Effect of Tetrathionate on the Stability and Immunological Properties of Muscle Triosephosphate Dehydrogenases*

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When three sulfhydryl groups of rabbit, turkey, sturgeon, and lobster triosephosphate dehydrogenases (TPD's) are modified by tetrathionate, the enzymes are completely inhibited. Depending on the temperature and length of incubation with tetrathionate, this inhibition can be reversed by the addition of thiols. The muscle TPD's become irreversibly inactivated in the presence of tetrathionate at 37°. The rate of this irreversible inactivation varies with different muscle TPD's. The immunological properties of the sturgeon enzyme are only slightly altered when it is inactivated with tetrathionate, while the immunological properties of the tetrathionate-inactivated turkey and lobster TPD's differ significantly from the native enzymes. The sulfhydryl groups in the different TPD's show a differential reactivity with *p*-mercuribenzoate in the presence and absence of 8.0 M urea. All of the TPD's studied appear to have no disulfide linkages, since all of the nonmethionine sulfur exists as sulfhydryl groups. It is concluded that the active conformations of the different TPD's are partly stabilized by the three active sulfhydryl groups per mole (120,000 g) of enzyme which react with tetrathionate, but the degree of stabilization varies with the different enzymes.

It is well established that the enzymatic activity of yeast and rabbit muscle triosephosphate dehydrogenases (TPD's)[1] depends on the state of the active sulfhydryl groups in the enzymes (Cori *et al.*, 1948; Krebs, 1955). The crystalline rabbit muscle enzyme prepared in the absence of reducing thiols or heavy metal-chelating agents such as EDTA becomes reversibly inactivated, but it may be reactivated by incubation with thiols such as cysteine or mercaptoethanol Velick, 1955). When the crystalline rabbit muscle enzyme is prepared in the presence of EDTA, it does not require activation with thiols and its thermal and *p*H stabilities are increased (Velick and Furfine, 1963). The work of Krimsky and Racker (1955), Velick and Hayes (1953) and Segal and Boyer (1953) has shown that the oxidative phosphorylation of D-glyceraldehyde-3-phosphate proceeds in two steps, the first of which is the oxidation of the substrate by bound DPN to form an *S*-acyl enzyme intermediate. The second step is the phosphorolysis of the *S*-acyl enzyme to form 1,3-diphosphoglyceric acid. Evidence indicates that there are at least three active—SH groups per 120,000 g of the rabbit muscle enzyme that are involved in the catalytic process (Velick, 1953; Perham and Harris, 1963). This evidence has been obtained by the use of—SH reagents such as *p*-mercuribenzoate (Velick, 1953), iodoacetate (Krimsky and Racker, 1952), and tetrathionate (Pihl and Lange, 1962).

Crystalline turkey, sturgeon, and lobster TPD's are quite similar to the rabbit muscle enzyme in their sensitivity to—SH reagents (Allison and Kaplan, 1964). Like the rabbit muscle enzyme, they are completely inhibited by 3 moles of tetrathionate per 120,000 g of enzyme at 0°, and this inhibition can be completely reversed by the addition of thiols under proper conditions. However, changes in the heat stability, susceptibility to proteolytic digestion, and immunological properties accompany inactivation of these dehydrogenases with tetrathionate. These changes are presented and discussed in this publication.

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[1] Abbreviation used in this work: TPD, triosephosphate dehydrogenase.

Experimental Procedure

Materials

The enzymes used in this study were prepared by the methods described elsewhere (Allison and Kaplan, 1964). Bound DPN was removed from the lobster TPD with acid-activated Norite A which had been water-washed to *p*H 4.5 and dried before use. Solutions (0.2–0.5%) of lobster TPD in 0.1 M sodium phosphate, *p*H 7.0, containing 10^{-3} M EDTA were stirred with the treated Norite A (100 mg/ml of enzyme solution) at 0° for 30 seconds. The Norite A was then removed by suction filtration through a sintered glass funnel previously chilled to 5°. At least 85% of the bound DPN was removed from the enzyme by this procedure as estimated by the increase in extinction at 340 mμ when substrate and arsenate were added to solutions of the enzyme under conditions which reduce bound DPN to $DPNH_2$.

DPN and *p*-mercuribenzoate were purchased from the Sigma Chemical Co. The dihydrate of sodium tetrathionate was prepared from thiosulfate by oxidation with iodine. Solutions of tetrathionate were prepared by weight and were stored at 5° for no longer than 3 weeks. The lithium salt of acetyl phosphate was prepared as described by Stadtman (1957). Solutions of acetyl phosphate were assayed with hydroxylamine by the procedure of Lipmann and Tuttle (1945).

Methods

Immunological Techniques.—The crystalline enzymes were dissolved in 10^{-3} M EDTA, *p*H 7.0, and dialyzed against the same solution before injection into rabbits. During the first course of immunization 20 mg of antigen in 1.0 ml of 10^{-3} M EDTA, *p*H 7.0, mixed with an equal volume of complete Freund's adjuvant, was injected into the toepads and intramuscularly. Three weeks after the initial injection rabbits were boosted by the intravenous injection of 2 mg of antigen a day for 5 days. Seven days after the last intravenous injection, 40 ml was bled from the ear. The antilobster

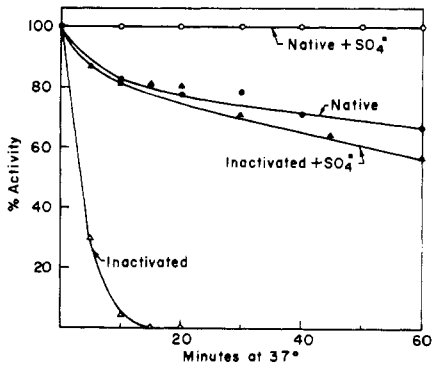

FIG. 1.—Effect of tetrathionate inactivation on the heat stability of turkey muscle TPD. Solutions (10^{-6} M) of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5, were incubated at 37° with the following reagents: ●, none; ○, 0.4 M $(NH_4)_2SO_4$, pH 8.5; △, 3×10^{-6} M $S_4O_6^{2-}$; ▲, 3×10^{-6} M $S_4O_6^{2-}$ and 0.4 M $(NH_4)_2SO_4$, pH 8.5. Samples (5 μg) were withdrawn at the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes, at which time they were assayed.

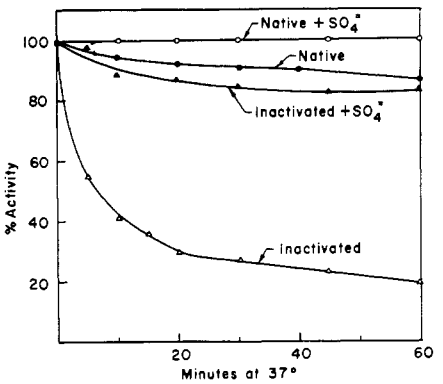

FIG. 2.—Effect of tetrathionate inactivation on the heat stability of sturgeon muscle TPD. Solutions (10^{-6} M) of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5, were incubated at 37° with the following reagents: ●, none; ○, 0.4 M $(NH_4)_2SO_4$, pH 8.5; △, 3×10^{-6} M $S_4O_6^{2-}$; ▲, 3×10^{-6} M $S_4O_6^{2-}$ and 0.4 M $(NH_4)_2SO_4$, pH 8.5. Samples (5 μg) were withdrawn at the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes, at which time they were assayed.

TPD used in this study was obtained after a single course of intravenous boosting, while the antiturkey and antisturgeon TPD's were obtained after three courses of intravenous boosting administered at 4-week intervals. All sera were tested for the presence of precipitating antibody by the gel-diffusion method of Ouchterlony (1949). Quantitative micro-complement (C′) fixation was performed as described by Wasserman and Levine (1961).

Trypsin Digestion.—Proteolytic digestion was carried out at 35° with twice-recrystallized salt-free trypsin purchased from Worthington Biochemical Corp. with a weight ratio of TPD to trypsin of 200:1. The rate of digestion was determined by measuring the increase in extinction at 280 mμ with time of samples of the digestion mixtures deproteinized with 5% trichloroacetic acid.

Cysteine Determinations.—Cysteine was determined spectrophotometrically with *p*-mercuribenzoate in the presence of 8.0 M urea (Boyer, 1954). A 0.10-ml volume of the enzymes (5–10 mμmoles) was added to 2.9 ml of 0.10 M sodium phosphate, pH 7.0, containing 8.0 M urea and 10^{-4} M *p*-mercuribenzoate. The increase in extinction at 250 mμ owing to mercaptide formation was then measured with time in a Zeiss Model PMQ II spectrophotometer. A correction factor for the effect of urea on mercaptide absorption at 250 mμ was determined with reduced glutathione. This correction factor was applied to the extinction coefficient of 7.6×10^3 reported by Boyer (1954) for mercaptide formation in 0.10 M phosphate, pH 7.0. The amount of non-methionine sulfur in the enzymes was determined as cysteic acid following performic acid oxidation at room temperature by the procedure of Schram *et al.* (1954). Cysteic acid was determined by automatic amino acid analyses in a Spinco amino acid analyzer by the procedure of Moore *et al.* (1958).

RESULTS

Effect of Tetrathionate Inactivation on the Heat Stability of Muscle TPD's.—Pihl and Lange (1962) have shown that rabbit muscle TPD is inactivated by the addition of 3 moles of tetrathionate per mole of enzyme. The evidence of Pihl and Lange (1962) suggests that tetrathionate reacts specifically with three thiol groups in the enzyme that appear to be involved in thioester formation at the active site of TPD during the catalytic oxidation of D-glyceraldehyde-3-phosphate. Similarly, turkey, sturgeon, and lobster TPD's are completely inactivated by tetrathionate within 2 minutes at 0° when 3 moles of inhibitor are added per mole of enzyme. As was observed with the rabbit muscle enzyme, this inhibition can be completely reversed by reducing thiols such as cysteine and mercaptoethanol if the inhibited enzymes are incubated at low temperatures and the activating thiol is added within an hour after the introduction of the inhibitor. After prolonged incubation at 0°, the tetrathionate-inactivated enzymes can be only partly reactivated with thiols, as shown in Table I for the lobster muscle TPD. The rate of irreversible inactivation of the enzymes by tetrathionate is much faster at higher temperatures and varies for different muscle TPD's. Figures 1–3 show the effect of tetrathionate inactivation on the stability of turkey, sturgeon, and lobster TPD's at 37°. Each of the tetrathionate-inactivated enzymes is much less stable at 37° than it is in the native state, although there are some significant differences in the stability of the three TPD's. The tetrathionate-inactivated lobster TPD is the most labile. It is irreversibly inactivated within 10 minutes at 37°. The treated turkey enzyme is somewhat more stable than the treated lobster enzyme; the sturgeon dehydrogenase is much less sensitive to ir-

TABLE I

STABILITY OF LOBSTER MUSCLE TRIOSEPHOSPHATE IN THE PRESENCE OF TETRATHIONATE AT 5°[a]

Time at 5° (hr)	Activity Recovered after Addition of Mercaptoethanol (%)
0	100
1	96
4	79
8	70
12	60
24	51

[a] The enzyme (10^{-6} M) was inactivated by the addition of 3×10^{-6} M $S_4O_6^{2-}$ in 0.05 M sodium pyrophosphate, pH 8.5, and was incubated at 5°. Samples (5 μg) were withdrawn in the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes at room temperature and then assayed.

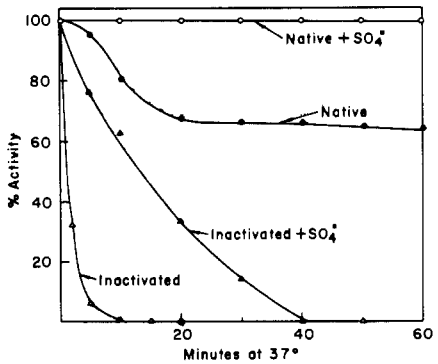

FIG. 3.—Effect of tetrathionate inactivation on the heat stability of lobster muscle TPD. Solutions (10^{-6} M) of the enzyme in 0.05 M sodium pyrophosphate, *p*H 8.5, were incubated at 37° with the following reagents: ●, none; ○, 0.4 M $(NH_4)_2SO_4$, *p*H 8.5; △, 3×10^{-6} M $S_4O_6^{2-}$; ▲, 3×10^{-6} M $S_4O_6^{2-}$ and 0.4 M $(NH_4)_2SO_4$, *p*H 8.5. Samples (5 μg) were withdrawn at the times indicated and were incubated with 10^{-3} M mercaptoethanol for 5 minutes, at which time they were assayed.

reversible inactivation following treatment with tetrathionate. Figures 1–3 also show that the heat stability of both the native and tetrathionate-inactivated TPD's depends on the ionic composition of the buffer. The presence of 0.4 M ammonium sulfate prevents the spontaneous denaturation of the native TPD's and increases the stability of the tetrathionate-inactivated TPD's at 37°. Preliminary experiments have indicated that the salt effect is due to the sulfate anion, since sodium sulfate produces the same effect as ammonium sulfate, whereas sodium chloride has little effect on the lability of the enzymes.

The decrease in stability of the tetrathionate-inactivated enzymes over the native enzymes in the absence and presence of sulfate suggests that the chemical modification of the active —SH groups of TPD by tetrathionate, which eventually leads to irreversible inactivation of the enzyme, proceeds in two steps as shown in the following scheme:

active state

$S_4O_6^{2-} \rightleftharpoons RSH$

reversibly inactivated state

↓

irreversibly inactivated state

The first step occurs rapidly even at 0°, while the second occurs slowly at low temperatures and at a much faster rate at moderate temperatures.

The Effect of Tetrathionate on the Antigenic Activity of Lobster Muscle TPD.—The strength of precipitin bands in two-dimensional gel-diffusion experiments between lobster TPD and a rabbit antiserum prepared against fully active TPD depends on the state of the catalytically active thiol groups of the enzyme. During gel-diffusion experiments conducted at room temperature, precipitin bands between wells containing a solution of the enzyme, which had lost 70% of its original activity after standing for 3 days at 5° in 0.01 M sodium phosphate, *p*H 8.0, in the absence of EDTA or thiols, were barely visible; while strong precipitin bands between wells containing the same antiserum and solutions of the aged enzyme, which had been partially reactivated with mercaptoethanol (85% of the original activity was recovered), were observed. A precipitin band between a well containing the tetrathionate-inhibited enzyme and a well containing the antiserum was not

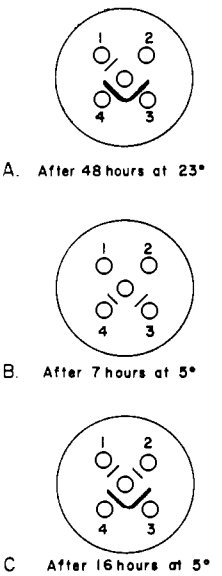

FIG. 4.—Effect of tetrathionate inactivation of lobster TPD on precipitin-band formation. Each enzyme well contained 0.10 ml of 3×10^{-6} M lobster muscle TPD in 0.05 M sodium pyrophosphate, *p*H 8.5, after the treatments indicated. Well 1, 3 days aging in 0.01 M sodium phosphate, *p*H 8.0; well 2, 10^{-5} M $S_4O_6^{2-}$; well 3, same as well 1 with the addition of 10^{-4} M mercaptoethanol; well 4, 10^{-5} M $S_4O_6^{2-}$ followed by the addition of 10^{-4} M mercaptoethanol. The center well contained 0.10 ml of a rabbit serum prepared against the active form of lobster muscle TPD. The time represents the period after the wells were filled.

observed even after 48 hours of incubation at room temperature, while a strong precipitin band appeared within 20 hours between a well containing the tetrathionate-inhibited enzyme, which was reactivated with mercaptoethanol, and a well containing the antiserum. Drawings of these observations are shown in Figure 4A. When agar-gel-diffusion experiments were conducted at 5°, the rate of formation of precipitin bands was decreased when the enzyme was partially or completely inactivated as shown in Figure 4B,C. Precipitin bands between wells containing the tetrathionate-inhibited enzyme or solutions of aged enzyme, which possessed 30% of maximal activity in the absence of thiols, and wells containing the antiserum appeared after 16 hours. However, precipitin bands between the fully reduced enzyme were observed within 7 hours under the same conditions as shown in Figure 4B. Losses in antigenic activity with antilobster TPD were observed when the lobster muscle enzyme was inactivated, as determined by quantitative C′-fixation analysis. Complete inactivation of the lobster muscle enzyme by tetrathionate resulted in a loss in C′-fixation activity, as shown in Figure 5. Compared to the C′-fixation curve for the fully activated enzyme in the presence of cysteine, the C′-fixation curve for the enzyme, which was completely inactivated with tetrathionate, is shifted toward higher antigen concentrations and has a lower peak height. The C′-fixation curve for the "aged" enzyme, which in this case possessed 70% of maximal activity in the absence of thiols, is slightly shifted toward higher antigen concentrations and has a lower peak height when compared to the curve for the fully activated enzyme. When the lobster muscle enzyme was irreversibly inactivated by incubation with tetrathionate for 30 minutes at 30°, antigenic activity was completely lost, as shown by curve 5 in Figure 5. The addition of cysteine to the irreversibly inactivated enzyme led to partial restora-

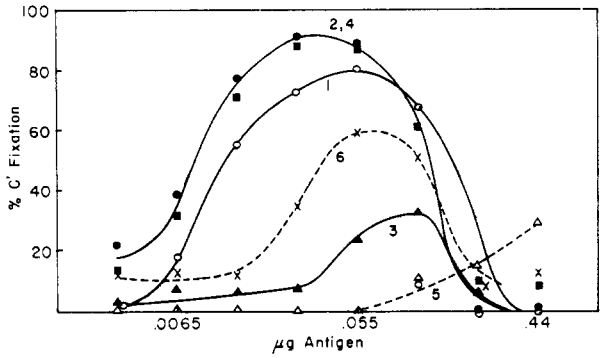

FIG. 5.—Effect of tetrathionate inactivation on the complement-fixation reaction of lobster muscle TPD with antilobster TPD. The following additions were made to 3 × 10^{-6} M solutions of the "aged" enzyme in 0.05 M sodium pyrophosphate, *p*H 8.5: curve 1, none; curve 2, 10^{-3} M cysteine; curve 3, 10^{-5} M $S_4O_6^{2-}$ at 0°; curve 4, 10^{-5} M $S_4O_6^{2-}$ at 0°, then 10^{-3} M cysteine; curve 5, 10^{-5} M $S_4O_6^{2-}$ at 30°; curve 6, 10^{-5} M $S_4O_6^{2-}$ at 30°, then 10^{-3} M cysteine. The mixtures were incubated at 0° for 20 minutes and at 30° for 30 minutes where indicated. Cysteine was added at the end of these incubations where indicated. A sample of each mixture was diluted to a final enzyme concentration of 0.44 μg/ml with isotonic Veronal buffer for the C'-fixation assay.

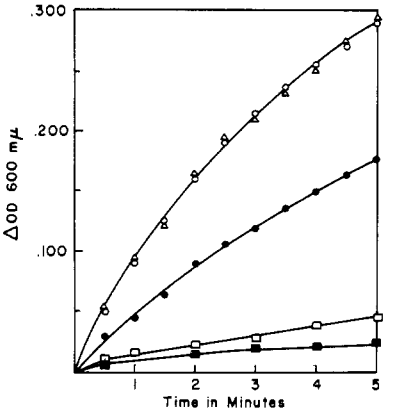

FIG. 6.—Effect of tetrathionate inactivation on precipitate formation between lobster TPD and antilobster TPD. Enzyme solutions (3 × 10^{-6} M) in 0.05 M sodium pyrophosphate, *p*H 8.5, contained the following additions: ○, 10^{-4} M mercaptoethanol; ●, 10^{-5} M $S_4O_6^{2-}$ at 0°; △, 10^{-5} M $S_4O_6^{2-}$ at 0°, then 10^{-4} M mercaptoethanol; ■, 10^{-5} M $S_4O_6^{2-}$ at 35°; □, 10^{-5} M $S_4O_6^{2-}$ at 35°, then 10^{-4} M mercaptoethanol. The mixtures were incubated with tetrathionate for 20 minutes at 0° or 35° where indicated. Mercaptoethanol was added to the mixtures at the end of the incubation period where indicated. After the incubation period, 0.5 ml of the enzyme solutions was mixed with a 2.5-ml solution of the antiserum (0.20 ml of the antiserum, 2.3 ml of buffer) in 0.05 M sodium pyrophosphate, *p*H 8.5, in a cuvet at 7°. Immediately after mixing, the optical density of the reaction mixtures was determined at 600 mμ at 30-second intervals.

tion of antigenic activity. However, after this treatment with cysteine, no enzymatic activity was recovered.

The results of both the gel-diffusion experiments at 5° and the C'-fixation experiments indicate that the reversibly inactivated enzyme (enzyme activity is restored by the addition of thiols) is more antigenic than the irreversibly inactivated enzyme (enzyme activity is not restored by thiols). However, a slow transition of the reversibly inactivated state to the irreversibly inactivated state occurred when the lobster muscle TPD was incubated with tetrathionate at 0° as shown in Table I. Therefore the losses in antigenic activity observed in the C'-fixation experiments and the gel-diffusion experiments that were conducted at 5° may be directly related to the blocking of the active sulfhydryl groups, or they may be caused by changes in antigenic determinants that accompany the irreversible inactivation of the enzyme that occurs slowly at 5°. To test between these two possibilities the antigenic activity of the fully active, the reversibly inactivated, and the irreversibly inactivated states of the enzyme was determined by measuring turbidity development owing to formation of an insoluble antigen-antibody complex at 600 mμ with a Zeiss Model PMQ II spectrophotometer equipped with a cell holder jacketed at 7°. This method has the advantage of measuring the antigenic activity of the reversibly inactivated state of the enzyme immediately after inactivation with tetrathionate. Under these conditions all of the enzyme will be in the reversibly inactivated state as shown in Table I. Figure 6 shows that the rate of formation of turbidity was decreased when the lobster enzyme was reversibly inactivated with tetrathionate before incubation with the antiserum. When the enzyme was irreversibly inactivated by heating in the presence of tetrathionate, the rate of formation of turbidity was decreased even further. This experiment indicates that the reversible inactivation of the lobster muscle enzyme leads to changes in antigenic determinants on the enzyme. Furthermore, the rate at which turbidity developed at 7° indicates that the formation of a three-dimensional complex between the antienzyme and the reversibly inactivated enzyme occurs long before an appreciable amount of the modified enzyme is irreversibly denatured in the C'-fixation experiments.

The Effect of Tetrathionate on the Antigenic Activity of Other Triosephosphate Dehydrogenases.—Inactivation of chicken and turkey TPD's with tetrathionate at 5° was accompanied by decreases in antigenic activity similar to those described for the lobster muscle enzyme. Addition of cysteine or mercaptoethanol to the enzymes that were inactivated with tetrathionate at 5° completely restored both antigenic and enzymatic activity. The changes in the C'-fixation reaction, which accompany both the reversible and irreversible inactivation of the turkey enzyme, are shown in Figure 7. Inactivation of the enzyme leads to a complete loss in the C'-fixation reaction at an antibody dilution of 1:8000, whether the inactivation was carried out under reversible or irreversible conditions. When mercaptoethanol was added to the irreversibly inactivated turkey TPD, no antigenic activity was recovered at an antiserum dilute of 1:8000.

When sturgeon muscle TPD was inactivated with tetrathionate, the reaction between the modified enzyme and an antiserum prepared against the active form of the sturgeon enzyme was only slightly altered as shown in Figure 8. The C'-fixation curve for the reversibly inactivated enzyme was slightly shifted toward higher antigen concentrations and has nearly the same peak height when compared to the C'-fixation curve for the active enzyme. Moreover, irreversible inactivation of the sturgeon enzyme failed to produce further changes in the C'-fixation reaction. The differences between the changes in the antigenic activity that accompany tetrathionate inactivation of the lobster and turkey TPD's, as compared to the sturgeon protein, may be the result of a structural difference in the sturgeon enzyme or to a difference in the specificity of the antibody prepared against the sturgeon enzyme. To test between these possibilities advantage was taken of the ability of the sturgeon enzyme to cross react

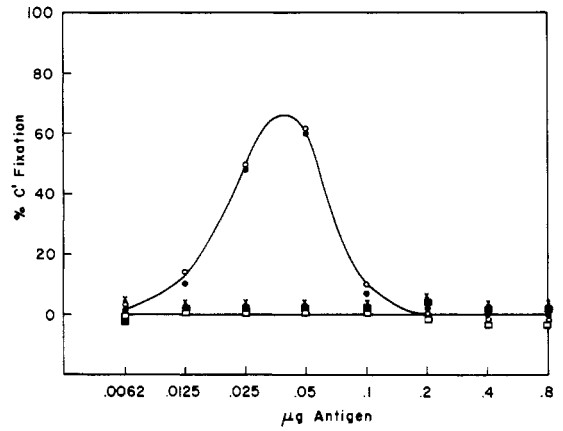

FIG. 7.—Effect of tetrathionate inactivation on the C'-fixation reaction of turkey muscle TPD with antiturkey TPD. The following additions were made to 3 × 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, *p*H 8.5: ○, none; ■, 10^{-5} M $S_4O_6^{2-}$ at 0°; ●, 10^{-5} M $S_4O_6^{2-}$ at 0° then 10^{-3} M mercaptoethanol; □, 10^{-5} M $S_4O_6^{2-}$ at 37°; ×, 10^{-5} M $S_4O_6^{2-}$ at 37°, then 10^{-3} M mercaptoethanol. The mixtures were incubated at 0° for 30 minutes where indicated and at 37° for 15 minutes where indicated. Mercaptoethanol was added at the end of these incubations where indicated. A sample of each mixture was diluted to a final enzyme concentration of 0.80 μg/ml with isotonic Veronal buffer for the C'-fixation assay.

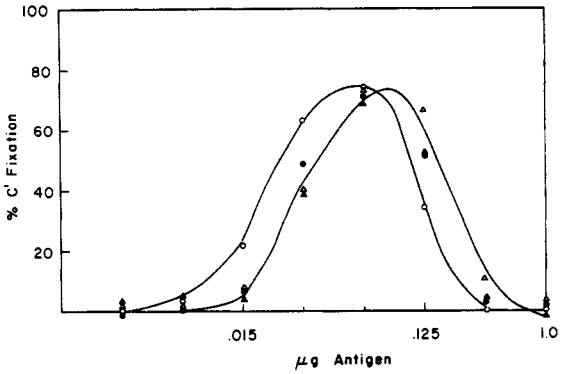

FIG. 8.—Effect of tetrathionate inactivation on the C'-fixation reaction of sturgeon muscle TPD with antisturgeon TPD. The following additions were made to 3 × 10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, *p*H 8.5: ○, none; ●, 10^{-5} M $S_4O_6^{2-}$ at 0°; △, 10^{-5} M $S_4O_6^{2-}$ at 37°; ▲, 10^{-5} M $S_4O_6^{2-}$ at 37°, then 10^{-3} M mercaptoethanol. The mixtures were incubated at 0° for 30 minutes where indicated and at 37° for 120 minutes where indicated. Mercaptoethanol was added at the end of the incubation period where indicated. A sample of each mixture was diluted to a final enzyme concentration of 1.0 μg/ml with isotonic Veronal buffer for the C'-fixation assay.

with the antiturkey TPD. The C'-fixation curve in Figure 9 between the tetrathionate-inactivated sturgeon TPD and the antiturkey enzyme is only slightly shifted toward higher antigen concentrations when compared to the curve for the reaction between active sturgeon enzyme and the antiturkey serum. This indicates that the difference in reactivity of the modified sturgeon TPD, as compared to the turkey and lobster dehydrogenases, is inherent in the enzyme itself rather than in the antibody.

The Effect of Bound DPN on the Antigenic Activity of Muscle Tricosephosphate Dehydrogenases.—Like the rabbit muscle enzyme (Cori *et al.*, 1948) lobster muscle TPD crystallized with approximately 2 moles of firmly bound DPN which cannot be removed by dialysis. On the other hand, turkey muscle TPD crystallizes with less than 0.2 mole of bound DPN per mole of enzyme and the sturgeon muscle enzyme crystallizes free of coenzyme (Allison and Kaplan, 1964). Solutions of the active lobster muscle TPD-DPN complex have the same broad absorption band between 320 and 400 mμ, as has been observed for the active rabbit muscle TPD-DPN complex. This absorption band has been attributed to an interaction between the coenzyme and the active —SH groups of the enzyme, since it is destroyed by —SH reagents such as iodoacetate (Krimsky and Racker, 1952), *p*-mercuribenzoate (Velick, 1953), and tetrathionate (Pihl and Lange, 1962). When exogenous DPN was added to solutions of the turkey and sturgeon muscle TPD's, this absorption band appeared.

When the bound DPN was removed from the lobster muscle enzyme with charcoal under conditions described under Experimental Procedure, both enzymatic and antigenic activity were retained. Moreover, the addition of exogenous DPN to solutions of the charcoal-treated apoenzyme did not increase either enzymatic or C'-fixation activity, as shown in Figure 10. Reversible inactivation of the apoenzyme with tetrathionate at 5° resulted in losses in C'-fixation activity similar to those observed when the native enzyme was reversibly inactivated. When mercaptoethanol was added to the $S_4O_6^{2-}$ and activated, a curve was obtained identical to that with the active apoenzyme. The addition of DPN to the tetrathionate-inactivated apoenzyme did not increase C'-fixation activity (Fig. 10) nor did it restore enzymatic activity. These observations indicate that the native lobster enzyme (containing bound DPN) and the lobster apoenzyme are immunologically indisinguishable. Similarly, when DPN was added to solutions of turkey and sturgeon muscle TPD's, no changes in the C'-fixation activity between the enzymes and their specific antienzymes were observed.

The Effect of Acetyl Phosphate on the Antigenic Activity of Lobster Muscle Triosephosphate Dehydrogenase.—Krimsky and Racker (1955) have shown that the catalytically active —SH groups of rabbit muscle TPD are acetylated when the charcoal-treated apoenzyme is treated with excess acetyl phosphate in the cold and that the addition of DPN and arsenate to the resulting acetyl enzyme leads to its arsenolysis. When a 1000 M excess of acetyl phosphate was added to the lobster muscle apoenzyme at 5°, approximately 90% of antigenic activity was lost, as estimated by the lateral displacement of the C'-fixation curve shown in Figure 11. When the apoenzyme was incubated with a 100 M excess of acetyl phosphate at 5°, the loss of C'-fixation activity was less marked, as shown in Figure 11. At both acetyl phosphate concentrations, incubation of the acetyl phosphate–treated apoenzyme with DPN and arsenate led to some recovery in antigenic activity, as shown in Figure 11.

The arsenolysis of acetyl phosphate by lobster muscle TPD has been studied at 37°. Table II shows that tetrathionate completely inhibits the arsenolysis reaction. The addition of DPN to the native enzyme enhances the arsenolysis reaction, while it has no effect on the tetrathionate-inhibited enzyme. The arsenolysis reaction of the native enzyme was also enhanced by the addition of cysteine. These data suggest that the addition of acetyl phosphate to lobster muscle TPD leads to the formation of an acetyl-enzyme intermediate. However, since nothing is known about the kinetics and equilibria involved during acetyl-enzyme formation and arsenolysis at 5° (the average temperature at which the previously described C'-fixation experiments were conducted), a more quantitative study must be completed in which the extent of acetyl-enzyme formation can be related to the observed losses in antigenic activity.

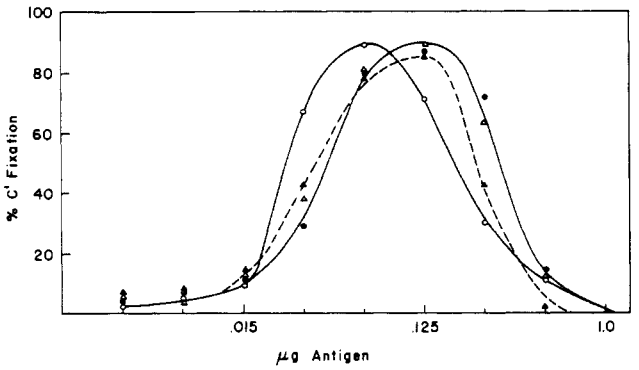

FIG. 9.—Effect of tetrathionate inactivation on the C'-fixation reaction of sturgeon muscle TPD with antiturkey TPD. The following additions were made to 3×10^{-6} M solutions of the enzyme in 0.05 M sodium pyrophosphate, pH 8.5: ○, none; ●, 10^{-6} M $S_4O_6^{2-}$ at 0°; △, 10^{-5} M $S_4O_6^{2-}$ at 37°; ▲, 10^{-5} M $S_4O_6^{2-}$ at 37°, then 10^{-3} M mercaptoethanol. The mixtures were incubated at 0° for 30 minutes where indicated and at 37° for 120 minutes where indicated. Mercaptoethanol was added at the end of the incubation period where indicated. A sample of each mixture was diluted to a final enzyme concentration of 1.0 μg/ml with isotonic Veronal buffer for the C'-fixation assay.

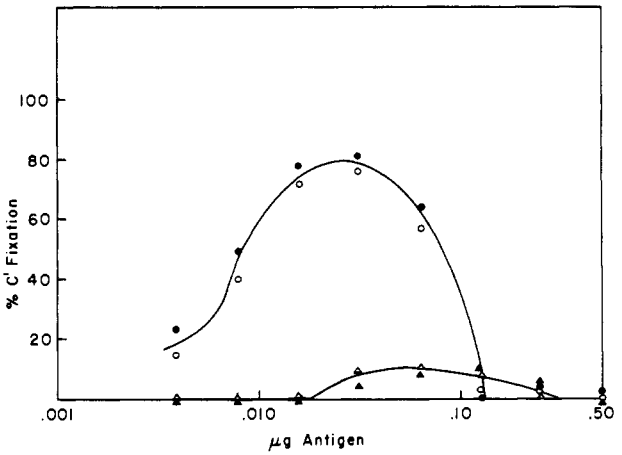

FIG. 10.—Effect of DPN and $S_4O_6^{2-}$ on the C'-fixation reaction of charcoal-treated lobster muscle TPD with antilobster TPD. The following additions were made to 3×10^{-6} M solutions of the charcoal treated apoenzyme in 0.05 M sodium pyrophosphate, pH 8.5: ●, none; ○, 10^{-5} M DPN; ▲, 10^{-5} M $S_4O_6^{2-}$ and △, 10^{-5} M DPN and 10^{-5} M $S_4O_6^{2-}$. The mixtures were incubated for 20 minutes at 0° at which time a sample of each was diluted to a final enzyme concentration of 0.50 μg/ml with isotonic Veronal buffer for the C'-fixation assay.

TABLE II

ARSENOLYSIS OF ACETYL PHOSPHATE BY LOBSTER MUSCLE TRIOSEPHOSPHATE DEHYDROGENASE[a]

Additions			Acetyl Phosphate Hydrolyzed in 30 min (μmoles)
10^{-3} M $S_4O_6^{2-}$	0.75 Mole DPN	5×10^{-3} M Cysteine	
−	−	−	4.0
−	−	+	5.6
−	+	+	7.4
+	−	−	0
+	−	+	4.5
+	+	+	6.7
+	+	−	0

[a] In a volume of 1 ml, reaction mixtures contained 100 μmoles of $KHCO_3$, 3 μmoles of dibasic sodium arsenate, 10 μmoles of acetyl phosphate, 3 mg of the lobster muscle enzyme, and the additions indicated in the table. Acetyl phosphate was assayed by the method of Lipmann and Tuttle (1945).

The Effect of Irreversible Tetrathionate Inactivation on the Proteolytic Digestion of Muscle TPD's.—Irreversible inactivation of lobster muscle TPD with tetrathionate leads to an increased susceptibility of the enzyme to proteolysis by trypsin. When the lobster muscle enzyme was irreversibly inactivated with tetrathionate and then incubated with trypsin at 37°, the rate of proteolysis, compared to that observed for the native enzyme, was increased as shown in Table III. The rate of proteolysis of the native enzyme was decreased in the presence of excess DPN, as was observed by Racker and Krimsky (1958) for the rabbit muscle enzyme and by Elödi and Szabolcsi (1959) for the swine muscle enzyme. When bound DPN was removed from lobster TPD with charcoal, the enzyme became more susceptible to tryptic digestion as observed by Racker and Krimsky (1958) and by Elödi and Szabolcsi (1959) for mammalian TPD's. Also, addition of DPN to the apoenzyme decreased the rate of digestion. Analogs of DPN which do not function as coenzymes in the oxidation of D-glyceraldehyde-3-phosphate also partly protect the lobster muscle enzyme against tryptic digestion as shown in Table III. The pyridine-3-aldehyde analog of DPN is a strong competitive inhibitor of lobster muscle TPD which, when bound to the enzyme, abolishes the 320–400 mμ absorption band associated with the active TPD-DPN complex (Kaplan *et al.*, 1957; Allison and Kaplan, 1964). The addi-

TABLE III

EFFECT OF COENZYME ON THE TRYPTIC DIGESTION OF LOBSTER MUSCLE TRIOSEPHOSPHATE DEHYDROGENASE[a]

Additions	Rate of Proteolysis Relative to the Native Enzyme	Rate of Proteolysis Relative to the Apoenzyme[b]
Expt 1		
None	1.0	
5×10^{-5} M DPN	0.50	
10×10^{-5} M DPN	0.50	
15×10^{-5} M DPN	0.35	
15×10^{-5} M Pyr-3-ald-DPN	0.65	
15×10^{-5} M TPN	0.70	
3×10^{-5} $S_4O_6^{2-}$	2.70	
3×10^{-5} $S_4O_6^{2-}$ + 15×10^{-5} M DPN	1.50	
Expt 2		
Apoenzyme		1.0
Apoenzyme + 3×10^{-5} M DPN		0.39
Apoenzyme + 6×10^{-5} M DPN		0.23
Apoenzyme + 3×10^{-5} M $S_4O_6^{2-}$		1.4

[a] Experiments were carried out in 0.05 M $NaPO_4$, pH 8.0, at 37°. Reaction mixtures contained 1×10^{-5} M enzyme or apoenzyme, the compounds listed in the concentrations indicated, and 36 μg of trypsin. (See Experimental Procedure for method of obtaining the apoenzyme.) The rate of proteolysis was estimated by the increase in optical density of aliquots deproteinized with 5% trichloroacetic acid. The values shown were recorded after 60 min of digestion. [b] The rate of proteolysis of the native enzyme was about 50% that of the apoenzyme.

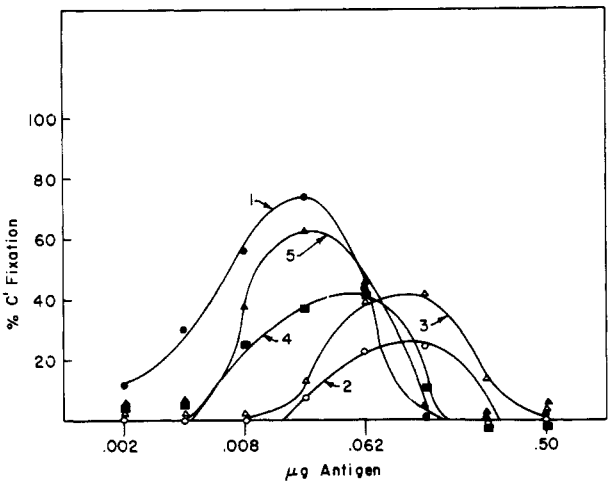

Fig. 11.—Effect of acetyl phosphate on the C′-fixation reaction of charcoal-treated lobster muscle TPD with antilobster TPD. The following additions were made to 10^{-5} M solutions of the apoenzyme in 0.05 M sodium pyrophosphate, *p*H 8.5: curve 1, 0.75 μmole DPN and 3 μmoles Na_2HAsO_4; curve 2, 0.01 M acetyl phosphate; curve 3, 0.01 M acetyl phosphate, 0.75 μmole DPN and 3 μmoles Na_2HAsO_4; curve 4, 0.001 M acetyl phosphate; curve 5, 0.001 M acetyl phosphate, 0.75 μmole DPN, and 3 μmoles Na_2HAsO_4. The apoenzyme was incubated with acetyl phosphate for 30 minutes at 5° before DPN and arsenate were added. After DPN and arsenate were added, the reaction mixtures were incubated at 5° for an additional 30 minutes, at which time samples were diluted with isotonic Veronal buffer to a final enzyme concentration of 0.50 μg/ml for the C′-fixation assay.

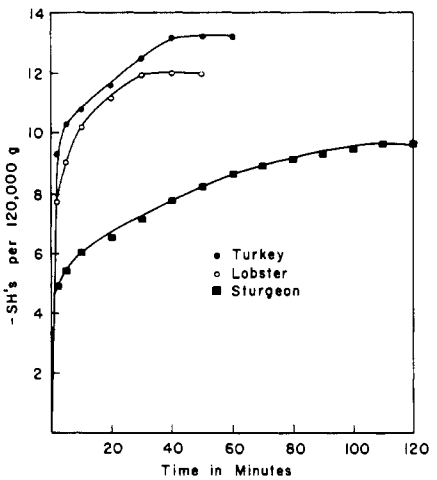

Fig. 12.—Rate of reaction of the —SH groups of various TPD's with *p*-mercuribenzoate in 8.0 M urea.

Table IV

Number of Sulfhydryl Groups in Various Muscle Triosephosphate Dehydrogenases

Enzyme	—SH's Reacting with *p*-Mercuribenzoate within 2 min in 0.1 M $Na\text{-}PO_4$, *p*H 7.0	Total —SH's Reacting with *p*-Mercuribenzoate in 0.10 M $Na\text{-}PO_4$, *p*H 7.0	Total —SH's Reacting with *p*-Mercuribenzoate in the Presence of 8.0 M Urea	Half Cystines Determined as Cysteic Acid
Rabbit	7.2	13.4	12.7	12
Turkey	6.2	8–10[a]	13.2	13
Sturgeon	2.5	2.5	9.6	10
Lobster	5.8	11.6	11.8	12

[a] Turbidity due to the formation of a precipitate developed before titration was complete; 8–10 —SH groups were titrated before the detectable turbidity was observed.

tion of pyridine-3-aldehyde DPN to the lobster muscle TPD decreases the rate of tryptic digestion of the enzyme. Furthermore, TPN, which is completely inactive as coenzyme, partly inhibits the rate of proteolysis of the lobster muscle TPD.

To a lesser degree DPN partly protects the tetrathionate-inactivated enzyme against tryptic digestion, as shown in Table III. Since DPN is less firmly bound in the inactive TDP-DPN complex (Velock and Furfine, 1963; Astrachan *et al.*, 1957) this would be expected. However, tetrathionate also increases the rate of tryptic digestion of the apoenzyme. Therefore it appears that inactivation of the active —SH groups of lobster muscle TPD leads to structural changes, other than decreasing its affinity for DPN, which increase its susceptibility to trypsin.

The Number of Free —SH Groups in Turkey, Sturgeon, and Lobster Muscle TPD's.—Spectrophotometric analysis with *p*-mercuribenzoate (Boyer and Schulz, 1959), amperometric titration with Ag^+ (Benesch *et al.*, 1955), and oxidation with iodosobenzoate (Rafter, 1957) have shown that there are 11 ± 2 free —SH groups per rabbit muscle TPD if one assumes a molecular weight of 120,000. Amino acid analysis for total cystine plus cysteine indicates that all of the nonmethionine sulfur of the rabbit muscle enzyme is equal to the number of the free —SH groups (Velick, 1954). Therefore the enzyme lacks stabilizing disulfide bonds. The —SH groups of the rabbit, turkey, sturgeon, and lobster muscle TPD's were determined in 0.10 M sodium phosphate, *p*H 7.0, both in the presence and absence of 8.0 M urea. A differential reactivity of the —SH groups in each of the TPD's was found in the experiments carried out in the absence of urea, as shown in Table IV. During experiments with the rabbit, turkey, and lobster TPD's there was a slow steady increase in the extinction at 250 mμ after the initial burst of mercaptide formation between the reactive —SH groups and *p*-mercuribenzoate. Turbidity caused by protein precipitation occurred after 8–10 —SH groups in the turkey muscle TPD had reacted with *p*-mercuribenzoate. However, during experiments in the absence of urea with the rabbit and lobster muscle TPD'S, the number of —SH groups that reacted with *p*-mercuribenzoate reached a maximum within an hour and no turbidity developed long after this maximum value was reached. The sturgeon enzyme behaves somewhat differently in its reactivity to the —SH reagent than do the other TPD's. No increase in extinction at 250 mμ over the initial burst of mercaptide formation was observed during experiments with the sturgeon enzyme in the absence of urea. The results of —SH determinations in the presence of 8.0 M urea are also shown in Table IV. The number of —SH groups in the rabbit enzyme that reacted with *p*-mercuribenzoate in 8.0 M urea reached a maximum value of 12.7 within 10 minutes, while the number of —SH groups that reacted with *p*-mercuribenzoate in the turkey and lobster enzymes reached maximum values of 13.2 and 11.8, respectively, within 30 minutes in 8.0 M urea as shown in Figure 12. Some of the —SH groups in the sturgeon muscle TPD reacted very slowly with *p*-mercuribenzoate, even in the presence of 8.0 M urea. The number of —SH groups in the sturgeon enzyme that reacted with *p*-mercuribenzoate in the presence of 8.0 M urea did not reach a maximum value until 100 minutes after the enzyme was mixed with the reagent. All these experiments were carried out at 23° under nearly identical

conditions of enzyme and *p*-mercuribenzoate concentrations. Therefore, the differences in the reactivity of the —SH groups in the various muscle TPD's with *p*-mercuribenzoate are not owing to the effects of concentration.

Automatic amino acid analyses of the rabbit, turkey, sturgeon, and lobster TPD's for cysteic acid following performic acid oxidation of the enzymes revealed that each of the TPD's lacks stabilizing disulfide bonds. Table IV shows that the number of cysteic acid residues determined by automatic amino acid analyses for each of the performic acid–oxidized enzymes is equal to the number of free —SH groups determined with *p*-mercuribenzoate in the presence of 8.0 M urea.

Discussion

The heat-stability studies show that the active —SH groups of muscle TPD's are involved in maintaining the tertiary structure of the molecule in an active configuration. Modification of the active —SH groups of the turkey, sturgeon, and lobster TPD's with tetrathionate decreased the heat stability of all of the enzymes, but to varying degrees with the different enzymes. There is a strong correlation between changes in the immunological properties of the enzymes and the decrease in heat stability after inactivation with tetrathionate. The turkey and lobster TPD's, when inactivated with tetrathionate, are quite susceptible to heat denaturation at 37°, while the tetrathionate-inactivated sturgeon TPD is much more stable. The immunological reactivity of the lobster and turkey TPD's with specific antisera prepared against the active form of the enzymes is decreased to a great extent when the enzymes are reversibly inactivated with tetrathionate. On the other hand, the immunological reactivity of the sturgeon enzyme with a specific antiserum prepared against the active form of the enzyme is only slightly decreased when the enzyme is reversibly inactivated with tetrathionate. These results indicate that the forces holding the tertiary structure of the lobster and turkey enzymes in a specific, active conformation are dependent on the state of the three active —SH groups of the two dehydrogenases. This is true to a lesser degree for the sturgeon enzyme. The fact that only the three active —SH groups of the sturgeon enzyme react with *p*-mercuribenzoate in the absence of urea supports the hypothesis that this dehydrogenase does not unfold to a great extent, even after modification of these three —SH groups. Furthermore, some of the —SH groups in the sturgeon enzyme react sluggishly with *p*-mercuribenzoate even in the presence of 8.0 M urea, indicating that part of the structure of the enzyme is stable.

The losses observed in antigenic activity accompanying chemical modification of the active —SH groups of the TPD's are subject to two interpretations. They may indicate that a specific antigenic determinant is modified by steric or charge effects introduced by covalent bonding of the —SH groups, or alternatively, they may indicate that the positions of several antigenic determinants in three-dimensional space are modified by a conformational change following chemical modification of the active —SH groups.

There is evidence that antisera prepared against protein antigens are specific for a specific three-dimensional conformation. Mills and Haber (1962) have shown that a rabbit antiserum prepared against bovine ribonuclease is specific for a certain spatial configuration of the protein. When ribonuclease is reduced and then reoxidized under conditions where there are formed protein molecules that differ from the native protein only in the arrangement of disulfide linkages, enzymatic activity is lost. These inactive ribonuclease derivatives failed to cross react with an antiserum prepared against the native enzyme as determined by agar-gel-diffusion analysis. Further evidence that antibodies are specific for a given tertiary structure of an enzyme has been obtained from immunologic studies of the pepsinogen system. Using the quantitative C′-fixation technique, Van Vunakis *et al.* (1964) and Gerstein *et al.* (1963) have shown that rabbit antisera prepared against native pepsinogen and denatured pepsin (both isolated from swine) are specific for the conformation of their homologous antigens. When pepsinogen is treated with alkali, urea, or heat, or is photooxidized with methylene blue, the pepsin moiety of the zymogen is unmasked. This conformational change is detected immunologically. Treatment of pepsinogen with alkali, urea, heat, or methylene blue leads to increased reactivity with the antipepsin serum. When pepsinogen was treated with these agents, the C′-fixation curve in experiments with the antipepsinogen serum was shifted to higher antigen concentrations, and decreases in peak height were observed after prolonged treatment. When the TPD's investigated in this study were inactivated with —SH reagents, both a lateral shift toward higher antigen concentrations and a decrease in the height of the C′-fixation curve were observed. Modification of the active —SH groups of the sturgeon TPD with tetrathionate leads to only a slight change in antigenic activity with both the antisturgeon and antiturkey TPD's. On the other hand, such modification of the turkey and lobster TPD's results in extensive losses in antigenic activity with the antisera prepared against the active form of the enzyme. The heat-stability studies and *p*-mercuribenzoate titrations indicate that the sturgeon TPD does not unfold to a great extent in the absence of urea after modification of its active —SH groups, while the same studies indicate that the turkey and lobster TPD's are altered significantly after chemical modification of their active —SH groups. Therefore it appears that the changes in antigenic activity accompanying chemical modifications of the active —SH groups of the TPD's are caused by changes in the tertiary structure of the enzymes and not primarily by the specific blocking of an antigenic determinant.

The possibility arises that tetrathionate inactivation of the TPD might be a result of a conformational change rather than a blocking of essential —SH groups. The fact that the sturgeon TPD apparently does not undergo structural change upon addition of tetrathionate appears to rule out this possibility.

Elödi and Szabolcsi (1959) have hypothesized that bound DPN stabilizes muscle TPD's in their native conformations, since removal of bound DPN from mammalian muscle TPD's with charcoal resulted in changes in optical rotation, intrinsic viscosity, and trypsin digestibility, all of which were reversed by the addition of coenzyme. They have noted, however, that the addition of more than 3 moles of DPN to the swine muscle apoenzyme increased the intrinsic viscosity over that of the native enzyme (2 moles of DPN/mole of enzyme) and reduced trypsin digestibility to very low values. During their original studies with the rabbit muscle enzyme, Taylor *et al.* (1948) observed that the charcoal-treated apoenzyme could not be crystallized, while it was easily crystallized after the readdition of DPN. Velick and Furfine (1963) have shown that excess DPN stabilizes rabbit muscle TPD against spontaneous inactivation at 39°. They found that the enzyme in the presence of a 5000 M excess of DPN was completely stabilized at 39°. Analysis of several crystalline muscle TPD's for bound DPN has revealed that the turkey, pheasant, and sturgeon dehydrogenases

are virtually free of coenzyme, while the halibut and chicken enzymes crystallize with less than 1 mole of DPN bound per mole of enzyme (Allison and Kaplan, 1964). All of these enzymes are as stable as the rabbit muscle enzyme when stored at 5° as suspensions in ammonium sulfate in the presence of 10^{-3} M EDTA. The observed stabilization of rabbit muscle TPD against spontaneous inactivation at 39° by excess DPN may be owing to a multivalent effect of DPN similar to the stabilization of the native and reversibly inactivated TPD's by the sulfate anion discussed in this study.

The results of the immunological experiments of this study indicate that bound DPN does not play a prominent role in the stabilization of the native conformations of the TPD's. The addition of DPN to the charcoal-treated lobster muscle enzyme or to the turkey and sturgeon enzymes does not alter their antigenicity with antisera prepared against the native enzymes.

The observation that the rate of tryptic digestion of mammalian TPD's is decreased by the presence of bound DPN (Racker and Krimsky, 1958; Elödi and Szabolcsi, 1959) has been confirmed for the lobster muscle enzyme. Since the rate of proteolysis of the tetrathionate-inactivated lobster muscle TPD, even in the presence of excess DPN, is greater than that of the native enzyme, it appears that the protection of TPD against proteolysis by DPN depends on the state of the active —SH groups of the enzyme. This does not necessarily mean that there is a direct interaction between the active —SH groups of TPD and bound coenzyme as proposed by Racker and Krimsky (1952). The proposal for a direct interaction between the active —SH groups of TPD and bound DPN is based on the observation that the broad absorption band between 320 and 400 mμ is destroyed when the active —SH groups of the enzyme are modified with iodoacetate or *p*-mercuribenzoate (Racker and Krimsky, 1952). Kosower (1958) has suggested that the 320–400 mμ absorption band is the result of charge transfer between the enzyme thiol group and the pyridine ring. However, recent evidence suggests that the broad absorption band is due to charge transfer from an indole ring of a tryptophan residue to the pyridinium moiety of DPN (Shifrin, 1964). Shifrin has prepared indolylethyl nicotinamide, which has an absorption spectrum in the visible region and is quite similar to that exhibited by the active TPD-DPN complex, which is not exhibted by a mixture of tryptamine hydrochloride and nicotinamide methochloride. Further evidence supporting the hypothesis that there is a charge-transfer interaction between tryptophan residues of TPD and bound coenzyme is provided by a comparison of the emission spectrum at 340 mμ of the model compound and that of the TPD-DPN complex. The pyridnium moiety of the model compound quenches the indole fluorescence apparently by a charge-transfer transition (Shifrin, 1964). Bound DPN partly quenches the tryptophan fluorescence of active TPD (Velick, 1958). Modification of the active —SH groups of rabbit muscle TPD with tetrathionate (W. S. Allison and M. O. Kaplan, unpublished observations) abolishes the quenching of the tryptophan fluorescence by DPN. This indicates that chemical modification of the active —SH groups of TPD leads to structural changes that affect the orientation of the coenzyme. Astrachan *et al.* (1957) observed that DPN bound to inactive TPD is more susceptible to other enzymes than is the DPN bound to the active enzyme. Moreover, Nygaard and Rutter (1956) found that DPN bound to rabbit muscle TPD was reduced more slowly than free DPN by beef heart lactic dehydrogenase in the presence of lactate, but the bound DPN was reduced at the same rate as free DPN when the TPD was inactivated by mercuribenzoate. From the evidence presented in the present study, it appears that the increased susceptibility of DPN to enzymes in the inactive TPD-DPN complex over that in the active TPD-DPN complex to enzymatic attack is due to a change in enzyme conformation following chemical modification of the active –SH groups.

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