Sayre, F. W., and Hill, B. H. (1957), *Proc. Soc. Exptl. Biol. Med. 96*, 695.
Schwimmer, S. (1953), *Nature 171*, 442.
Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65*, 132.
Waksman, A., and Roberts, E. (1963), *Biochem. Biophys. Res. Commun. 12*, 263.
Wood, T. (1964), *Biochem. J. 91*, 453.

Photooxidation with Rose Bengal of a Critical Histidine Residue in Yeast Enolase*

Edward W. Westhead

ABSTRACT: Rose bengal has been used as a photoactivated oxidant to inactivate yeast enolase. The pH dependence of the rate of inactivation shows that oxidation of a histidine group is responsible for the loss of activity, and since the inactivation is first order to less than 0.1% residual activity either a single group or two or more exactly equivalent groups are involved. Amino acid analyses and other data support this interpretation but also show that oxidation of other, noncritical, amino acids also occurs. The enzyme is partially protected by Mg^{2+} and maximally protected by Mg^{2+} and substrate. Substrate alone does not protect. Rose bengal was found to be far more specific and less generally damaging to the enzyme than methylene blue.

The description of the active site of any enzyme is interesting as a contribution to our knowledge of chemical reaction mechanisms. In addition we may hope that the accumulation of such descriptions for a variety of enzymes with diverse catalytic properties may show a pattern of mechanism that will be interesting in terms of biological development. Although direct chemical evidence for the participation of a specific group in catalysis is most convincing, this approach suffers from a lack of reagents specific for "active site" groups. Weil *et al.* (1953, 1955) have shown that for some enzymes photooxidation with methylene blue is sufficiently specific to implicate a histidine residue in the catalytic mechanism. This reagent has since been used successfully by others (*e.g.*, Ray *et al.*, 1960), but more frequently investigators have found that so many groups were oxidized at similar rates that no sure interpretation could be placed on the results.

The present work was initiated in the hope that replacement of the cationic methylene blue by an anionic photoactive dye would lead to greater specificity for the active sites of enzymes that bind anionic substrates. Rose bengal was chosen on the basis of studies by Oster *et al.* (1959) which describe the photooxidizing efficiencies of a large number of dyes. Brake and Wold (1960) had previously described the photooxidation of yeast enolase by methylene blue and concluded that the reagent lacked sufficient specificity to allow assignment of a critical role to any particular residue. The present work therefore provides a clear test of the relative usefulness of rose bengal in this particular case.

Experimental Section

Materials. Rose bengal was obtained from the Fisher Scientific Co. and was manufactured by the National Aniline Division of Allied Chemical Corp. Although it was found to contain several components by chromatography on alumina (Jirsa and Raban, 1962), the dye was used without purification except for passage through a column of Dowex 50 resin in the sodium form to remove other cations. This dye is a highly halogenated fluorescein dye and in its pure form is tetraiodotetrachlorofluorescein. Amino acids and their derivatives were the best grades obtainable from Calbiochem. Enolase was prepared and assayed as previously described (Westhead and McLain, 1964); chromatography on TEAE-cellulose was carried out as described in that paper except that it was done at room temperature rather than at 4°. D-Phospholactic acid samples were gifts from Drs. Clinton Ballou and Finn Wold.

Methods. Amino acid analysis was done with a Spinco Model 120 analyzer. Samples were hydrolyzed in 6 N hydrochloric acid at 110° for 25 and 40 hr in an atmosphere of oxygen-free argon. The values for the leucine, arginine, and phenylalanine content of enolase were found to be very reproducible and were routinely used as standards in calculating μmoles of other amino acids $mole^{-1}$ of enzyme when small amounts of protein were hydrolyzed.

Oxidation was followed using a Clark polarographic

* From the Department of Biochemistry, Dartmouth Medical School, Hanover, N. H. *Received April 12, 1965; revised July 19, 1965.* This work was supported by a career development award (5-K3-GM-5479) and a research grant (GM 08474) from the United States Public Health Service.

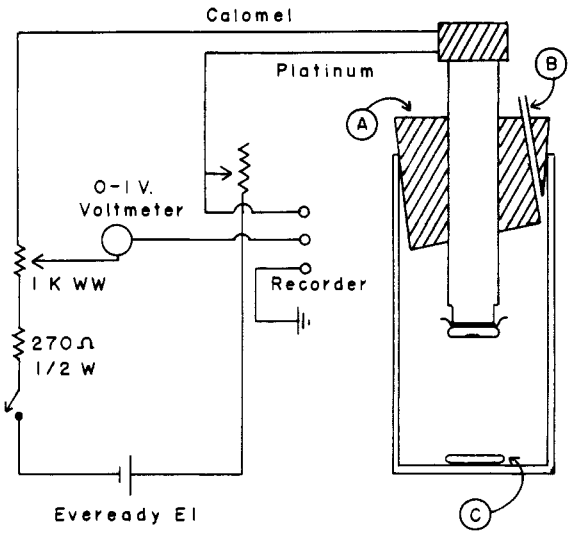

FIGURE 1: Diagram of the oxygen electrode system. The electrode is inserted through a polyethylene stopper (A) cut on a bias to allow air bubbles to be forced out through tubing (B). The electrode requires rapid stirring by the magnetic stirrer (C).

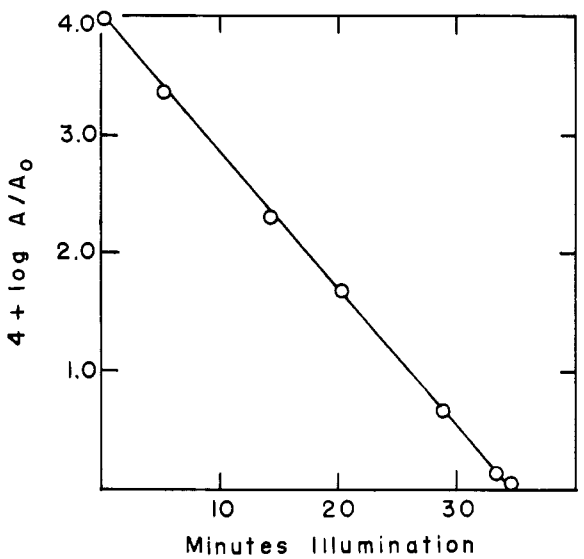

FIGURE 2: First-order plot of the loss of activity as a function of length of time of illumination. Conditions were: 25°, pH 8.3, Tris–HCl buffer 0.05 M in HCl, 10^{-5} M EDTA, enolase at 10 mg/ml.

oxygen electrode made by Yellow Springs Instrument Co., Yellow Springs, Ohio. A 10-mv recorder was used to measure the decrease in oxygen uptake with time. Figure 1 is a diagram of the oxygen measuring system. To calibrate the recorder for oxygen concentration, it was assumed that at 25° air-saturated water was 0.25 mM in oxygen and that the concentration of oxygen was reduced to zero upon addition of small amounts of sodium hydrosulfite (sodium dithionite). A 300 or 500

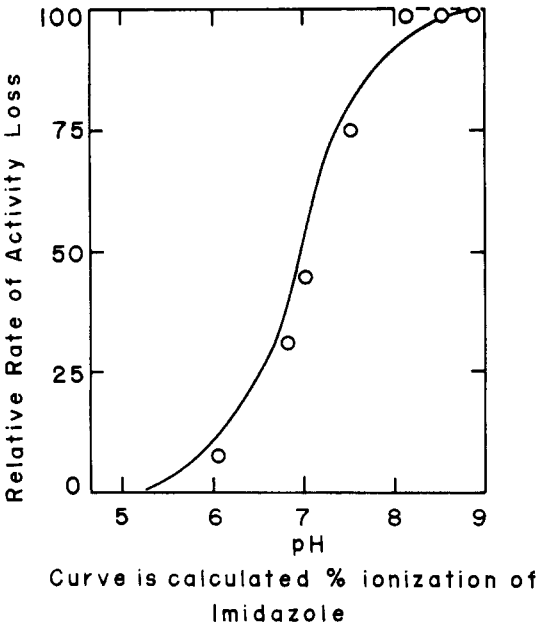

FIGURE 3: Initial rate of activity loss as a function of pH at 25°. Above pH 7, Tris–HCl buffer, 0.05 M in HCl. Below pH 7, phosphate buffer, 0.05 M. All solutions 0.50 M KCl, 10^{-5} M in EDTA. Enzyme at 3.0 mg/ml.

w slide projector was used as a light source; when the projector was operated on a stabilized voltage source rates of photooxidation were found to be accurately reproducible over a period of 1 or 2 days. The lens of the projector was placed 10 cm from the sample and focused to give the most concentrated light possible. The sample was usually in a 12-mm tube which was enclosed in a glass water jacket through which water at 25° was circulated. When experiments were repeated weeks apart, the relative rates of oxidation of two substrates were found to be reproducible, but absolute rates showed large variability. Aging of rose bengal solutions seemed to be a major cause of variation in rates.

In order to compare the rates of oxygen uptake with the rate of loss of enzymic activity, it was necessary to prevent air entering the system when the sample was taken (see Figure 1). To do this, the oxygen electrode was pushed down in its collar (A) far enough to force solution up into the polyethylene tubing (B). A Hamilton microliter syringe was then inserted through tube (B) into the main body of solution and a sample was removed. When a large sample was needed, the electrode was further depressed during sampling to maintain the liquid seal against the entrance of air. The diffusion of air into the liquid through the narrow tube (B) was negligible.

The system was routinely tested for oxygen leakage by interrupting the light beam during the course of oxidation. When no air bubbles or leaks were present, the uptake of oxygen ceased at once and the current remained constant indefinitely.

Rose bengal was usually used at concentrations of less than 0.01 mg/ml. At this concentration the dye has an absorbance of 0.60/cm at 550 mμ, where it absorbs maximally in the visible region. Thus in a reaction chamber of 1-cm diameter there is little to gain by using higher concentrations of the dye. In an air-saturated solution with enolase at 6.7 mg/ml and rose bengal at 3×10^{-3} mg/ml, the concentrations of oxygen, enzyme, and dye are 240, 100, and 3 μM, respectively.

Results

Early experiments showed that rose bengal is far more efficient than methylene blue in destroying the activity of enolase. Under similar conditions of dye concentration the loss of activity with rose bengal is about 500 times as fast as with methylene blue. The artificial light in the laboratory is sufficient to cause an appreciable rate of photooxidation at the lowest rose bengal concentrations used. In the dark, however, several hours' incubation of enzyme with dye causes no loss of enzymic activity.

Evidence for Histidine Oxidation as the Cause of Inactivation. When a metal ion free solution of enolase is photooxidized, the rate of activity loss is first order in active enzyme. Figure 2 shows a plot of the logarithm of per cent residual activity as a function of time. In this experiment the initial enzyme concentration was 10 mg/ml, and a current of water-saturated air was blown over the surface of the solution to maintain a sufficiently high oxygen concentration in the solution. Aliquots removed early in the oxidation were diluted to a suitable concentration for assay. Since the assay itself is easily valid over a 100-fold range of activity it was possible to obtain the data of Figure 2 which show that the loss of activity is characterized by a single first-order rate constant down to $^1/_{10,000}$ of the original activity.

Measurement of the first-order rate of activity loss in a series of buffered solutions from pH 5.6 to 9.2 gave the data shown in Figure 3. At all pH values the initial loss of activity was first order, but at the lower three points the rate began to deviate from the straight line after about 30% loss of activity. It will be seen that the rate of loss of activity is markedly dependent upon the hydrogen ion concentration.

When amino acids and their derivatives are photooxidized under the same conditions, the effect of pH is as shown in Figure 4. These data are obtained from the rates of uptake of oxygen in each case, using the oxygen electrode. The results are not given as rate of O_2 uptake but as rate of oxidation of the amino acid derivative. To convert from the former to the latter, one must determine the stoichiometry of the oxidation by running the reaction to completion with limiting amounts of oxidizable compound. It was found that in the case of indole compounds (tryptophan, *N*-acetyltryptophan, and *N*-acetyltryptophan ethyl ester) there was 1.5 moles of O_2 used/mole of indole group. In the case of tyrosine, *N*-acetyltyrosine, tyrosine methyl ester, histidine, imidazole, histidine methyl ester, and *N*-acetylhistidine,

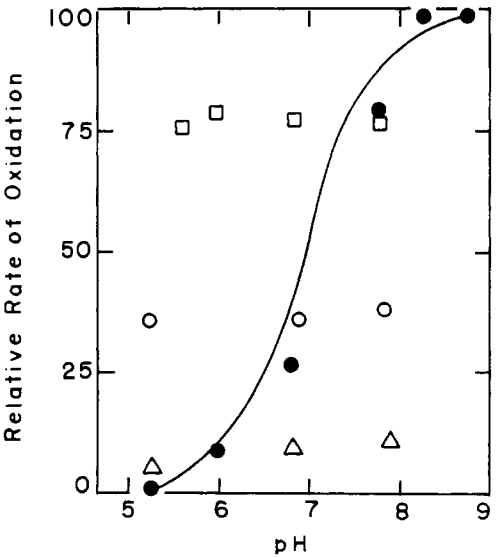

FIGURE 4: Relative rates of oxidation of imidazole, ●; tryptophan and *N*-acetyltryptophan, ○; methionine and *N*-acetylmethionine, □; and tyrosine, △. Conditions as in Figure 3, amino acids at 0.10 mM.

1 mole of O_2 was taken up for each mole of reductant. Methionine and methionylalanine each took up 1 mole of oxygen, going to the sulfone. (The product was verified for methionine using the amino acid analyzer and was assumed for the dipeptide.) *N*-Acetylmethionine, however, was found to take up only 0.5 mole of O_2, going presumably to the sulfoxide. After acid hydrolysis of enzyme samples that were photooxidized for longer times, traces of sulfoxide but not of sulfone were found in the analytical amino acid chromatograms. Others have previously shown (*e.g.*, Ray and Koshland, 1962) that photooxidation of methionine in a protein results predominantly in formation of the sulfoxide with negligible amounts of sulfone appearing at low degrees of oxidation. All of the model compound reactions were first order in reductant and zero order in oxygen to at least 90% completion of the oxidation and to oxygen concentrations below 5% of the initial concentration. It is apparent that, of the amino acid side chains likely to be oxidized, only histidine and its derivatives show a pH dependence of oxidation similar to that found for the loss of enzymic activity. Yeast enolase, incidentally, has neither sulfhydryl nor disulfide groups (Malmström *et al.*, 1959).

Amino acid analysis gave confirmatory evidence for the oxidation of a single histidine; Figure 5 shows the correspondence between reduction of histidine content and degree of inactivation by photooxidation. It will be noted that some nonessential histidine(s) appear to be oxidized at a slower rate than the one(s) which cause loss of activity. Titration evidence has indicated that of the 14 histidines in enolase approximately 12 of them do not titrate in the normal pH range and are presumably inaccessible or otherwise "abnormal" (Westhead, 1964).

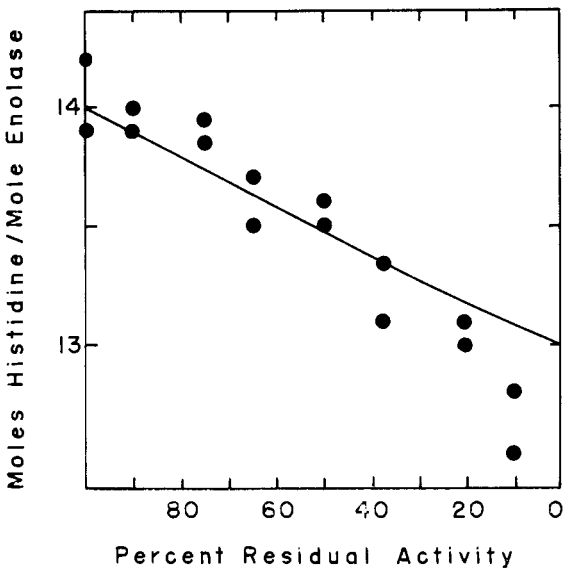

FIGURE 5: Moles of histidine/mole of enolase found after photooxidation as a function of residual activity. Photooxidation at pH 8.3, 25°, Tris-HCl buffer 0.05 M in HCl, 10^{-5} M in EDTA. Line drawn is theoretical for oxidation of a single critical histidine residue.

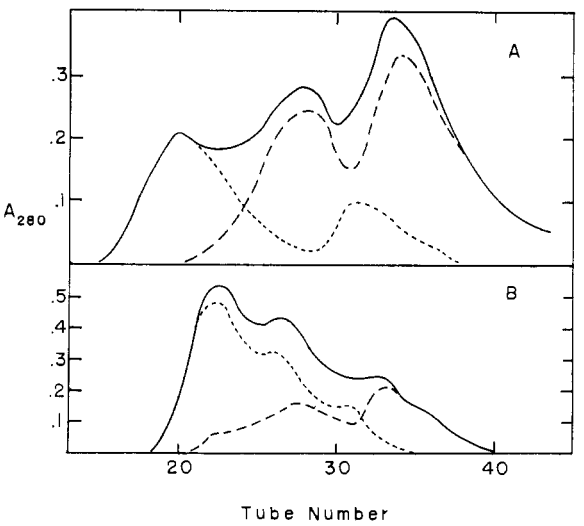

FIGURE 6: Chromatography on TEAE-cellulose of photooxidized enzyme. (A) Under conditions of Figure 5 taken to 70% loss of activity, (B) similar conditions but with EDTA replaced by 10^{-3} M Mg^{2+} and 10^{-3} M phosphoglyceric acid, taken to 17% loss of activity. ——— Protein measured at 280 mμ; ····· active enzyme; --------- inactive enzyme (see text).

Specificity of the Photooxidation. Amino acid analysis has also shown that no tyrosine is oxidized, but the standard methods of analysis are not suitable for determining the extent of oxidation of tryptophan or methionine. Evidence that no tryptophan was oxidized was obtained from spectral data. Upon photooxidation of tryptophan and its derivatives, a large broad absorption peak appeared near 305 mμ. After photooxidation of enolase to 90% inactivation, however, the ultraviolet spectrum of enolase was indistinguishable from that of the native enzyme.

It was found earlier that chromatography on TEAE-cellulose at pH 8.5 is a sensitive test for heterogeneity of enolase (Westhead, 1964). Using the same method of chromatography but increasing the volume of the eluent buffers to 100 ml each, we obtained the patterns shown in Figure 6. The enolase used in these experiments was completely homogeneous when chromatographed in the same manner before oxidation. The first peak of these patterns corresponds in position with the native enolase used for the experiment. It can be seen in Figure 6A that the pattern can be resolved most simply into four components, unchanged enolase, oxidized active enolase, and two species of oxidized inactive enzyme. The dotted and dashed curves showing active and inactive protein are calculated from the protein concentration and specific activity of each fraction, assuming that all molecules are either fully active or completely inactive. This assumption is justified to some extent by kinetic data obtained from the fractions of Figure 6B. In this experiment the amount of oxidized, but active, enzyme was increased by prolonging the oxidation time but protecting the crucial histidine with magnesium ion and substrate. Michaelis constants were measured for each fraction and were found to *decrease* with increasing degree of oxidation. Identical substrate kinetics were obtained whether magnesium or manganese was used as activating ion.

TEAE-cellulose chromatography is also very effective in removing the rose bengal from the enzyme. No other method, including dialysis or precipitation by trichloroacetic acid, has been found to separate the dye from the protein.

Evidence for an Active-Site Location of the Critical Histidine. Brake and Wold (1960) showed that when methylene blue was used to inactivate enolase there was an increase in the diffusion constant of the enzyme of 37% at 60% residual acivity and that there was an 11% increase in the sedimentation constant at 27% residual activity. In contrast, there was no detectable difference in the rates of sedimentation when a solution of enolase photooxidized to 10% residual activity with rose bengal was compared with an unoxidized sample at the same concentration. The experiment was performed by centrifuging both solutions at the same time, using a wedge-shaped window in one cell to displace the image on the photographic plate. This shows that no major change in the protein structure takes place as a result of the inactivation.

When the enzyme is oxidized in the presence of 10^{-3} M Mg^{2+} and different concentrations of substrate, there is specific protection of the active site as shown in Figure 7. These data also show that considerable oxidation of the enzyme can take place at unprotected sites with little or no loss of activity. Very similar data were ob-

tained with the competitive inhibitor D-phospholactic acid in place of the equilibrium mixture of phosphoglyceric acid and phosphopyruvic acid. These compounds were found not to inhibit the photooxidation of imidazole under the same conditions.

Table I is a brief summary of data obtained on the effect of magnesium and substrate concentration on the rate of inactivation. It is clear that the highest rate of inactivation occurs in the absence of metal ions and that in this condition substrate gives no protection even at $15K_m$. The assumption that EDTA completely removes metal ions has been validated in this laboratory by Dr. D. P. Hanlon with radioactive isotopes of Mn^{2+} and Mg^{2+}. Magnesium alone protects substantially, but even at 10^{-2} M it does not protect as fully as metal plus substrate. (The kinetic constant for magnesium activation is 2×10^{-4} at a substrate concentration of $5K_m$.) A marked trend toward saturation by metal is evident between 2×10^{-4} and 10^{-2} M. It is not possible to fit the data to a theoretical binding equation without making unjustified assumptions. A plot of rate of activity loss *vs.* metal ion concentration does not give a rectangular hyperbola. Control experiments have shown that magnesium, at a concentration of 2×10^{-3} M, has no effect on the oxidation of imidazole by rose bengal.

TABLE I: Photooxidation with Activating Ion and Substrate.[a]

Concentration of Reagent (M)			Rate Constant for Activity Loss
EDTA	Mg^{2+}	Substrate	(M^{-1})
4×10^{-4}	0	0	0.99
4×10^{-4}	0	2.4×10^{-3}	0.99
0	1.0×10^{-4}	0	0.45
0	2.5×10^{-4}	0	0.28
0	10×10^{-4}	0	0.23
0	80×10^{-4}	0	0.20
	80×10^{-4}	2.4×10^{-3}	0.07

[a] Enolase concentration 3.5 mg/ml, other conditions as in Figure 5 except as described.

Discussion

The original premise that an anionic dye should be more specific for the catalytic site than a cationic dye has clearly led to greatly improved results in this case. The premise itself cannot be generally substantiated until the dyes have been compared on other enzymes. The fact that the histidine residue must be in the *uncharged* form for oxidation to occur has little bearing on this question since there may well be other positively charged groups to attract the substrate. It seems well established (*e.g.*, Blum, 1941; Sluyterman, 1961) that a short-lived complex is formed between the activated dye and its substrate prior to transfer of the oxygen, and it may be that a rational study of photooxidants and conditions of photooxidation will lead to increased specificity for other enzymic sites. An excellent start has been made in this direction by Sluyterman (1961, 1962).

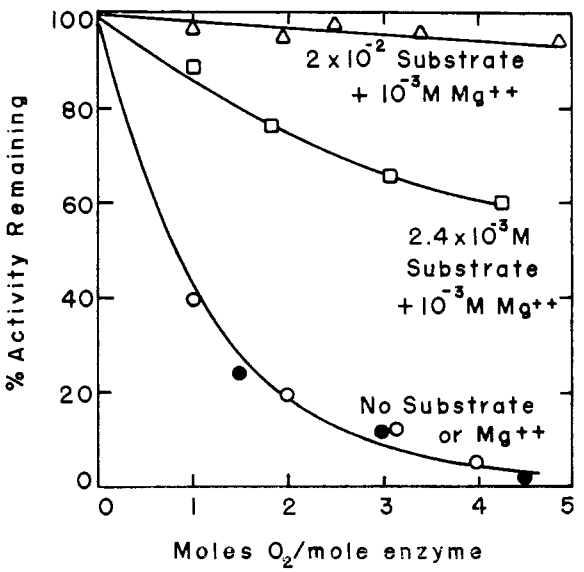

FIGURE 7: Loss of activity compared with oxygen uptake. Enzyme at 3.0 mg/ml (45 μM), other conditions as in Figure 5. Absorbance at 280 mμ ———; active enzyme · · · · · ·; inactive protein ---------; see text for details.

No attempt has yet been made to define the role of the critical histidine, but experiments in this direction are planned. A role in the binding of activating metal ion might be expected in view of the protection offered by Mg^{2+}. The role of the activating metal ions is complex (Brewer and Weber, 1965; Hanlon and Westhead, 1965), but direct binding measurements show that two atoms of Mg^{2+} or Mn^{2+} are bound to the enzyme in the absence of substrate. When substrate is present, a third mole of the divalent ion is bound.

This, plus the fact that substrate protects from photooxidation only in the presence of the divalent ion, suggests that the third ion bound serves to bind the substrate to the enzyme. The same conclusion has been reached by Cohn (1963) using a combination of electron spin resonance and nuclear magnetic resonance techniques. Brake and Wold (1962) have showed that carboxymethylation of yeast enolase on a particular methionine residue leads to inactivation. In this case, neither metal ion nor substrate alone protected, but the combination of the two did. The substrate thus appeared responsible for the protection but did not bind in the absence of metal ion.

The data of Figures 6 and 7 show that the oxidation is not completely specific for the critical histidine. It seems most likely in view of other evidence presented here that the other oxidation occurs at methionine residues which take up only 0.5 mole of oxygen.

In an earlier report (Westhead, 1963), data were shown that indicated that there was a small but definite level of activity which disappeared only slowly during prolonged photooxidation. Further experiments, however, showed that in the presence of sufficient EDTA this residual activity was not found. Apparently some tightly bound metal ion can protect the active site. Although this may be an inhibitory metal, in the assay medium this would be replaced by magnesium to yield a low level of activity.

Although the data of Figure 2 do not distinguish between the oxidation of one critical group and several such groups which oxidize at the same rates, we have now obtained a peptide from cyanogen bromide cleavage of the TEAE-fractionated enzyme which contains 1 mole of oxidized histidine. The identification of this peptide has been facilitated by the finding that photooxidation of histidine gives rise to two new peaks on the amino acid chromatogram appearing earlier than aspartic acid in the system of Spackman *et al.* (1958). This chemical evidence for the involvement of a histidine residue in enolase activity is in accord with earlier indirect evidence which indicated that a histidine residue was involved in the catalysis (Wold and Ballou, 1957) and in binding of metal ions (Malmström and Westlund, 1956).

References

Blum, H. (1941), Photodynamic Action and Diseases Caused by Light, New York, Reinhold.

Brake, J. M., and Wold, F. (1960), *Biochim. Biophys. Acta 40*, 171.

Brake, J. M., and Wold, F. (1962), *Biochemistry 1*, 386.

Brewer, J. M., and Weber, G. (1965), *Federation Proc. 24*, 285.

Cohn, M. (1963), *Biochemistry 2*, 623.

Hanlon, D. P., and Westhead, E. W. (1965), *Federation Proc. 24*, 285.

Jirsa, M., and Raban, P. (1962), *Nature 195*, 1100.

Malmström, B. G., Kimmel, J. R., and Smith, E. L. (1959), *J. Biol. Chem. 234*, 1108.

Malmström, B. G., and Westlund, L. E. (1956), *Arch. Biochem. Biophys. 61*, 186.

Oster, G., Bellin, J. S., Kimball, R. W., and Schrader, M. E. (1959), *J. Am. Chem. Soc. 81*, 5095.

Ray, W. J., and Koshland, D. E. (1962), *J. Biol. Chem. 237*, 2493.

Ray, W. J., Latham, H. G., Katsoulis, M., and Koshland, D. E. (1960), *J. Am. Chem. Soc. 82*, 4743.

Sluyterman, L. A. A. E. (1961), *Rec. Trav. Chim. Pays-Bas 80*, 989.

Sluyterman, L. A. A. E. (1962), *Biochim. Biophys. Acta 60*, 557.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Weil, L., James, S., and Buchert, A. R. (1953), *Arch. Biochem. Biophys. 46*, 266.

Weil, L., and Seibles, T. S. (1955), *Arch. Biochem. Biophys. 54*, 368.

Westhead, E. W. (1963), *Federation Proc. 22*, 594.

Westhead, E. W. (1964), *Biochemistry 3*, 1062.

Westhead, E. W., and McLain, G. (1964), *J. Biol. Chem. 239*, 2464.

Wold, F., and Ballou, C. E. (1957), *J. Biol. Chem. 227*, 313.