A Soluble 17β-Hydroxysteroid Dehydrogenase from Human Placenta. The Binding of Pyridine Nucleotides and Steroids*

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ABSTRACT: The molecular weight of the 17β-hydroxysteroid dehydrogenase of human placenta, estimated by gel filtration and reduced nicotinamide–adenine dinucleotide phosphate titration, is 62,000–65,000. Fluorescence measurements indicate that the Michaelis constants of nicotinamide–adenine dinucleotide phosphate and reduced nicotinamide–adenine dinucleotide phosphate are 0.89 and 0.83 μM, respectively, and that the dissociation constants of these pyridine nucleotides are 51 and 46 mμM, respectively. When the enzyme is cooled in the presence of 1% glycerol, it loses its activity rapidly and this loss is paralleled by a decrease in the ability of the enzyme to bind reduced nicotinamide–adenine dinucleotide phosphate. Because of the rather broad substrate specificity of this enzyme, various compounds were examined for their effect on 17β-estradiol oxidation. Structural analogs of 17β-estradiol (17-desoxyestradiol and 17α-estradiol), as well as certain other steroid hormones (androst-4-en-3,17-dione and progesterone) and nonsteroidal compounds (2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane and diethylstilbestrol), were found to be competitive inhibitors of 17β-estradiol oxidation, while corticosteroids did not inhibit it. Binding studies by ultracentrifugation indicate a single binding site on the enzyme for 17β-estradiol and estrone. When examined by gel filtration the binding of 17β-estradiol to the enzyme was found to be reduced by diethylstilbestrol as well as by a synthetic estrogen antagonist, 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl)pyrrolidine. Conditions which inactivate the enzyme (cold sodium dodecyl sulfate, *p*-hydroxymercuribenzoate, and guanidine hydrochloride) also reduce its estrogen binding capacity.

In an attempt to characterize estrogen "receptors" several groups (Toft *et al.*, 1967; Jensen *et al.*, 1968; Talwar *et al.*, 1968) have investigated the binding of 17β-estradiol to macromolecular substances present in the bovine and rat uterus. To date these studies have been limited by the small amounts of estrogen binding material which may be extracted from the uterus and the instability of this material.

The 17β-hydroxysteroid dehydrogenase of human placenta catalyzes reaction I. Like the uterine estrogen

$$17\beta\text{-estradiol} + NAD^+ (NADP^+) \rightleftharpoons \text{estrone} + NADH (NADPH) + H^+ \quad (I)$$

"receptors," this enzyme binds both steroidal and nonsteroidal estrogens (Langer *et al.*, 1959; Adams *et al.*, 1962). Since significant quantities of the 17β-hydroxysteroid dehydrogenase may be obtained in an apparently homogeneous state (Jarabak *et al.*, 1962; Jarabak, 1969), it seemed that certain problems related to estrogen–protein interactions might be approached advantageously by a study of the binding of steroids to this enzyme.

The 17β-hydroxysteroid dehydrogenase has a dual specificity for pyridine nucleotide cofactors. Additional studies of pyridine nucleotide binding to this enzyme were undertaken to further characterize the cofactor–enzyme interaction and to permit a comparison of cofactor and substrate binding.

Methods

Materials. Deionized glass-distilled water was used for all solutions. Crystalline bovine serum albumin was obtained from Armour, crystalline ovalbumin from Sigma, and three-times-crystallized ribonuclease and α-chymotrypsin from Worthington. Pyridine nucleotides were obtained from P-L Laboratories and used without further purification. Spectroscopic grade glycerol (Matheson Coleman and Bell) was used without further purification but other organic solvents were redistilled prior to their use. The concentration of all organic solvents is expressed as a percentage by volume. The steroids and other compounds tested for their inhibitory properties were purified when necessary so that physical constants corresponded to the best values given in the literature. 17β-Estradiol-6,7-^{3}H (42.4 Ci/mmole) and estrone-6,7-^{3}H (42.2 Ci/mmole) were obtained from New England Nuclear Corp., and had radiochemical

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purity of greater than 97%. When necessary, steroids formed in enzymatic reactions were identified by thin-layer chromatography.

The 17β-hydroxysteroid dehydrogenase used in these studies was prepared by the method of Jarabak (1969) and had a specific activity of 2.05 units/mg of protein when assayed by the rate of NAD reduction. Discontinuous polyacrylamide gel electrophoresis (Davis, 1964) of this preparation revealed a single band of protein at pH 7.0 and 8.3. No inhomogeneity was detected when portions of this preparation were subjected to gel filtration on Sephadex G-100 or chromatography on DEAE-cellulose. When the protein concentration of a solution containing the purified enzyme is determined by the method of Warburg and Christian (1941), the value obtained is only 0.48 that obtained when the protein concentration is determined by either the Lowry (Lowry *et al.*, 1951) or biuret (Gornall *et al.*, 1950) methods. This probably is a reflection of the low tryptophan content of the enzyme: analysis by the method of Goodwin and Morton (1946) indicates that two molecules of tryptophan are present in each molecule of enzyme. The protein concentrations given in this paper are values obtained by the Lowry method, unless otherwise indicated.

Fluorescence Measurements. An Aminco-Bowman spectrophotofluorometer equipped with a Varian F-80 X-Y recorder was used for fluorescence measurements. The cuvet temperature was maintained at 25 ± 0.5° by using a hollow cell holder through which water from a constant-temperature bath was circulated. No corrections have been applied to either the activation or emission spectra and none have been made for variations in the response of the photomultiplier tube during experiments of short duration. In longer experiments, quinidine standards were included to permit minor corrections for instability of the instrument.

Enzyme Assays. Enzyme activity was measured spectrophotometrically (with a Gilford recording spectrophotometer) or fluorometrically at 25 ± 0.5°. In all assays enzyme activity was estimated from the initial linear change in absorbance or fluorescence. Spectrophotometric assays for dehydrogenation of 17β-estradiol have been described previously (Jarabak *et al.*, 1962), but the definition of one unit of enzyme activity has been modified since that time. In the present study one unit of enzyme is defined as the amount of enzyme which reduces 1 μmole of pyridine nucleotide/min in a cuvet of 1.0-cm light path at 25 ± 0.5°. Because spectrophotometric assays were not sufficiently sensitive for some measurements, fluorometric assays, described below, were used also. Since arbitrary units were used for all fluorometric measurements, no units are given for reaction velocities determined fluorometrically.

Michaelis Constants of NADP and NADPH. The cuvets contained in a final volume of 1.0 ml, 50 μmoles of Tris-HCl at pH 7.4, 0.075 μmole of 17β-estradiol or estrone, added in 0.1 ml of 95% ethanol, and varying quantities of NADP or NADPH. Appropriate quantities of enzyme, added in 25 μl of medium A (20% glycerol, 5 mM potassium phosphate, and 1 mM EDTA at a final pH of 7.0), initiated the reactions. The reactions were followed fluorometrically at 460 mμ (340-mμ wavelength of excitation).

Michaelis Constants of Steroids. The cuvets in which *steroid oxidation* occurred contained, in a final volume of 1.0 ml, 50 μmoles of Tris-HCl at pH 7.4, 0.45 μmole of NAD, or 0.37 μmole of NADP, and varying quantities of steroid in 0.1 ml of 95% ethanol. Appropriate quantities of the enzyme, added in 25 μl of medium A, initiated the reactions. The reactions were followed fluorometrically at 460 mμ (340-mμ wavelength of excitation). The reaction cuvets in which *steroid reduction* occurred contained either 0.6 mμmole of NADH or NADPH when the reactions were followed fluorometrically at 460 mμ; either 0.09 μmole of NADH or 0.07 μmole of NADPH was present when the reactions were followed spectrophotometrically at 340 mμ. The Michaelis constants of both pyridine nucleotides and steroids were estimated graphically by the method of Lineweaver and Burk (1934).

$[I_{50}]$[1] for Inhibitors of 17β-Estradiol Oxidation. The reaction cuvets contained, in a final volume of 1.0 ml, 50 μmoles of sodium pyrophosphate at pH 10.2, 0.45 μmole of NAD, 0.75 mμmole of 17β-estradiol added in 0.05 ml of 95% ethanol, and varying amounts of inhibitor added in 0.05 ml of 95% ethanol. The reaction was initiated by the addition of 0.12 μg of enzyme in 0.02 ml of medium A and followed fluorometrically at 460 mμ.

K_i for Inhibitors of 17β-Estradiol Oxidation. The assay system used was identical with that already described for determining the $[I_{50}]$ of various inhibitors with the exception that varying quantities of 17β-estradiol were added in 0.05 ml of 95% ethanol. The inhibitor constants were determined graphically by the method of Dixon (1953).

K_i for Inhibitors of Estrone or Progesterone Reduction. The assay system used was identical with that already described for the determination of the Michaelis constants of estrone and progesterone with the exception that inhibitors were also present in varying concentrations. Both substrate and inhibitor were added in 0.05 ml of 95% ethanol.

Ultracentrifugal Determination of Estrone and 17β-Estradiol Binding. The method described by Hayes and Velick (1954) was modified as follows: each 10-ml polycarbonate centrifuge tube contained, in a total volume of 2.25 ml, 0.14 mg of enzyme, 5.2 mmoles of glycerol, 160 mmoles of potassium phosphate buffer at pH 7.0, 0.4 μmole of EDTA, 0.68–16.8 mμmoles of estrone (specific activity 310 cpm/mμmole) or 0.90–22.5 mμmoles of 17β-estradiol (specific activity 461 or 10,557 cpm/mμmole). A 0.2-ml aliquot was removed from each tube before centrifugation and analyzed for radioactivity and enzymatic activity. Centrifugation was for 21–24 hr at 40,000 rpm in a Spinco Model L ultracentrifuge equipped with a 40 rotor. When the centrifugation was completed, the rotor was allowed to come to a stop without braking and a 0.2-ml aliquot

[1] $[I_{50}]$ is the concentration of inhibitor required to produce a 50% reduction in the velocity of 17β-estradiol oxidation.

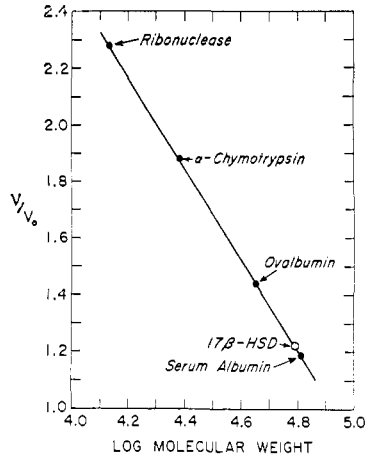

FIGURE 1: Molecular weight estimation by gel filtration. A 10 × 880 mm column of Bio-Gel P-100 was equilibrated with a solution of 0.2 M potassium phosphate and 1 mM EDTA at pH 7.0 and elution was with the same buffer. The column was operated at room temperature. Fractions were assayed for enzyme activity and for absorption at 280 mμ in order to establish the elution volume, V, of each protein. The void volume of the column, V_0, was determined with Blue Dextran 2000.

was taken from the upper surface of the solution and analyzed for radioactivity and enzymatic activity. If the enzyme activity of the sample removed after centrifugation was greater than 0.1% of that for the sample removed prior to centrifugation, it was assumed that some mixing had occurred inadvertently after the centrifugation and the results for that tube were discarded. Controls indicated that there was no significant decrease in the concentration of steroid due to adsorption on the walls of the centrifuge tubes or to sedimentation of the steroid. Glycerol was added to prevent the loss of enzyme activity which occurs at lower temperatures in its absence.

Gel Filtration. A 10 × 265 mm Bio-Gel P-60 column was thoroughly equilibrated at 23° with buffer which contained 0.2 M potassium phosphate, 1 mM EDTA, and 10 μM 17β-estradiol-6,7-^{3}H (specific activity 365 cpm/mμmole) at pH 7.0. Samples of enzyme, containing 0.6 mg of protein, were applied directly to the top of the column in 0.5–0.6 ml of buffer or were allowed to react with denaturing agents for varying periods and then applied to the column. The enzyme was eluted with the same buffer which had been used to equilibrate the column and the eluate was collected in 1.6-ml fractions. When U-11,100A[2] or diethylstilbestrol was added to the filtration buffer, the column was reequilibrated with buffer before a sample of enzyme was applied.

[2] Trivial names used are: metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone ditartrate; U-11,100A, 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl)pyrrolidine hydrochloride; amphenone B, 3,3-bis(*p*-aminophenyl)-2-butanone; chlomiphene, 2-[*p*-(2-chloro-1,2-diphenylvinyl)phenoxy]-triethylamine dihydrogen citrate; MER-25, 1-(*p*-2-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenyl ethanol; cyproterone acetate, 1,2α-methylene-6-chloro-Δ^{4,6}-pregnadien-17α-ol-3,20-dione-17α-acetate.

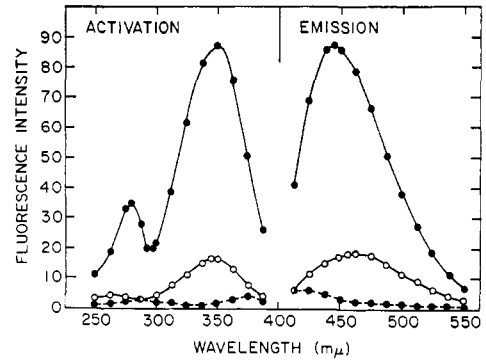

FIGURE 2: Activation and emission spectra of free NADPH and NADPH bound to the 17β-hydroxysteroid dehydrogenase. The fluorescence activation spectra of NADPH (○——○), the enzyme (●- - -●), and the nucleotide–enzyme complex (●——●) were measured at 460 mμ; while the emission spectra were measured at 340 mμ. The cuvet contained, in a final volume of 1.0 ml, 4.9 μmoles of potassium phosphate at pH 7.0, 47.2 μg of enzyme added in 0.04 ml of medium B (50% glycerol, 5 mM potassium phosphate, and 1 mM EDTA at a final pH of 7.0), and 86 mμmoles of NADPH.

Results

Estimation of Molecular Weight. When the molecular weight of the purified 17β-hydroxysteroid dehydrogenase was estimated by gel filtration (Andrews, 1964) on a calibrated polyacrylamide column (Bio-Gel P-100), a value of 62,000 was obtained (Figure 1). A similar gel filtration on a calibrated dextran column (Sephadex G-200) yielded a value of 65,000.

The Binding of Pyridine Nucleotides. As noted previously (Jarabak *et al.*, 1963), addition of NADPH to solutions containing the 17β-hydroxysteroid dehydrogenase causes intensification of the fluorescence maximum of the nucleotide and a shift to a lower wavelength (Figure 2). These changes, which indicate binding of the nucleotide to the enzyme, may be used to determine both the amount of nucleotide bound to the enzyme and the binding constant of the nucleotide (Velick, 1958). The results of one such determination are shown in Figure 3; 1 mole of NADPH is bound to 63,800 g of enzyme (agreeing closely with the molecular weight estimated by gel filtration) with a binding constant of 46 mμM.

The fluorescence of NADH is not significantly increased when it is added to the 17β-hydroxysteroid dehydrogenase nor is the protein fluorescence at 350 mμ significantly quenched by the addition of NAD.

The addition of NADP or NAD to solutions containing both the enzyme and NADPH reduces the enhanced fluorescence of the NADPH, suggesting competition for the nucleotide binding site. The dissociation constant of NADP, determined from this effect on NADPH fluorescence (Velick, 1958), was 51 mμM while that of NAD was 1.9 μM.

Talalay *et al.* (1958) reported Michaelis constants for NAD and NADH of 30 and 80 μM, respectively, while those for NADP and NADPH were below 1 μM. (The latter two constants were too small to determine spectrophotometrically with accuracy.) In the current stud-

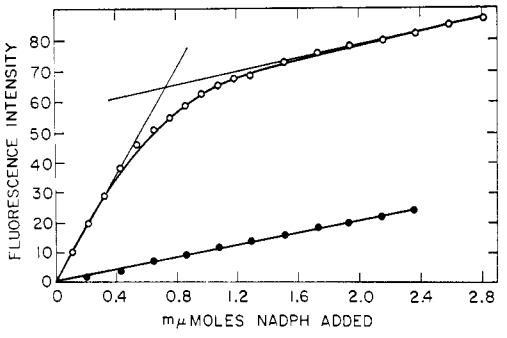

FIGURE 3: Fluorometric titration of the 17β-hydroxysteroid dehydrogenase with NADPH. The cuvet contained, in an initial volume of 1.0 ml, 4.9 μmoles of potassium phosphate at pH 7.0 and 47.2 μg of enzyme added in 0.04 ml of medium B (O——O) or 0.04 ml of medium B containing no enzyme (●——●). Additions of NADPH were made with a micropipet. Corrections have been made for the dilution due to these additions. Activation was at 340 mμ and the fluorescence emission was measured at 460 mμ. The equivalence point is 0.74 mμmole, of NADPH which corresponds to a minimal combining weight for the 17β-hydroxysteroid dehydrogenase of 63,800 g. The curved line is the theoretical curve for K_{NADPH} = 46 mμM.

ies, fluorometric measurements permitted the Michaelis constants for NADP (0.89 μM) and for NADPH (0.83 μM) to be determined.

Warren and Crist (1967) observed nonlinearity in double-reciprocal plots of NAD reduction by the 17β-hydroxysteroid dehydrogenase at NAD concentrations above 16.6 μM and suggested that these results were compatible with a second site binding NAD. This phenomenon is not apparent in the data of Talalay *et al.*

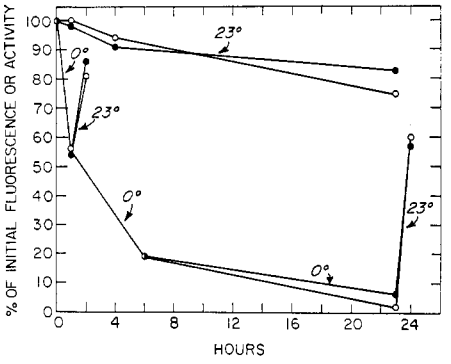

FIGURE 4: Effect of cold inactivation of the 17β-hydroxysteroid dehydrogenase on the activity of the enzyme and the fluorescence of the NADPH–enzyme complex. The enzyme was diluted with 5 mM potassium phosphate buffer (pH 7.0) at either 23 or 0°. Protein and glycerol concentrations after dilution were 23.6 μg/ml and 1%, respectively. At appropriate times two aliquots were removed from each of these solutions. One was assayed for enzymatic activity (O——O) while the other was mixed rapidly with 1 mμmole of NADPH and its fluorescence at 460 mμ was determined immediately (●——●). These measurements were also performed on aliquots that had been removed from the 0° sample (at 1 and 23 hr) and warmed to 23° for 1 hr. In order to correct for differences in fluorescence intensity due to differences in temperature, the fluorescence intensity of aliquots which had been cooled and then warmed was compared with the initial fluorescence of the sample at 23° rather than the sample at 0°.

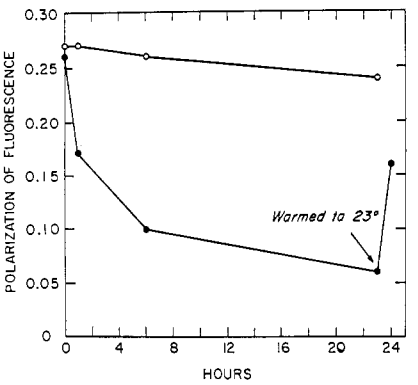

FIGURE 5: Effect of cold inactivation of the 17β-hydroxysteroid dehydrogenase on the fluorescence polarization of the NADPH–enzyme complex. The enzyme was diluted as described in Figure 10; enzyme stored at 23° is represented by O——O while that stored at 0° is represented by ●——●. At appropriate times aliquots were mixed with 1 mμmole of NADPH and the polarization of fluorescence, P, at 460 mμ was determined immediately. $P = (F_{||} - F_{\perp})/(F_{||} + F_{\perp})$, where $F_{||}$ is the fluorescence intensity measured when the electrical vectors of the polarizing and analyzing prisms are parallel and $F_{\perp}$ is the fluorescence intensity when the electrical vectors of these prisms are crossed. Although the largest contribution to the polarization of fluorescence intensity is made by the nucleotide–enzyme complex, the contributions made by the free nucleotide and enzyme are appreciable. Thus the polarization of fluorescence of the completely dissociated nucleotide–enzyme complex is not zero. When the polarization of fluorescence of enzyme and NADPH were determined independently at 460 mμ and then summed, a value of 0.06 was obtained.

(1958) but the assay conditions employed by the two groups differed somewhat. In order to investigate this discrepancy, we have repeated these measurements, using the assay system of Warren and Crist. A double-reciprocal plot of the results did not reveal any deviation from linearity, even at NAD concentrations above 16.6 μM. The Michaelis constant obtained for NAD under these conditions was 56 μM.

When the 17β-hydroxysteroid dehydrogenase is stored at temperatures below 11° in aqueous solutions or in solutions of low glycerol concentration, it loses activity. High concentrations of glycerol (20% or greater) prevent this inactivation. The loss of activity appears to be biphasic, with an initial rapid phase followed by a slower phase. Results from gel filtration and electrophoretic studies suggested that the initial, partially reversible, loss of activity is due to a change in the shape of the molecule and that this is followed by a slower, irreversible aggregation (Jarabak *et al.*, 1966). In an attempt to obtain more detailed information about the changes occurring during cold inactivation, fluorometric studies were performed.

As noted previously, the addition of NADPH to a solution of the 17β-hydroxysteroid dehydrogenase produces a marked intensification in the fluorescence of the nucleotide. If NADPH is added to aliquots removed from a solution of the 17β-hydroxysteroid dehydrogenase during the process of cold inactivation, the fluorescence intensification and the enzyme activity decrease at the same rate (Figure 4). Warming a solution

TABLE I: K_m and V_{max} for Substrates of the 17β-Hydroxysteroid Dehydrogenase.

Substrate	Pyridine Nucleotide	$K_m \times 10^6$ M	V_{max}
17β-Estradiol	NAD	6.0[a]	100
17β-Estradiol	NADP	1.9	88
Testosterone	NAD	400	3.8
Testosterone	NADP	860	5.6
20α-Hydroxy-pregn-4-en-3-one	NAD	490	0.32
20α-Hydroxy-pregn-4-en-3-one	NADP	270	0.20
Estrone	NADH	6.2	
Estrone	NADPH	0.71	
Androst-4-ene-3,17-dione[b]	NADH	1000	
Progesterone[b]	NADH	c	
Progesterone[b]	NADPH	820	

[a] A value of 4.0 μM was obtained for the K_m when 50 μmoles of sodium pyrophosphate at pH 10.2 was used to buffer the reaction rather than 50 μmoles of Tris-HCl at pH 7.4. [b] Values determined by spectrophotometric assay. [c] This value could not be accurately determined since the rate of reduction with NADH was very slow, less than 1/100 the rate of reduction with NADPH.

of the enzyme which has been partially cold inactivated, increases the fluorescence intensification and the enzyme activity to the same extent. The two most likely interpretations for these observations are: (1) NADPH is not bound to the cold inactivated enzyme, or (2) NADPH is bound to the cold inactivated enzyme but in such a way that its fluorescence is no longer intensified.

To permit a choice between these alternatives, the polarization of fluorescence of the nucleotide, which occurs when the nucleotide binds to the enzyme (Velick, 1958), was measured at intervals during the course of cold inactivation. The polarization of NADPH fluorescence decreased at the same rate as the enzyme lost activity and increased when the partially inactivated enzyme was warmed (Figure 5), indicating that the reversible phase of cold inactivation of the enzyme is accompanied by a loss of its ability to bind NADPH. Warming solutions of the cold inactivated enzyme leads to a partial recovery of its ability to bind NADPH and also a partial return of enzymatic activity.

Although the fluorescence intensification of NADPH decreases by more than 90% after the enzyme has been stored at 0° for 23 hr, when the enzyme (to which no NADPH had been added) was activated at 280 mμ, its fluorescence at 350 mμ did not change significantly (<5%) in this same period.

Michaelis Constants and Maximum Velocities of Substrates. The 17β-hydroxysteroid dehydrogenase has a rather broad substrate specificity, catalyzing the interconversion of 17β-estradiol and estrone, of testosterone and androst-4-ene-3,17-dione, and of 20α-hydroxy-pregn-4-en-3-one and progesterone (Langer *et al.*, 1959; Purdy *et al.*, 1964). The Michaelis constants and maximum velocities for these reactions are listed in Table I. The data in this table show that 17β-estradiol and estrone have much lower Michaelis constants than the other substrates and that the Michaelis constant for a given steroid may be affected by the pyridine nucleotide present in the reaction mixture.

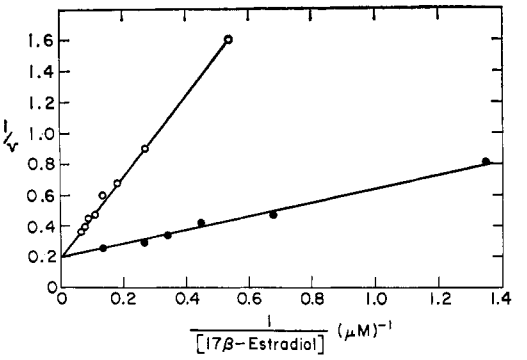

FIGURE 6: Inhibition of 17β-estradiol oxidation by diethylstilbestrol at varying concentrations of 17β-estradiol. The assays were performed fluorometrically in the presence (○——○) and absence (●——●) of 18.7 μM diethylstilbestrol.

[I_{50}] for Inhibitors of 17β-Estradiol Oxidation. In order to obtain a more complete picture of the compounds bound to the 17β-hydroxysteroid dehydrogenase than could be gathered from further structure–activity studies, a number of steroids and nonsteroidal compounds, many of which are not substrates for the enzyme, were tested for their effect on 17β-estradiol oxidation. The results (Table II) indicate that 17-desoxyestradiol and 17α-estradiol are stronger inhibitors of 17β-estradiol oxidation than some compounds which are substrates for the enzyme. In addition many nonsteroidal compounds inhibit 17β-estradiol oxidation. None of the corticosteroids tested was inhibitory.

K_i for Inhibitors of 17β-Estradiol Oxidation and Estrone and Progesterone Reduction. When the inhibition of 17β-estradiol oxidation by diethylstilbestrol was examined in detail, it was found to be competitive with respect to 17β-estradiol (Figure 6) and noncompetitive with respect to NAD (Figure 7). The inhibition of 17β-estradiol oxidation by many of the other compounds listed in Table II was examined kinetically and in all instances it was found to be competitive. This was also true for the inhibition of estrone reduction by 17β-estradiol and estriol and of progesterone reduction by testosterone. The inhibitor constants of these compounds, listed in Table II, provide one measure of their affinity for the enzyme.

Ultracentrifugal Studies. An independent determination of the dissociation constants of certain steroids was made by the ultracentrifugal technique. This method also permitted the number of binding sites per enzyme molecule to be determined. At 25° the dis-

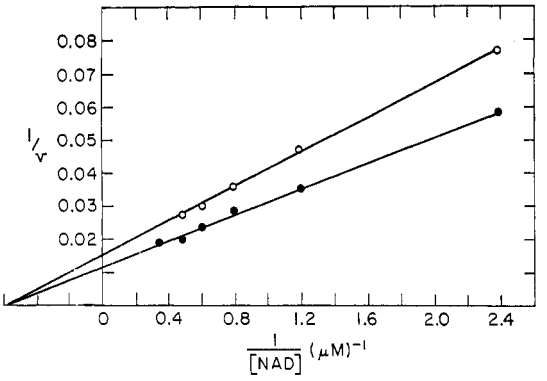

FIGURE 7: Inhibition of 17β-estradiol oxidation by diethylstilbestrol at varying concentrations of NAD. The reactions were performed fluorometrically in the presence (○——○) and absence (●——●) of 3.7 μM diethylstilbestrol.

sociation constant for 17β-estradiol is 1.9 μM (Figure 8), while at 4° the dissociation constants for 17β-estradiol and estrone are 2.1 and 3.4 μM, respectively. A straight line drawn through the experimental points in Figure 8 intersects the ordinate at approximately 1.0 (the same is true for 17β-estradiol and estrone at 4°), indicating that the enzyme has a single binding site for 17β-estradiol and estrone in the absence of pyridine nucleotide cofactors.

Factors Affecting Substrate Binding. When the 17β-hydroxysteroid dehydrogenase is passed through a Bio-Gel P-60 column which has been equilibrated with buffer containing radioactive 17β-estradiol, the effluent fractions which possess enzymatic activity also contain increased radioactivity, indicating that 17β-estradiol has been bound to the enzyme (Figure 9A). The effect on this binding of various conditions which produce enzyme denaturation or inhibition was determined by performing a series of gel filtration experiments. When samples of the enzyme were reacted with 2 mM sodium dodecyl sulfate (1 and 16 hr at 23°), 2 mM *p*-hydroxy-

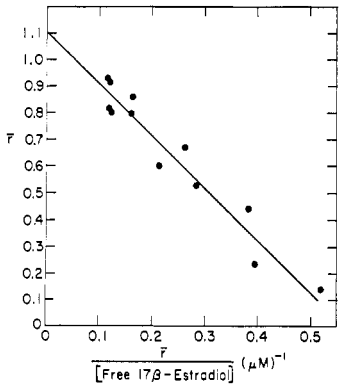

FIGURE 8: Ultracentrifugal determination of 17β-estradiol binding to the 17β-hydroxysteroid dehydrogenase at 25°. The slope, *K*, and intercept, *n*, of the plot were determined by least-squares analysis of the data obtained from two experiments; $\bar{r}$ is the moles of 17β-estradiol bound per mole of enzyme. The results indicate that *K*, the dissociation constant, has a value of 1.9 μM, while *n*, the number of 17β-estradiol binding sites per mole of enzyme, has a value of 1.1.

TABLE II: [I_{50}] and K_i for Inhibitors of 17β-Hydroxysteroid Dehydrogenase.[a]

Reaction Inhibited and Compound Tested	[I_{50}] × 10^6 M	$K_i \times 10^6$ M
17β-Estradiol Oxidation		
17-Desoxyestradiol	0.12	0.19
o,p'-DDD	0.16	0.22
U-11,100A	1.5	0.61
Equilin	1.9	
m,p'-DDD		2.8
Diethylstilbestrol	2.6	3.3
Estrone	2.6	3.7 (2.6)[b]
16-Difluoroestrone	2.8	2.5
17α-Estradiol	3.7	4.5
16-Difluoroestradiol-17β	8.5	9.7
trans-Clomiphene	13	9.3
cis-Clomiphene	13	11
p,p'-DDD		12
Hexestrol	17	
Estrone-3-methyl ether	18	
Metyrapone	33	
17α-Ethynylestradiol	42	63
Progesterone	64	130
Cyproterone acetate	90	
19-Norandrost-4-ene-3,17-dione	200	320
Amphenone B	220	
Androst-4-ene-3,17-dione	450	
Estrone Reduction		
17β-Estradiol		3.3
Estriol		5.0
Progesterone Reduction		
Testosterone		160[b]

[a] Unless specified the pyridine nucleotide used in the reactions was either NAD or NADH. The [I_{50}] was not reached for the following compounds at the concentrations (× 10^6 M) listed in parentheses: 17α-ethynylestradiol-3-methyl ether (13), MER-25 (48), 17α-ethynyl-19-nortestosterone (200), 11-deoxycortisol (860), cortisol (1100), and corticosterone (1100). Most of these compounds were insoluble at higher concentrations. [b] Value obtained with NADP or NADPH. *o,p'*-DDD = 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane; *m,p'*-DDD = 2-(3-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane; *p,p'*-DDD = 2,2-bis(4-chlorophenyl)-1,1-dichloroethane.

mercuribenzoate (30 min and 16 hr at 23°), and 5 M guanidine hydrochloride (2 hr at 23°), or were cold inactivated by a 2-week dialysis at 4° against 5 mM Tris-HCl at pH 7.4, similar results were obtained. The results demonstrate that 17β-estradiol binding is reduced by conditions which produce denaturation but that the

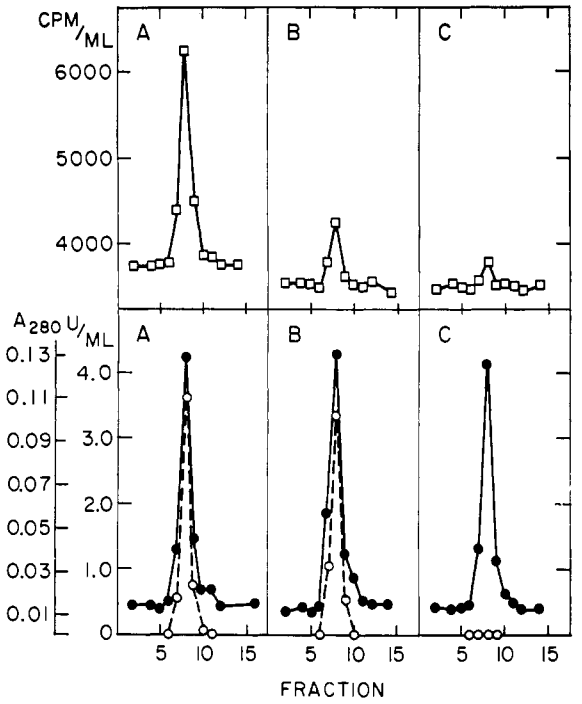

FIGURE 9: Factors affecting the binding of 17β-estradiol to the 17β-hydroxysteroid dehydrogenase. Samples of enzyme were applied on a Bio-Gel P-60 column which had previously been equilibrated with radioactive 17β-estradiol. The enzyme was eluted and each fraction was analyzed for radioactivity (□——□), absorbance at 280 mμ (●——●), and enzyme activity (○——○). In expt A, both the buffer and enzyme were unmodified. In B, diethylstilbestrol (10 μM) was added to the buffer and the column was reequilibrated with the new buffer before the sample of enzyme was applied. In C, the enzyme was reacted with *p*-hydroxymercuribenzoate (2 mM) for 16 hr at 23° and then applied to the column. Further experimental details may be found in the Methods section.

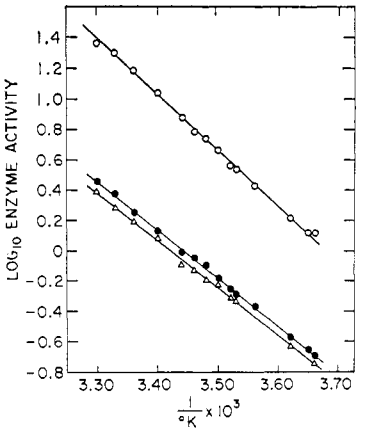

FIGURE 10: Arrhenius plots of 17β-estradiol oxidation by the 17β-hydroxysteroid dehydrogenase. Assays were performed with NAD (●——●), NADP (△——△), or 3-acetylpyridine-adenine dinucleotide (○——○). Sodium pyrophosphate (100 μmoles) was present in the assay cuvets because the higher concentration (440 μmoles) generally used in these assays tended to result in precipitation when the cuvet temperature was lowered.

inactivated enzyme still may bind significant quantities of 17β-estradiol. (Denatured samples of the enzyme bound between 0.5 and 4.9 mμmoles of 17β-estradiol, whereas a sample which had not been denatured bound 10.5 mμmoles of the steroid.) A representative example is shown in Figure 9C. Diethylstilbestrol or U-11,100A, the competitive inhibitors of 17β-estradiol oxidation, also lower 17β-estradiol binding when present at concentrations of 10 μM, but full enzyme activity may be recovered after the enzyme has been exposed to these compounds (Figure 9B). Thus their effect is reversible. Although the binding of radioactive 17β-estradiol to the enzyme may be demonstrated by using Bio-Gel (or Sephadex) columns which have been equilibrated with 17β-estradiol, a single passage of the enzyme to which radioactive 17β-estradiol has been added through a similar column which has not been equilibrated with 17β-estradiol removes all of the bound steroid from the enzyme.

Arrhenius Plots. As already noted, one factor affecting the activity of the 17β-hydroxysteroid dehydrogenase is the temperature at which it is stored. In order to determine whether changes in the *assay* temperature also produced unusual effects on the activity of the enzyme, the 17β-hydroxysteroid dehydrogenase was assayed over the temperature range in which cold inactivation occurs and Arrhenius plots were prepared from the results (Figure 10). The slope of the Arrhenius plots with 3-acetylpyridine-adenine dinucleotide, NAD, and NADP did not show inflection points, indicating that assay temperatures below 11–12° were not accompanied by an unusual decrease in activity. The activation energy with 3-acetylpyridine-adenine dinucleotide, NAD, and NADP was 17,000, 14,600, and 14,400 cal, respectively.

Discussion

The molecular weight of the 17β-hydroxysteroid dehydrogenase, estimated from gel filtration data, is 62,000–65,000. This value is in good agreement with the minimum molecular weight (63,800) obtained by titration of the enzyme with NADPH and suggests that, in the absence of substrate, a single molecule of NADPH is bound per molecule of enzyme. Ultracentrifugal data indicate that a single molecule of 17β-estradiol or estrone is bound to the enzyme in the absence of cofactor.

The binding of NADPH to the enzyme is characterized by an increase in the fluorescence of the reduced nucleotide as well as a shift in the fluorescence maximum to a lower wavelength. Neither of these phenomena is noted when NADH is added to the 17β-hydroxysteroid dehydrogenase. While this may indicate that the binding constant for NADH is much greater than for NADPH (the Michaelis constant for NADH is 36 times larger than the same constant for NADPH), additional binding studies will be required to demonstrate that NADH is bound to the enzyme in the absence of added substrate.

Although NAD and NADP compete with NADPH for a single binding site, the total number of binding sites for each of these nucleotides remains to be determined. Warren and Crist (1967) observed stimulation

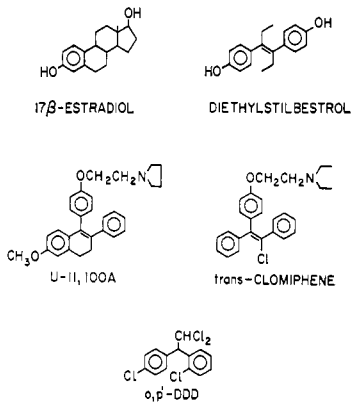

FIGURE 11: Structure of 17β-estradiol and of certain nonsteroidal inhibitors of the 17β-hydroxysteroid dehydrogenase. *o,p'*-DDD = 2,2-bis(4-chlorophenyl)-1,1-dichloroethane.

of the 17β-hydroxysteroid dehydrogenase by NAD at concentrations greater than 16.6 μM and suggested that this effect might be due to the binding of NAD at a second cofactor site. We have not been able to confirm their observation nor have we succeeded in measuring the total number of NAD sites by fluorescence measurements.

A number of enzymes which are inactivated by cold undergo a change in molecular weight during their inactivation (Jarabak *et al.*, 1966). While it is likely that this loss of enzymatic activity results from alterations in the geometry of the active site, it is not clear whether these alterations have (1) changed the orientation of the reactants so that they can no longer be brought into favorable juxtaposition, (2) affected the binding of the reactants, or (3) produced a combination of these effects. During cold inactivation the 17β-hydroxysteroid dehydrogenase loses its ability to bind NADPH at the same rate that it loses its activity. The cold inactivated enzyme also binds less steroid. While these findings indicate that the binding of cofactor and substrate to the enzyme are affected by cold inactivation, they do not completely exclude the possibility that the orientation of bound reactants is altered also at low temperatures.

In addition to a loss of activity with *storage* at reduced temperatures, some cold inactivated enzymes (Graves *et al.*, 1965; Kayne and Suelter, 1966; Massey *et al.*, 1966) show an inordinate reduction in activity when *assayed* at reduced temperatures (the Arrhenius plot of their activity contains a discontinuity at some critical temperature). Although the 17β-hydroxysteroid dehydrogenase loses activity when stored at reduced temperatures, the Arrhenius plot of its activity does not contain a discontinuity. This suggests that the presence of bound substrate and cofactor prevent the enzyme from rapidly assuming less active configurations at reduced temperatures.

The extensive structure–activity studies performed by Langer *et al.* (1959) revealed that the most reactive substrates for the 17β-hydroxysteroid dehydrogenase were C_{18} steroids which possessed both a phenolic A ring and a 17β-hydroxyl or a 17-keto function. Some compounds lacking one of these structural features act as substrates for the enzyme but are converted into product at a much slower rate, *e.g.*, testosterone was found to be oxidized by the enzyme, but at only 1% the rate of 17β-estradiol (Langer *et al.*, 1959; Adams *et al.*: 1962) while progesterone was reduced at 2% the rate of estrone (Purdy *et al.*, 1964).

The present studies demonstrate that both 17β-estradiol and estrone have lower Michaelis constants for the enzyme than do testosterone, 20α-hydroxypregn-4-en-3-one, androst-4-ene-3,17-dione, or progesterone and that the maximum velocity for the oxidation of 17β-estradiol is much higher than that for testosterone or 20α-hydroxypregn-4-en-3-one. While these results confirm previous observations that testosterone, androst-4-ene-3,17-dione, 20α-hydroxypregn-4-en-3-one, and progesterone may be substrates for the enzyme *in vitro*, the relatively high Michaelis constants and low maximum velocities of these compounds make it seem unlikely that the soluble 17β-hydroxysteroid dehydrogenase plays a major role in their oxidation or reduction in the human placenta. Additional support for this contention is the presence of a microsomal 17β-hydroxysteroid dehydrogenase in human placenta (Lehmann and Breuer, 1967) which oxidizes testosterone and 17β-estradiol at similar rates.

Fluorometric methods were used to determine the dissociation constants of the pyridine nucleotide cofactors for the 17β-hydroxysteroid dehydrogenase. Similar methods could not be used to measure substrate binding since there were no apparent spectral changes which accompanied binding of the substrate to the enzyme. Consequently substrate binding was evaluated by kinetic (inhibitor) and ultracentrifugal techniques. The inhibitor constants for 17β-estradiol, estrone, testosterone, androst-4-ene-3,17-dione, 20α-hydroxypregn-4-en-3-one, and progesterone are only somewhat smaller than the Michaelis constants for these compounds. The dissociation constants for 17β-estradiol and estrone determined by ultracentrifugation are also smaller than the Michaelis constants for these compounds. Despite some differences in experimental conditions there is fair agreement between the inhibitor constants for 17β-estradiol and estrone determined kinetically and the dissociation constants determined by ultracentrifugal means. It would appear from this data that the Michaelis constants and the dissociation constants of various substrates of the 17β-hydroxysteroid dehydrogenase are similar, if not identical; while the same constants for various pyridine nucleotide cofactors of the enzyme often differ by more than an order of magnitude.

As noted in this and in previous investigations, structural modifications at a number of locations on the steroid nucleus result in major changes in both the reaction rate of a steroid in an enzymatic reaction and its affinity for the enzyme. These changes are not necessarily parallel, however. For example, the oxidation of 17α-estradiol is not catalyzed by the 17β-hydroxysteroid dehydrogenase but 17α-estradiol competitively inhibits the oxidation of 17β-estradiol and has a K_i almost as small as that of estrone.

While a competitive inhibitor is not necessarily bound at the same site as the substrate with which it competes, the structural similarities (Figure 11) between 17β-es-

tradiol and certain of the nonsteroidal compounds which inhibit 17β-estradiol oxidation suggest that these inhibitors (diethylstilbestrol, U-11,100A, and the clomiphene isomers[3]) are bound at the 17β-estradiol binding site of the enzyme. In this regard it is particularly noteworthy that diethylstilbestrol and U-11,100A bind to the enzyme with affinities as great as that of estrone. The isomers of 2,2-bis(chlorophenyl)-1,1-dichloroethane do not bear as great a structural resemblance to 17β-estradiol as do the other nonsteroidal inhibitors and with few exceptions (Levin *et al.*, 1968) they do not possess any appreciable estrogenic or antiestrogenic activity *in vivo*. Further information will be necessary in order to determine whether the inhibition of 17β-estradiol oxidation[4] by the isomers of 2,2-bis(chlorophenyl)-1,1-dichloroethane is due to the binding of these compounds at the 17β-estradiol binding site.

While protein–steroid interactions are affected by modifications of the steroid nucleus, they are also affected by alterations of the protein. Inactivation of the 17β-hydroxysteroid dehydrogenase by cooling or by treatment with *p*-hydroxymercuribenzoate, guanidine hydrochloride, or sodium dodecyl sulfate results in a decrease in the amount of 17β-estradiol bound to the enzyme. Significant quantities of 17β-estradiol (but not NADPH) still are bound to the fully inactivated enzyme in some instances, however. The fact that a number of denaturing agents all decrease the amount of 17β-estradiol bound to the enzyme suggests that this is not due to a specific alteration in the enzyme but rather to nonspecific changes occurring with denaturation. Similar observations have been made with the estrogen "receptor" of the rat uterus (Toft *et al.*, 1967).

Although the 17β-hydroxysteroid dehydrogenase binds estrogenic compounds and as such might be considered an estrogen "receptor" (Jensen, 1968), the present study reveals that the protein–steroid interactions of this enzyme differ quantitatively in a number of respects from those of estrogen "receptors" isolated from the uterus. The 17β-hydroxysteroid dehydrogenase has approximately the same affinity for 17β-estradiol (2.1 μM at 4°) and estrone (3.4 μM at 4°). Toft *et al.* (1967) noted that the apparent 17β-estradiol dissociation constant for the 9.5S estrogen binding component from rat uterus is 70 mμM, and Jensen and Jacobson (1962) demonstrated that rat uterine tissue possesses a much greater affinity for 17β-estradiol than for estrone. While the K_i for 17α-estradiol (4.5 μM) suggests that this compound has a somewhat smaller affinity for the 17β-hydroxysteroid dehydrogenase than does 17β-estradiol, both *in vivo* (Toft and Gorski, 1966) and *in vitro* (Talwar *et al.*, 1968) studies indicate that the affinity of 17α-estradiol for the uterine estrogen "receptor" is considerably lower than that of 17β-estradiol. Testosterone and progesterone are both bound to the 17β-hydroxysteroid dehydrogenase but the Michaelis and inhibitor constants of these compounds suggest that their affinity for the enzyme is only $^1/_{50}$ to $^1/_{100}$ that of 17β-estradiol. *In vivo* studies (Toft *et al.*, 1967) performed with tritium-labeled compounds did not demonstrate testosterone or progesterone bound to the 9.5S component obtained from rat uterus; however, it is unlikely that the sensitivity of these measurements was great enough to observe whether testosterone or progesterone was bound with $^1/_{100}$ the affinity of 17β-estradiol.

[3] Although the clomiphene isomers inhibit 17β-estradiol oxidation *in vitro*, there is no evidence to indicate that this is their mechanism of action *in vivo*. The results of the present study, however, would support the contention that some effects of these isomers may be due to a competition with 17β-estradiol at estrogen binding sites (Roy *et al.*, 1964).

[4] The most effective isomer of 2,2-bis(chlorophenyl)-1,1-dichloroethane in producing adrenal necrosis is 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane while 2,2-bis(4-chlorophenyl)-1,1-dichloroethane is the least effective. Although the mechanism by which these compounds produce adrenal necrosis and the mechanism by which they inhibit the oxidation of 17β-estradiol may be totally unrelated, it is of interest that 2-(2-chlorophenyl)-2-(4-chlorophenyl)-1,1-dichloroethane is also the most effective inhibitor of 17β-estradiol oxidation while 2,2-bis(4-chlorophenyl)-1,1-dichloroethane is the least effective.

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Comparison of the Esterase Activities of Trypsin, Plasmin, and Thrombin on Guanidinobenzoate Esters. Titration of the Enzymes*

T. Chase, Jr., and Elliott Shaw

ABSTRACT: The acyl-enzyme intermediate, *p*-guanidinobenzoyl-trypsin, has been shown to deacylate unusually slowly, permitting the use of *p*-nitrophenyl *p'*-guanidinobenzoate as a convenient titrant of trypsin. It is now found that thrombin and plasmin, key enzymes in blood clotting physiology which have a trypsin-like esterase specificity, can also be titrated by *p*-nitrophenyl *p'*-guanidinobenzoate, showing similar "burst" behavior. This indicates the formation of an acyl-enzyme intermediate in normal plasmin catalyzed reactions, as has been established for the other two enzymes, and strengthens the hypothesis that all three proteolytic enzymes have evolved from a common ancestral form. The kinetic properties of the esterase action of trypsin thrombin, and plasmin on the ethyl and *p*-nitropheny esters of *p'*-guanidinobenzoic acid have been characterized. Noteworthy quantitative differences have been found distinguishing thrombin from trypsin and plasmin: the first named is the slowest acylated and the most rapid in deacylation. The esterase action of thrombin appeared even more disparate from the other two proteolytic enzymes in its relative ease of hydrolysis of the isomeric ester, *p*-nitrophenyl *m'*-guanidinobenzoate. These findings suggest that the active center of thrombin has greater geometric adaptibility to simple substrates than that of trypsin and plasmin.

We have recently reported on the use of *p*-nitrophenyl *p'*-guanidinobenzoate hydrochloride (*p*-NPGB[1]) as a titrant for trypsin (Chase and Shaw, 1967); we wish to extend these observations and describe the reaction of this and related compounds with the proteolytic enzymes of the blood clotting system, plasmin and thrombin. Current interest in these enzymes derives not only from their very important physiological role but also from the possibility that such specialized enzymes may have evolved from trypsin or from a common

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[1] The following abbreviations are used: DIP-trypsin, diisopropylphosphoryl-trypsin; E*p*GB, ethyl *p*-guanidinobenzoate·HCl; GB, guanidinobenzoyl (as, *p*-guanidinobenzoyl-trypsin); *m*-NPGB, *p*-nitrophenyl *m'*-guanidinobenzoate·HCl; *p*-NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate·HCl; NPZL, *p*-nitrophenyl N^{α}-benzyloxycarbonyl-L-lysinate·HCl; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone·HCl.