Latt, S. A., Auld, D. S., and Vallee, B. L. (1972), *Biochemistry 11,* 3015.
Lipscomb, W. N. (1973), *Proc. Nat. Acad. Sci. U. S. 71,* 3797.
Lipscomb, W. N., Hartsuck, J. A., Reeke, G. N., Quiocho, F. A., Bethge, P. H., Ludwig, M. L., Steitz, T. A., Muirhead, H., and Coppola, J. C. (1968), *Brookhaven Symp. Biol. 21,* 24.
Lumry, R., and Smith, E. L. (1955), *Discuss. Faraday Soc. 20,* 105.
McClure, W. O., Neurath, H., and Walsh, K. A. (1964), *Biochemistry 3,* 1897.
Navon, G., Shulman, R. G., Wyluda, B. J., and Yamane, T. (1968), *Proc. Nat. Acad. Sci. U. S. 60,* 86.
Navon, G., Shulman, R. G., Wyluda, B. J., and Yamane, T. (1970), *J. Mol. Biol. 51,* 15.
Riordan, J. F. (1973), *Biochemistry 12,* 3915.
Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2,* 1460.
Smith, E. L. (1949), *Proc. Nat. Acad. Sci. U. S. 35,* 80.
Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181,* 789.
Vallee, B. L., and Riordan, J. F. (1968), *Brookhaven Symp. Biol. 21,* 91.
Vallee, B. L., Riordan, J. F., Auld, D. S., and Latt, S. A. (1970), *Phil. Trans, Roy. Soc. London, Ser. B 257,* 215.
Vallee, B. L., Riordan, J. F., Bethune, J. L., Coombs, T. L., Auld, D. S., and Sokolovsky, M. (1968), *Biochemistry 7,* 3547.
Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49,* 109.
Waldschmidt-Leitz, E. (1931), *Physiol. Rev. 11,* 358.
Whitaker, J. R., Menger, F., and Bender, M. L. (1966), *Biochemistry 5,* 386.

# Functional Arginyl Residues as NADH Binding Sites of Alcohol Dehydrogenases†

Louis G. Lange, III, James F. Riordan, and Bert L. Vallee*

ABSTRACT: Two specific arginyl reagents, 2,3-butanedione and phenylglyoxal, inactivate the alcohol dehydrogenases from human liver, horse liver, and yeast. These arginyl residues have been identified as components of the NADH binding sites in these three enzymes as substantiated by the protective effect of NADH against loss of activity. Coenzyme binding to the modified horse enzyme is virtually abolished as revealed by absorption and circular dichroic spectroscopy and gel filtration studies. Protection experiments with a series of coenzyme analogs further indicate that these arginyl residues interact, most likely, with the pyrophosphate moiety of the coenzyme. Specific, reversible modifications of the arginyl residues by butanedione and of the catalytically essential thiol residue, Cys-46, by *p*-mercuribenzoate differentiate arginine from cysteine modification. Moreover, differences in the rates of specific irreversible carboxymethylation of Cys-46 by iodoacetate in native and butanedione-modified liver alcohol dehydrogenase indicate that the functional arginyl residues are in close proximity to the active center cysteines.

At least 1000 of the more than 1500 enzymes now known act on negatively charged substrates or require anionic cofactors. As part of a general study on the mode of binding of such ligands to these enzymes, we have found that arginyl residues often serve as the complementary, positively charged recognition sites. Thus, a single arginyl residue participates in binding the terminal carboxyl group of peptide substrates to carboxypeptidase A (Vallee and Riordan, 1968; Riordan, 1970, 1973) and arginines are critical for substrate binding to *Escherichia coli* alkaline phosphatase (Daemen and Riordan, 1974). Moreover, metabolic regulation of enzyme function by hormones or other means frequently involves interaction with phosphate, cyclic AMP, ATP, their derivatives, or related compounds. Recognition of these anionic ligands might constitute a very important function of arginyl residues in proteins.

The present study examines such concepts particularly as they may pertain to binding of NADH to the alcohol dehydrogenases. We have studied the role of arginine in binding this coenzyme to the enzymes from human liver, horse liver, and yeast. Arginine-specific, $\alpha$-dicarbonyl reagents inactivate all three of these dehydrogenases, and loss of activity correlates both with arginine modification and loss of coenzyme binding. These results identify arginyl residues as NADH binding sites in these alcohol dehydrogenases and may bear on the binding of NADH and other nucleotide coenzymes to enzymes, in general.

## Materials and Methods

Horse liver alcohol dehydrogenase (Boehringer-Mannheim Corporation) was obtained as a crystalline suspension in 0.02 M phosphate buffer (pH 7.5) and 10% ethanol. Concentrated enzyme solutions were prepared by dialyzing the suspension for 3 days *vs.* daily changes of a 1000-fold volume excess of 0.1 M phosphate (pH 7.5, 4°); centrifugation at 5000 rpm for 15 min, 4°, removed insoluble material. Protein concentration, using a molar absorptivity at 280 nm of $3.57 \times 10^4$ $M^{-1}$ $cm^{-1}$, and enzymatic

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115. *Received May 13, 1974.* This work was supported by Grants-in-Aid GM-15003, GM-02123, and GM-00451 from the National Institutes of Health, of the Department of Health, Education and Welfare.

activity, which varied from 12.9 to 13.6 $\Delta A_{340}$/min per mg, were determined by the method of Drum *et al.* (1969).

Yeast alcohol dehydrogenase isolated from *Saccharomyces cerevisiae* was obtained as a lyophilized powder from Worthington Biochemical Co. Stock solutions were prepared by dissolving the protein in 0.1 M phosphate buffer (pH 7.5) and dialyzing vs. a 100-fold volume excess of the same buffer overnight at 4°. Protein concentration was determined from absorbance at 280 nm using molar absorptivity of $2.02 \times 10^5$ $M^{-1}$ $cm^{-1}$ (Hayes and Velick, 1954). Activity was determined as reported previously (Vallee and Hoch, 1955).

Human liver alcohol dehydrogenase was isolated according to Blair and Vallee (1966), and fraction II resulting from the carboxymethylcellulose chromatography step with a specific activity of 1.9 $\Delta A_{340}$/min per $A_{280}$ was used in these studies. Activity was assayed with a Unicam SP-800 spectrophotometer by recording the absorbance at 340 nm after addition of 25 $\mu$l of stock enzyme to 3 ml of reaction mixture containing 142 $\mu$mol of pyrophosphate, 100 $\mu$mol of ethanol, and 5.7 $\mu$mol of $NAD^+$ at pH 8.8, 25°.

The oxidized and reduced forms of $\beta$-diphosphopyridine nucleotide from yeast and adenosine diphosphate were obtained from Sigma. Solutions were prepared fresh daily and kept on ice. ADP-ribose and 3-CHO-$NAD^+$ (P-L Biochemicals), adenosine (Schwarz) phenylglyoxal monohydrate, and 2,3-butanedione were purchased from Aldrich Chemical Co. and *p*-HgBzO[1] from Sigma. Phenylglyoxal-7-$^{14}C$, prepared from acetophenone-7-$^{14}C$ (ICN) according to Riley and Gray (1943), had a specific activity of 113,500 cpm/mol.

Experiments examining dependence on butanedione concentration and pH as well as protection by substrates, NAD(H), or inhibitors were performed by diluting the alcohol dehydrogenases with butanedione–borate solutions of appropriate concentration and pH at 25° to give final concentrations of 1 mg/ml of horse liver alcohol dehydrogenase, 120 $\mu$g/ml of yeast alcohol dehydrogenase and 0.4 $A_{280}$ unit/ml of human liver alcohol dehydrogenase. Aliquots were assayed at timed intervals.

Other protection experiments employing the heavy metal derivatives $K_2Pt(CN)_4$ (Pfaltz and Bauer) and $KAu(CN)_2$ (Fisher) were carried out by first incubating horse liver alcohol dehydrogenase, 2.65 mg, with 900 $\mu$l of 22.2 mM protective agent in 50 mM borate (pH 8.5) followed by addition of 100 $\mu$l of 50 mM butanedione in 50 mM borate (pH 8.5).

Essential sulfhydryl groups were protected by adding 1 mM *p*-HgBzO in 50 mM borate (pH 8.5, 25°) to horse liver alcohol dehydrogenase, 4.82 mg/ml, in the same buffer. A *p*-HgBzO liver alcohol dehydrogenase–butanedione derivative was prepared by incubating *p*-HgBzO liver alcohol dehydrogenase for 1 hr, 25°, after addition of an appropriate aliquot of butanedione diluted 100-fold (116 mM) into borate buffer. Unreacted butanedione was removed by dialysis for 2–3 hr *vs.* a 1000-fold volume excess of 50 mM borate (pH 8.5, 4°) without change in activity after dialysis. Concentrated dithiothreitol sufficient to give a final dithiothreitol concentration of 2 mM was added to regenerate free sulfhydryl groups.

The converse experiment was carried out by incubating horse liver alcohol dehydrogenase, 4.82 mg/ml, with 390 mM butanedione in 50 mM borate (pH 8.5). The resulting liver alcohol dehydrogenase–butanedione was then treated with *p*-HgBzO in borate (pH 8.5) and gel filtered within 5 min through Bio-Gel P-4 equilibrated with 20 mM Veronal (pH 8.5). A control experiment, in which enzymatic activity was regenerated, consisted of gel filtration in Veronal without prior reaction with *p*-HgBzO.

The three alcohol dehydrogenases were modified with phenylglyoxal by adding a small aliquot of concentrated stock enzyme to 5 mM phenylglyoxal in 125 mM bicarbonate (pH 7.9, 25°). Aliquots were removed at timed intervals and assayed both for enzymatic activity, and for horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase, after gel filtration through Bio-Gel P-4 (0.8 × 8 cm) equilibrated with 125 mM bicarbonate (pH 7.5), for $^{14}C$ incorporation. The fraction containing the protein was diluted into Bray's solution and radioactivity was determined by standard liquid scintillation techniques using a Packard Tricarb instrument.The concentration of the modified protein was determined both by the method of Lowry *et al.* (1951) and by $A_{280}$ as described above; identical results were obtained. A stoichiometry of two phenylglyoxals per arginyl side chain was used for calculations, in line with previous observations (Takahashi, 1968).

Horse liver alcohol dehydrogenase, 0.83 mg/ml in 50 mM borate (pH 8.5), was carboxymethylated with 6.1 mM iodoacetate-$^{14}C$ (198,000 cpm/$\mu$mol, New England Nuclear) at 20°. Aliquots were removed at intervals and assayed for activity and $^{14}C$ incorporation, the latter after gel filtration through Bio-Gel P-4 equilibrated with 50 mM borate. The same procedure was employed for butanedione–liver alcohol dehydrogenase.

Absorption and CD[1] spectra were recorded with a Cary 14 spectrophotometer and a Cary 61 spectropolarimeter, respectively, while analyses at single wavelengths were performed with a Zeiss PMQ II spectrophotometer. All pH determinations were made with a Radiometer PHM 63 meter equipped with a GK 2321C electrode.

Binding of reduced coenzyme to both native and phenylglyoxal-modified horse liver alcohol dehydrogenase was measured by the gel filtration procedure of Hummel and Dreyer (1962) using a Bio-Gel P-4 column (1.7 × 21 cm, flow rate = 2.9 ml/min) equilibrated at 20° with 24.5 $\mu$M NADH–50 mM phosphate (pH 7.5). An Isco Model UA-5 absorbance monitor equipped with a 340-nm filter was used to follow absorbance at this wavelength. In a control experiment, 30 $\mu$l of liver alcohol dehydrogenase solution containing 790 $\mu$g of native enzyme in phosphate buffer gave the same elution profile as did a 300-$\mu$l sample containing 790 $\mu$g of liver alcohol dehydrogenase equilibrated against eluting buffer. Hence 30-$\mu$l aliquots of the liver alcohol dehydrogenase phenylglyoxal reaction mixture were applied to the column directly. Integrated areas of the peak and trough regions were self-consistent in assessing coenzyme binding.

Amino acid analyses of native and modified enzymes were carried out on samples hydrolyzed for 24 hr in 6 N HCl in sealed, evacuated ampoules at 110°, according to the method of Spackman *et al.* (1958) using a Beckman 120C amino acid analyzer.

## Results

*Human Liver Alcohol Dehydrogenase.* Butanedione, 50 mM, in 50 mM borate (pH 9.0) rapidly inactivates human liver alcohol dehydrogenase to 50% of the native activity

[1] Abbreviations not listed in *Biochemistry 5,* 1445 (1966) are as follows: *p*-HgBzO, *p*-mecuribenzoate; CD, circular dichroism.

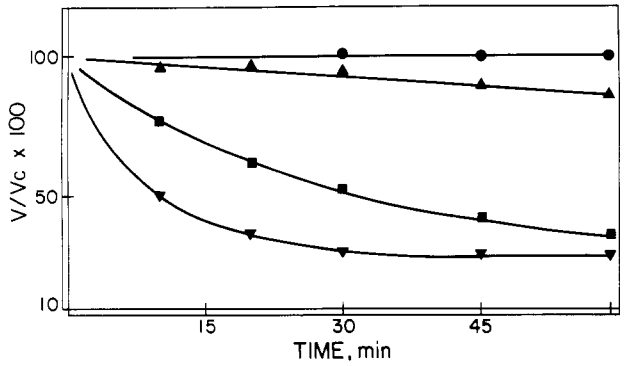


FIGURE 1: Change in the activity of human liver alcohol dehydrogenase, 0.4 $A_{280}$/ml, pH 9.0, *vs.* time on modification with 50 mM butanedione–50 mM borate (▼), and in the presence of 1 mM $NAD^+$ (■) and 1 mM NADH (▲). The presence of 20 mM ethanol (▼) or 20 mM acetaldehyde (▼) affords no protection. The native enzyme (●) (control) retains full activity over this time period.

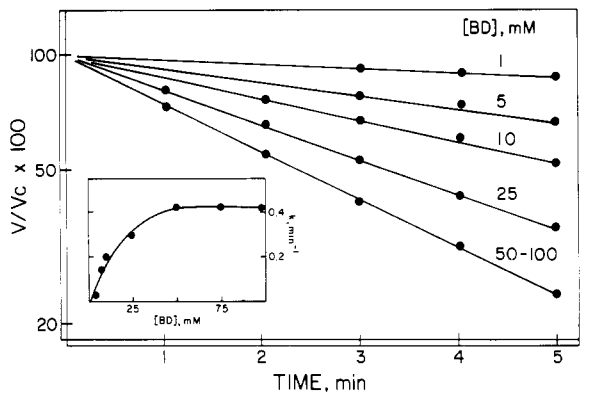


FIGURE 3: Semi-log plot of changes in the activity of horse liver alcohol dehydrogenase, 1 mg/ml, in 50 mM borate (pH 8.5) *vs.* time as a function of butanedione concentration. Insert: a plot of $k$, the reciprocal of the half-life of inactivation, *vs.* [butanedione].

after 10 and to 28% after 45 min. The presence of ethanol, 20 mM, or acetaldehyde, 20 mM, in the modification mixture does not affect the rate of inactivation (Figure 1). On the other hand, NADH, 1 mM, affords nearly complete and $NAD^+$, 1 mM, moderate protection against loss of activity.

Inactivation of human liver alcohol dehydrogenase with butanedione proceeds to 28% of the native activity but no further. Alterations of pH and butanedione concentration only affect the rate but not the extent of inactivation. However, modification with 10 mM phenylglyoxal, another arginyl reagent (Takahashi, 1968), in 125 mM bicarbonate (pH 7.9) inactivates the human enzyme to 8% of native activity after 60 min. Since human liver alcohol dehydrogenase is as yet defined inadequately with respect to both homogeneity and the nature of its isozymes, decisive interpretation of results obtained with the present preparation must be deferred. The alcohol dehydrogenases from horse liver and yeast are better characterized and, hence, were selected for more detailed examination.

*Horse Liver Alcohol Dehydrogenase.* Under conditions identical with those used above, *i.e.*, 50 mM butanedione in 50 mM borate (pH 9.0), the inactivation of horse liver alcohol dehydrogenase proceeds even more rapidly than that of the human enzyme. Only 24% of the native horse liver alcohol dehydrogenase activity remains after 5 min and 12% after 30 min. To optimize inactivation conditions the effects of pH and reagent concentration were studied. The rate of inactivation is the same at pH 8.5 and 9.5 ($t_{1/2}$ = 8 min) but is less at pH 7.5 ($t_{1/2}$ = 39 min) and is even slower at pH 6.5 ($t_{1/2}$ = 85 min) (Figure 2). In 50 mM borate (pH 8.5) a plot of log residual activity *vs.* time is linear for up to 70% of inactivation (Figure 3) and, hence, reciprocal initial half-lives can serve to quantitate the rate of reaction. When butanedione concentrations vary from 1 to 10 mM, inactivation with respect to reagent concentration is first order, but at higher concentrations the reaction becomes zero order (Figure 3, insert) indicative of saturation kinetics. Borate does not become a limiting factor under these conditions since inactivation by 50 mM butanedione proceeds as rapidly in 50 mM as in 100 mM borate. For subsequent studies, 5 mM butanedione, 50 mM borate, and pH 8.5 were adopted for the site-specific modification of horse liver alcohol dehydrogenase (*vide infra*).

Under these conditions, neither ethanol, 20 mM, nor acetaldehyde, 20 mM, affects the rate of inactivation of horse liver alcohol dehydrogenase (Figure 4). However, NADH, 1 mM, affords virtually complete protection against loss of activity while $NAD^+$, 1 mM, is only partially effective.

Under conditions where butanedione modifies the enzyme such that 22% of the native activity remains, NADH protects maximally, $NAD^+$, ADP-ribose, and 3-pyridinecarboxaldehyde-$NAD^+$ to a lesser extent, while adenosine affords virtually no protection at all (Table I).

The inactivation by butanedione is reversible. Incubation with 5 mM reagent in 50 mM borate (pH 8.5) for 50 min reduces activity to 18% of the control (Table II, line 2). Gel filtration through a Bio-Gel P-4 column equilibrated with

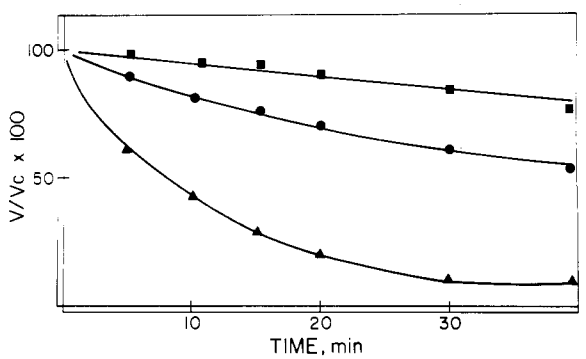


FIGURE 2: Changes in the activity of horse liver alcohol dehydrogenase, 1 mg/ml, *vs.* time on modification with 5 mM butanedione–50 mM borate at pH 6.5 (■), 7.5 (●), 8.5 (▲), and 9.5 (▲).

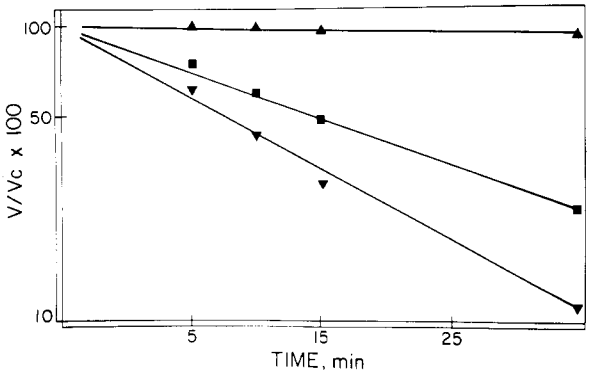


FIGURE 4: Semi-log plot of changes in the activity of horse liver alcohol dehydrogenase, 1 mg/ml, *vs.* time on modification with 5 mM butanedione–50 mM borate (pH 8.5) (▼) and in the presence of 1 mM $NAD^+$ (■) or 1 mM NADH (▲). The presence of 20 mM ethanol (▼) or 20 mM acetaldehyde (▼) affords no protection.

TABLE I: Protection of Horse Liver Alcohol Dehydrogenase Against 2,3-Butanedione Inactivation by Coenzyme; Its Analogs and Constituents.[a]

| Protective Agent | $V/V_c \times 100$[b] |
|---|---|
| NADH | 78 |
| $NAD^+$ | 45 |
| ADP-ribose | 40 |
| 3-Pyridinecarboxaldehyde-$NAD^+$ | 33 |
| Adenosine | 25 |
| None | 22 |

[a] Horse liver alcohol dehydrogenase, 1 mg/ml, was incubated in 5 mM butanedione–50 mM borate (pH 8.5), 25°, in the presence of 1 mM protective agent for 25 min. [b] $V_c$ = activity of native enzyme.

20 mM Veronal buffer (pH 8.5) separates the enzyme both from unreacted reagent and borate; activity then returns to 90% of that of the native enzyme over a period of about 3 hr (Table II, line 3). The degree of inactivation, however, depends upon the time of exposure to butanedione. The amount of activity that the modified enzyme recovers after gel filtration is inversely proportional to the length of time that it remains in contact with excess butanedione beyond about 30 min. Thus, only about 10% activity returns after 3 hr of reaction.

Phenylglyoxal also inactivates horse liver alcohol dehydrogenase. Reagent (5 mM) in 125 mM bicarbonate (pH 7.9) decreases enzyme activity to 20% of that of the native enzyme in 30 min. Again, the reduced form of the coenzyme, 1 mM, protects fully and the oxidized form, 1 mM, to a lesser extent.

*Amino Acid Analyses.* Amino acid analyses of butanedione and phenylglyoxal-modified horse liver alcohol dehydrogenase demonstrate loss of arginyl residues (Table III). Modification by 5 mM butanedione in 50 mM borate (pH 8.5) or 5 mM phenylglyoxal in 125 mM bicarbonate (pH 7.9) results in the loss of 5.4 and 4.2 of the 22 arginyl residues per molecule, *i.e.*, 2.7 and 2.1 per subunit, respectively (Table III, column 2, lines 1 and 2). The presence of NADH, 1 mM, reduces the loss to only 0.5 and 0.3 arginyl residue per subunit (Table III, column 3, lines 1 and 2). Thus, NADH protects 2.2 and 1.8 residues of arginine per subunit from modification (column 4). The lysine and histidine contents of the modified enzymes are the same as those of native liver alcohol dehydrogenase.

*Phenylglyoxal-$^{14}C$ Incorporation.* Phenylglyoxal-$^{14}C$ is incorporated rapidly into horse liver alcohol dehydrogenase. Loss of activity is progressive and relates linearly to the incorporation of radioactivity over the entire course of the reaction (Figure 5). Extrapolation to zero activity again indicates that two arginyl residues per subunit are modified (Table III, column 5).

*Exclusion of Cysteine Modification as a Basis for Inactivation.* Since modification of cysteinyl residues is known to abolish the activity of horse liver alcohol dehydrogenase (Li and Vallee, 1964), it was important to rule out sulfhydryl modification as a possible basis for the inactivation by butanedione. The reversibilities of both the butanedione and the *p*-HgBzO inactivation of horse liver alcohol dehydrogenase were used to advantage to investigate this possibility.

Butanedione-inactivated horse liver alcohol dehydrogenase can be restored to virtually full activity by the removal of borate using gel filtration (Table II lines 2 and 3). Similarly, the virtually instantaneous inactivation of horse liver alcohol dehydrogenase with *p*-HgBzO in borate buffer at pH 8.5 (Table II, line 4) is also reversible. The degree of inactivation is proportional to the concentration of *p*-HgBzO employed and, if the *p*-HgBzO modification is not allowed to proceed beyond 70% inactivation, full native activity can be restored by addition of dithiothreitol, 2 mM (Table II, line 5). This time-dependent process generally requires 1–2 hr for maximal reactivation. When such a modified enzyme has less than 30% activity only partial reactivation can be

TABLE II: Reversible Modifications of Horse Liver Alcohol Dehydrogenase with Butanedione and *p*-HgBzO.[a]

| Enzyme | $V/V_c \times 100$ |
|---|---|
| 1. Native | 100 |
| 2. + butanedione | 18 |
| 3. + butanedione, + P-4 | 90 |
| 4. + *p*-HgBzO | 34 |
| 5. + *p*-HgBzO, + dithiothreitol | 100 |
| 6. + butanedione, + *p*-HgBzO | 5 |
| 7. + butanedione, + *p*-HgBzO, + P-4 | 15 |
| 8. + *p*-HgBzO, + butanedione | 9 |
| 9. + *p*-HgBzO, + butanedione, + dithiothreitol | 14 |
| 10. + *p*-HgBzO, + butanedione, + dithiothreitol, + P-4 | 48 |

[a] Horse liver alcohol dehydrogenase, $3.74 \times 10^{-5}$ M, was treated with butanedione, 5 mM, in 50 mM borate (pH 8.5) for 50 min. One aliquot was assayed directly (line 2) and a second was gel filtered through Bio-Gel P-4 equilibrated with 20 mM Veronal (pH 8.5) before assay, line 3. Native enzyme was also treated with 0.35 mM *p*-HgBzO, dialyzed *vs.* a 1000-fold volume of 50 mM borate (pH 8.5, 4°), and assayed before (line 4) and after addition of 2 mM dithiothreitol (line 5). The butanedione-modified liver alcohol dehydrogenase was also treated with *p*-HgBzO and then assayed before (line 6) and after (line 7) gel filtration. The activities reported after gel filtration are the final values attained after 3 hr at 20°. The *p*-HgBzO liver alcohol dehydrogenase sample was further modified with butanedione and again assayed before (line 8) and after addition of dithiothreitol (line 9). Dithiothreitol does not affect the activity of native horse liver alcohol dehydrogenase. Finally, the dithiothreitol-treated sample was gel filtered and then assayed (line 10).

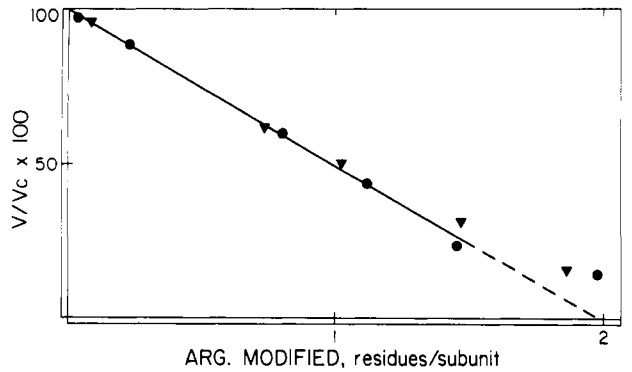


FIGURE 5: Correlation of inactivation of horse liver alcohol dehydrogenase (●) and yeast alcohol dehydrogenase (▼) with arginine modification on incubation with 5 mM phenylglyoxal-$^{14}C$ in 125 mM bicarbonate (pH 7.9).

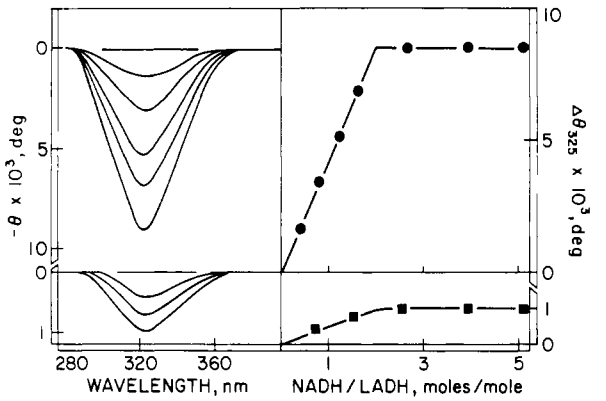


FIGURE 6: (A) Left panel (upper): Effect of NADH on the CD spectrum of native horse liver alcohol dehydrogenase, $1 \times 10^{-5}$ M, in 0.1 M phosphate (pH 7.5), 21°. The magnitude of the negative CD trough at 325 nm increases as the NADH concentration is varied stepwise from 0 to $2 \times 10^{-5}$ M. There is no further change with higher concentrations of NADH. (Lower): The corresponding CD spectra for horse liver alcohol dehydrogenase modified with phenylglyoxal to 8% activity. (B) Right panel: CD spectral titrations of native (upper) and modified (lower) horse liver alcohol dehydrogenase.

TABLE III: Stoichiometry of Arginine Modifications in Horse Liver and Yeast Alcohol Dehydrogenase.[a]

| Modified Alcohol Dehydrogenase | Arginine Residues Modified per Subunit | | | |
|---|---|---|---|---|
| | −NADH | +NADH | Δ | $^{14}C$ Incorp. |
| Butanedione horse liver | 2.7 | 0.5 | 2.2 | |
| Phenylglyoxal horse liver | 2.1 | 0.3 | 1.8 | 2.0 |
| Butanedione yeast | 2.1 | | | |
| Phenylglyoxal yeast | 1.8 | | | 2.3 |

[a] Data in columns 2, 3, and 4 derived from amino acid analyses of horse liver alcohol dehydrogenase allowed to react for 30 min and of yeast alcohol dehydrogenase allowed to react for 60 min with butanedione or phenylglyoxal under conditions described under Materials and Methods. Data in column 5 are extrapolated values for complete inactivation. Amino acid analyses of butanedione- and phenylglyoxal-modified horse liver alcohol dehydrogenase demonstrate loss of arginyl residues. Modification by 5 mM butanedione in 50 mM borate (pH 8.5) or 5 mM phenylglyoxal in 125 mM bicarbonate (pH 7.9) results in the loss of 5.4 and 4.2 of the 22 arginyl residues per molecule, *i.e.*, 2.7 and 2.1 per subunit, respectively (column 2, lines 1 and 2). The presence of NADH, 1 mM, reduces the loss to only 0.5 and 0.3 arginyl residue per subunit (column 3, lines 1 and 2). Thus, NADH protects 2.2 and 1.8 residues of arginine per subunit from modification (column 4). The lysine and histidine contents of the modified enzymes are the same as those of native liver alcohol dehydrogenase.

accomplished. Importantly, treatment of horse liver alcohol dehydrogenase with butanedione does not prevent subsequent reaction of the enzyme with *p*-HgBzO. If butanedione-modified liver alcohol dehydrogenase exhibiting 18% of the native activity (Table II, line 2) is allowed to react with *p*-HgBzO, its activity is further reduced instantaneously to 5% (Table II, line 6). Removal of borate from this reaction mixture by gel filtration returns activity only to about 15% rather than 90% activity of the control (Table II, line 7). Similarly, modification with *p*-HgBzO does not prevent subsequent modification with butanedione. Addition of butanedione, 5 mM, in borate (pH 8.5) to the 34% active *p*-HgBzO liver alcohol dehydrogenase preparation further decreases activity to 9% (Table II, line 8). Dithiothreitol (2 mM) now restores activity to only 14% (Table II, line 9). The time required for dithiothreitol reactivation precludes restoration of full native activity to the sample treated successively with *p*-HgBzO, butanedione, and dithiothreitol after subsequent gel filtration. However, this procedure does allow recovery of almost half of the original activity (Table II, line 10).

*Carboxymethylation of Native and Butanedione-Liver Alcohol Dehydrogenase.* Incubation of horse liver alcohol dehydrogenase with iodoacetate-$^{14}C$ in phosphate buffer (0.1 M, pH 7.5) preferentially carboxymethylates the active-site cysteinyl residues and abolishes enzymatic activity. Inactivation ($t_{1/2}$ = 11 min) correlates with the incorporation of 2.0 carboxymethyl groups per molecule of enzyme (Li and Vallee, 1963, 1964). The results of modification in phosphate (0.1 M, pH 8.5) or borate (0.05 M, pH 8.5) were quite similar. Butanedione-modified horse liver alcohol dehydrogenase with 14% remaining activity also reacts with iodoacetate-$^{14}C$, but at a rate nine times slower ($t_{1/2}$ = 100 min) than native liver alcohol dehydrogenase.

*Coenzyme Binding to Modified Horse Liver Alcohol Dehydrogenase.* On formation of the active liver alcohol dehydrogenase-NADH complex, the absorption maximum of the free NADH shifts from 340 to 325 nm (Theorell and Bonnichsen, 1951). This shift fails to occur on addition of NADH to a solution containing phenylglyoxal-modified horse liver alcohol dehydrogenase. Spectral broadening at shorter wavelengths may arise from small amounts of unmodified, native liver alcohol dehydrogenase capable of forming an NADH complex. NADH alone does not exhibit significant circular dichroism in this spectral region but its complex with horse liver alcohol dehydrogenase generates a characteristic negative ellipticity band centered at 325 nm (Figure 6A). A CD titration, performed by addition of increasing concentrations of coenzyme to the liver alcohol dehydrogenase solution, Figure 6A, demonstrates that 2 mol of NADH binds to 1 mol of enzyme. This finding is entirely consistent with earlier rotatory dispersion titration data of native horse liver alcohol dehydrogenase with NADH (Li *et al.*, 1962): a plot of $\Delta\theta_{325}$ *vs.* NADH/liver alcohol dehydrogenase increases linearly and then breaks sharply at a

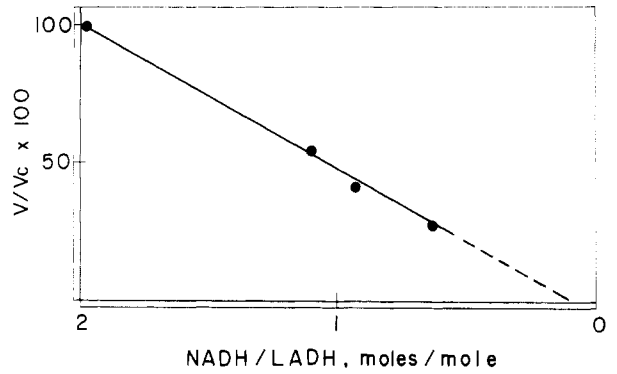


FIGURE 7: Correlation of activity loss with loss of NADH binding as determined by the method of Hummel and Dreyer (1962). A 30-$\mu$l aliquot of horse liver alcohol dehydrogenase (26.7 mg/ml) incubated with 5 mM phenylglyoxal for varying times was passed through a Bio-Gel P-4 column (1.7 × 21 cm) and coenzyme binding was assessed as described under Materials and Methods.

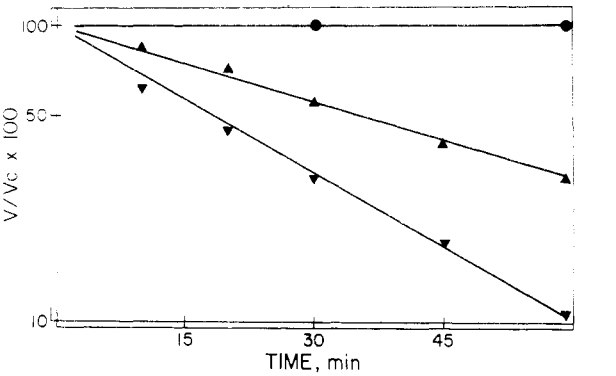


FIGURE 8: Semi-log plot of changes in activity of yeast alcohol dehydrogenase, 120 $\mu$g/ml, *vs.* time on modification with 25 mM butanedione–50 mM borate (pH 8.0) alone (▼), in the presence of 3 mM $NAD^+$ (▼) or 1 mM NADH (▲) or in the absence of butanedione (●) (control). The presence of 430 mM ethanol (▼) or 20 mM acetaldehyde (▼) affords no protecting.

molar ratio of about 2 with a maximum of $\Delta\theta_{325} = -8.6 \times 10^{-3}$ deg. Importantly, on addition of NADH to phenylglyoxal liver alcohol dehydrogenase exhibiting only 8% activity of the native enzyme, the maximum $\Delta\theta_{325}$ is $-9.8 \times 10^{-4}$ deg, *i.e.*, only 11% of that of the native enzyme (Figure 6B). Moreover, the intensities of the trough generated by titration with NADH of phenylglyoxal–horse liver alcohol dehydrogenase inactivated to varying degrees are directly proportional to the remaining enzymatic activities.

Thus, the capacity of phenylglyoxal-modified horse liver alcohol dehydrogenase to bind the coenzyme is greatly decreased, as is also apparent from the results of gel filtration experiments (Figure 7) which demonstrate independently that 2 mol of NADH binds to 1 mol of native enzyme. This is apparent both from the amount of NADH present in the protein fraction and from that removed from the included fraction. Clearly, the loss of activity and loss of coenzyme binding capacity of the enzyme modified with phenylglyoxal to different extents correlate linearly.

Both $Au(CN)_2^-$ and $Pt(CN)_4^{2-}$ exert protective effects against modification of horse liver alcohol dehydrogenase by 5 mM butanedione in 50 mM borate (pH 8.5). After 30 min, 100 and 90%, respectively, of native activity remains in the presence of 20 mM protective agent. Amino acid analysis of the gel-filtered product indicates modification of only 0.3 and 0.2 arginyl residues per subunit for $Au(CN)_2^-$ and $Pt(CN)_4^{2-}$, respectively.

*Yeast Alcohol Dehydrogenase.* Modification of the alcohol dehydrogenase from Baker's yeast with butanedione (25 mM) in 50 mM borate (pH 8.0) reduces enzymatic activity to 10% of the control within 60 min (Figure 8). Under these conditions, the inactivation reaction is kinetically first order with a half-life of 17 min.

Changes in pH and reagent concentration alter the rate, as shown for horse liver alcohol dehydrogenase (*vide supra*). The presence of either ethanol, 430 mM, acetaldehyde, 20 mM, or $NAD^+$, 3 mM, is without effect, but NADH, 1 mM, affords moderate protection against inactivation, the half-life now being 35 min. Phenylglyoxal, 5 mM, in bicarbonate (pH 7.9) also inactivates yeast alcohol dehydrogenase, and the patterns of protection by substrates and coenzymes are similar to those seen for modification with butanedione.

Amino acid analyses of butanedione- or phenylglyoxal-modified yeast alcohol dehydrogenase show the loss of 2.1 and 1.8 of the 8 arginyl residues per subunit, respectively (Table III, column 2, lines 3 and 4). Since NADH provides only partial protection against modification of yeast alcohol dehydrogenase, experiments carried out in its presence would not yield more definitive information regarding the correlation of arginyl residues modified with loss of activity.

Up to a loss of 82% of the native activity a plot of residual activity *vs.* the number of arginyl residues modified on exposure of the enzyme to phenylglyoxal-$^{14}C$, 5 mM, is linear (Figure 5). Extrapolation to zero activity indicates modification of 2.3 arginyl residues per subunit (Table III, column 5).

## Discussion

Despite persistent search for many years, the identity of NADH binding sites to enzymes such as the alcohol dehydrogenases has essentially remained unknown. The present studies are part of a general investigation of the hypothesis that arginyl residues of all three of the alcohol dehydrogenases examined indeed serve as NADH binding sites, as evidenced by their inactivation by $\alpha$-dicarbonyl reagents. The known specificity of these reagents for arginine (Riordan, 1973), confirmed in the present instance by amino acid analysis, radioactivity incorporation, and differential labeling experiments using NADH, demonstrates that arginyl residues are essential to this enzyme–coenzyme interaction.

Horse liver alcohol dehydrogenase, the best characterized of the alcohol dehydrogenases generally available, was used to establish the procedures for modification and analysis. Amino acid analyses of horse liver alcohol dehydrogenase modified with either butanedione or phenylglyoxal indicate that two arginyl residues per subunit are responsible for loss of NADH binding and, hence, activity (Table III). Furthermore, NADH prevents both loss of these two arginyl residues and the concomitant loss of activity. Importantly, butanedione modifies neither lysyl nor histidyl residues.

Incorporation of phenylglyoxal-$^{14}C$ provides further quantitative evidence for the number of essential arginyl residues (Table III). In the completely inactive horse liver alcohol dehydrogenase, 2.0 arginyl residues per subunit are modified. Moreover, there is a linear correlation between loss of activity and modification of these arginyl residues. In spite of the correlation between loss of activity and modification of two arginyl residues both of them need not participate in coenzyme binding. Thus, *e.g.*, one of them could be involved in coenzyme binding, and the other could be protected adventitiously. NADH binding is known to induce

substantial conformational changes in horse liver alcohol dehydrogenase (Zeppezauer *et al.*, 1967) and, hence, even a residue remote from the active-site region could thereby become inaccessible to reagent.

Other amino acid residues have been variously proposed, considered, and rejected as NADH binding sites previously. Both zinc and sulfhydryl groups, known to be critical to catalysis, were suggested as likely coenzyme recognition sites. However, removal of zinc to form the apoenzyme has little effect on NADH binding (Iweibo and Weiner, 1972). Further, carboxymethylation of the active center sulfhydryl groups significantly weakens but does not abolish NADH binding (Li and Vallee, 1965). Consistent with this finding, X-ray crystallographic studies of horse liver alcohol dehydrogenase reveal that this thiol group does not interact directly with ADP-ribose (Brändén *et al.*, 1973). Thus, it would appear that neither of these functional components of alcohol dehydrogenase is critical for coenzyme binding.

The idea has also been advanced that some positively charged residue attracts the phosphate group of AMP- or ADP-ribose to the coenzyme binding site (Fisher *et al.*, 1967) and accounts for the spectral changes occurring on binding NADH to liver alcohol dehydrogenase (Kosower, 1962). Although recent studies have pointed to a functional role for lysyl residues in liver alcohol dehydrogenase (Plapp, 1970), there is no evidence that lysines are essential for NADH binding. Increases in activity have been observed on methylation of up to 12 lysyl residues per subunit of liver alcohol dehydrogenase by means of formaldehyde and borohydride reduction (Jörnvall, 1973a). Modification in the presence of coenzymes and inhibitors prevents both the labeling and the increase in activity. On the other hand, similar treatment with pyridoxal phosphate inactivates the enzyme completely, due to modification of a single lysyl residue per active center with consequent loss of coenzyme binding (McKinley-McKee and Morris, 1972). It has not been established as yet in this instance if loss of activity is a result of modification of a lysyl residue not affected by the other reagents or whether the characteristics of phosphopyridoxyllysine are the determining factor. Thus, while such studies indicate that lysyl residues appear to be of importance for the activity of liver alcohol dehydrogenase the mode of their participation has not been delineated. It should be noted that X-ray structure analyses of D-glyceraldehyde-3-phosphate dehydrogenase have been interpreted to show that in this enzyme lysyl residue 183 assists in binding coenzyme (Buehner *et al.*, 1973).

Since X-ray analyses (Brändén *et al.*, 1973) demonstrate close physical proximity of the "functional" cysteinyl residue (Li and Vallee, 1964) to other, possible recognition sites such as arginine (*vide infra*), it seemed imperative to rule out that butanedione or phenylglyoxal might actually bring about their effects by modification of Cys-46 rather than of arginine. The modification of sulfhydryl groups of horse liver alcohol dehydrogenase with *p*-HgBzO, reversible by addition of dithiothreitol (Table II), provided a means to eliminate this possibility. Rendering the -SH group inaccessible by interaction with *p*-HgBzO does not prevent further inactivation of the enzyme by butanedione, and subsequent addition of dithiothreitol to butanedione-treated *p*-HgBzO liver alcohol dehydrogenase fails to regenerate activity (Table II). Hence, butanedione must inactivate the enzyme by modifying active-site residues other than cysteine. The possibility that butanedione modifies *both* cysteinyl and arginyl residues has also been excluded. Removal of borate from horse liver alcohol dehydrogenase, which had been modified with butanedione and then treated with *p*-HgBzO, regenerates activity to that characteristic of *p*-HgBzO liver alcohol dehydrogenase. The sulfhydryl groups must remain accessible to *p*-HgBzO even after butanedione modification. Therefore, butanedione inactivates horse liver alcohol dehydrogenase only by modifying its arginyl, not its cysteinyl residues. It should be pointed out, however, that arginyl modification does affect subsequent carboxymethylation of the sulfhydryl groups, a finding which is likely related to the proximity of these two functional residues (*vide infra*).

Until recently, reagents for the modification of arginyl residues in proteins under mild conditions have not been available and, hence, their role in the mechanism of alcohol dehydrogenase could not be examined by this means. In the present study, both butanedione and phenylglyoxal were employed, much as they have served previously to study alkaline phosphatase (Daemen and Riordan, 1974). Butanedione has the advantage of reacting with protein arginyl residues *via* a process which is often freely reversible on removal of borate and thus allowing the facile performance of protection studies.

Phenylglyoxal, on the other hand, forms a much more stable adduct with arginine; the $^{14}C$-labeled form is readily available and may be better suited for quantitation and, possibly, sequence work, though the latter requires further study. Thus, each reagent has its own merits (Riordan, 1973).

Several factors may contribute to the effect of pH on the rate of inactivation of horse liver alcohol dehydrogenase by butanedione (Figure 2). It is unlikely that the reduced effectiveness of modification at lower pH values merely reflects the ionization of arginyl residues, since free arginine ($pK > 12$) is modified readily even at pH 7.5. The $pK$ of boric acid is 9.0 and since $B(OH)_4^-$ is more effective than $B(OH)_3$ in stabilizing the *cis*-diol adduct (Weser, 1967) postulated as an intermediate in the arginine–butanedione reaction, higher pH values would favor inactivation. Alternatively, or in addition, the interaction between butanedione and borate could be pH dependent and lowering the pH could lead to the formation of butanedione monohydrate. The effect of pH on the absorption spectrum points to the presence of several distinct components of butanedione in aqueous solutions, and, if borate is added, yet other products could be generated (L. G. Lange, J. F. Riordan, and B. L. Vallee, unpublished observations). The actual species that reacts with arginyl residues is not as yet identified. Ionizations of other groups on the enzyme which could regulate the reactivity or accessibility of the active center arginyl residues toward butanedione cannot be ruled out, of course. Over this pH range there are changes in the catalytic activity both of the forward and reverse reactions of horse liver alcohol dehydrogenase which share features in common with those of the inactivation reaction.

The dependence of the rate of inactivation of horse liver alcohol dehydrogenase on butanedione concentration suggests that the reagent binds reversibly to the enzyme to form a Michaelis complex, with an apparent binding constant of ~14 mM, accounting perhaps, in part, for the high degree of selectivity of arginine modification. Since there is no increase in the inactivation rate on doubling the borate concentration, the zero-order dependence of inactivation on butanedione concentration above 50 mM does not reflect a decrease in the effective concentration of borate caused by

complexation with the diketone.

The reduced coenzyme binds more firmly to all three enzymes than does the oxidized species (Sund and Theorell, 1963; L. G. Lange, J. F. Riordan, and B. L. Vallee, unpublished observations). Hence, at equimolar concentrations, NADH would be expected to protect more fully than $NAD^+$. The decreased effectiveness of ADP-ribose and 3-pyridinecarboxaldehyde-$NAD^+$ in preventing loss of activity (Table I) is also consistent with their much lower affinities for the enzyme. Moreover, the almost complete lack of protection by adenosine suggests that the functional arginyl residue serves to bind the pyrophosphate bridge of the coenzyme.

Other factors may be relevant to the interpretation of these experiments, however, and their further delineation is important. Thus, borate is known to react with simple *cis*-diols of hexoses and pentoses as well as with the ribose moieties of pyridine nucleotides (Ulmer and Vallee, 1965). Addition of high concentrations of coenzyme to the modification mixture therefore disturbs the borate–butanedione–coenzyme equilibrium and alters the concentration of the reactive butanedione species to unknown levels. For yeast alcohol dehydrogenase particularly ($K_d^{NAD^+}$ = 0.3 mM), the results of experiments with saturating concentrations of $NAD^+$ would not be comparable with those performed at lower coenzyme concentrations. Therefore, protection experiments performed at 1 mM coenzyme concentration show that protection for horse and human liver alcohol dehydrogenase is complete but not for yeast alcohol dehydrogenase.

The function of arginyl residues as NADH recognition sites is substantiated by examining the binding of NADH to the modified horse enzyme. The absorption spectrum of phenylglyoxal-modified horse liver alcohol dehydrogenase mixed with NADH is characteristic of the *free* coenzyme. Moreover, a CD titration of modified liver alcohol dehydrogenase having 8% of the original activity demonstrates that the $\Delta\theta_{325}$ maximum is only about 11% that of the native enzyme, indicating a loss of about 90% of the NADH binding capacity (Figures 6A and 6B). Indeed, a plot of the activity of liver alcohol dehydrogenase preparations modified to different degrees *vs.* moles of NADH bound per mole of enzyme is linear and suggests that the binding of coenzyme to the modified liver alcohol dehydrogenase is so weak that a complex cannot be detected under the conditions employed. Based on identical molar absorptivities for the modified and native enzyme–NADH complexes dissociation constants of at least $8 \times 10^{-4}$ and $2\text{–}4 \times 10^{-7}$ M, respectively, can be calculated. Hence, NADH binding to the modified enzyme is at least 2000 times weaker than to native liver alcohol dehydrogenase. The results of gel filtration experiments substantiate the fact that the modified enzyme has effectively lost the capacity to bind coenzyme (Figure 7). The technique of Hummel and Dreyer (1962) depends neither on a spectral shift nor binding anisotropy to assess formation of the complex. Instead, the amount of coenzyme bound to protein is estimated from the absorption profile of the chromatography eluent. The linear correlation between loss of binding and activity (Figure 7) corroborates the spectral findings and provides conclusive evidence that the modification of arginine residues abolishes coenzyme binding.

In the case of yeast alcohol dehydrogenase, loss of activity correlates with modification of eight essential arginyl residues per molecule, as determined by amino acid analysis and incorporation of phenylglyoxal-$^{14}C$. Yeast alcohol dehydrogenase is composed of four similar or identical subunits (Harris, 1964) and binds four coenzyme molecules per tetramer (Hayes and Velick, 1954; Sund and Theorell, 1963; Temler and Kägi, 1973) implying two functional arginyl residues per coenzyme binding site, in agreement with the stoichiometry of horse liver alcohol dehydrogenase. It has been suggested recently that only two yeast alcohol dehydrogenase coenzyme binding sites exist (Yamada and Yamato, 1973; Dickinson, 1974), based, in part on the fact that carboxymethylation of only two cysteinyl residues completely inactivates the yeast enzyme. It is difficult to reconcile these results without assuming alternate mechanisms, *e.g.*, such as a half-site reactivity model similar to that suggested for yeast glyceraldehyde-3-phosphate dehydrogenase (Stallcup and Koshland, 1973). Clearly, further details on the numbers of coenzyme binding sites of yeast alcohol dehydrogenase are required. Due to the incomplete protection of yeast alcohol dehydrogenase afforded by NADH under the conditions employed, it did not prove possible to obtain an accurate value for the number of essential arginyl residues since some of those of yeast alcohol dehydrogenase modified under the present conditions might prove not to be involved in NADH binding.

Comparison of the results of X-ray diffraction studies on horse liver alcohol dehydrogenase (Brändén *et al.*, 1973) as well as on lactate (Rossman *et al.*, 1971) and malate dehydrogenases (Hill *et al.*, 1972) have been interpreted to denote that the main features of the structures of the NADH binding sites may be common to many or all dehydrogenases although no really significant sequence similarities exist between them. The primary sequences of alcohol dehydrogenases from different species, on the other hand, exhibit marked homology (Jörnvall and Markovic, 1972; Jörnvall, 1973b). Since such homologies are particularly striking in those regions which participate in substrate, coenzyme binding, or catalysis, it has been suggested that the same overall mechanistic features, including the amino acid residues essential to catalysis, have been maintained during evolution. On such grounds then, human liver alcohol dehydrogenase would also be expected to employ arginyl residues to bind NADH. The present data reinforce this expectation since it, too, is inactivated by butanedione while being protected from inactivation by NADH.

While butanedione reduces the activity of human liver alcohol dehydrogenase to only about 30%, phenylglyoxal lowers it to 8% of the native value. Though noted earlier (Riordan, 1973; Daemen and Riordan, 1974), the variation in effectiveness of these two reagents is as yet unexplained, as is the lack of complete inactivation of human liver alcohol dehydrogenase by butanedione. The former may be intrinsic to features of the two reagents but the possibility that phenylglyoxal may modify residues in addition to arginine has not been ruled out. Further, the preparation of human liver alcohol dehydrogenase employed has not been purified to homogeneity (see Materials and Methods) and definitive conclusions regarding the basis of residual activity cannot be drawn. Since its isolation in this laboratory 10 years ago, the existence of multiple isoenzymes has become apparent, but neither their functional nor structural interrelationships have been defined adequately. More detailed studies of the modified human enzyme have been deferred pending complete purification and characterization, now in progress. Instead, such studies were carried out on the far better characterized horse liver and yeast enzymes.

The present data, suggesting that arginyl residues of al-

cohol dehydrogenase bind NADH, perhaps through an interaction with the coenzyme's pyrophosphate bridge, are entirely consistent with sequence determinations (Jörnvall, 1973b) and X-ray analyses at 2.9-Å resolution (Brändén *et al.*, 1973) demonstrating that ADP-ribose—and by implication the coenzyme—binds in a characteristic pocket on the surface of the protein. Neither zinc nor Cys-46 appear to participate directly in coenzyme binding. It had been shown previously (Li and Vallee, 1964) that the cysteinyl residue, now known to be residue 46 (Jörnvall, 1970), is adjacent to an arginine in the octapeptide isolated from the active center of *S*-carboxymethyl horse liver alcohol dehydrogenase. It now appears that this arginyl residue 47 is suitably located and, indeed, interacts with the pyrophosphate bridge of ADP-ribose (C. I. Brändén, personal communication).

The speed and selectivity of carboxymethylation using iodoacetate-$^{14}C$ compared to the much slower reaction with iodoacetamide (Li and Vallee, 1965) have led to the suggestion that the free carboxyl group of iodoacetate aids in the rate acceleration and preferential labeling of Cys-46. Moreover, instead of second-order kinetics the inactivation by iodoacetate follows a Michaelis–Menten scheme with binding apparently preceding covalent modification (Reynolds and McKinley-McKee, 1969). We find that arginine modification markedly alters the rate of incorporation of iodoacetate into alcohol dehydrogenase suggesting that the free carboxyl group of iodoacetate could bind to the guanidinium side chain of an arginine in the vicinity of Cys-46 in the manner of an affinity label. Butanedione modification of alcohol dehydrogenase would block this guanidinium side chain precluding this interaction thereby reducing the rate of iodoacetate inactivation. Such considerations and the present experiments would tend to place an arginine at the active site near Cys-46 in the tertiary structure of the protein.

Further evidence that arginine is the NADH binding site is provided by the protection experiments with $Pt(CN)_4^{2-}$ and $Au(CN)_2^-$. These heavy metal derivatives, employed in the X-ray diffraction studies of Brändén *et al.* (1973), have recently been shown to inhibit horse liver alcohol dehydrogenase by competing with coenzyme (Gunnarsson *et al.*, 1974). Since they also prevent butanedione inactivation and arginine modification, it would seem that the arginyl residues indeed participate in coenzyme binding. Moreover, X-ray crystallographic results have identified Arg-47 as the sole binding site for $Pt(CN)_4^{2-}$ (C. I. Brändén, personal communication). Thus, at least one of the arginyl residues modified would seem to be Arg-47 and hence close to Cys-46. Furthermore, this is entirely consistent with the results of sequence and X-ray analyses which identify Arg-47 as a component of the horse liver alcohol dehydrogenase binding site and, in addition, as a general anion binding site (C. I. Brändén, personal communication). The corresponding residue in the sequence of yeast alcohol dehydrogenase, on the other hand, is histidine (Jörnvall, 1973b), suggesting that an arginine from another region of the molecule makes contact with NADH.

Yet other X-ray crystallographic studies independently support the postulated interaction between arginine residues and the pyrophosphate bridge of NADH. Suitable models containing methylguanidinium cations can readily form a stable, cyclic, electrostatic 2:1 complex with a phosphate anion (Cotton *et al.*, 1973).

The location both of the arginine participating in NADH binding in the primary sequence and in the three-dimensional structure of the enzyme is of great interest. Other reagents more optimal for sequence studies than those currently employed are being evaluated to relate the functional observations to the sequence and crystal-structure analysis of horse liver alcohol dehydrogenase.

The present results bear on the mechanism of NADH binding to dehydrogenases in general. Despite intensive study of these enzymes, the identities of the protein residues responsible for NADH binding have remained largely unknown. Arginine seems to be one of the residues which contribute to NADH binding to the alcohol dehydrogenases, probably through interaction with the coenzyme's pyrophosphate bridge. The role of arginyl residues might even extend to the binding of other phosphate-containing coenzymes such as NADP, FAD, ATP, CoA, UDPG, etc. The details of their mechanism of binding are also largely unknown, but based on the results reported here, arginine side chains may be expected to play essential roles in the binding of some or all of these.

It has long been appreciated that the majority of coenzymes are nucleotides or their analogs, a systematic feature of their structures which has evoked considerable speculative comment but whose basis has remained unexplained. In the context of the present studies, it is, however, of some interest to recall Dixon and Webb's of necessity philosophical comment on this problem, considering knowledge available at the time it was made (1958). To quote: if enzymes form on nucleic acid templates (sic) "it may well be that the enzyme protein may retain some impress of the nucleic structure, so that certain parts of it are particularly adapted to combine with nucleotide-like molecules. Although no definite evidence can be adduced, it is possible that this may be the reason that so many coenzymes have this type of structure."

While it would be unwarranted to consider the present data a substantiation of such views, it is nevertheless desirable to point to the abundance of arginine in histones (DeLange and Smith, 1971) where they are postulated to interact with phosphate groups of nucleic acids, while involved in regulating gene activity and protein synthesis. The frequency of occurrence of arginines in these proteins contrasts with their relative scarcity in most others, including enzymes, where they are found far less frequently than would be expected from the relative number of arginine codons in the genetic code (King and Jukes, 1969). This relative scarcity has been proposed to reflect on evolutionary pressure restricting arginine to certain important biological functions, *i.e.*, regulating translation and controlling protein structure and degradation (Wallis, 1974).

The extension of the demonstration that arginines participate in the recognition of anionic substrates as well as of coenzymes may denote a beginning of the appreciation of a pervading role of this amino acid which, judging by the rapidly emerging information, will prove involved both in catalytic and hormonal processes and their control at all levels.

Acknowledgment

We would like to thank Drs. H. Jörnvall and C. Brändén for making their data available to us prior to publication and for valuable discussions.

References

Blair, H. A., and Vallee, B. L. (1966), *Biochemistry 5*, 2026.

Brändén, C. I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I., and Åkeson, Å. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2439.

Buehner, M., Ford, G. C., Moras, D., Olsen, K. W., and Rossman, M. G. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3052.

Cotton, F. A., Day, V. W., Hazen, E. E., Jr., and Larsen, S. (1973), *J. Amer. Chem. Soc. 95,* 4834.

Daemen, F., and Riordan, J. F. (1974), *Biochemistry 13,* 2865.

DeLange, R. J., and Smith, E. L. (1971), *Annu. Rev. Biochem. 40,* 279.

Dickinson, M. (1974), *Eur. J. Biochem. 41,* 31.

Dixon, M., and Webb, E. C. (1958), *in* Enzymes, 1st ed, New York, N. Y., Academic Press, p 447.

Drum, D. E., Li, T.-K., and Vallee, B. L. (1969), *Biochemistry 8,* 3783.

Fisher, R. F., Haine, A. C., Mathias, A. P., and Rabin, B. R. (1967), *Biochim. Biophys. Acta 139,* 169.

Gunnarsson, P.-O., Pettersson, G., and Zeppezauer, M. (1974), *Eur. J. Biochem. 43,* 479.

Harris, I. (1964), *Nature (London) 203,* 30.

Hayes, J. E., and Velick, S. F. (1954), *J. Biol. Chem. 207,* 225.

Hill, E., Tsernoglou, D., Webb, L., and Banaszak, L. J. (1972), *J. Mol. Biol. 72,* 577.

Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.

Iweibo, I., and Weiner, H. (1972), *Biochemistry 11,* 1003.

Jörnvall, H. (1970), *Eur. J. Biochem. 14,* 521.

Jörnvall, H. (1973a), *Biochem. Biophys. Res. Commun. 51,* 1069.

Jörnvall, H. (1973b), *Proc. Nat. Acad. Sci. U. S. 70,* 2295.

Jörnvall, H., and Markovic, O. (1972), *Eur. J. Biochem. 29,* 167.

King, J. L., and Jukes, T. H. (1969), *Science 164,* 788.

Kosower, E. M. (1962), *Biochim. Biophys. Acta 56,* 474.

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

Li, T.-K., and Vallee, B. L. (1963), *Biochem. Biophys. Res. Commun. 12,* 44.

Li, T.-K., and Vallee, B. L. (1964), *Biochemistry 3,* 869.

Li, T.-K., and Vallee, B. L. (1965), *Biochemistry 4,* 1195.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

McKinley-McKee, J. S., and Morris, D. L. (1972), *Eur. J. Biochem. 28,* 1.

Plapp, B. V. (1970), *J. Biol. Chem. 245,* 1727.

Reynolds, C. H., and McKinley-McKee, J. S. (1969), *Eur. J. Biochem. 10,* 474.

Riley, H. A., and Gray, A. R. (1943), in Organic Syntheses, Collect. Vol. II, Blatt, A. H., Ed., New York, N. Y., Wiley, p 509.

Riordan, J. F. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29,* 462 Abstr.

Riordan, J. F. (1973), *Biochemistry 12,* 3915.

Rossman, M. G., Adams, M. J., Buehner, M., Ford, G. C., Hackert, M. L., Lentz, P. J., Jr., McPherson, A., Jr., Schevitz, R. W., and Smiley, I. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 179.

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

Stallcup, W. B., and Koshland, D. E., Jr. (1973), *J. Mol. Biol. 80,* 41.

Sund, H., and Theorell, H. (1963), *Enzymes, 2nd Ed., 7,* 25.

Takahashi, K. (1968), *J. Biol. Chem. 243,* 6171.

Temler, R. S., and Kägi, J. H. R. (1973), Abstracts, IXth International Congress on Biochemistry, Abstract 2r4, p 109.

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

Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. Relat. Areas Mol. Biol. 27,* 37.

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

Vallee, B. L., and Riordan, J. F. (1968), *Brookhaven Symp. Biol. 21,* 91.

Wallis, M. (1974), *Biochem. Biophys. Res. Commun. 56,* 711.

Weser, U. (1967), *Struct. Bonding (Berlin) 2,* 160.

Yamada, T., and Yamato, M. (1973), *J. Biochem. (Tokyo) 74,* 971.

Zeppezauer, E., Söderberg, B. O., Brändén, C. I., Åkeson, Å., and Theorell, H (1967), *Acta Chem. Scand. 21,* 1099.