A Study of the Mechanism by Which Steroid Hormones Influence Rabbit Liver Aldehyde Dehydrogenase

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It has recently been reported (1) that rabbit liver contains a diphosphopyridine nucleotide-linked aldehyde dehydrogenase that is sensitive to a variety of steroid hormones and to the estrogen analogue diethylstilbestrol. One group of steroids uniformly inhibited the reaction; another group of steroids and diethylstilbestrol either inhibited or stimulated, depending upon the experimental conditions; a third group containing many structurally related steroids was not effective in the system.

Yielding and Tomkins (2) have reported that steroids inhibit glutamic dehydrogenase by favoring a dissociation of the enzyme into subunits. The present investigation was undertaken in an effort to determine whether steroids act similarly in the aldehyde dehydrogenase system by altering the configuration of the enzyme. Since a pure preparation of this protein is not available, an indirect approach was necessary. The rate of the reaction was measured in the presence of compounds known to alter the secondary or tertiary structure of proteins in aqueous solution, and the effect of these compounds on the response to steroids was studied. Progesterone, one of the steroids that uniformly inhibit the reaction, and diethylstilbestrol, which either inhibits or stimulates depending upon the experimental conditions, were examined in detail. In the standard assay with propionaldehyde and diphosphopyridine nucleotide (1), a low concentration of urea, guanidine hydrochloride, or sodium dodecyl sulfate altered the rate of the reaction in a manner similar to diethylstilbestrol, while the effects of higher concentrations mimicked those of progesterone. Furthermore, selected concentrations of any one of the three compounds abolished both the stimulation by diethylstilbestrol and the inhibition by progesterone. In addition, the effect of selected concentrations of urea on the rate of the reaction was studied under a variety of conditions. Without exception, the analogy between a low concentration of urea and diethylstilbestrol and between a higher concentration of urea and progesterone was maintained. The data suggest that both diethylstilbestrol and progesterone may indeed act in this system by altering the secondary or tertiary structure of the enzyme or enzyme substrate complex.

Centrifugation in sucrose gradients did not indicate a change in sedimentation behavior of the steroid-treated enzyme, as was observed by Yielding and Tomkins (2) with glutamic dehydrogenase.

EXPERIMENTAL PROCEDURE

Progesterone, diethylstilbestrol, DPN, and DPNH were obtained from Sigma Chemical Company; 3-acetylpyridine-DPN was from Pabst Laboratories; lactic dehydrogenase was from Worthington Biochemical Corporation; ion exchange cellulose compounds were from the California Corporation for Biochemical Research. Enzymic activity was assayed as previously described (1) by the rate of DPN reduction in the presence of diphosphopyridine nucleotide. Progesterone or diethylstilbestrol was added, where indicated, in 5 µl of methanol; methanol alone was added to the control reaction mixtures. The rate of the reaction was proportional to enzyme concentration and to time for at least 10 minutes under all the conditions studied. Centrifugation in sucrose gradients was performed by the method of Martin and Ames (3).

Preparation of Enzyme—Fresh rabbit livers were homogenized, centrifuged, and fractionated with ammonium sulfate as previously described (1), except that the incubation at 37° was omitted. Further purification was carried out at 3-5° by the following modified procedure. The ammonium sulfate fraction (Fraction 2 (1)) containing the steroid-sensitive activity derived from one liver (in a volume of about 50 ml) was dialyzed for 15 hours against 4 liters of glass-distilled water. The resulting precipitate was centrifuged and discarded. To the clear supernatant fluid were added 10 g of carboxymethyl cellulose that previously had been washed with water on a Buchner funnel. The slurry was stirred for 10 minutes and filtered through the funnel. The cellulose cake was washed with 50 ml of distilled water, and the wash liquor was combined with the original filtrate. The resulting turbid solution was centrifuged, and the precipitate was discarded. To the clear supernatant fluid were added 1 m Tris buffer, pH 7.4, to a final concentration of 0.002 M, and the pH of the mixture was adjusted to 7.0 by the addition of a few drops of 2 N HCl. The solution was passed through a column (2 x 15 cm) containing 9 g of diethylaminoethyl cellulose thoroughly equilibrated with 0.002 M Tris, pH 7.4. The column was washed with 80 ml of the Tris buffer. Activity was eluted, usually between the 7th and 13th 8 ml fraction, by a linear salt gradient. The mixing flask contained 200 ml of 0.001 M phosphate buffer, pH 7.0, and the reservoir, 200 ml of 0.5 M NaCl in the same buffer. The most active fractions were pooled, and solid ammonium sulfate was added to 60% saturation (36.1 g/100 ml of solution). The precipitate was centrifuged and dissolved in 10 ml of distilled water containing 5 x 10^-4 M EDTA. The preparation used in the present investigation had a specific activity of approximately 1000 and catalyzed the reduction of 0.12 µmole of DPN per minute per mg of protein in the standard assay with propionaldehyde (1). Activity remained constant for 2 months when the preparation was stored at approximately -20°.
RESULTS

Experiments with Urea, Guanidine-HCl, Sodium Dodecyl Sulfate, and NaCl—The rate of the reaction as a function of urea concentration, without added steroid and in the presence of \(10^{-5}\) M diethylstilbestrol or progesterone is shown in Fig. 1. Urea alone (Fig. 1, \(\cdots\cdots\)), at concentrations below 3 M, stimulates the reaction. Maximal stimulation occurs at a concentration of approximately 2 M, and increasing the concentration above 3 M results in inhibition. The initial points on the three curves show that under the conditions of this experiment, 10\(^{-5}\) M diethylstilbestrol, in the absence of urea, stimulates the reaction 130%, whereas the same concentration of progesterone inhibits 70%. The degree of stimulation elicited by diethylstilbestrol (Fig. 1, \(\cdots\cdots\)) decreases with increasing urea concentration and becomes insignificant at about the point where maximal stimulation by urea alone occurs. The percentage of inhibition by progesterone (Fig. 1, \(\cdots\cdots\)) remains nearly constant up to 2 M urea and then gradually decreases to zero at about 3.6 M urea.

Under the conditions of the present experiment, diethylstilbestrol, in the absence of urea, stimulates at all concentrations where any effect is observed and, similarly, progesterone always inhibits. Thus, urea differs from both the hormones in that it can either stimulate or inhibit, depending upon its concentration. As reported previously (1), progesterone and diethylstilbestrol do not compete with each other in the system and presumably act at different sites. Urea at concentrations above 2 M abolishes the stimulation by diethylstilbestrol and at 3.6 M the inhibition by progesterone as well.

Similar experiments were carried out with guanidine-HCl and sodium dodecyl sulfate. These reagents, like urea, stimulate the reaction at low concentrations, inhibit at higher concentrations, and alter the response to added progesterone and diethylstilbestrol in an analogous fashion.

Fig. 2 shows the rate of the reaction as a function of NaCl concentration. Inhibition occurs as ionic strength is increased but, in contrast to the results shown in Fig. 1, the response to progesterone and diethylstilbestrol is not altered.

Thus, urea, guanidine-HCl, and sodium dodecyl sulfate, but not NaCl, can mimic both progesterone and diethylstilbestrol and abolish their effects on the rate of the reaction. These results suggest that the mechanism of action of progesterone and diethylstilbestrol in this system might be similar to that of urea. Consequently, the effects of selected concentrations of urea on the rate of the reaction under a variety of conditions were compared with those of diethylstilbestrol and progesterone.

Effect of Aldehyde Concentrations—As reported previously (1), 10\(^{-5}\) M diethylstilbestrol stimulates the reaction with high glyceraldehyde concentrations, inhibits when aldehyde concentration is decreased and brings about an increase in the apparent \(K_m\) for this aldehyde. The results shown in Fig. 3 indicate that 1.8 M urea has a qualitatively similar but less marked effect. In the previous study, diethylstilbestrol (10\(^{-5}\) M) increased the glyceraldehyde concentration on response of aldehyde dehydrogenase to urea. Reaction mixtures of 0.5 ml volume contained varying amounts of glyceraldehyde, 5 \(\times\) 10\(^{-4}\) M DPN, 5 \(\times\) 10\(^{-4}\) M EDTA, 0.1 M glycine buffer, pH 9.0, and enzyme (60 \(\mu\)g of protein). \(\cdots\cdots\), without urea; \(\cdots\cdots\), with 1.8 M urea. Incubation was at 25° for 5 minutes.
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FIG. 4. Effect of urea on aldehyde dehydrogenase as a function of pH. Reaction mixtures of 0.5 ml volume contained $5 \times 10^{-4}$ M DPN, $5 \times 10^{-4}$ M propionaldehyde, $5 \times 10^{-3}$ M EDTA, $2 \times 10^{-4}$ M mercaptoethanol (mixed 0.1 M glycine-0.1 M phosphate at the indicated pH), and enzyme (30 µg of protein). --- without urea; O---O, with 1.8 M urea. Incubation was at 25° for 5 minutes.

Effect of pH—Fig. 4 shows the rate of the reaction in 1.8 M urea and the control rate as a function of pH. The pH optimum is shifted to a lower value in urea and no stimulation by this compound is observed at pH 10. With respect to both these observations, similar results were obtained with diethylstilbestrol (1).

Partial Inhibition by HMB—The effects of two concentrations of urea and of progesterone and diethylstilbestrol on the rate of the reaction partially inhibited by HMB are shown in Table I. In agreement with the results shown in Fig. 1, both diethylstilbestrol ($10^{-5}$ M) and urea (1.8 M) stimulate the reaction in the absence of the sulfhydryl reagent. In contrast, with $2 \times 10^{-3}$ M HMB, which itself inhibits the reaction about 30%, both diethylstilbestrol and 1.8 M urea bring about substantial additional inhibition. On the other hand, both progesterone ($10^{-5}$ M) and urea (3.5 M) inhibit the reaction without HMB, and this inhibition is abolished or decreased on the addition of HMB to the reaction mixture. Thus, the analogy between steroids and urea is maintained in the system partially inhibited by HMB.

Experiments with 3-Acetylpyridine-•DPN—The initial rate of the reaction at 25° with optimal concentration of substrates and in the absence of steroids is 2 to 3 times faster with acetylpyridine-•DPN than with DPN. The result of an experiment similar to that shown in Fig. 1, with 3-acetylpyridine-•DPN substituted for DPN, is shown in Fig. 5. The initial points on the curves show that diethylstilbestrol does not stimulate the reaction with this analogue of DPN, but, in contrast, brings about a decrease in the rate. Similarly, urea at concentrations that stimulate the reaction with DPN, inhibits with acetylpyridine-•DPN. Progesterone, on the other hand, maintains its inhibitory effect and the percentage of inhibition decreases with increasing concentration of urea.

1 The abbreviation used is: HMB, p-hydroxymercuribenzoate.

Table I

<table>
<thead>
<tr>
<th></th>
<th>Without HMB</th>
<th>With HMB ($2 \times 10^{-2}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>∆ O.D. 340</td>
<td>% change</td>
</tr>
<tr>
<td>Control</td>
<td>0.230</td>
<td></td>
</tr>
<tr>
<td>Stilbestrol, $10^{-5}$ M</td>
<td>0.480</td>
<td>+110%</td>
</tr>
<tr>
<td>Urea, 1.8 M</td>
<td>0.390</td>
<td>+70%</td>
</tr>
<tr>
<td>Progesterone, $10^{-5}$ M</td>
<td>0.060</td>
<td>-73%</td>
</tr>
<tr>
<td>Urea, 3.5 M</td>
<td>0.170</td>
<td>-26%</td>
</tr>
</tbody>
</table>

Ultracentrifugation in Sucrose Gradients—The analogy between the effects of steroids and those of urea suggests that the observed changes in catalytic activity might be the result of alterations in the configuration of the enzyme. In view of the finding of Yielding and Tomkins (2) that certain steroids inhibit glutamic dehydrogenase and favor a dissociation of the enzyme into subunits, ultracentrifugation of the aldehyde dehydrogenase in sucrose gradients was performed. The results of one experiment in which the enzyme was placed on the top of gradients containing the complete reaction mixture with no steroid and with progesterone or diethylstilbestrol are shown in Fig. 6. Crystal-lattice dextrin dehydrogenase was added to all three tubes as a marker. Apparently, neither progesterone nor diethylstilbestrol significantly altered the sedimentation velocity. The same amount of enzyme was used in all three tubes and the apparent difference in activity recovered is due to progesterone or stilbestrol in the aliquot used for assay. The effect of these compounds on the enzymic activity is reversible upon dilution or

FIG. 5. Effect of urea (●—●), urea plus diethylstilbestrol, $10^{-4}$ M (○—○), and urea plus progesterone, $10^{-3}$ M (△—△) on the rate of aldehyde dehydrogenase with acetylpyridine-•DPN. Conditions were the same as those described in Fig. 1, except that $1 \times 10^{-4}$ M acetylpyridine-•DPN was substituted for DPN, and 10 µg of enzyme was used.
Steroid-sensitive Aldehyde Dehydrogenase

Fig. 6. Recovery of aldehyde dehydrogenase after centrifugation in sucrose gradients without hormone (●●●●), with $10^{-4}$ M diethylstilbestrol (▲▲▲▲), and with $10^{-4}$ M progesterone (○○○○). An aqueous solution (100 μl), containing 1 mg of aldehyde dehydrogenase and 100 μg of lactic dehydrogenase, was layered on the top of sucrose gradients containing, in addition to the indicated hormone, $1 \times 10^{-2}$ M DPN, $1 \times 10^{-2}$ M propionaldehyde, $1 \times 10^{-2}$ M EDTA, $4 \times 10^{-2}$ M mercaptoethanol, and 0.1 M glycine, pH 9.0. Centrifugation was for 7 hours at 39,000 r.p.m. in the swinging bucket rotor of the Spinco model L preparative ultracentrifuge. After centrifugation, fractions were collected from the bottom of the tube, and aliquots were assayed for aldehyde dehydrogenase by the standard assay with propionaldehyde and for lactic dehydrogenase (○○○○) by the oxidation of DPNH in the presence of pyruvate. The location of the peaks of lactic dehydrogenase activity were essentially identical in all three tubes.

dialysis, but in this case, the final concentration in the assay mixture was $10^{-3}$ M. Similar experiments in which various components of the reaction mixture were omitted from the sucrose gave the same results. In all cases, single peaks of activity with essentially the same sedimentation velocity were obtained. The approximate sedimentation coefficient ($s_{20,w}$) of the enzyme determined from these experiments is 9.5 S. On the assumption that the enzyme has the same frictional characteristics as lactic dehydrogenase, the approximate molecular weight is 200,000.

Discussion

Under a variety of conditions, the activity of the aldehyde dehydrogenase investigated in these studies was altered in a qualitatively similar way by either low concentrations of urea or by diethylstilbestrol. Higher concentrations of urea appeared to mimic progesterone in this respect. Furthermore, selected concentrations of either urea, guanidine-HCl, or sodium dodecyl sulfate abolished both the stimulation by diethylstilbestrol and the inhibition by progesterone. These observations suggest that steroids may act in this system by a mechanism similar to that of urea or detergents. The mechanism by which urea affects proteins has been considered in a number of recent reports and reviews (4-9). There is general agreement that the reagent alters the secondary and tertiary structure and induces configurational changes in proteins. Both hydrophobic and hydrogen bonds are probably present in native globular proteins in aqueous solution, and it is not certain how these bonds are initially disrupted by urea. Tanford et al. (9) has concluded that the free energy responsible for the native structure of a globular protein in water is contributed largely by hydrophobic bonds between the apolar side chains. Steroids, because of their nonpolar structure might be expected to participate in hydrophobic bonding and to disrupt such bonding in proteins. Thus, proteins in which hydrophobic bonding is important for maintaining configuration could be altered by the addition of steroids. The aldehyde dehydrogenase investigated in these experiments may be such a protein. The data are, of course, only suggestive, and any definite conclusion regarding configurational changes in the enzyme must await investigation of a more highly purified preparation by physicochemical techniques.

Inhibition of the aldehyde dehydrogenase by sodium chloride indicates that ionic interactions may also be important for catalytic activity. Sodium chloride, unlike urea, does not alter the response to steroids, indicating that the sites of action are probably different.

Rajagopalan, Fridovich, and Handler (10) have recently reported that a number of enzymes are inhibited by urea in a competitive manner. It is also known that ribonuclease is unfolded by urea, although the activity is not diminished (11). In the latter case the substrate is believed to induce refolding of the enzyme. As reported previously (1), inhibition of aldehyde dehydrogenase by progesterone is not competitive with either aldehyde or DPN. However, the inhibition by 2 M urea and by diethylstilbestrol with low glyceraldehyde concentrations can be overcome by increasing the aldehyde concentration. In this case, the kinetics are complicated by the fact that stimulation occurs with higher concentrations of aldehyde.

Experimental evidence that the pyridine nucleotide substrate of a bacterial succinic semialdehyde dehydrogenase alters the configuration of the enzyme has been presented by Nirenberg and Jakoby (12). The attractive theory proposed by Koshland (13) and Levy et al. (14) that configurational changes induced in enzymes by their substrates can be reversed or altered by other substances, perhaps including hormones, could explain both the increased rate of the aldehyde dehydrogenase reaction when acetylpyridined*DPN is substituted for DPN and the differences in response to diethylstilbestrol with the two pyridine nucleotide substrates. If DPN, by virtue of its amine group, induces a configurational change in the enzyme which is unfavorable for catalytic activity, this change might be prevented by diethylstilbestrol. No experimental support for such an hypothesis has been obtained with the aldehyde dehydrogenase system. In any case, the data indicate that the amide group of DPN plays an essential role in the stimulatory mechanism. It is also probable from the results obtained with HMB that sulfhydryl groups on the enzyme participate in the stilbestrol-stimulated reaction.

No significant change in the sedimentation behavior of the steroid-treated enzyme was observed on centrifugation in sucrose gradients. In contrast, under similar conditions, steroids favor a dissociation of glutamic dehydrogenase into subunits with a...
lower sedimentation velocity (2). Thus, steroids may alter the configuration of aldehyde dehydrogenase without favoring its dissociation into subunits. Because of the difference in protein concentrations required for kinetic and ultracentrifugation experiments, it is not possible to rule out dissociation of the enzyme by steroids under conditions used in the kinetic studies.

**SUMMARY**

The rate of a steroid-sensitive aldehyde dehydrogenase reaction was measured in the presence of compounds known to alter the configuration of proteins in aqueous solution, and the effects of these compounds on the response to steroids were studied. With optimal concentrations of propionaldehyde and diphosphopyridine nucleotide, low concentrations of urea, guanidine hydrochloride, or sodium dodecyl sulfate stimulate the reaction, as does diethylstilbestrol, whereas higher concentrations inhibit, as does progesterone. Urea, in concentrations above 2 M, abolishes the stimulation by diethylstilbestrol and at 3.6 M the inhibition of progesterone as well.

The reaction is inhibited rather than stimulated by 1.8 M urea, as well as by diethylstilbestrol, under the following conditions: (a) with limiting concentrations of glyceraldehyde; (b) with the acetylpyridine analogue of diphosphopyridine nucleotide rather than with diphosphopyridine nucleotide; (c) in the system partially inhibited by p-hydroxymercurnbenzoate. Under the first of these conditions, both progesterone and 3.5 mM urea maintain their inhibitory effects. In the system with the acetylpyridine analogue of diphosphopyridine nucleotide, progesterone inhibits and the extent of inhibition decreases with increasing urea concentration. In the system partially inhibited by p-hydroxymercurnbenzoate, the inhibition by progesterone or by 3.5 mM urea is decreased or abolished.

The pH optimum of the reaction is shifted to a lower value by both 1.8 M urea and by diethylstilbestrol. No stimulation by these compounds is observed at pH 10.

The analogy between the effects of steroids and those of urea, together with the fact that selected concentrations of urea abolish the response to steroids, suggests that steroids act in this system by altering the configuration of the enzyme.

No significant change in the sedimentation behavior of the steroid-treated enzyme was observed on ultracentrifugation in sucrose gradients.

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