the *p*H range indicated, a reasonable accuracy is obtainable. The increase of the reaction period to 48 hr. caused an additional uptake of 0.01 meq. hydroxide ion/g. indicating that the reaction is essentially completed in 24 hr.

TABLE II

THE EFFECT OF HYDROXIDE ION CONCENTRATION ON THE METHYL ESTER DETERMINATION OF METGEL

Expt. no.	Equilibrium[a] *p*H	Meq. OH^- consumed / g. Metgel
1	7.75	0.90
2	8.00	.88
3	8.4	.90
4	8.5	.90
5	8.5	.93
6	9.0	.94
		.91

[a] The *p*H of the system after 24 hr. incubation.

A check of the validity of this technique is offered by a comparison of these results with those derived by other methods. The methyl ester content of Metgel may be calculated from the difference in the titration curves of the initial and final materials. The validity of these results has been discussed in a previous section. Still another method for methoxyl analysis is the micro-Zeisel determination.[25] A compilation of these analyses for two different preparations of Metgel is given in Table III.

TABLE III

ANALYTICAL VALUES FOR THE METHOXYL CONTENT OF METGEL (MEQ./G. METGEL)

Method	Lot 2	Lot 3
Alkaline hydrolysis	0.71	0.91
Titration (difference)	.71	.94
Micro-Zeisel[a,b]	..	.87[c]

[a] Analyses performed by Elek Microanalytical Labs., L. A., Calif. [b] The value for gelatin was 0.10 meq./g. as compared to the calculated value of 0.04 meq./g. (computed for the methionine content). [c] Not corrected for the gelatin blank.

From these data it may be concluded that the alkaline hydrolysis method for methoxyl analysis is relatively simple and reliable to ± 4%.

Acknowledgment.—The authors wish to acknowledge the able assistance of Mr. Trayon Onett in the latter phase of this problem.

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PITTSBURGH, PA.

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Sulfhydryl Groups in Relation to Aldolase Structure and Catalytic Activity[1]

BY A. D. SWENSON AND P. D. BOYER

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Spectrophotometric measurements readily reveal a graded reactivity of *p*-mercuribenzoate with the –SH groups of rabbit muscle aldolase; some react rapidly at *p*H 7 and 25°, some react much more slowly, and others react only in the presence of urea. With 4.8 *M* or higher urea concentrations, 28 *p*-mercuribenzoate molecules react rapidly per aldolase molecule. The catalytic activity is progressively decreased to 0 as urea concentration is increased up to 3.6 *M*; inactivation by up to 4 *M* urea is completely reversible upon dilution. The rate and extent of reaction with *p*-mercuribenzoate is not influenced by the presence of an excess of substrates. Up to 10 –SH groups per mole of aldolase can react with *p*-mercuribenzoate without decrease in the catalytic activity. Additional reaction with *p*-mercuribenzoate causes a reversible loss of catalytic activity which appears to be associated with structural changes in the aldolase.

Introduction

Considerable information has been accumulated about the status of sulfhydryl groups in enzymes and their catalytic activity.[2] For example, the pioneering studies of Hellerman, *et al.*,[3] with urease showed that the most reactive –SH groups were not essential for catalytic activity but that reaction of additional –SH groups resulted in marked activity loss. Quantitative observations on the relationships between enzyme structure, number of –SH groups reacted with a particular reagent, and the catalytic activity have been limited. Particularly lacking are results which give a clear indication of whether the essential –SH groups have a primary role in the catalysis or whether reaction of the –SH groups results in secondary changes leading to loss of activity. The latter possibility has been suggested by others, including Desnuelle and Rovery[4] and Nygaard.[5]

The rate and extent of the reaction of –SH groups of proteins with the mercaptide forming agent, *p*-mercuribenzoate, at neutral *p*H and under mild conditions, may be measured conveniently by a spectrophotometric technique.[6] This paper gives results of application of the spectrophotometric procedure to measurements of the –SH groups of rabbit muscle aldolase together with catalytic activity measurements in the presence and absence of various urea concentrations. Rabbit muscle aldolase was chosen for study because it is readily obtainable in pure form,[7] conveniently assayed, and has been reported to be inhibited by reaction with

(1) Supported in part by research grant 1783 of the National Science Foundation and by the Hill Family Foundation.

(2) E. S. G. Barron, *Advances in Enzymol.*, **11**, 201 (1951).

(3) L. Hellerman, F. P. Chinard and V. R. Dietz, *J. Biol. Chem.*, **147**, 443 (1943).

(4) P. Desnuelle and M. Rovery, *Biochem. Biophys. Acta*, **8**, 26 (1949).

(5) A. P. Nygaard, *Acta Chem. Scand.*, **10**, 397 (1956).

(6) P. D. Boyer, THIS JOURNAL, **76**, 4331 (1954).

(7) J. F. Taylor, A. A. Green and G. T. Cori, *J. Biol. Chem.*, **147**, 591 (1948).

p-mercuribenzoate[8] and to contain 29 –SH groups per mole as measured by amperometric titration.[9]

Results

Reactive Sulfhydryl Groups of Aldolase.—The addition of increasing amounts of aldolase to a fixed concentration of *p*-mercuribenzoate at *p*H 7 results in a progressive increase in the absorbance of the *p*-mercuribenzoate at 250 mμ until all the *p*-mercuribenzoate has reacted with –SH groups. Results obtained when absorbance measurements were made 3 and 60 minutes following addition of the aldolase are shown in Fig. 1A.

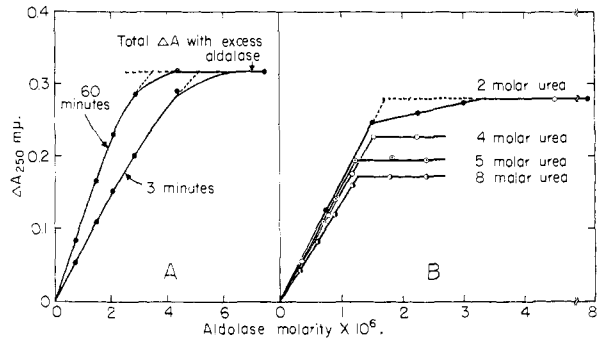

Fig. 1.—Spectrophotometric shift from addition of aldolase to *p*-mercuribenzoate in (A) 0.05 *M* phosphate buffer and 3.5 × 10^{-5} *M* *p*-mercuribenzoate *p*H 7.0, 25°, and (B) as in A but with indicated urea concentrations.

The presence of urea increases both the rate and the extent of reaction of aldolase –SH groups with *p*-mercuribenzoate. Figure 1B shows results obtained with various urea concentrations after the maximum spectral shift had been obtained. This was 3 minutes or less when 5 *M* or greater urea was present; with 2 *M* urea readings were at 180 minutes. Data showing the cause of the decrease in the total ΔA_{250} in the presence of urea will be presented later.

The fraction of the *p*-mercuribenzoate which has reacted with aldolase under a given set of conditions may be taken as approximately equal to the ratio of the observed ΔA_{250} to the total possible ΔA_{250}. This calculation will give an accurate value for the number of –SH groups reacted only if the absorbance at 250 mμ is the same for each –SH which reacts or if all –SH groups have a statistically equal reactivity with *p*-mercuribenzoate. Different proteins show a variation in the spectral shift at 250 mμ accompanying mercaptide formation,[2] and it thus seems feasible that variation may be encountered with different –SH groups on the same protein. One or both of the aforementioned requisites is met within experimental error for aldolase in the presence of 4 *M* or greater urea concentration, as shown by the straight line relationship between ΔA_{250} and aldolase concentration until a maximum is reached (for example, curve 4, Fig. 1). The lack of a straight line relationship for the experiments in the absence of urea is principally and perhaps entirely a reflection of the decrease in rate of mercaptide formation as the concentration of free *p*-mercuribenzoate and reactive –SH groups decreases; with increase in time of reaction the curves progressively approach linearity. The same total ΔA_{250} is reached when aldolase is allowed to react with a slight excess of *p*-mercuribenzoate for 60 minutes and then an excess of aldolase added as when an excess of aldolase is added immediately. This suggests that the –SH groups which react slowly in the absence of urea show the same ΔA_{250} within experimental error, as those which react rapidly. Alternatively, the more reactive groups of the excess aldolase added may displace the *p*-mercuribenzoate residues from some of the less reactive –SH groups of the aldolase preincubated with excess *p*-mercuribenzoate.

From the fraction of the *p*-mercuribenzoate which has reacted, the number of –SH groups per mole of aldolase which have formed mercaptides can be calculated readily. Figure 2 shows the –SH groups reacted per mole of aldolase as a function of urea concentration and of time. Readings made under comparable conditions 15 seconds after addition of an excess of *p*-mercuribenzoate to aldolase without urea present showed, in various trials, 5 to 7 –SH groups per mole which reacted within this period. These groups are clearly distinct in their reactivity from those reacting slowly over a several hour period. They are likely those responsible for the positive nitroprusside test mentioned by Racker.[10]

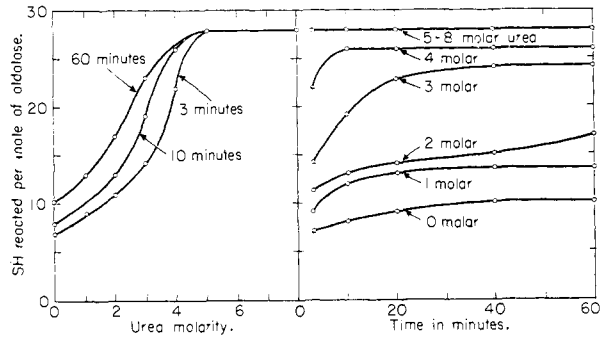

Fig. 2.—Effect of urea on rate and extent of reaction of aldolase SH groups with *p*-mercuribenzoate. Reaction mixtures contained 0.05 *M* phosphate buffer, 3.5 × 10^{-5} *M* *p*-mercuribenzoate, and urea as indicated, *p*H 7.0, 25°.

The effect of urea in decreasing the ΔA_{250} noted in Fig. 1B results from an increase in the absorbance of the *p*-mercuribenzoate in the presence of the urea, as shown in Fig. 3. The absorbance of the mercaptide of *p*-mercuribenzoate at 250 mμ is not increased by urea; this is illustrated in Fig. 3 for the mercaptide with glutathione. The increase in the absorbance of the *p*-mercuribenzoate undoubtedly results from formation of a urea-*p*-mercuribenzoate compound, very likely involving the mercury, which, on the appearance of the SH compound, gives way to the formation of the mercaptide with the *p*-mercuribenzoate.

Role of Aldolase –SH Groups in the Catalytic Activity.—The data in Table I summarize the

(8) K. Bailey and B. B. Marsh, *Biochim. Biophys. Acta*, **9**, 133 (1952).

(9) R. E. Benesch, H. A. Lardy and R. Benesch, *J. Biol. Chem.*, **216**, 663 (1955).

(10) E. Racker in Colowick, *et al.*, "Glutathione," Academic Press, New York, N. Y., 1954, p. 101.

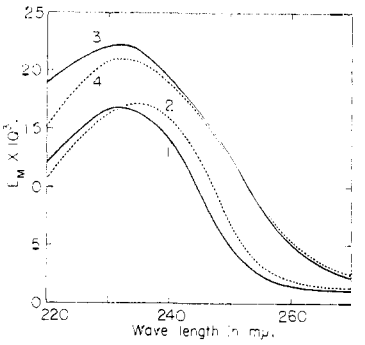

Fig. 3.—Effect of urea on the absorbance of *p*-mercuribenzoate and its glutathione mercaptide: curve 1, 5.7×10^{-5} *M* *p*-mercuribenzoate in 0.05 *M* phosphate *p*H 7.0; curve 2, as 1 but with 8 *M* urea added; curve 3, as 1 but with 10^{-4} *M* glutathione added; curve 4, as 3 but with 8 *M* urea added.

relation between the catalytic activity and the number of –SH groups reacted with *p*-mercuribenzoate at *p*H 7. The results show clearly that mercaptidation of all the most reactive –SH groups of aldolase at *p*H 7 in phosphate buffer does not significantly decrease the catalytic activity. Exposure at 37° for 1 hour was necessary to get 12 –SH groups to react in the absence of urea and to show a definite decrease (30%) in catalytic activity.

TABLE I

RELATION BETWEEN EXTENT OF REACTION WITH *p*-MERCURIBENZOATE AND CATALYTIC ACTIVITY OF ALDOLASE

Moles *p*-mercuribenzoate reacted per mole of aldolase	% of initial catalytic activity
0	100
6	98
8	99
10.5	98
12	70
14	32

In Fig. 4 are shown data on the rate of reaction of aldolase with *p*-mercuribenzoate in the presence and absence of substrates. With the concentration of enzyme used, the 0.07 *M* added fructose 1,6-diphosphate would be converted readily into an equilibrium mixture of the fructose and triose phosphates.[11] Clearly the presence of substrates in relatively high concentration had no effect within experimental error on the rate or extent of reaction of aldolase –SH with *p*-mercuribenzoate.

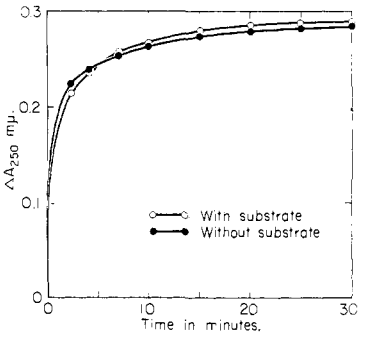

Fig. 4.—Lack of effect of substrate on the reaction of aldolase with *p*-mercuribenzoate. Reaction mixtures contained 0.05 *M* phosphate buffer, 2.6×10^{-6} *M* aldolase, 3.6×10^{-5} *M* *p*-mercuribenzoate with or without 0.07 *M* fructose, 6-diphosphate, *p*H 7.0, 25°.

Reversibility of Aldolase Inactivation.—Table II gives results obtained when aldolase was incubated with or without *p*-mercuribenzoate present, followed by activity tests in the presence and absence of added glutathione. A control sample without *p*-mercuribenzoate incubated 1 hour at 0° showed no loss of activity, and that incubated at 37° a slight loss of activity. This loss was restored by glutathione addition. The marked activity loss following incubation with excess *p*-mercuribenzoate at 37° was largely reversed by the glutathione addition.

TABLE II[a]

REVERSAL OF THE *p*-MERCURIBENZOATE INHIBITION OF ALDOLASE

Incubation temp., °C.	*p*-Mercuribenzoate, *M* × 10^5	Relative activity after incubation: No glutathione added	Relative activity after incubation: Glutathione added
0	0	100.0	99.0
37	0	88.5	100.0
37	3	12.0	87.5
37	3	12.5	88.0

[a] See Experimental section for details.

Effect of Urea on Aldolase Activity.—The catalytic activity of aldolase when assayed in the presence of urea progressively decreases as the urea concentration increases and falls rather abruptly to zero as the urea level reaches 3.6 *M* (Fig. 5).

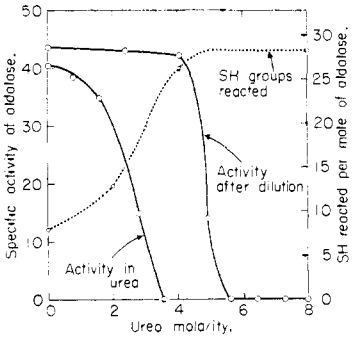

Fig. 5.—The effect of urea on the catalytic activity of aldolase. For details see the experimental section.

The loss in activity resulting from exposure to urea up to 4.0 *M* is essentially completely reversible as shown by the activity measured after a 100-fold dilution of the urea (Fig. 5). The extent of reversal of urea denaturation fell off sharply above 4.0 *M;* only 30% of the original activity was regained after exposure to 4.8 *M* urea, and exposure to 5.6 *M* urea or higher resulted in irretrievable activity loss. The loss in activity with exposure to 5.6 *M* urea varied with aldolase concentration; with $^1/_{10}$ as much aldolase as used for the experiments reported in Fig. 5, approximately $^1/_4$ of the activity was regained following dilution.

(11) D. Herbert, A. H. Gordon, V. Subrahmanyan and D. E. Green, *Biochem. J.*, **34**, 1108 (1940).

Discussion

The data show that in the presence of 5.0 *M* or higher urea concentration 28 moles of *p*-mercuribenzoate react rapidly with one mole of aldolase. This figure likely represents the total number of –SH present in the aldolase, and agrees well with the value of 29 –SH per mole reported by Benesch, *et al.*, from amperometric Ag titration measurements in 8 *M* urea solution[9] using the same values for molecular weight and absorbance at 280 mμ for determination of aldolase molarity. These values for total –SH considerably exceed the number of reported half-cysteine residues per mole,[12] and suggest that few if any disulfide bonds are present in the native aldolase. Further measurements are necessary to establish this important structural feature of the molecule.

As noted by others,[2] –SH groups of proteins vary considerably in reactivity. The –SH groups of rabbit muscle aldolase show a wide gradation in reactivity detectable by spectrophotometric observations with the single reagent, *p*-mercuribenzoate. At *p*H 7 and 25°, 5 to 7 –SH groups per mole react rapidly, the reaction being completed within 15 seconds. An additional 3 to 4 –SH groups react in the absence of urea in a 90-minute period. The rate of reaction of these –SH groups is sufficiently slow to distinguish them clearly from those which react in 15 seconds. The addition of urea is required to bring about reaction of the remaining –SH groups. Further, differences in the reactivity of these –SH groups can also readily be recognized. This is shown by the increase in total number of –SH groups reacting in the presence of 3, 4 and 5 *M* urea (Fig. 2).

The graded reactivity of the aldolase –SH groups and the ability of urea to increase their reactivity strongly suggests that the lack of reactivity of the aldolase –SH groups results from their unfavorable steric position within the molecule. Similar interpretations have been suggested earlier for "sluggish" –SH groups of proteins.[2,9] Although Benesch, Benesch and Rogers[13] have shown that urea may increase the reactivity of the –SH of glutathione toward nitroprusside, glutathione and other low molecular weight thiols react very rapidly with *p*-mercuribenzoate. Therefore structural features of the protein molecule likely account for reduced reactivity of some –SH groups.

That the –SH groups of the muscle aldolase may not participate directly in the enzyme catalysis is suggested by three facets of the experimental data. First, up to 10 –SH groups per mole, which includes all the rapidly reacting –SH groups, can react with *p*-mercuribenzoate without decrease in the catalytic activity; second, an excess of substrate fails to diminish the reactivity of the –SH groups with *p*-mercuribenzoate; and third, the decrease in catalytic activity occurs only when the less reactive –SH groups combine with *p*-mercuribenzoate. Such combination could logically result in changes of the secondary structure of the aldolase molecule. This possibility also draws support from correlations of the effects of urea and *p*-mercuribenzoate on the enzyme. Such correlations are well shown by the data of Fig. 5, which further define the relationship between structure, –SH reactivity and catalytic activity. The loss of activity resulting from increase in urea concentration is roughly paralleled by an increase in reactivity of the –SH groups. Sufficient structural change to cause complete loss of catalytic activity occurs well before complete reaction of the –SH groups. Exposure to 4.0 *M* urea allows reaction of all but approximately 3 of the –SH groups with *p*-mercuribenzoate, yet the effect on catalytic activity is entirely reversible. Thus the aldolase molecule in 4.0 *M* urea retains sufficient structure to allow the molecule to regain the original configuration or a comparably effective configuration of the active center. Likewise, aldolase inactivated by reaction with *p*-mercuribenzoate at 37° retains sufficient structure for regaining of catalytic activity (Table II). The urea concentration required for complete reaction of the –SH corresponds approximately to that for irreversible inactivation of the aldolase.

The activity of enzymes is variably affected by urea. Aldolase is more sensitive than trypsin to urea; the loss of trypsin activity in 8 *M* urea is completely recovered on dilution.[14] Ribonuclease maintains catalytic activity in 8 *M* urea solution,[15] pepsin in 4 *M* urea solution,[16] and carboxypeptidase in 6 *M* urea.[17] The pronounced effect of urea on aldolase catalytic activity is indicative of the importance of secondary and tertiary protein structure in the enzymic function of aldolase.

The ability of rabbit muscle aldolase to react with 10 *p*-mercuribenzoate molecules without loss of catalytic activity (Table I) was not an expected result. However, as mentioned earlier the most reactive groups of urease are not essential for catalytic activity.[3] The type of result obtained with aldolase and urease may prove to be a more general phenomenon. Desnuelle and Rovery noted that the loss of activity of urease occurred concomitantly with combination of the less reactive groups with phenyl isocyanate.[4] They argued cogently for the interpretation similar to that presented herein for aldolase, namely, that the inactivation resulted from reversible structural changes accompanying reaction of the –SH groups. Nygaard has suggested that some of the effects of *p*-mercuribenzoate on lactic dehydrogenase may result from structural alteration.[5] The results with aldolase are in contrast to the finding of Wallenfels and Sund[18] that the loss of activity of liver alcohol dehydrogenase is strictly proportional to the extent of reaction with *p*-mercuribenzoate.

The frequent interpretation that inactivation accompanied by blocking of –SH group implies a primary role of the –SH groups in enzyme catalysis is open to serious question. Enzymes showing such inactivation, particularly where the inactivation is

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(14) J. I. Harris, *Nature*, **177**, 471 (1956); H. O. Michel, *Federation Proc.*, **15**, 315 (1956).

(15) C. B. Anfinsen, W. F. Harrington, A. Hoidt, K. Linderstrom-Lang, M. Ottenson and J. Schellman, *Biochem. Biophys. Acta*, **17**, 141 (1955).

(16) J. Steinhardt, *J. Biol. Chem.*, **123**, 543 (1938).

(17) Y. Halsey and H. Neurath, *ibid.*, **217**, 247 (1955).

(18) K. Wallenfels and H. Sund, *Angew. Chem.*, **67**, 517 (1955).

reversible, have been termed "sulfhydryl enzymes."[2] Such classification should not carry the implication of a primary function of the –SH groups in catalysis. The demonstration of a primary function for –SH groups is difficult. The probability of a primary function would follow from information as to how the –SH participates, as has been obtained for glyceraldehyde 3-phosphate dehydrogenase,[19] or the establishment that the inactivation is not accompanied by secondary structural changes. Such information is lacking for most "sulfhydryl enzymes."

Experimental

Special Reagents.—Aldolase was crystallized from rabbit muscle by the method of Taylor, *et al.*,[7,20] except that all solutions for extraction and crystallization contained 10^{-4} *M* ethylenediaminetetraacetate and were prepared in distilled water passed through a mixed ion-exchange resin bed (Rohm and Haas Amberlite Monobed 3) to remove ionic impurities. The enzyme used was crystallized 4 times to a constant specific activity. When stored as a suspension in the crystallizing medium at 5° the enzyme maintained full activity for at least three months. The molar concentration of aldolase was determined from its absorbance at 280 mμ (A_{280}/0.910 equal grams of aldolase per liter[20]) and its molecular weight of 147,000.[20] The more recent figure of 149,000[21] would not significantly change the values of –SH groups per mole reported herein.

Fructose 1,6-diphosphate solutions were prepared from the magnesium salt (Nutritional Biochemicals) by passing a 0.02 *M* solution through a Dowex 50-H column after which the *p*H was brought to 7.4 with 2 *M* NaOH.

Purification of urea was deemed advisable to minimize possible acceleration of –SH oxidation by any heavy metal contaminants present. For most of the studies urea was purified by passage of 8 *M* solutions through small beds of mixed ion-exchange resin. However, this procedure resulted in a prominent initial elution followed by a slower continuous elution from the column of material giving small absorption at 220–260 mμ. This material did not give a spectral shift with *p*-mercuribenzoate. Also, different batches of urea of the same grade and source differed in their content of ultraviolet absorbing impurities. These could be removed by recrystallization twice from hot 95% ethanol, and urea purified in this manner rather than by the ion exchange resins was used for some later studies.

Aldolase Catalytic Activity Assay.—The catalytic activity assay used is a modification[22] of that of Sibley and Lehninger.[23] One ml. of aldolase solution, recently brought to 30° and containing approximately 10 μg. of aldolase, was added to 0.5 ml. of 0.02 *M* fructose 1,6-diphosphate, *p*H 7.4, and 0.5 ml. of 0.5 *M* hydrazine sulfate, *p*H 7.4, at 30°. At the end of 10 minutes the reaction was stopped with 1 ml. of 0.005 *M* 2,4-dinitrophenylhydrazine in 2 *M* HCl. The development of chromogen proceeded for another 10 minutes at 30° at which time 7 ml. of 0.375 *M* NaOH was added and the system removed from the bath to stand at room temperature. After standing exactly 10 minutes at room temperature the absorbance of 525 mμ was read using a blank in which the sequence of addition of enzyme and 2,4-dinitrophenylhydrazine was reversed. The absorbance at 525 mμ divided by the calculated absorbance of the enzyme at 280 mμ in the assay system is defined herein as the specific activity of the enzyme. For values as measured on the Beckman DU spectrophotometer, using 1 cm. light paths, the 4 times crystallized enzyme had a specific activity of 46–50. Absorbance measurements in routine assays were made with a Bausch and Lomb Spectronic 20 colorimeter and converted to the equivalent Beckman readings by use of appropriate conversion factors.

Although the aldolase assay procedure used has only approximately $1/_{10}$ the sensitivity of Beck's modification[24] of the Sibley-Lehninger[23] method, it is considerably simpler, readily reproducible, and could be used because sensitivity was not a limitation of the experimental procedures. Removal of protein by trichloroacetic acid precipitation is not necessary when working with the purified enzyme; the acidic 2,4-dinitrophenylhydrazine solution serves to stop the enzyme action.

Urea caused a marked depression of color development in the aldolase assay. For example, the presence of 4 *M* urea in the assay mixture decreased the absorbance at 525 mμ by 79%. To accommodate this effect when assay samples contained urea, the procedure was altered to include the same concentration of urea in all of a given series of samples during color development. This was done as follows. At the end of the 10-minute incubation period of the enzyme and substrate, a 1-ml. aliquot was removed and added to a solution containing 1 ml. of the 2,4-dinitrophenylhydrazine reagent and 1 ml. of a urea solution of sufficient concentration to bring all samples to the urea level of that of the highest urea content of the series under investigation. Color development then proceeded as previously described.

Reactive Sulfhydryl Determinations.—The spectrophotometric method of Boyer[5] was used for measurement of the amount of *p*-mercuribenzoate reacting with –SH groups. The *p*-mercuribenzoate was reprecipitated four times,[5] and a sufficient amount dissolved in 0.05 *M* tetrasodium pyrophosphate to give stock solutions containing approximately 2×10^{-3} *M* *p*-mercuribenzoate. The concentration of solutions was accurately determined from the absorbance at 232 mμ[5] of appropriate dilutions. In all –SH determinations, requisite corrections were made for the absorbance of aldolase at 250 mμ.

Assays in the presence of urea were made by the same procedure, with consideration of the effect of the urea on the extent of the spectrophotometric shift accompanying mercaptide formation, as noted in the section on results. Assays in 8 *M* urea necessitated making all solutions to be used 8 *M* in urea.

Effect of Urea on Catalytic Activity of Aldolase.—For measurements of the catalytic activity of aldolase in the presence of urea, as reported in Fig. 5, the following conditions were used: 0.1 ml. of aliquots of an aldolase solution, 400 μg. per ml., were incubated 30° for 10 minutes with 0.9 ml. of 0 to 8 *M* urea solution and 0.5 ml. of 0.5 *M* hydrazine, *p*H 7.4. Then 0.5 ml. of 0.02 *M* fructose 1,6-diphosphate, *p*H 7.4, was added, with the resultant urea concentrations during the period of enzyme action as shown in Fig. 5. After 10 minutes a 1-ml. aliquot was removed from the reaction mixture and added to a mixture of 1.0 ml. of 0.005 *M* 2,4-dinitrophenylhydrazine in 2 *N* HCl, and 1.0 ml. of a urea solution of sufficient concentration to bring the urea level of each mixture to that of the highest in the series under study. This was necessary to have all tubes at equal urea concentration for subsequent color development. This procedure was checked using glyceraldehyde in place of enzyme and substrate and was shown to be accurately reproducible.

The experiments on recovery of activity following dilution of urea (Fig. 5) were performed as follows: 2-mg. portions of crystalline aldolase, freshly centrifuged from ammonium sulfate suspension and well drained, were dissolved in 1 ml. of urea solution of the concentration indicated by Fig. 5. The small volume of the centrifuged aldolase suspension would decrease the molarity of the urea by considerably less than 5%. The solutions were allowed to stand at 30° for 10 minutes, and then diluted 100 times with water containing sufficient urea to bring all samples to 0.08 *M* urea; this concentration of urea was known from other studies to have no effect on catalytic activity. Catalytic activity measurements were made 10 minutes after dilution. Longer periods of standing after dilution did not result in increase in catalytic activity. One-ml. aliquots of the diluted solutions were added to the substrate and hydrazine and the assay carried out as previously described. The data reported in Fig. 5 are for solutions with no added buffer; the *p*H of these solutions was approximately 7.2. Closely similar results were obtained when the urea solutions contained 0.05 *M*

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(23) John A. Sibley and A. L. Lehninger, *J. Biol. Chem.*, **177**, 859 (1949).

(24) William S. Beck, *ibid.*, **212**, 847 (1955).

phosphate buffer *p*H 7.4 and 10^{-4} *M* ethylenediaminetetraacetate.

Correlation of Loss of Catalytic Activity with Extent of Reaction with *p*-Mercuribenzoate.—For these experiments, from 1 to 5 × 10^{-6} *M* aldolase was incubated with 3 × 10^{-5} *M* *p*-mercuribenzoate and 0.15 *M* phosphate buffer, *p*H 7.0. At room temperature, only approximately 8 –SH groups per mole of aldolase reacted within a 3-hour period. Exposure at 37° for 1 hour was necessary to obtain reaction with 12 –SH groups and exposure for 3 hours resulted in reaction of 14 groups. Longer exposure periods usually resulted in appearance of turbidity from protein precipitation. Subsequent to the desired incubation with the *p*-mercuribenzoate, samples were diluted with water to give solutions containing 20 μg. of aldolase per ml., and the activity was determined by the usual procedure. No compensation was necessary for differences in unreacted *p*-mercuribenzoate in the diluted samples because the low residual concentrations did not affect the catalytic assay. Aldolase samples incubated at 37° for three hours in the phosphate buffer without addition of *p*-mercuribenzoate showed little or no loss of catalytic activity. The loss observed in the presence of *p*-mercuribenzoate could thus be ascribed to reaction of the enzyme with the mercurial.

Reversal of the *p*-Mercuribenzoate Inactivation of Aldolase.—Samples of 1 ml. total volume containing 1 × 10^{-6} *M* aldolase and 0.12 *M* phosphate buffer *p*H 7.2 with or without 3 × 10^{-5} *M* *p*-mercuribenzoate were incubated at 0 or 37° as indicated in Table II. Then either 0.2 ml. of water or of 0.05 *M* glutathione, *p*H 7, was added, the samples allowed to stand 20 minutes at room temperature, and placed in an ice-bath. Catalytic activity assays were then made on appropriate aliquots as described previously.

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The α-Chymotrypsin-catalyzed Hydrolysis of a Series of Hydrazides. I. Determination of *p*H Optima and their Dependence upon Temperature[1]

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It has been found that the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide and of six α-N-acylated-L-tyrosinhydrazides can be followed quantitatively by the spectrophotometric determination of the liberated hydrazine in the form of a protonated bis-*p*-dimethylaminobenzalazine. With this procedure it has been shown that for aqueous solutions at 25° the *p*H optimum for the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide is 7.05 ± 0.15 and that the *p*H optima for the comparable reactions involving the six α-N-acylated-L-tyrosinhydrazides lie in a more basic region, *i.e.*, *ca.* 7.7 to 8.0. It also has been observed that the *p*H optima for the α-chymotrypsin-catalyzed hydrolysis of three representative α-N-acylated-L-tyrosinhydrazides are strikingly temperature dependent in the region between 25 and 40° and that an increase in temperature from 25 to 40° causes the *p*H optima to be shifted to a more acidic region, *i.e.*, from *ca.* 7.8 to *ca.* 6.8.

The knowledge that α-N-nicotinyl-L-tyrosinhydrazide and presumably α-N-acetyl-L-phenylalaninhydrazide are hydrolyzed in the presence of α-chymotrypsin[5-7] led us to consider the usefulness of the hydrazides of certain α-amino acids and acylated α-amino acids as specific substrates in studies involving the above enzyme. While MacAllister and Niemann[5] had followed the α-chymotrypsin-catalyzed hydrolysis of α-N-nicotinyl-L-tyrosinhydrazide, in aqueous solutions at 25° and *p*H 7.9 and 0.02 *M* in the EDA[8] component of an EDA–HCl buffer, with the aid of a formol titration[9] and Goldenberg, Goldenberg and McLaren[6] had determined the extent of the α-chymotrypsin catalyzed hydrolysis of α-N-acetyl-DL-phenylalaninhydrazide, in aqueous solutions at 24.6° and *p*H 7.3 and 0.05 *M* in an unspecified phosphate buffer, with a Grassmann–Heyde titration[10] neither of these procedures was employed in the present study because it was anticipated that for many α-amino acid and α-N-acylated amino acid hydrazides any titrimetric procedure based upon the determination of liberated carboxyl groups would not be sufficiently sensitive to be used for determining their rates of hydrolysis by α-chymotrypsin.

Goldenberg, Goldenberg and McLaren[7] were aware of the limitations of the above titrimetric procedures when applied to the hydrazides and these investigators devised a colorimetric procedure for the qualitative recognition of the α-chymotrypsin-catalyzed hydrolysis of α-N-acetyl-DL-phenylalaninhydrazide, which was based upon the reduction of phosphomolybdate ion to the so-called molybdenum blue by the liberated hydrazine While it was stated[7] that this latter procedure was capable of detecting an extent of hydrolysis of 1 to 2% at specific substrate concentrations of 0.03 to 0.05 *M* no attempt appears to have been made to develop the procedure to the point where it could be used for quantitative measurements.

An alternative colorimetric procedure for determining the extent of the α-chymotrypsin-catalyzed hydrolysis of α-amino acid and α-N-acylated α-amino acid hydrazides, and one that promised exceptional sensitivity, was suggested by the observation of Pesez and Pelit[11] that hydrazine reacts with *p*-dimethylaminobenzaldehyde in aqueous acidic media to give a protonated bis-*p*-dimethylaminobenzalazine with an absorption maximum

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