Active-site directed inactivation of rat ovarian 20 α -hydroxysteroid dehydrogenase

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Rat ovarian 20 α -hydroxysteroid dehydrogenase plays a pivotal role in leuteolysis and parturition by catalysing the reduction of progesterone to give the progestationally inactive steroid 20 α -hydroxyprogesterone. Putative mechanism based inhibitors of this enzyme were synthesized as potential progestational maintaining agents, including the epimeric allylic alcohol pair 3 β -hydroxy- α -vinyl-5 α -androstane-17 β -methanol and the related vinyl ketone 1-(3 β -hydroxy-5 α -androstane-17 β -yl)-2-propen-1-one. The vinyl ketone inactivates rat ovarian 20 α -hydroxysteroid dehydrogenase, semi-purified by poly(L-lysine)-agarose column chromatography, in a rapid time-dependent manner. Analysis of the pseudo-first-order inactivation plots gave a K_i of 2.0 μ M for the inhibitor and a $t_{1/2}$ for the enzyme of 20 s at saturation. These data indicate that the vinyl ketone is a potent and efficient inactivator of the ovarian dehydrogenase. Neither dialysis in the presence or absence of a competing nucleophile nor gel filtration reverses the inactivation, suggesting that a stable covalent bond is formed between the enzyme and steroid ligand. Both substrates (20 α -hydroxyprogesterone and NADP⁺) protect the enzyme from inactivation; moreover, initial velocity measurements in the presence of saturating concentrations of both substrates indicate that the vinyl ketone can behave as a competitive inhibitor, yielding a K_i value identical with that obtained in the inactivation experiments. Our results imply that the vinyl ketone is an active-site directed alkylating agent. By contrast the allylic alcohol pair 3 β -hydroxy- α -vinyl-5 α -androstane-17 β -methanol are neither substrates nor inhibitors of the ovarian enzyme and appear to be excluded from the catalytic site. The rapid inactivation observed with the vinyl ketone suggests that this compound may be useful as a progestational maintaining agent.

INTRODUCTION

20 α -Hydroxysteroid dehydrogenase plays a central role in progesterone metabolism, catalysing the NADP⁺ linked reduction of progesterone (Wiest, 1959; Wiest & Wilcox, 1961) to yield the progestationally inactive metabolite 20 α -hydroxyprogesterone (Talwalker *et al.*, 1966). The ovarian enzyme plays a major role in the termination of pregnancy in the rat (Wiest, 1968), where a 50-fold increase in activity is observed at parturition (Kuhn & Briley, 1970). Similarly a 1.5-fold post-partum increase has been noted in human placental 20 α -hydroxysteroid dehydrogenase (Diaz-Goya *et al.*, 1979). These increases in 20 α -hydroxysteroid dehydrogenase activity rather than decreased progesterone synthesis contribute to the lower plasma progesterone levels associated with the termination of pregnancy. The ovarian enzyme also catalyses the decline in progesterone levels which occurs during leuteolysis (Lamprecht *et al.*, 1969). Because of its apparent role as a regulator of progesterone levels in the cycling and pregnant female, 20 α -hydroxysteroid dehydrogenase represents a target enzyme for drug action. Potent selective inhibitors of the dehydrogenase have the potential to maintain progesterone levels and perhaps prevent miscarriage.

In considering candidates that might promote irreversible inhibition of the ovarian 20 α -hydroxysteroid dehydrogenase we were attracted to the previous studies

of MacInnes on alcohol dehydrogenase (MacInnes *et al.*, 1981). These studies showed that alcohol dehydrogenase catalyses the oxidation of allylic alcohols to yield the corresponding Michael acceptors (α,β -unsaturated aldehydes), which, when generated within the proximity of the active site, can undergo alkylation to produce inactivated enzyme. Employing a similar strategy Covey (1979) synthesized an epimeric pair of 17 β -propargylic alcohols: 17 β -[(1*S*)-1-hydroxy-2-propynyl]androst-4-en-3-one and 17 β -[(1*R*)-1-hydroxy-2-propynyl]androst-4-en-3-one as potential suicide substrates of 20 α -hydroxysteroid dehydrogenase. It was subsequently found that the 1*S* stereoisomer acts as a suicide substrate of 20 β -hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (Strickler *et al.*, 1980), whereas the 1*R* stereoisomer acts as a suicide substrate of the 17 β /20 α -hydroxysteroid dehydrogenase of human term placenta (Tobias *et al.*, 1982). The ability of these compounds to inactivate ovarian 20 α -hydroxysteroid dehydrogenase has never been reported. One drawback to these studies is that the propargylic alcohols contain conjugated ketones in their A-rings and may display affinity for the progesterone receptor. By contrast steroids in the 5 α - and 5 β -pregnane series have no progestational activity (Ogle & Beyer, 1982) and have been reported to display a high affinity for rat ovarian 20 α -hydroxysteroid dehydrogenase (Wilcox & Wiest, 1966). We have exploited these earlier findings and

Abbreviations used: 20 α -hydroxyprogesterone, 20 α -hydroxypregn-4-en-3-one; vinyl ketone, 1-(3 β -hydroxy-5 α -androstane-17 β -yl)-2-propen-1-one; allylic alcohol, 3 β -hydroxy- α -vinyl-5 α -androstane-17 β -methanol.

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Scheme 1. Synthesis of the racemic allylic alcohol pair (II) and the vinyl ketone (III)

describe here the synthesis and evaluation of three 5α -steroids which contain either a vinyl ketone or an allylic alcohol in place of the side chain at C-17. One such compound, 1-(3β -hydroxy- 5α -androstane-17 β -yl)-2-propen-1-one (Scheme 1: III), is an exceedingly potent and efficient inhibitor of the rat ovarian 20α -hydroxysteroid dehydrogenase; its potential as a progestational maintaining agent is discussed.

MATERIALS AND METHODS

3β -Hydroxy- 5α -pregnan-20-one (allopregnanolone) was purchased from Steraloids Inc. (Wilton, NH, U.S.A.). NADP⁺ and 20α -hydroxyprogesterone were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Poly(L-lysine)-agarose was purchased from P and L Biochemicals (Milwaukee, WI, U.S.A.) and Sephadex G-25F was obtained from Pharmacia (Piscataway, NJ, U.S.A.). All solvents were purchased from Burdick and Jackson (Muskegon, MI, U.S.A.), except toluene, which was obtained from Fisher Scientific. Other reagents were products of Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

All n.m.r. were recorded on a Bruker WM-360 MHz spectrometer, in C^2HCl_3 with a deuterium field lock relative to tetramethylsilane. Infrared spectra were recorded on a Perkin-Elmer 521 spectrometer with an extended range interchange. Absorption bands are listed as strong (s), medium (m), weak (w) and broad (b). Melting points were taken on a Fisher-Johns melting-point apparatus and were not corrected. Reactions were followed by t.l.c. carried out on Analtech 02521 plates (Analtech Inc., Newark, DE, U.S.A.). Semi-preparative t.l.c. was performed on 20 cm $\times$ 20 cm E. Merck 5763 plates (Darmstadt, E. Germany) in the solvents indicated.

Steroid synthesis

Preparation of the intermediate aldehyde 3β -hydroxy-17 β -formyl-androstane (I) (Scheme 1) was achieved by using a modification of the method described by Gelbart & Thomas (1978). In order to increase the yield of the crucial acetoxylation product ($3\beta,21$ -diacetoxy- 5α -pregnane-20-one) to 80%, the ratio of $\text{Pb}(\text{OAc})_4$ to

steroid was increased by a factor of 3. N.m.r. indicated that aldehyde I had been formed via this route, since an aldehyde proton with a chemical shift of 9.66 p.p.m. was observed. Other chemical shifts agree with those reported by Gelbart & Thomas (1978).

Synthesis of 3β -hydroxy- α -vinyl- 5α -androstane-17 β -methanol (II) and 1-(3β -hydroxy- 5α -androstane-17 β -yl)-2-propen-1-one (III)

Aldehyde I (200 mg, 6.6 mmol) and 15 ml of dry dioxane (CaH_2 distilled) were placed in an oven-dried, round-bottomed flask fitted with a septum and the system was purged with dry N_2 . Vinyl magnesium bromide (4 ml of 1.0 M solution in tetrahydrofuran) was introduced via a syringe. Once hydrogen evolution had ceased (3 min) the reaction was placed on ice and quenched with 2 ml of water and 0.1 ml of 2.0 M-sulphuric acid. After the bulk of solvent had been removed under reduced pressure, the reaction mixture was poured into water and extracted with chloroform. Extracts were washed with water and concentrated to dryness. The total products were dissolved in methanol, applied to semi-preparative t.l.c. plates and developed in toluene/acetonitrile (10:3, v/v). Product II, which consisted of both epimeric alcohols, was eluted with methanol. Ir.: (cm^{-1}) —OH, 3610 m, 3400 s and b, 1028 s; —CH=CH₂, 991 s, 953 m, 926 m and b; steroid nucleus, 2930 s, 2861 s, 1448 s, 1380 s. N.m.r.: δ (p.p.m.) ($[\text{}^2\text{H}]\text{chloroform}$) 0.66 and 0.77 (2 s, C-18 —CH₃, peak height ratios 4:5), 0.80 and 0.82 (2 s, C-19 —CH₃, peak

Fig. 1. Purification of rat ovarian 20α -hydroxysteroid dehydrogenase by poly(L-lysine)-agarose chromatography

Rat ovarian cytosol (4 ml obtained from 10 ovaries) was applied to a 2 ml column of poly(L-lysine)-agarose equilibrated in 10 mM-potassium phosphate containing 12 mM-monothioglycerol and 1 mM-EDTA, pH 6.0. The column was washed in equilibration buffer followed by 100 mM-NaCl dissolved in the equilibration buffer as indicated. The absorbance at 280 nm (—) and the enzyme activity (O—O) were monitored, and the fractions indicated were pooled.

Fig. 2. Time-dependent inactivation of rat ovarian 20 α -hydroxysteroid dehydrogenase mediated by vinyl ketone (III)

Portions (192 μl ; 0.52 mg of protein/ml) of semi-purified thiol-free rat ovarian 20 α -hydroxysteroid dehydrogenase were incubated in a final volume of 200 μl containing the concentration of vinyl ketone indicated in 4% acetonitrile. Aliquots (10–20 μl) were removed at various times and diluted directly into the standard 1.0 ml assay for measurement of enzyme activity (a). The times required to promote 50% loss of enzyme activity ($t_{1/2}$) were determined from estimates of the initial velocities of inactivation; these values were then plotted against the concentration of vinyl ketone (b). The line shown was drawn by regression; $r = 0.94$.

height ratio 4:5), 3.6 (m, CHOH , C-3); chemical shifts for the side chains are: 5.84 (1H, m, $-\text{CH}=\text{C}$), 5.06 (1H, q, $\text{C}=\text{CH}_2$), 5.02 (1H, q, $\text{C}=\text{CH}_2$), 3.98 (1H, m, $-\text{CH}-\text{CH}=\text{C}$).

Oxidation of the allylic alcohols (II) (100 mg) to the vinyl ketone (III) was performed in acetone with excess Jones reagent ($\text{CrO}_3/\text{H}_2\text{SO}_4/\text{water}$). The reaction was quenched with methanol followed by dilution with water. The product was extracted in chloroform, concentrated to dryness and applied to semi-preparative t.l.c. plates in a methanol solution. Plates were developed in toluene/acetonitrile (10:2, v/v). The vinyl ketone product (III) was visualized under u.v. light, eluted with methanol and crystallized from hexane/acetone. I.r.: (cm^{-1}) $\text{C}=\text{O}$, 1690 s and b (shoulder at 1658), 1102 s; $\text{C}=\text{CH}_2$, 1603 s, 983 s, 965 m; steroid nucleus, 2925 s, 2852 s, 1438 s, 1369 m. U.v. maximum 216 nm in methanol, ϵ_{216} 7000. N.m.r.: δ (p.p.m.) ($[\text{H}^2]\text{chloroform}$) 0.61 (3H, s, C-18 $-\text{CH}_3$), 1.03 (3H, s, C-19 $-\text{CH}_3$), 6.44 (1H, q, $J_{2,3}$ *cis* 10.8 Hz, *trans* 16.6 Hz, $-\text{CO}-\text{CH}=\text{C}$), 6.22 (1H, d, $J_{2,3}$ *trans* 16.6 Hz, $\text{CH}=\text{CH}_2$), 6.44 (1H, d, $J_{2,3}$ *cis* 10.8 Hz, $\text{CH}=\text{CH}_2$). M.p. 165–167 $^\circ\text{C}$.

Enzyme studies

Preparation of 20 α -hydroxysteroid dehydrogenase. In a typical preparation five female Sprague-Dawley rats (150–200 g; Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) were killed by cervical dislocation at random phases of the oestrus cycle. Their ovaries were excised, washed in ice-cold 0.25 M-sucrose and homogenized in 4.0 ml of 10 mM-potassium phosphate containing 12 mM-monothioglycerol and 1 mM-EDTA, pH 6.0. Cytosol was then prepared by differential centrifugation and chromatographed on a 2 ml column of poly(L-lysine)-agarose equilibrated in homogenization buffer. After the column was washed free of non-absorbed protein the dehydrogenase was eluted with homogenization buffer containing 100 mM-NaCl. The active fractions were pooled and dialysed against three changes (1:100,

v/v) of 10 mM-Tris/HCl containing 12 mM-monothioglycerol, 1 mM-EDTA and 20% (v/v) glycerol, pH 8.0. Aliquots (1.0 ml) containing 0.6 mg of protein/ml were frozen in liquid N_2 and stored at -80°C for future use. Several separate preparations of enzyme were used in this study and their specific activities ranged from 105 to 180 nmol of 20 α -hydroxyprogesterone oxidized/min per mg of protein. For details of the enzyme assay see below.

Assay of 20 α -hydroxysteroid dehydrogenase activity. Enzyme activity was measured in 1.0 ml systems containing 0.1 ml of 1.0 M-Tris/HCl, pH 8.0, 20 μl of 115 mM-NADP $^+$, 20 μl of 2.2 mM-20 α -hydroxyprogesterone, 20 μl of acetonitrile and water. The final concentration of organic solvent was 4% (v/v). Reactions were initiated by the addition of enzyme and followed by measuring the production of reduced pyridine nucleotide at 340 nm (ϵ_{340} 6270 $\text{M}^{-1}\cdot\text{cm}^{-1}$) on a Beckman DU-7 spectrophotometer at 25 $^\circ\text{C}$. Activities were corrected for the non-enzymatic reduction of nucleotide; this rate was typically no more than 2–5% of the enzyme-catalysed reaction.

Inactivation of 20 α -hydroxysteroid dehydrogenase. Samples of frozen enzyme (1.0 ml, 0.6 mg of protein/ml) were thawed and dialysed against three changes (1:1000, v/v) of 10 mM-Tris/HCl, pH 8.0, to remove the monothioglycerol. After dialysis the thiol-free enzyme retained 80% of its original activity. Portions of the dialysed enzyme (192 μl) were placed in a final volume of 200 μl containing various concentrations of steroid inactivator (0–33.3 μM) in 4% acetonitrile at 25 $^\circ\text{C}$. At time intervals thereafter portions (10–20 μl) were withdrawn from the incubation and diluted directly into the 1.0 ml assay system. The high concentration of 20 α -hydroxyprogesterone (44 μM) and NADP $^+$ (2.3 mM) in the assay and the extensive dilution (50–100-fold) effectively quench any further alkylation events. The determination of

Table 1. Dialysis of rat ovarian 20 α -hydroxysteroid dehydrogenase inactivated by the vinyl ketone (III)

Two identical (500 μ l, 0.77 mg of protein/ml) samples of thiol-free rat ovarian 20 α -hydroxysteroid dehydrogenase were treated with either 4% acetonitrile (Control) or 40 μ M-vinyl ketone in 4% acetonitrile (Experimental). Aliquots of enzyme were removed after 20 min and assayed for enzyme activity. Each sample was then divided into two portions and dialysed extensively against either 10 mM-Tris/HCl, pH 8.0, or 10 mM-Tris/HCl containing 0.25 mM-2-mercaptoethanol, pH 8.0. Aliquots were withdrawn from the dialysis after 5 and 14 h and assayed for enzyme activity. All measurements have s.d. of 9% or less.

Manipulation	Enzyme activity (%)	
	Control	Experimental
No addition	100	100
4% acetonitrile after 20 min	94	—
40 μ M-vinyl ketone after 20 min	—	7
Dialysis		
5 h	88	4
14 h	82	3
5 h (plus 2-mercaptoethanol)	97	6
14 h (plus 2-mercaptoethanol)	90	10

enzyme activity in these assays is thus a measure of enzyme inactivation. Semi-logarithmic plots of initial enzyme activity (per cent) versus time gave estimates of $t_{1/2}$ for inactivation. Secondary plots of $t_{1/2}$ versus $1/[I]$ gave a $t_{1/2}$ for the enzyme at saturating inhibitor concentration and a K_i for the inhibitor. Control incubations, which contained acetonitrile only, indicated that the enzyme retains full activity during the time course of enzyme inactivation.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), employing crystalline bovine serum albumin (Armour Pharmaceuticals, Kankakee, IL, U.S.A.) as a standard.

RESULTS

Purification and properties of 20 α -hydroxysteroid dehydrogenase

When rat ovarian cytosol is subjected to poly(L-lysine)-agarose chromatography the 20 α -hydroxysteroid dehydrogenase is separated from the majority of cytosolic protein (Fig. 1). The result is similar to that reported by Mori & Wiest (1979). The pooled active fractions routinely gave a specific activity of 120 nmol of 20 α -hydroxyprogesterone oxidized/min per mg, which represents a 5–7-fold protein purification. Specific activities reported for the purified enzyme range from 1990 to 8317 nmol of 20 α -hydroxyprogesterone oxidized/min per mg (Pongsawadi & Anderson, 1984; Mori & Wiest, 1979). Based on these measurements our preparation is 1–5% pure. Determination of kinetic constants for our preparation gave a K_m of 2.0 μ M for 20 α -hydroxyprogesterone, which is in agreement with the value reported

Fig. 3. Gel exclusion chromatography of rat ovarian 20 α -hydroxysteroid dehydrogenase inactivated by the vinyl ketone (III)

Portions (0.55 ml, 0.45 mg of protein/ml) of semi-purified thiol-free rat ovarian 20 α -hydroxysteroid dehydrogenase were incubated in the presence of 40 μ M-vinyl ketone dissolved in 4% acetonitrile or 4% acetonitrile alone for 20 min at 25 °C. At this time less than 10% of the initial enzyme activity remained in the sample containing vinyl ketone. The incubation mixtures were then applied to a column of Sephadex G-25F (0.8 cm $\times$ 46 cm) equilibrated in 10 mM-Tris-HCl containing 12 mM-monothioglycerol and 1 mM-EDTA, pH 8.0. The absorbance at 280 nm was monitored (—) and enzyme activity in each fraction determined (●—●). The elution profiles observed with enzyme incubated in the absence of vinyl ketone (a) and the presence of vinyl ketone (b) are shown. In both cases the enzyme elutes in the void volume, which corresponds to fraction no. 7.

by Pongswadi & Anderson (1984), and a V_{max} value identical with our specific activity measurement.

Inactivation of 20 α -hydroxysteroid dehydrogenase

Upon incubation of the semi-purified 20 α -hydroxysteroid dehydrogenase with various concentrations of the vinyl ketone (III), rapid time-dependent loss of enzyme activity is observed (Fig. 2a). This inactivation is concentration-dependent and demonstrates saturation kinetics. To obtain reliable kinetic constants for the inactivation event, a large number of time points were taken in the first minutes of each time course (complete data not shown). When these results are transformed by plotting the $t_{1/2}$ (s) against the reciprocal of the inhibitor concentration the resulting plot indicates that the vinyl ketone is a potent inhibitor ($K_i = 2.0 \mu$ M) and a very rapid inactivator, yielding a $t_{1/2}$ for the enzyme of 20 s at saturation (Fig. 2b). This method of data analysis is essentially identical with that employed by Kitz & Wilson (1962).

Fig. 4. 20 α -Hydroxyprogesterone and NADP⁺ protect rat ovarian 20 α -hydroxysteroid dehydrogenase from inactivation mediated by the vinyl ketone (III)

Portions (20 μ l, 0.47 mg of protein/ml) of semi-purified thiol-free rat ovarian 20 α -hydroxysteroid dehydrogenase were incubated in the presence of 5.0 μ M-vinyl ketone and increasing amounts of 20 α -hydroxyprogesterone (0–25 μ M) as indicated. Enzyme activity in the absence of any steroid is also shown. The concentration of organic solvent was maintained at 4% (v/v) acetonitrile. Aliquots (20 μ l) were removed with time and diluted directly into the standard 1.0 ml assay for determination of enzyme activity (a). Portions (20 μ l, 0.44 mg of protein/ml) of semi-purified thiol-free rat ovarian 20 α -hydroxysteroid dehydrogenase were incubated in the presence of 8.6 μ M-vinyl ketone and increasing amounts of NADP⁺ (0–200 μ M) as indicated. Enzyme activity in the absence of any additions is also shown. Aliquots (20 μ l) were removed at the indicated time and diluted directly into the standard 1.0 ml assay for determination of enzyme activity (b).

Evidence that the vinyl ketone (III) forms a stable covalent bond with 20 α -hydroxysteroid dehydrogenase

The time-dependent loss of enzyme activity mediated by the vinyl ketone (III) shown in Fig. 2(a) was followed after substantial dilution of the reaction mixture (50–100-fold). To establish that this inactivation event resulted in covalent bond formation, preparations of the dehydrogenase inactivated by this compound were subjected to exhaustive dialysis (Table 1). These experiments indicate that enzyme activity does not return after this treatment. Moreover, dialysis in the presence of a powerful competing nucleophile (2-mercaptoethanol) was unable to displace the steroid from the protein and restore enzyme activity. During these manipulations control enzyme retained at least 80% of its initial activity.

Additional evidence for covalent bond formation was obtained by subjecting samples of control enzyme and enzyme inactivated with vinyl ketone (III) to gel-exclusion chromatography (Fig. 3). In both instances the enzyme elutes in the void volume but activity is not restored to the inactivated preparation.

The vinyl ketone (III) is an active-site directed alkylating agent

Both the substrates, 20 α -hydroxyprogesterone and pyridine nucleotide (NADP⁺), provide protection from inactivation mediated by the vinyl ketone (III) (Fig. 4a

and 4b). Secondary plots of $1/k_{app}$ versus the concentration of protecting ligand provide estimates of the dissociation constants of 20 α -hydroxyprogesterone ($K_d = 1.6 \mu$ M) and NADP⁺ ($K_d = 1.6 \mu$ M). Although we have not independently obtained a K_d for the steroidal substrate, the K_d for NADP⁺ is in close agreement with that obtained by Pongsawadsi & Anderson (1984). To substantiate the idea that the vinyl ketone competes for the active site of the enzyme we have also shown that the competitive inhibitor of the dehydrogenase, 17 β -oestradiol, will protect the enzyme from inactivation. In these protection studies a K_d of 5.0 μ M was obtained for 17 β -oestradiol, which is identical with that obtained for this compound in our competitive inhibition studies (data not shown). Furthermore, under initial velocity conditions in which the concentrations of 20 α -hydroxyprogesterone (44 μ M) and pyridine nucleotide (2.3 mM) are able to provide complete protection against inactivation, the vinyl ketone is able to act as a competitive inhibitor of the dehydrogenase (Fig. 5). Under such conditions the vinyl ketone is presumably binding to the enzyme in a rapid reversible fashion, but it does not occupy the active site long enough to initiate an alkylation event. In these competitive inhibition studies the K_i value determined for the vinyl ketone is 2.0 μ M and is identical with the K_i determined in the inactivation experiments. Together these data provide a strong case for the view that the vinyl ketone is an active-site directed agent.

Fig. 5. Competitive inhibition of the initial rate of 20α -hydroxyprogesterone oxidation by the vinyl ketone (III)

Initial velocities of NADP^+ reduction at fixed concentrations of 20α -hydroxyprogesterone (5 – $40\ \mu\text{M}$) were determined in the presence of various concentrations of vinyl ketone (0 – $40\ \mu\text{M}$); in each instance the reaction was initiated with $11\ \mu\text{g}$ of enzyme. The data were analysed by a primary Dixon plot (Dixon, 1953). The inset shows the secondary Dixon plot (Segal, 1975) from which the K_i was determined. Abscissa values of the secondary plot are the slopes determined for the lines of the primary plot represented as $(\text{nmol of NADP}^+/\text{min per mg of protein})^{-1}/\mu\text{M-vinyl ketone}$; ordinate values refer to the concentration of 20α -hydroxyprogesterone (μM^{-1}).

Evaluation of the C-20 epimeric alcohols 1-(3β -hydroxy- 5α -androst- 17β -yl)prop-2-en-1-one (II) as inhibitors of 20α -hydroxysteroid dehydrogenase

The C-20 epimeric alcohols (II) are synthetic intermediates on the pathway to the vinyl ketone (III). It was felt that the C-20 α -allylic alcohol could be oxidized by the dehydrogenase to the corresponding vinyl ketone, which

could then act as a Michael acceptor and inactivate the enzyme. In this manner the allylic alcohols would act as suicide substrates in a fashion similar to that described by MacInnes *et al.* (1981) for alcohol dehydrogenase. Evaluation of the racemic mixture of C-20 epimeric alcohols indicated that they cannot be utilized as substrates even when highly favourable conditions for oxidation were employed (i.e. pH 10.0 and 0.4 mg of enzyme protein to initiate the reaction). Moreover, the racemic mixture was unable to inhibit the enzyme-catalysed oxidation of 20α -hydroxyprogesterone even at the limit of steroid solubility (approximately $50\ \mu\text{M}$), suggesting that these compounds are excluded from the active site at these concentrations. Similarly the vinyl ketone (III) was examined as a substrate by optimizing conditions for reduction (i.e. pH 6.0 and 0.4 mg of protein to initiate the reaction): under these conditions no consumption of NADPH could be measured.

DISCUSSION

Decreases in plasma progesterone levels generally precede the termination of pregnancy. This is true whether progesterone at term is produced by the corpus luteum as in the rat (Caspo & Wiest, 1969) or in the placenta as in sheep (Bassett *et al.*, 1969) and humans (Caspo, 1977). Evidence exists that 20α -hydroxysteroid dehydrogenase is a major enzyme responsible for the decline in progesterone levels since it catalyses the reduction of this hormone to give the inactive 20α -hydroxyprogesterone. A rationale thus exists for the development of selective inhibitors of the 20α -hydroxysteroid dehydrogenase as potential progestational agents. This paper indicates that the vinyl ketone (III) is a potent ($K_i = 2.0\ \mu\text{M}$) and exceedingly efficient inactivator of the dehydrogenase, yielding a $t_{1/2}$ for the enzyme of 20 s at saturation. Moreover, the compound is active-site directed and forms a stable covalent bond with the enzyme. The mechanism of inactivation is believed to occur via nucleophilic attack of the vinyl ketone (Scheme 2). This represents another example of how the introduction of Michael acceptors placed on the steroid nucleus can yield highly reactive alkylating agents (Penning *et al.*, 1981). Further it can be speculated that the reason for the rapid inactivation may be polarization of the C-20 carbonyl, which may be assisted by a positively charged amino acid at the active site. Such a mechanism would facilitate hydride transfer in the normal catalytic cycle of the enzyme.

It will be recalled that Covey (1979) has synthesized a progesterone analogue (17β -[(1*R*)-1-hydroxy-2-propynyl]androst-4-en-3-one) containing a propargylic al-

Scheme 2. Proposed mechanism of inactivation of the ovarian 20α -hydroxysteroid dehydrogenase by the vinyl ketone

cohol in its side chain; this compound appears to act as a suicide substrate for the human placental 17 β -/20 α -hydroxysteroid dehydrogenase (Tobias *et al.*, 1982). Thus the acetylenic alcohol is oxidized by the enzyme to yield the corresponding acetylenic ketone, which can then promote a rapid time-dependent inactivation of the enzyme ($t_{1/2}$ = 1 min at 50 μ M inhibitor). A more thorough examination of this earlier work, however, reveals that the acetylenic alcohols are oxidized by the enzyme under non-physiological conditions (0.5 mM-steroid), suggesting that these compounds may act as suicide substrates only under forced conditions. It should be emphasized that the ability of the acetylenic alcohols and ketones to inactivate the ovarian 20 α -hydroxysteroid dehydrogenase has not been assessed; moreover, the affinity of these progesterone analogues (agonist or antagonist) for the human progesterone receptor has not been reported.

The vinyl ketone (III) described here represents an exciting development since, unlike the acetylenic alcohol or ketone synthesized by Covey (1979), it is not a progesterone analogue since it contains a saturated A-ring of the *trans* configuration. Moreover, it inactivates the dehydrogenase with an exceedingly short half-life and it displays a low K_i , suggesting that this compound may be a superior alkylating agent. The ability of the vinyl ketone to inhibit the ovarian 20 α -hydroxysteroid dehydrogenase *in vivo* and act as an anti-leuteolytic agent and maintain pregnancy in the rat remains to be determined. The success of such studies will depend upon the selectivity of the vinyl ketone for the dehydrogenase and how rapidly this Michael acceptor will be scavenged by endogenous nucleophiles.

The ability of the vinyl ketone (III) to inactivate the human placental 20 α -hydroxysteroid dehydrogenase also remains to be determined. At the present time there is reason to believe that the 20 α -hydroxysteroid dehydrogenase activity of rat ovarian cytosol and human placenta may be catalysed by quite different proteins. In the placental enzyme both the 17 β - and 20 α -hydroxysteroid dehydrogenase activities co-exist on the same protein (Strickler *et al.*, 1981); there is no evidence to suggest that the same is true for the rat ovarian enzyme.

Unlike the propargylic alcohol and acetylenic ketone synthesized by Covey (1979) neither the allylic alcohols nor the vinyl ketone described here appear to be substrates for the rat ovarian 20 α -hydroxysteroid dehydrogenase. This may not be an unexpected finding since the K_m values reported for the propargylic alcohols were close to 0.5 mM. At this concentration the allylic

alcohol pair and vinyl ketone described here are insoluble in the assay system.

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