaction really proceeded to II was established by isolation of II from the medium. In the pH region of 2·5 to 0·5 the reaction followed first order



I II

kinetics in both steroid and hydrogen ion. The order of reaction in steroid was determined by the observation that the logarithm of the steroid (I) concentration, obtained by difference from the concentration of II, when plotted against time of reaction followed a straight line (Fig. 1). The order of reaction in hydrogen ion was obtained

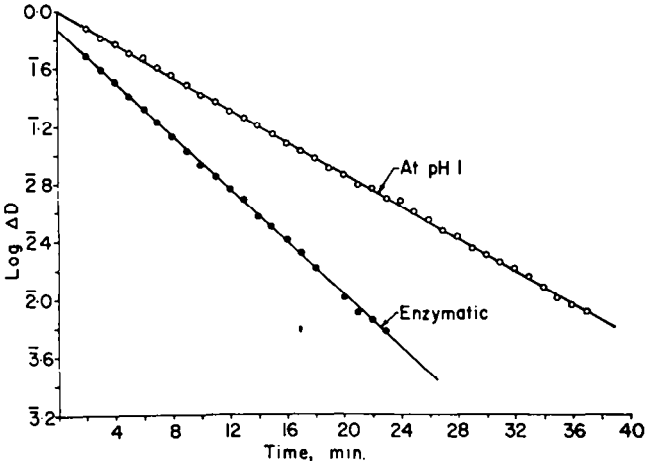


FIG. 1. Disappearance of 5-androstene-3,17-dione (I) as a function of time at 30° under acid catalysis (open circles) and enzymatic catalysis (solid dots). In both cases the concentration of steroid was 5×10^{-5}M; the acid used was HCl at pH 1·0; the enzyme used was obtained from *Pseudomonas testosteroni* and 2·0 ml of our standard preparation (see Experimental section) was diluted to 25 ml and 1·0 ml of this was added to 49 ml solution of the steroid in aqueous phosphate buffer (0·10M) at pH 7·0. Log ΔD is the logarithm of the optical density (D_t) at 248 mμ subtracted from the optical density (D_{100}) at 248 mμ at the end of the reaction, i.e., $\text{Log } \Delta D = \log (D_{100} - D_t)$ and ΔD is a function of the concentration of I. In both cases (acid and enzyme) the absorption-maximum which developed was at 248 mμ and II was isolated as the product of reaction.

from the slope of the line when the logarithm of the reaction rate constant (k', obtained from the half-time of reaction) was plotted against pH (Fig. 2).

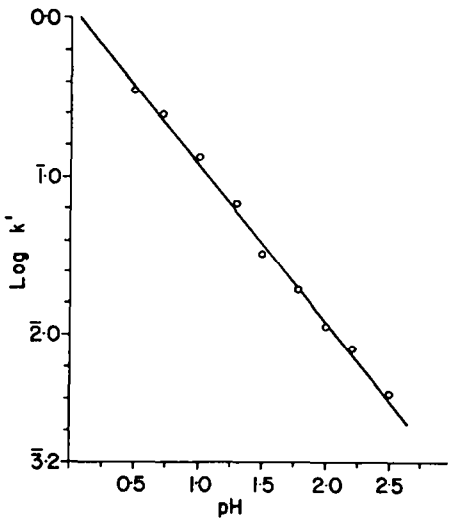


FIG. 2. Dependence of acid-catalyzed isomerization of 5-androstene-3,17-dione (I) to the Δ^4-3-ketone (II) on pH at 30°. The rate constant (k') at each pH was obtained graphically from the straight line produced when log ΔD (cf. Fig. 1) was plotted against time for different concentrations of hydrochloric acid. The line drawn through the experimental points in the graph above (log k' vs pH) is the theoretical line for first order kinetics in hydrogen ion. The pH measured was on a Beckman pH-meter ("Zeromatic") and was not calculated from the conc of HCl added; consequently, no correction was necessary for the activity coefficient of hydrogen ion.

The half-time of reaction for I at pH 1·0 was 5·2 minutes. This was a convenient time for measurements, and 1·0 was taken as a standard pH at which to measure the rate of isomerization of other compounds and to compare the influence of structure on the rate of reaction. A pH of 1·0 was also approximately in the middle of the region where the reaction was both measurable and first order in hydrogen ion. The results of such measurements on 12 different steroidal β,γ-unsaturated ketones are listed in Table 1 in which $Rt_{1/2}$ expresses the half-time period relative to that of 5-androstene-3,17-dione.

The isomerization of I was also measured both in the region near neutrality and under basic conditions. The behavior of the compound, however, was quite different from that observed under strongly acid conditions. While II could be isolated easily from isomerization at pH 1·0, difficulty was experienced in attempts to isolate it when the pH was greater than about 2·5. Under strongly alkaline conditions II could be obtained but not in a good yield. The rate of reaction based on increased absorption near 248 mμ in the pH region of 3–6 was somewhat variable, but $t_{1/2}$ was of the order of magnitude of several hours. At pH 7·1, I was converted to U.V.-absorbing materials with a $t_{1/2}$ of approximately 29 minutes. The efficacy of base catalysis increased slowly above pH 8; at pH 9 the rate was approximately the same as at pH 1·0, and at pH 12 it was slightly greater than twice this value. Since the products were not well defined, we made no attempt to measure the rates precisely in the pH region of 3–12.

Experiments with D_2O. The isomerization of I to II was carried out at 30° in a solution at pH 1·0 of DBr in D_2O (90·6 atom % of excess deuterium). The reaction was allowed to proceed for 23 half-life periods (120 min) which insured complete

conversion. The α,β-unsaturated ketone (II) was isolated and analyzed[9] for deuterium by combustion to D_2O—H_2O and measurement of the latter's rate of fall through a viscous medium. The D-content of II was 3·45 atom % of excess deuterium which is equivalent to the incorporation of 96·5% of one proton from the medium. The difference from 100% is only slightly greater than the additive error of the two (D_2O, steroid) determinations. A similar experiment was carried out in which II was the starting material instead of I, and no deuterium (0·03 ± 0·04 atom %) was incorporated during 124 min at pH 1·0 (DBr, D_2O).

Talalay and Wang[4] have reported that when I is isomerized in 0·23N HCl in 89% D_2O at 25° and the product is diluted with carrier-I and analyzed by mass spectrometry, there is an incorporation of 0·95 atoms per mole. This is slightly more than expected on the basis of the D_2O purity of their medium, and the excess may represent enolization-ketonization of II during their experiment. However, our result is in substantial agreement with theirs.

Enzymatically catalyzed isomerization. While Talalay[1] has measured the enzymatically catalyzed reaction of six of the compounds which we studied non-enzymatically, we felt it might be of interest to extend his findings to all of the substances which we had.

The rate of isomerization of the various β,γ-unsaturated ketones listed in Table 1 was measured by observing the rate of appearance of absorption (at 248 mμ) for the Δ^4-3-ketones produced. The rate of disappearance of the β,γ-unsaturated ketones was obtained by difference from the rate of appearance of the α,β-unsaturated ketones. All of the isomerizations were allowed to proceed to completion without interference. Proof that isomerization occured was afforded by the isolation of II when I was used as substrate. The isomerizations very closely approximated first order kinetics in steroid under the conditions we used as shown for a typical case in Fig. 1. A few of the $\Delta^{5(10)}$-compounds deviated slightly from first order kinetics during the disappearance of the last 25% of β,γ-unsaturated ketone, and a half-time period was calculated in all cases from the data obtained during the first 50–75% of reaction. The results are listed in Table 1 both as found experimentally and as a relative value ($Rt_{1/2}$) to the half-time period of 5-androstene-3,17-dione (I) which we have arbitrarily taken as a standard.

The clear, homogeneous, enzymatic solution was prepared essentially according to the procedure of Talalay *et al.* from *Pseudomonas testosteroni* which had been adapted to growth in the presence of testosterone.[4,10] Contrary to earlier reports,[4] we found it to be relatively stable when stored at +5°; during a period of one month the activity, as measured by its influence on the rate of conversion of I to II, was the same within 50%. The enzymatic isomerizations were carried out at pH 7·0 in a 0·10M phosphate buffer at 30°. The activity of the enzyme was assayed with I each time a series of kinetic measurements was made. No correction was applied for a non-enzymatic reaction, but in the standard (I) case, the enzymatic reaction at our usual concentration of enzyme was five times faster than the non-enzymatic reaction. That the reactions studied were really enzymatically catalyzed was shown by a

[9] Analyses were carried out by Mr. Josef Nemeth, 303 W. Washington Street, Urbana, Illinois. The experimental error is ±2% of the true value. Deuterium oxide of 99·5% purity obtained from General Dynamics Corporation was used as a standard.

[10] P. I. Marcus and P. J. Talalay, *J. Biol. Chem.* **218**, 661 (1956).

dependence of the rate on the concentration of enzyme; thus, as shown in Table 1, the reaction was first order in enzyme, *i. e.*, the half-time period was inversely proportional to the enzyme concentration, for 5-androstene-3,17-dione as well as for 5(10)-estrene-3,17-dione.

DISCUSSION

It is apparent from Table 1 that the $\Delta^{5(6)}$-compounds isomerize under acid-catalysis at a rate 10–38 times as great as do the corresponding $\Delta^{5(10)}$-compounds. Under enzymatic catalysis the $\Delta^{5(6)}$-compounds also react the faster, but the difference in rate between the two series is magnified to a factor varying from 29 to a few hundred. Talalay *et al.* have reported that the $\Delta^{5(10)}$-steroids which they measured (5-estren-17β-ol-3-one, 5-estrene-3,17-dione, and the 17α-methyl, ethyl, and ethynyl derivatives of 5-estren-17β-ol-3-one) isomerize under enzymatic catalysis approximately 0·25–0·75% as fast as does I, and our results are in essential agreement with this. In both types of catalysis we found the slowest reacting substances were the pair of 17-oxygenated $\Delta^{5(10)}$-3-ketones lacking a 17-alkyl group, i.e., 5(10)-estrene-3,17-dione and 5(10)-estren-17β-ol-3-one, but in general there was only a small influence of the 17-substituent. Within any one series ($\Delta^{5(10)}$ or $\Delta^{5(6)}$) what differences there are usually amount to less than a factor of one, and the maximal difference is only five-fold. The energy differences reflected by these rate figures are too small to warrant our analyzing them. What seems to us to be significant, however, is that the differences induced by changes in C-17 are of the same order of magnitude in both types of catalysis. It is also of interest that 5-estrene-3,17-dione reacted non-enzymatically and enzymatically at about the same rate (within a factor of one) as did the analogous derivative (5-androstene-3,17-dione) with the angular methyl group, and steric hindrance by C-19 seems to play a small role in both types of catalysis.

The mechanism of the acid-catalyzed reaction can be viewed as proceeding by a rate-determining protonation through a transition-state which approximates the structure of the dienol. This mechanism would account for the pH-dependence, for the incorporation of deuterium, and for the faster rate of isomerization of the $\Delta^{5(6)}$-compounds compared to the $\Delta^{5(10)}$-compounds, since the heteroannular distribution of the electrons should be more stable than the homoannular one by analogy to the well known stabilities of the corresponding types of steroidal diene.

We should like to point out that there is as yet no clear reason why the enzymatic reaction could not take an analogous course in which the enzyme functions as the protonating-deprotonating agent. The generally parallel affect of structural features on the enzymatic and non-enzymatic reactions is consistent with a parallel mechanism in which the chemistry of the substrate rather than substrate specificity[1] of the enzyme determines, for instance, that the $\Delta^{5(6)}$-compounds isomerize faster than the $\Delta^{5(10)}$-compounds. On the other hand, Talalay has argued[1,5] that an intra-molecular H- transfer occurs, because he found that enzyme previously equilibrated with D_2O catalyzed isomerization of I in D_2O without incorporation of label into the steroid. This information, however, can be readily accommodated without the assumption of an intramolecular transfer.

Suppose that an NH_2-group on the enzyme acts as a protonating-deprotonating agent, itself undergoing a displacement reaction, i.e., one of the two protons attacks C-6 (or C-10, depending on the series) of the steroid while the proton at C-4 of the steroid attacks the nitrogen atom of the enzyme. Suppose further that the NH_2-group

equilibrates with the medium slower than steroid is bound and isomerized. These suppositions are reasonable, because Talalay[1] has pointed out that the enzyme has the highest turnover rate of any known enzyme, and he has shown[1] that the amino acid content of the enzyme allows, e.g., through lysine, an NH_2-group to be present on its surface. If, then, a molecule of steroid isomerizes on the enzyme, it will incorporate label and the ND_2-group will become NHD. In the next few encounters with steroid, the NHD-group will become an NH_2-group, and all subsequent steroid molecules will react without incorporation of deuterium. Except for a small percentage of molecules, the isomerized product will be unlabelled as a consequence of an *overall* intermolecular exchange of a proton from C-4 of one molecule to C-6 of another.

EXPERIMENTAL

M.p.'s were determined on a Kofler hot stage, and rotations in ethanol in ca. 1% solutions on a "High Precision" polarimeter.* I.R. spectra were determined on a Perkin–Elmer Infracord and U.V. spectra on a Cary Model 14 PM Recording Spectrophotometer to which was attached a constant temp bath for the thermostatting of the cell-holder and an automatic programming device for automatic scanning at fixed intervals of time. Measurements of pH were made on a Beckman Zeromatic instrument. The sonic oscillator used in the preparation of the enzymatic solution had an output of 250 W and 10 KC. Alumina was activity grade I of Woelm deactivated as indicated. Microanalyses are by Schwarzkopf Microanalytical Laboratories.

Preparation of 5-*androstene*-3,17-*dione* (I). To a solution of 2·0 g 5-androsten-3β-ol-17-one in 265 ml acetone at 4° was added to 1·84 ml CrO_3-reagent. The acetone used had been successively refluxed 3 times for 1 hr with 1/20th its volume of CrO_3-reagent and each time distilled. The CrO_3-reagent was composed of 134 g CrO_3, 125 ml conc H_2SO_4, and sufficient water to make 500 ml solution. The reaction of the steroid with the CrO_3-reagent was carried out with stirring under nitrogen for 10 min. The mixture was then poured into 1400 ml water and refrigerated at 5° for 1 hr during which time the precipitated product solidified. The solid was filtered, washed well with water, dried, and chromatographed in portions.

Chromatography was carried out on alumina ("neutral") which had been deactivated with 20% of its weight of water. In a typical case, 100 mg crude product from oxidation was dissolved in 0·30 ml chloroform which had been just subsequently allowed to remain 30 min at room temp with $\frac{1}{6}$th its weight of alumina ("basic"). The solution of the steroid in chloroform to which was added 0·70 ml redistilled iso-octane was then passed through a column of 20 g deactivated alumina and elution was continued with $CHCl_3$–iso-octane (3/7; v/v). Pure 5-androstene-3,17-dione (I) was eluted in the first 8 fractions of 3·0 ml each. The product which crystallized when the solvents were removed slowly by a stream of nitrogen, melted at 119–125° (Lit.,[6] m.p. 119–125°) and weighed 25 mg. It showed no absorption-maximum in the U.V. between 220–400 mμ. Further elution with $CHCl_3$–iso-octane removed more I of slightly less purity and finally unchanged 5-androsten-3β-ol-17-one was removed. When chloroform containing 0·75% ethanol was passed through the column, the effluent formed 2 phases as the result of elution of water from the alumina.

The m.p. of our 5-androstene-3,17-dione was consistently near 120° as reported[6] and not the higher m.p. (m.p. 158°) reported earlier.[11] This difference in the m.p.'s is apparently due to polymorphism[6] or to the manner in which the m.p. is determined.

Isolation of 4-*androstene*-3,17-*dione* (II) *from the isomerization of* 5-*androstene*-3,17-*dione* (I). A solution of 40 mg 5-androstene-3,17-dione (I) in 10 ml methanol was added dropwise with stirring to 1 l. water which was brought to pH 1·0 with HCl. The mixture was kept at 30° for 1·5 hr. The acid was carefully neutralized with potassium hydroxide, and the steroid extracted 3 times with 200 ml portions ether. The extracts were combined, washed, dried, and the solvents evaporated. The crude 4-androstene-3,17-dione (II) was crystallized from methanol–water. The crystals (18 mg) m.p. 172–175°, λ_{max}^{EtOH} 240 (ε 17,700). The I.R. spectrum was identical with an authentic sample kindly supplied by Syntex, S.A., which did not depress the m.p. of our sample.

Incubation of 40 mg I with 0·4 ml enzyme preparation in 1 l. 0·10M phosphate buffer at pH 7·0,

* Purchased from A. E. Thompson, Inc.

[11] A. Butenandt and J. Schmidt-Thomé, *Ber. Dtsch. Chem. Ges.* **69**, 882 (1936).

extraction, and crystallization yielded 23 mg 4-androstene-3,17-dione (II), m.p. 171–174°, λ_{max}^{EtHO} 240 mμ (ε 16,300). The I.R. spectrum was identical to that obtained from an authentic sample.

5-Estrene-3,17-dione. To a solution of 5-esterene-3β,17β-diol[14] (200 mg) in 140 ml acetone at 3–5° was added 0·4 ml CrO_3-reagent under nitrogen. After 20 min, another 0·2 ml reagent was added. Stirring was continued at 4° for 15 min. Isolation of the product (by addition of water and extraction with ethyl acetate) and chromatography as described for I yielded 5-estrene-3,17-dione which after 3 crystallizations from acetone–hexane m.p. 104–106°, $[\alpha]_D^{21}$ −117°, and showed no selective absorption in the U.V.; λ_{max} 5·78 and 5·85 μ. (Found: C, 79·13%; H, 8·77%; Calc. for $C_{18}H_{24}O_2$: C, 79·37%; H, 8·88%).

17α-Methyl-3,5-estradiene-3,17β-diol diacetate. A solution of 17α-methyl-19-nor-testosterone[13] (1·0 g, m.p. 155–156°, Lit.[13] 156–158°) in acetic anhydride (4 ml) and acetyl chloride (4·5 ml) was refluxed under nitrogen for 2 hr and then for 2 hr further with an additional 1·0 ml acetic anhydride. At the end of the reaction, most of the solvents were evaporated by an increase in the rate of flow of nitrogen through the apparatus. The contents of the flask were then dried *in vacuo* until there was no detectable odor of the reagents. The crude enol acetate was chromatographed on 100 g silica gel. The material was applied in benzene and the column washed with the same solvent. A fraction which was eluted with 5% ether in benzene possessed absorption maxima at 236 mμ, 1750 cm^{-1} and 1210 cm^{-1}; this by-product was not further investigated. 17α-Methyl-3,5-estradiene-3,17β-diol diacetate (880 mg) was eluted with 10% ether in benzene. Crystallization from ether and then from cold methanol yielded the analytical sample m.p. 65–67° with the formation of a viscous oil which did not flow freely until 94° was reached; λ_{max}^{EtOH} 235 mμ (ε 20,100), $\nu_{max}^{CS_2}$ 1750, 1725, 1260, 1240, and 1210 cm $^{-1}$, $[\alpha]_D^{20}$ −89°. (Found: C, 74·10; H, 8·76; Calc. for $C_{23}H_{32}O_4$: C, 74·16; H, 8·66%).

17α-Ethynyl-5-estrene-3β,17β-diol. A solution of sodium borohydride (600 mg) in water (1·5 ml) was added dropwise with stirring to a solution of 17α-ethynyl-3,5-estradiene-3,17β-diol diacetate[8] (500 mg, m.p. 165–167°, Lit.[8] m.p. 167–169°) in tetrahydrofuran (15 ml) and methanol (30 ml) and left overnight. Addition of water, filtration, and recrystallization of the precipitate from acetone–water yielded 145 mg *17α-ethynyl-5-estrene-3β,17β-diol 17-acetate* m.p. 91–93° (Lit.,[8] m.p. 98–102°), $\nu_{max}^{CS_2}$ 3600 (free hydroxyl), 3320 (ethynyl band), 1750 (C=O stretch for 17-acetate), and 1240 cm^{-1} (C—O stretch for the 17-acetate), no selective absorption in the U.V. Chromatography of this material on silica gel did not improve the m.p. nor did drying in a high vacuum, and it was used directly for the next step.

The mono-acetate (m.p. 91–93°, 225 mg) in 10 ml ether was added dropwise with stirring to 10 ml of a solution of lithium aluminium hydride in ether (25 mg per ml), and the mixture was allowed to stand overnight. Excess hydride was decomposed by the addition of ethyl acetate and a saturated solution of sodium sulfate was added followed by solid sodium sulfate. Decantation of the organic solvent, thorough washing with ethyl acetate, evaporation of the solvents, and crystallization from methanol yielded 160 mg of the diol m.p. 207–209° (Lit.,[8] m.p. 207–210°).

Preparation of other β,γ-unsaturated ketones. The various compounds which we synthesized were prepared from the corresponding 3β-alcohols by procedures similar to that described for the preparation of I. and the 3β-alcohols were either obtained by gift, purchase, or synthesis according to the methods previously described in the literature. Although our starting materials and intermediates possessed the reported m.p.'s and expected I.R. spectra, the m.p. of several of our final β,γ-unsaturated ketones were significantly lower than what has been previously reported. However, all of the compounds were chromatographed as discussed for I and the 3β-alcohols should not have been contaminants. Based on the U.V. spectra prior to isomerization, none of the samples could have contained more than a few per cent of the Δ^4-3-ketone as contaminants. Moreover, the β,γ-unsaturated ketones were all isomerized to substances with the expected U.V. absorption of the Δ^4-3-ketones, and we feel the structures assigned must be correct. The discrepancies in m.p. are presumably due to polymorphism or to isomerization (or oxidation) during heating on the Kofler block. The problem of m.p. in the case of 5-androstene-3,17-dione has already been discussed in this paper as well as in the literature.[6,11]

Our m.p.'s (given first) and those recorded in the literature (given second) are as follows: 17α-methyl-5-androstene-17β-ol-3-one (123–129°, 156–157°[8]); 17α-ethyl-5-androstene-17β-ol-3-one

[12] F. B. Colton, U.S. Patent No. 2,729,654, Jan. 3, 1956; *Chem. Abstr.* **50**, 11372c (1956).

[13] C. Djerassi, L. Miramontes, G. Rosenkrantz, and F. Sondheimer, *J. Amer. Chem. Soc.* **76**, 4092 (1954).

[14] R. Villotti, C. Djerassi, and H. J. Ringold, *J. Amer. Chem. Soc.* **81**, 4566 (1959).

(118–128°, 149°[11]); 17α-methyl-5-estren-17β-ol-3-one (132–134°; 138–139°[8]); 17α-ethynyl-5-estren-17β-ol-3-one (144–146°, 151–153°[8]); 5(10)-estrene-3,17-dione (134–142°, 144–146°[12]).

Kinetic experiments. The kinetic experiments under acid-catalysis were carried out in the following manner. A solution of the steroid in methanol (7 mg/10 ml) was freshly prepared and 1·0 ml added with stirring to 48·0 ml water at 30° which had been adjusted to the desired pH with hydrochloric acid A clear, homogenous solution resulted. The U.V. absorption of the thermostatted (30°) solution was measured continuously in glass-stoppered cells at 248 mμ for short-lived reactions and intermittently (programmed) for long-lived reactions on a Cary Model 14 PM recording spectrophotometer; the "blank" was an HCl solution of the same pH lacking steroid. The maximum which developed was at 248 mμ. The reaction was allowed to proceed until the optical density did not change, and the final optical density at 248 mμ (D_{100}) due to the α,β-unsaturated ketone, e.g., II, was used as an exact measure of the initial concentration of β,γ-unsaturated steroid, e.g., I, and the difference between the optical density at a given time (D_t) and D_{100} was called ΔD and taken to be a measure of the concentration of β,γ-unsaturated ketone at the given time. The solutions of I (and similarly for its analogs) showed initially an optical density of about 0·03 at 248 mμ which was neglected, since the final optical densities were near 1·0 and the percentage error was small from this initial absorption. The logarithm of ΔD when plotted against time (cf. Fig. 1) yielded a straight line from which the half-time period ($t_{1/2}$) and the reaction rate constant (k') were obtained graphically. The half-time values in min for various pH's (in parentheses) for the conversion of I to II were 2·0 (0·50), 2·8 (0·72), 5·3 (1·00), 10·3 (1·28), 21·6 (1·50), 36·0 (1·78), 61·9 (2·00), 84·0 (2·20), and 161 (2·50). The half-time period at pH 1·0 for the other β,γ-unsaturated ketones is listed in Table 1. As is illustrated in Fig. 1 for 5-androstene-3,17-dione, the isomerization obeyed first order kinetics for 7 half-time periods, and, since 4-androstene-3,17-dione was easily isolable, we feel the isomerization was at least the major reaction occurring.

The enzymatic experiments were carried out in the same manner as described for the acid-catalyzed reactions except that 0·10M phosphate buffer (pH 7·0) was used in place of the HCl solution. The non-enzymatic reaction was allowed to proceed for a few min in order to establish a base line, and then to 49·0 ml of the solution was added 1·0 ml standard enzyme preparation, 2·0 ml of which had been just previously diluted to 25·0 ml with water. The "blank" solution in the spectrophotometer was prepared in the same way except the steroid was omitted. The half-time periods were obtained graphically from a plot of log ΔD vs time as described for the acid-catalyzed reaction except that D_t was corrected by the subtraction of the optical density at 248 mμ which obtained at the time immediately preceding the addition of the enzyme. This correction was always small (a few per cent of the D_{100}), and in all cases the addition of the enzyme markedly accelerated the increase in absorption at 248 mμ.

Enzyme preparation. A starter-culture* (10 ml) was inoculated with *Pseudomonas testosteroni* (American Type Culture No. 11996). After the culture was shaken for 8 hr at 30°, flasks containing 45 ml each of culture medium were inoculated with 0·25 ml bacterial suspension from the starter culture. The flasks were shaken at 30° for 16 hr, and 2·5 ml of a testosterone suspension in water was added to each. The testosterone suspension was prepared by sonic oscillation (30 min) of 500 mg testosterone in 50 ml water. Incubation was then continued for 24 hr and was followed by centrifugation at 13,500 r.p.m. (in an International Centrifuge, Model HT) for 10 min in the cold (4°). The sediments were washed twice by resuspension (in a loose Potter homogenizer) in 45 ml 0·001M EDTA solution in 0·03M phosphate buffer (pH 7·2), and then centrifuged as described above. After the second wash, the cakes were each resuspended in 45 ml EDTA solution and subjected for 30 min to the maximum output of a 10 kilocycle, 200 watt sonic oscillator. The cell debris was sedimented by centrifugation for 30 min at 13,500 r.p.m. in the cold. The combined supernatants were used as the enzyme preparation to catalyze isomerization of the β,γ-unsaturated ketones. This preparation was diluted as indicated e.g., in Table 1 and Fig. 1.

Acknowledgement—Support of this investigation through Research Grant No. 6019 and Research Training Grant No. 5001 of the National Institutes of Health is gratefully acknowledged. We should also like to thank Dr. Frank B. Colton of G. D. Searle and Company for the generous gift of several steroids.

* 1·0 g $NH_4H_2PO_4$, 1·0 g $(NH_4)_2HPO_4$, 2·0 g KH_2PO_4, 10 g Difco yeast extract, 10 ml trace element solution, and sufficient water to make 1 l. solution. The trace element solution was composed of 2·0 g $MgSO_4 \cdot 7H_2O$, 100 mg NaCl, 50 mg $FeSO_4 \cdot 7H_2O$, 50 mg $ZnSO_4 \cdot 7H_2O$, 50 mg $MnSO_4 \cdot 3H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, 1·0 ml 0·1N M_2SO_4, and sufficient water to bring the volume to 100 ml.