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Steroid–Protein Interaction at Sites Which Influence Catalytic Activity*

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ABSTRACT: The effects of 14 steroids (representing major classes in terms of chemical structure and biological activity) on the catalytic activity of 6 enzymes have been studied. Activities of fumarase, lactate dehydrogenase, and isocitrate dehydrogenase were essentially unchanged (<5%) in the presence of steroid concentrations as high as 1.3×10^{-4} M. On the other hand, activities of bovine liver glutamate dehydrogenase, glucose 6-phosphate dehydrogenase from bovine corpus luteum, and rabbit liver aldehyde dehydrogenase were inhibited by a variety of steroids. With the latter three enzymes, inhibition was effective immediately on addition of steroid, reversible on dilution, and not competitive in regard to substrate and cofactor. The experimental data were found to fit a mathematical model applied to determine the association constant, K_A, for steroid–protein interaction at those sites which influence catalytic activity. In the case of glucose 6-phosphate dehydrogenase, the reversible combination of a single steroid molecule with the sensitive site was sufficient to completely eliminate activity at the affected catalytic site. Further, different steroids effect inhibition by binding at identical sensitive sites. Findings are similar in the case of aldehyde dehydrogenase. With glutamate dehydrogenase, the data are consistent with a mechanism whereby the combination of two steroid molecules with the sensitive site is necessary to completely eliminate activity, although a mechanism with interacting inhibitor binding sites (allosteric) would give similar results.

Determination of K_A values of the several steroids tested indicates that the sensitive sites of glutamate dehydrogenase have the highest affinity for estradiol-17β while those of glucose 6-phosphate dehydrogenase have the highest affinity for Δ^5-3β-hydroxy steroids and those of aldehyde dehydrogenase have the highest affinity for 3-keto steroids.

Characterization of the interaction of steroids with appropriate receptor sites on macromolecules which ultimately mediate the biological response lies at the heart of current investigation dealing with the mechanism of hormone action. Several models of steroid allosteric effects on the activity of key enzymes have been presented. Three important examples are glutamate dehydrogenase (Yielding and Tomkins, 1960; Tomkins *et al.*, 1961; Warren *et al.*, 1964), glucose 6-phosphate dehydrogenase (Marks and Banks, 1960; McKerns and Kaleita, 1960; Nielson and Warren, 1965; Warren and Betz, 1965), and aldehyde dehydrogenase (Maxwell and Topper, 1961).

This investigation was designed to provide a general, quantitative evaluation of the steroid binding sites of these and various other enzymes. By determination of steroid–enzyme association constants with a kinetic method, we thought to selectively evaluate steroid binding at those sites which influence catalytic activity. We considered the important aspects of these steroid–protein interactions to be: (1) the affinity of steroid binding, (2) the stoichiometry of steroid binding, (3) whether various steroids bind at identical sites on a given enzyme, and (4) binding as a function of steroid structure.

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Experimental Section

Materials. Reagent grade steroids: NAD^+,[1] $NADP^+$, D-isocitric acid, lactic acid, L-glutamic acid, glucose 6-phosphate, DL-glyceraldehyde, and Tris base were purchased from the Sigma Co. Potassium phosphate salts (reagent grade) were purchased from the Mallinckrodt Co. and used without further purification. Malic acid and the DEAE-cellulose utilized in the purification of rabbit liver aldehyde dehydrogenase were products of Calbiochem Co; the latter was prepared according to the method of Sober and Peterson (1958). All pH values were determined with a Beckman Model 76 pH meter.

Enzymes and Assays. Assays of glutamate dehydrogenase, lactate dehydrogenase, isocitric dehydrogenase, and glucose 6-phosphate dehydrogenase were carried out by monitoring the generation of reduced cofactor spectrophotometrically at 340 mμ with a Beckman DU monochrometer equipped with a Gilford power supply and a 10-mV Honeywell chart recorder. Fumarase was assayed in this instrument by monitoring fumarate production at 295 mμ. A water jacket surrounding the cuvet chamber maintained a temperature of 30° with all the enzymes above. Rabbit liver aldehyde dehydrogenase was assayed at 27° by following generation of NADH with a Farrand Model A fluorometer utilizing an exciting wavelength of approximately 360 mμ (Corning color filter no. 7–37) and an emission wavelength of approximately 460 mμ (Corning color filters nos. 4–70 and 3–72). Strict attention was paid to securing initial linear velocities in all cases. All reactions were initiated by the addition of enzyme. Steroids were added in 0.1 ml of propylene glycol or in 25–50 μl of ethanol as indicated. Control assays contained vehicle alone. Final volume of all assay solutions was 3.0 ml.

Bovine liver glutamate dehydrogenase (EC 1.4.1.3) was obtained from the Sigma Co. as an ammonium sulfate suspension of crystals (type I). Aliquots were dissolved, fresh daily, in cold 0.075 M potassium phosphate buffer (pH 7.3)–5 mM NaCl. The assay solutions contained 130 μmoles of potassium phosphate buffer (pH 7.0), 50 μmoles of L-glutamic acid (previously adjusted to pH 7.0 with potassium hydroxide), and 2.0 μmoles of $NADP^+$. The reaction was started by addition of 10.0 μg of enzyme which in the absence of steroid (control) generated 12.0 mμmoles of reduced cofactor/min.

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) was prepared from bovine corpora lutea as described by Nielson and Warren (1965) who modified Kirkman's method (Kirkman, 1962). The purified (but not homogeneous) preparation generated 1.7 μmoles of NADPH/min per mg of protein and was free of 6-phosphogluconate dehydrogenase (EC 1.1.1.44) activity. The enzyme was stored in 20% glycerol containing 0.1 mM $NADP^+$. Aliquots were diluted to a concentration of 0.10 mg of protein/ml each day. The assay solution contained 100 μmoles of potassium phosphate buffer (pH 7.0), 5.0 μmoles of NaCl, 15 μmoles of $MgSO_4$, 2.0 μmoles of glucose 6-phosphate, and 1.0 μmole of $NADP^+$. The reaction was started by addition of 10.0 μg of the enzyme preparation which in the absence of steroid (control) generated 17.0 mμmoles of reduced cofactor/min.

Rabbit liver aldehyde dehydrogenase (EC 1.2.1.3) was prepared according to the method of Maxwell and Topper (1961) with omission of the CM-cellulose chromatography step. The preparation generated 0.75 μmole of reduced cofactor/min per mg. The assay solution contained 100 μmoles of sodium glycine buffer (pH 9.1), 30 μmoles of DL-glyceraldehyde, and 1.0 μmole of NAD^+. The reaction was initiated by the addition of 30–70 μg of protein. Velocity data in the text are in arbitrary units which depend upon the convenient settings on the Farrand spectrofluorometer. For each experiment, of course, these settings remained constant.

Isocitrate dehydrogenase (EC 1.1.1.41) was obtained from the Sigma Co. and assayed with and without steroids according to the method of Ochoa (1955). Lactate dehydrogenase (EC 1.1.1.27), crystalline from rabbit muscle, was obtained from C. F. Boehringer and Sons in 2.0 M ammonium sulfate and assayed as described by Warren *et al.* (1966). Crystalline fumarase (EC 4.2.1.2) from heart muscle, also obtained from C. F. Boehringer and Sons in 2.0 M ammonium sulfate, was assayed with malate as substrate as described by Warren *et al.* (1966).

The concentrations of cofactor and substrate used in all the routine assays above were shown to be at or near saturation by the fact that doubling the concentrations of both, in all cases (with and without steroids), did not change the velocity of the reaction in excess of 5%.

Steroid Solubility. Solubility of representative steroids was evaluated in both water and the phosphate buffer described above using a Farrand Model A fluorometer with both excitation and emission wavelength at 360 mμ (in essence using the instrument for evaluation of light scatter). Increasing amounts of steroid were added with the solubility limit being designated as that point where a definite increase in emission energy was noted. Estradiol-17β, dehydroisoandrosterone, and progesterone were tested and found, in both the water and the phosphate buffer solution, to be soluble to the extent of 70, 250, and 250 μM, respectively.

Binding Capacity. Evaluation of the binding of androstenedione and progesterone by glutamate dehydrogenase and glucose 6-phosphate dehydrogenase preparations were carried out spectrophotometrically as described by Westphal *et al.* (1958). Evaluation of the binding of estradiol-17β to these same proteins was carried out as described by Wang *et al.* (1963). Proteins were used at concentrations of 330 μg/ml and the quantity of steroid bound at total steroid concentrations of 66 μM in no instance exceeded 9 μg/100 μg of protein. Considering the very small quantity of protein used in the actual assays, the quantity of steroid bound (in terms of that present) becomes negligible so that total steroid added $\cong$ [I].

Results

As a preliminary step, the activities of the 6 enzymes were determined in the presence and absence of 14 steroids representing the major classes in terms of chemical

[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

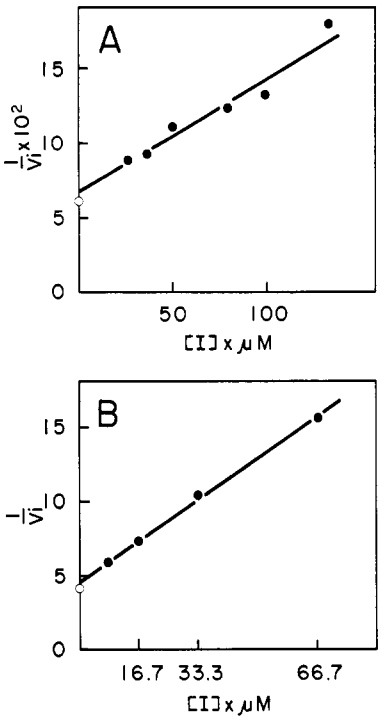

FIGURE 1: Inhibition of glucose 6-phosphate dehydrogenase by pregnandione (A) and inhibition of rabbit liver aldehyde dehydrogenase by desoxycorticosterone (B). Lines fitted to points by least squares. Open circles on ordinates represent reciprocals of initial velocities ($1/V_0$) in the absence of inhibiting steroid. Pearson correlation coefficients are (A) 0.068 and (B) 0.999. Conditions of assay listed in text.

TABLE 1: Steroids Utilized in Kinetic Experiments Represented by Figures 1–4.[a]

Enzyme	Steroid	r
Glucose 6-phosphate dehydrogenase	Dehydroisoandrosterone	0.984
	Androstendione	0.867
	Pregnandione	0.968
	Progesterone	0.974
Aldehyde dehydrogenase	Progesterone	0.962
	Pregnandione	0.971
	Deoxycorticosterone	0.999
	Corticosterone	0.999
	Epitestosterone	0.985
	Testosterone	0.955
Glutamate dehydrogenase	Estradiol-17β	0.985
	Estradiol-17α	0.980
	Dehydroisandrosterone	0.956
	Testosterone	0.988
	Progesterone	0.993

[a] The values listed under "r" are the correlation coefficients of the Dixon plots (see Figures 1 and 2) for each steroid. For glutamic acid dehydrogenase "r" refers to correlation coefficient of $1/V_i$ *vs.* $[I]^2$ plots as in Figure 2B.

structure and hormonal activity. Estradiol-17β, estradiol-17α, estrone, estriol, Δ^5-pregnenolone, dehydroepiandrosterone, progesterone, pregnandione, androstenedione, testosterone, epitestosterone, desoxycorticosterone, corticosterone, and cortisol were used at concentrations of 66 and 132 μM. No significant effect (<5%) of any of these steroids on the activity of isocitric dehydrogenase, fumarase, or lactate dehydrogenase was noted.

On the other hand certain of the steroids tested, at 66 μM concentrations, inhibited glucose 6-phosphate dehydrogenase, glutamate dehydrogenase, and aldehyde dehydrogenase. Pregnenolone and dehydroepiandrosterone were the most effective inhibitors of glucose 6-phosphate dehydrogenase while estradiol-17β and desoxycorticosterone were relatively ineffective (producing only 15 and 3% inhibition, respectively). Estradiol-17β was the most effective inhibitor of glutamate dehydrogenase while dehydroepiandrosterone and desoxycorticosterone were relatively ineffective (producing only 8 and 1% inhibition, respectively). Pregnandione and desoxycorticosterone were the most effective inhibitors of aldehyde dehydrogenase while estradiol-17β and desoxycorticosterone were ineffective (both producing less than 5% inhibition). Further studies were confined to these enzymes.

The mechanism of inhibition of glutamate dehydrogenase by estradiol-17β and progesterone, glucose 6-phosphate dehydrogenase by progesterone and dehydroepiandrosterone, and aldehyde dehydrogenase by androstenedione and cortisol was evaluated by variation of cofactor and substrate levels with and without steroids present. Initial velocity values analyzed by the double-reciprocal method of Lineweaver and Burk (1934) revealed, in all cases, that inhibition was not competitive in terms of either cofactor or substrate. All subsequent assays were done with cofactor and substrate at saturating concentrations.

Steroid inhibition was effective immediately on addition of steroid to the reaction mixture and shown to be reversible by dilution and when analyzed by the method of Ackerman and Potter (1949). Thus a simple, reversible expression can be written as

$$E + nI \rightleftharpoons EI_n \quad (1)$$

In this expression, E represents an enzyme site which is capable of binding steroid with resultant change in enzymatic activity, I represents free steroid, and EI_n represents the associated complex. It follows that the enzyme–steroid association constant, K_A, may be written as

$$K_A = \frac{[EI_n]}{[EI][I]^n} \quad (2)$$

Assuming that steroid, on binding at the appropriate site, completely inactivates the affected catalytic site and because the inhibition is not competitive with respect to

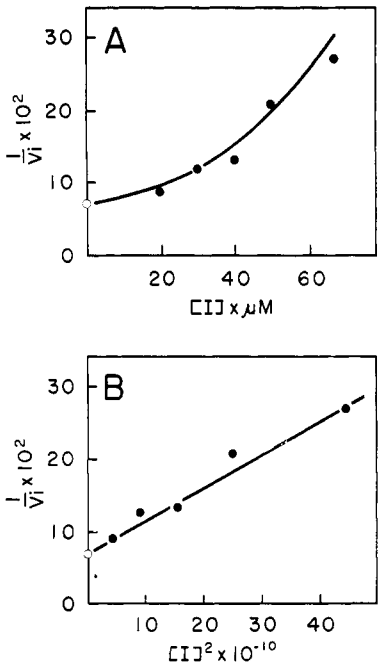

FIGURE 2: Inhibition of glutamic dehydrogenase by estradiol-17β. The data (A) are plotted according to the method of Dixon and Webb (1964) and in a variation (B) utilizing the square of the inhibitor concentration, $[I]^2$, on the abscissa. In A, the line containing the reciprocal of the initial velocity of the controls ($1/V_0$), represented by the open circle, is curved. In B, the straight line fitted to the data points by least squares predicts ($1/V_0$) and has a Pearson correlation coefficient of 0.985.

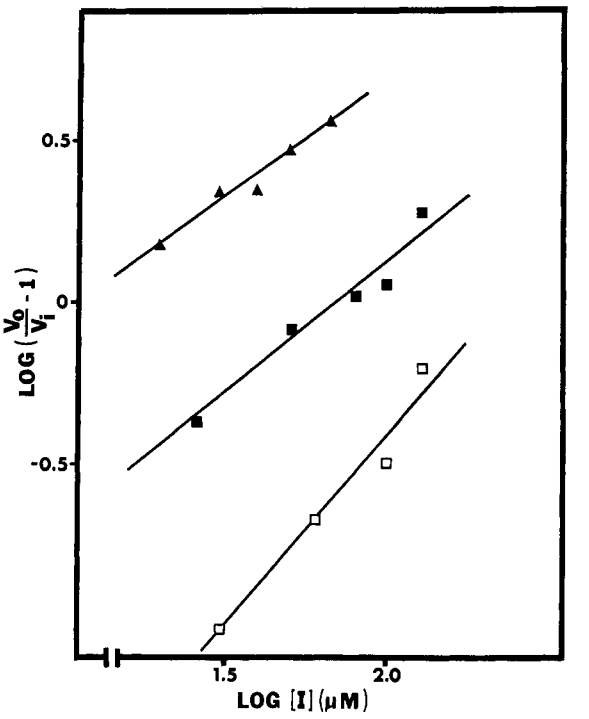

FIGURE 3: Inhibition of glucose 6-phosphate dehydrogenase by dehydroisoandrosterone (▲—▲), pregnandione (■—■), and progesterone (□—□). Data plotted by method of Johnson *et al.* (1942) and lines fitted by least-mean-squares method. Correlation coefficients listed in Table II.

substrate or cofactor, it follows that the activity remaining in the presence of the inhibiting steroid, V_i, is proportional to [E]. With activity in the absence of inhibitor designated as V_0, it follows that activity lost as a result of the inhibiting steroid, $V_0 - V_i$, is proportional to $[EI_n]$. The association constant then may be written in the kinetic form

$$K_A = \frac{V_0 - V_i}{V_i[I]^n} \qquad (3)$$

Spectrophotometric studies described above indicated that only a small fraction (<2%) of the steroid present was bound to enzyme so that total steroid added ≅ [I]. It must be pointed out that K_A derived above is pertinent *only* for steroid binding at sites which affect activity and has nothing to do with any possible binding at sites which do not influence activity.

The results of kinetic experiments utilizing the conditions of assay given above and a series of differing steroid concentrations were graphed according to the method of Dixon and Webb (1964) with the reciprocal velocity on the ordinate, inhibitor concentration on the abscissa, and n assumed = 1. The intercept on the abscissa gives the negative reciprocal of K_A, indicating the steroid concentration required for 50% inhibition. Steroids representing each class found to inhibit a given enzyme were utilized in inhibition experiments of this kind. These were as listed in Table I. The following remarks apply to this list.

Figure 1 shows representative curves for glucose 6-phosphate dehydrogenase and aldehyde dehydrogenase fitted to the data points by the method of least-mean squares. Each data point is the average of at least three assays. A product moment formula was used to calculate the linear correlation coefficient, r, as a measure of the goodness of fit of these points to the line depicted (Spiegel, 1961). For these two enzymes the data are consistent with $n = 1$. For the steroids tested, the linear (Pearson) correlation coefficients are listed in Table I.

When the steroid inhibition of glutamate dehydrogenase was plotted according to this method, a nonlinear curve was obtained for all the steroids tested. Figure 2A illustrates this result for a representative steroid. When the steroid concentrations for each level of inhibition were squared and the result was plotted as in Figure 2B, a straight line was obtained. For all the steroids tested (Table I), the correlation coefficients with the Dixon plot utilizing $[I]^2$ on the abscissa ranged from 0.956 to 0.993. These data are consistent with $n = 2$ for steroid interactions with glutamate dehydrogenase as given by eq 2.

When the reciprocal of the noninhibited velocity, $1/V_0$, was not included in the least-mean-squares calculations for the graphical determinations of $1/K_A$, its value was included without exception within the limits of two standard errors of estimate (Spiegel, 1961) of the least-mean-squares line on each side of its actual intercept on the ordinate. This was true for all the enzyme–steroid pairs listed in Table I. Thus, the least-mean-squares lines accurately predicted $1/V_0$ in each instance,

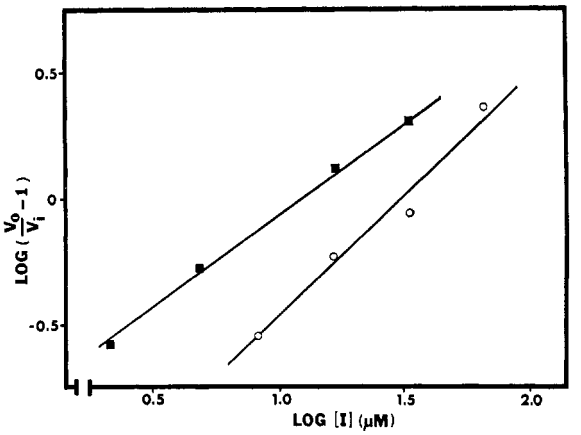

FIGURE 4: Inhibition of aldehyde dehydrogenase by corticosterone (■—■) and epitesterone (○—○). Data plotted as in Figure 3 and lines fitted by least-mean-squares method. Correlation coefficients listed in Table II.

which corroborates the validity of the kinetic approach as do the uniformly high correlation coefficients.

In an effort to quantitate n more directly, the graphical method of Johnson *et al.* (1942) was utilized. The linear equation is derived from eq 3 and is expressed as

$$\log \frac{V_0}{V_i} - 1 = n \log [I] + \log K_A \qquad (4)$$

When $\log (V_0/V_i) - 1$ is graphed as a function of log [I], the resultant straight line has a slope of n and a log [I] value of $1/K_A$ at $\log (V_0/V_i) - 1 = 0$. Results with a few representative steroids with each of the three enzymes are graphed according to this method in Figures 3–5. Lines were fitted to the data points (each the average of at least three assays) according to the method of least-mean squares. With glucose 6-phosphate dehydrogenase, the mean value for n, ($\bar{n}$), was found to be 0.940, with a standard deviation corrected for small samples ($\hat{\sigma}$) of 0.206. With aldehyde dehydrogenase, $\bar{n} = 0.848$ and $\hat{\sigma} = 0.110$; with glutamate dehydrogenase, $\bar{n} = 2.19$ and $\hat{\sigma} = 0.276$. Thus, the nearest integer values for n are: glucose 6-phosphate dehydrogenase and aldehyde dehydrogenase, $n = 1$; glutamate dehydrogenase, $n = 2$. The $1/K_A$ values derived from this graphical method are listed in Table II as the concentration of steroid required to cause 50% inhibition of the enzyme indicated. The correlation coefficients of the lines from which these values were derived are listed. With both methods of calculation of $1/K_A$ in many inhibition experiments, this value was generally reproducible within 10–15% for each enzyme–steroid system tested. The value for $n = 2$ for glutamate dehydrogenase–steroid interaction supports the use of the $[I]^2$ plot in Figure 2B and suggests that 2 moles of steroid is required to completely inhibit 1 mole of catalytic site.

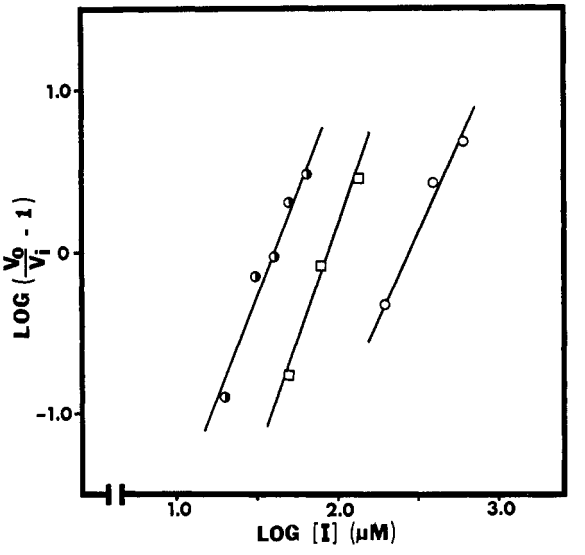

FIGURE 5: Inhibition of glutamate dehydrogenase by estradiol-17β (◑—◑), progesterone (□—□), and dehydroisoandrosterone (▲—▲). Data plotted as in Figure 3 and lines fitted by least-mean-squares method. Correlation coefficients listed in Table II.

TABLE II: Steroid Concentrations Causing 50% Inhibition of Glutamate Dehydrogenase, Glucose 6-Phosphate Dehydrogenase, and Aldehyde Dehydrogenase.[a]

Enzyme	Steroid	Concn Causing 50% Inhibn (μM)	r
Glucose 6-phosphate dehydrogenase	Dehydroisoandrosterone	11.2	0.997
	Androstendione	54.4	0.992
	Pregnandione	71.0	0.977
	Progesterone	179.0	0.981
Aldehyde dehydrogenase	Pregnandione	12.0	0.998
	Deoxycorticosterone	21.3	0.999
	Corticosterone	23.8	0.989
	Epitestosterone	30.8	0.986
	Progesterone	44.6	0.983
	Testosterone	68.6	0.967
Glutamate dehydrogenase	Estradiol-17β	30.4	0.974
	Progesterone	89.0	0.994
	Estradiol-17α	106.0	0.949
	Dehydroisoandrosterone	128.0	0.972
	Testosterone	268.0	0.991

[a] Concentrations determined by graphical method of Johnson *et al.* (1942). "r" is correlation coefficient of least-mean-squares lines obtained as shown in Figures 3–5.

TABLE III: Site Specificity of Steroid Binding to Glucose 6-Phosphate Dehydrogenase.[a]

Steroids and Concentrations Utilized (μM)	K_A Values Used in Calculations ($M^{-1} \times 10^{-4}$)	Calculated V_i		Exptl V_i
		Nonidentical Sites	Identical Sites	
Dehydroisoandrosterone (60) Pregnandione (133)	6.05 1.94	1.82	4.19	6.10
Androstendione (60) Progesterone (133)	1.66 0.39	9.96	12.0	11.6
Androstendione (133) Progesterone (133)	1.66 0.39	6.20	8.10	8.60
Dehydroisoandrosterone (60) Progesterone (133)	6.05 0.39	4.30	5.90	6.10

[a] When the velocities calculated for identical and nonidentical sites are compared with the observed velocity, the data are consistent with identical sites of binding for the combinations of steroids tested. Velocity data are in millimicromoles of cofactor generated per minute.

Observations with all the steroids at 66 μM concentrations and the more effective inhibitors at variable concentrations (Table II) reveal: (a) dehydroepiandrosterone is the most efficient inhibitor of glucose 6-phosphate dehydrogenase while it is relatively ineffective with aldehyde dehydrogenase and glutamate dehydrogenase, (b) desoxycorticosterone is a very effective inhibitor of aldehyde dehydrogenase while it is almost without effect on glutamate dehydrogenase and glucose 6-phosphate dehydrogenase, (c) estradiol-17β is the most effective inhibitor of glutamate dehydrogenase but is considerably less effective with the other two enzymes, and (d) estradiol-17α is considerably less effective than estradiol-17β in the case of glutamate dehydrogenase. While these observations do not permit extensive correlation of steroid structure and binding they do indicate some specificity of the steroid binding sites of each of the three enzymes.

We considered that for a given enzyme, various steroids effect inhibition by combination with the same sensitive site. To test this assumption, mixed inhibition studies were carried out with glucose 6-phosphate dehydrogenase and aldehyde dehydrogenase. For an identical binding site, steroids A and B would act additively, giving the following expression for the activity in the presence of both

$$V_{i_{A+B}} = \frac{V_0}{1 + K_A[A] + K_B[B]} \quad (5)$$

In contrast, for independent and noninteracting sites, the activity remaining after the binding of steroid A is essentially the initial velocity for steroid B inhibition. The final activity in the presence of both is expressed as

$$V_{i_{AB}} = \frac{V_0}{(1 + K_A[A])(1 + K_B[B])} \quad (6)$$

Table III gives the results of such experiments with glucose 6-phosphate dehydrogenase and Table IV with aldehyde dehydrogenase. The K_A values utilized for the calculations were determined on the day of the experiment by averaging K_A values determined with at least two different steroid concentrations, using eq 3. The observed values for V_i are compared with the values calculated from eq 5 and 6 for identical and nonidentical noninteracting sites. For each enzyme, the data are consistent with the hypothesis that different steroids bind at identical sites.

Discussion

It is immediately apparent that the steroids used in this study do not change the catalytic activity of lactate dehydrogenase, isocitrate dehydrogenase, or fumarase. It can be concluded that either these enzymes do not have steroid binding sites active at the steroid concentrations used or that they do but the binding exerts no effect on catalytic activity.

These general studies of steroid–protein binding offered the possibility of establishing an ordered series of steroid inhibitors applicable to a variety of enzymes in a manner analogous to the lyotropic series of anions which inhibit a variety of enzymes in a generally repeatable sequence (Warren *et al.*, 1966; Warren and Cheatum, 1966) or to the "polarity rule" governing relative binding affinity of steroids to sites on human and bovine serum albumins (Bischoff and Pilhorn, 1948; Eik-Nes *et al.*, 1954). As indicated in Table II, such an ordered series does not exist for the enzymes studied. Rather, each enzyme is characterized by a unique class sensitivity, and the order of binding, as determined by the kinetic parameters utilized, does not bear an inverse relationship to the number of polar groups on the steroid nucleus.

The utilization of the kinetic scheme given above pre-

TABLE IV: Site Specificity of Steroid Binding to Aldehyde Dehydrogenase.[a]

Steroids Utilized	K_A Values Used in Calculations ($M^{-1} \times 10^{-4}$)	Calculated V_i		Exptl V_i
		Nonidentical Sites	Identical Sites	
Progesterone Desoxycorticosterone	5.16 7.16	0.052	0.082	0.081
Progesterone Testosterone	5.16 2.76	0.086	0.112	0.101
Testosterone Desoxycorticosterone	2.76 6.17	0.071	0.096	0.107

[a] The data are most consistent with identical binding sites for the combinations of steroids tested (see also Table III). Velocity data are in arbitrary fluorometer units as described in the text. All steroids are at a concentration of 25 μM.

sents certain pitfalls related to steroid solubility, the ratio of bound steroid to free steroid, and the kinetic idiosyncrasies of each particular enzyme. Establishing K_A values depends upon a precise knowledge of the steroid concentration. The formation of steroid micelles or larger aggregations would lower the effective concentration of steroid in an indeterminate fashion, resulting in an erroneously low value for the apparent association constant. Our confidence that we had not exceeded steroid solubility in the aqueous reaction mixtures utilized was accrued by light-scattering studies of such mixtures containing increasing concentrations of several representative steroids. Optical studies described above established that with both glucose 6-phosphate dehydrogenase and glutamate dehydrogenase the total steroid bound never exceeded 5% of the total steroid added even at the lowest steroid concentrations used.

The assumption that $n = 2$ with glutamate dehydrogenase (*i.e.*, that 2 moles of steroid bind to completely inactivate 1 mole of catalytic site giving rise to the complex EI_2) raises the question as to the existence of an EI species and its effect on the catalytic site. The consistent demonstration of $n = 2$ by Eyring plots with extremely high linear correlation coefficients indicates that such an EI species is present in insignificant amounts to be detected by our kinetic method. Finally, it must be pointed out that the conclusion "$n = 2$" is valid only if there is no interaction between steroid binding sites. Clearly, allosteric effects (interaction between two or more spatially separated sites on the protein so that binding of steroid at one changes binding affinity of the other) could be responsible for the results shown in Figures 2 and 5.

The present work offers something of a quantitative kinetic approach to a middle ground of protein–steroid interaction. Steroid binding sites of high affinity are characterized by association constants in the range 10^6–10^9 M^{-1}. These sites may occur in molecules such as transcortin (Muldoon and Westphal, 1967) and the uterine receptor protein (Toft and Gorski, 1966) or they may be the catalytic site in steroid-metabolizing enzymes such as human placental 17β-hydroxy steroid dehydrogenase (Warren and Crist, 1967). They are relatively specific, demonstrating high affinity for only a few steroids, which compete with one another for the binding site. In contrast, steroid binding sites of moderate affinity are characterized by association constants in the range of 10^5–10^8 M^{-1}. They are found in plasma proteins such as albumin and enzymes like those studied above. They may exhibit moderate "class" specificity.

It is attractive to assume that binding at sites of moderate affinity is due to Van der Waal's forces and hydrophobic bonding between the steroids and areas of the protein containing tyrosine, phenylalanine, tryptophan, and other hydrophobic residues. Indeed, Wildnauer and Canady (1966) have put forth elegant evidence that formation of an enzyme–inhibitor complex by α-chymotrypsin and low-polarity hydrocarbon inhibitors is analogous to an extraction process with the binding site on the enzyme acting as a nonaqueous phase.

Fisher *et al.* (1962) have suggested that tyrosyl hydrogen bonding is important in maintaining the structural integrity of glutamate dehydrogenase subunits. While the steroid A ring should be capable of stacking with the tyrosyl residue to effect a disruption leading to an inhibition that kinetically would be noncompetitive, the comparative effectiveness of estradiol-17β, progesterone, and estradiol-17α indicate clearly that factors other than the A ring are involved.

Nevertheless, for several enzymes of metabolic importance, the existence of steroid binding sites of moderate affinity and specificity which are capable of affecting catalytic activity with a physiologic significance as yet to be fully evaluated, is beyond doubt.

Acknowledgment

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Spectrophotometric Measurements of the Kinetics of Ca^{2+} and Mn^{2+} Accumulation in Mitochondria*

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ABSTRACT: The cation-sensitive indicator murexide was used to measure Ca^{2+} and Mn^{2+} accumulation by rat liver mitochondria. The absorbance change of murexide caused by Ca^{2+} or Mn^{2+} was measured in a dual-wavelength spectrophotometer at 540–510 mμ. Close to 100% of the indicator was found to be in the medium outside the mitochondria and thus murexide gives a direct measure of the extramitochondrial Ca^{2+} concentration. It is shown that the half-time of the accumulation of 380 μM Ca^{2+} in the absence of permeant anions is about 15 sec. Mn^{2+} accumulation is three to four times slower than Ca^{2+} accumulation. However, 25 μM Ca^{2+} added to the mitochondrial suspension together with 380 μM Mn^{2+} accelerates the Mn^{2+} uptake considerably. The permeant anion acetate facilitates the accumulation of the divalent cations. The kinetics of the Ca^{2+} accumulation are compared with the kinetics of the concomitant intramitochondrial pH change. The murexide technique as a kinetic method for measuring divalent cation accumulation in mitochondria is discussed.

The respiratory carriers respond to divalent cation accumulation very rapidly. The half-time of the oxidation of cytochrome *b* due to Ca^{2+}-activated electron transport is about 30–50 msec (Mela, 1968a). The Ca^{2+}-induced change of the intramitochondrial pH measured by bromothymol blue (Chance and Mela, 1966a) is rather slow with a half-time of approximately 20 sec. It has been difficult to correlate these changes to the actual cation accumulation by the mitochondria, because of the lack of adequate techniques for measuring the kinetics of the ion movements. Cation-sensitive electrodes are not ideal due to their slow response time (2–10 sec) (Chance and Yoshioka, 1966). Atomic absorption and radioactive ^{45}Ca measurements require 15–30

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