Inactivation of Lactate Dehydrogenase by Butanedione†

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ABSTRACT: Arginine residues of the H_4 isozyme of bovine lactate dehydrogenase (EC 1.1.1.27) were modified by treatment with butanedione in borate buffers by the method of Riordan. Modification of residues other than arginine could not be detected and, following extensive derivatization with butanedione, no arginine-containing peptides were released by tryptic hydrolysis. The time course of inactivation of the enzyme by butanedione was complex and suggested that an initial reversible combination of the guanidinium group with butanedione is followed by an irreversible reaction and that arginyl residues of differing reactivities are essential for the action of the enzyme. Measurements of incorporation of [2,3-^{14}C]butanedione into the enzyme indicated that there are not more than three essential arginyl residues per subunit. Fluorescence titrations indicate that partially inactivated enzyme retains most of its capacity for binding NADH. However, the inactivated enzyme–NADH complex binds inhibitors and pyruvate less firmly than does the native enzyme–NADH complex. It is concluded from these results and from a previous study of the effects of guanidination on the enzyme that both an arginyl and a lysyl residue are involved in binding the carboxylate group of substrates to the enzyme–coenzyme complex.

In 1960 Karnes *et al.* concluded from inhibition measurements carried out with rat liver lactate dehydrogenase that the AMP moiety of NAD^+ provides the major binding force for the coenzyme molecule. Adams *et al.* (1970) extended this conclusion by showing that AMP is the smallest fragment of the coenzyme molecule which induces the change in conformation of dogfish lactate dehydrogenase which accompanies the binding of the coenzyme molecule. These workers reasoned that binding of the adenosine end of the coenzyme molecule allows the pyrophosphate group to interact with a charged group on the enzyme surface. This interaction was presumed to induce a conformation change in the enzyme molecule and to generate a binding site for the nicotinamide end of the molecule.

Kinetic measurements have indicated that coenzyme binding is relatively independent of pH between values of 5 and 10. This finding suggests that the pyrophosphate moiety of the coenzyme may interact with the guanidinium of arginine (Winer and Schwert, 1958; Schwert *et al.*, 1967). This paper reports the changes in the properties of beef heart lactate dehydrogenase which result from modification of guanidinium groups by reaction with 2,3-butanedione.

Yankeelov *et al.* (1966) first described the conditions under which butanedione could be converted into a form which would react with the guanidinium group of arginine under nondenaturing conditions. Grossberg and Pressman (1968) extended these studies and used the reagent to modify the arginine residues in antibody molecules. Subsequently, Yankeelov (1970) demonstrated that both the dimer and the trimer of butanedione react with arginine to form products which lack a guanidinium group. The oligomers are presumably the active forms in the reagent described in his earlier publications.

The use of monomeric butanedione as a reagent for the modification of arginine was first reported by Vallee and Riordan (1968). If the initial reaction between butanedione and a guanidinium group involved the addition of two N–H bonds across the carbonyls of butanedione, the adduct would have vicinal hydroxyl groups (Riordan, 1970). If these were in the cis configuration, the adduct should be stabilized by borate. This hypothesis was confirmed by the finding that butanedione inactivated the peptidase activity of carboxypeptidase more effectively in 50 mM borate than in 20 mM barbital at pH 7.5. Riordan found the peptidase activity of the enzyme to disappear when three arginine residues had reacted with butanedione and further observed that, when borate and butanedione were removed by gel filtration, one of the three adducts reverted to arginine with concomitant recovery of peptidase activity.

Because the reaction in borate does not require preparation of the dimer or trimer of butanedione, we have used Riordan's procedure for the study reported here.

Experimental Section

The preparation of the H_4 isozyme of bovine lactate dehydrogenase and of pyruvate, the methods for determining enzyme concentration and enzymatic activity and the procedure of fluorescence titration were those described by Yang and Schwert (1970). A Perkin-Elmer Model SPF-2A fluorescence spectrophotometer was employed for fluorescence titrations.

NADH was obtained from Sigma Chemical Co. and butanedione from the Aldrich Chemical Co. [2,3-^{14}C]Butanedione with a specific activity of 1.62 mCi/mmole was obtained from Mallinckrodt Nuclear.

Derivatization of the enzyme was carried out at 25° by adding lactate dehydrogenase to solutions of butanedione in sodium borate buffers. At intervals aliquots were removed and diluted 200-fold with 0.1 M sodium phosphate, pH 7.0. Over a period of a few hours no change could be detected in the enzymatic activity of the diluted reaction system. Routinely, however, 10 to 20 μl of the diluted enzyme was immediately mixed into 3 ml of 0.1 M sodium phosphate, pH

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7.0, containing 0.13 mM NADH and 0.267 mM pyruvate, and enzymatic activity was estimated from the initial rate of decrease at 27° of the 340-nm absorbance of NADH.

The derivatized lactate dehydrogenase used for fluorescence titrations was prepared by allowing the enzyme, at a concentration of 4 mg/ml, to react for 30 min at 25° with 0.1 M butanedione in 0.25 M sodium borate buffer, pH 7.7. The enzyme was then dialyzed against 3 changes of a large excess of 0.1 M sodium borate buffer, pH 7.7. A control, treated in the same way except that butanedione was omitted from the reaction system, showed unchanged specific activity.

Amino acid analysis was carried out on a 0.6 × 75 cm column of Technicon Type C Chromobeads with use of the elution gradient described in Technicon Technical Publication No. TA0-0155-00 for 5.5-hr analysis. Reaction mixtures of butanedione and free amino acids were loaded directly onto the column. Unreacted butanedione eluted with the solvent front as indicated by a large absorbance in the 450-nm tracing. Derivatized protein was freed of butanedione by dialysis or by precipitation with ammonium sulfate before it was hydrolyzed for amino acid analysis.

Assays of [^{14}C]butanedione incorporation into the enzyme were carried out by the procedure described by Bollum (1968). Aliquots of the reaction mixture (50 μl) were pipetted onto 2.3-cm disks of Whatman No. 3MM paper, dried, washed for 20 min with 500 ml of 10% trichloroacetic acid, rinsed with two 250-ml volumes of 5% trichloroacetic acid, and dried with 95% ethanol and ether. This washing procedure removed approximately 99.99% of free [^{14}C]butanedione from paper disks.

Counting was carried out in vials containing 4 ml of 0.4% 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene in toluene by use of a Packard Model 2002 liquid scintillation spectrometer. The mean counting efficiency of 10 aliquots containing 2–20 nmoles of butanedione delivered directly into counting vials containing a paper disk was 66.4 ± 0.5%. Comparison of identical samples of derivatized enzyme counted by the method of Bollum and counted after combustion by the procedure of Kalberer and Rutschmann (1961) indicated that no measurable quenching resulted from the presence of the paper disks in the counting vials.

Sedimentation analyses were carried out with a Spinco Model E analytical ultracentrifuge.

Results

Preliminary Experiments. The extent of reaction of 10 mM arginine with butanedione in 0.5 M sodium borate buffer, pH 7.5, was determined by measuring the disappearance of arginine with the amino acid analyzer. With 10 mM butanedione the concentration of arginine fell to 58% of the initial concentration in 2.5 hr and to 5% of the initial concentration after 5 days. With 100 mM butanedione only about 5% of the arginine initially present remained after 2.5 hr. As arginine and butanedione disappeared, a new peak, which was eluted behind ammonia and ahead of arginine, appeared in the chromatographic tracing. The sum of the areas under this peak and under the arginine peak remained essentially constant at all stages of derivatization. When 10 mM lysine or histidine, and 1 mM tyrosine or 2 mM tryptophan were treated separately with 10 mM butanedione for 48 hr, no adduct and no decrease in butanedione could be detected in any case.

The system in which 10 mM arginine and butanedione had been allowed to react for 10 days was subjected to hydrolysis for 20 hr in 6 N HCl at 110°. Amino acid analysis at the end of this period revealed that the new compound had disappeared, that approximately half the arginine had been regenerated, and that no other ninhydrin-reactive product could be detected.

As indicated below, there is reason to believe that prolonged exposure to an excess of butanedione derivatizes all the arginine residues in lactate dehydrogenase Acid hydrolysis of enzyme treated in this way is also accompanied by regeneration of approximately half the arginine of the protein. No anomalous peaks appeared in the chromatogram and there was no measurable alteration in the amount of any amino acid residue other than arginine.

Time Course of Inactivation. The rate of loss of enzymatic activity in 0.25 M borate, pH 7.9, in the presence of varying concentrations of butanedione is shown in Figure 1. If the weight of 1 mole of the active subunit is taken as 36,000 g and the arginine content as 9 residues per subunit (Millar, 1962; Pesce *et al.*, 1964), the butanedione concentrations are approximately 25-, 50-, 100-, and 200-fold excesses over total arginine.

It is evident that these curves are kinetically anomalous. The initial rates of inactivation are not proportional to the butanedione concentration and the inactivation rates decrease very markedly with time. The latter fact suggests that enzymatic activity depends upon groups which have at least two levels of reactivity with butanedione. The fact that the initial rate of inactivation is not proportional to butanedione concentration suggests that the initial reaction between guanidinium groups and butanedione is reversible and that the concentrations of butanedione used were sufficient to convert most of the reactive groups into the combined form. The curves shown in Figure 1 were calculated for the scheme[1]

$$E \underset{-1}{\overset{1I}{\rightleftharpoons}} EI \xrightarrow{2} E'I \underset{-3}{\overset{3I}{\rightleftharpoons}} (E'I)I \xrightarrow{4} E''I_2 \qquad (1)$$

in which E is enzyme and I is butanedione using the values for the various rate constants which are shown in the legend to the figure. No great weight can be attached to the fitting of the curves to the experimental points. It does seem reasonable to conclude, however, that the scheme shown in eq 1 is probably the simplest which will describe the results.

Effects of pH and of Ionic Strength upon Inactivation. Measurements of the rate of inactivation were made in 0.25 and 0.44 M borate at four pH values between 7.1 and 8.5. Within this range, pH has no measurable effect upon the rate of inactivation. However, at every pH value inactivation proceeded more slowly in 0.25 M than in 0.44 M buffers. If Riordan's formulation of the reaction is correct, it would be anticipated that an increase in borate concentration would be accompanied by an increase in the rate of inactivation. It was also found that the rate of inactivation was slowed in borate buffer containing 0.7 M KCl. Thus, it seems possible that the effect of the higher borate concentration on stabilization of the initial adduct was more than offset by an ionic strength effect.

Reversibility of Inactivation. The reversibility of inactivation was tested by allowing enzyme at a concentration of 4 mg/ml to react with 0.109 M butanedione in 0.1 M sodium borate, pH 8.0, until only 15% of the initial activity remained. The reaction system was then divided into two parts. One was

[1] Details of the derivation of this expression will be supplied upon request.

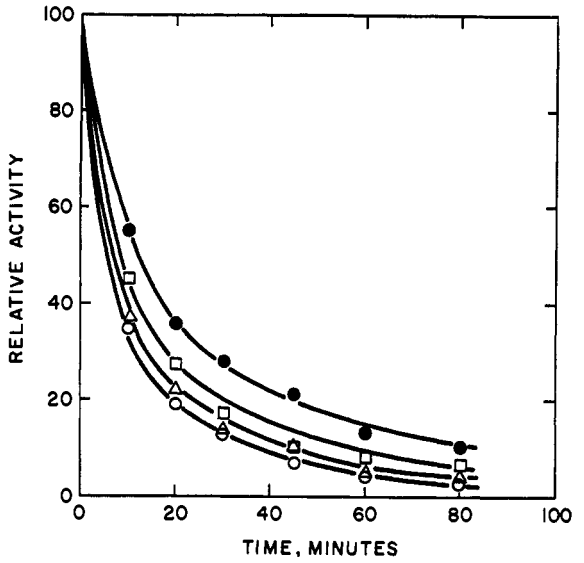

FIGURE 1: Plot of loss of enzymatic activity with time at varying concentrations of butanedione. The enzyme at a concentration of 2 mg/ml in 0.25 M borate, pH 7.9, was treated with: (●) 0.012 M, (□) 0.024 M, (△) 0.049 M, and (○) 0.098 M butanedione at 25°. The lines were calculated for the reaction shown in eq 1 using values of 1.67 sec^{-1} for k_1 and k_3, 3.3×10^{-2} sec^{-1} for k_{-1} and k_{-3}, 4.8×10^{-3} sec^{-1} for k_2, and 7.2×10^{-4} sec^{-1} for k_4. The rate constants are numbered to correspond to the reactions shown in eq 1. Because butanedione was present in large excess over total arginine, the reactions corresponding to k_1 and k_3 were treated as first-order reactions. It was assumed that E and EI, which would be expected to dissociate to E upon dilution for assay, have full activity, that E'I and (E'I)I retain one-third of the activity of the native enzyme and that $E''I_2$ is inactive.

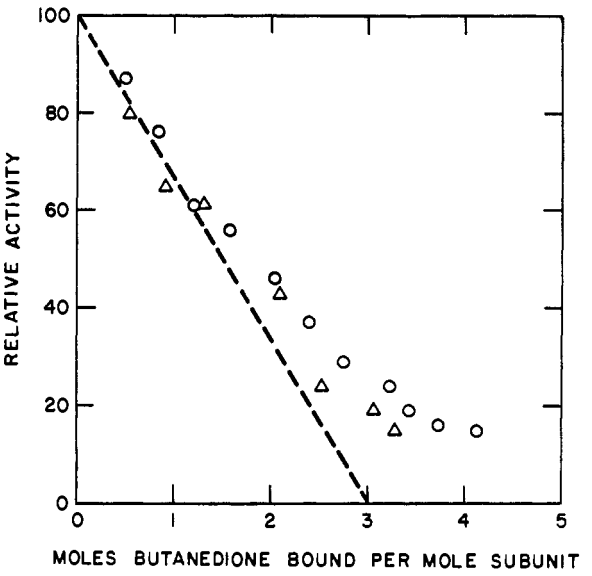

FIGURE 2: Plot of remaining enzymatic activity against moles of [^{14}C]butanedione bound per mole of enzyme subunit (36,000 g). The two types of symbol represent duplicate experiments. In each experiment lactate dehydrogenase at a concentration of 1 mg/ml was incubated with 27.3 mM [^{14}C]butanedione (1.62 mCi/mmole) in 0.47 M borate, pH 8.0. At intervals 50-μl samples were withdrawn for counting and 20-μl samples were diluted 100-fold with 0.1 M phosphate, pH 7.0, for activity determinations.

freed of reagent by passage through a 0.95 × 19.5 cm column of G-20 Sephadex equilibrated with 0.1 M borate, pH 8.0, and the other was freed of butanedione and borate by passage through a similar column which had been equilibrated with 0.1 M phosphate. Enzymatic activity was determined on each of these solutions immediately after they emerged from the column and at daily intervals for a 5-day period. During this period the enzyme in borate lost about half of its residual enzymatic activity while that in phosphate doubled in activity. Controls, treated in the same way, except that they were not allowed to react with butanedione, retained essentially constant activity during the 5-day period.

Properties of the Derivatized Enzyme. A sample of lactate dehydrogenase was allowed to react with butanedione until 96% of its activity had disappeared. The protein was dialyzed free of butanedione against 0.1 M sodium phosphate–0.1 M sodium borate, pH 8.1. Upon ultracentrifugation in the same buffer only a single boundary, which sedimented at 7.4 S (uncorrected for buffer viscosity), was observed. Native enzyme shows similar sedimentation behavior (Millar, 1962). Electrophoresis on Cellogel strips in Tris-borate buffer, pH 8.9, followed by staining for enzymatic activity, indicated that the derivatized protein migrated as a single band and that the anodic mobility of the derivatized enzyme is higher than that of the native enzyme.

When similarly derivatized protein was separated from butanedione by ammonium sulfate precipitation followed by dialysis against phosphate buffer, sedimentation analysis revealed additional boundaries with sedimentation velocities of approximately 4.3 and 11 S. Electrophoresis of samples prepared in this way revealed two enzymatically active bands both of which displayed a higher anodic mobility than that of the native enzyme.

Incorporation of [^{14}C]Butanedione. Because of the uncertainty attending the estimation of arginine in the derivatized enzyme by the usual methods of amino acid analysis, recourse was taken to measurements of the incorporation of [^{14}C]-butanedione into the enzyme. The results of these measurements are shown in Figure 2. From these results it is concluded that not more than three arginine residues are essential to enzymatic activity.

Following 6 days of reaction of enzyme with [^{14}C]butanedione present in approximately 100-fold excess over total arginine, 8.2–8.5 moles of butanedione were incorporated per mole of enzyme subunit. This numerical correspondence to the total arginine content is probably meaningless because incorporation continued until at 3 weeks of reaction 13–14 moles of butanedione had been incorporated.

Tryptic Hydrolysis of Derivatized Enzyme. If, in fact, all of the arginines of lactate dehydrogenase can be derivatized with butanedione, it would be anticipated that there would be large differences in the tryptic peptides derived from native and inactivated enzyme. To test this hypothesis, 8 mg of lactate dehydrogenase, which had been reacted with butanedione until less than 3% of the activity remained, was dialyzed against 0.1 M sodium borate–0.1 M sodium phosphate, pH 8.1, and was treated with 0.2 mg of trypsin in 0.3 M borate–0.3 M phosphate, pH 7.5, for 8 days. During proteolysis approximately 40% of the protein precipitated and, when the pH of the digest was adjusted to 5 for application to the peptide separation column, a further precipitate formed. This was removed by centrifugation. Native enzyme, subjected to the same treatment, exhibited similar behavior.

The peptides which were soluble at pH 5 were resolved on a 100 × 0.6 cm column of Technicon Chromobeads, Type P, according to the procedure of Catravas (1964). The effluent stream was split and one portion was hydrolyzed with 3 N

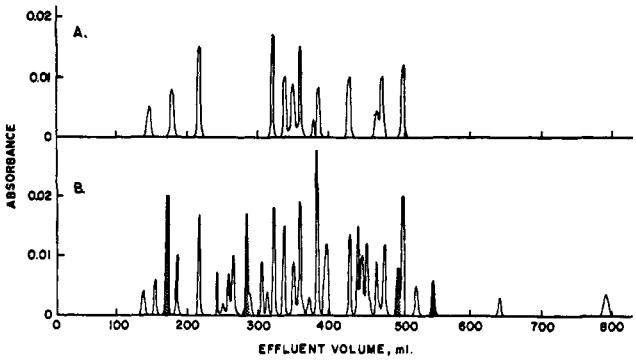

FIGURE 3: Elution diagram of tryptic peptides derived from: (A) lactate dehydrogenase reacted with butanedione until 97% of the initial activity had been lost and (B) native enzyme. The stippled areas indicate peptides which contain arginine.

NaOH for 1 hr at 105°. This stream was neutralized and the ninhydrin color developed.

The other stream was passed through delay coils to compensate for the time of hydrolysis and color development and was treated with the modification of the Sakaguchi reagent which has been developed for use with the AutoAnalyzer. The ninhydrin absorbancy was measured at 570 nm and the Sakaguchi absorbancy at 505 nm.

The recorder tracing obtained at 570 nm with tryptic peptides of derivatized enzyme is shown in the upper part of Figure 3 and that with native enzyme in the lower part of the figure. The peptides which contain arginine are stippled. It is evident that the tryptic hydrolysate of the derivatized enzyme contains fewer peptides and none in which arginine is free to react with the Sakaguchi reagent.

Protection against Inactivation by NADH and by Inhibitors. The effects of added oxamate, oxalate, NADH, NADH plus oxamate, and NADH plus oxalate on the rate of inactivation of lactate dehydrogenase by butanedione are shown in Figure 4. It is evident that oxamate and oxalate have no effect upon the rate of inactivation. This is consistent with the finding that these inhibitors are not bound by the enzyme but only by the enzyme–coenzyme complexes (Novoa *et al.*, 1959; Novoa and Schwert, 1961). Added NADH exerts a protective effect and this effect is augmented in the presence of oxamate or oxalate. Pyruvate at a concentration of 24 mM had no effect upon the rate of inactivation.

Fluorescence Titrations. The interactions of lactate dehydrogenase with NADH and of the enzyme–NADH complex with inhibitors can be studied independently of the enzymatic reaction by means of fluorescence titrations (Winer *et al.*, 1959; Winer and Schwert, 1959). The left-hand side of Figure 5 shows the augmentation of fluorescence which results when small volumes of NADH solution are added to solutions containing equal amounts of native enzyme and of enzyme which had lost 80% of its activity as a result of reaction with butanedione.

It has been established that oxamate is an inhibitor which acts primarily as a competitor against pyruvate (Novoa *et al.*, 1959) and that quenching of the fluorescence of the enzyme–NADH complex by oxamate results from the formation of the same enzyme–NADH–oxamate complex which is responsible for the action of oxamate as an inhibitor (Winer and Schwert, 1959). The right-hand side of Figure 5 shows the effects of added oxamate on the complexes formed between NADH and native or derivatized enzyme. The upper axis refers to additions made to the native enzyme–NADH system and the lower axis to additions made to the inactivated enzyme–NADH system.

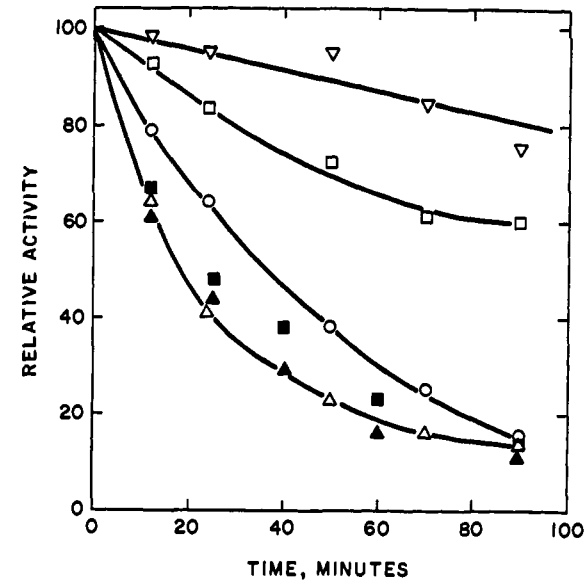

FIGURE 4: Rate of inactivation of lactate dehydrogenase (1 mg/ml) in 0.2 M borate, pH 7.5, in the presence of 23.6 mM butanedione and: (△) no other addition; (▲) 0.32 mM potassium oxamate; (■) 10.6 mM potassium oxalate; (○) 0.33 mM NADH; (□) 0.33 mM NADH and 10.6 mM oxalate; and (▽) 0.33 mM NADH and 0.32 mM oxamate.

Although oxalate acts principally as a competitive inhibitor with respect to lactate, it can also form an enzyme–NADH–oxalate complex. The formation of this complex is attended by an augmentation of the fluorescence of enzyme-bound

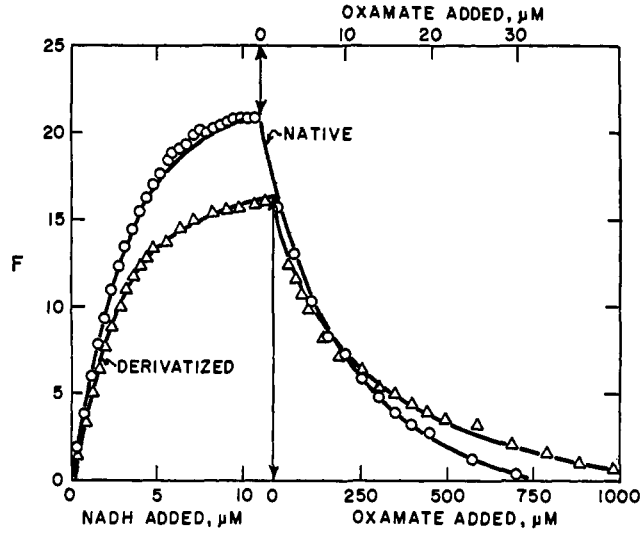

FIGURE 5: Plot of augmentation of fluorescence observed at 27° when 0.4-μl additions of 1.97 mM NADH are made to 2 ml of 0.1 M phosphate, pH 7.0, containing 0.115 mg/ml of native (○) or 80% inactivated (△) enzyme. The subsequent effect of making 0.4-μl additions of 10 mM oxamate to the native and of 100 mM oxamate to the inactivated enzyme–NADH system is shown in the right-hand side of the figure. The augmentation, *F*, is in arbitrary recorder scale units measured at sensitivity setting 1 on the instrument. The excitation wavelength was 346 nm with a 6-nm slit and the emission wavelength was 436 nm with a 20-nm slit. The curves were calculated from the constants given in Table I.

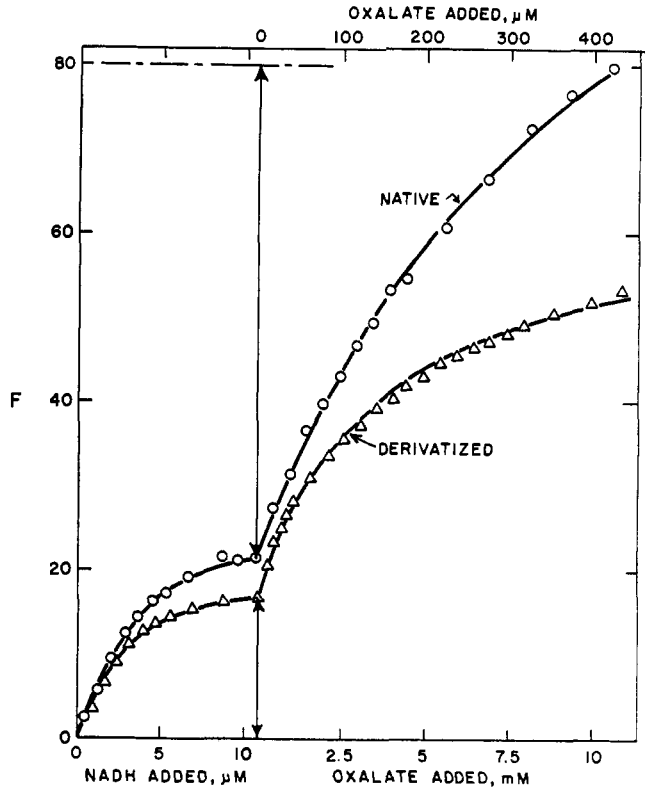

FIGURE 6: Effect of oxalate on the fluorescence of enzyme–NADH complexes. The conditions and symbols are the same as those used in Figure 5 with the exception that 0.1 M oxalate was added to the cuvet containing native enzyme and 1 M oxalate was added to the cuvet containing inactivated enzyme.

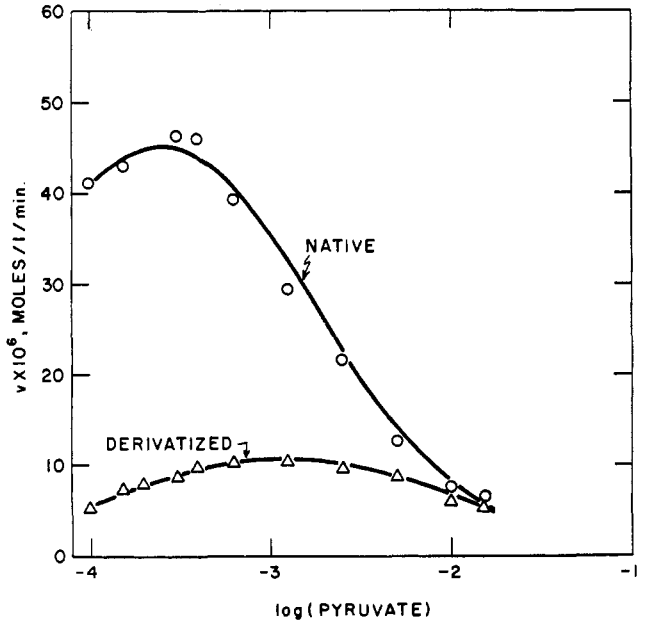

FIGURE 7: Plot of initial reaction velocity against logarithm of pyruvate concentration for native enzyme and enzyme which has been treated with butanedione until only 15% of its activity remains. Pyruvate inhibition was assumed to result from formation of an absorptive enzyme–NAD^+–pyruvate complex. The curve was fitted to the expression $v = V^{app}/[1 + (K_p/P) + (P/K_I^{app})]$ in which v is reaction velocity, V^{app} the maximum velocity (in the absence of inhibition by pyruvate) at infinite pyruvate and a finite NADH concentration, and K_I^{app} the apparent dissociation costant for pyruvate from the enzyme–NAD^+–pyruvate complex. K_I^{app} is not a simple dissociation constant but contains other kinetic terms (Novoa *et al.*, 1959).

TABLE I: Constants Characterizing Fluorescence Titrations of Native Lactate Dehydrogenase and of Enzyme Which Has Lost 80% of Its Activity Following Reaction with Butanedione.

Constant[f]	Native Enzyme	Inactivated Enzyme
	From results shown in Figure 5	
n^a	0.97	0.75
$K_R{}^{*b}$	1.1 μM	1.25 μM
γ^c	7.7×10^6 mole^{-1} l.$^{-1}$	7.7×10^6 mole^{-1} l.$^{-1}$
$K_{I(oxamate)}{}^d$	6.7 μM	277 μM
	From results shown in Figure 6	
n^a	1.0	0.8
$K_R{}^{*b}$	1.25 μM	1.35 μM
δ^e	44×10^6 mole^{-1} l.$^{-1}$	24×10^6 mole^{-1} l.$^{-1}$
$K_{I(oxalate)}{}^d$	0.37 mM	3.9 mM

[a] Number of binding sites for NADH per mole of enzyme subunit. [b] Dissociation constant for the dissociation of NADH from the enzyme–NADH complex. [c] Augmentation of fluorescence when 1 M NADH is converted to 1 M enzyme–NADH complex, expressed in scale units. [d] Dissociation constant for the dissociation of oxamate or oxalate from the enzyme–NADH–inhibitor complex. [e] Augmentation of fluorescence when 1 M enzyme–NADH complex is converted to 1 M enzyme–NADH–oxalate complex. [f] The curves shown in Figures 5 and 6 were calculated by use of these constants.

NADH. The right-hand side of Figure 6 shows the effects of added oxalate on the fluorescent emission of complexes of native and of derivatized enzyme with NADH. In this figure the upper axis refers to additions made to the system containing native enzyme and the lower to additions made to the inactivated enzyme–NADH system.

Table I lists the dissociation constants derived from the results shown in Figures 5 and 6. Calculations were made by the method of Yang and Schwert (1970). The values shown in Table I for the molar emissitivity of the enzyme–NADH–oxalate complex and for the dissociation constant for the dissociation of oxalate from this complex are less reliable than the other values which are shown. In the case of the native enzyme the maximal emission could not be approached sufficiently closely at the enzyme concentration used to get a good estimate of the molar emission of the complex. In the case of the inactivated enzyme such large concentrations of oxalate were required to get a response that nonspecific affects may have had an effect on fluorescent emission. Despite these uncertainties, it is evident that the loss of enzymatic activity which results from reaction with butanedione can not be accounted for by a loss of ability to interact with NADH but must result principally from the failure of the enzyme–NADH complex to bind substrate.

Estimation of Michaelis Constants. Determination of the apparent Michaelis constant for NADH in 0.1 M phosphate, pH 7.0 at 27° at single pyruvate concentration of 1 mM, yielded values of 5.5 μM for native enzyme and for enzyme which retained only 25% of its initial activity.

In contrast, measurements of initial reaction velocities at varying pyruvate concentrations but at a single NADH concentration of 97 μM yielded markedly different results for the two proteins. The results of these measurements are shown in Figure 7. The curves in Figure 7 were drawn using values of the

Michaelis constant for pyruvate of 39 and 133 μM for native and inactivated enzyme, respectively, and values of 1.58 and 10.2 mM for the apparent dissociation constant for the dissociation of pyruvate from the enzyme–NAD^+–pyruvate complex.

Discussion

Although the results reported here of studies of the reaction of arginine and of lactate dehydrogenase with butanedione in the presence of borate are generally consistent with those reported by Yankeelov (1970) for derivatization by oligomers of butanedione, there are points of significant difference. Arginine reacts more rapidly with butanedione in borate than with the oligomeric reagent and also the product formed in this study appears to be less stable to acid hydrolysis than that formed with the oligomers. Yankeelov (1970) found only about 12% of the product to be reconverted to arginine during 24 hr of hydrolysis with 6 N HCl at 110° while we estimate about half the adduct to be converted under these conditions. The resolution of this discrepancy may lie in the fact that the products formed under these two sets of reaction conditions are not the same.

It is evident that the use of vicinal diketones for the modification of arginine residues in proteins will be more useful after the chemistry of the reaction is better understood. Riordan (1970) has visualized a 1:1 reaction between butanedione and the guanidinium group. This view is supported by the finding that most of the arginine disappears when equimolar amounts of arginine and butanedione are allowed to react for extended periods of time.

Riordan has also proposed, from the fact that the reaction is promoted in borate, that the initial adduct contains cis hydroxyl groups. One would expect such an adduct to be unstable and, in fact, Riordan did find that one arginine of carboxypeptidase to be regenerated when borate and butanedione were removed. In view of the fact, however, that two other arginines of carboxypeptidase and all the arginines which react in lactate dehydrogenase form products which are reasonably stable under mild conditions, it must be assumed that some further reaction, possibly a dehydration to form a substituted imidazole ring, occurs with most adducts. This proposal is consistent with the kinetic interpretation which has been given in this paper.

The finding that incorporation of [^{14}C]butanedione continues at a slow rate until incorporation exceeds the arginine content of lactate dehydrogenase may result from formation of oligomers of butanedione during the extended period of these experiments. Grossberg and Pressman (1968) reported that the oligomer is formed on standing in borate buffer as well as in phosphate buffer as originally reported by Yankeelov and his collaborators (1966). The reaction of the oligomer with arginine would result in the incorporation of more than 1 mole of butanedione per mole of arginine modified and, in addition, lysyl residues react at a slow rate with the oligomers (Yankeelov, 1970).

The kinetic results indicate that the arginine residues of lactate dehydrogenase vary widely in their accessibility to or reactivity with butanedione. The results of the incorporation measurements indicate that not more than three arginines are essential to enzymatic activity. If groups which are not essential to enzymatic reactivity are reactive with butanedione, the number of essential groups could be less than three. The fact that the binary enzyme–NADH complex and the enzyme–NADH–inhibitor complexes are resistant to inactivation by butanedione suggests either that the essential arginine residues are physically masked by bound coenzyme or that they are rendered inaccessible by the change in conformation which accompanies combination of enzyme with coenzyme.

The findings that inactivation with butanedione causes the number of binding sites for NADH to decrease much less rapidly than does activity, that the enzyme–NADH dissociation is unchanged in inactive enzyme, and that inactivation causes little change in the fluorescent emissivity of the enzyme–NADH complex all indicate that the binding site for coenzyme is not altered by reaction with butanedione. This is clearly not the result that was anticipated at the outset of this investigation.

The fact that a reagent directed against the guanidinium group has little effect on coenzyme binding does not discredit the hypothesis that a guanidinium group binds the pyrophosphate bridge of the coenzyme. X-Ray crystallographic analysis (Adams *et al.*, 1970) indicates that the coenzyme binding site is in a deep crevice. Our results show that this site is well protected against attack by butanedione. It has been shown previously that the site resists photooxidation (Millar and Schwert, 1963) and guanidination (*vide infra*). It is possible that the slowly reacting group which is indicated by kinetic measurements is that involved in coenzyme binding. This hypothesis is consistent with the finding, shown in Table I, that the number of binding sites for NADH is reduced to about 0.8 per subunit in enzyme which retains only 20% of its activity in the assay system used.

The present results clearly suggest that at least part of the force which binds pyruvate and inhibitors to the enzyme–NADH complex results from the interaction of the carboxylate groups of these substances with a guanidinium group. If this conclusion is accepted, a substantial revision of previous conclusions is required. It seems desirable to review, briefly, the evidence upon which the earlier conclusions were based.

Measurements of the variation of kinetic constants (Winer and Schwert, 1958; Schwert *et al.*, 1967) and of inhibition constants (Winer and Schwert, 1959) with pH have led to three hypotheses concerning the role of ionic groups in the action of the enzyme.

1. A group with a pK value of approximately 7 acts as the source and sink of the proton involved in the reversible oxidation of lactate by NAD^+. This group was assumed to be an imidazolium of histidine. This identification is supported by the finding (Woenckhaus *et al.*, 1969) that alkylation of a single histidine of pig heart lactate dehydrogenase results in loss of activity.

2. As discussed previously, a guanidinium group is involved in coenzyme binding.

3. The charged form of a group with a pK value of approximately 9.4, assumed to be an ϵ-ammonium of lysine, is required to bind the anionic substrates, lactate and pyruvate, to the enzyme–coenzyme complexes.

Yang and Schwert (1970) treated lactate dehydrogenase with *O*-methylisourea in an attempt to show that substrate binding is altered when lysyl groups are converted to homoarginyl residues. It was found that guanidination had little effect upon the interaction of enzyme with coenzyme. However, substrates and inhibitors are bound much more firmly by the enzyme–coenzyme complex formed with guanidinated enzyme than by the complex formed with native enzyme.

The simplest interpretation of the effects of guanidination and of the results reported here is that both an ϵ-ammonium and a guanidinium group are involved in binding the car-

boxylate group of substrates and of inhibitors.[2] The interaction of an anion with two positively charged sites might serve to immobilize the substrate molecule in the specific orientation required for reaction. Displacement of one center of positive charge by guanidination might well cause firmer substrate binding and masking of the other by reaction with butanedione could result in diminished substrate binding.

This rationalization of results leaves one observation which must be accounted for. The concept that an ϵ-ammonium group is involved in substrate binding rests principally upon the observation that the Michaelis constant for pyruvate increases very sharply at pH values greater than 7 (Winer and Schwert, 1958; Schwert *et al.*, 1967). Evidently if this group were converted to a guanidinium group, substrate binding would not be diminished until the pH was increased to values greater than 9. In fact, however, Yang and Schwert (1970) found that with the guanidinated enzyme the Michaelis constant for pyruvate still increased by about two orders of magnitude between pH 7 and pH 9. Evidently one must conclude either that the lysine involved in substrate binding is not attacked by *O*-methylisourea and that increased interaction between pyruvate and the guanidinated enzyme–NADH complex results from other effects of guanidination or that there is another group on the enzyme surface which is not susceptible to guanidination but which has a p*K* value of about 9.4. At the present time the latter alternative seems more attractive provided that an identity can be established for the hypothetical other group.

Dr. Janos Südi[3] has recently proposed a general reaction scheme for dehydrogenases which provides a resolution to this problem. Südi visualizes the group which acts as a source and sink for protons (presumably an imidazolium in the case of lactate dehydrogenase) to be close to the binding site for the nicotinamide ring of the coenzyme. The charge-transfer complex which is formed when NAD^+ is bound results in a decrease in the p*K* value of the imidazolium group. Conversely, in the enzyme–NADH complex the p*K* value of the imidazolium increases to a value of about 9. The catalytic step is proposed to be a push-pull mechanism identical with that proposed by Winer and Schwert (1959).

One of the most attractive features of Südi's hypothesis is that it accounts in a simple way for the dependence of the equilibrium of the complete reaction upon proton concentration. From a more limited viewpoint, it also provides a resolution of the dilemma posed by our results. Our current conclusion is that both arginyl and lysyl residues bind the carboxylate group of substrates and inhibitors to the enzyme–coenzyme complexes and that the increase in the magnitude of the Michaelis constant for pyruvate at high pH values results principally from deprotonation of the proton-donating imidazolium group in the enzyme–NADH complex.

[2] Dr. W. W. Cleland first directed our attention to this possibility.

[3] Personal communication.

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