Rotatory dispersion titration provides a convenient method for the study of site-specific interactions of chromophoric coenzymes, coenzyme analogs, and inhibitors with appropriate enzymes (Ulmer and Vallee, 1961; Ulmer *et al.*, 1961). The effect of optically inactive competitive agents on these optically active chromophoric complexes greatly extends the usefulness of this procedure, which can thus be employed to advantage for the study of enzymatic mechanisms.

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Structural and Catalytic Alterations of Dehydrogenases After Photooxidation*

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The properties of three dehydrogenases have been studied after photooxidation with methylene blue. The resulting changes in catalytic activity include changes in coenzyme specificity and substrate inhibition characteristics, accompanied by changes in coenzyme binding and in some physical properties. Although significant changes in catalytic activity were seen after destruction of less than 2 to 3 moles of histidine per mole of enzyme, there also were evidences at this point of changes in enzyme structure. The structural changes produced after photooxidation in these and, perhaps, in other enzymes limit the usefulness of this technique for studying the role of histidine at the active sites of enzymes. However, some changes in catalytic properties can be correlated with changes in enzyme structure.

Methods have been sought for the specific destruction of amino acids in proteins in order to determine the functional role of certain amino acids in enzymes. Photooxidation of proteins with methylene blue destroys several amino acids, but generally destruction of histidine and tryptophan begins first and is followed by destruction of tyrosine, cysteine, and methionine as photooxidation is continued (Weil and Buchert, 1951; Weil *et al.*, 1951, 1952, 1953).

Previous studies with photooxidation have implicated histidine in the active site of certain enzymes (Weil *et al.*, 1952, 1953; Millar and Schwert, 1962), since loss of enzyme activity occurred as histidine was being destroyed (Weil *et al.*, 1952, 1953). However, it has been pointed out that changes in protein structure which can accompany photooxidation may also account for the changes in enzyme activity (Brake and Wold, 1960).

We have subjected three dehydrogenases, horse liver alcohol dehydrogenase (ADH),[1] beef heart muscle lactic dehydrogenase (beef H LDH), and chicken heart muscle lactic dehydrogenase (chicken H LDH), to photooxidation with methylene blue and have observed changes in catalytic activity as measured by the ability of the enzymes to react with NAD and analogs of NAD

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as well as changes in physical properties. A preliminary report of part of this work has appeared (Robinson and Stollar, 1962).

Materials and Methods

Enzymes.—Beef heart LDH was prepared by a modification of the Straub method described by Neilands (1955). After calcium phosphate gel adsorption and ammonium sulfate fractionation, the enzyme was obtained in crystalline form by DEAE column chromatography and was recrystallized three times prior to use. The preparation used contained only one electrophoretic band and corresponded to the H_4 molecular species (Cahn *et al.*, 1962). We shall refer to this enzyme as beef H LDH.

The chicken H LDH was prepared by a method described elsewhere (Cahn *et al.*, 1962; Pesce *et al.*, in preparation). This enzyme also showed only one electrophoretic band on either starch grain or starch gel.

Horse liver ADH was purchased as a crystalline suspension from C. F. Boehringer and Sons.

All of the enzyme preparations used in this study were dialyzed overnight against 0.1 M potassium phosphate buffer, *p*H 7.0, before use.

Enzyme Assays.—Beef H and chicken H LDH were assayed in a cuvet containing lithium lactate, 1.3×10^{-2} M, unless otherwise specified; NAD or analog of NAD, 2×10^{-4} M; and K_2HPO_4, 0.1 M at *p*H 8.8, in a final volume of 3.0 ml. The reaction was started by addition of enzyme, and the change in optical density at 340 mμ was followed at room temperature. Enzyme activity is defined by the initial rate of change in optical density.

ADH was assayed in a cuvet containing ethanol, 6×10^{-2} M, NAD or analog of NAD, 2×10^{-4} M, glycine-sodium hydroxide buffer, 0.1 M at *p*H 9.4, in a final volume of 3.0 ml. The reaction was started by the addition of enzyme and the rate followed from changes in optical density at 340 mμ.

Photooxidation.—Photooxidation was done manometrically in Warburg respirometer flasks with an Aminco constant-temperature illuminated water bath and shaker. The bath was illuminated through a glass bottom by a bank of eight Sylvania 150 W projector spot lamps, delivering a light intensity of 3800 foot-candles, as measured at the water surface. Each flask contained 10 mg of enzyme in 0.1 M potassium phosphate buffer, *p*H 7.0, in a final volume of 3.0 ml. Ten μg of purified methylene blue was added from a side-arm just before illumination was begun; 0.2 ml of 20% potassium hydroxide was placed in the center well. All reactions were carried out at 25° in the presence of air. The reaction was stopped by taking the flask away from the light and immediately removing the methylene blue by adsorption to freshly washed Dowex 50W-X2 resin (J. T. Baker Chemical Co.). The resin was removed by centrifugation.

Amino acid analysis was done with the Beckman Model 120 automatic amino acid analyzer (Spackman *et al.*, 1958). The protein was previously hydrolyzed in 6 N HCl for 24 hours at 110° *in vacuo*.

Tryptophan content was determined by analysis of the ultraviolet spectrum of the intact protein in 0.1 N sodium hydroxide, according to the method of Goodwin and Morton (1946), using the relation: $M_{tyr}/M_{tryp} = (0.592\ D_{2944} - 0.263\ D_{2800})/(0.263\ D_{2800} - 0.170\ D_{2944})$, where M = moles per gram protein and D is optical density.

Coenzyme binding was determined by measuring fluorescence intensification and fluorescence polarization of the coenzyme in the presence of the enzyme by methods previously described (Velick, 1958; Shifrin *et al.*, 1959). Measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with a Glan-Thompson prism adjacent to the cuvet holder in the path of the emitted light. All measurements were made at room temperature. Solutions were excited at 345 mμ and fluorescence was observed at 445 mμ.

Coenzyme binding to the beef heart LDH was measured by titrating 13 mμmoles of NADH or AcPyADH with increments of enzyme in 0.1 M potassium phosphate buffer at *p*H 7.0. The observed fluorescence intensification was plotted in arbitrary units against enzyme concentration. The stoichiometry of the coenzyme-enzyme complex was the ratio of moles of coenzyme to moles of enzyme at the inflection point of the resulting plot.

Coenzyme binding to liver ADH was measured by titrating 13 mμmoles of NADH or AcPyADH with increasing amounts of enzyme. Fluorescence measurements were made with the Glan-Thompson prism in both positions. Fluorescence polarization was calculated and plotted against enzyme concentration. The stoichiometry of the coenzyme-enzyme complex was determined by the inflection point of the plot (Velick, 1958).

Optical rotation was determined with a Rudolph spectropolarimeter, Model 80, with a General Electric A-H6 mercury lamp.

Ultracentrifugation was done in a Spinco Model E ultracentrifuge.

The reaction of PCMB was measured by determining mercaptide formation spectrophotometrically. One mg of enzyme was mixed with a 10-fold molar excess of PCMB in 0.1 M potassium phosphate buffer, *p*H 7.0, in a final volume of 3.0 ml. The reaction mixture was kept at a constant temperature of 25°. The total change in absorbancy at 250 mμ over the initial 20 minutes of the reaction was used to estimate the amount of PCMB reacting with the enzyme with $\epsilon_M = 7.6 \times 10^3$ for mercaptide formation of PCMB with cysteine at *p*H 7.0 (Boyer, 1954).

Results

Prolonged photooxidation ultimately leads to complete inactivation of the enzymes. This is illustrated in Figure 1, where photooxidation of the liver ADH, as measured by oxygen uptake, is plotted against both the per cent activity of the enzyme remaining and the amount of histidine in the enzyme remaining. When less than 40% of the original amount of histidine remains in the enzyme, inactivation is essentially complete.

Coenzyme Specificity.—Attention was next turned to the early effects of photooxidation, since these effects would most likely reveal the results of destruction of small amounts of histidine alone. Figure 2 shows that after mild photooxidation there is an early change in catalytic activity of ADH, which is indicated by the change in relative ability of the enzyme to react with NAD and analogs of NAD.

The next two figures compare the changes in co-

[1] Abbreviations: ADH, horse liver alcohol dehydrogenase; H LDH, heart-type lactic dehydrogenase; M LDH, muscle-type lactic dehydrogenase; NAD and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; AcPyAD and AcPyADH, 3-acetylpyridine adenine dinucleotide, oxidized and reduced forms, respectively; Pyr-3-ald-AD, pyridine-3-aldehyde adenine dinucleotide; NHxD, nicotinamide hypoxanthine dinucleotide; and PCMB, *p*-chloromercuribenzoate.

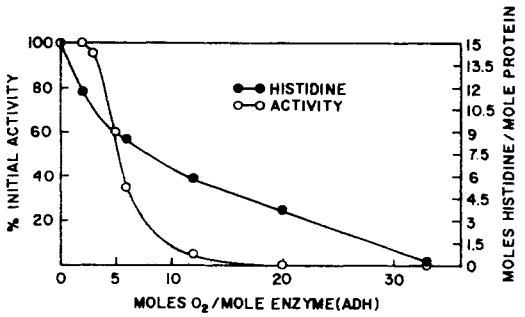

FIG. 1.—The effect of extensive photooxidation on the activity and on the histidine content of horse liver ADH. The per cent initial activity is the ratio of the activity of the photooxidized enzyme to the activity of the untreated enzyme under the same assay conditions. See Methods for details of procedures. Assumed mw of ADH, 84,000.

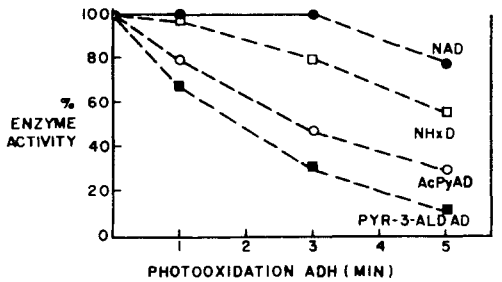

FIG. 2.—Effect of photooxidation of horse liver ADH on the relative activity with NAD and analogs of NAD. All determinations of activity with concentration of NAD and analogs of NAD of 2×10^{-4} M at 26°. Activity of the photooxidized enzyme with each coenzyme is compared to activity of the untreated enzyme with the same coenzyme to give the per cent activity.

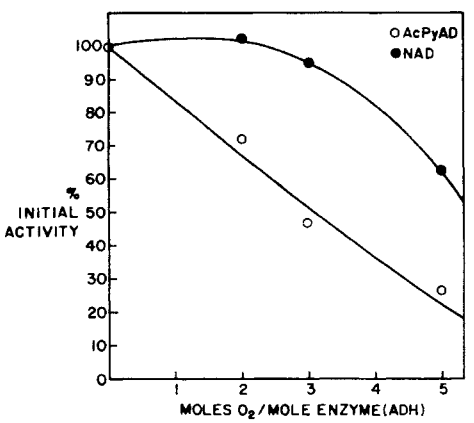

FIG. 3.—Change in coenzyme specificity of horse liver ADH after mild photooxidation.

enzyme specificity of two of the enzymes after mild photooxidation. With ADH (Fig. 3), there is no loss of activity with NAD until oxygen uptake exceeds 3 moles per mole of enzyme. The activity of the enzyme with AcPyAD declines more rapidly and is reduced to nearly 50% of its initial activity at the point where activity with NAD is just beginning to decrease.

A converse situation exists with the beef heart LDH (Fig. 4). Here activity with NAD is more labile to photooxidation and is decreased to 50% of its initial value before the activity with AcPyAD becomes affected.

The activity of both untreated and photooxidized beef H LDH was assayed at varying substrate concentrations. The data are shown in Table I. With NAD used as coenzyme with the untreated enzyme, there is seen to be no significant change in activity over the range of lactate concentration tested. Photooxidation leads to some (25%) increase in activity ratio. On the other hand, the coenzyme AcPyAD gives considerable inhibition of enzyme activity at high substrate concentration. This substrate inhibition becomes much less effective after photooxidation.

TABLE I

RELATION OF THE ACTIVITY OF UNTREATED AND PHOTOOXIDIZED BEEF H LDH TO SUBSTRATE CONCENTRATION

	Untreated Beef H LDH	Moles O_2/Mole Enzyme		
		3.5	5	8
NAD	1.04[a]	1.24	1.25	1.24
AcPyAD	0.42	0.69	0.69	0.80

[a] The numbers represent the ratio of the enzyme activity in the presence of 0.2 M lithium lactate to enzyme activity in the presence of 0.013 M lithium lactate. Concentration of NAD and AcPyAD was 2×10^{-4} M in 0.1 M K_2HPO_4 buffer at *p*H 8.8.

Chicken H LDH.—In order to study the substrate inhibition characteristics more fully, we turned to chicken H LDH because of its stability and known substrate inhibition. Preliminary studies revealed that on photooxidation changes in coenzyme activity similar to those in H LDH occurred, except that a large increase in activity with AcPyAD occurred accompanying slight loss of activity with NAD. This is shown in Table II.

TABLE II

ACTIVITY OF UNTREATED AND PHOTOOXIDIZED CHICKEN H LDH

Chicken H LDH	NAD	AcPyAD
Untreated	100[a]	100
Photooxidized[b]	86	190

[a] Activity is expressed in relative terms, with 100 equalling the activity of the untreated enzyme. All assays done in presence of 0.013 M lithium lactate. [b] Photooxidation: 5 moles O_2 per mole enzyme.

The relation between enzyme activity and substrate concentration is illustrated by the next two figures. The light-oxidized chicken H LDH shows very little substrate inhibition when compared to the untreated enzyme (Fig. 5). Although there is some difference in the affinities for pyruvate of the treated and untreated enzymes, this difference is not nearly as striking as the change in substrate inhibition.

The chicken H LDH shows a marked inhibition with excess lactate when AcPyAD is the coenzyme, as illustrated in Figure 6. As can be seen, there is little inhibition by lactate with the treated enzyme.

Oxalate is known to be an inhibitor of lactic dehydrogenases. It has been found recently in our laboratory that oxalate differently inhibits the two types of LDH (Bieber, Thorne, and Kaplan, in preparation). This is illustrated in Table III. Photooxidation of the chicken heart LDH makes this enzyme considerably less sensitive to the oxalate inhibition (Table III). The data in Figures 5 and 6 as well as the results of Table III suggest that photooxidation of the LDH brings about a change in the substrate inhibition characteristics of the enzyme.

Amino Acid Analyses [2,3].—Amino acid analysis was done first with beef H LDH. The results for untreated enzyme and enzyme at two levels of photooxidation are

TABLE III
PER CENT INHIBITION WITH 3 × 10^{-4} M OXALATE[a]

Chicken M LDH	33
Chicken H LDH	78
Chicken H LDH, photooxidized[b]	55

[a] At *p*H 7.5. [b] Photooxidation: 5 moles O_2 per mole enzyme.

shown in Table IV. All 18 amino acids were examined, and the table includes those amino acids known to be affected by photooxidation. With photooxidation to the extent of 3.5 moles oxygen per mole of enzyme, histidine was the only amino acid affected. With photooxidation to the extent of 8 moles oxygen per mole of enzyme further destruction of histidine occurred, accompanied presumably by destruction of tryptophan to account for the additional oxygen uptake, although tryptophan content was not measured at the latter point.

TABLE IV
AMINO ACID CONTENT OF UNTREATED AND PHOTOOXIDIZED BEEF H LDH

Amino Acid[a]	Untreated	Photooxidized: 3.5 moles O_2/mole enzyme	Photooxidized: 8 moles O_2/mole enzyme
Histidine	24.3[d]	21.8	20.3
Tyrosine	27.3	28.1	28.1
Tryptophan[b]	30.0	31.2	—
Cysteine[c]	12.5	14.8	12.3
Methionine	38.1	37.8	37.3

[a] Amino acids were determined chromatographically with a Beckman automatic amino acid analyzer after 24-hr. hydrolysis in 6 N HCl. [b] Tryptophan was determined by analysis of the ultraviolet spectrum in alkali. [c] The figures for cysteine are of no absolute significance. PCMB titration in 6 M urea reveals 17 moles of cysteine per mole of beef H LDH (Di Sabato *et al.*, 1963). The data reported in the table may be of some relative significance, indicating no large change in cysteine on photooxidation. [d] The numbers represent the moles of amino acid per mole of enzyme, based on the MW of beef H LDH of 140,000. No significant changes were found in the other 13 amino acids analyzed.

The data point out that histidine was the only amino acid affected until photooxidation exceeded 3.5 moles of oxygen per mole of enzyme. This is consistent with the findings of Weil, who studied in detail the relative rates of disappearance of amino acids during photooxidation with methylene blue. With lactoglobulin (Weil *et al.*, 1951), lysozyme (Weil *et al.*, 1952), and chymotrypsin (Weil *et al.*, 1953), it was found that histidine was the most sensitive amino acid to photooxidation, followed by tyrosine and tryptophan, in order of sensitivity. Cysteine and methionine were much less sensitive and were destroyed in significant amounts only with fairly prolonged exposure to methylene blue and light. Because of this and also because of the experimental error present in the analysis of cysteine and methionine in proteins by the chromatographic technique used, amino acid analysis done on the remaining two dehydrogenases was much more limited. Table V shows the changes in

[1] We are grateful to Mr. Fred G. Castillo for performing the amino acid analyses with the automatic amino acid analyzer.

[2] It has been shown that in both beef H LDH and chicken H LDH there are no disulfide bridges; all cysteine is present in its reduced state (Di Sabato *et al.*, 1963).

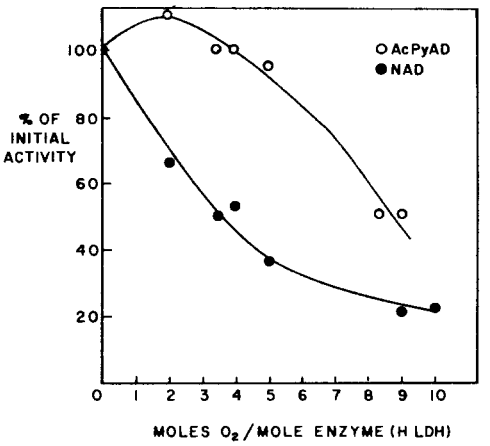

FIG. 4.—Changes in coenzyme specificity of beef H LDH after mild photooxidation. Assumed mw of beef H LDH, 140,000. Activity assayed at lithium lactate concentration of 0.013 M.

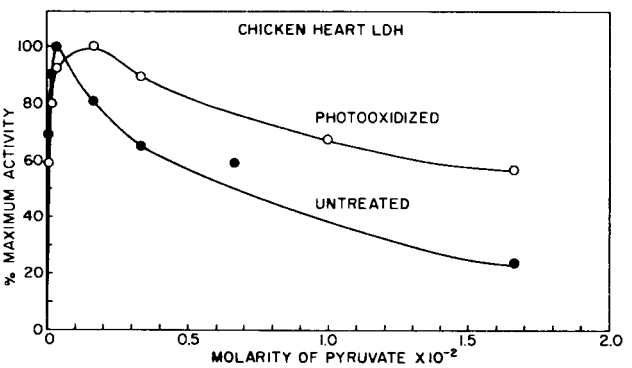

FIG. 5.—Rate of reaction of different levels of pyruvate with native and photooxidized chicken H LDH. The concentration of NADH used in all cases was 1.5 × 10^{-4} M. Approximately 0.1 μl of enzyme was used in a volume of 3 ml at 26°. The *p*H of the reaction was 7.5 (PO_4 buffer). Photooxidation: 5 moles O_2/mole enzyme.

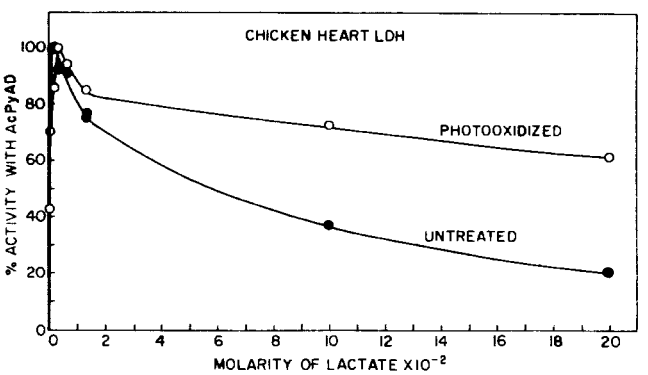

FIG. 6.—Effect of lactate concentration on the rate of reaction of native and photooxidized chicken H LDH with AcPyAD as coenzyme. In all cases, the concentration of pyruvate was 1.3 × 10^{-2} M and of AcPyAD 1 × 10^{-4} M. Reaction was started at *p*H 8.0 in a volume of 3 ml at 26°. 2.5 μl of the native enzyme and 5 μl of the photooxidized enzyme were used. Photooxidation of chicken H LDH: 5 moles O_2/mole enzyme.

histidine, tyrosine, and tryptophan content of liver ADH at several levels of photooxidation. Here, histidine is the first amino acid to be affected, with no appreciable change in tyrosine or tryptophan contents until the extent of photooxidation exceeds 3 moles of oxygen per mole of enzyme.

Photooxidized chicken H LDH was studied at one

TABLE V
CHANGES IN AMINO ACID CONTENT OF HORSE LIVER ADH AFTER PHOTOOXIDATION

	Moles O_2 per Mole Enzyme					
	0	2	3	4	5	6
Histidine	15.2[a]	12.7	11.8	—	—	8.6
Tryptophan	5.1	—	4.8	4.1	2.8	—
Tyrosine	9.3	—	9.0	7.7	8.0	—

[a] The numbers represent moles of amino acid per mole of enzyme, assuming the mw of ADH to be 84,000. Histidine was determined chromatographically as previously described. Tyrosine and tryptophan were determined from the ultraviolet spectrum of the intact protein in 0.1 N NaOH.

TABLE VI
CHANGE IN HISTIDINE CONTENT OF CHICKEN H LDH AFTER PHOTOOXIDATION

Amino Acid	Untreated	Photooxidized 5 moles O_2/mole enzyme
Histidine	30[a]	25
Tryptophan	24	24
Cysteine	28	27

[a] The numbers represent moles of amino acid per mole of enzyme based on the mw of chicken H LDH of 140,000. Histidine and tryptophan content were determined as described for beef H LDH. Cysteine was determined by PCMB titration in 6 M urea (Di Sabato *et al.*, 1963).

level of oxidation, where 5 moles of histidine per mole of enzyme were destroyed. Tryptophan was unaffected at this point, as was cysteine (Table VI).

The limitation in the accuracy of determination of the amino acids is such that one cannot rule out small changes. This especially applies to cysteine and methionine determined chromatographically and tryptophan and tyrosine determined by analysis of the ultraviolet spectrum. This precludes claiming absolute specificity for destruction of histidine, even when photooxidation is limited to 3 moles of oxygen per mole of enzyme. However, all the data taken together indicate that the major change in amino acid content of these enzymes is in histidine when photooxidation is limited to 3 moles of oxygen per mole of enzyme.

TABLE VII
COENZYME BINDING TO UNTREATED AND PHOTOOXIDIZED ENZYMES CORRELATED WITH ACTIVITY[a]

	NAD Activity (%)	NADH Binding (%)	AcPyAD Activity (%)	AcPyADH Binding (%)
	ADH			
Untreated	100	100	100	100
Photooxidized				
3 moles O_2/mole enzyme	86	100	60	60
5 moles O_2/mole enzyme	71	50	24	25
	Beef H LDH			
Untreated	100	100	100	100
Photooxidized				
3 moles O_2/mole enzyme	66	60	100	91
8 moles O_2/mole enzyme	23	33		

[a] The figures represent the per cent activity or binding compared to that of the untreated enzymes.

Effect of Coenzyme Binding.—The coenzyme binding is reported as the molar ratio of coenzyme to enzyme in the coenzyme-enzyme complex with the enzyme in the presence of excess coenzyme. In Table VII, the ratio with the untreated enzymes is considered to be 100% binding, and the per cent deviation from this is given for the photooxidized enzymes. The coenzyme binding is compared to the activity of the enzyme with the same coenzyme. Although the agreement is not always exact, there is generally a fair correlation of the loss in enzyme activity with a given coenzyme and the ability of the enzyme to bind the same coenzyme.

The results of our study of coenzyme binding after photooxidation are at variance with the results of others (Millar and Schwert, 1962). These investigators reported that with fluorescence techniques no change in the NAD-binding ability of beef H LDH was seen after photooxidation until more than 55% of the activity of the enzyme with NAD had been destroyed. However, in their results, further photooxidation did decrease NAD binding, and at 75% inactivation the NAD binding was also reduced to 75% of the value of the untreated enzyme, which agrees with our data.

Our results show that the ability of the beef H LDH to bind NADH is not changed until more than 20% of the initial activity with NAD has been lost. However, when more than 35% of the initial activity with NAD has been lost, the ability to bind NADH has been decreased to a similar extent.

Other Properties of the Photooxidized Enzymes.—After establishing that the catalytic properties of the enzymes could be altered by destruction of small amounts of histidine, it was necessary to investigate certain properties of the treated enzymes which might reflect changes in secondary and/or tertiary structure, since structural changes themselves might affect the catalytic properties of the enzymes.

Optical Rotation.—Effects of photooxidation on optical rotation are shown in Table VIII. The specific rotation was determined at three wave lengths for both enzymes, and values for untreated and photooxidized ADH and H LDH were compared. Photooxidation was limited to the extent of 3 moles of oxygen per mole of ADH and 4 moles of oxygen per mole of beef H LDH. Even with this limited treatment, a definite change in specific rotation is seen at each wave length for both enzymes.

TABLE VIII
SPECIFIC ROTATION OF UNTREATED AND PHOTOOXIDIZED ENZYMES[a]

λ (mμ)	ADH Control	ADH Photooxidized	Beef H LDH Control	Beef H LDH Photooxidized
367	−48	−86	−192	−165
405	−32	−62	−134	−124
546	−14	−27	− 50	− 35

[a] Photooxidation of ADH: 3 moles O_2/mole enzyme. Photooxidation of beef H LDH: 4 moles O_2/mole enzyme. Control enzymes were exposed to the same conditions in the absence of light.

Ultracentrifugation.—Further evidence of change in protein structure after photooxidation is given by ultracentrifugation. Both untreated beef H and horse liver ADH move as a single sharp peak. After photooxidation to the extent of 3–4 moles of oxygen per mole of enzyme, the peak in each case becomes less sharp and with the beef H LDH a slower-moving component is present as well.

PCMB Reaction with LDH.—We have also observed

the effect of photooxidation on the rate of the reaction of beef H LDH with PCMB. During the initial 20 minutes of the reaction, untreated LDH was found to react with 4.5 moles of PCMB per mole of enzyme, whereas photooxidized LDH (4 moles of oxygen per mole of enzyme) reacted with 8 moles of PCMB per mole of enzyme under the same experimental conditions.

Change in Immunologic Properties.—The effect of photooxidation on the immunologic properties was studied with a rabbit antiserum to the crystalline chicken H enzyme (Cahn *et al.*, 1962). As indicated in Figure 7, the complement-fixation curve of the treated chicken H LDH is considerably altered when compared to that of the untreated enzyme. The alteration is observed as approximately a 3-fold lateral displacement for maximal fixation. The immunologic change can also be seen from the data in Table IX. Under conditions where more than 70% of the normal chicken H LDH is precipitated by the antibody, only a small amount of the photooxidized enzyme is precipitated (Table IX). Photooxidation of the beef H enzyme (4 μmoles O_2 per mole enzyme) also results in a change of its antigenic properties; under conditions where 90% of the native beef H enzyme is precipitated by the antibody against the chicken H LDH, only traces of the photooxidized beef H enzyme are precipitated.

TABLE IX
EFFECT OF PHOTOOXIDATION ON PRECIPITATION BY ANTI-CHICKEN H LDH

Vol. of Dilution of Anti-Chick H LDH (ml)	% Enzyme Activity Removed by Precipitation with Anti-Chick H Antibody	
	Untreated	Photooxidized[a]
0	0	0
0.1	13	0
0.2	72	11
0.4	100	47
1.0	100	100

[a] Photooxidation: 5 moles O_2/mole enzyme.

Thermostability.—It had been noted that the photooxidized enzymes lost activity upon storage more rapidly than the untreated enzymes. Chicken H LDH, the most stable of these enzymes to heat, was studied to determine the effect of temperature on activity. The differences in stability at 57° between untreated and photooxidized enzyme are shown in Figure 8. The chicken H LDH is completely stable for 15 minutes at 70°. The treated enzyme loses 95% of its activity after 5 minutes at 65°.

Turnover Number of Chicken H LDH.—The turnover number of the treated chicken H LDH is approximately 40% of the untreated enzyme. We are certain, however, that this drop may not be attributed to the presence of denatured, completely inactive protein. We have been unable to fractionate out any possible inactive protein by DEAE chromatography, ammonium sulfate precipitation, or electrophoresis on starch grain. It is of interest to note that the migration of the photooxidized chicken H on starch gel is essentially the same as that of the native enzyme.

DISCUSSION

In the present work, NAD and analogs of NAD have enabled us to determine a change in the catalytic properties of dehydrogenases after destruction of less

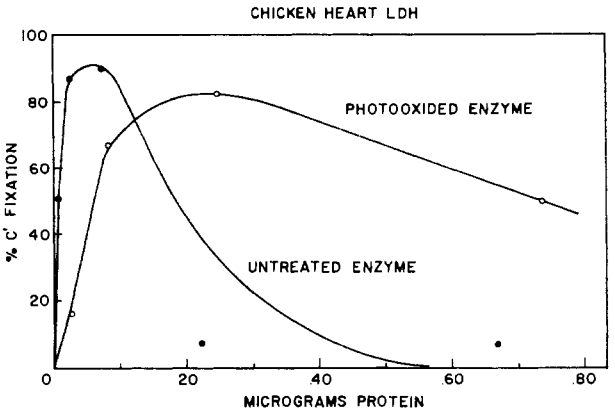

FIG. 7.—Complement-fixation curves of native and photooxidized chicken H LDH. The anti-chicken H antibody was used for this study (Cahn *et al.*, 1962). The complement-fixation procedure was that described by Wasserman and Levine (1961).

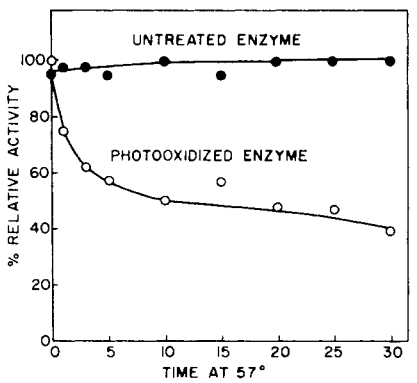

FIG. 8.—Thermostability of untreated and photooxidized chicken H LDH at 57°. Enzyme assay carried out with 1.5×10^{-4} M NADH. Photooxidation: 5 moles O_2/mole enzyme.

than 3 moles of histidine per mole of enzyme. By confining the effects of photooxidation to as few amino acid groups as possible, one might expect the least change in enzyme structure. However, the data presented indicate that the secondary and/or tertiary structures are easily affected by photooxidation. Distinct changes in several properties of the enzymes after destruction of only 3 moles of histidine per mole of enzyme make it difficult to attribute these effects of photooxidation to a change in an amino acid at the active site. It seems equally likely that a change in structure might modify the conformation of the active site of the enzymes and in this way result in a change in catalytic properties. If one assumes, as indicated previously, that there are 4 subunits per mole of LDH (mw 140,000), then it is possible that only one histidine is destroyed per subunit in the early photooxidation of this enzyme. In liver ADH, where there appear to be 2 subunits per mole (Vallee, 1960), only 3 moles of histidine are destroyed. This would suggest that as long as roughly one histidine per subunit is destroyed in this enzyme, the NAD activity of the enzyme is maintained (see Table I).

Brake and Wold (1960), in their study of the photooxidation of yeast enolase, point out that a significant change in sedimentation and diffusion coefficients occurs after photooxidation. They indicate that the chemically modified group (histidine or tryptophan) could not be assumed to be part of the "active site" of the enzyme if other physical properties of the enzyme

were known to have changed. Evidence is cited that in enolase 12 imidazole groups are involved in intramolecular H-bonding, stabilizing the tertiary structure of the molecule (Malmstrom *et al.*, 1959). The results of others as well as our own suggest that histidine residues may be generally important in maintaining the tertiary structure of proteins.

As indicated by studies with chicken H LDH, the photooxidation results in a loss of the substrate inhibition characteristics of the enzyme. This has been observed with the substrate inhibition both of excess pyruvate in the presence of NADH and of excess lactate with AcPyAD. It is our feeling that the inhibition caused by pyruvate is due to a ternary complex formation between the pyruvate, the reduced coenzyme, and LDH, since reactions with different reduced coenzyme analogs give different degrees of inhibition with pyruvate. It has been shown recently by Dr. C. Thorne in our laboratory that a ternary complex exists between AcPyADH, lactate, and chicken H LDH. A complex between oxalate, AcPyADH, and this enzyme has also been detected. The concentrations required for complex formation parallel the concentrations required for the substrate inhibition when AcPyAD is used as coenzyme. The marked effects observed on the substrate inhibition characteristics indicate that this property of the LDH is closely linked to the structure of the enzyme and that one of the prime effects of modification of the structure is the change in the substrate inhibition. We have found recently that other protein-modifying reagents, such as bromosuccinimide and nitrous acid, also alter the chicken H LDH, and a prime effect of these reagents is a loss of substrate inhibition characteristics of the enzyme. These observations strongly point to the fact that the substrate inhibition phenomenon is intimately linked with the structure of the dehydrogenase.

The changes in immunologic properties, heating stabilities, optical rotation, and other physical characteristics of the dehydrogenases after photooxidation indicate the importance of the individual amino acids in maintaining the native structure of the enzyme. Our studies suggest that at least one histidine is important in maintaining the tertiary structure of the three dehydrogenases studied. The present work also supports the suggestion of Levine (1962) that the immunologic reactivity of proteins is due largely to the tertiary structure of the proteins themselves. It would appear also from our studies that, although modification of the structure of proteins by specific amino acid reagents may not give direct information in regard to the active site, the information gained may be useful in correlating enzyme structure with catalytic activity.

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