The Interaction of Mammary Glucose 6-Phosphate Dehydrogenase with Pyridine Nucleotides and 3\(\beta\)-Hydroxyandrost-5-en-17-one* 

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Glucose 6-phosphate dehydrogenase was first described in yeast by Warburg and Christian (1), who showed that nicotinamide adenine dinucleotide phosphate was the specific coenzyme. The enzyme was later prepared from liver and again shown to be specific for the same coenzyme (2). Subsequent preparations (see Cheldelin (3)) from various microbial sources have included two which react with both nicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide (4, 5). Recently, glucose 6-phosphate dehydrogenase has been crystallized from yeast (6) and bovine udder (7) and obtained in highly purified form from human erythrocytes (8).

The inhibition of glucose-6-P dehydrogenase by certain steroids was reported by McKerns (9) for the adrenal enzyme and by Marks and Banks (10) for the enzyme isolated from human erythrocytes. The latter authors showed that sensitivity to steroid inhibition was exhibited by glucose-6-P dehydrogenase from other mammalian sources, but not from spinach or yeast. A particularly effective inhibitor was 3\(\beta\)-hydroxyandrost-5-en-17-one (dehydroepiandrosterone).

We have recently reported (11) that under suitable conditions NAD could replace NADP in the oxidation of glucose-6-P catalyzed by glucose-6-P dehydrogenase from mammalian sources, but not with the yeast enzyme. Preliminary evidence suggested that the reactions with NAD and NADP were catalyzed by the same protein (11).

This paper describes experiments designed to elucidate the relationship between the NAD-linked and NADP-linked activities of glucose-6-P dehydrogenase and the mechanism of steroid inhibition. Evidence is presented that NAD and NADP compete for one site on the enzyme and that, in addition, NAD is bound at a second site. Dehydroepiandrosterone inhibits the NADP-linked reaction without altering the activity with respect to NAD. Under a variety of conditions, the two activities could be affected differentially and these findings have been interpreted as resulting from alterations in the spatial configuration of the enzyme.

EXPERIMENTAL PROCEDURE

Materials—Pyridine and adenine nucleotides were obtained from Pabst Laboratories or the Sigma Chemical Company. The sodium salts of glucose 6-phosphate and 6-phosphogluconate were obtained from the Sigma Chemical Company. Dehydroepiandrosterone was a commercial sample, recrystallized from methanol and sublimed in a vacuum. Urea was recrystallized from ethanol. Glycerol and \(\beta\)-mercaptoethanol were of analytical reagent quality. DEAE-cellulose and Ecteola-cellulose were obtained from Bio-Rad Laboratories and washed successively with 1 m KOH, 1 m potassium phosphate, pH 7.4, and distilled water to remove colored material and "fines." Sephadex G-50 was obtained from Pharmacia, Uppsala, Sweden, yeast alcohol dehydrogenase from Worthington Biochemicals Corporation, and yeast glucose-6-P dehydrogenase from C. F. Boehringer. The 6-phosphogluconic dehydrogenase was a partially purified preparation separated during the DEAE-cellulose chromatography of glucose-6-P dehydrogenase (11). Ammonium sulfate solution saturated at 4°C was neutralized with ammonium hydroxide and contained 0.001 M EDTA. Calcium phosphate gel was prepared by the method of Tsuboi and Hudson (12) and suspended in 20% (by volume) glycerol to a concentration of 30 mg (dry weight) per ml. All concentrations of glycerol are given by volume. When ammonium sulfate was added to solutions containing glycerol, the resulting degree of saturation was assumed to be independent of the glycerol concentration and was calculated as for aqueous solutions. The composition of the various buffers used during the purification of the enzyme is given in Table I.

Sprague-Dawley rats, 3 to 4 months old, were bred in our laboratory. Seven days after birth, all litters were reduced to 6 to 8 young. The mothers were killed 14 to 19 days post partum, at which time the activity of glucose-6-P dehydrogenase in the mammary glands is at its peak (13, 14).

Methods—Enzymatic assays were performed in a Beckman model DU spectrophotometer at 25°C unless otherwise noted. Assay mixtures contained the following components: 0.1 M Tris, pH 8.6; 3.3 mM glucose-6-P; and either 0.083 mM NADP or 5.0 mM NAD. During early stages of the enzyme purification, corrections for 6-phosphoglucuronid dehydrogenase were made by noting the difference in reaction rate obtained with two cuvettes, one with 3.3 mM 6-phosphogluconate and the other containing glucose-6-P and 6-phosphogluconate, each 3.3 mM (15). The reactions were initiated with glucose-6-P or enzyme and followed by noting the increase in absorbancy at 340 \(\mu\)m with time. One unit is defined as the amount of enzyme required to reduce 1 \(\mu\)mole of NADP per minute at 25°C. Reaction velocities with NAD analogues were measured at the appropriate \(\lambda_{max}\). All assays with NADP, NAD, and NAD analogues were performed under conditions of zero order kinetics. The
Composition of solutions used in enzyme purification

All media also contained 0.007 M β-mercaptoethanol and 0.001 M EDTA.

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>Potassium phosphate</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.5</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>7.5</td>
<td>0.005</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>7.5</td>
<td>0.005</td>
<td>10</td>
</tr>
<tr>
<td>D</td>
<td>7.5</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>7.5</td>
<td>0.005</td>
<td>20</td>
</tr>
</tbody>
</table>

The following values, at the indicated λ<sub>max</sub>, were used for the molar absorbancy coefficients: NADH and NADPH, 6,220 liters per mole cm at 340 mp; reduced nicotinamide hypoxanthine dinucleotide, 11,300 liters per mole cm at 395 mp (17). Reduced thionicotinamide adenine dinucleotide, 9,100 liters per mole cm at 363 mp; reduced acetylpyridine adenine dinucleotide, 6,200 liters per mole cm at 338 mp; and reduced thionicotinamide adenine dinucleotide, 13,300 liters per mole cm at 395 mp (17).

Protein concentrations were determined from absorbancy measurements at 260 mp and 290 mp (18).

Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer. Fluorescence intensities are given in arbitrary units. Fluorescence activation and emission spectra were obtained with an Electra Instruments XY recorder.

RESULTS

Isolation of Enzyme

Stabilization- The partially purified preparations of glucose 6-P dehydrogenase previously described (11) proved to be relatively unstable. When stored at -20°, such preparations retained full activity for approximately 1 week. After 2 weeks, 67% and after 3 weeks, 25% of the activity remained. The success in stabilizing the highly labile placental 17β-hydroxy-steroid dehydrogenase with glycerol (19) and conducting its entire purification in aqueous glycerol solutions (20) prompted us to try this method with glucose-6-P dehydrogenase. The enzyme was greatly stabilized by 20% or 50% glycerol in crude extracts of lactating mammary gland. Furthermore, when a partially purified preparation of glucose-6-P dehydrogenase with a specific activity of 33.9 units per mg of protein was stored in Medium B at a protein concentration of 20 µg per ml, at approximately 4°, the enzyme maintained its full activity for at least 6 months. No loss in activity could be detected on storing the enzyme solution at 23° for 1 week. Thus, glycerol affords striking protection to the enzyme, even at high dilution and in the absence of detectable quantities of bound NADP or NADPH (see below). The erythrocyte enzyme is stabilized by low concentrations of NADP or NADPH (8, 21, 22).

The procedure for purifying the enzyme is detailed below and data for one preparation are summarized in Table II.

Step 1. Preparation of Homogenate-Abdominal and inguinal mammary glands, obtained from 36 lactating rats 14 to 19 days post partum, were homogenized with 2 volumes of ice cold Medium A for 1 minute at full speed in a Waring Blender. All subsequent operations were performed at approximately 4°, except where noted otherwise. The homogenate was centrifuged for 10 minutes at 2,000 × g, and the supernatant fluid was filtered through cotton. The residue and supernatant fat pad were resuspended in one-half the initial volume of Medium A in a Waring Blender for 30 seconds. After low speed centrifugation, the supernatant solution was again filtered. The combined supernatant fractions were centrifuged for 30 minutes at 20,000 × g, the solution was carefully decanted and filtered through cotton.

Step 2. Ammonium Sulfate Precipitation—Ammonium sulfate was added, 1 g for every 3 ml, to bring the solution to 55% of saturation. The precipitate was centrifuged off and dissolved in Medium B to give three-fourths of the volume of the 20,000 × g supernatant solution (320 ml). The enzyme was reprecipitated by adding saturated ammonium sulfate solution to 55% of saturation. The precipitate was suspended in Medium B to give 570 ml and dialyzed against three aliquots of 5 liters of this medium for a total of 17 hours.

Step 3. Calcium Phosphate Gel—The dialyzed enzyme was diluted to a protein concentration of 25 mg per ml and an equal volume of calcium phosphate gel added. The suspension was allowed to stand for 1 hour and then centrifuged, and the clear supernatant solution was discarded. The enzyme was eluted from the gel with a mixture consisting of 4 volumes of Medium B and 1 volume of saturated ammonium sulfate.

Step 4. Ammonium Sulfate—The ammonium sulfate concentration of the calcium phosphate gel eluate was increased to 35% of saturation. The precipitate was allowed to accumulate overnight, centrifuged, and discarded. The ammonium sulfate

Table II

Purification of glucose-6-P dehydrogenase

<table>
<thead>
<tr>
<th>Step</th>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity*</th>
<th>Specific activity*</th>
<th>Yield</th>
<th>NAD+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Homogenate, 20,000 × g supernatant</td>
<td>700</td>
<td>25,800</td>
<td>5,550</td>
<td>0.215</td>
<td>100</td>
<td>8.7</td>
</tr>
<tr>
<td>2</td>
<td>(NH₄)₂SO₄ precipitate (55%)</td>
<td>600</td>
<td>15,300</td>
<td>5,010</td>
<td>0.328</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Calcium phosphate gel eluate</td>
<td>560</td>
<td>3,230</td>
<td>5,100</td>
<td>1.58</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>(NH₄)₂SO₄ precipitate (35-60%)</td>
<td>204</td>
<td>1,590</td>
<td>3,570</td>
<td>2.25</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>DEAE-cellulose eluate</td>
<td>155</td>
<td>507</td>
<td>2,700</td>
<td>5.33</td>
<td>49</td>
<td>10.5</td>
</tr>
<tr>
<td>6</td>
<td>Ecteola cellulose eluate</td>
<td>7.0</td>
<td>88</td>
<td>1,930</td>
<td>15.4</td>
<td>94</td>
<td>7.5</td>
</tr>
<tr>
<td>7</td>
<td>(NH₄)₂SO₄ precipitate (30-40%)</td>
<td>5.0</td>
<td>34.5</td>
<td>750</td>
<td>21.8</td>
<td>13.5</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>(NH₄)₂SO₄ precipitate (37-47%)</td>
<td>4.8</td>
<td>7.9</td>
<td>355</td>
<td>44.6</td>
<td>6.4</td>
<td>10.1</td>
</tr>
</tbody>
</table>

* Activities refer to NADP-linked reaction.
† % NAD = activity of NAD-linked, as a percentage of activity of NADP-linked glucose-6-P dehydrogenase, under the conditions given in the text. Note that the NAD concentration used in these assays is well below the K<sub>m</sub>.
concentration of the supernatant solution was brought to 60% of saturation and the precipitate was allowed to accumulate overnight.

Step 5. DEAE-cellulose—The precipitate was dissolved in Medium C, and the solution was dialyzed against large volumes of Medium C and applied to a column (3.2 × 21 cm) of DEAE-cellulose previously equilibrated with Medium C. The enzyme was eluted from the column at room temperature with a gradient of increasing phosphate concentration. The top of the column was connected to a constant volume (300 ml) mixing chamber containing Medium C; this was connected to a reservoir filled with Medium D. Fractions of 20 ml were collected. Most of the enzyme emerged in Fractions 11 to 17, which were combined and dialyzed against large volumes of Medium E overnight in the cold. A precipitate which formed on dialysis was removed by centrifugation.

Step 6. Ecteola-cellulose—The enzyme was applied to an Ecteola-cellulose column (1.6 × 16 cm) previously equilibrated with Medium E and eluted at room temperature with a gradient of increasing KCl concentration. This gradient was achieved by allowing a solution of 1 M KCl in Medium E to flow into a mixing chamber connected to the top of the column and filled with Medium E. Fractions of 15 ml were collected. The bulk of the enzyme emerged in Fractions 3 and 4 and was precipitated with ammonium sulfate (60% of saturation).

Step 7. Ammonium Sulfate Fractionation—The enzyme protein was redissolved in Medium B and fractionated with saturated ammonium sulfate. Most of the enzyme precipitated between 30 and 40% of saturation. It was dissolved in Medium B, and the ammonium sulfate was removed by passing the solution through a column (1.2 × 11.2 cm) of Sephadex G-50, previously equilibrated with Medium B. On careful refractionation of this preparation with ammonium sulfate, the bulk of the enzyme precipitated between 37 and 47% of saturation.

The overall purification was approximately 200-fold over the 20,000 g supernatant solution of the initial homogenate. The specific activity was 4.6 units per mg of protein. This value may be compared with a specific activity of 67.5 units per mg of protein for the crystalline enzyme from bovine udder (7) and 670 units per mg of protein for the crystalline enzyme from yeast (6). The purified mammary enzyme was devoid of 6-phosphogluconic dehydrogenase activity. The ratio of absorbancies at 280 μg to 260 μg was 1.56.

Properties of Enzyme

Several experimental approaches were used to investigate the interrelationship between the NAD-linked and NADP-linked reactions and the mechanism of steroid inhibition of the latter. These include the effects of different reagents on the two activities; kinetic analyses; the effects of various means of altering the three-dimensional structure of the enzyme; and fluorescence measurements.

Products of NAD-linked Reaction—The products of the oxidation of glucose-6-P by NAD were identified in two experiments with enzyme of specific activity = 5.0 units per mg of protein and devoid of any measurable 6-phosphogluconic dehydrogenase activity (11). In one experiment, glucose-6-P was used to reduce a limiting amount (111 μmole) of NAD in the presence of the enzyme. When the optical density change indicated that 95% of the NAD had been reduced, excess acetaldehyde and yeast alcohol dehydrogenase were added which led to the rapid and quantitative discharge of the absorbancy at 340 μm. In the second experiment, a limiting quantity of glucose-6-P (49.9 μmole) was added to a mixture containing excess SAD and enzyme. The change in absorbancy at 340 μm indicated that a quantity of NAD had been reduced equivalent to the quantity of glucose-6-P added. The further addition of NADP and 6-phosphogluconic dehydrogenase now resulted in a change in absorbancy at 340 μm, equivalent to 103% of the initial change. Under these conditions, 6-phosphogluconic dehydrogenase does not interact with NAD. These experiments indicate that the enzyme catalyzes a stoichiometric reaction between glucose-6-P and NAD which leads to the formation of equimolar quantities of 6-phosphogluconate and NADH.

Kinetic Constants—The Michaelis constants of the enzyme for NAD, NADP, and for glucose-6-P in the presence of NAD and NADP are summarized in Table III. The Kₘ for glucose-6-P in the NAD-linked reaction was shown to be the same at two NAD concentrations, both below the Kₘ. The Michaelis constant for NAD is greater by some three orders of magnitude than it is for NADP; for glucose-6-P it is 16 times higher in the presence of NAD than it is with NADP.

The Michaelis constant for NADP reported for the normal erythrocyte enzyme is 2.1 × 10⁻⁶ M (23) or 4.2 × 10⁻⁶ M (22); Lowry et al. (24) found a value of 2.79 × 10⁻⁶ M for the enzyme from rabbit brain. The observed Kₘ for glucose-6-P is in good agreement with determinations of 3.9 × 10⁻⁴ M (23) and 3.5 × 10⁻⁴ M (22) reported for the erythrocyte enzyme, and in fair agreement with McLean's value of 5.8 × 10⁻⁵ M (14) for the crude mammary enzyme.

Earlier experiments (11) indicated that certain NAD analogues could also react in the glucose-6-P dehydrogenase reaction. When 5.0 μm nucleotides were used, the relative rates of glucose-6-P oxidation at pH 8.5 were: NAD, 100; thionicotinamide adenine dinucleotide, 220; nicotinamide hypoxanthine dinucleotide, 11, acetylpyridine adenine dinucleotide, 0 (11). The Michaelis constants for these analogues have not been determined.

Phosphate and Magnesium—The inhibition, by phosphate, of the NADP-linked reaction (25) is less marked (83% at 0.33 M) than the inhibition of the NAD-linked reaction (81% at 0.33 M).
Magnesium ion stimulates the NADP-linked reaction slightly (26) but inhibits the NAD-linked reaction (Fig. 1).

**Effect of pH**—The rate of the NADP-linked reaction is substantially unaltered between pH 7 and 10 in Tris buffer (Fig. 2). In either phosphate or bicarbonate buffers, the NADP-linked reaction is somewhat slower than it is in Tris at the same pH. In contrast to these results, the rate of the NAD-linked reaction is strongly dependent on pH (Fig. 2). In Tris buffer, at pH 8.8, the reaction is 6 times as fast as at pH 7.0. A similar pH-dependence was observed when thionicotinamide adenine dinucleotide was used in place of NAD. The reaction with NAD is considerably slower in phosphate buffer than in Tris. In carbonate-bicarbonate buffer, however, the NAD reaction is strongly stimulated (Fig. 2).

**Bicarbonate**—The striking stimulation of the NADP-linked reaction by bicarbonate was investigated further. Fig. 3 shows the effect of bicarbonate concentration on the rate of pyridine nucleotide reduction. NADP reduction is not stimulated even at low bicarbonate concentrations and is inhibited at higher concentrations. NAD reduction is stimulated optimally at approximately 0.1 M bicarbonate. Potassium bicarbonate, but not sodium chloride, elicits the same effect as sodium bicarbonate. The stimulation by a given concentration of bicarbonate does not depend on the concentration of NAD. The $K_m$ for NAD is not substantially altered in the presence of bicarbonate (Fig. 4).

**Inhibition of NAD-linked Reaction by NADPH**—Very low concentrations of NADP strongly inhibit the reduction of NAD by glucose-6-P. These experiments were performed by adding NADP, which was rapidly reduced under the experimental conditions. Thus, NADP added to give final concentrations of 1.6, 4.0, and 20.8 mM produced 33, 58, and 97% inhibition, respectively, when 5.0 mM NAD was used. The effect of varying the NAD concentration at different levels of NADP indicated (Fig. 5) that small quantities of NADP behaved as a competitive inhibitor at high concentrations of NAD, but that
as the NADP concentration was increased, or the level of NAD diminished, the reaction kinetics were anomalous. Since the quantities of NAD used were all below $K_m$, these results suggest that in the presence of low concentrations of NADP raising the NAD concentration leads to a disproportionate increase in activity for two reasons: a displacement of inhibitory NADP by NAD on one site, and binding of NAD at a second site.

Dehydroepiandrosterone—The nature of the dehydroepiandrosterone inhibition of erythrocyte glucose-6-P dehydrogenase was investigated by Marks and Banks (10) who showed that the inhibition by the steroid was not competitive with respect to either glucose-6-P or NADP. We have confirmed these findings with the mammary enzyme.

The reaction with NAD is unaffected by dehydroepiandrosterone, at steroid concentrations up to $1 \times 10^{-4}$ M, where the reaction with NADP is 86% inhibited. When $2 \times 10^{-4}$ M dehydroepiandrosterone was used, the NADP-linked reaction was 20% inhibited. Table IV summarizes these data and includes experiments indicating that the steroid effect is not dependent on pyridine nucleotide concentration.

Since the inhibition of the NADP-linked reaction by dehydroepiandrosterone is not competitive with respect to either NADP or glucose-6-P, it would seem that the steroid may be bound at a separate site on the enzyme, closely juxtaposed on the active site in the folded three-dimensional protein structure. Various means are available for altering this spatial configuration and, thus, for testing such a possibility by noting the effect on dehydroepiandrosterone inhibition. The addition of urea or glycerol and the alteration of the pH or temperature of the reaction were examined in this respect.

**Fig. 5.** Plot of reciprocal of initial velocity of NAD-linked reactions (v) versus reciprocal of molarity of NAD; O—O, without NADP; O—O, with 1.0 µM NADP; O—O, with 8.33 µM NADP. Specific activity of enzyme = 21.8 units per mg of protein. Assay conditions as in text.

**Table IV**

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Concentration of coenzyme</th>
<th>Concentration of dehydroepiandrosterone</th>
<th>Activity of controls</th>
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</thead>
<tbody>
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<td>Experiment 1</td>
<td></td>
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<td>%</td>
</tr>
<tr>
<td>NADP</td>
<td>0.083</td>
<td>0.1</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>86</td>
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<tr>
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<td></td>
<td>10</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>NADP</td>
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<td>10</td>
<td>50</td>
</tr>
<tr>
<td>NADP</td>
<td>5.0</td>
<td>10</td>
<td>47</td>
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<tr>
<td>Experiment 3</td>
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<td></td>
<td>%</td>
</tr>
<tr>
<td>NAD</td>
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<td>100</td>
<td>107</td>
</tr>
<tr>
<td>NAD</td>
<td>5.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NAD</td>
<td>5.0</td>
<td>200</td>
<td>81</td>
</tr>
</tbody>
</table>

**Urea**—The inhibitory effect of urea on the NADP-linked reaction was found to depend both on the urea concentration and the time of initial incubation. In the experiment illustrated in Fig. 6, the enzyme was incubated with urea for exactly 5 minutes and then assayed in the presence of the same concentration of urea. Both the NAD-linked and NADP-linked reactions are sensitive to urea inhibition. The addition of urea leads to a decrease in the steroid sensitivity of NADP-linked glucose-6-P dehydrogenase; the NAD-linked reaction remains insensitive to dehydroepiandrosterone. Since dehydroepiandros-
Fig. 6. The effect of urea and dehydroepiandrosterone on the rate of reaction. Enzyme was incubated at 23°C with the indicated concentrations of urea. Aliquots, removed at exactly 5 minutes, were added to cuvettes which contained the usual assay components plus the same concentrations of urea present in the incubation mixture, and 10 μl of dioxane, with or without dehydroepiandrosterone (final concentration = 10 μM). O—O, with NADP, 0.038 unit of enzyme; □—□, with NADP, dehydroepiandrosterone, 0.038 unit of enzyme; ◦—◦, with NAD, either with or without dehydroepiandrosterone, 0.39 unit of enzyme; x—x, rate of NADP-linked reaction in the presence of dehydroepiandrosterone as a percentage of rate in absence of steroid. Specific activity of enzyme = 38.1 units per mg of protein for the NADP-linked, and 21.8 units per mg of protein for the NAD-linked reaction.

When the log of the reaction velocity was plotted against the reciprocal of the absolute temperature, straight lines were obtained from which the Arrhenius heat of activation, $\Delta H^\circ$, was determined. The NADP-linked reaction gave a value of 11,400 calories without, and 13,700 calories with added dehydroepiandrosterone; with NAD or thionicotinamide adenine dinucleotide the value (determined between 15°C and 35°C) was 14,900 calories.

High pH—The effect of high pH on the NADP-linked reaction was tested. It is clear (Fig. 9) that dehydroepiandrosterone partially prevents the loss of activity with increased pH; but at higher temperatures the reverse was true (Fig 8B). This may be a reflection of the protective effect on the enzyme exerted by NADP but not by NAD (8, 22). At none of the temperatures tested did dehydroepiandrosterone inhibit the reaction with NAD or with thionicotinamide adenine dinucleotide. When the log of the reaction velocity was plotted against the reciprocal of the absolute temperature, straight lines were obtained from which the Arrhenius heat of activation, $\Delta H^\circ$, was determined. The NADP-linked reaction gave a value of 11,400 calories without, and 13,700 calories with added dehydroepiandrosterone; with NAD or thionicotinamide adenine dinucleotide the value (determined between 15°C and 35°C) was 14,900 calories.

Glycerol—The addition of increasing concentrations of glycerol to the reaction cuvette leads to a progressive diminution of NADP-linked activity, but causes some stimulation in the reaction with NAD (Fig. 7). Like urea, glycerol appears to overcome the inhibitory effect of dehydroepiandrosterone on the NADP-linked reaction; in 50% glycerol, in which the enzyme exhibits only 10% of its original NADP-linked activity, this inhibition is entirely abolished. The rate of the NAD-linked reaction in glycerol is not affected by dehydroepiandrosterone. Furthermore, the inhibition by NADP of the NAD-linked reaction can be partially overcome in glycerol as shown in Table V. That this is not the result of a decrease in NADP-binding by the enzyme in glycerol solution is suggested by the identical fluorescence titrations in aqueous and 40% glycerol solutions shown in Fig. 12 (see later).

Temperature—The effect of dehydroepiandrosterone on the NADP-linked reaction was examined at temperatures between 15 and 48°C. The results suggest that the steroid becomes a less effective inhibitor at higher temperatures (Fig. 8A). Between 15 and 35°C, the increase in reaction rate with NAD and thionicotinamide adenine dinucleotide was greater than with NADP.

**TABLE V**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Optical density change per minute</th>
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<tbody>
<tr>
<td></td>
<td>Aqueous solution</td>
</tr>
<tr>
<td>None</td>
<td>0.023</td>
</tr>
<tr>
<td>NADP, 2.0 μM</td>
<td>0.004</td>
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<tr>
<td>NADPH, 0.5 μM</td>
<td>0.001</td>
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</table>
Fluorescence Studies—Fluorescence activation and emission spectra for glucose-6-P dehydrogenase and NADPH suggest that there is very little, if any, bound NADPH in the purified enzyme (Fig. 10). The addition of glucose-6-P to the enzyme does not lead to a detectable increase in fluorescence near 470 nm, showing that little, if any, NADP is bound to the enzyme. The fluorescence of added NADPH is markedly enhanced when it is bound to the enzyme (Fig. 10B, and see Kirkman (8)). Thus, glucose-6-P dehydrogenase behaves like

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Fig. 8. The effect of temperature and dehydroepiandrosterone on the rate of reaction. All components of the assay mixture, except enzyme, were incubated in cuvettes in the cell housing of the spectrophotometer at the indicated temperature for 15 minutes, until the cuvettes had reached temperature equilibrium. Enzyme was added to initiate the reaction. Temperatures were measured at the end of the reaction time. Velocities (v) are expressed in terms of Δ O.D. per minute per unit of enzyme. A, NADP-linked reaction, symbols same as for Fig. 6. B, ○—○, with NAD, either with or without dehydroepiandrosterone; △—△, with 0.3 mM thionicotinamide adenine dinucleotide, either with or without dehydroepiandrosterone. Steroid was added in dioxane and steroidless controls received the same volume of dioxane. Specific activity of enzyme = 38.1 units per mg of protein.

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Fig. 9. The effect of high pH and dehydroepiandrosterone on the rate of NADP-linked reaction. Reactions were carried out in 0.033 M carbonate-bicarbonate buffer, of the indicated pH, otherwise assay conditions as in text. ■—■, with 10 μM dehydroepiandrosterone. Specific activity of enzyme = 38.1 units per mg of protein. Other symbols same as for Fig. 6.

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Fig. 10. The fluorescence spectra of enzyme (○—○), NADPH (■—■), and enzyme plus NADPH (●—●). Spectra were taken before and after addition of 2 μl of 0.2 mM NADPH in a total volume of 1.0 ml and are all corrected for the "blank" spectrum given by Medium B and buffer only. A, activation spectra, measured at 470 nm. Cuvettes contained 0.1 M Tris-acetate, pH 7.2, and 0.05 ml of Medium B or 0.05 ml (0.1 mg of protein) of enzyme in Medium B. B, emission spectra, with exciting light of λ = 340 nm. Cuvettes contained 0.1 M Tris-acetate, pH 8.6, and 0.1 ml of Medium B or 0.1 ml (0.2 mg of protein, specific activity = 38.1 units per mg of protein) of enzyme in Medium B.

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most pyridine nucleotide dehydrogenases examined so far in conferring an enhanced fluorescence on bound, reduced pyridine
nucleotide (27). The activation spectrum of NADPH in the presence of enzyme (Fig. 10A) shows the presence of a large, new peak with a maximum near 285 nm, a spectral region where absorption by NADPH is minimal but protein absorption is maximal. This is strongly suggestive of activation of NADPH fluorescence by transfer of energy from excited aromatic amino acid residues in the enzyme to the bound NADPH (28). No such energy transfer could be detected when 20 times as much NADH was used in place of NADPH.

Advantage may be taken of the enhanced fluorescence of bound NADPH (Fig. 10B) to titrate the enzyme (Fig. 11). On the assumptions that the molecular weight of the enzyme is 100,000 and that 1 mole of enzyme binds 1 mole of NADPH, the enzyme used in this particular experiment was 55% pure. An enzyme preparation of lower specific activity gave a correspondingly lower end point and degree of purity. Comparison of the initial slopes in Fig. 11 indicates that bound NADPH fluoresces 3.0 times as intensely as the free nucleotide. Kirkman found a ratio of 2.7 with the erythrocyte enzyme (8). From the extrapolated end point at 0.55 mmole of NADPH, it can be calculated that 1 unit of enzyme binds $0.11 \times 10^{-4}$ mole of NADPH; Kirkman found that 0.12 $\times 10^{-4}$ mole of NADPH is bound per unit of erythrocyte glucose-6-P dehydrogenase. When similar measurements were made in the presence of 0.1 mm dehydroepiandrosterone, the fluorescence of NADPH and of the mixture of NADPH and enzyme was unaltered by the steroid.

Protein fluorescence is quenched when either NAD or NADH is bound by glyceraldehyde 3-phosphate dehydrogenases (28). The binding of either NADP or NADPH by glucose-6-P dehydrogenase also leads to the quenching of protein fluorescence. Thus, the binding of NADPH is accompanied by a simultaneous decrease in protein fluorescence (350 nm) and increase in fluorescence of the reduced pyridine nucleotide (460 nm), whereas NADP binding affects only the protein fluorescence. Since free NADPH is less fluorescent than the bound form, its release from the enzyme results in a decrease in fluorescence near 460 nm. Advantage was taken of these circumstances to observe the displacement of enzyme-bound NADPH by excess NADP and to show that NADPH is more tightly bound to glucose-6-P dehydrogenase than the oxidized nucleotide.

Titration of the enzyme with NADPH in 40% glycerol solution indicated that the presence of glycerol did not interfere with binding of the nucleotide (Fig. 12). Thus, it may be concluded that the inhibitory effect of glycerol on the NADP-linked reaction (Fig. 7) and the reversal of NADP inhibition of the NAD-linked reaction by glycerol (Table V) cannot be attributed to changes in NADP binding induced by the glycerol.

FIG. 12. Fluorescence titrations of enzyme with NADPH in aqueous and 40% glycerol solutions. Cuvettes contained 0.1 M Tris-acetate, pH 8.6, glycerol where indicated, 0.1 ml of Medium B, or 0.1 ml of enzyme (0.1 mg of protein; specific activity = 49.0 units per mg of protein) in Medium B, in a total volume of 1.0 ml. The NADPH (0.02 mm) was added in 1-μl increments. Exciting light of $\lambda = 300$ nm was used and fluorescence was measured at 400 nm (upper curves) and 350 nm (lower curves). Cuvette containing Medium B instead of enzyme.

The discovery that glucose-6-P dehydrogenase can react with NAD prompted an investigation of the nature of this interaction to determine its relationship to the NADP-linked activity. The following observations afford strong evidence for the fact that the oxidation of glucose-6-P by NAD or by NADP is catalyzed by a single protein. (a) The ratio of NAD- and NADP-linked
activities remains constant throughout the course of purification. (b) This ratio is the same in the glucose-6-P dehydrogenase preparations obtained from five different mammalian sources which were examined earlier (11). In these experiments, enzymes of widely differing specific activities were used and, in addition, the ratios of activities with NAD-salogenes and the effects of pH, dehydroepiandrosterone, and 2'-AMP were examined. In every respect, the five preparations gave closely similar results which were in sharp contrast to data obtained with a commercial preparation of yeast glucose-6-P dehydrogenase (11). Kirkman (8) has found that highly purified glucose-6-P dehydrogenase from erythrocytes will also react with NAD.

(c) Heat inactivation of the enzyme results in a parallel loss of both activities (11). (d) Kinetic measurements are not consistent with the idea that separate proteins are responsible for the NAD-linked and NADP-linked reactions.

It is a striking fact that in almost every respect, the NAD-linked and NADP-linked activities respond differently to various reagents and conditions. Thus, the addition of 2'-AMP (11) and the alteration of pH have little effect on the NADP-linked reaction but result in considerable changes in NAD-linked activity; dehydroepiandrosterone, on the other hand, and progesterone (11), inhibit the NADP-linked reaction at steroid concentrations which do not affect the reaction with NAD. The effect of Mg++ is to stimulate, slightly, the NADP-linked, but to inhibit the NAD-linked activity; glycerol, however, has the opposite effect, stimulating the NAD-linked, but inhibiting the NADP-linked reaction; and bicarbonate produces a strong stimulation of the NAD-linked reaction and an inhibition of the reaction with NADP.

Although all the experiments with NAD were performed at non-saturating concentrations, it seems unlikely that this fact alone could explain all the differential responses observed. Thus, bicarbonate stimulates the NAD-linked reaction without altering the $K_m$ for NAD (Fig. 4). An explanation that would be consistent with catalysis of both reactions by a single enzyme but allow for the observed differences between the two reactions is that the active sites for NAD and NADP are different.

Kinetic measurements (Fig. 5) suggest that NAD competes with NADP at one site but that it is also bound elsewhere. The data in Table V strongly suggest that the active site for NAD is not the site at which NADP is bound. The potent inhibition, by NADP, of the NAD-linked reaction in aqueous solution is substantially diminished in 40% glycerol. Since 40% glycerol apparently does not affect the binding of NADP to the enzyme (Fig. 12), this diminished inhibition occurs in spite of the fact that NADP is still bound to its active site, thus precluding access by NAD to this site. Since glycerol does not inhibit the reaction with NAD, it is unlikely that glycerol affects glucose-6-P binding.

The competitive nature of the NADP inhibition of the NAD-linked reaction at high NAD concentrations (Fig. 5) suggests that the binding of NAD at the NADP site must be involved in enzymatic activity. Possibly, the NADP site acts as an "activating" site for NAD, where binding of a second molecule of NAD enhances the reactivity of the NAD bound to its active site. This fact might be expected to yield aberrant reaction kinetics with NAD (29). However, if the affinity for NAD is greater at the "activating" than at the active site, normal kinetics should obtain at all but very low NAD concentrations.

Freden has reported (29) that beef liver glutamic dehydrogenase, which reacts with NAD and NADP at comparable rates, also binds NAD at two sites and NADP at only one. The active site for the two nucleotides in glutamic dehydrogenase, however, is identical and the second NAD-binding site serves as an "activating" site for the NAD-linked reaction (29). Other NAD-linked enzymes have been shown to bind 2 or 4 moles of NAD per mole of enzyme (see (30)) and a mechanism of dehydrogenase action has been proposed which requires that an even number of moles of NAD be bound per mole of enzyme (31).

Four procedures which can result in alteration in the three-dimensional structure of proteins (cf. Koshland (32)) were tested for their effect on the inhibition of the NADP-linked reaction by dehydroepiandrosterone. The addition of either urea or glycerol, raising the pH, and increased temperature all served, in varying degrees, to diminish the relative inhibition by the steroid. These results are all consistent with the concept that a change in the three-dimensional structure of the protein, induced by these various means, precludes the dehydroepiandrosterone from exerting its inhibitory effect by preventing the steroid-binding site from assuming a position sufficiently close to the active center. It is also possible that the various means here employed diminish the affinity of the enzyme for dehydroepiandrosterone and thus decrease the inhibitory effect directly.

The effect of glycerol is of particular interest since, like dehydroepiandrosterone, glycerol inhibits only the NADP-linked reaction. Furthermore, as in the case of dehydroepiandrosterone, glycerol does not inhibit by competing with NADPH for binding (Fig. 12). Thus, glycerol may lead to a deformation of the active center similar to that produced by dehydroepiandrosterone. This would explain the reversal of NADP inhibition of the NAD-linked reaction by glycerol (Table V). This deformation may also be related to the stabilizing effect of glycerol on the enzyme.

Recent studies by Maxwell and Topper (33, 34) on liver aldehyde dehydrogenase indicated that certain steroids, urea, guanidine hydrochloride, sodium dodecyl sulfate, and diethylstilbestrol alter the three-dimensional structure of the enzyme. The experiments here reported also suggest that dehydroepiandrosterone acts by deforming the geometry around the active site of glucose-6-P dehydrogenase. Maxwell (34) showed that, unlike glutamic dehydrogenase (35), the aldehyde dehydrogenase is not cleaved by subunits by steroids or diethylstilbestrol. Glucose-6-P dehydrogenase from erythrocytes is not cleaved by dehydroepiandrosterone (21). Experiments have not, so far, been undertaken to test whether mammary glucose-6-P dehydrogenase is cleaved by dehydroepiandrosterone, but the deformation may occur either with or without cleaving of the protein.

The significance of the interaction of glucose-6-P dehydrogenase with NAD, on the one hand, and dehydroepiandrosterone, on the other, is not yet clear. Preliminary experiments indicate that the incorporation of acetate-1-14C into long chain fatty acids, catalyzed by centrifuged homogenates of lactating rat mammary glands, is inhibited by dehydroepiandrosterone. These experiments were performed under conditions in which the glucose-6-P dehydrogenase reaction served to generate NADPH required for fatty acid synthesis.

Recent investigations on the mechanism of feedback inhibition in microorganisms are of interest in connection with the steroid

1 H. R. Levy, unpublished experiments.
effect because they demonstrate the interaction of enzymes with compounds structurally unrelated to the substrates. Gerhart and Pardee (36) showed that the inhibition of aspartate transcarbamylase in *Escherichia coli* by CTP resulted from the binding of CTP at a site distinct from the aspartate binding site, but that competition between CTP and aspartate occurred. Changeux (37) analyzed the feedback inhibition, by isoleucine, of 1-threonine deaminase in *E. coli*, and showed that kinetic and other experiments were compatible with the presence of two distinct, but interacting, sites on this enzyme. Monod and Jacob (38) have referred to this type of inhibition as “allosteric” inhibition and state that end product inhibition may merely be one type of allosteric effect. These authors, in discussing the glutamic-alanine dehydrogenase conversion observed by Tomkins (39), point out that such mechanisms are not observed in bacteria and that bacteria do not synthesize steroids. They suggest that the type of regulatory mechanisms which steroids control may, perhaps, occur only in differentiated organisms. It may, therefore, be significant that yeast glucose-6-P dehydrogenase does not respond to dehydroepiandrosterone (10, 11), and that perhaps this inhibition in the mammalian enzyme may reflect an allosteric inhibition with possible physiological importance.

**SUMMARY**

1. Glucose 6-phosphate dehydrogenase was isolated from mammary glands of lactating rats and purified 200-fold in the presence of glycerol as a stabilizing agent.

2. The oxidation of glucose 6-phosphate, normally associated with nicotinamide adenine dinucleotide phosphate (NADP), can also be effected by nicotinamide adenine diphosphate (NAD) and certain NAD analogues. This activity is not the result of a separate enzyme but is catalyzed by the same protein. The products of the oxidation of glucose 6-phosphate by NADP were identified as stoichiometric quantities of reduced NAD and 6-phosphogluconate.

3. The Michaelis constants for NADP and NAD were found to be 8.9 μM and 0.015 μM, respectively. The Michaelis constant for glucose 6-phosphate with NAD as coenzyme is 16 times higher than when NADP is the coenzyme.

4. The NAD-linked and NADP-linked activities were affected in different ways by various conditions or reagents. Thus, bicarbonate and glycerol stimulated the NADP-linked but inhibited the NADP-linked activity; Mg++, which produces a slight stimulation of the NADP-linked reaction, inhibited the activity with NAD. The NAD-linked reaction was strongly pH-dependent with an optimum around pH 8.8: the NADP-linked activity was relatively insensitive to pH changes. NADP and its reduced form are potent inhibitors of the NAD-linked activity and dehydroepiandrosterone inhibits the NADP-linked, but not the NAD-linked reaction.

5. Urea, glycerol, high pH, and increased temperature reverse dehydroepiandrosterone inhibition of the NADP-linked reaction. This is interpreted as resulting from alterations in the three-dimensional structure of the protein, thereby removing the steroid-binding site from proximity to the active center of the enzyme.

6. The interpretation of the interaction of glucose 6-phosphate dehydrogenase with NADP, NAD, and dehydroepiandrosterone is discussed.
The Interaction of Mammary Glucose 6-Phosphate Dehydrogenase with Pyridine Nucleotides and 3 β-Hydroxyandrost-5-en-17-one

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