Anomalous Rotatory Dispersion of Enzyme Complexes. IV. Mechanism of Inhibition of Liver Alcohol Dehydrogenase by Buffer Anions and Bases*

TING-KAI LI,† DAVID D. ULMER,‡ AND BERT L. VALLEE

From the Biophysics Research Laboratory of the Department of Medicine, Harvard Medical School, and the Peter Bent Brigham Hospital, Boston, Massachusetts

Received December 6, 1962

The interaction of various buffer anions and bases with complexes which DPNH and 1,10-phenanthroline form with liver alcohol dehydrogenase has been studied by means of rotatory dispersion titration. At *p*H 7.5, pyrophosphate, chloride, formate, tris(hydroxymethyl)aminomethane, and imidazole reversibly displace equivalent amounts of both DPNH and 1,10-phenanthroline, apparently through interaction with the zinc atoms of the enzyme. These agents do not alter the optical rotatory dispersion of the enzyme itself. Borate ions, in addition to interacting at the zinc sites, also react with the ribosyl residues of DPNH. These effects upon the formation of the enzyme-coenzyme complex appear to underlie the inhibitory action of buffer ions upon the catalytic activity of liver alcohol dehydrogenase.

The catalytic activity of horse liver alcohol dehydrogenase is inhibited by a variety of reagents, including buffer anions and bases (Theorell *et al.*, 1955). Mechanisms by which these inhibitions may be brought about have been inferred from kinetic studies. Measurements of the physicochemical effects of such agents upon the interaction of the enzyme with its coenzyme and/or substrate at equilibrium constitute an alternate approach to this problem.

We have reported previously that highly specific Cotton effects result from the formation of binary complexes of liver alcohol dehydrogenase with chromophoric coenzymes, coenzyme analogs, and site-specific reagents (Ulmer *et al.*, 1961). The magnitudes of these Cotton effects serve as direct measures of the degree of binding of such agents to the active centers of the enzyme at equilibrium—a method which we have termed *rotatory dispersion titration* (Li *et al.*, 1962a). We have now employed this procedure to study the mechanism of inhibition of liver alcohol dehydrogenase by buffer anions and bases. The results indicate that such inhibitions by buffer ions may be brought about through the reversible displacement of DPNH from the zinc atoms of the enzyme. A preliminary account of these data has been given (Li *et al.*, 1962b).

MATERIALS AND METHODS

Crystalline alcohol dehydrogenase of horse liver was obtained from C. F. Boehringer and Soehne, Mannheim, West Germany. Prior to use, the enzyme was dialyzed for 5 days against 0.1 M sodium phosphate buffer, *p*H 7.5, at 4° to remove low-molecular-weight impurities which absorb radiation at 280 mμ. The concentration of protein was determined by measurement of the optical density at 280 mμ, based upon an absorbancy index of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950). Molar concentrations were based on a molecular weight of 83,300 (Ehrenberg and Dalziel, 1958). The catalytic activity of the enzyme for both the oxidation of ethanol and the reduction of acetaldehyde was measured spectrophotometrically as described previously (Vallee *et al.*, 1959).

The concentration of reduced diphosphopyridine nucleotide (β-DPNH, Sigma Chemical Company) was determined by measurement of the absorbancy at 340 mμ (Kaplan, 1960). Solutions of DPNH in metal-free distilled water were prepared fresh every few days from the desiccated stock powder and stored at *p*H 6 and 4°. Under these conditions no significant formation of inhibitors due to decomposition of DPNH (Fawcett *et al.*, 1961) was noted. 1,10-Phenanthroline monohydrate (G. F. Smith and Company) was dissolved in 0.1 M phosphate at *p*H 7.5. Sodium dihydrogen phosphate, sodium pyrophosphate, sodium chloride, sodium borate, and imidazole (Eastman) were all reagent-grade chemicals and were used without further purification. Tris(hydroxymethyl)aminomethane (Sigma Chemical Company), primary standard grade, was extracted with sodium dithizone in carbon tetrachloride at *p*H 8 to remove contaminating metals prior to use (Thiers, 1957). D-Ribose was obtained from the Nutritional Biochemicals Company. Stock solutions of all reagents were adjusted to *p*H 7.5 with sodium hydroxide or hydrogen chloride. Dialyses were carried out in precleaned (Hughes and Klotz, 1956) cellulose casings (Visking Company). The purification of water and glassware has been described (Vallee and Hoch, 1955).

Optical rotation was measured by means of a model 200S-80Q photoelectric spectropolarimeter with oscillating polarizer prism (O. C. Rudolph and Sons); a high-intensity, high-pressure mercury lamp (A-H6, General Electric Company) was used as a light source. Excellent stability of the lamp could be maintained by circulating cooled, deionized water through the quartz-jacketed lamp housing. All measurements were performed in wide-bore, 5-cm semi-micro polarimeter cells with quartz end-plates (O. C. Rudolph and Sons). The temperature of the solutions was maintained at 10 ± 1° by circulating cooled water through the polarimeter housing from an external bath. A stream of nitrogen gas directed onto the polarimeter cell end-plates prevented fogging. Rotational angles were measured by the method of symmetrical angles (Rudolph, 1955). Rotatory dispersion titrations of alcohol dehydrogenase with DPNH[1] and 1,10-phenanthroline

* This work was supported by the Howard Hughes Medical Institute and by grants-in-aid from the National Institutes of Health of the Department of Health, Education and Welfare, No. H-3117 (C4) and GM-K3-8260 C1B, and the National Science Foundation.

† Fellow of the Helen Hay Whitney Foundation.

‡ Research Career Development Awardee of the National Institutes of Health, Department of Health, Education and Welfare.

[1] β-DPNH itself is optically active. However, within the spectral range and at the concentrations employed in these titrations, the optical rotation of the coenzyme is barely detectable, and is not altered by the addition of the inhibitors.

in the presence and absence of inhibitors were performed as described previously (Li *et al.*, 1962a). The corresponding polarimeter blanks did not contain enzyme but were otherwise identical with the experimental samples. All measurements were performed with a constant symmetrical angle of 5°. The slit width of the monochromator was restricted to less than 0.2 mm. The maximum optical density of the sample solutions within the wave length regions examined was less than 1.8 units per 5-cm pathlength. The optical rotatory dispersion of the liver alcohol dehydrogenase–DPNH complex was measured over a range of wave lengths varying from 300 to 450 mμ, and that of the liver alcohol dehydrogenase–1,10-phenanthroline complex from 295 to 340 mμ. Specific rotations were calculated on the basis of enzyme concentrations and are precise to ± 1° in the titrations of the enzyme with DPNH and to ± 3° in the titrations of the enzyme with 1,10-phenanthroline at the lower wave lengths.

Results

The amplitude[2] of the Cotton effect of the liver alcohol dehydrogenase–DPNH complex at 327 mμ is directly proportional to the concentration of the bound DPNH (Li *et al.*, 1962a). Pyrophosphate does not affect the optical rotatory dispersion of the enzyme, but it markedly alters that of the enzyme-coenzyme complex. The magnitude of the Cotton effect, characteristic of the complex, is reduced in proportion to the concentration of pyrophosphate present. Thus, in the presence of 0.2 M pyrophosphate at *p*H 7.5, the reduction in amplitude of the Cotton effect from 67° to 28° is equivalent to a displacement of 58% of the DPNH bound to the enzyme at saturation (Fig. 1).

The displacement of DPNH by pyrophosphate is reversible. As the concentration of DPNH is increased to 6×10^{-5} M, the amplitude of the Cotton effect concomitantly increases from 28° to 48°, an increase of 30% in binding (Table I). Since DPNH binds at or near the zinc atoms of alcohol dehydrogenase (Vallee *et al.*, 1959; Mahler *et al.*, 1962) and pyrophosphate is known to complex zinc, it was thought that pyrophosphate and DPNH might compete for the metal site of the enzyme. This hypothesis was examined further.

Table I

Effect of Pyrophosphate and Varying Concentrations of DPNH on the Amplitude of the Cotton Effect of the Liver Alcohol Dehydrogenase–DPNH Complex[a]

Pyrophosphate (M)	DPNH (M × 10^5)	Amplitude of Cotton Effect (degrees)
0.2	4	28
0.2	6	48
0	4	67
0	6	67

[a] Liver alcohol dehydrogenase: 1.66 mg/ml in 0.1 M phosphate, *p*H 7.5, 10°.

The chelating agent 1,10-phenanthroline binds specifically to the zinc atoms of alcohol dehydrogenase, inducing a characteristic Cotton effect at 297 mμ (Vallee and Coombs, 1959; Ulmer *et al.*, 1961). Upon addition of 0.2 M pyrophosphate, the amplitude of this Cotton effect is reduced from 80° to 36°, demonstrating that pyrophosphate also displaces 1,10-phenanthroline from the zinc atoms of liver alcohol dehydrogenase (Fig. 2).

Chloride, formate, Tris, and imidazole do not alter the optical rotatory dispersion of the enzyme itself, but they reduce the magnitudes of the Cotton effects of the enzyme-DPNH and of the enzyme 1,10-phenanthroline complexes (Tables II and III). The decreases in the magnitudes of these Cotton effects are direct functions of the concentration of these agents.

[2] The terminology is in accord with that of Djerassi (1960).

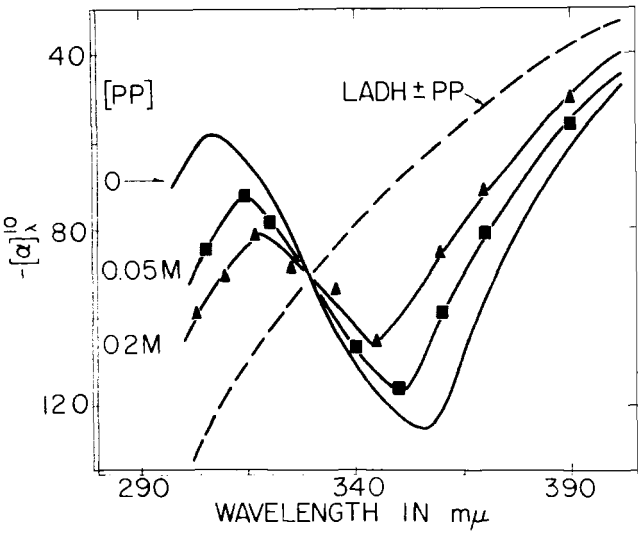

Fig. 1.—The effect of pyrophosphate ion concentration on the optical rotatory dispersion of the liver alcohol dehydrogenase·DPNH complex. Specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, is plotted against wave length. Liver alcohol dehydrogenase, 1.66 mg/ml in 0.1 M phosphate, *p*H 7.5, 10°, served as the control (- - -). Identical concentrations of enzyme plus 4×10^{-5} M DPNH (——) were exposed to 0.05 M pyrophosphate (■) and 0.2 M pyrophosphate (▲). In each instance, identical concentrations of all the reagents in the absence of the enzyme served as the polarimeter blanks. The addition of pyrophosphate to the control did not alter its optical rotatory dispersion.

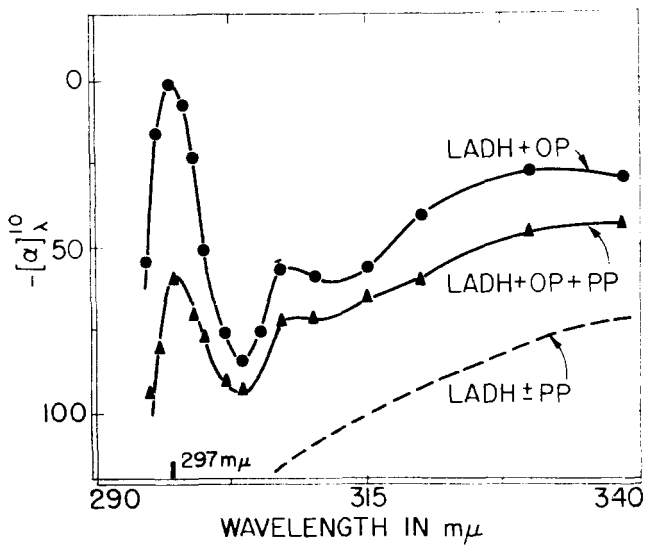

Fig. 2.—The effect of pyrophosphate ions on the magnitude of the Cotton effect of the liver alcohol dehydrogenase·1,10-phenanthroline complex. The specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, is plotted against wave length. Liver alcohol dehydrogenase, 0.5 mg/ml, in 0.1 M phosphate, *p*H 7.5, 10°, served as the control (- - -). Identical concentrations of enzyme plus 6×10^{-5} M 1,10-phenanthroline (●) were exposed to 0.2 M pyrophosphate (▲). Addition of pyrophosphate to the control did not alter its optical rotatory dispersion.

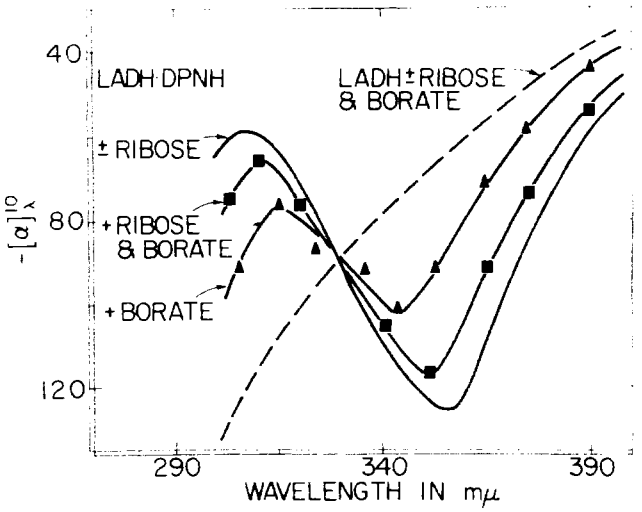

FIG. 3.—The effect of borate and D-ribose on the optical rotatory dispersion of liver alcohol dehydrogenase and on the magnitude of the Cotton effect of the liver alcohol dehydrogenase·DPNH complex. The specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, is plotted against wave length. Liver alcohol dehydrogenase, 1.66 mg/ml, in 0.1 M phosphate, *p*H 7.5, 10°, was the control (- - -). Identical concentrations of enzyme plus 4×10^{-5} M DPNH (——) were exposed to 0.01 M borate (▲) and 0.01 M borate plus 0.07 M ribose (■). Ribose, 0.07 M, did not alter the Cotton effect of the enzyme-coenzyme complex (——). Addition of both ribose and borate to the control did not alter its optical rotation.

The resultant changes in the degree of enzyme-coenzyme binding are proportional to the degree to which these agents affect enzyme–1,10-phenanthroline binding. Again, in each instance, an increase in the concentration of DPNH concomitantly increases the magnitude of the Cotton effect of the enzyme-coenzyme complex, and the reversibility of the process is thereby demonstrated. Phosphate ions, employed as a control, neither inhibit enzymatic activity nor alter the magnitudes of the Cotton effects of alcohol dehydrogenase complexes with DPNH and 1,10-phenanthroline (Tables II and III).

TABLE II

EFFECT OF BUFFER ION AND DPNH CONCENTRATIONS ON THE AMPLITUDE OF THE COTTON EFFECT OF THE LIVER ALCOHOL DEHYDROGENASE–DPNH COMPLEX[a]

Buffer	Buffer Concentration (M)	DPNH Concentration (M × 10^5)	Amplitude of Cotton Effect (degrees)
Chloride	0.5	4	34
	0.2	4	42
	0.2	6	61
Formate	0.5	4	29
	0.2	4	35
	0.2	6	54
Tris	0.4	4	32
	0.2	4	38
	0.2	6	57
Imidazole	0.1	4	26
	0.1	6	40
	0.05	4	37
Borate	0.1	4	0
	0.01	6	32
	0.01	4	25
Phosphate	0.4	6	67
	0.4	4	67
	0.005	6	67

[a] Liver alcohol dehydrogenase: 1.66 mg/ml in 0.1 M phosphate, *p*H 7.5.

TABLE III

THE EFFECT OF BUFFER IONS ON THE AMPLITUDE OF THE COTTON EFFECT OF THE LIVER ALCOHOL DEHYDROGENASE–1,10-PHENANTHROLINE COMPLEX[a]

Buffer	Buffer Concentration (M)	Amplitude of Cotton Effect (degrees)
Chloride	0.5	44
Formate	0.5	33
Tris	0.4	40
Imidazole	0.1	29
Borate	0.1	44
Phosphate	0.005	80
	0.4	80

[a] Liver alcohol dehydrogenase, 0.5 mg/ml, and 1,10-phenanthroline, 6×10^{-5} M, in 0.1 M phosphate, *p*H 7.5.

The effect of borate on these systems differs from that described for the ions above. The concentrations of borate which are required to displace DPNH from alcohol dehydrogenase are much lower than those needed to displace 1,10-phenanthroline (Tables II and III). The cause of this differential effectiveness was investigated further.

Borate ions from complexes with sugars containing hydroxyl groups in the *cis*-configuration (Boeseken, 1949). Though specific data demonstrating the interaction of ribose with borate could not be found in the literature, such a reaction, demonstrated in the course of these studies (*vide infra*), might have been anticipated. Thus the interaction of borate with the ribosyl-hydroxyl groups of DPNH might well interfere with the formation of the enzyme-coenzyme complex. Consistent with this hypothesis, D-ribose does not affect the binding of DPNH to alcohol dehydrogenase but it markedly curtails the effectiveness of borate ions in displacing bound DPNH (Fig. 3).

The stoichiometry of the ribose-borate and DPNH-borate complexes was determined by means of the difference in the optical rotation of the sugars and of their borate complexes (Zittle, 1951). At *p*H 7.5, one mole of borate reacts with each mole of ribose. Titration of DPNH with borate shows similar changes in optical rotation, which become maximal at 2 moles of borate per mole of DPNH (Fig. 4). Apparently one mole of borate reacts with each of the two ribosyl residues of DPNH, in accord with the stoichiometry of the ribose-borate complexes.

DISCUSSION

Inhibition of horse liver alcohol dehydrogenase by buffer anions and bases has been studied extensively. The kinetics of the inhibitions resulting from various buffer ions differ quantitatively and in certain instances even qualitatively (Theorell *et al.*, 1955; Theorell and McKinley McKee, 1961; Plane and Theorell, 1961). Common mechanistic denominators to reconcile these apparent discrepancies have been sought (Theorell and McKinley McKee, 1961). The effects of these inhibiting ions upon the interaction of the coenzyme and other site-specific reagents with the enzyme are here examined at equilibrium, an approach similar to that utilized to advantage previously in the study of the Old Yellow Enzyme (Theorell and Nygaard, 1954; Nygaard and Theorell, 1955).

DPNH binds at or near, and the chelating agent 1,10-phenanthroline binds directly to, the zinc atoms

of alcohol dehydrogenase (Vallee *et al.*, 1959; Vallee and Coombs, 1959). Binding of these chromophoric molecules at the active centers of the enzyme results in characteristic Cotton effects, the magnitudes of which are directly proportional to the concentrations of the complexes formed (Ulmer *et al.*, 1961; Li *et al.*, 1962a). Hence, these specific Cotton effects may be employed to measure the effect of a variety of inhibitors upon the binding of DPNH and of 1,10-phenanthroline to alcohol dehydrogenase, and to discern the interaction of these inhibitors at the zinc-containing active centers of the enzyme.

Pyrophosphate, chloride, formate, Tris,[3] and imidazole, ions which inhibit the enzyme, all reversibly decrease the binding of DPNH and of 1,10-phenanthroline (Figs. 1 and 2, Tables I, II, III). A given concentration of each inhibiting ion displaces DPNH and 1,10-phenanthroline to the same degree; none of them alters the optical rotatory dispersion of the protein itself. It seems improbable, therefore, that the dissociation of the enzyme-coenzyme and enzyme-chelate complexes is brought about through alterations in protein structure (Schellman and Schellman, 1958; Blout, 1960). Phosphate ions, which do not inhibit alcohol dehydrogenase, do not alter the characteristics of the Cotton effects. Hence, nonspecific effects, *e.g.* ionic strength, also do not seem to underlie these observations. It would appear that the reversible displacement of both DPNH and 1,10-phenanthroline from the enzyme is the consequence of the interaction of the anions and bases with the zinc atoms of the enzyme. Apparently all of these ions compete with the coenzyme for binding at the active centers of alcohol dehydrogenase.

The displacement of DPNH by these buffer ions is reminiscent of the effects of alkaline *p*H on the binding of DPNH. At *p*H 10, only one mole of DPNH is bound to each mole of the enzyme. The ionization of a specific group on the protein, perhaps a sulfhydryl group, which might be responsible for binding of the coenzyme was considered to be a possible explanation for this decrease in binding (Theorell and Bonnichsen, 1951). Detailed examination of the binding of DPNH to liver alcohol dehydrogenase as a function of *p*H has recently been performed by means of optical rotatory dispersion titration and fluorescence polarimetry (Li *et al.*, 1962a; Brand *et al.*, 1962). Two moles of DPNH remain bound to each mole of enzyme between *p*H 7.0 and 9.5, the amount decreasing sharply thereafter until none is bound at *p*H 11. The present data suggest that the dissociation of the liver alcohol dehydrogenase–DPNH complex at these *p*H values might be attributable to the interaction of hydroxyl ions with the zinc atoms of the enzyme in a manner similar to that shown here for buffer anions and bases. In fact, in the presence of increasing concentrations of Tris the enzyme-coenzyme dissociation curve measured as a function of *p*H shifts to progressively lower *p*H values, as might be expected from the combined effects of both hydroxyl and Tris ions when interacting with the zinc atoms of the enzyme (Fig. 5).

Compared to the other anions and bases studied, borate ions are disproportionately more effective in displacing DPNH than 1,10-phenanthroline from the enzyme. The dual action of this agent can be held accountable for the disparity. Borate appears to interfere with the interaction of the enzyme with the coenzyme both by competing for the zinc atoms of the enzyme (Table III) and by interacting with the ribose moiety of the coenzyme itself (Fig. 4). The latter

[3] Unpublished data.

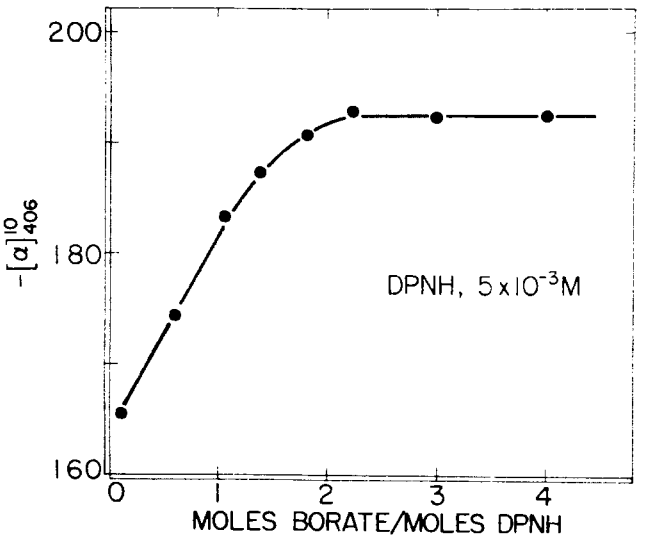

FIG. 4.—Rotatory dispersion titration of DPNH with borate ions. 5×10^{-3} M DPNH in water at 10° was titrated with increments of 0.2 M sodium borate. The specific levorotation of the reaction mixture at 406 mμ and 10°, $-[\alpha]^{10}_{406}$, is plotted against the moles of borate added per mole of DPNH.

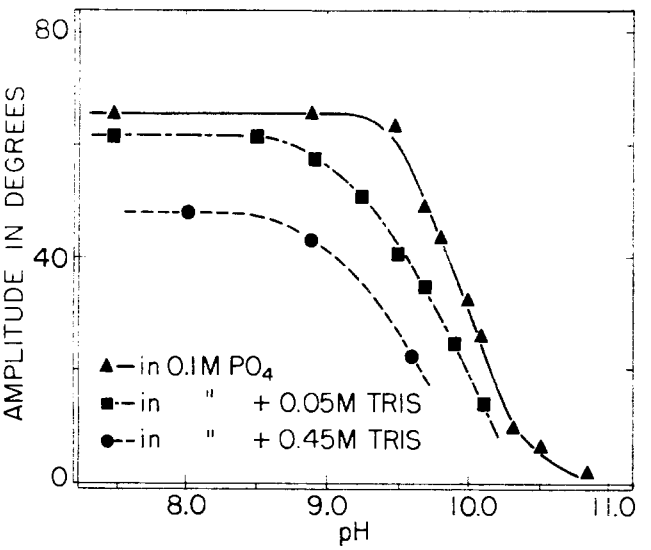

FIG. 5.—The effect of alkaline *p*H and varying concentrations of tris(hydroxymethyl)aminomethane on the binding of DPNH to liver alcohol dehydrogenase. The amplitude of the Cotton effect of the enzyme·DPNH complex (see Fig. 1) is plotted against *p*H. Liver alcohol dehydrogenase, 1.66 mg/ml, and 6×10^{-6} M DPNH in 0.1 M PO_4, 10° (▲), were exposed to 0.05 M Tris (■) and 0.45 M Tris (●) at the *p*H values indicated.

reaction has also been suggested previously as a possible mechanism for the inhibition of borate ions in the yeast alcohol dehydrogenase system (Roush and Gowdy, 1961).

The striking effectiveness of imidazole in displacing DPNH from liver alcohol dehydrogenase under equilibrium conditions bears upon previous studies. Imidazole has been shown to compete with DPN, ethanol, and acetaldehyde. Even though imidazole forms complexes with zinc (Koltun *et al.*, 1958), it does not compete with DPNH under conditions appropriate for kinetic measurements (Theorell and McKinley McKee, 1961). Under conditions of equilibrium, the competition of imidazole with DPNH for binding to the enzyme becomes apparent, as shown by the present data (Tables II and III).

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.

References

Blout, E. R. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., editor, New York, McGraw-Hill Book Company, p. 238.

Boeseken, J. (1949), *Advan. Carbohydrate Chem. 4*, 189.

Bonnichsen, R. K. (1950), *Acta Chem. Scand. 4*, 715.

Brand, L., Everse, J., and Kaplan, N. O. (1962), *Biochemistry 1*, 423.

Djerassi, C., editor (1960), Optical Rotatory Dispersion, New York, McGraw-Hill Book Company.

Ehrenberg, A., and Dalziel, K. (1958), *Acta Chem. Scand. 12*, 465.

Fawcett, C. P., Ciotti, M. M., and Kaplan, N. O. (1961), *Biochim. Biophys. Acta 54*, 210.

Hughes, T. R., and Klotz, I. M. (1956), *in* Methods of Biochemical Analysis, vol. 3, Glick, D., editor, New York, Interscience Publishers, Inc., p. 265.

Kaplan, N. O. (1960), *in* The Enzymes, ed. 2, vol. 3, Boyer, P. D., Lardy, H., and Myrbäck, K., editors, New York, Academic Press, Inc., p. 105.

Koltun, W. L., Dexter, R. N., Clark, R. E., and Gurd, F. R. N. (1958), *J. Am. Chem. Soc. 80*, 4188.

Mahler, H. R., Baker, R. H., and Shiner, V. J. (1962), *Biochemistry 1*, 47.

Li, T. K., Ulmer, D. D., and Vallee, B. L. (1962a), *Biochemistry 1*, 114.

Li, T. K., Ulmer, D. D., and Vallee, B. L. (1962b), Abstracts of Papers, 141st Meeting of the American Chemical Society, p. 55C.

Nygaard, A. P., and Theorell, H. (1955), *Acta Chem. Scand. 9*, 1587.

Plane, R. A., and Theorell, H. (1961), *Acta Chem. Scand. 15*, 1866.

Roush, A. H., and Gowdy, B. B. (1961), *Biochim. Biophys. Acta 52*, 200.

Rudolph, H. (1955), *J. Opt. Soc. Am. 45*, 50.

Schellman, C. G., and Schellman, J. A. (1958), *Compt. rend. trav. lab. Carlsberg. Ser. Chim. 30*, 463.

Theorell, H., and Bonnichsen, R. (1951), *Acta Chem. Scand. 5*, 1105.

Theorell, H., and McKinley McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1834.

Theorell, H., and Nygaard, A. P. (1954), *Acta Chem. Scand. 8*, 1649.

Theorell, H., Nygaard, A. P., and Bonnichsen, R. (1955), *Acta Chem. Scand. 9*, 1148.

Thiers, R. (1957), *in* Methods of Biochemical Analysis, vol. 5, Glick, D., editor, New York, Interscience Publishers, p. 273.

Ulmer, D. D., Li, T. K., and Vallee, B. L. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1155.

Ulmer, D. D., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 730.

Vallee, B. L., and Coombs, T. L. (1959), *J. Biol. Chem. 234*, 2615.

Vallee, B. L., and Hoch, F. L. (1955), *Proc. Nat. Acad. Sci. U. S. 41*, 327.

Vallee, B. L., Williams, R. J. P., and Hoch, F. L. (1959), *J. Biol. Chem. 234*, 2621.

Zittle, C. A. (1951), *Advan. Enzymol. 12*, 493.

Structural and Catalytic Alterations of Dehydrogenases After Photooxidation*

DWIGHT ROBINSON, DAVID STOLLAR, SHIRLEY WHITE, AND NATHAN O. KAPLAN

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts†

Received September 26, 1962

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

* This work was supported in part by grants from the National Institutes of Health (CY-3611 and 2G-212), the National Science Foundation (G20,C29), and from the American Cancer Society (P-77E, the Thomas S. Miller Memorial Grant for Cancer Research).

† Publication No. 211 of the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass.