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STRUCTURE AND REACTIVITY OF STEROIDS—VI

LONG RANGE EFFECTS IN A SERIES OF $\Delta^{1,3,5(10)}$-ESTRATRIENE COMPOUNDS*

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Abstract—The kinetics of the oxidation of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes which differ in D ring size, in C_3 substituent and in the character of fusion of the B and C rings by CrO_3 in AcOH has been studied. An increase in size of the D ring in the substrate diminishes reactivity of the 17β-hydroxy group in the oxidation. It has been found that the reactivities of the 17β- and 17aβ-hydroxy groups of the $\Delta^{1,3,5(10)}$-estratriene derivatives depend upon the electronic nature of the substituent at C_3, explained by a long range effect operating from the A to D ring. The pK' a's of phenolic steroids have been measured and it was shown that introduction of the $\Delta^{8(9)}$ double bond increases the degree of dissociation of the phenolic hydroxyl. Expansion of the D ring and also reduction of the 17(17a)-keto group to 17β (17aβ) hydroxy group decreases the degree of dissociation of the phenolic hydroxyl as a result of the long range effect from the D to A ring. A linear correlation has been established between the oxidation and dissociation rate constants, confirming the existence of an interaction between C_3 and $C_{17(17a)}$ at the expense of the long range effects from the A to D ring and *vice versa*. The long range effect apparently arises from a combination of inductive and conformational effects of the substituent. The kinetics of enzymic oxidation of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes by soluble 17β-estradiol dehydrogenase from human placenta has been investigated. In the case of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes of natural configuration, a linear correlation has been established between the values of the maximum rate constant and the oxidation rate constant. An assumption can thus be made about the major role of the contribution of the specific reactivity of the substrate to that of the enzyme-substrate complex, involving the size of the D ring and the long range effect of the substituent at C_3 upon the reaction center at $C_{17(17a)}$. For 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes of the 8-isoconfiguration it is the specific reactivity of the substrate that makes the major contribution to the maximum rate values; this specific activity is a result of the substrate's configuration, which is responsible for the stability of the enzyme-substrate complex.

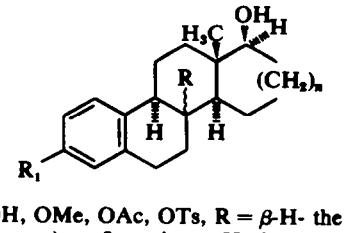
Knowledge of the dependences between the reactivity of the functional groups of steroids and the structure of the latter provides an approach to their metabolism and the mechanism of their action in living organisms.

In this connexion the long range effect in polycyclic compounds discovered by Barton in 1955 is most interesting.[1-4] Barton and his associates introduced the notion of conformational transmission[2] which is realised in a polycyclic system at the expense of a change in the dihedral angles resulting from some structural distortions. A detailed analysis of the conformational transmission effect and its semiquantitative estimation has been performed by Bucourt[5-7] and Robinson and Whalley.[8] Since, quite a number of papers have appeared dealing with the effects of conformational transmission.[9-13]

The problem acquires added complexity when substituents, capable of displaying an inductive effect are introduced into a polycyclic system; no wonder therefore that opinions differ. For instance, Schwarz *et al.* favour the long range inductive effect[14-18] which was subjected to a theoretical analysis by Peterson.[19] In some papers the "field effect" is believed to play an essential role.[20-22] However, the more complicated nature of this phenomenon has been advanced in later studies.[23-24] At present, it seems feasible to differentiate between the conformational and inductive long range effects in order to clarify the latter, a difficult task which may be accomplished by a careful and wise choice of the series of polycyclic compounds and experimental schemes.

The present work is a comparative study of the structure of $\Delta^{1,3,5(10)}$-estratriene derivatives and the reactivity of their hydroxy groups. The latter are known to be involved in the metabolism of estrogens (a phenolic hydroxyl at C_3 and a secondary hydroxyl at $C_{17(17a)}$.

Compounds of the following general formula have been studied:



R_1 = H, OH, OMe, OAc, OTs, R = β-H- the "natural" (*trans-anti-trans*) configuration. α-H- the "8-*iso*" (*cis-sin-trans*) configuration. $n = 1$ the normal series, $n = 2$—the D-homo-series.

*A preliminary report of these findings was given at the 7th International Symposium on the Chemistry of Natural Products. Riga, July, 1970, pp. 404–405.

The kinetics of the chemical oxidation of these compounds by CrO_3 in AcOH has been studied[25,26] and $pK'a$'s of the 3-hydroxy groups compared. Finally, the kinetics of oxidation of the 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratriene by soluble 17β-estradiol dehydrogenase from human placenta was investigated as an enzymic model of metabolic transformation.

The data on the chemical and enzymic oxidations were subjected to comparative analysis.[27,28]

The kinetics of oxidation of 17β-*hydroxy*-$\Delta^{1,3,5(10)}$-*estratrienes with* CrO_3 *in* 90% AcOH

At present,[29–32] the mechanism of oxidation of secondary carbinols by chromic acid is visualized as Scheme I, where step 1 is a steady-state with $K_m^{-1} = K_s = k_{-1}/k_{+1}$ and step 2 is rate-determining and involves decomposition of the secondary carbinol chromate, most probably, *via* a cyclic transitional complex.[33]

SCHEME 1.

$$R_2CHOH + HCrO_4^- + H^+ \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} R_2CHOCrO_3H + H_2O \quad (1)$$

$$R_2CHOCrO_3H \xrightarrow{k_2} R_2C=O + Cr(IV) \quad (2)$$

$$Cr(IV) + Cr(VI) \xrightarrow{k_3} 2Cr(V) \quad (3)$$

$$R_2CHOH + Cr(V) \xrightarrow{k_4} R_2C=O + Cr(III) + 2H^+ \quad (4)$$

Steps 3 and 4 are rapid. A mathematical analysis of Scheme 1 has been performed.[26] Starting from steady-state conditions we derived a rate equation for the oxidation reaction

$$V = -\frac{d[Cr(IV)]}{dt} = \quad (I)$$

$$\frac{2k_2[R_2CHOH]_o\left\{[HCrO_4^-]_o - \frac{k_2}{k_3}K_s[R_2CHOH]_o\right\}}{K_s^{-1} + \frac{2k_2}{k_4} + [R_2CHOH]_o}$$

where $K_s = k_1[H^+]/(k_{-1}[H_2O] + k_2) = \text{const.}$, when $[H^+]$ and $[H_2O] = \text{const.}$ The steroid carbinols were oxidized by CrO_3 in 90% AcOH. Special TLC experiments revealed one and the same transformation, i.e. oxidation of the 17β-hydroxy group, to occur in all the substrates investigated. The rate of oxidation was determined spectrophotometrically by a decrease in the absorption maximum of the acidic chromate ion ($HCrO_4^-$) at 345 nm and 23 ± 0·5°C. Each sample contained $1{\cdot}0 \cdot 10^{-3}$ M CrO_3 solution, the carbinol concentration was varied from $2{\cdot}0 \cdot 10^{-3}$ M to $2{\cdot}0 \cdot 10^{-2}$ M (the ratio of $[CrO_3]_o$ to $[R_2CHOH]_o$ from 1:2 to 1:20). The data obtained from an analysis of the optical density vs. time ($D = f(t)$) curves obey the following linear dependence:

$$1/V = a + b/[R_2CHOH] \quad (II)$$

Therefore in equation (1) term $(k_2/k_3)K_s[R_2CHOH]_o \ll [HCrO_4^-]_o$ and can be neglected. This enabled us to determine k_2 and $k_m = K_s^{-1} + (2k_2/k_4)$ from plot (II), where $a = \frac{1}{2}k_2[HCrO_4^-]_o$ and $b = K_m/2k_2[HCrO_4^-]_o$. Special experiments were performed to show that, unlike k_2, constant K_m depends upon the hydrogen ions and water concentrations. An analysis ot these results shows that for all the compounds investigated $K_m \simeq K_s^{-1}$, e.g. $2k_2/k_4 \ll K_s^{-1}$ and $k_1[H_2O] \gg k_2$. Therefrom it follows that $K_m \simeq k_{-1}[H_2O]/k_{+1}[H^+]$.

Thus it has been established that the reactivity of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes in the oxidation reaction may well be described in terms of two constants: k_2, the kinetic constant and K_m, the thermodynamic constant.

We have studied the kinetics of the oxidation of secondary steroid carbinols by CrO_3 in AcOH; these 17β-hydroxy-$\Delta^{1,3,5(10)}$ estratrienes had substituents at C_3 of different electronic nature and which also differed as to the size of the D ring and the character of fusion of the B and C rings. The values of k_2 and K_m are listed in Tables 1 and 2.

Since the protonation of the secondary carbinol chromate is known to enhance the rate of its degradation[34], the values of k_2 should be higher for oxidation in the acidic medium. The data of Table 1 and 2 shows that this was the case.

Table 1. The values of k_2 and K_m for 17β-hydroxy-$\Delta^{1,3,5(10)}$ estratrienes (solvent – 90% AcOH, pH 1·76)

No.	Compound	k_2 min^{-1}	$K_m \cdot 10^2$ M
1	D-estradiol	0·37 ± 0·01	3·0 ± 0·3
2	D-estradiol 3-methyl ether	0·79 ± 0·03	3·6 ± 0·3
3	D-estradiol 3-acetate	0·28 ± 0·01	1·2 ± 0·1
4	D,L-8-isoestradiol	0·28 ± 0·01	1·8 ± 0·2
5	D,L-8-isoestradiol 3-methyl ether	0·80 ± 0·03	5·0 ± 0·4
6	D,L-8-isoestradiol 3-acetate	0·14 ± 0·01	0·60 ± 0·05
7	D,L-D-homoestradiol	0·042 ± 0·001	0·83 ± 0·07
8	D,L-D-homoestradiol -methyl ether	0·27 ± 0·01	2·1 ± 0·2
9	D,L-D-homoestradiol 3-acetate	0·016 ± 0·001	0·18 ± 0·02
10	D,L-8-iso-D-homo-estradiol	0·096 ± 0·012	0·25 ± 0·02
11	D,L-8-iso-D-homo-estradiol 3-methyl ether	0·37 ± 0·01	3·3 ± 0·3
12	D,L-8-iso-D-homo-estradiol 3-acetate	0·036 ± 0·001	0·71 ± 0·06

The relationship between the oxidation rate and the substituent at C_3 *in* 17β-*hydroxy*-$\Delta^{1,3,5(10)}$-*estratrienes.* As seen from Tables 1 and 2, the values of k_2 for 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes of the normal and D-homo series, having natural and 8-

Table 2. The k_2 and K_m for D-estradiol derivatives (solvent 90% AcOH, pH 0·37)

No.	Compound	k_2 min^{-1}	$K_m \cdot 10^2$ M
1	D-estradiol	0·75 ± 0·01	3·2 ± 0·1
2	D-estradiol 3-methyl ether	1·35 ± 0·02	5·7 ± 0·1
3	D-estradiol 3-acetate	0·46 ± 0·11	2·6 ± 0·7
13	D-estradiol 3-tosylate	0·24 ± 0·05	2·9 ± 0·6
14	D-3-desoxyestradiol	0·67 ± 0·11	3·4 ± 0·6

iso-configurations, are determined by the electronic nature of the substituent at C_3. The values of k_2 were found to decrease in the following order depending on whether the substituent was an electron donor or acceptor: k_2(OMe) > k_2(OH) > k_2(H) > k_2(OAc > k_2(OTs), the order being the same whatever the pH of the medium.

The changes in the K_m values, although of the same order, are not so clear-cut.

An attempt to carry out McDanial and Brown's analysis between log k_2, log K_m and the σ constants for the substituents at C_3 in 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratriene did not prove successful. It should be noted, however, that the reaction constant was negative, in agreement with the literature.[34–36]

Thus we assume that the relationship between the oxidation rate of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes and the character of the substituent at C_3 is determined by the long range effect from ring A to D (A → D). The absence of a correlation between log k_2 and the σ constants does not permit a conclusion to be made about the effect being purely inductive, although certain authors support the idea of the inductive character of the long range effect[14–18,37] We believe that the long range effect in 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes is determined by a combination of both conformational and inductive effects.

As seen from Dreiding models, estradiol (D-homoestradiol) has a planar conformation, whereas in 8-isoestradiol (8-iso-D-homoestradiol) the A, B and C, D rings are localized in the different planes (Fig 1). In spite of the difference in geometry of these molecules, their C_3–C_{17}(C_{17a}) distances are approximately the same and sufficiently great. Moreover, the presence of planar aromatic ring A, renders a spatial interaction between the substituents at C_3 and C_{17}(C_{17a}) (the so-called "field effect") improbable. We believe therefore that the interaction of these substituents takes place due to a long range effect *via* the tetracyclic steroid system.

The relationship between oxidation rate and the D ring size. As seen from Table 1, when steroids of the normal series are replaced with D-homo analogues, a decrease in k_2 and K_m values is observed for compounds having both the natural and the 8-iso configuration. Since in the normal compounds the five-membered D ring is more highly strained

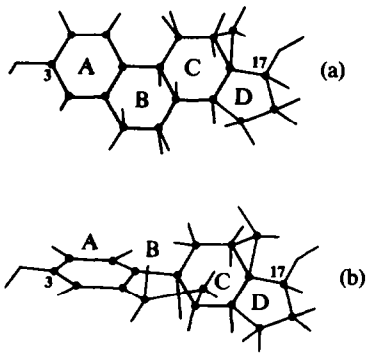


Fig 1. Dreiding's molecular model of estradiol (a) and 8-isoestradiol (b).

than the six-membered ring of the D-homo analogues, the decomposition of the cyclic transitional complex (Scheme 1, step 2) will proceed at a higher rate in the former, which is expressed in higher values of k_2. A similar regularity is involved in the changes of $K_m \simeq K_s^{-1} = k_{-1}/k_{+1}$, which expresses the lower stability of chromates of the normal series carbonyls with respect to their D-homo analogues in the steady state step 1 (Scheme 1).

The relationship between the oxidation rate and the character of fusion of the B and C rings. It was found that both in the normal and D-homo series, transition from the normal to the 8-iso configurations ensues changes in the total oxidation rate and the k_2 and K_M values. This is due to the fact that the altered character of fusion between the B and C rings results in deformation of the dihedral angles shared by the B, C and D rings. This entails a change in the strain of the D ring. 3-Methyl ethers of estradiol (2) and its 8-epimer (5), whose k_2 values are practically the same, proved to be the only exceptions. This is apparently due to comparatively high reactivity of compounds 2 and 5 in the oxidation reaction ($k_2 \simeq 0{\cdot}8$) determined by the substituent at C_3; hence the conformational effect on the reaction center, associated with the deformation of the dihedral angles is not so distinct. For the 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes having comparatively low values of k_2 ($k_2 < 0{\cdot}37$), the replacement of the compounds of natural configuration with 8-epimers results in changes in k_2. For instance, a changed C_8 configuration in estradiol (1) and its 3-acetate (3) brings about a decrease in k_2 (compounds 4 and 6, respectively). Contrarywise, with compounds 7–9, a similar transfer to 8-epimers (10–12) leads to a greater k_2.

The pK'a*'s of phenolic steroids*

In estrogens not only the 17β-hydroxy group, but also the phenol hydroxyl behaves as a metabolic transformation site. It would be interesting to show how the degree of C_3 hydroxyl dissociation depends on the structure of 3-hydroxy-$\Delta^{1,3,5(10)}$-

estratrienes and, in particular, on the structural changes of the D ring. To this end, ten phenolic steroids (**1**, **4**, **7**, **10**, **15**, **16**, **17**–**20**) have been studied.

A comparative study of *pKa*'s of phenolic steroids has been carried out,[37,38] and has been found that the difference in *pKa*'s depends upon the character of substitution in the D ring. This fact was explained by a long range polar effect from the D to A ring (D → A).[37] Moreover, it has been shown which groups when conjugated with aromatic ring A (for example, a keto group at C_6 or the $\Delta^{6(7)}$ double bond) increase the degree of dissociation of the phenolic hydroxyl.[38]

UV spectroscopy was used[38] to determine the apparent *pK'a* values for the ten phenolic steroids.

The UV spectra of nonionized steroid were taken in 0·1 N HCl in the region of 220–340 nm, those of the practically ionized form – in the 0·1 N NaOH. In phosphate buffer (0·01 M $Na_3PO_4 \times 12\,H_2O$ and 0·01 M $NaH_2PO_4 . 2\,H_2O$) the steroid is in equilibrium with its ionized form, therefore the UV curves have a transient character: as pH grows, the λ_{max} of the ionized form appears (238, 298 nm) and that at 280 nm corresponding to the non-ionized form disappears (Fig. 2).

Kirdani *et al.*[38] used the 300 nm wavelength but absorption of the non-ionized form of the steroid at 300 nm was not taken into account when calculating the *pKa*.

However, as was established with 0·1 N HCl

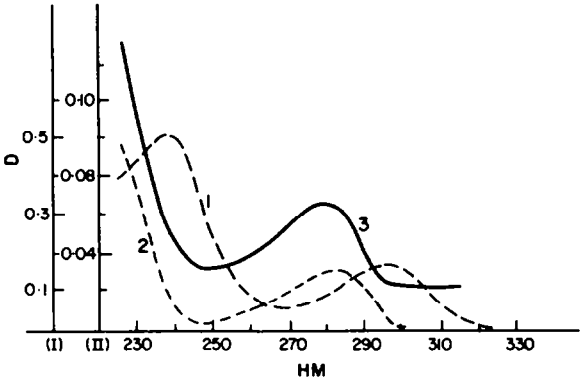


Fig 2. UV spectra of D-estradiol: (1) 0·1 N NaOH, 6·05 . 10^{-5} M, (2) ethanol, 6·10 . 10^{-5} M scale I, 0·1 N HCl, 3·02 . 10^{-5} M, scale II.

solutions of all the compounds investigated, the non-ionized form of the steroid does absorb at 300 nm. Therefore, another formula was used for estimating *pK'a*:[39]

$$pK'a = \mathrm{pH} + \log \frac{(\epsilon_1 - \epsilon_2)}{(\epsilon_2 - \epsilon_3)},$$

where ϵ_1 is the molar extinction of the steroid at the analytical wavelength in 0·1 N NaOH, ϵ_2 is the molar extinction of the steroid at the analytical wavelength in the buffer, ϵ_3 is the molar extinction of the steroid at the analytical wavelength in 0·1 N HCl. Since the question to be clarified was the *pK'a* alterations associated with structural changes in the steroid molecule, apparent *pKa* values were estimated, e.g. the ionic strength of the solution was not taken into account. $\lambda = 240$ nm was decided upon as the analytical wavelength for 3-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes and $\lambda = 290$ nm for 3-hydroxy-$\Delta^{1,3,5(10),8(9)}$-estratetraenes. It should be noted that $\lambda = 300$ nm is less convenient because of low values of optical density (0·1 optical density units and less at the concentrations employed, $2 \cdot 10^{-5}$–$5 \cdot 10^{-5}$ M). So in some cases a satisfactory reproducibility was hard to obtain and mean square errors were great. The *pK'a*'s are in Table 3.

A pairwise comparison of *pK'a*'s for compounds

Table 3. The *pK'a*'s of 3-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes

No.	Compound	the number of runs	*pK'a*
1	D-estradiol	17	10·30 ± 0·10
4	D,L-8-isoestradiol	9	10·35 ± 0·13
7	D,L-D-homoestradiol	4	11·31 ± 0·24
10	D,L-8-iso-D-homoestradiol	4	10·89 ± 0·09
15	D,L-$\Delta^{8(9)}$-dehydroestradiol	2	9·97 ± 0·14
16	D,L-$\Delta^{8(9)}$-dehydroestrone	4	9·50 ± 0·12
17	D-estrone	12	10·26 ± 0·14
18	D,L-8-isoestrone	8	10·28 ± 0·10
19	D,L-D-homoestrone	8	10·63 ± 0·10
20	D,L-8-iso-D-homoestrone	6	11·05 ± 0·19

1 and **15**, **17** and **16** shows that introduction of the additional $\Delta^{8(9)}$ double bond conjugated with aromatic A ring decreases the *pK'a* due to delocalization of the negative charge of the phenoxy ion, as reported.[38] For compounds of the normal series, **1** and **17**, **4** and **18**, no noticeable difference in the *pK'a* values depending on the character of fusion of rings B and C was found. At the same time for the D-homo series the *pK'a* values increase in the case of **19** and **20** and decrease for **7** and **10** when they were substituted with 8-epimers. There is some controversy in the literature as to the differences between the *pKa*'s of estrone and estradiol.[37,38] We revealed no noticeable difference in the *pK'a*'s of either compounds **1** and **17** or of their D-homo analogues of the 8-iso-configuration, (**10**) and (**20**). However, a pairwise comparison of the *pK'a*'s of phenolic steroids **7** and **19**, **16** and **15** shows that with the introduction of a keto group to $C_{17}(C_{17a})$ the degree of the phenol hydroxyl dissociation becomes greater than that of their $C_{17}(C_{17a})$-hydroxy derivatives. From a comparison of the *pK'a*'s of compounds **1** and **7**, **4** and **10**, **17** and **19**, **18** and **20** it is evident that an expansion of the D ring decreases the acidity of the 3-hydroxy group.

We believe that the differences in the $pK'a$'s associated with the structural changes in the D ring should be ascribed to a long range effect from the D to A ring (D → A) of a predominantly conformational character.

It should be noted that a linear correlation is observed (with a correlation coefficient $r = 0{\cdot}992$) between the k_2 constants, for compounds **1**, **4**, **7** and **10** which differ in the size of the D ring, the character of fusion of rings B and C (Table 1) and dissociation constants (K_{diss}) of 3-hydroxyls (Fig 3). This fact should be taken to mean that in 3,17β-dihydroxy compounds the substituents at C_3 and C_{17}(C_{17a}) affect each other in a specific fashion. An electron donor or acceptor at C_3 causes a deformation of the aromatic ring bonds and hence conformational changes in the B ring. This distortion is conformationally transmitted to the D ring and results in a change in the reactivity of the 17 functional group.

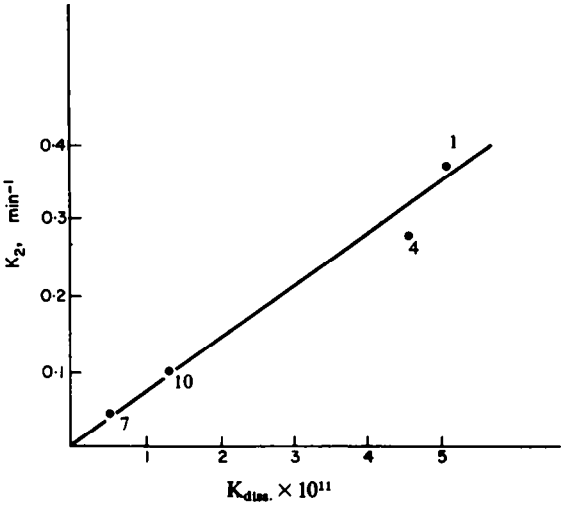


Fig 3. k_2 *vs* K_{diss} for 3·17β-dihydroxy-$\Delta^{1,3,5(10)}$-estratrienes.

On the other hand, structural changes in the steroid molecule (expansion of the D ring, introduction of an additional double bond or replacement of a 17-hydroxy group with a 17-keto group) causing a distortion of the dihedral angles in the D, C and B rings, lead to a deformation of the bonds in the aromatic ring and hence to redistribution of its electron density. The latter is manifested as a change in the acidity of the 3-hydroxy group associated with the structure of the molecule. The conclusion may be made that the nature of the long range effect is the combined effect of the inductive and conformational factors transmitted from ring A to D and back.

Oxidation of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes by soluble 17β-estradiol dehydrogenase from human placenta

Oxidation of estradiol with a preparation of soluble 17β-estradiol dehydrogenase from human placenta was used as an enzymic model for metabolic transformation of this substance in organism.

17β-estradiol + NAD^+ ⇌ estrone + NADH.

The enzyme was prepared as described by Engel.[40] Special TLC assays showed that in the 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes investigated only oxidation of 17β-hydroxy group takes place. Experimental conditions were designed so as to choose the enzyme concentration lying in the linear part of the curve, which is a plot of the initial rate (V) vs the enzyme concentration (E) at a saturating concentration of NAD^+.

The results of a study of the oxidation kinetics of some substrates and their inhibition with the reaction product, performed with an unpurified enzyme preparation, permitted some conclusions to be made as to the nature of the enzyme and the character of its interaction with the substrate and the reaction product.[27] It has been found that with racemic compounds, the L-enantiomer does not take part in the reaction.[27,28]

Table 4 shows the Michaelis (K_m) and maximum rate (V_{max}) constants for the enzymic oxidation of 17β-hydroxy compounds.

As is seen from the data of Table 4, 17β-hydroxy compounds of the normal series possess a higher reactivity than their D-homo analogues. This becomes evident from a comparison of the V_{max} for the substrates of natural configuration, (**1**) and **7**, **2** and **8** as well as their 8-epimers, **4** and **10** and **5** and **11**.

Table 4. The K_m and V_{max} values for 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes

No.	Compound	$K_m \cdot 10^5$ M	$V_{max} \cdot 10^5$ mole l^{-1} min^{-1}
1	D-estradiol	0·48 ± 0·10	0·407 ± 0·023
2	D-estradiol 3-methyl ether	1·06 ± 0·13	0·972 ± 0·053
3	D-estradiol 3-acetate	1·66 ± 0·28	0·282 ± 0·021
13	D-estradiol 3-tosilate	1·63 ± 0·28	0·355 ± 0·032
14	D-3-desoxyestradiol	0·53 ± 0·05	0·362 ± 0·010
4	D,L-8-isoestradiol*	1·25 ± 0·22	0·893 ± 0·102
5	D,L-8-isoestradiol 3-methyl ether	0·10 ± 0·04	0·461 ± 0·035
7	D,L-D-homoestradiol	0·10 ± 0·04	0·181 ± 0·018
8	D,L-D-homoestradiol 3-methyl ether	0·27 ± 0·04	0·219 ± 0·010
10	D,L-8-iso-D-homo-estradiol	0·26 ± 0·05	0·196 ± 0·014
11	D,L-8-iso-D-homo-estradiol methyl ether	0·10 ± 0·01	0·153 ± 0·004

*Account is taken only of the D-enantiomar concentration.

For the substrates of the natural configuration of the normal and D-homo series, 1–3, 13–14 and 7–8, the rate of degradation of the enzyme-substrate complex (V_{max}) depends upon the electron nature of the substituent at C_3 and decreases in the following order as the electron donating groups are replaced with electron acceptors:

$$V_{max}(OMe) > V_{max}(OH) > V_{max}(H) > V_{max}(OTs) > V_{max}(OAc).$$

For the compounds of the natural configuration, 1–3, 7, 8, a satisfactory linear correlation ($r = 0{\cdot}945$) was observed between the values of V_{max} and the rate constant k_2 for the chemical oxidation (Fig 4).

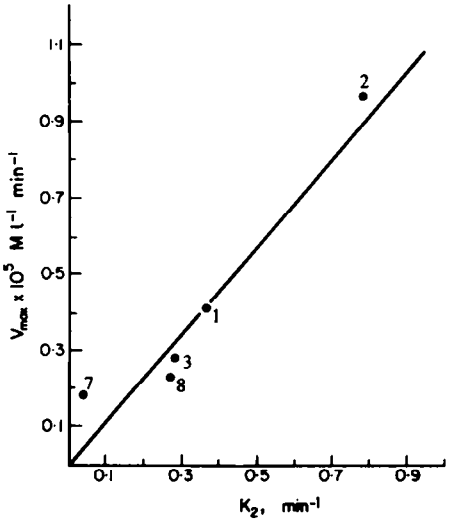


Fig 4. k_2 *vs* V_{max} for 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes of the natural configuration.

This indicates that it is the specific reactivity of the substrate (k_2) determined by the size of ring D and the long range effect of the substituent at C_3 on the reaction centre $C_{17}(C_{17a})$, that makes the major contribution to the degradation rate (V_{max}) of the enzyme-substrate complex.

It is known that K_m and V_{max} do not always change symbasically.[41, 42, 43]

However, as follows from our data, the electronic effect of the substituent at C_3 upon the rate of degradation of the enzyme-substrate complex (V_{max}) is parallel to a decrease in the substrate's affinity for the enzyme, which is expressed as in an increase in K_m. Thus in the series of compounds with the natural configuration, 1–3, 13–14, 7–8, replacement of the C_3 hydroxyl with other substituents results in a decrease in the substrate's affinity for the enzyme (growth of K_m) with a simultaneous increase or decrease in V_{max} depending on the electronic behaviour of the substituent at C_3.

Any changes in the molecular geometry of the substrate should affect drastically the affinity of the substrate for the enzyme and, consequently, govern the behavior of the enzyme-substrate complex.

When estradiol (1) is replaced with 8-iso-estradiol (4), K_m increases with V_{max}. In the case of estradiol 3-methyl ether, (2), a transfer to 8-epimer (5) leads to a decrease in K_m with a simultaneous decrease in V_{max}. A similar parallelism may be observed in the changes in K_m and V_{max} for the D-homo analogues, 7–10, 8–11.

Due to the steric hindrances associated with the geometry of 8-iso substrate (Fig 1), the C_3 hydroxyl would presumably be bound to the enzyme surface to a lesser degree than the OMe group. In an alkaline medium it would be the stronger electron donor, which is expressed in higher V_{max} for 4 than for 5. On the contrary, for estradiol (1) and D-homo-estradiol (7), the affinity of the substrate for the enzyme is higher (lower K_m) than that of their 3-methyl ethers, 2 and 8, V_{max} being higher for the latter.

Thus, with compounds of the 8-iso-configuration the electronic behaviour of the substituent at C_3 has a decisive effect on the rate of degradation of the enzyme-substrate complex. This effect is determined by the affinity of the substrate for the enzyme and ultimately, by the geometry of the whole substrate molecule.

EXPERIMENTAL

Measurement of the rate of oxidation of 17β-*hydroxy*-$\Delta^{1,3,5(10)}$-*estratrienes by* CrO_3 *in* 90% AcOH

The rate of oxidation was determined in thermostated cuvettes (overall capacity-3 ml) at 23 ± 0·5° by measuring the change in optical density with time. All solutions were preliminary thermostated for 2–3 min at the given temperature. The sample contained 1·5 ml of $1{\cdot}10^{-3}$ M CrO_3 in 90% AcOH. Steroid concentrations were varied from $2{\cdot}10^{-2}$ to $2{\cdot}10^{-3}$ M. The measurements were taken against a standard solution (3 ml of steroid solution in 90% AcOH) and commenced 20–30 sec after mixing the reagents.

The initial rate (V opt. dens. units/min) was determined graphically from the slope of the optical density vs time plot. 2–3 measurements were performed for each steroid concentration (mean arithmetical error in determining V was within 0·4–10%).

Constants k_2 and K_m were estimated from the linear plot $1/V\text{-}1/[R_2CHOH]_o$. An intersept "a" in the ordinate is equal to $\frac{1}{2}k_2$, that in the abcissa—to $1/K_m$. The rate constants of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes are reproducible. The mean-square error in determining k_2 and K_m was 3·19 and 7·5% respectively.

The measurements of pK'a *of* 3-*hydroxy*-$\Delta^{1,3,5(10)}$-*estratrienes*

The UV spectra were taken on a Hitachi EPS-2. The final values of pH were obtained on a Beckmann pH-meter using a standard buffer at pH 9·250 (25°).

The phosphate buffer (0·01 M) was prepared by adding 0·01 M $Na_3PO_4 . 12\ H_2O$ to 0·01 M $Na\ H_2PO_4 . 2\ H_2O$.

"Comparative" spectra of the nonionized and almost completely ionized estrogen were obtained respectively in 0·1 N HCl and 0·1 N NaOH. A 0·05 ml sample of the

steroid solution in EtOH was added to the stirred buffer, acid or alkali. The UV spectra were taken after balancing the instrument with an appropriate solvent. Then accurate pH values of the solutions were measured in a Beckmann pH-meter. The apparent *pKa* values were estimated from the formula:

$$pK'a = \text{pH} + \log (\epsilon_{NaOH} - \epsilon_{buffer})/(\epsilon_{buffer} - \epsilon_{HCl})$$

where ϵ_{NaOH}, ϵ_{buffer}, ϵ_{HCl} are the molar extinction coefficients respectively in alkali, buffer and acid at the analytical wavelength (240 nm, 290 nm).

Measurement of the rate of enzymic oxidation of 17β-hydroxy-$\Delta^{1,3,5(10)}$-estratrienes

Soluble 17β-estradiol dehydrogenase was isolated from human placenta as described by Engel, using the first four purification steps.[40]

The measurement technique has been described.[27,28] The reaction sample contained 0·015–0·15 μ mole of substrate in 0·05 ml of 95% EtOH, 1 ml of 0·2 M glycine buffer, 0·1 ml of the enzyme preparation in a total volume of 2,8 ml, the final pH 9·53, the protein content 0·289 mg. The reaction was initiated by adding 1·5 μ mole of NAD^+ to 0·2 ml of twice distilled water and an increase in the optical density of the reaction solution measured at 340 nm against a control containing all components except substrate. Measurements were taken 20 sec. after the NAD^+ addition and usually continued for 1·5–2 min.

The initial reaction rates were estimated from the slope of the optical density *vs* time plot. The mean arithmetical error in determining the initial rate (from 2–3 runs for each substrate concentration) did not exceed 10%. The Michaelis constants (K_m) and the maximal reaction rates (V_{max}) were found by the method of Lineweaver-Burk using the least squares technique.

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