IV. 1. SOME ASPECTS OF PROTEIN STRUCTURE IN RELATION TO THE ROLE OF —SH AND —S—S— GROUPS IN ENZYMIC CATALYSIS *

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I. Present Concepts of the Role of —SH and —S—S— Groups

It is our privilege and obligation at this symposium to examine the present knowledge about the role of —SH and —S—S— groups in enzyme catalysis. Ours is not a new undertaking; since the classical and pioneering studies of Hopkins, of Rapkine, and others over 20 years ago, the —SH group has received much attention. The extent of present interest is indicated by results tabulated in a recent review (1). In a period of a little over 2 years, about 135 reports were noted concerning effects of agents reacting with —SH groups on enzymes or enzyme systems and, of these, about 87% showed definite inhibition by —SH group reagents. Likely, in most instances this inhibition did not reflect a primary role of the —SH group in the catalytic process. The role of —S—S— groups in enzyme catalysis has received less attention, although there has been increasing appreciation and interest in the past several years of the structural role of —S—S— groups.

At the outset, there may be some value in presenting briefly an ap-

praisal of various possible modes of action of —SH and —S—S—. What appear to be the most likely possibilities are given in the next sections.

1. POSSIBLE CAUSES OF ENZYME INHIBITION
UPON REACTION OF —SH GROUPS

Causes of inhibition resulting from mercaptide formation, oxidation, alkylation, or other reaction of —SH groups may be conveniently divided into four categories.

a. The —SH Groups May Have a Primary Role in the Catalysis

This has been the most frequently suggested explanation for the inhibition of catalytic activity by —SH group reagents. Unfortunately, such a role has been often suggested but little proved. Means by which —SH groups might participate directly in the catalysis are as follows:

(1) *As an Acyl Acceptor.* The participation of an acyl-S-enzyme in glyceraldehyde-3-phosphate dehydrogenase catalysis is perhaps the best established function of —SH groups in an enzymic reaction. It is in some way fitting that this finding should be made with the enzyme whose inhibition by iodoacetate played a prominent role in early studies of muscle contraction. Evidence for the participation of —SH as the acyl acceptor rests principally upon demonstrations that an acyl-enzyme is an intermediate (*1, 2*), the known chemical properties of acyl-S-R compounds, and the demonstration that approximately 2 —SH per mole of enzyme become unreactive toward *p*-mercuribenzoate upon acyl-enzyme formation (*3*). The sum total of the evidence is convincing but not conclusive.

The manner by which the acyl-enzyme is formed remains to be elucidated. Concepts involving hemiacetal formation (*2, 4*) or "aldehydolysis" of an enzyme-S-DPN linkage (*2, 5*) have gained temporary acceptance but fallen to the category of attractive suggestions with the considered appraisal of time and more detailed scrutiny.

Although it is logical that covalent acyl-S-enzyme compounds might participate in other enzymic catalyses, good evidence for such function has not yet been obtained.

(2) *In Cofactor Binding.* Inherent in a function of cofactor binding is also the possibility that the —SH participating in the binding may aid electronic rearrangements promoting catalysis. Good evidence for the participation of —SH in binding of metal cofactors and of coenzymes has been obtained by various investigators, including Velick, Racker, Kaplan, Wallenfels, Hoch, and Vallee and others. Undoubtedly more evidence bearing on this possibility will be discussed by these and other speakers at this symposium.

SH groups may participate in cofactor as well as substrate binding by

formation of covalent addition products, by ionic attraction to a mercaptide ion, by participation of an —S⁻ or —SH in H-bonding, or by formation of charge transfer complexes. With regard to the latter, Kosower (6) calls attention to the fact that mercaptide ions should behave as good donors for charge transfer complexes. Such a complex could be responsible for the intriguing broad spectral band at 365 mμ observed in glyceraldehyde-3-phosphate dehydrogenase by Racker and Krimsky (5).

(3) *In Substrate Binding.* As with cofactors, such binding might also be accompanied by activation. In one sense the formation of an acyl-S-enzyme represents binding of a portion of a substrate. A related interesting suggestion is that an —SH of transamidinase may participate in the formation of an acyl-amidine derivative (7). No good evidence appears to be at hand for participation of —SH groups in substrate binding by non-covalent bonding.

(4) *Alternate Oxidation and Reduction.* —SH participation in this manner has been frequently suggested, and still remains a good possibility. Such suggestions gain support from the ease with which —SH groups may be oxidized and —S—S— groups reduced. Participation does not necessarily involve bivalent oxidation and reduction as transient free-radical formation appears plausible. However, evidence sufficient to give good support to an oxidation-reduction role appears singularly lacking.

(5) *Participation as Phosphoryl Acceptor.* The occasional suggestion that an —SH group might act as a transient acceptor of a phosphoryl group in enzymatic phosphate transfer does not as yet have experimental support.

(6) *Other Modes of Action.* Likely, as with most areas where our understanding is meager, other modes of action will be found. For example an —SH group might not participate in substrate or cofactor binding, but could interact with the substrate or cofactor subsequent to binding so as to promote catalysis.

b. —SH *May Help Maintain the Requisite Tertiary Structure*

This is a possibility that can be neither dismissed nor regarded as probable on the basis of present knowledge. The types of bonding mentioned above for possible participation of —SH groups in binding of substrates and cofactors might also be involved in the maintenance of tertiary structure. The H of —SH groups may act as donors for weak hydrogen bonds (1, 8), and such bonds might contribute critical extra stabilization when other factors favoring a particular tertiary structure are also present. The possibility of charge transfer complexes between —SH groups and tyrosine residues should not be overlooked. Ionic attractions or even covalent bonds other than —S—S— remain possible.

c. Reaction of —SH May Cause Structural Changes Resulting in Inactivation

Such changes might be independent of any direct role of —SH in the tertiary structure or primary catalytic role of the —SH. If this type of change, or that mentioned under *d* below, occurs, the —SH groups responsible should be regarded as a liability to the enzyme, not as an asset.

The fine studies of Madsen and co-workers (*9-11*) on the cleavage of phosphorylase subsequent to reaction with *p*-mercuribenzoate give the best demonstration of structural change resulting from —SH reaction. The cause for this interesting structural change remains to be elucidated. It may be pertinent to the observed precipitation of some proteins at neutral pH and room temperature upon reaction with *p*-mercuribenzoate. Structural changes, caused by either removal of a bond aiding maintenance of tertiary structure or by spatial displacement, appear to be the most likely explanations. Proteins may be regarded as in a dynamic state with minor fluctuations in structure in different portions of the molecule. Such fluctuations may temporarily expose an —SH group with resultant mercaptide formation, prevention of return to other configurations, and promotion of a weakening of tertiary structure leading to exposure of other groups and continued structural change.

Another type of structural change, that accompanying intramolecular or intermolecular disulfide formation, may form the basis for the activation of many enzymes by low molecular weight thiols. In evaluation of possible structural changes accompanying disulfide formation or cleavage, attention should be given not only to the bringing together of portions of the peptide chain, but to the restricted rotation of disulfide bonds, favoring the occurrence of a dihedral angle of 90° (*12*).

d. Substances Reacting with —SH Groups May Interfere by Their Proximity to a Catalytic Site

Such interference might be steric, or might possibly result from the chemical properties of the moiety attached to the S. The former possibility was recognized by Singer, who presented evidence that the extent of inhibition of wheat germ lipase by —SH group reagents increased with size of the substrate (*13*).

It has proven to be a difficult problem to distinguish between actions as given in subsections *a* and *b*, where the —SH may be regarded as having an essential role, and subsections *c* and *d*, where the —SH does not have any role in the catalysis. Progress in this area may come from studies directed primarily towards elucidation of the catalytic mechanism of

various enzymes, rather than from studies directed primarily toward the action of —SH groups.

2. The Role of —S—S— Groups in Enzymic Catalysis

The primary and basic contribution of —S—S— groups to the tertiary structure of enzymes and other proteins is well-established. The structural role of —S—S— in other proteins has already been mentioned, and will be discussed at length in this symposium. In all instances, admittedly few, where —S—S— bonds are known to occur in enzymes, loss of enzyme activity results from cleavage of all the —S—S— bonds, although activity may be retained when some —S—S— bonds are cleaved (see reference 1). Other possible direct roles of —S—S— groups have been suggested, but these appear so tenuous as to not warrant further discussion at this time.

II. Some Studies on Structure and Catalysis with Aldolase and Glyceraldehyde-3-phosphate Dehydrogenase

The remainder of this presentation will concern some recent studies on the effect of urea and p-mercuribenzoate on the activity of rabbit muscle aldolase and glyceraldehyde-3-phosphate dehydrogenase,[*] together with some additional observations pertinent to the structure and catalysis by these proteins. Both enzymes appear to contain no —S—S— linkages (although this has not been firmly established), are active in the presence of relatively high concentrations of low molecular weight thiols, and may be inhibited by reaction with p-mercuribenzoate. The aldolase has nearly twice as many —SH groups per unit weight as the dehydrogenase. However, with aldolase, unlike with the dehydrogenase, no essential role of the —SH has been demonstrated and indeed, as noted above, the —SH groups may actually be a liability to the enzyme. Other important differences will be discussed shortly.

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[*] The rabbit muscle aldolase and glyceraldehyde-3-phosphate dehydrogenase were conventional preparations (14, 15) crystallized in presence of $5 \times 10^{-4} M$ ethylenediaminetetraacetate (EDTA). For the data reported, extinction coefficients and molecular weights given by Taylor et al. (16) were used (aldolase molecular weight: 149,000; glyceraldehyde-3-phosphate dehydrogenase molecular weight: 120,000). There is uncertainty about the molecular weight of the dehydrogenase; Dandliker and Fox have reported measurements leading to a value of 138,000 to 140,000 (17).

Measurements of the catalytic activity of the dehydrogenase with glyceraldehyde-3-phosphate were made essentially as described previously (3) but with use of 0.03 M fructose-1,6-diphosphate previously incubated for 10 minutes at 50° with 4.5×10^{-7} aldolase at pH 7.6 as a source of the substrate. Measurements of the extent of reaction with p-mercuribenzoate were made spectrophotometrically (18).

1. Effect of Urea on Enzymic Catalysis

The data in Fig. 1 show the effects of urea on the catalytic activity of aldolase. When the activity is measured in the presence of urea, all activity is lost at 3.5 M urea concentration. The most likely interpretation is that urea has caused structural change leading to displacement of
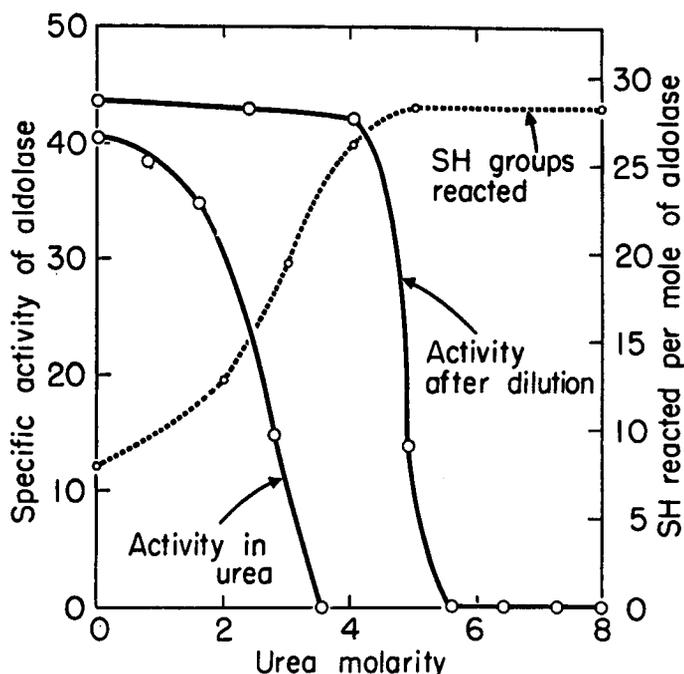

Fig. 1. Effect of urea on the catalytic activity and reactivity of —SH groups of aldolase. The figure is taken from Swensen and Boyer (19). The solid curves show the activity of aldolase at 30°, pH 7.4, in the presence of the urea concentration indicated, or upon exposure (at higher aldolase concentrations) to the urea concentration indicated at 30° for 10 minutes, followed by 100 fold dilution and activity measurement. The dotted curve shows the number of —SH groups reacted in 3 minutes at pH 7.0 and 25°, with $3.5 \times 10^{-5} M$ p-mercuribenzoate and $0.75 \times 10^{-6} M$ aldolase.

groups forming a catalytic site. That aldolase is not irreversibly inactivated by this concentration of urea is shown by the results obtained when aldolase is exposed to urea, then the urea concentration markedly lowered by dilution, and the activity measured (Fig. 1). More than 5 M urea was required for irreversible inactivation.

Glyceraldehyde-3-phosphate dehydrogenase is much more sensitive to urea than aldolase, and indeed appears to be among the most sensitive

enzymes. Alcohol dehydrogenase also shows pronounced sensitivity to urea (20). The effect of urea on the catalytic activity of glyceraldehyde-3-phosphate dehydrogenase is shown in Fig. 2. All activity is lost at 1.5 M urea with glyceraldehyde-3-phosphate as a substrate, and irreversible inactivation occurs at a urea concentration of 2.5 M. This striking sensitivity to urea was noted earlier by Sekuzu et al. (20). As with the aldolase,
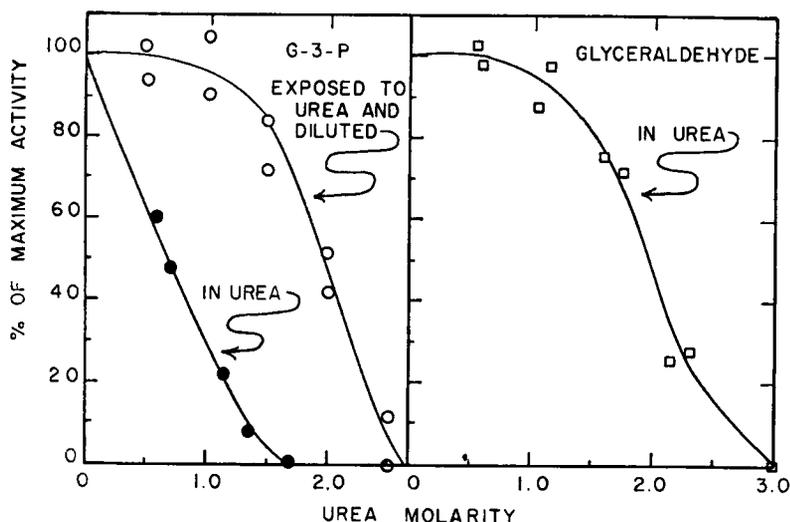

Fig. 2. Effect of urea on catalysis by glyceraldehyde-3-phosphate dehydrogenase. Activity measurements with glyceraldehyde-3-phosphate as a substrate were made at 30° in 0.023 M pyrophosphate buffer, pH 8.0. Measurements in urea were made with $3 \times 10^{-9} M$ dehydrogenase. For measurements after urea dilution, $3.2 \times 10^{-6} M$ dehydrogenase was exposed to the urea concentration indicated, then diluted to $3.2 \times 10^{-9} M$ dehydrogenase for measurement. Measurements with glyceraldehyde as a substrate were made with $2.12 \times 10^{-6} M$ dehydrogenase in the urea concentration as indicated.

the most probable, but at this stage by no means the certain, explanation is that the effect of urea results from structural changes.

Of definite interest is the finding that activity with glyceraldehyde is less sensitive to urea than is activity with the phosphorylated substrate. As shown by the right hand curve of Fig. 2, approximately 3 M urea was required for inactivation with glyceraldehyde.† The reaction rate with

† The difference with the two substrates cannot at this time be ascribed definitely to differences in susceptibility to structural change of the enzyme. Because the rate of reaction with glyceraldehyde is much slower than that with 3-phosphoglyceraldehyde, much more enzyme was present in the trials with glyceraldehyde, and it is possible that enzyme concentration might affect the extent of structural change.

glyceraldehyde-3-phosphate in the absence of urea is much more rapid than that with glyceraldehyde under the assay conditions used. It is plausible that 1.5 M urea makes activity toward the phosphorylated substrate similar to that with glyceraldehyde; such slight activity would not have been detected under the assay conditions. Both glyceraldehyde and 3-phosphoglyceraldehyde oxidation probably proceeds through formation of an acyl-S-enzyme intermediate, thus it is unlikely that 1.5 M urea is interfering with this —SH function.

The sensitivity of glyceraldehyde-3-phosphate oxidation to urea recalls an earlier observation in this laboratory that Veronal inhibits catalysis with the phosphorylated substrate but not with glyceraldehyde (4). The differential effect of urea on activity toward the two substrates is also somewhat analogous to some recent observations on aldolase degradation and activity. With aldolase, removal of three terminal tyrosines markedly decreases the catalytic activity towards fructose-1,6-diphosphate, but slightly increases the activity towards the poorer substrate, fructose-1-phosphate (21).

2. EFFECT OF UREA ON REACTION WITH p-MERCURIBENZOATE

Shown in Fig. 1 is the effect of urea concentration on the extent of reaction of the —SH groups of aldolase with p-mercuribenzoate in a three minute period. The concentration of urea required to bring about reaction of all the —SH groups is approximately that required to cause reversible inactivation of the protein. It appears, however, that considerable structural change can occur without irreversible activity loss.

The effect of urea on the time course of mercaptide formation with glyceraldehyde-3-phosphate dehydrogenase is shown in Fig. 3. In contrast to the effects on catalytic activity, the effect of urea on promotion of reaction of the —SH groups is similar to the results obtained with aldolase. Of importance is the fact that, within limits of experimental error, 1.5 M urea did not increase the reactivity of those —SH groups which react slowly at pH 7. As with aldolase, concentrations of urea somewhat above 4 M were necessary for all the —SH groups to react. With glyceraldehyde-3-phosphate dehydrogenase, unlike aldolase, irreversible inactivation resulted at urea concentration considerably below those necessary for maximal reactivity of the —SH groups. These differences emphasize that at this stage of development of our knowledge, each protein appears in many respects to be a story unto itself.
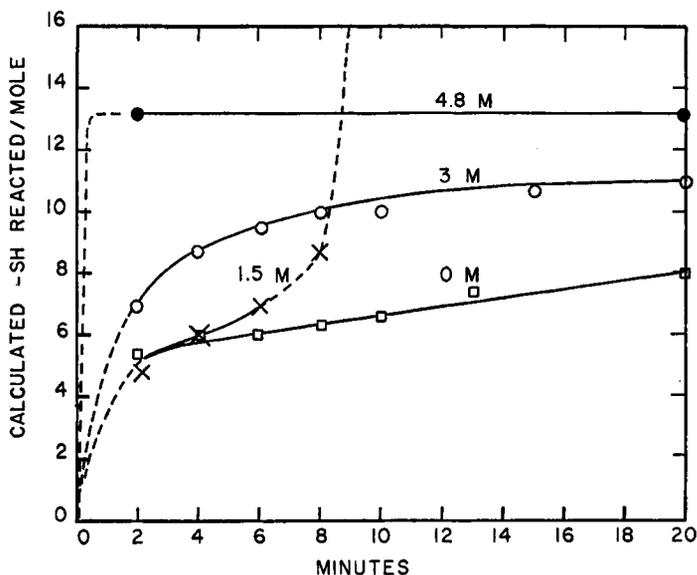
FIG. 3. Effect of urea on the time course of mercaptide formation between p-mercuribenzoate and glyceraldehyde-3-phosphate dehydrogenase. Measurements of the amount reacted were made spectrophotometrically at 250 mμ, using $2.35 \times 10^{-6}\ M$ dehydrogenase and $4.05 \times 10^{-5}\ M$ p-mercuribenzoate in 0.05 M phosphate buffer, at approximately 25°, pH 7.0. The rapid increase in apparent reaction in presence of 1.5 M urea resulted from marked turbidity formation. Other samples remained visibly clear during the 20 minute reaction period.

3. EFFECT OF UREA, MERCAPTOETHANOL, AND p-MERCURIBENZOATE ON OPTICAL ROTATION*

The striking difference in susceptibility of the two enzymes to urea inactivation made of interest comparisons of the effect of urea on the optical rotation of the enzymes, in as much as subtle changes in structure may be reflected in rotation changes. In Fig. 4 are shown the specific rotations of aldolase at various urea concentrations. Comparable experiments for the dehydrogenase are shown in Fig. 5. With both enzymes changes in rotation were nearly complete within several minutes after addition of the higher urea concentrations, thus they resemble β-lactoglobulin in this respect (22). The sensitivity of both enzymes to urea is considerably

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* Optical rotation measurements were made with a Rudolph photoelectric polarimeter through the cooperation of Dr. John Schellman, using the 5461 Å line of mercury, and a 1 decimeter cell holding about 2.5 ml. Optical rotations as reported are not corrected for the refractive index increment of the urea; this correction would reduce the measured values in 6 M urea by about 4%.
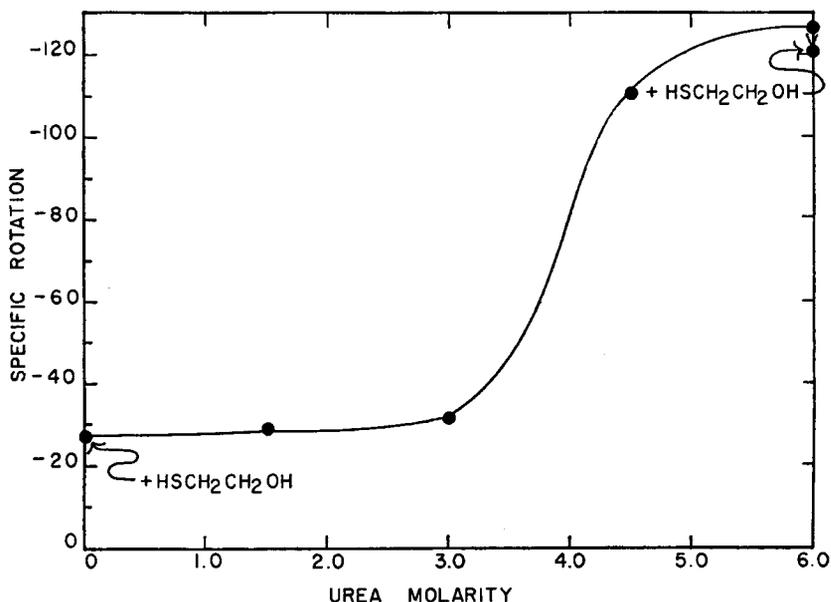
FIG. 4. The effect of urea concentration on the optical rotation of aldolase. Measurements in the absence of urea were made with a solution containing $1.8 \times 10^{-4}\,M$ aldolase in $0.05\,M$ phosphate buffer, pH 7.4, and $1.5 \times 10^{-4}\,M$ EDTA at 25.0°. Measurements in presence of urea were made by appropriate dilution of the initial solution with $8\,M$ urea, in order to conserve protein. Solutions stood approximately 15 minutes at each urea concentration. Measured rotations were constant during this period, except for the $4.5\,M$ sample which showed a slow, continued increase in rotation. The points indicated show the effect of addition of sufficient mercaptoethanol to make the solution $0.1\,M$ in mercaptoethanol on the rotation in the presence of 0 and $6\,M$ urea.

greater than that noted by Kauzmann and Simpson for serum albumin, β-lactoglobulin, ovalbumin, and pepsin (*22, 23*).

Comparison of Figs. 4 and 5 shows that both enzymes gave strikingly similar increases in optical rotation with increase in urea concentration, although the initial levorotation of the dehydrogenase (−35.6°) is definitely greater than that of the aldolase (−26.9°). Thus the marked sensitivity of glyceraldehyde-3-phosphate dehydrogenase to urea does not result from any marked structural change as revealed by optical rotation. It must be recalled, however, that small changes in structure may be sufficient to obviate enzymatic catalysis, particularly a complicated catalysis involving interaction with 3 different substrates. Slight optical rotation increases are caused by $1.5\,M$ urea.

Also shown on Figs. 4 and 5 are the effects on rotation following addition of mercaptoethanol to the enzyme solutions when $6\,M$ urea was present or absent. In the absence of urea, no change in optical rotation was
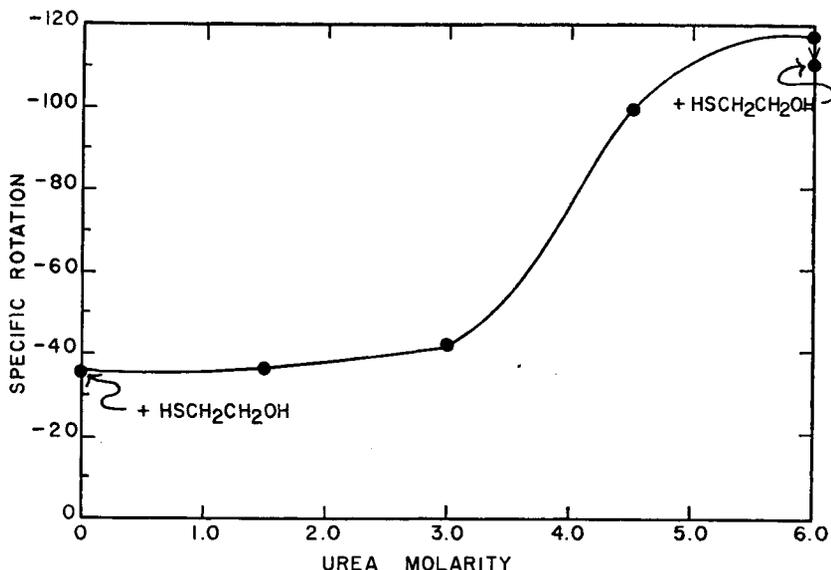
FIG. 5. The effect of urea concentration on the optical rotation of glyceraldehyde-3-phosphate dehydrogenase. The experimental procedure was, as given in Fig. 4, with $1.9 \times 10^{-4}\ M$ protein in the initial solution without urea. Measured rotations were essentially constant, except for the 4.5 M sample which showed a slow continued increase in rotation, and the 3.0 M sample for which only initial readings were possible because of the marked turbidity which developed after several minutes.

observed. This likely reflects an absence of —S—S— linkages in these enzymes. When 6 M urea was present, addition of mercaptoethanol resulted in a slight drop in rotation with both enzymes. Perhaps some disulfide linkages had formed in the solutions and these were cleaved by the thiol.

Some measurements on the effect of p-mercuribenzoate on the optical rotation in presence and absence of urea were attempted. Turbidity formation accompanying reaction with p-mercuribenzoate limited measurements with aldolase and made measurement with the dehydrogenase impractical except at high urea concentrations. Results obtained with aldolase are shown in Fig. 6. It may be seen that in the absence or the presence of 4.5 M urea, the observed rotations were only slightly increased by the reaction with the p-mercuribenzoate. Clearly any structural changes that may be induced by the p-mercuribenzoate appear to be small, yet sufficient change was occurring in the aldolase solution without urea present to give rise to considerable turbidity after about 10 minutes. In the relatively concentrated protein solution, only a small fraction of the protein need precipitate to cause turbidity formation. It may be that an "all or none"
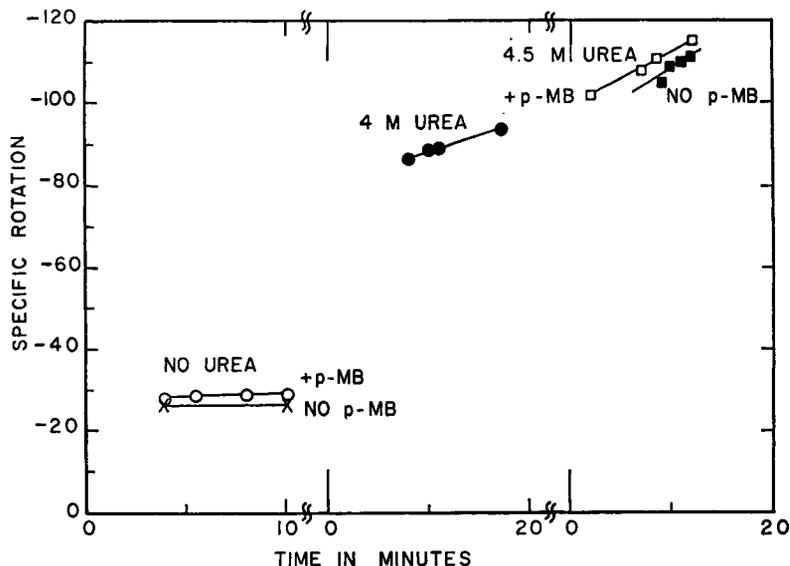
Fig. 6. The effect of *p*-mercuribenzoate on the optical rotation of aldolase at different urea concentrations. Conditions were as given in Fig. 4; *p*-mercuribenzoate when present was 0.004 *M*. Measurements were not feasible at 1.5 or 3.0 *M* urea because of the rapid development of turbidity.

phenomenon is operative, as suggested by Madsen for the cleavage of phosphorylase by reaction with *p*-mercuribenzoate (*9–11*).

With glyceraldehyde-3-phosphate dehydrogenase, as with aldolase, no change in rotation resulted when *p*-mercuribenzoate was added to a solution in 4.5 *M* urea. This is similar to the result reported with ovalbumin in 7.5 *M* urea (*23*). The effect of *p*-mercuribenzoate on the rotation of aldolase at lower urea concentrations appears to be somewhat less than that noted for ovalbumin (*23*).

4. DPN Binding and the Optical Rotation
of Glyceraldehyde-3-Phosphate Dehydrogenase

An important consideration relative to enzymatic catalysis is whether binding of a substrate or cofactor might be accompanied by structural changes in the enzyme protein. Various investigators have suggested that such structural or conformation changes could have an important role in catalysis (*24, 25*), and Klotz and Heiney have shown change in the rotation of an hemocyanin upon oxygenation (*26*). Glyceraldehyde-3-phosphate dehydrogenase as normally prepared crystallizes with 2 molecules of bound DPN per mole of enzyme, and this DPN can be removed by treatment with charcoal. Rotation measurements on such dehydrogenase prepara-

TABLE I

EFFECT OF DPN ON THE SPECIFIC ROTATION OF
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE[a]

Enzyme Preparation	Specific Rotation
Crystallized with DPN	−35.6°
Charcoal treated	−38.4°
Charcoal treated DPN	−36.0°[b]

[a] Rotations were measured under conditions as given in Fig. 4. Measurements were made with $2.06 \times 10^{-4} M$ enzyme as crystallized with DPN (15), with the same enzyme solution treated with charcoal to remove DPN (27), and with the charcoal treated enzyme solution to which $3 \times 10^{-4} M$ DPN was added. Protein concentrations were determined by ultraviolet absorption at 290 mμ using values given by Velick et al. (27).

[b] Value corrected for rotation of added DPN. Without correction, the rotation would be −35.6°.

tions are given in Table I. The slight and reversible increase in levorotation upon removal of the DPN is of definite interest. It appears likely that the increased rotation reflects a slight loosening of structure when DPN dissociates from the enzyme, and regaining of the original structure upon addition of DPN. Some properties of the dehydrogenase may be a reflection of this structural change, such as the observation that DPN is required for acetyl phosphate hydrolysis and acetyl transfer reactions by the enzyme, although it does not appear to be reduced and oxidized in such catalysis (27, 28), and that DPN removal renders the enzyme more susceptible to tryptic digestion (29). Structural change upon binding of cofactors or substrates may be pertinent to a number of observations on enzyme catalysis, such as the interesting report that binding of DPNH to lactic dehydrogenase appears necessary for formation of a binding site for pyruvate (30).

III. Summary

Various possible roles of —SH groups and —S—S— groups in enzyme catalysis have been reviewed. Attention has been directed to the limited number of instances where good evidence is available for an essential role of —SH groups, and to the possibility that for some enzymes the presence of —SH groups may be a liability.

Structural features of enzymes in relation to their catalytic activity and the role of —SH groups have been briefly considered, together with data from recent studies with muscle aldolase and glyceraldehyde-3-

phosphate dehydrogenase. The catalytic activity of the dehydrogenase is much more sensitive to urea than that of aldolase, although urea has similar marked effects on the two enzymes in increasing the optical rotation and the reactivity of —SH groups toward p-mercuribenzoate. Over $4\ M$ urea is required for irreversible inactivation of aldolase, but less than $3\ M$ results in irreversible inactivation of the dehydrogenase. The effect of urea on optical rotation increase of aldolase is only slightly increased by p-mercuribenzoate, although the p-mercuribenzoate induces rapid appearance of turbidity at low urea concentrations.

At $1.5\ M$ urea, dehydrogenase activity with glyceraldehyde-3-phosphate as a substrate is blocked, but activity with glyceraldehyde as a substrate is only slightly impaired.

Glyceraldehyde-3-phosphate dehydrogenase shows a slight increase in levorotation upon removal of bound DPN by charcoal, and this increase is reversed by the addition of DPN.

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