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INHIBITION OF β-HYDROXYSTEROID DEHYDROGENASE

II. KINETICS AND pH EFFECT*

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SUMMARY

1. An investigation was conducted on the kinetics of inhibition of β-hydroxysteroid dehydrogenase (3(or 17)-β-hydroxysteroid: NAD(P) oxidoreductase, EC 1.1.1.51) from *Pseudomonas testosteroni*. The natural estrogen, estradiol-17β ($K_i = 1 \cdot 10^{-5}$ M) was a non-competitive inhibitor as were 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one ($K_i = 3 \cdot 10^{-7}$ M) and 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one ($K_i = 7 \cdot 10^{-7}$ M). 4,4-Dimethyl-17β-hydroxyandrost-5-eno[3,2-c]pyrazole ($K_i = 5 \cdot 10^{-7}$ M) and 17β-hydroxy-4,4,17α-trimethylandrost-5-eno[2,3-d]isoxazole ($K_i = 4 \cdot 10^{-6}$ M) were competitive inhibitors. These four synthetic androstane or androstene derivatives were more tightly bound to the enzyme than was estradiol-17β.

2. The degree of inhibition by estradiol-17β and by diethylstilbestrol was found to vary with pH. Both estrogenic compounds were between 4 and 20 times more effective inhibitors when the pH was made 4 units more alkaline. Possible reasons for this behavior are a change in the degree of ionization of groups at or near the active site(s) of the enzyme and a contribution of phenolate ion by these estrogenic compounds.

INTRODUCTION

In the preceding paper[2], it was reported that certain substituents on C-2 of the androstane nucleus, as in 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one and 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one, inhibited β-hydroxysteroid dehydrogenase at low concentrations. Bridged ring structures at C-2 and C-3 such as steroidal 2,3-isoxazoles and 2,3-pyrazoles were also efficient enzyme inhibitors. A similar type of investigation was conducted by MARCUS AND TALALAY[3] with 1,3,5-estratriene derivatives. They found that the phenolic A ring with a 3-hydroxyl substituent resulted in effective inhibition.

This communication is concerned with further delineating the mode of inhibition and with determining the dissociation constants of inhibitor–enzyme complexes.

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Estradiol-17β was compared with four androstane or androstene derivatives found by us to be effective inhibitors of the β-enzyme at low concentrations. An observation that estradiol-17β and diethylstilbestrol inhibited to greater extent at pH 9.5 than at pH 5.5 led to a study of the possible reasons for this behavior.

MATERIALS

The enzyme, β-hydroxysteroid dehydrogenase (3(or 17)-β-hydroxysteroid:NAD(P) oxidoreductase, EC 1.1.1.51), was prepared from testosterone-induced cultures of *Pseudomonas testosteroni* by the method of MARCUS AND TALALAY[4] with slight modifications by us as reported before[2]. NAD and $NADH_2$ were obtained from Sigma Chemical Company. Testosterone, androst-4-ene-3,17-dione, estradiol-17β and diethylstilbestrol were products of the Vitamerican Oil Corporation, Mann Research Laboratories, Eli Lilly and Nutritional Biochemicals, respectively. The other steroids were obtained from Dr. R. O. CLINTON and his associates of this Institute[5-8].

METHODS

The enzyme system contained 0.1 μmole of $NADH_2$ or 0.5 μmole of NAD, 0.05 μmole of the substrate which was either androst-4-ene-3,17-dione or testosterone dissolved in absolute methanol, test steroid of varying amounts dissolved in absolute methanol, 100 μmoles of Sørensen's phosphate buffer (pH 5.5) or potassium phosphate or sodium pyrophosphate buffers of given pH values, and β-hydroxysteroid dehydrogenase in a total volume of 3.0 ml. The reaction mixtures were contained in 1-cm silica cuvettes. The reaction was initiated by the addition of enzyme and the initial reaction rate was measured at 340 mμ on the Beckman DU spectrophotometer for the first minute.

Kinetic measurements were made with androst-4-ene-3,17-dione as substrate and $NADH_2$ as cofactor in Sørensen's phosphate buffer (pH 5.5) with 50 μl (250 β units) of steroid dehydrogenase. For studying the effect of pH on estrogen inhibition of β-hydroxysteroid dehydrogenase, potassium phosphate or sodium pyrophosphate buffers within their appropriate buffering ranges were used.

RESULTS

Mode of inhibition and K_i values

Four of the compounds which were among the most effective inhibitors of steroid dehydrogenase, as given in the preceding paper[2], were further characterized according to their mode of inhibition by the method of LINEWEAVER AND BURK[9]. The data for 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one are presented graphically in Fig. 1, those for 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one in Fig. 2, those for 4,4-dimethyl-17β-hydroxyandrost-5-eno[3,2-c]pyrazole in Fig. 3, and those for 17β-hydroxy-4,4,17α-trimethylandrost-5-eno[2,3-d]isoxazole in Fig. 4. These compounds were compared with estradiol-17β (Fig. 5) which had been shown by MARCUS AND TALALAY[3] to be tightly bound to the enzyme surface. From the K_i values summarized in Table I, it may be seen that estradiol-17β, a non-competitive inhibitor, was less tightly bound to the enzyme than any of the other synthetic androstane or

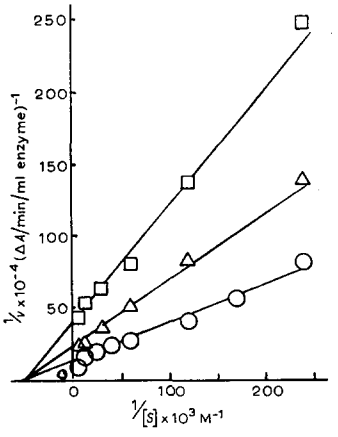

Fig. 1. Inhibition characteristics of 2-hydroxymethylene-17α-methylandrostan-17β-ol-3-one on β-hydroxysteroid dehydrogenase. The inhibitor concentrations were: none (○—○); 0.167 μM (△—△); and 0.833 μM (□—□).

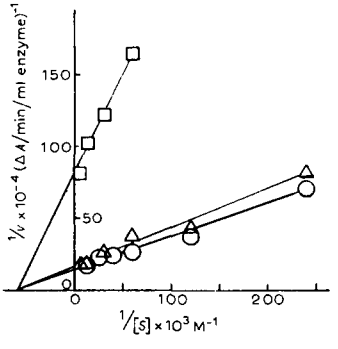

Fig. 2. Inhibition characteristics of 2α-cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one on β-hydroxysteroid dehydrogenase. The inhibitor concentrations were: none (○—○); 0.167 μM (△—△); and 1.67 μM (□—□).

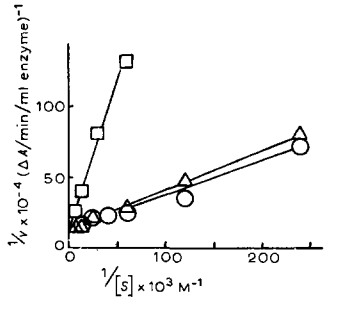

Fig. 3. Inhibition characteristics of 4,4-dimethyl-17β-hydroxyandrost-5-eno[3,2-c]pyrazole on β-hydroxysteroid dehydrogenase. The inhibitor concentrations were: none (○—○); 0.167 μM (△—△); and 1.67 μM (□—□).

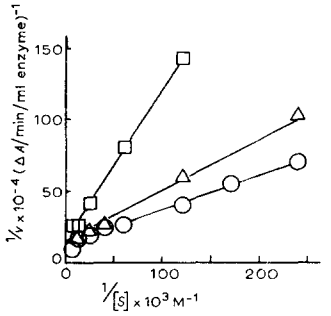

Fig. 4. Inhibition characteristics of 17β-hydroxy-4,4,17α-trimethylandrost-5-eno[2,3-d]isoxazole on β-hydroxysteroid dehydrogenase. The inhibitor concentrations were: none (○—○); 1.67 μM (△—△); and 16.7 μM (□—□).

androstene derivatives irrespective of whether they were competitive or non-competitive inhibitors.

2-Hydroxymethylene-3-keto steroids exist as hydrogen-bonded, 6-membered ring structures. Reaction of these compounds with hydroxylamine hydrochloride gives the corresponding 2,3-isoxazole[7]. This change in the ring system to the 2,3-conjugated isoxazole resulted in the conversion of an androstane derivative which was a non-competitive inhibitor to one which was competitive, but with less affinity for the enzyme surface. Condensation of the 2-hydroxymethylene-3-keto-steroid with hydrazine[5] to give the pyrazole derivative also yielded a competitive inhibitor, but this time affinity for the enzyme surface was approximately the same as for the parent compound.

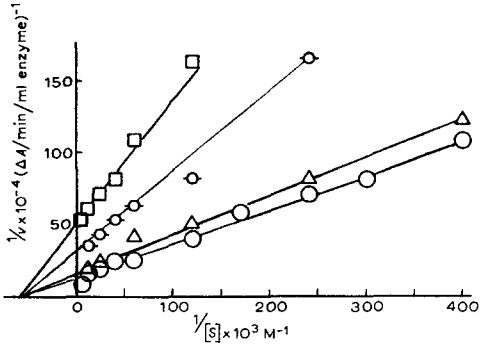

Fig. 5. Inhibition characteristics of estradiol-17β on β-hydroxysteroid dehydrogenase. The inhibitor concentrations were: none (○—○); 1.67 μM (△—△); 41.7 μM (⊖—⊖); and 16.7 μM (□—□).

The effect of pH on inhibition

The relative effectiveness of various steroids as inhibitors and the kinetic determinations reported above were compared using androstenedione as substrate in a medium buffered at pH 5.5. Estradiol-17β, tested as an inhibitor of steroid dehydrogenase with testosterone as substrate in a medium buffered at pH 9.5, which favors steroid oxidation, exhibited a 3-fold increase in effectiveness as an inhibitor

TABLE I

KINETICS OF INHIBITION OF β-HYDROXYSTEROID DEHYDROGENASE BY SYNTHETIC ANDROSTANE DERIVATIVES COMPARED WITH ESTRADIOL-17β

Steroid	I_{50} *(μM)*	*Type of inhibition*	K_i *(μM)*
Estradiol-17β	11	Non-competitive	10
2-Hydroxymethylene-17α-methylandrostane-17β-ol-3-one	0.9	Non-competitive	0.3
2α-Cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one	0.78	Non-competitive	0.7
4,4-Dimethyl-17β-hydroxyandrost-5-eno[3,2-c]pyrazole	0.83	Competitive	0.5
17β-Hydroxy-4,4,17α-trimethylandrost-5-eno[2,3-d]-isoxazole	8.5	Competitive	4

compared with its action at pH 5.5. Diethylstilbestrol, tested under the same conditions, showed a 10-fold increase in the concentration necessary to produce 50% inhibition at pH 5.5 with androstenedione compared with testosterone as substrate at pH 9.5. In each case, better inhibition was observed at the more alkaline pH. An explanation for this behavior was sought.

The participation of NAD carries with it uptake or release of a H^+ ion from solution. The reaction, therefore, is pH-dependent. Oxidation of testosterone is favored at alkaline pH values while androstenedione reduction is favored at more acid pH values; so, the reaction systems are buffered according to which direction the reaction is to proceed. The increased degree of inhibition of steroid dehydrogenase with estradiol-17β or with diethylstilbestrol noted above at pH 9.5 was in each case compared with the rate of reaction without either test agent at that pH. Therefore, this observed difference was in addition to any change in rate due to pH of the reaction medium alone.

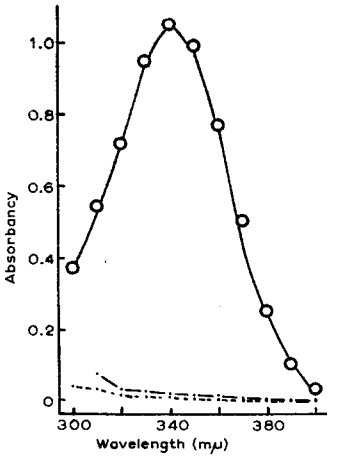

Fig. 6. Absorption spectrum of NAD and dihydroxyacetone (○—○) contrasted with that of NAD and diethylstilbestrol (·——·) and of NAD alone (·— —·).

From work in KAPLAN's laboratory[10], it is known that NAD can combine with carbonyl reagents at an alkaline pH. Also, MONDER[11] reported that cortisone and other allied steroids complex with NAD at high pH. Estradiol was found unreactive here. Fig. 6 illustrates the results obtained by us with diethylstilbestrol and NAD. Diethylstilbestrol did not complex with NAD in pyrophosphate buffer (pH 9.8) while dihydroxyacetone did. Therefore, complex formation with NAD at pH 9.5 using testosterone as substrate can be eliminated as an explanation for a greater degree of inhibition at alkaline pH values.

The dependence of the degree of inhibition upon pH was investigated further using androstenedione and $NADH_2$ as the respective substrate and cofactor. Diethylstilbestrol and estradiol-17β were compared as inhibitors of the reaction as illustrated in Fig. 7. Since the reaction rate itself is pH-dependent, the rate of androstenedione

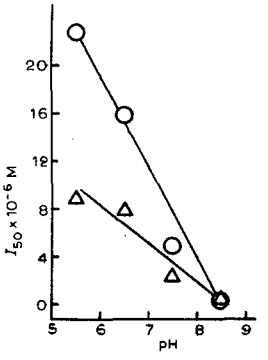

Fig. 7. Estrogen effect on androstenedione reduction as a function of pH: diethylstilbestrol (○—○), and estradiol-17β (△—△).

reduction decreased with increasing pH. Independent of this rate change, there was an increased degree of inhibition by estrogen at progressively more alkaline pH values compared with the reaction rate determined without the estrogenic compound in each case. In addition, with androstenedione as substrate, diethylstilbestrol inhibition varied more with pH than did estradiol inhibition.

Using testosterone as substrate and NAD as cofactor, as shown in Fig. 8, the

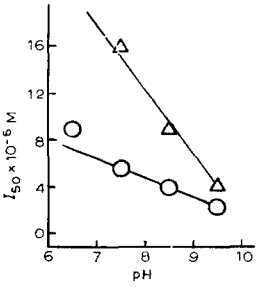

Fig. 8. Estrogen effect on testosterone oxidation as a function of pH: diethylstilbestrol (○—○), and estradiol-17β (△—△).

degree of inhibition with the two estrogens also varied with pH and this inhibition was more pronounced as the pH became progressively more alkaline. In this case, estradiol inhibition was more affected by an alteration in pH than was diethylstilbestrol inhibition. This situation was the reverse of that observed with androstenedione as substrate.

DISCUSSION

In the case of the phenolic, estrogenic compounds dealt with here, phenolate ion may be one of the factors responsible for tight attachment to the enzyme because of the pH dependency of inhibition potential. The pK values of estradiol-17β and diethylstilbestrol were found to be 11.8 and 12.2, respectively, in 50% ethanol. (The pK values of 11.8 and 12.2 may be high compared with that of 9.9 for phenol. The reason for this may lie in the solvents used for the two sets of determinations. Since estradiol-17β and diethylstilbestrol are insoluble in water, they were dissolved in 50% ethanol for the pK determinations. Phenol is more water-soluble. Changing the solvent from water to 50% ethanol may alter the degree of ionization by 1 or 2 pK units.) As the pH of medium increases, so will the contribution by phenolate ion. A major factor in any discussion of pH and reaction rates is, of course, the influence of pH on the ionizable groups of the enzyme. The relative importance of a series of pH-dependent factors is contingent on the dissociation constants of the various enzyme, cofactor, substrate and inhibitor complexes as a function of pH.

Another similarity between natural and synthetic estrogens was stated by GRUNDY[12], who reviewed data obtained by X-ray crystallography. The dimensions of estrone and diethylstilbestrol are quite similar. The lengths of the two molecules were found to be 8.55 Å and the widths 3.88 Å. Also, the thicknesses of the two molecules are quite similar. The diethylstilbestrol molecule is not planar. The benzene rings are out of the plane formed by the aliphatic chains. Therefore, it has thickness and that thickness is approximately the same as that produced by the conformational puckering of the steroid nucleus.

MARCUS AND TALALAY[3] have shown that stilbenes and estratrienes are firmly bound to the enzyme. Phenol is not an inhibitor, but this may be due to the necessity for multiple attachment to the enzyme, since oxidation–reduction occurs at positions 3 and 17, and to a lesser degree at position 16 of the steroid nucleus. The influence of pH on binding of these estrogens represents an additional factor in this complex system.

ACKNOWLEDGEMENTS

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