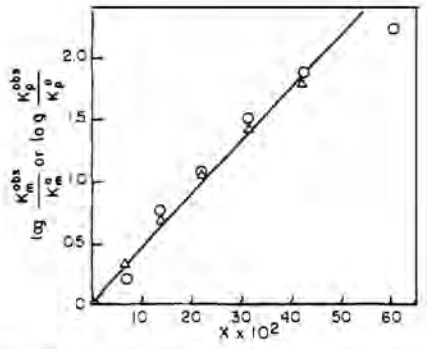

FIGURE 5: The data for K_m (○) and K_p (△) plotted according to eq 5. The solid line is calculated according to eq 5 with $A = 1.74$ and $K_D = 10.0$ M.

of dimethyl sulfoxide. As shown in Figure 4 binding of proflavine to α-chymotrypsin in aqueous dimethyl sulfoxide is essentially temperature independent.

The results of this investigation lend further support to the theory that α-chymotrypsin-catalyzed reactions in 65% aqueous dimethyl sulfoxide and at subzero temperatures follow essentially the same reaction pathway as under "normal" conditions.

References

Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18*, 405.
Blow, D. M. (1971), *Enzymes, 3rd Ed., 3*, 185.
Clement, G. E., and Bender, M. L. (1963), *Biochemistry 2*, 836.
Fersht, A. R., and Requena, Y. (1971), *J. Mol. Biol. 60*, 279.
Fink, A. L. (1973), *Biochemistry 12*, 1736.
Glazer, A. N. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 171.
Hess, G. P. (1971), *Enzymes, 3rd Ed., 3*, 213.
Himoe, A., Brandt, K. G., DeSa, R. J., and Hess, G. P. (1969), *J. Biol. Chem. 244*, 3483.
Mares-Guia, M., and Figueiredo, A. F. S. (1972), *Biochemistry 11*, 2091.
Schonbaum, G. R., Zerner, B., and Bender, M. L. (1961), *J. Biol. Chem. 236*, 2930.
Zerner, B., Bond, R. P. M., and Bender, M. L. (1964), *J. Amer. Chem. Soc. 86*, 3674.

Changes in Sulfhydryl Groups of Honeybee Glyceraldehyde Phosphate Dehydrogenase Associated with Generation of the Intermediate Plateau in Its Saturation Kinetics†

W. G. Gelb,‡ J. F. Brandts,§ and J. H. Nordin*

ABSTRACT: Experiments with honeybee (*Apis mellifera*) and rabbit muscle glyceraldehyde phosphate dehydrogenases were conducted to obtain information at the chemical level regarding anomolous saturation kinetics of the honeybee enzyme. Results demonstrate that the enzyme's sulfhydryl groups are implicated in the process. Measured by 5,5′-dithiobis(nitrobenzoic acid) titration, native honeybee glyceraldehyde phosphate dehydrogenase has one less active sulfhydryl than the native rabbit muscle enzyme and displays changes in overall sulfhydryl reactivity after preincubation with glyceraldehyde 3-phosphate or glyceraldehyde 3-phosphate plus NAD^+. The total 5,5′-dithiobis(nitrobenzoic acid) reactive sulfhydryls of rabbit muscle glyceraldehyde phosphate dehydrogenase are not changed by preincubation with NAD^+ or glyceraldehyde 3-phosphate; honeybee glyceraldehyde phosphate dehydrogenase, under certain conditions of preincubation with these ligands, shows a decrease of two total 5,5′-dithiobis(nitrobenzoic acid) reactive sulfhydryl groups. This difference has been confirmed by an independent experiment in which the two enzymes were carboxymethylated with [^{14}C]bromoacetic acid. The loss of sulfhydryl groups in honeybee glyceraldehyde phosphate dehydrogenase is not a result of its acylation by glyceraldehyde 3-phosphate. After generation of the stable form of the honeybee enzyme with glyceraldehyde 3-phosphate plus NAD^+ it is possible to regenerate the anomolous kinetic curve by treatment of the enzyme with dithiothreitol. It is proposed that in honeybee glyceraldehyde phosphate dehydrogenase, an intrachain disulfide bond forms in conjunction with the conversion of the enzyme from the "metastable" to the stable state.

Intermediate plateau and transition regions in ligand saturation curves have been reported for a number of enzymes. These include glutamate dehydrogenase (LeJohn and Jackson, 1968), phosphoenolpyruvate carboxylase (Corwin and Fanning, 1968), adenosine diphosphoglucose pyrophosphorylase (Gentner and Preiss, 1968), cytidine triphosphate synthetase (Levitski and Koshland, 1969), pyruvate kinase (Somero, 1969), lactate dehydrogenase (Somero and Hochachka, 1969), acetylcholinesterase (Kato *et al.*, 1972), and L-threonine dehydratase (Kagan and Dorozhko, 1973). However, except for glutamate dehydrogenase, no satisfactory explanation for these anomalous kinetics is presently available (Teipel and Koshland, 1969). A similar transition in substrate saturation curves of glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) obtained from the honeybee *Apis mellifera* and car-

† From the Department of Biochemistry, University of Massachusetts, Amherst, Massachusetts 01002. *Received July 9, 1973.* Supported by grants from the National Aeronautics and Space Administration (NGL 22-010-029) and the Herman Frasch Foundation.
‡ Present address: Pharmacia Fine Chemicals Inc., Piscataway, N. J.
§ Department of Chemistry, University of Massachusetts.

penter ant *Camponotus pennyslvanicus* have also been reported by us (Gelb and Nordin, 1969; Nordin *et al.*, 1970).

Inspection of the transition regions of many of these curves (*cf.* inset of Figure 1 of Gelb *et al.*, 1970) shows that simple binding phenomena or "isozymes" cannot explain such cooperativity since the Hill coefficient of the transition is approximately 40. Honeybee glyceraldehyde phosphate dehydrogenase has been found byd irectexperiment to have a maximum of four NAD^+ binding sites (Gelb *et al.*, 1970). This value equals the number reported for rabbit muscle glyceraldehyde phosphate dehydrogenase (Conway and Koshland, 1968).

The anomolous kinetics of trout lactate dehydrogenase (Somero and Hochachka, 1969) and Alaskan king crab pyruvate kinase (Somero, 1969) as well as carpenter ant and honeybee glyceraldehyde phosphate dehydrogenases occur in organisms known to adapt to a wide environmental temperature range. It is therefore of interest to examine the chemical nature of these transitions to attempt any assessment of their possible significance in thermal compensatory mechanisms.

Based on the results of earlier studies (Gelb *et al.*, 1970) it was proposed that honeybee glyceraldehyde phosphate dehydrogenase exists in two forms: one, obtained when the enzyme is isolated, that displays the transition, and a second stable form, that does not display this phenomenon, obtained by a preincubation of the first ("metastable") form with NAD^+ plus D,L-glyceraldehyde 3-phosphate.

In the case of honeybee glyceraldehyde phosphate dehydrogenase, abolition of the transition by preincubation of the "metastable" form of the enzyme with *N*-ethylmaleimide (Gelb *et al.*, 1970) suggested the possibility that the enzyme's sulfhydryl groups are somehow associated with the interconversion between the two forms of the enzyme.

This communication details the results of experiments with honeybee glyceraldehyde phosphate dehydrogenase which provide additional evidence for the participation of the enzyme's sulfhydryl groups in the transition from the "metastable" to the stable form. A preliminary report of this work has been presented (Gelb and Nordin, 1971).

Materials and Methods

D,L-Glyceraldehyde 3-phosphate diethyl acetal (monobarium salt), β NAD^+, iodoacetic acid, Nbs_2,[1] 2-mercaptoethanol, and Tris were purchased from Sigma Chemical Co. Urea was a product of Mann Laboratories. Uniformly labeled [^{14}C]-fructose 1,6-bisphosphate (69 mCi/mmol) was obtained from Amersham-Searle, and [^{14}C]bromoacetic acid (methyl labeled, 5 mCi/mmol) was obtained from International Chemical and Nuclear Corporation. Dithiothreitol was purchased from Calbiochem. Honeybees were either purchased from commercial suppliers or were a gift from the Entomology Department, University of Massachusetts. Rabbit muscle glyceraldehyde phosphate dehydrogenase and fructose bisphosphate aldolase and bovine liver catalase were obtained from Sigma, trypsin from Nutritional Biochemicals, and bovine serum albumin from Pentex Inc. All were crystalline preparations.

Preparation of Glyceraldehyde Phosphate. D,L-Glyceraldehyde 3-phosphate diethyl acetal monobarium salt was converted to the free acid according to suppliers instructions. D-[^{14}C]Glyceraldehyde 3-phosphate was prepared by a modification of the method of Hall (1960), using 50 μCi of [U-^{14}C]-fructose 1,6-bisphosphate and crystalline rabbit muscle fructose bisphosphate aldolase. The specific radioactivity of the isolated D-[^{14}C]glyceraldehyde 3-phosphate was estimated by determining the chemical concentration of D-glyceraldehyde 3-phosphate enzymatically, using rabbit muscle glyceraldehyde phosphate dehydrogenase and excess NAD^+ followed by a radioactivity determination in a Packard Tri-Carb liquid scintillation spectrometer. The product had a specific activity of 1.76×10^9 cpm/mmol.

Glyceraldehyde Phosphate Dehydrogenase Assay. Measurements of the specific activities of honeybee and rabbit muscle glyceraldehyde phosphate dehydrogenases were performed at 25° and pH 8.5 by the method of Velick (1955) as described elsewhere (Gelb *et al.*, 1970). The standard assay buffer contained 5 mM sodium pyrophosphate, 5 mM sodium arsenate, and 1 mM EDTA. (No sulfhydryl reagents were included in the assay buffer.) For certain experiments the pH of this buffer was 9.0. Total protein concentrations were obtained from absorbance measurements at 280 nm assuming an extinction coefficient of 1.06 cm^2 mg^{-1} (Murdock and Koeppe, 1964; Marquardt *et al.*, 1968.) In some experiments protein was determined by the method of Lowry *et al.* (1951).

Preparation of Honeybee Glyceraldehyde Phosphate Dehydrogenase. Crystalline honeybee enzyme was prepared by a modification of the procedure of Marquardt *et al.* (1968). The details of the method employed and the specific activities of preparations have been published earlier (Gelb *et al.*, 1970; Nordin *et al.*, 1970).

Polyacralymide Gel Electrophoresis. Electrophoresis was conducted as described by Davis (1964). A modification of the procedure described by Weber and Osborn (1969) was employed for electrophoresis in the presence of sodium dodecyl sulfate using a 10% gel. The stable form of honeybee glyceraldehyde phosphate dehydrogenase was prepared by incubating 0.5 ml of "metastable" enzyme (5 mg/ml) in a pH 9.0 assay buffer which contained 2 mM NAD^+ and 3.6 mM D,L-glyceraldehyde 3-phosphate. Enzyme in each state and standards of bovine serum albumin, catalase, aldolase, and trypsin (also 5 mg/ml in the above buffer), were diluted tenfold in 0.01 M sodium phosphate–1% sodium dodecyl sulfate–0.01 M iodoacetic acid at pH 7.0 and allowed to react for 2 hr at 37°. Approximately 12 μg of each protein was subjected to electrophoresis for 4 hr at a constant current of 8 mA/tube. Following electrophoresis the gels were stained and destained according to the procedure of Chrambach *et al.* (1967). Bromophenol Blue (0.05%) was used as tracking dye. While the method of Weber and Osborn (1969) employs 2-mercaptoethanol, it was omitted in these experiments since it would lead to reduction of any disulfide bonds present in the glyceraldehyde phosphate dehydrogenase.

Nbs_2 Studies. For the titration of *native* rabbit muscle and honeybee glyceraldehyde phosphate dehydrogenases with Nbs_2, enzyme crystals were harvested by centrifugation and dissolved in 1 ml of assay buffer to give protein concentrations between 1.0 and 1.4 mg/ml. To these solutions, appropriate additions of D,L-glyceraldehyde 3-phosphate (50 mg/ml) and/or 0.1 M NAD^+ were made and they were then incubated for 2 min at 20°. Each mixture was then applied to a Sephadex G-25 (coarse) column (1.0 × 45 cm) which had been previously equilibrated with 0.1 M imidazole–1 mM EDTA (pH 7.0). The fractions containing glyceraldehyde phosphate dehydrogenase activity were pooled and additional imidazole buffer was added to bring the final enzyme concentration to 0.2–0.3 mg/ml. One milliliter of the enzyme solution was placed in a cuvet and the reaction initiated by the addition of 5 μl of 0.01 M Nbs_2. The reaction was monitored at 412 nm

[1] Abbreviation used is: Nbs_2, 5,5′-dithiobis(nitrobenzoic acid).

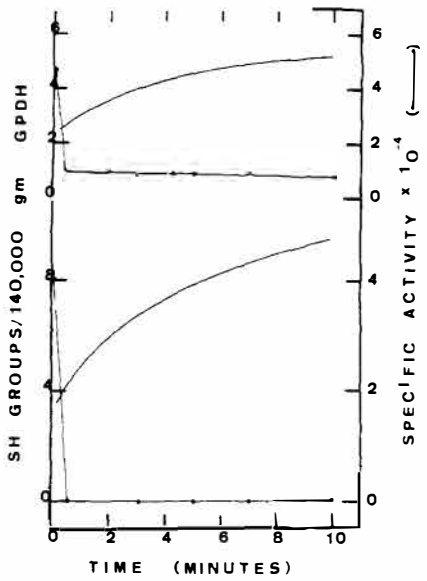

FIGURE 1: Effect of Nbs_2 on enzymatic activities of glyceraldehyde phosphate dehydrogenases. Each reaction mixture contained enzyme, 0.3 mg/ml, in 0.1 M imidazole–1 mM EDTA at pH 7.0 and 20° in a final volume of 1.0 ml. The reaction was initiated by the addition of 5 μl of 0.01 M Nbs_2. Enzyme activity measurements were then made on suitable aliquots either before addition of Nbs_2 or after adding it to the reaction mixture: upper frame, "metastable" honeybee enzyme; lower frame, rabbit muscle enzyme. The continuous curve represents the Nbs_2 titration and the curve with the closed circles, enzymatic activity (GPDH, glyceraldehyde phosphate dehydrogenase).

with a Gilford Model 240 spectrophotometer equipped with a Honeywell Electronic 19 recorder.

To determine the total Nbs_2-reactive sulfhydryl content of the *denatured* enzyme, 0.46 g of solid urea was added per ml to another aliquot of the pooled diluted enzyme solution eluted from the Sephadex G-25 column, described above. This solution was then incubated for 15 min at 20° and divided into four 1-ml samples. Nbs_2 (0.01 M; 10 μl) was added to each sample and the optical density changes at 412 nm measured periodically for 1 hr, at which time the reactions had reached completion. After subtraction of the appropriate blank readings at 412 nm the average concentration of reacted Nbs_2 in the four cuvets was determined using a molar extinction coefficient for the thionitrobenzoate ion of 13,600 l. mol^{-1} cm^{-1} at 412 nm (Ellman, 1959).

Specific Activity of [^{14}C]*Bromoacetic Acid.* [^{14}C]Bromoacetic acid was assayed for specific activity by its reactivity with urea-denatured rabbit muscle glyceraldehyde phosphate dehydrogenase. (Independent titration of the urea-denatured enzyme with Nbs_2 indicated it to contain 13.0 sulfhydryl groups/mol.) Two milligrams of crystalline rabbit muscle enzyme were dissolved in 1 ml of assay buffer (pH 9.0), followed by the addition of 0.46 g of solid urea. After 15 min at 20°, 20 μl of [^{14}C]bromoacetic acid was added. The mixture was incubated for 1 hr and then applied to a Sephadex G-25 column (1.0 × 45 cm) which was preequilibrated with 0.1 M imidazole–6 M urea (pH 7.0). Fractions (1 ml) were collected and the eluate assayed for radioactivity by liquid scintillation spectrometry and for protein by the method of Lowry *et al.* (1951). The standard curve of protein *vs.* absorbance at 500 nm was linear in all regions examined for the carboxymethylated enzyme in urea. Based on protein concentration and radioactivity, a specific activity of 6.23×10^9 cpm/mmol of [^{14}C]bromoacetic acid was obtained for the ^{14}C-labeled carboxymethylated enzyme.

Studies with [^{14}C]*Bromoacetic Acid.* The procedure followed is a modification of the method of Zahler and Cleland (1968) for the specific determination of disulfide bonds. Crystalline honeybee or rabbit muscle glyceraldehyde phosphate dehydrogenases were harvested by centrifugation at 12,000*g* for 10 min and the pelleted crystals dissolved in 1 ml of assay buffer (pH 9.0) (final protein concentrations, 1.3–1.8 mg/ml). Next, the appropriate additions, if any, of D,L-glyceraldehyde 3-phosphate (50 mg/ml) and/or of 0.1 M NAD^+ were made to obtain the desired state of honeybee enzyme, and the mixture incubated for 2 min at 20°. Then 0.46 g of solid urea was added followed by a 5-min incubation at 20°. Next 13 μl of 0.1 M iodoacetic acid was added to carboxymethylate all free sulfhydryl groups, and the mixture was allowed to incubate an additional 1 hr at 20°. The solution was then dialyzed against two changes (250 ml each) of 0.05 M Tris (pH 9.0) for 2 hr to remove excess iodoacetate and unreacted substrates and products of the enzymic reaction. The enzyme solution was concentrated to approximately 0.5 ml by rolling the dialysis membrane in dry Sephadex G-200. Urea (0.18 g) was added to 0.3 ml of concentrated enzyme solution followed by 0.1 ml of 3 mM dithiothreitol and the mixture was incubated 40 min at 20° to allow reduction of any disulfide bonds in the enzyme. Next, 0.2 ml of 1.0 M Tris (pH 8.1), 0.5 ml of 5 mM sodium arsenite (in 1.0 M Tris (pH 8.1), and 0.32 g of urea were added to the enzyme solution followed by a 15-min incubation at 20°. The sodium arsenite added in this step serves to complex reduced dithiothreitol rendering it less accessible to reaction with the bromoacetic acid (Zahler and Cleland, 1968). Twenty microliters of [^{14}C]bromoacetic acid solution were then added and the mixture allowed to incubate 15 min at 20° and then an aliquot was applied to a Sephadex G-25 (fine) column (1.0 × 50 cm) preequilibrated with 0.1 M Tris–6 M urea at pH 8.1. One-milliliter fractions were collected and the eluate assayed for protein (Lowry *et al.*, 1951) and radioactivity.

Studies with D-[^{14}C]*Glyceraldehyde 3-Phosphate.* A 0.7-ml suspension of honeybee glyceraldehyde phosphate dehydrogenase crystals (5 mg/ml) was centrifuged at 12,000*g* for 10 min and the pellet dissolved in 1 ml of assay buffer (pH 9.0). Twenty-five microliters of D-[^{14}C]glyceraldehyde 3-phosphate (4 mg/ml) or 25 μl of D-[^{14}C]glyceraldehyde 3-phosphate plus 10 μl of 0.1 M NAD^+ were added followed by an incubation for 2 min at 20°. Solid urea (0.46 g) was then added and the mixture incubated an additional 5 min. Next, 13 μl of 0.1 M iodoacetic acid was added and the enzyme solution was allowed to incubate for 1 hr at 20°. The protein solution was applied to a Sephadex G-25 (fine) column (1.0 × 50 cm), previously equilibrated with 0.1 M Tris–6 M urea at pH 8.1. One-milliliter fractions were collected and each fraction assayed for protein concentration (Lowry *et al.*, 1951) and radioactivity.

Results

A comparison of the effects of Nbs_2 on *native* rabbit muscle and honeybee glyceraldehyde phosphate dehydrogenases indicates fundamental differences exist between the two enzymes with regard to their relative sulfhydryl reactivities.

Figure 1 compares the number of fast reacting sulfhydryls in the two enzymes as well as the effect of thionitrobenzoate addition on the proteins' catalytic activities. The rabbit muscle enzyme contains about four sulfhydryl groups capable of reacting within 30 sec with Nbs_2; all enzymatic activity is

TABLE I: Effect of Added Ligands on the Total Nbs_2-Reactive Sulfhydryl Groups of Urea-Denatured Rabbit Muscle and Honeybee Glyceraldehyde Phosphate Dehydrogenases.

Addition[a]	Sulfhydryl Groups/140,000 g of Glyceraldehyde Phosphate Dehydrogenase[b]	
	Rabbit Muscle	Honeybee
None	12.9 ± 0.2 (2)	16.0 ± 0.1 (2)
NAD^+ (2 mM) plus glyceraldehyde 3-phosphate (3.6 mM)	12.7 ± 0.2 (4)	14.1 ± 0.3 (3)
NAD^+ (3 mM) plus glyceraldehyde 3-phosphate (1.8 mM)	13.1 ± 0.2 (4)	14.5 ± 0.1 (3)
Glyceraldehyde 3-phosphate (3.6 mM)	13.6 ± 0.3 (2)	14.0 ± 0.1 (2)
Glyceraldehyde 3-phosphate (3.6 mM); after Sephadex G-25, NAD^+ (0.02 mM)	13.2 ± 0.2 (2)	13.7 ± 0.1 (2)
NAD^+ (2 mM)	13.2 ± 0.3 (4)	15.9 ± 0.1 (4)
NADH (2 mM)	13.1 ± 0.2 (4)	15.8 ± 0.3 (4)

[a] All glyceraldehyde 3-phosphate concentrations expressed as D,L racemic mixtures. [b] Numbers in parentheses refer to the number of determinations.

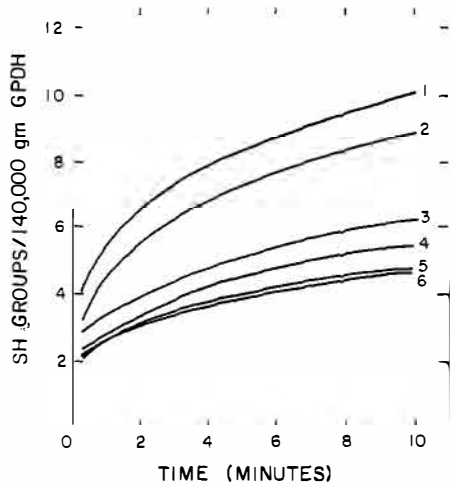

FIGURE 2: Titration of native rabbit and honeybee glyceraldehyde phosphate dehydrogenases with Nbs_2. Rabbit muscle or "metastable" honeybee enzymes, 1.0–1.4 mg/ml, were preincubated at 20° in 1 ml of assay buffer (pH 9.0) for 2 min with or without ligands at the final concentrations shown below. The entire reaction mixture was then applied to a Sephadex G-25 column equilibrated with 0.1 M imidazole–1 mM EDTA (pH 7.0). The pooled enzyme fractions were adjusted to 0.3 mg of protein/ml with the above imidazole buffer and a 1.0-ml aliquot was allowed to react with 5 μl of 0.01 M Nbs_2. The curves obtained were from enzyme samples subjected to the following preincubations: (1) three superimposable curves which represent rabbit muscle enzyme alone or with 2.0 mM NAD^+ or 3.6 mM D,L-glyceraldehyde 3-phosphate; (2) rabbit muscle enzyme and 2 mM NAD^+ plus 3.6 mM D,L-glyceraldehyde 3-phosphate or rabbit muscle enzyme and 3.0 mM NAD^+ plus 1.8 mM D,L-glyceraldehyde 3-phosphate (two superimposable curves); (3) honeybee enzyme and 3.6 mM D,L-glyceraldehyde 3-phosphate; (4) honeybee enzyme alone or with 2.0 mM NAD^+; (5) honeybee enzyme and 2.0 mM NAD^+ plus 3.6 mM D,L-glyceraldehyde 3-phosphate; (6) honeybee enzyme and 1.8 mM D,L-glyceraldehyde 3-phosphate plus 3.0 mM NAD^+ (GPDH, glyceraldehyde phosphate dehydrogenase).

destroyed within this same period. However, honeybee glyceraldehyde phosphate dehydrogenase displays only 2.5–3 fast reacting sulfhydryls. There was a concomitant loss of 75% of the enzyme's catalytic activity but 25% remained even after titration of several additional sulfhydryl groups during the 10-min experimental period.

Additional differences in sulfhydryl reactivities of the *native* enzymes were noted when comparing Nbs_2 titrations after exposure to various combinations of D,L-glyceraldehyde 3-phosphate and NAD^+ followed by gel filtration on Sephadex G-25 to remove the ligands. Figure 2 illustrates the results of these experiments. Curve 4 in Figure 2 shows the Nbs_2 titration of "metastable" honeybee glyceraldehyde phosphate dehydrogenase preincubated alone and after preincubation in 2 mM NAD^+. Experimentally, the two curves were indistinguishable and are shown as one. Identical results, with respect to addition of NAD^+, were also obtained with rabbit muscle enzyme as shown in curve 1. Similarly, when honeybee or rabbit muscle enzymes were preincubated with 2 mM NAD^+ plus 3.6 mM D,L-glyceraldehyde 3-phosphate prior to titration with Nbs_2, a decrease in sulfhydryl reactivity relative to incubation without ligands was observed for both enzymes (curve 2, rabbit, and curves 5 and 6, honeybee). Incubation of honeybee glyceraldehyde phosphate dehydrogenase with NAD^+ plus glyceraldehyde 3-phosphate converts it from the "metastable" to the stable state (Gelb *et al.*, 1970). The conversion occurs regardless of which ligand, NAD^+ or glyceraldehyde 3-phosphate, is in excess. Since the rabbit muscle enzyme does not show any change in kinetic properties with this treatment (Gelb *et al.*, 1970) it served as a control for the honeybee enzyme. Preincubation of "metastable" honeybee enzyme with 3.6 mM D,L-glyceraldehyde 3-phosphate alone (Figure 2, curve 3) caused a marked *increase* in the sulfhydryl reactivity over that obtained when "metastable" honeybee enzyme was incubated either alone or with NAD^+ (curve 4). However, the rabbit muscle enzyme exhibited no corresponding increase under the same conditions (curve 1).

Table I lists the total Nbs_2 reactive sulfhydryl groups, obtained with *denatured* rabbit muscle and honeybee glyceraldehyde phosphate dehydrogenases subjected first to various preincubation conditions as described under Materials and Methods. The total Nbs_2 reactive sulfhydryl titer of rabbit muscle glyceraldehyde phosphate dehydrogenase was unchanged by the various incubations. In contrast, the total Nbs_2 reactive sulfhydryl titer of honeybee enzyme was changed under certain conditions. When the "metastable" enzyme is preincubated alone its total Nbs_2 reactive sulfhydryl content was 16/140,000 g of enzyme. When it was pretreated with NAD^+ plus glyceraldehyde 3-phosphate, glyceraldehyde 3-phosphate alone, or with glyceraldehyde 3-phosphate and then after Sephadex G-25 chromatography with NAD^+, its sulfhydryl content decreased to approximately 14. Upon incubation of the "metastable" enzyme with either NAD^+ or NADH prior to reaction with Nbs_2 no decrease in total sulfhydryl titer was observed as compared to control values. Although the data in Table I suggest that with honeybee glyceraldehyde phosphate dehydrogenase certain preincubation conditions may result in disulfide bond formation, a change in sulfhydryl titer from 16 to 14 represents a small absolute difference. Therefore, another experiment using an independent method was conducted to confirm these results. A modification of the method of Zahler and Cleland (1968) was employed to quantitate changes in disulfide bonds in the two dehydrogenases. With this method, the protein is completely

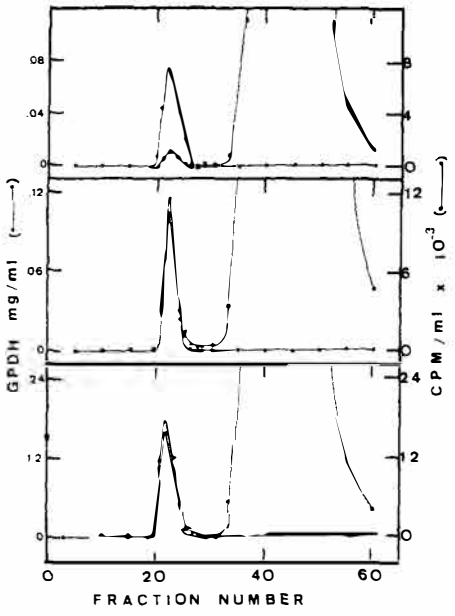

FIGURE 3: Reactivity of [^{14}C]bromoacetic acid with the two states of honeybee glyceraldehyde phosphate dehydrogenase. See Materials and Methods section for experimental details: upper frame, "metastable" enzyme; middle frame, stable enzyme (prepared by preincubating "metastable" glyceraldehyde phosphate dehydrogenase with 2 mM NAD^+ plus 3.6 mM D,L-glyceraldehyde 3-phosphate; lower frame, metastable enzyme preincubated with 3.6 mM D,L-glyceraldehyde 3-phosphate only; (○) protein; (■) radioactivity (GPDH, glyceraldehyde phosphate dehydrogenase).

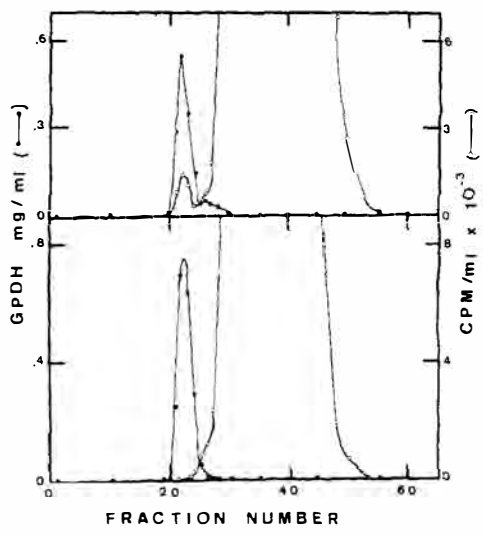

FIGURE 4: Reactivity of D-[^{14}C]glyceraldehyde 3-phosphate with honeybee glyceraldehyde phosphate dehydrogenase. See Materials and Methods section for experimental details: upper frame, enzyme preincubated in 0.59 mM D-[^{14}C]glyceraldehyde 3-phosphate; lower frame, enzyme preincubated in 1 mM NAD^+ plus 0.59 mM D-[^{14}C]-glyceraldehyde 3-phosphate; (○) protein; (●) radioactivity (GPDH, glyceraldehyde phosphate dehydrogenase).

carboxymethylated with iodoacetic acid to block any sulfhydryl groups present. After removal of unreacted iodoacetate, any disulfide bonds are reduced with dithiothreitol. The protein is then subjected to a second carboxymethylation using [^{14}C]bromoacetic acid. The absolute number of sulfhydryl groups determined using this technique is much smaller and larger percentage changes can be seen. The choice of [^{14}C]bromoacetic acid rather than Nbs_2, as described by Zahler and Cleland (1968), was made to alleviate interference by the simultaneous reaction of Nbs_2 with the reducing agent dithiothreitol. Figure 3 shows the elution profiles on Sephadex of urea-denatured honeybee enzyme after carboxymethylation with iodoacetic acid and [^{14}C]bromoacetic acid. Approximately five times more [^{14}C]carboxymethyl is incorporated into protein with both the stable enzyme and "metastable" enzyme preincubated with glyceraldehyde 3-phosphate than with "metastable" enzyme which had been preincubated alone. Quantitative data obtained from these profiles (moles of [^{14}C]carboxymethyl groups incorporated per 140,000 g of honeybee and rabbit muscle enzymes under various conditions) are in excellent agreement with the evidence presented in Table I and suggest a disulfide bond is formed when honeybee enzyme is (1) incubated with 2 mM NAD^+ plus 3.6 mM D,L-glyceraldehyde 3-phosphate (change from 0.4 to 2.2 sulfhydryl groups per 140,000 g of enzyme) or (2) incubated with 3.6 mM D,L-glyceraldehyde 3-phosphate (change from 0.4 to 2.0 sulfhydryl groups per 140,000 g of enzyme). The first mentioned treatment showed no affect on rabbit muscle glyceraldehyde phosphate dehydrogenase while the second resulted in a change from 0.8 to 0.6 mol of [^{14}C]carboxymethyl group incorporated per 140,000 g of enzyme. A control experiment was also conducted to test the possibility that the incorporation of [^{14}C]carboxymethyl groups into honeybee enzyme as shown in Figure 3 was a result of prior removal of acyl groups (of glyceraldehyde 3-phosphate) from glyceraldehyde phosphate dehydrogenase by transesterification to dithiothreitol prior to carboxymethylation with [^{14}C]bromoacetic acid. Reaction of glyceraldehyde phosphate dehydrogenase with certain acyl compounds has been shown to occur *via* certain of its sulfhydryl and lysine residues (Matthew *et al.*, 1967). Figure 4 shows the elution profiles obtained when honeybee glyceraldehyde phosphate dehydrogenase is incubated with either 0.59 mM D-[^{14}C]glyceraldehyde 3-phosphate alone (top frame) or with 1 mM NAD^+ plus 0.59 mM D-[^{14}C]glyceraldehyde 3-phosphate. Neither sample was subjected to incubation with dithiothreitol as in the experiment illustrated in Figure 3. Therefore, any covalent bonds formed between glyceraldehyde 3-phosphate and the enzyme could not be destroyed by this reagent. Calculations using the data from Figure 4 show that only 0.2 mol of D-[^{14}C]glyceraldehyde 3-phosphate was introduced per mol of enzyme when it was incubated with radioactive substrate alone and no incorporation was observed when D-[^{14}C]glyceraldehyde 3-phosphate plus NAD^+ were incubated with the enzyme.

If, in fact, the conversion from the "metastable" to stable form of glyceraldehyde phosphate dehydrogenase occurs in conjunction with disulfide bond formation it should be possible to regenerate the transition in the kinetic saturation curve by subjecting the stable form to reduction. The upper frame of Figure 5 shows the saturation kinetics obtained with "metastable" honeybee glyceraldehyde phosphate dehydrogenase. The middle frame shows kinetic data obtained with stable enzyme prepared by incubation of an aliquot of metastable enzyme (assay in upper frame) with 3.6 mM D,L-glyceraldehyde 3-phosphate and 2 mM NAD^+. As was reported previously (Gelb and Nordin, 1969; Gelb *et al.*, 1970), the transition is abolished under these conditions. When stable enzyme is incubated with 2.4 mM dithiothreitol for 1 hr and the kinetic saturation curve again analyzed, the results shown in the lower frame of Figure 5 are obtained. Although the magnitude of the observed transition (ΔV_{max}) varied somewhat from experiment to experiment (Figure 5, top and bottom frames; see also Gelb *et al.*, 1970) the regeneration of the transition always occurred when the stable form of the

enzyme was incubated with dithiothreitol. These results indicate that the conversion from metastable to the stable state can be caused by incubation with the ligands glyceraldehyde 3-phosphate plus NAD^+ and that the conversion back to the "metastable" state occurs when the stable form is treated with dithiothreitol, a reagent capable of reducing disulfide bonds.

Polyacrylamide gel electrophoresis of "metastable" and stable honeybee glyceraldehyde phosphate dehydrogenases at pH 8.3 (Davis, 1964) failed to reveal any difference in their mobilities. It has been shown previously that the two forms were also inseparable by gel filtration (Gelb *et al.*, 1970). Attempts by Marquardt *et al.* (1968) to distinguish between holo and apo forms of honeybee glyceraldehyde phosphate dehydrogenase by electrophoretic techniques were also unsuccessful. Relative mobilities of the subunits of the two states of honeybee glyceraldehyde phosphate dehydrogenase were determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Weber and Osborn, 1969) but in the *absence* of thiols. The marker proteins employed and their minimum molecular weights were: bovine serum albumin, 68,000 (Tanford *et al.*, 1967); catalase, 60,000 (Sund *et al.*, 1967); aldolase, 40,000 (Kawahara and Tanford, 1966); and trypsin, 23,000 (Dayhoff and Eck, 1967). From the data the subunit molecular weights from both states were estimated to be between 35,000 and 36,000, a value in agreement with the literature for glyceraldehyde phosphate dehydrogenase protomers (Harrington and Karr, 1965; Harris and Perham, 1965). Disulfide bond formation is not occurring between subunits of honeybee glyceraldehyde phosphate dehydrogenase since higher molecular weight bonds could not be detected by this technique. This suggests that the disulfide bond is *intra subunit* in nature.

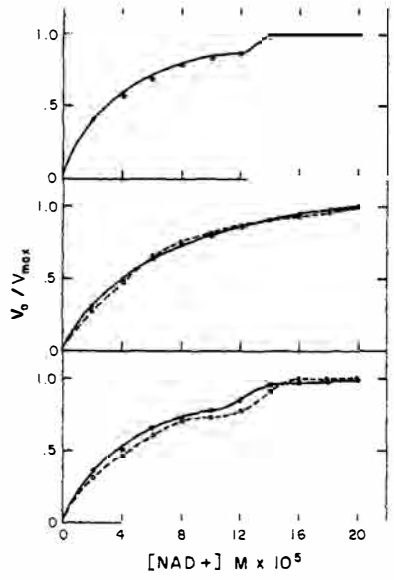

FIGURE 5: Regeneration of the transition in the kinetic saturation curve of honeybee glyceraldehyde phosphate dehydrogenase. Honeybee glyceraldehyde phosphate dehydrogenase, 0.25 mg/ml, in assay buffer (pH 9.0) was first analyzed for the kinetic transition by diluting suitable aliquots 200-fold in the assay cuvet. Then a 1.0-ml aliquot of the former solution was treated with D,L-glyceraldehyde 3-phosphate (3.6 mM final concentration) and NAD^+ (2.0 mM final concentration). After incubating 5 min at 20°, 5-μl aliquots were transferred to an assay cuvet for analysis of enzymatic activity at this point. The remaining reaction mixture was applied to a 1.4 × 25 cm column of Sephadex G-25 to remove substrates and products. To a 0.3-ml fraction of the column eluate was added 0.2 ml of 6 mM dithiothreitol. After 60 min at 0°, assays were performed on aliquots of this mixture. Each data point in the three frames represents the average of two–four separate velocity measurements. The dotted lines represent the results obtained in a repeat experiment in which another sample of "metastable" enzyme was preincubated with glyceraldehyde 3-phosphate and NAD^+ as described above. V_0/V_{max} = the initial velocity of the reaction at various concentrations of NAD^+; V_{max} = the initial velocity of the reaction at 20×10^{-5} M NAD^+.

Discussion

The present study extends the view that oxidation of sulfhydryl groups occurs in conjunction with kinetic transition in honeybee glyceraldehyde phosphate dehydrogenase and provides some insight into this difference in behavior between the two enzymes. It remains to be determined whether similar transitions in other enzymes also occur with changes in their sulfhydryl group chemistry.

Several lines of evidence demonstrate conclusively that the sulfhydryl reactivities of both *native* and *denatured* honeybee and rabbit muscle enzymes differ. Results in Figure 1 clearly indicate both a difference in the number of "fast" sulfhydryl groups between the two *native* enzymes and in the effect Nbs_2 has in destroying their catalytic activities. The relative reactivities of the sulfhydryls of honeybee enzyme have not been investigated previously, but results of the present studies regarding the number of "fast" sulfhydryls in the rabbit muscle enzyme are in excellent agreement with the observations of others. Studies with *o*-iodosobenzoate (Olson and Park, 1964), fluorodinitrobenzene (Shaltiel and Soria, 1969), and *p*-mercuribenzoate (Boross *et al.*, 1969) show that there are four fast reacting sulfhydryl groups in pig and rabbit muscle glyceraldehyde phosphate dehydrogenases. It is assumed that four such groups exist in honeybee glyceraldehyde phosphate dehydrogenase also. Because Nbs_2 reacts with only 2.5 sulfhydryls/mol of *native* honeybee glyceraldehyde phosphate dehydrogenase within 30 sec, it seems one "reactive" sulfhydryl is protected from reaction with this reagent under the conditions used in Figure 1. That this result is real is reinforced by the fact that only 75% of the enzyme's catalytic activity is destroyed in the same time period (as opposed to complete loss of activity in the rabbit muscle enzyme). The slow inactivation of honeybee glyceraldehyde phosphate dehydrogenases during the following 10 min could be explained either by slow reactivity of one additional "active" sulfhydryl group with Nbs_2 (after 3 mol of thiol reagent couple) or slow denaturation of the enzyme under the experimental conditions employed. The structural environment allowing for Nbs_2 reactivity of the "fast" sulfhydryl group is clearly different in the two enzymes, however. In support of this view, kinetic experiments by Boross *et al.* (1969) with pig muscle glyceraldehyde phosphate dehydrogenase and by Kemp and Forest (1968) with phosphofructokinase show that sulfhydryl groups in the native enzymes vary in their Nbs_2 reactivities, presumably because of activation or conformational restraints imposed by neighboring amino acids.

Further evidence for differences in sulfhydryl reactivity between the two *native* glyceraldehyde phosphate dehydrogenases is seen in Figure 2. Rabbit muscle enzyme shows no significant changes either in the number of "fast" sulfhydryls titrated with Nbs_2 or in sulfhydryl reactivity with Nbs_2 under the conditions employed. Furthermore, preincubation of *either* enzyme with NAD^+ alone resulted in no change while preexposure to NAD^+ plus glyceraldehyde 3-phosphate caused a small decrease in sulfhydryl reactivity. However,

while rabbit muscle glyceraldehyde phosphate dehydrogenase was not affected by preincubation with glyceraldehyde 3-phosphate, this treatment resulted in a marked increase in the reactivity of honeybee enzyme toward Nbs_2 as compared to preincubation with NAD^+. It is possible that exposure to glyceraldehyde 3-phosphate causes a partial unfolding of honeybee glyceraldehyde phosphate dehydrogenase, allowing greater reactivity of its sulfhydryl groups. This property is not seen with the rabbit muscle enzyme.

The total number of Nbs_2-reactive sulfhydryls in the *denatured* enzymes is also affected differently by preincubation of the native dehydrogenases with ligands. The data in Table I show that rabbit muscle glyceraldehyde phosphate dehydrogenase does not undergo significant change in this parameter as a result of various preincubation conditions. However, preincubation with certain combinations of ligands results in a decrease of *two* total Nbs_2-reactive sulfhydryls in the honeybee enzyme. This suggests the possibility of a disulfide bond being formed in honeybee glyceraldehyde phosphate dehydrogenase. The results with [^{14}C]bromoacetic acid indicate that, per mole of enzyme, 1.6–1.8 sulfhydryls are unavailable for S-carboxymethylation by iodoacetate after honeybee glyceraldehyde phosphate dehydrogenase is preincubated with either glyceraldehyde 3-phosphate or glyceraldehyde 3-phosphate plus NAD^+. This value is in excellent agreement with the change in total Nbs_2-reactive sulfhydryl groups (Table I). In agreement with the data in Table I, the sulfhydryl titer of rabbit muscle glyceraldehyde phosphate dehydrogenase was not *changed* by preincubation as measured by S-carboxymethylation. The base value of 0.6–0.8 mol of [^{14}C]carboxymethyl group incorporated per mol of rabbit muscle enzyme may represent an endogenous amount of mixed disulfides present after its isolation (Parker and Allison, 1969).

Conversion of honeybee glyceraldehyde phosphate dehydrogenase from the "metastable" to the stable form causes a disappearance of the transition region in the saturation curve (Figure 5). Regeneration of the anomolous kinetic curve can be accomplished by incubation of the newly formed stable enzyme with dithiothreitol. Coupled with evidence presented in Table I this experiment strongly suggests preincubation of "metastable" glyceraldehyde phosphate dehydrogenase causes the formation of a disulfide bond. Which sulfhydryls are involved is not known but curves 4 and 5 of Figure 2 tend to rule out the "fast" titratable sulfhydryl groups since essentially the same number are present in both forms of the *native* enzyme.

That variability exists in both the presence and position of sulfhydryl groups of representative mammalian (pig muscle) and arthropod (lobster) glyceraldehyde phosphate dehydrogenases has been conclusively shown by sequencing studies (Davidson *et al.*, 1967; Harris and Perham, 1968). Therefore, it is possible that structural differences in the locations of sulfhydryl groups permit disulfide bond formation in honeybee enzyme but prohibit it in rabbit muscle enzyme under identical conditions of exposure to glyceraldehyde 3-phosphate and glyceraldehyde 3-phosphate plus NAD^+. Intramolecular disulfide bond formation has been demonstrated after chemical treatment of enzymes. Guinea pig liver transglutaminase undergoes a dithiothreitol reversible inactivation when preincubated with Nbs_2 in the absence of calcium ion. Loss of activity in the enzyme coincides with the loss of two detectable sulfhydryl groups (Connellan and Folk, 1969). On the basis of mapping experiments the sulfhydryl groups involved were concluded to be those that bind glutamine while the single sulfhydryl group required for all catalytic activities of the enzyme was *not* part of the disulfide bond. This is similar to the observations noted in the present study. Perham and Harris (1964) reported the formation of intrachain disulfide bonds in mammalian glyceraldehyde phosphate dehydrogenase upon treatment of the native enzyme with *o*-iodosobenzoate. This resulted in complete inactivation of the enzyme. Parker and Allison (1969) showed that the immediate product of the reaction between *o*-iodosobenzoate and the active-site sulfhydryls of pig muscle glyceraldehyde phosphate dehydrogenase is not a disulfide but probably a sulfenyl derivative. This decomposes when the enzyme's conformation is changed to permit formation of a disulfide bond. Interestingly, the present studies show that the loss of two sulfhydryls occurs with only slight changes in enzymatic activity and the loss in sulfhydryl groups is induced by the natural substrates of the enzyme.

Honeybee and carpenter ant glyceraldehyde phosphate dehydrogenases display another striking difference with respect to rabbit muscle glyceraldehyde phosphate dehydrogenase. The two former enzymes are not inactivated or dissociated by physiological levels of adenosine triphosphate at 0° as is rabbit muscle enzyme (Gelb and Nordin, 1970; Oliver *et al.*, 1971). Low-temperature stability of their glyceraldehyde phosphate dehydrogenases probably plays a role in overwintering (Gelb *et al.*, 1971; Oliver *et al.*, 1971). Greene and Feeney (1970) have reported similar stability of the glyceraldehyde phosphate dehydrogenase from the cold-adapted Antarctic fish *Dissostichus mawsonii*.

References

Boross, L., Cseke, E., and Vas, M. (1969), *Acta Biochim. Biophys. Acad. Sci. Hung. 4*, 301.

Chrambach, A., Reisfeld, R., Wyckoff, M., and Zaccari, J. (1967), *Anal. Biochem. 20*, 150.

Connellan, J. M., and Folk, J. E. (1969), *J. Biol. Chem. 244*, 3173.

Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.

Corwin, L. M., and Fanning, G. K. (1968), *J. Biol. Chem. 243*, 3517.

Davidson, B. E., Sajgo, M., Noller, H., and Harris, J. (1967), *Nature (London) 216*, 1181.

Davis, B. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dayhoff, M., and Eck, R. (1967), Atlas of Protein Sequence and Structure, Silver Springs, Md., National Biomedical Research Foundation.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Gelb, W. G., and Nordin, J. H. (1969), Abstracts, 158th National Meeting of the American Chemical Society, New York, N. Y., Sept, No. BIOL 31.

Gelb, W. G., and Nordin, J. H. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 666 Abstr.

Gelb, W. G., and Nordin, J. H. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1132 Abstr.

Gelb, W. R., Oliver, E. J., Brandts, J. F., and Nordin, J. H. (1970), *Biochemistry 9*, 3228.

Gelb, W. G., Oliver, E. J., Brandts, J. F., and Nordin, J. H. (1971), *Cryobiology 8*, 474.

Gentner, N., and Preiss, J. (1968), *J. Biol. Chem. 243*, 5882.

Greene, F., and Feeney, R. (1970), *Biochim. Biophys. Acta 220*, 430.

Hall, L. M. (1960), *Biochem. Biophys. Res. Commun. 3*, 239.

Harrington, W. F., and Karr, G. M. (1965), *J. Mol. Biol. 13*, 885.

Harris, J., and Perham, R. (1965), *J. Mol. Biol. 13*, 876.
Harris, J., and Perham, R. (1968), *Nature (London) 219*, 1025.
Kagan, Z. S., and Dorozhko, A. I. (1973), *Biochim. Biophys. Acta 302*, 110.
Kato, G., Tan, E., and Yung, J. (1972), *J. Biol. Chem. 247*, 3186.
Kawahara, K., and Tanford, C. (1966), *Biochemistry 5*, 1578.
Kemp, R. G., and Forest, P. B. (1968), *Biochemistry 7*, 2596.
LeJohn, H. B., and Jackson, S. (1968), *J. Biol. Chem. 243*, 3447.
Levitski, A., and Koshland, D. E., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.
Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951), *J. Biol. Chem. 193*, 265.
Marquardt, R. R., Carlson, C. W., and Brosemer, R. W. (1968), *J. Insect. Physiol. 14*, 317.
Matthew, E., Merriwether, B., and Park, J. H. (1967), *J. Biol. Chem. 242*, 5024.
Murdock, A., and Koeppe, O. (1964), *J. Biol. Chem. 239*, 1983.
Nordin, J. H., Duffield, R., Freedman, N., Gelb, W. G., and Brandts, J. F. (1970), *Cryobiology 6*, 373.
Oliver, E. J., Gelb, G. W., Evans, F., Brandts, J. F., and Nordin, J. H. (1971), *Cryobiology 8*, 465.
Olson, E., and Park, J. H. (1964), *J. Biol. Chem. 239*, 2316.
Parker, D., and Allison, W. S. (1969), *J. Biol. Chem. 244*, 180.
Perham, R., and Harris, J. (1964), *Proc. Int. Congr. Biochem., 6th*, 293.
Shaltiel, S., and Soria, M. (1969), *Biochemistry 8*, 4411.
Somero, G. N. (1969), *Biochem. J. 114*, 237.
Somero, G. N., and Hochachka, P. W. (1969), *Nature (London) 223*, 194.
Sund, H., Weber, K., and Molbert, C. (1967), *Eur. J. Biochem. 1*, 400.
Tanford, C., Kawahara, K., and Lapanje, S. (1967), *J. Amer. Chem. Soc. 89*, 729.
Teipel, J., and Koshland, D. E., Jr. (1969), *Biochemistry 8*, 4656.
Velick, S. (1955), *Methods Enzymol. 1*, 401.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Zahler, W., and Cleland, W. W. (1968), *J. Biol. Chem. 243*, 716.

Lactate Racemase. Direct Evidence for an α-Carbonyl Intermediate†

Allan Cantwell and Don Dennis*

ABSTRACT: Evidence in support of a direct internal hydride transfer mechanism for lactic acid racemization is presented. The proposed symmetrical α-carbonyl intermediate has been trapped as an enzyme-bound oxime in the presence of hydroxylamine and as a dissociable reduced derivative lactaldehyde when the reaction is conducted in the presence of sodium borohydride.

Studies in our laboratory have presented evidence in support of the hypothesis that a direct internal hydride shift is involved in the racemization of lactic acid as catalyzed by the enzyme lactate racemase (EC 5.1.2.1.) derived from *Clostridium butylicum* (Dennis and Kaplan, 1963; Shapiro and Dennis, 1965, 1966). This report describes the effect of hydroxylamine as a potent inhibitor of the racemase reaction resulting in the trapping of bound [1-^{14}C]lactate on the enzyme as an oxime. The inhibition is reversed and the [^{14}C]lactate is released upon the addition of a competing carbonyl compound, namely pyruvic acid.

The reaction intermediate involving an α-carbonyl of lactic acid can be reduced, dissociated, and isolated when borohydride reduction is performed during the reaction in the presence of lactic acid. This dissociable reduction product has been identified as lactaldehyde derived from lactic acid.

This evidence strongly supports the hypothesis that a direct internal hydride shift of the α-hydrogen of the substrate occurs with the resultant formation of a symmetrical α-carbonyl intermediate which can undergo reduction with borohydride or oxime formation upon the addition of hydroxylamine.

† From the Department of Chemistry, The University of Delaware, Newark, Delaware 19711. *Received August 8, 1973.* This work was supported by the National Science Foundation (Grant GB-7886). This work was carried out in partial fulfillment of the requirements for the Ph.D. degree (A. C.).

Experimental Procedure

Materials. D(−)- and L(+)-lithium lactate salts (grade A) were obtained from either Calbiochem or Miles laboratories. Sodium pyruvate was the product of Sigma Chemical Co. A crystalline ammonium sulfate suspension of beef heart lactate dehydrogenase type III was obtained from Sigma Chemical Co. Tritiated sodium borohydride, D,L-sodium [1-^{14}C]lactate, sodium [2-^{14}C]pyruvate, [1-^{3}H]ethanol, and [U-^{14}C]benzoic acid were purchased from the New England Nuclear Corporation. Tritiated H_2O was the product of Packard. Aquasol liquid scintillation counting solution and low potassium content vials were obtained from the New England Nuclear Corporation. Thin layer plates (type K 301 R silica gel with fluorescent indicator) were purchased from Eastman Chemical Corp. Methylglyoxal and 1,2-propanediol were purchased from Aldrich Chemical Co. and were redistilled upon receipt. Sodium borohydride was the product of Metal Hydrides Inc. Egg white lysozyme was purchased from Worthington Biochemical Corporation. Bio-Gel 150 and Sephadex G-100 resins were obtained from Bio-Rad Laboratories and Pharmacia Fine Chemicals, respectively. Acrylamide bisacrylamide, ammonium persulfate, and Coomassie Blue were the products of Canalco. Lactaldehyde was made by the method of Abeles (1959).

Methods. STANDARD RACEMASE REACTION ASSAY SYSTEM. The reaction contained 1.0 ml of 0.1 M sodium acetate buffer