Reversible Inactivation of Dehydrogenases*

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SUMMARY

1. The reversible dissociation of lactic and malic dehydrogenases has been studied in detail and compared with the dissociation properties of triosephosphate dehydrogenase, α-glycerophosphate dehydrogenase, and liver alcohol dehydrogenase.

2. Evidence is presented for the existence of an intermediate form of lactic dehydrogenase with catalytic properties altered during reactivation.

3. The technique of reversible dissociation has been used to prepare interspecies hybrids of lactic, malic, and triosephosphate dehydrogenases.

4. Some of the catalytic and immunological properties of hybrid malic dehydrogenases have been examined.

5. The effects of salts on the reactivation of malic dehydrogenases have been investigated and related to previous results on lactic dehydrogenases. It is proposed that the observed salt effects may result from changes in the activity coefficients of the exposed peptide and amide groups.

A previous report from this laboratory described the effects of various factors which modify the rates and extents of reactivation of lactic and malic dehydrogenase after these enzymes had been inactivated by guanidine hydrochloride, urea, or low pH (1). It was found that certain ions inhibited the reactivation process but that pyridine nucleotides markedly increased both the rate and extent of reactivation. Studies of binding of coenzyme to lactic dehydrogenase during reactivation from urea and comparisons of treated and untreated lactic dehydrogenase and malic dehydrogenase with regard to their physical, catalytic, and immunological properties were briefly discussed. The techniques of reversible inactivation were used to produce hybrids of mitochondrial malic dehydrogenases from different species supernatant and mitochondrial malic dehydrogenase from a single species, as well as hybrids of lactic dehydrogenases. Preliminary evidence for the existence of at least two catalytically active forms of lactic dehydrogenase during reactivation from guanidine hydrochloride was also presented. Various aspects of these studies have been examined in further detail and reversible inactivation by lithium chloride, as well as guanidine, acid, and urea, has now also been demonstrated. In our more recent investigations of malic dehydrogenase, particular emphasis has been placed on the effects of salts and reducing agents on reactivation. The nature of acid-dissociated malic dehydrogenase has been examined and the kinetic and immunological properties of hybrid malic dehydrogenases have been studied. In addition, the catalytic and physical properties of the forms of lactic dehydrogenase which are intermediates during reactivation have been further characterized. Also, we have extended our studies of reversible inactivation of dehydrogenases to include a comparison of lactic dehydrogenase and malic dehydrogenase with triosephosphate dehydrogenase, α-glycerophosphate dehydrogenase, and liver alcohol dehydrogenase.

EXPERIMENTAL PROCEDURE

Materials—AcPyDPN; DP, and DPNH were purchased from P-L Biochemicals, Inc. AcPyDPN was produced by enzymatic reduction of AcPyDPN according to the general method described previously (3). Sodium pyruvate and oxaloacetic acid were purchased from Nutritional Biochemicals, β-mercaptoethanol and guanidine hydrochloride from Eastman Kodak, urea from Fisher, and dithiothreitol and dihydroxyacetone phosphate from Calbiochem. Sephadex was purchased from Pharmacia, bovine serum albumin from Armour, and hydrolyzed starch from Connaught Medical Laboratories. Urea and guanidine hydrochloride were recrystallized from methanol.

Methods—Beef H , chicken M , and chicken H lactic dehydrogenases were prepared as described by Pesce et al. (2). Lactic dehydrogenase from dogfish (Squalus acanthias) muscle and bullfrog (Rana catesbiana) muscle were prepared in this laboratory.

1 The abbreviations used are: beef H , beef heart lactic dehydrogenase; chicken H , chicken heart lactic dehydrogenase; AcPyDPN, 3-acetylpyridine analogue of DPN; AcPyDPNH, 3-acetylpyridine analogue of DPNH; dogfish M , dogfish muscle lactic dehydrogenase; chicken M , chicken muscle lactic dehydrogenase; bullfrog M , bullfrog muscle lactic dehydrogenase. The nomenclature used for lactic dehydrogenases indicates both the tetrameric structure and subunit composition of the enzymes (2). Thus, H enzymes have four H-type subunits, M enzymes have four M-type subunits, and the hybrid lactic dehydrogenases H M , H M , and H M have the indicated subunit composition.

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Reversible Inactivation of Dehydrogenases

Effect of DPNH on reactivation of various lactic dehydrogenases from guanidine hydrochloride

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ratio of per cent reactivation with DPNH to that without DPNH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bullfrog Mdc.</td>
<td>1.0</td>
</tr>
<tr>
<td>Dogfish Mdc.</td>
<td>1.3</td>
</tr>
<tr>
<td>Chicken Mdc.</td>
<td>1.0</td>
</tr>
<tr>
<td>Rabbit Mdc.</td>
<td>1.0</td>
</tr>
<tr>
<td>Beef H4</td>
<td>2.0</td>
</tr>
<tr>
<td>Chicken H4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* All samples were inactivated in 5.3 M guanidine hydrochloride + 0.1 M β-mercaptoethanol + the indicated buffer (pH 7.5). Protein concentration varied from 0.6 to 0.9 mg per ml and samples were diluted 50 times in the indicated buffer + 0.1 M β-mercaptoethanol to reactivate. The DPNH concentration varied from 5.76×10⁻⁴ M to 8.85×10⁻⁴ M.

† In 0.1 M sodium phosphate, pH 7.5.

‡ In 0.1 M potassium phosphate, pH 7.5.

§ In 0.1 M Tris-HCl, pH 7.5. This was a commercial preparation containing hybrid lactic dehydrogenases, but was predominantly M4.

* This was a purified sample of beef heart lactic dehydrogenase containing H3M and some H2M2, but the predominant species was H4.

† The rates of reactivation of chicken H4 and beef H4 were markedly elevated by DPNH when the experiment was carried out in either Tris HCl or phosphate buffer.

Enzyme activity of α-glycerophosphate dehydrogenase was determined by measuring the initial rate of oxidation of DPNH (1.0×10⁻⁴ M) in the presence of 4.1×10⁻⁴ M dihydroxyacetone phosphate, in a solution containing 7.5×10⁻⁴ M triethanolamine hydrochloride + 0.001 M EDTA + 0.001 M β-mercaptoethanol, pH 7.5. Enzyme activities of malic dehydrogenase and lactic dehydrogenase were determined by measurement of initial rates of oxidation of DPNH as described by Thorne, Grossman, and Kaplan (8) and Fine, Kaplan, and Kufiniec (9), respectively. The degree of substrate inhibition of lactic dehydrogenases is expressed as a ratio (DPNHactivity/DPNHcontrol) of rates with low (3.3×10⁻⁴ M) and high (1×10⁻² M) concentrations of pyruvate. This ratio is extremely dependent on the DPNH preparation used, values between 3.0 and 4.2 being obtained with the native chicken H4 enzyme with different batches of the reduced coenzyme. This variation in DPNH appears to be due to an inhibitor which is present in different amounts in different batches and which accounts for the variation in ratios given in the text and figures for the native enzymes. Starch gel electrophoresis was performed as described by Fine and Costello (10).

Lactic Dehydrogenase

Effect of DPNH on Reactivation of Various Lactic Dehydrogenases from Guanidine Hydrochloride—We have previously reported that DPNH causes a marked increase in the rate and extent of reactivation of chicken H4 after denaturation in guanidine hydrochloride (1). The stimulatory effect of DPNH was most pronounced during the initial stages of reactivation. Several additional lactic dehydrogenases have been examined with respect to the effect of DPNH on reactivation. The data are presented in Table I and are expressed as the ratio of the extent of reactivation in the presence of DPNH to that of a control without DPNH. In all cases the enzymes were compared 30 min after reactivation was started. The heart lactic dehydrogenase, beef H4 and chicken H4, reactivated much faster in the presence of DPNH. Of the four muscle enzymes tested, only dogfish M3 reacted more rapidly in the presence of coenzyme, and the effect was small. This difference between heart and muscle lactic dehydrogenases may reflect tighter binding of DPNH to the heart enzymes.

It has been shown that the reduced coenzyme does not bind to urea-denatured lactic dehydrogenase in the presence of high concentrations of urea (11). The stimulatory effect of DPNH on reactivation raised the possibility that on dilution the dissociated enzyme would begin to refold and that DPNH might stimulate reactivation by binding to these intermediate forms of the enzyme. One might expect to demonstrate binding of coenzyme prior to detection of enzyme activity. In order to test this possibility, chicken H4 was inactivated in urea and then diluted in order to permit reactivation. At various times duplicate aliquots were withdrawn and assayed for both enzyme activity and ability to bind coenzyme. Coenzyme binding was determined by measuring enhancement of fluorescence on addition of AcPyDPNH. The data summarized in Fig. 1 show that there was no detectable lag between coenzyme binding and appearance of enzyme activity. If AcPyDPNH did interact with inactive lactic dehydrogenase, it did so without a detectable enhancement of fluorescence.
Evidence for Intermediate Forms of Enzyme during Reactivation

The results of several experiments suggest that there are at least two active forms of the enzyme which are present in varying proportions during reactivation. We have previously shown that the degree of substrate inhibition by pyruvate increases as enzyme activity reappears and eventually approaches a level comparable to untreated enzyme; this change in catalytic property was accompanied by a change in the degree of hybridization of beef H4 and chicken H4 (1). These two forms were denatured separately in guanidine hydrochloride and diluted separately to reactivate. Aliquots of the separately reactivating enzymes were mixed at various times during reactivation. A progressive decrease in the extent of hybridization was observed as reactivation proceeded. These observations still left open the possibility that hybridization was occurring between molecules which had not yet regained enzymatic activity.

This phenomenon has been investigated in further detail by studying the heat stability of reactivating chicken H4. Untreated enzyme, as well as maximally reactivated enzyme, is stable for several hours at 54°; however, during the course of reactivation the enzyme shows activity of at least two types with regard to heat stability. The results of a typical experiment are described in Fig. 2. At various times during reactivation aliquots were withdrawn and transferred to a water bath at 54°. After 1 min aliquots of heated enzyme were assayed. Aliquots of enzyme which had been allowed to reactivate at room temperature were also assayed, and the activity of the heated enzyme was expressed as a percentage of the activity of unheated enzyme. Fig. 2 shows that an increasing fraction of enzyme activity becomes heat stable as reactivation proceeds.

These results led us to test the possibility that the enzyme activity with the low degree of pyruvate inhibition, which predominates during the early phase of reactivation, might consist of a mixture of two or more forms; one type with a low DPNHlow:DPNHhigh ratio and another type with a high ratio. The data summarized in Fig. 3 show that the enzyme that has been allowed to reactivate at room temperature initially has a low degree of substrate inhibition and as reactivation proceeds the ratio approaches that for the untreated enzyme (3.5 in this experiment). Heating to 54° destroys approximately 50% of this activity and the heat-stable fraction has the same DPNHlow:DPNHhigh ratio as the native enzyme.

In a subsequent experiment it was shown that the heat-labile fraction of chicken H4 will hybridize with reactivating beef H4, but that the heat-stable fraction does not hybridize. At 20 min after initiation of reactivation, samples of chicken H4 were heated to 54° for 5 min (destroying 50% of the enzyme activity) and mixed with reactivating beef H4. Samples of unheated reactivating chicken H4 were also mixed with separate samples of reactivating beef H4. Hybrids were not formed between heated chicken H4 and unheated beef H4, but hybrids were formed between unheated enzymes. The starch gel patterns are shown in Fig. 4.

Effect of Reducing Agents—Under the conditions used, the optimal concentration of β-mercaptoethanol required for reactivation of lactic dehydrogenase was in the range 0.1 M to 0.2 M and no reactivation was observed in 0.003 M β-mercaptoethanol. Dithiothreitol was effective at somewhat lower concentrations, the optimal concentration being 0.01 M, while 0.003 M dithiothreitol was 80% as effective.

Reversible Inactivation of Lactic Dehydrogenase by Lithium Chloride—A previous report from this laboratory indicated that addition of lithium chloride increased the rate of inactivation of...
Reversible Inactivation of Dehydrogenases

Fig. 3. Effect of heat on enzyme activity and pyruvate inhibition of reactivating chicken H4. Chicken H4 (1.06 mg per ml) was inactivated in 5.3 M guanidine hydrochloride + 0.1 M Tris-hydrochloride + 0.1 M β-mercaptoethanol (pH 7.5). After 25 min of reactivation a sample was withdrawn and transferred to a water bath at 54°. This is indicated by the vertical arrows. Aliquots of heated and unheated enzyme were assayed at the times indicated with both low pyruvate (3.3 × 10^-3 M) and high pyruvate (1 × 10^3 M). The degree of substrate inhibition is expressed as a ratio (DPN / DPNH) of rates with low and high pyruvate (4). The initial rates of enzyme activity are expressed as the percentage of the activity of an untreated control measured at low pyruvate concentration. All activities were measured at room temperature.

lactic dehydrogenase by urea (12), but little is known concerning the effect of lithium chloride alone. We have found that lactic dehydrogenase is inactivated by lithium chloride alone and that this inactivation is largely reversible by dilution in the presence of β-mercaptoethanol. The data in Table II show that in the complete absence of β-mercaptoethanol activity is not regained after inactivation in 5.3 M lithium chloride. In the presence of 0.1 M β-mercaptoethanol, 70 to 80% of the enzyme was reactivated. Inactivation was somewhat faster at 25° than at 0° and was accompanied by heavy precipitation of protein but very little turbidity was observed when enzyme was inactivated at 0°. Similar rates and extents of reactivation were obtained with enzyme inactivated at either temperature; the percentage reactivation was generally in the range of 60 to 80%. The kinetics of reactivation was followed in some detail and a marked temperature effect was observed. Fig. 5A shows that reactivation of chicken H4 after inactivation by lithium chloride treatment is much faster at 0° in the presence of DPNH. Reactivation of chicken H4 was affected by DPNH in a similar manner. These results led us to test the possibility that inactivation by lithium chloride might be accompanied by some degree of dissociation similar to the inactivation by urea and guanidine. This possibility was investigated by testing for hybrid formation between beef H4 and chicken H4. Beef and chicken H4 were inactivated separately in lithium chloride and reactivated separately or in equal mixture by dilution, initially at 0°, and then transferred to 25° and allowed to reanimate. Fig. 6 shows that hybrids were formed between enzymes which were allowed to reanimate together. It was found also that, when the separately reactivating enzymes were maintained at 0°, hybrids were formed when the enzymes were mixed and transferred to room temperature; however, if the enzymes were allowed to reanimate separately at 25° and were then mixed, hybrids were not observed.

Properties of Native and Reactivated Chicken H4—Native (untreated) chicken H4 and chicken H4 which has been reactivated after inactivation in guanidine hydrochloride are stable at 55° and their electrophoretic mobilities on starch gel are identical. It was also found that reactivated and untreated enzymes showed similar properties with respect to substrate saturation; they were inhibited to the same extent by excess pyruvate, and the K_m values for pyruvate for native and reactivated enzyme were the same within experimental error, being 12.5 × 10^{-4} M and 14.7 × 10^{-4} M, respectively.

It was of interest to compare the immunological properties of native enzyme, enzyme reactivated in the absence of nucleotide, and enzyme reactivated in the presence of DPN or DPNH. Chicken H4 was inactivated in 7.8 M guanidine hydrochloride + 0.1 M β-mercaptoethanol + 0.1 M Tris-acetate, pH 7.3, at a final enzyme concentration of 2.4 mg per ml. Reactivation was initiated by diluting 20-fold in 0.1 M Tris-acetate + 0.1 M β-mercaptoethanol, pH 7.3; all operations were performed at room temperature. Some of these reactivation mixtures contained 4.75 × 10^{-4} M DPN or DPNH. Enzyme was also diluted at room temperature in the same buffer (in the absence of guanidine hydrochloride) and to the same protein concentration that existed in the reactivation mixtures. After maximal reactivation was obtained, β-mercaptoethanol and residual guanidine hydrochloride were removed by dialysis or filtration through Sephadex G-25. The immunological properties of these preparations were then compared by the technique of microcomplement fixation as described previously (13). As shown in Fig. 8, untreated enzyme and enzyme reactivated in the absence or presence of DPN or DPNH were identical by this criterion.

Malic Dehydrogenase

Inactivation by Guanidine

It was reported previously that malic dehydrogenases could be reversibly inactivated by high concentrations of guanidine hydrochloride (1). Further details of this type of inactivation will be presented here.

Effect of Buffers on Reactivation and Heat Denaturation—It was considered that those buffers that were most effective in promoting the reactivation of the guanidine hydrochloride-dissociated
enzymes might show a similar effect in preventing the heat denaturation of these enzymes. Parallel experiments were set up in which the rate and extent of denaturation of pig mitochondrial malic dehydrogenase in the selected buffers (0.1 M) could be directly compared with the reactivation of this enzyme after guanidine hydrochloride treatment, with the same protein concentration in both cases. The results are given in Table III; details of the experimental procedure are described in "Methods." There is an excellent correlation between the salt effects on heat inactivation and on reactivation. Although not indicated in Table III, further experiments have shown that the final extent, as well as the rate of reactivation, is dependent on the type of buffer used.

**Effect of Salt Concentration**—Of the salts used in the previous section, citrate was the most effective in promoting reassociation; therefore, the dependence of the reactivation on salt concentration was determined with this buffer at pH 7.0. The results given in Fig. 9 show that although 0.5 M citrate gave a faster initial rate of reactivation, the extent of reactivation after 2 hours was identical with that observed with 0.2 M citrate. A concentration of 1.0 M citrate proved inhibitory. Similar results were obtained with malate at pH 7.0.

**Effect of Fluoride, Chloride, Bromide, and Iodide on Reactivation**—It was noted earlier that certain ions could inhibit the reactivation of guanidine hydrochloride-treated lactic dehydrogenase (1). These ions have now been examined for their effect on the reactivation of malic dehydrogenase. The results indicate that 0.1 M solutions of iodide, bromide, and chloride, in order of

**Table II**

<table>
<thead>
<tr>
<th>Concentration of β-mercaptoethanol during</th>
<th>Reactivation</th>
<th>% initial activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivation</td>
<td>Reactivation</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>74</td>
</tr>
<tr>
<td>0.1</td>
<td>0.002</td>
<td>33</td>
</tr>
<tr>
<td>0</td>
<td>0.1</td>
<td>81</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4. Hybridization of beef H₄ and chicken H₄. Chicken H₄ and beef H₄ (each at 1.06 mg per ml) were inactivated separately in 0.1 M guanidine hydrochloride + 0.1 M Tris-hydrochloride + 0.1 M β-mercaptoethanol. After 20 min separate aliquots were withdrawn and diluted 20-fold to start reactivation. Aliquots of the separately reactivating enzymes were mixed at 0, 20, and 30 min (Samples 1, 2, and 3, respectively). At 20 min an aliquot of reactivating chicken H₄ was partially heat-inactivated at 54° and mixed with unheated reactivating beef H₄ (Sample 4). The hybrid mixtures were resolved by starch gel electrophoresis as described previously (10).
decreasing effectiveness, inhibit the malic dehydrogenase reactivation, whereas fluoride is without effect.

**pH Dependence of Reactivation**—In the presence of 0.5 M acetate and 0.1 M β-mercaptoethanol virtually identical rates and extents of reactivation of guanidine hydrochloride-treated pig heart mitochondrial malic dehydrogenase were observed at pH 7.0 and 8.0, respectively; whereas at pH 6.0 and 9.0 less than 1% of enzyme reactivation could be detected.

**Requirement for Reducing Agent**—It was previously noted that the presence of β-mercaptoethanol was required both in the dissociation and reactivation steps to obtain satisfactory reactivation, the optimal concentration being 0.1 M. Cleland’s reagent, dithiothreitol, has been shown to be a most effective reducing reagent and the effectiveness of this reagent in the reversible dissociation of pig heart mitochondrial malic dehydrogenase in guanidine hydrochloride was determined (14). The results are illustrated in Fig. 10. Although low concentrations of dithiothreitol are effective in the inactivation step, considerably higher concentrations are required for reactivation. For the reactivation, 0.05 M dithiothreitol was found to be about twice as effective as 0.1 M β-mercaptoethanol.

**Acid Dissociation of Malic Dehydrogenases**

It was shown previously that malic dehydrogenase can be reversibly dissociated by exposure to low pH and subsequent reneutralization, both the inactivating and reactivating steps requiring the presence of β-mercaptoethanol (1). Initial studies were carried out at a pH of 2.0 or less. It was found that even after storage of the dissociated enzyme for 24 hours at these low pH values it was possible to obtain essentially the same rate and degree of reactivation. An ultracentrifugal study of the pig and chicken heart mitochondrial malic dehydrogenases, which in the native state have an s20,w of 4.0, revealed that at pH 2.0 or below the enzyme was converted into material moving as a single peak with an s20,w of 1.7. When the same enzymes were dialyzed against 0.1 M sodium citrate, pH 2.8, in the absence of β-mercaptoethanol, two peaks were detected in the ultracentrifuge with s20,w values of 6.0 and 1.7, respectively. With time the peak with an s20,w of 6.0 was converted to the slower sedimenting form, little of the faster form remaining after 24 hours at 4°. Assays at various times after the initial dialysis revealed that neither form had enzymatic activity. Enzyme treated in this manner could not be reactivated by neutralization, either in the presence or absence of β-mercaptoethanol. If either pig or chicken mitochondrial malic dehydrogenases were dialyzed against 0.1 M sodium citrate, pH 2.8, in the presence of 0.1 M β-mercaptoethanol, only material with an s20,w of 4.0 (corresponding to the native enzyme) and an s20,w of 1.7 was detected, with conversion of the native enzyme to the slower sedimenting form occurring with time. The material with an s20,w of 1.7 was enzymatically inactive but could be reactivated on reneutralization by dialysis with 0.1 M sodium citrate, pH 7.0, containing 0.1 M β-mercaptoethanol. Chicken heart supernatant malic dehydrogenase which, like the mitochondrial enzyme, has an s20,w of 4.0 in the native state, was also examined at pH 2.0 in the presence of 0.1 M β-mercaptoethanol. Under these conditions the enzyme was converted to a form which moved as a single peak in the ultracentrifuge with an s20,w of 1.7. An ultracentrifugal analysis was also carried out on a malic dehydrogenase crystallized from *Bacillus subtilis*. This enzyme in the native state, unlike vertebrate malic dehydrogenases, has an s20,w of 6.3 and is converted by dialysis for 2 hours against 0.1 M β-mercaptoethanol, pH 2.0, into material sedimenting as a single peak in the ultracentrifuge (preparation).
Fig. 7. Hybridization of beef H₁ and chicken H₄ in lithium chloride. Beef H₁ and chicken H₄ were inactivated separately by incubation in 5.3 M lithium chloride + 0.1 M Tris-hydrochloride + 0.1 M β-mercaptoethanol, pH 6.9, at 0°. After 43 min in lithium chloride, the samples were diluted 50-fold in 0.1 M Tris-hydrochloride + 0.1 M β-mercaptoethanol, pH 7.5, at 0°. Sample 1 contained only chicken H₄, Sample 2 an equal mixture of chicken H₄ and beef H₁, and Sample 3 only beef H₁. After 32 min at 0°, the diluted samples were brought up to 25° and allowed to reactivate for a total of 120 min before electrophoresis was carried out. Approximately 80% of the original catalytic activity was recovered in all cases.

Fig. 8. Immunological comparison by complement fixation of native and reactivated chicken H₄. Untreated enzyme, ▲; reactivated + DPNH, ◇; reactivated + DPN, ◊; reactivated without additions, □. The enzyme was reactivated from 7.6 M guanidine hydrochloride. LDH, lactic dehydrogenase.
Comparison of effect of salts on reactivation and on heat inactivation of malic dehydrogenase

Table III

<table>
<thead>
<tr>
<th>Salt</th>
<th>Heat inactivation&lt;sup&gt;a&lt;/sup&gt; (activity remaining after 10 min)</th>
<th>Reactivation&lt;sup&gt;b&lt;/sup&gt; (relative amounts of enzyme reactivated after 60 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Citrate</td>
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<tr>
<td>Malate</td>
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<td>100</td>
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<td>Phosphate</td>
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<td>95</td>
</tr>
<tr>
<td>Sulfate</td>
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</tr>
<tr>
<td>Tris</td>
<td>2</td>
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</tr>
<tr>
<td>Acetate</td>
<td>1</td>
<td>18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Details in "Experimental Procedure."

<sup>b</sup> Inactivation: 7.3 M guanidine hydrochloride, 0.1 M citrate, 0.1 M β-mercaptoethanol. Reactivation (25°C): 0.1 M β-mercaptoethanol, salts as indicated in table.

Peak in the ultracentrifuge with a s<sub>20,w</sub> of 3.5. If dialysis was carried out for an extended period or if the pH was reduced below 2.0, only material moving as a single peak with an s<sub>20,w</sub> of 1.7 could be detected. The materials with s<sub>20,w</sub> values of 3.5 and 1.7 were not enzymatically active, but both could be partially reactivated by dilution or dialysis against 0.1 M citrate, pH 7.0, containing 0.1 M β-mercaptoethanol.

Reversible Inactivation of Malic Dehydrogenases with Other Media

By the use of techniques essentially as described for the studies with guanidine hydrochloride it was possible to show that mitochondrial malic dehydrogenases from pig and chicken can be reversibly inactivated in 8 M urea or 6 M lithium chloride. In both cases the presence of DPNH considerably enhanced both the rate and extent of reactivation.

By the use of extended dialysis to remove salts and in the presence of 0.1 M β-mercaptoethanol, we were able to observe reversible inactivation of pig mitochondrial malic dehydrogenase from alkaline solution (pH 11.5).

Nature of Dissociated and Reactivated Malic Dehydrogenases

The malic dehydrogenases obtained by reactivation from either acid or guanidine hydrochloride treatment appear to be identical, in all parameters studied, with the native enzymes. Crystalline mitochondrial malic dehydrogenases, when subjected to starch gel electrophoresis at pH 7.0, show several separable bands of activity (8). Identical electrophoretic mobilities and the same patterns of bands were observed in reactivated and native mitochondrial malic dehydrogenases. The degree of substrate inhibition by oxalacetate of the fully reactivated enzymes is identical with that of the native enzymes. The possibility that intermediate forms may exist during reactivation, as described above for lactic dehydrogenases, is at present being investigated. Because the reactivation from acid or guanidine hydrochloride normally involved a 50-fold dilution of the enzyme...
FIG. 11. Immunological comparison by complement fixation of native and reactivated chick mitochondrial malic dehydrogenase. Untreated enzyme, X; reactivated enzyme, O. The enzyme was reactivated from 7.6 M guanidine hydrochloride. MDH, malic dehydrogenase.

It was not possible to determine the molecular weight of the reactivated enzyme by ultracentrifugal techniques. However, with Sephadex G-100 gel filtration columns calibrated for the determination of molecular weight (4, 15), both native and reactivated malic dehydrogenases exhibited identical elution patterns, corresponding to a molecular weight of 67,000 for the vertebrate enzymes. By the immunological technique of semi-microcomplement fixation, which is capable of detecting extremely small changes in protein structure (16), both native and reactivated chicken mitochondrial malic dehydrogenases gave identical reactions with a rabbit antibody directed against native chick mitochondrial malic dehydrogenase (Fig. 11). When acid-treated chick mitochondrial malic dehydrogenase with an s_{20,w} of 1.7, prepared in the absence of a reducing agent, was examined for reaction with the same antibody used above, no reaction could be observed at the dilution of antibody used to obtain a reaction with the native enzyme (1:8500). At a considerably smaller dilution of antibody (1:1000) a reaction could be observed with the acid-treated material, although a several fold increase in antigen concentration was required. Whether this actually represents a reaction of the antibody with a subunit of malic dehydrogenase is equivocal, as the immunological reaction was carried out in the presence of salts, which may give rise to nonspecific aggregation of subunits resembling in some degree the native enzyme. Addition of salt to acid-treated material has, on occasion, been noted to produce precipitation or to induce the formation of a species that sediments more rapidly than the native enzymes when it is examined in the ultracentrifuge.

Hybridization of Malic Dehydrogenases

Although the ultracentrifugal studies with acid-treated malic dehydrogenases suggested that this treatment results in the dissociation of malic dehydrogenase into discrete subunits, it was conceivable that the observed changes in sedimentation coefficients were due to different conformations of the same molecule. It was considered that the preparation of hybrid malic dehydrogenases, when considered with the evidence of the hybridization of other proteins (17-19), would constitute a relatively unequivocal demonstration of subunit formation. Hybrid malic dehydrogenases were prepared by mixing aliquots of enzymes from different sources, previously inactivated either in acid or guanidine hydrochloride, and reactivating such a mixture by dilution, with separate reactivations of the parent enzymes as controls. The presence of hybrid enzymes was detected by starch gel electrophoresis. By such means it has been possible to obtain hybrids of mitochondrial and supernatant enzymes from the same species (Fig. 12A) and between mitochondrial malic dehydrogenases of different species (Fig. 12, B and C). In the former case, the yield of hybrid enzyme is relatively low, presumably because of the different rates of reactivation of the two types of malic dehydrogenase (1). In the latter case a sufficient quantity of a hybrid between chicken and tuna mitochondrial malic dehydrogenases was obtained to enable it to be isolated from the reactivated parent enzymes by starch block electrophoresis (1). As shown in Fig. 12C, the catalytic properties of the hybrid are intermediate between those of the parent types. With semimicrocomplement fixation (13) with a rabbit antibody directed against chicken mitochondrial malic dehydrogenase, the antibody dilutions required for 50% complement fixation were 1:8500, 1:2000, and 1:150 for native (or reactivated) chick mitochondrial malic dehydrogenase, tuna-chicken hybrid, and native (or reactivated) tuna mitochondrial malic dehydrogenase, respectively. The molecular weight of the hybrid enzyme was determined by the use of a calibrated gel filtration column. The elution volume of the hybrid enzyme was identical with that obtained with the parent enzymes corresponding to a molecular weight of 67,000. The possibility that the new component detected on electrophoresis might be an aggregate of two intact enzymes is thus unlikely, although dissociation of such an aggregate during gel filtration cannot be ruled out.
Triosephosphate Dehydrogenase

Inactivation by Urea—The enzymes were dissolved in a solution containing 8 mM urea + 0.05 M sodium phosphate + 0.001 M EDTA; the final protein concentration was 1 mg per ml. The inactivation mixture was left at room temperature for 15 to 60 min; during this time the enzymes were completely inactivated. Reactivation mixtures were prepared by dilution of the inactive enzyme 25- to 30-fold in 0.05 M sodium phosphate + 0.001 M EDTA + 0.1 M β-mercaptoethanol. The rates of the extent of reactivation were followed with respect to time, at room temperature. The data presented in Fig. 13 show that, with the exception of the yeast and halibut enzymes, 35 to 85% of the enzyme activity was regained. The sturgeon enzyme reactivated initially but, as is shown in Fig. 13, the activity later began to decay.

Inactivation by Guanidino—The enzymes were dissolved in a solution containing 5 mM guanidine hydrochloride + 0.05 M sodium phosphate + 0.001 M EDTA; the final protein concentration was 1 mg per ml. The inactivation mixtures were left at room temperature for 15 to 60 min and reactivation was carried out as described for reactivation of triosephosphate dehydrogenase from urea. The data are summarized in Fig. 14. The halibut and yeast enzymes also failed to reactivate from guanidine hydrochloride. The lobster enzyme, like the reactivation of the sturgeon enzyme from urea, lost activity after an initial increase in activity.

Inactivation by Acid—Solutions of pheasant triosephosphate dehydrogenase containing 1 mg, 2.5 mg, 5 mg, 7.5 mg, and 10 mg per ml were dialyzed exhaustively against 1.0 × 10⁻³ M EDTA + 1 × 10⁻³ M β-mercaptoethanol, pH 7.0. The solutions were then brought to pH 2.0 by dialysis against 0.01 M HCl and left for 3 hours at 4° before assaying in 0.05 M sodium phosphate + 1.0 × 10⁻³ M EDTA with and without 0.1 M β-mercaptoethanol. The data are presented in Fig. 15.

Inactivation by Sodium Dodecyl Sulfate—A solution containing 1 mg per ml of pheasant triosephosphate dehydrogenase + 1.0 × 10⁻³ M EDTA (pH 7.5) was treated with 4 × 10⁻⁶ M sodium dodecyl sulfate. The rate of inactivation was determined and the enzyme was found to be completely inactivated within 5
min. Attempts to reactivate the enzyme by the procedures described above were unsuccessful.

Hybridization of Lobster and Pheasant Triosephosphate Dehydrogenases—It has been reported that triosephosphate dehydrogenase contains subunits (20-22). In order to obtain evidence that some degree of dissociation is associated with inactivation in urea, the pheasant and lobster enzymes were inactivated separately in 8 M urea and reactivated in an equimolar mixture by 27-fold dilution in 0.1 M Tris + 1 × 10⁻³ M EDTA + 0.1 M β-mercaptoethanol. After standing for 15 min at room temperature the enzymes were concentrated by vacuum dialysis; most of the protein precipitated. For comparison, aliquots of urea-denatured pheasant and lobster triosephosphate dehydrogenases were inactivated separately, and then reactivated and concentrated as described for the mixture. The individual enzymes were much more stable to vacuum dialysis than the mixture; the yield of active enzyme from the mixture was relatively low.

The formation of a hybrid was detected by starch gel electrophoresis. At pH 7.5 and 8.9, respectively, the mixture had a component with a mobility intermediate between the pheasant and lobster enzymes. The intermediate nature of the pheasant-lobster hybrid of triosephosphate dehydrogenase was also established by immunological methods, with double diffusion in agar and quantitative semimicrocomplement fixation.

Studies are in progress to further characterize this hybrid enzyme and to rule out the possibility that the new component detected on electrophoresis is not merely an aggregate of two intact enzymes. From the observation of hybrid forms of other enzymes, it would seem unlikely that the hybrid is simply an aggregate, since the intermediate electrophoretic form is found only with agents which promote dissociation.

Effect of Coenzyme—The observation that malic and lactic dehydrogenases became reactivated more rapidly when coenzyme was added to the reactivating enzyme led us to test the effect of DPN. The addition of DPN had no effect on the reactivation of pheasant triosephosphate dehydrogenase.

Effect of Temperature—A marked temperature dependence has been observed for the reactivation of lactic and malic dehydrogenases (1). In the present studies it was found that triosephosphate dehydrogenase also does not reactivate at 0°C.

α-Glycerophosphate Dehydrogenase

Deal and Holleman (23) reported that rabbit muscle α-glycerophosphate dehydrogenase can be dissociated into subunits by 9 M guanidine hydrochloride. It was of interest to see whether this process was reversible. After 15 min in 6.9 M guanidine...
hydrochloride, 0.1% of the enzyme activity remained and less than 0.1% remained after 20 hours. The enzyme was also rapidly inactivated in 4 M guanidine hydrochloride. The time course of reactivation at two concentrations of β-mercaptoethanol after 20 hours in guanidine hydrochloride is shown in Fig. 16. The degree of reactivation is markedly dependent on the concentration of β-mercaptoethanol. Approximately 75 to 90% of the enzyme activity could be regained, depending on how long the enzyme was allowed to stand in guanidine before reactivation.

Alcohol Dehydrogenase

In contrast to the other enzymes studied, horse liver alcohol dehydrogenase when subjected to acid treatment showed no change in sedimentation coefficient. Incubation of the enzyme with 1,10-phenanthroline prior to, during, or after acid treatment also failed to dissociate the enzyme. These treatments caused a complete loss of activity, as did treatment with urea or guanidine hydrochloride. None of the many methods used, including additions of zinc or reducing agent or both elicited reactivation. A possible explanation is that during inactivation by such procedures the zinc moiety may be released from the enzyme activity reappears and eventually approaches a level as that which is present in concentrated urea or guanidine hydrochloride. The data presented in Fig. 1 appear to rule out this possibility. It is possible that there is an interaction between coenzyme and the unfolded peptide chain which cannot be detected by the methods used. However, the most reasonable explanation seems to be that during reactivation there is an equilibrium between active and inactive forms of the enzyme and that DPNH enhances reactivation by stabilizing the active form.

Several laboratories have studied denaturation of lactic dehydrogenase by urea (11, 12, 27-29) and there has been some discussion concerning whether urea denaturation of lactic dehydrogenase is an “all-or-none” phenomenon (12, 29). More recently, Epstein, Carter, and Goldberger (30) demonstrated reversible denaturation of rabbit muscle lactic dehydrogenase by both urea and guanidine hydrochloride, but the kinetics of the process was not reported in any detail. It was of considerable interest to test whether reactivation of lactic dehydrogenase from guanidine hydrochloride, urea, and lithium chloride occurred via formation of intermediate species which had modified catalytic activity. During reactivation from each of the above reagents the degree of substrate inhibition by pyruvate increases as enzyme activity reappears and eventually approaches a level comparable to untreated enzyme; it was found, also, that the reaction of guanidine hydrochloride and lithium chloride is accompanied by a decrease in the extent of hybridization of beef 

\[ \text{DPNH}^{\frac{1}{2}} \text{H}_4 \] and chicken \[ \text{H}_4 \]. Studies of heat stability of reactivating enzyme in conjunction with an examination of pyruvate inhibition and degree of hybridization with unheated enzyme showed rather conclusively that there are at least two species of active enzyme during reactivation from guanidine hydrochloride (Figs. 3 to 8). To the extent that the kinetics of reactivation, stimulation of reactivation by DPNH, and changes in \[ \text{DPNH}_{\text{high}} \text{ ratios} \] are suggestive of modified forms of enzyme, it is possible that reactivation from both urea and lithium chloride also proceeds through intermediate forms.

Although the catalytic properties and ease of hybrid formation are similar, it does not seem likely that the inactivated form of lactic dehydrogenase which exists in lithium chloride is the same as that which is present in concentrated urea or guanidine hydrochloride, because the extent of reactivation is much higher after inactivation by lithium chloride. Hybridization experiments suggest very strongly that dissociation occurs in all three cases, but the lower degree of reactivation from urea and guanidine hydrochloride indicates that the protein may be more extensively unfolded in these media than in lithium chloride.

A variety of experimental conditions will modify the substrate inhibition. A decrease in temperature increases the extent of substrate inhibition, and it has been suggested that a configurational alteration in protein structure may be involved (31). There is also a marked increase in substrate inhibition as pH is lowered (32). Several chemical modifications which are known to modify the structure of lactic dehydrogenase also lower the
extent of inhibition by high substrate concentration; these experimental treatments include photo-oxidation, oxidation by N-bromosuccinimide, and deamination by nitrous acid (31). In the present studies we have observed a good correlation between a low degree of pyruvate inhibition and the degree of apparent dissociation as evidenced by hybrid formation. After complete reactivation the degree of inhibition is the same as for untreated enzyme and the enzyme will no longer hybridize. Therefore, it seems that pyruvate inhibition of lactic dehydrogenase is somehow associated with an interaction between the subunits. In this connection, it is of interest that substrate inhibition by pyruvate appears to be due to a ternary complex between the pyruvate, oxidized coenzyme, and the lactic dehydrogenase. Hence the formation of the complex may be quite dependent on the conformation of the enzyme.

Although by several criteria vertebrate malic dehydrogenases are multichain enzymes, the reversible dissociation of these enzymes into subunits has not previously been demonstrated. Harrison (33) found that bovine malic dehydrogenase could be dissociated with either 1,10-phenanthroline or with a polymeric substance present in lipoid acid preparations, zinc or DPNH protecting against such dissociation. Attempts to reaggregate the enzyme were unsuccessful. Thorne and Kaplan (34) demonstrated that pig heart mitochondrial malic dehydrogenase was inactivated by acid, alkali, and urea with concomitant changes in fluorescence, but were unable to reaggregate the enzyme. The reactivation of malic dehydrogenase following various inactivation procedures, obtained in the present study, is no doubt due to the inclusion of a relatively high concentration of a reducing agent such as β-mercaptoethanol in both the inactivation and reactivation steps. This conclusion is strengthened by finding that, without such a reducing agent, the addition of acid to pig or chick heart mitochondrial malic dehydrogenase causes a 50% increase in the sedimentation coefficient (as observed by Thorne and Kaplan (34)), with later conversion to a slower sedimenting form and an irreversible inactivation of the enzyme. The inclusion of a reducing agent prevents the formation of the entity having an S20,w of 6.0 and enables the enzyme to be reactivated.

As a working hypothesis we consider that the effect of DPNH on the reactivation of malic dehydrogenase may be similar to that proposed for lactic dehydrogenase reactivation, namely, that the subunits can interact to form a variety of polymeric structures, the native enzymatically active form being stabilized by DPNH.

The hybridization of malic dehydrogenases was attempted with a 3-fold purpose. (a) It would provide evidence that the inactivating agents were resulting in the dissociation of the enzymes into subunits rather than just altering their conformation. (b) If subunits were formed by such treatment, could hybrid mitochondrial malic dehydrogenases be formed between such disparate species as chick and tuna (the latter enzyme being chosen for such a study on the basis of its unusual electrophoretic mobility)? (c) There are two types of malic dehydrogenase, one located in the mitochondria and the other in the supernatant fraction and, although from several criteria, such as amino acid analysis, electrophoretic mobility, and susceptibility to substrate inhibition they are different proteins (35-37), it seemed possible that they might possess sufficient similarity to enable hybrids to be formed. Indeed, both the mitochondrial and supernatant enzymes show multiple bands on electrophoresis (8, 38, 39), suggesting that some of the bands might have resulted from natural hybrids of the supernatant and mitochondrial forms. The results shown in Fig. 12 provided an answer to these problems. Hybrid enzymes can be formed between mitochondrial enzymes from different species and between supernatant and mitochondrial enzymes from a single species. The position of the supernatant-mitochondrial hybrid on electrophoresis did not correspond to any of the bands observed with the native enzymes. Perhaps different rates of aggregation of the supernatant and mitochondrial enzymes after synthesis of the separate poly peptide chains or different intracellular sites of synthesis of the two enzymes precludes the formation of natural hybrids. Because the catalytic and immunological properties of the artificial hybrids are intermediate between those of the parent enzymes, it appears that, as in the hybrid forms of lactic dehydrogenases, the subunits can act independently.

In all experiments with triosephosphate dehydrogenase, where activity was recovered, there was an absolute requirement for the β-mercaptoethanol. Of the seven triosephosphate dehydrogenases studied, only the yeast and halibut enzymes failed to become reactivated under the conditions that were used. Since dithiothreitol was somewhat more effective than β-mercaptoethanol in the reactivation of lactic and malic dehydrogenases, this reagent was tested to see whether it might aid the reactivation of the halibut and yeast enzymes; however, all attempts to reaggregate these enzymes were unsuccessful. Studies by Mr. J. Everse of this laboratory indicate that triosephosphate dehydrogenase probably dissociates in acid. Peaks with apparent sedimentation constants of 1.47, 1.43, 1.40, 1.35, and 1.38 were observed for acid-treated turkey, chicken, pheasant, beef, and rabbit triosephosphate dehydrogenases, respectively.

In our studies on the inactivation and reactivation of triosephosphate dehydrogenase in acid, it was found that the enzyme inactivated much more rapidly at high protein concentration, and the yield of reactivated enzyme was also less. Acid-treated enzyme at a concentration of 5 to 10 mg per ml yielded 18 to 26% of reactivated enzyme after 24 hours, while at a concentration of 1 to 2.5 mg per ml, approximately 40% of the original activity was regained. Greater yields of reactivated enzyme at lower protein concentration were also observed by Epstein et al. (30) in their studies of the reactivation of urea- and guanidine-denatured lactic dehydrogenase. Apparently, at high protein concentration, much potential enzyme activity is lost due to nonspecific aggregation, perhaps by the formation of disulfide interchain bridges.

The observation of hybrid formation between lobster and pheasant triosephosphate dehydrogenases provides strong evidence that urea-denatured triosephosphate dehydrogenase is dissociated into subunits. The preliminary ultracentrifugal studies also suggest that the enzyme is dissociated in acid. Although ultracentrifugal and hybridization studies were not performed on the guanidine hydrochloride-inactivated triosephosphate dehydrogenase, the similarity in the kinetics of inactivation and reactivation with urea-denatured enzyme indicate that this reagent causes dissociation. More recently, we have shown that the enzyme is apparently irreversibly dissociated by treatment with succinic anhydride. Similar observations have been made by Haas in his studies on aldolase (40).

Van Eys, Judd, Ford, and Womack (41) have observed reversible inactivation of α-glycerophosphate dehydrogenase by freezing and thawing and by acid at high ionic strength. From studies of the effects of dilution on kinetics of inactivation and
Reversible Inactivation of Dehydrogenases

In previous studies of the mechanism of hybridization of lactic dehydrogenases in vitro (44), it was found that the order of effectiveness of sodium halides as inducers of hybrid formation was SCN\(^-\) and I\(^-\) > Br\(^-\) > Cl\(^-\), and it was suggested that hybridization was partially due to increased salt concentration in the eutectic mixture. In other experiments not reported here, inactivation of lactic dehydrogenase by 6.0 M salts was examined and the order of effectiveness was found to be NaCl < LiCl and NaBr < NaI and NaSCN.

Chicken \(H_2\) was stable for several hours at room temperature in sodium chloride, but was unstable in lithium chloride and sodium bromide and was very rapidly inactivated by sodium thiocyanate and sodium iodide. The rates and extents of reactivation paralleled the effects on enzyme activity, i.e. only 15% of the original activity was recovered from sodium thiocyanate, but 75 to 85% recovery was obtained from sodium bromide and lithium chloride. For sodium thiocyanate and sodium iodide, the extent of reactivation was very dependent on the temperature at which inactivation had occurred. No activity was recovered if inactivation was not carried out at 0°C. The catalytic properties of chicken \(H_2\), with regard to substrate inhibition during reactivation from sodium thiocyanate, are very similar to the properties observed during reactivation from lithium chloride, guanidine, and urea, i.e. the ratio for untreated enzyme as reactivation proceeded was SCN\(^-\) > I\(^-\) > Br\(^-\) > Cl\(^-\), and it was suggested that reversible dissociation of F-actin and amide groups and amide groups. These salts which increase the activity coefficients of these groups would favor reaggregation, and those salts which decrease the activity coefficients, or salt in, would inhibit the reactivation.

The results obtained to date with the lactic dehydrogenase and malic dehydrogenase system are in excellent agreement with those predicted from the work of Robinson, Nagy, and Jencks. The halides inhibit reactivation of lactic dehydrogenase and malic dehydrogenase in the order, I\(^-\) > Br\(^-\) > Cl\(^-\) > F\(^-\), which is the order of effectiveness in dissociating F-actin (49) and an inhibitor of a diphosphopyridine nucleotide from \(B. subtilis\) (50) inhibiting an antigen-antibody reaction (51, 52); decreasing the activity coefficient of acetyltetraglycine ethyl ester (47), and inhibiting fumarase (53). Citrate, which was the most effective salt for the reactivation of malic dehydrogenase, was also the most effective in increasing the activity coefficient of acetyltetraglycine ethyl ester (47), for activating acetyl coenzyme A carboxylase (54), and it also serves to activate fumarase (53). Further, in the parallel experiments on the reactivation and heat denaturation of malic dehydrogenase, there was excellent agreement between protection against denaturation and effectiveness in reactivation.

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