Modulation of Heart Muscle Mitochondrial Malate Dehydrogenase Activity. II. *p*-Mercuribenzoate Activation, Model of a Possible Allosteric Control Mechanism for Substrate Homeostasis*

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ABSTRACT: Some properties of *p*-mercuribenzoate-activated heart muscle mitochondrial malate dehydrogenase were investigated with a view to elucidating the mechanism and possible significance of the activation. The pH optimum was not altered by activation, but catalytic rate disparity between activated and native enzyme was progressively diminished above pH 8. Activated enzyme was inactivated more rapidly at 38° than native enzyme until the two rates were equal; thereafter the rates of inactivation were the same. Neither subunit dissociation nor enzyme aggregation was detected with activating or inactivating *p*-mercuribenzoate levels by sucrose density gradient centrifugation.

The presence of activation was dependent on substrate concentration. At all coenzyme concentrations studied, activation was found at saturating and inhibition at low concentration of substrate due to elevation of both V_{max} and K_m. Activation of initial net velocity of oxalacetate reduction and of the equilibrium rates nicotinamide–adenine dinucleotide $\rightleftarrows$ reduced nicotinamide–adenine dinucleotide and oxalacetate $\rightleftarrows$ malate were parallel. Net and equilibrium kinetic study suggested that activation results from increase in rate-limiting coenzyme dissociation and hence in V_{max}, while substrate dissociation rate and K_m are simultaneously increased. Decrease in the rate of chemical transformation to near rate limiting by levels of *p*-mercuribenzoate resulting in about 98% inhibition of activity was suggested by the marked decrease in the oxalacetate $\rightleftarrows$ malate rate to near equality with the nicotinamide–adenine dinucleotide $\rightleftarrows$ reduced nicotinamide–adenine dinucleotide rate. Mitochondrial malate dehydrogenase activation at saturating and inhibition at low substrate concentration by reaction with 3–4 moles of *p*-mercuribenzoate/mole of enzyme at an allosteric site suggests an homeostatic mechanism for control of substrate concentration which may be mediated by a physiological effector *in vivo*.

The activating and inactivating effect of *p*-mercuribenzoate on heart muscle mitochondrial malate dehydrogenase activity was described in a preceding paper (Silverstein and Sulebele, 1970). In this communication some properties of PMB-activated M-MDH[1] were investigated with a view to elucidating the mechanism of activation and its possible physiologic significance. It is shown by initial net and equilibrium kinetics that the PMB reaction results in a substrate-specific potential homeostatic mechanism for control of substrate concentration by increasing both V_{max}[2] and K_m due to increase in coenzyme and substrate dissociation rates. These alterations result in activation at saturating and inhibition at low substrate concentration.

Materials and Methods

All chemicals used were of reagent grade. Sources have been given (Silverstein and Sulebele, 1970).

Activation with p-Mercuribenzoate. Unless otherwise stated, M-MDH was activated by reaction with 4 equiv of PMB/mole of enzyme in 0.4 ml of 0.1 M K phosphate or 0.2 M Tris-NO_3 (pH 8.0) for 18–20 hr at 0°. Overnight incubation was used only for convenience; activation stable at 0° resulted after 1–2-hr incubation.

Initial Velocity Measurements. Measurement of initial velocity of malate dehydrogenase reaction was done by monitoring the rate of change of NADH concentration as reflected in the absorbance at 340 mμ in a Gilford spectrophotometer equipped with a chart recorder. Temperature was maintained constant at 5° in a thermostatted cell compartment by circulation of water from a Forma water bath. Reaction was initiated by addition of enzyme with an adder–mixer (Boyer and Segal, 1954). Unless otherwise indicated, assay conditions were identical with those already described (Silverstein and Sulebele, 1970).

Equilibrium Velocity Measurements. Measurements of reaction velocities at equilibrium were performed at 1° by means of tracer concentrations of [^{14}C]oxalacetate and [^{14}C]NADH, prepared as described previously (Silverstein and Sulebele,

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[1] Abbreviations used are: PMB, *p*-mercuribenzoate; PHMB, *p*-hydroxymercuribenzoate (the general designation PMB and the more specific designation PHMB have been used interchangeably; the major species under the experimental conditions used is the *p*-hydroxy form (Boyer, 1954)); M-MDH, mitochondrial malate dehydrogenase.

[2] V_{max} and K_m refer to these constants obtained at and referable to one optimum concentration of the second reactant and were determined identically with native and PMB-activated M-MDH.

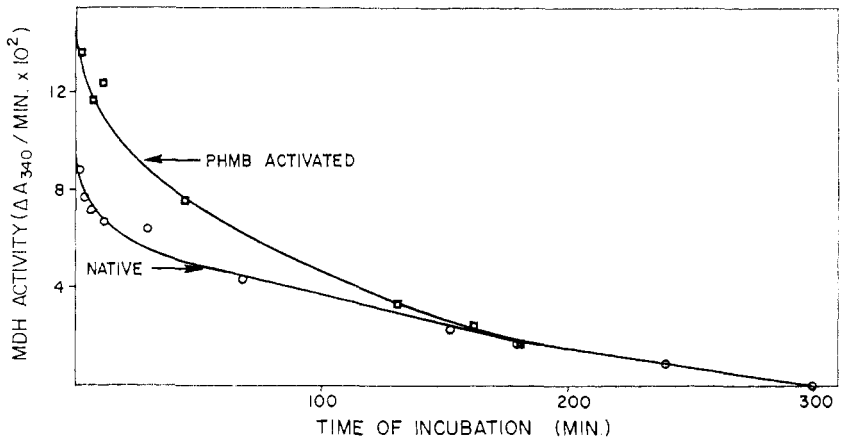

FIGURE 1: Heat inactivation of native and *p*-mercuribenzoate-activated mitochondrial malate dehydrogenase. Bovine heart M-MDH (143 $\mu\mu$moles) was activated by incubation with 4 equiv of PMB/mole of enzyme at 0° for 20 hr in 0.4 ml of 0.2 M Tris-NO_3 (pH 8.0). A control M-MDH solution was similarly incubated in the absence of PMB. Both solutions were then incubated in a water bath at 38°. Aliquots of solution were assayed for activity before and during the 38° incubation.

1969a; Silverstein and Boyer, 1966). Separation of oxalacetate and malate was done by precipitation of oxalacetate as the 2,4-dinitrophenylhydrazone, while NAD and NADH were separated on DEAE-cellulose columns. Reaction was terminated with $AgNO_3$ after 40–80% of isotopic equilibrium had been attained. The general procedure for measurement of oxalacetate $\rightleftarrows$ malate and NAD $\rightleftarrows$ NADH rates has been described (Silverstein and Sulebele, 1969a).

Sucrose Density Gradient Centrifugation. Continuous gradients between 2.4 ml of 6% sucrose and 2.3 ml of 25% sucrose in 50 mM K phosphate (pH 8.0) were prepared at 4° in a Büchler gradient former. Pig heart mitochondrial MDH (3.2 mμmoles) was activated with 3.2 equiv of PMB/mole of enzyme and inactivated with 20 equiv of PMB/mole of enzyme in 0.4 ml of 0.1 M K phosphate (pH 8.0) by reaction for 20 hr at 0°. Sucrose density gradients were layered with 0.3 ml of native, activated, or inactivated enzyme and centrifuged for 16 hr at 5° at 37,000 rpm in a SW-50 rotor in an L-2 Spinco ultracentrifuge. Three drop fractions were assayed for protein (Lowry *et al.*, 1951) and activity (Silverstein and Sulebele, 1970).

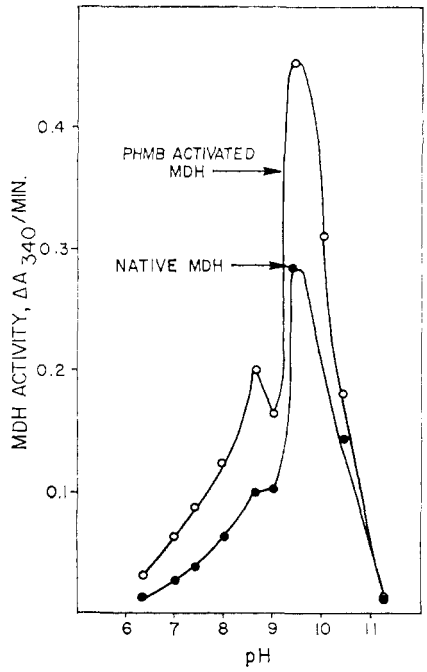

FIGURE 2: pH and activity profile of native and *p*-mercuribenzoate-activated mitochondrial malate dehydrogenase. Bovine heart M-MDH (286 $\mu\mu$moles) was activated by reaction with 4 equiv of PMB/mole of enzyme in 0.8 ml of 0.2 M Tris-NO_3 (pH 8.0) at 0° for 20 hr. Enzyme without PMB was similarly incubated. Samples of both enzyme solutions were then assayed for enzymatic activity from pH 6.4 to 11.25 as described in Materials and Methods with the substitution of the following buffers: 83 mM K phosphate (pH 6.4); 90 mM K phosphate (pH 7.0); 120 mM Tris-Cl (pH 7.4); 170 mM Tris-Cl (pH 8.0); 370 mM Tris-Cl (pH 8.6); 762 mM Tris-Cl (pH 9.0); 310 mM glycine-Na (pH 9.4); 150 mM glycine-Na (pH 10.0); 210 mM glycine-Na (pH 10.4), and universal buffer (pH 11.25), 28.6 mM with respect to diethylbarbituric acid, citric acid, KH_2PO_4, and boric acid.

Results

Heat Inactivation. PMB-activated M-MDH was inactivated more rapidly than native MDH at 38° until the two rates became equal at 3 hr. Thereafter both enzymic activities declined at the same rate until almost total inactivation occurred at 5 hr (Figure 1).

pH Optimum. The pH optimum for the reaction of NADH with oxalacetate was 9.4 for both native and PMB-activated bovine heart M-MDH (Figure 2), which is close to the figure of 9.2 reported previously (Grimm and Doherty, 1961). The pH profiles were also generally similar. The disparity in activity tended to diminish with increasing pH above 8 and was almost completely abolished at pH 11.25.

Comparison of Kinetic Properties with Oxalacetate and Mesoxalate. Studies of the effect of oxalacetate concentration on the initial velocity of PMB-activated and native M-MDH revealed an interesting property of the PMB–enzyme. The enzyme is activated when oxalacetate concentration is at or near saturation, but inhibited at low oxalacetate concentration (Figure 3). The reason for this unusual behavior is that reaction of 3–4 moles of PMB/mole of M-MDH increases the maximum velocity (V_{max})[2] by 36% enabling activation, while simultaneously increasing K_m for oxalacetate by 131%, thus requiring increased oxalacetate concentration for saturation. The combination of the two alterations results in decreased initial velocity at low oxalacetate concentration and increased velocity at saturating concentration (Table I). This property has the interesting effect of tending to stabilize oxalacetate

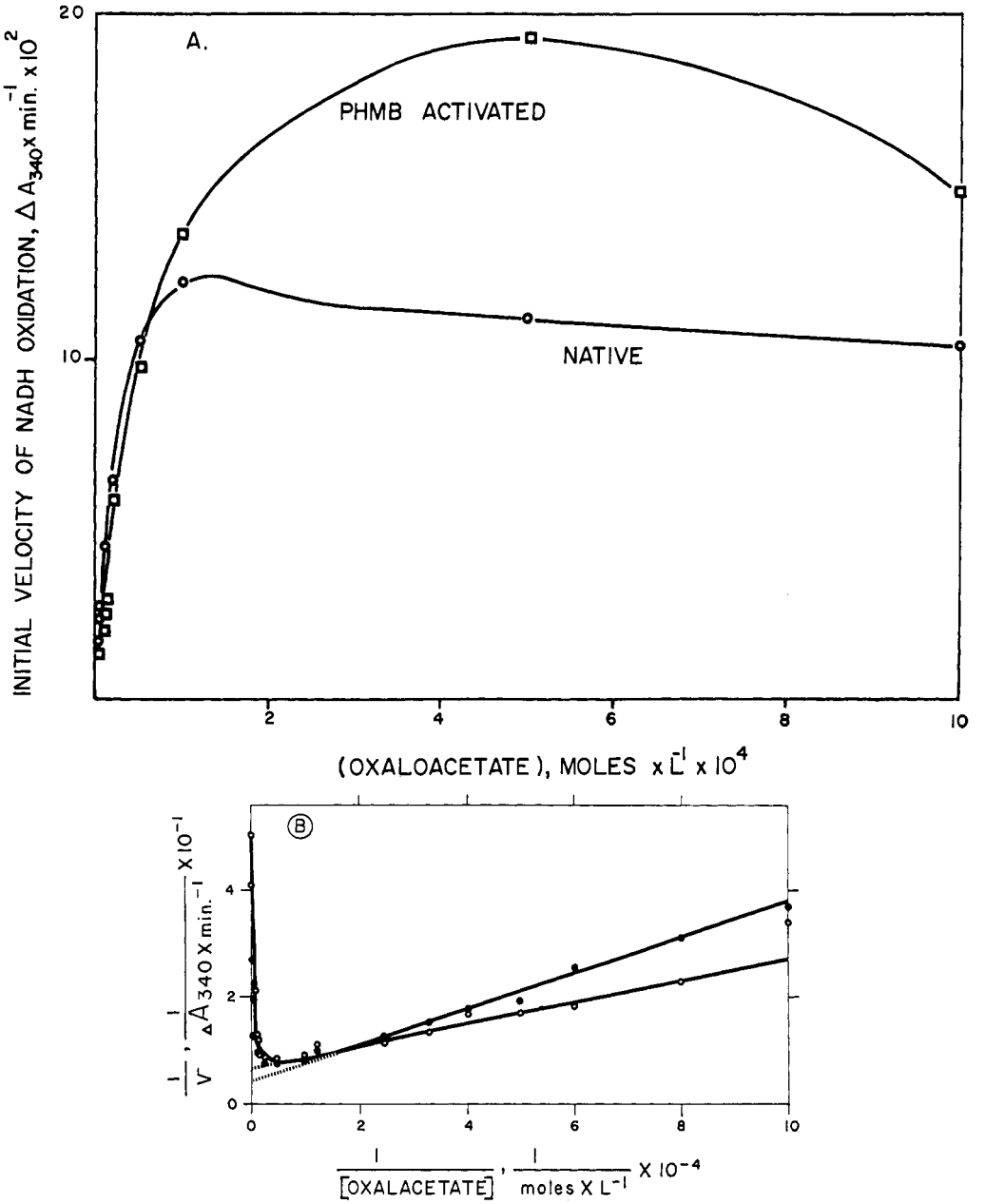

FIGURE 3: Effect of oxalacetate concentration on native and *p*-mercuribenzoate-activated mitochondrial malate dehydrogenase catalyzed initial rate of NADH oxidation. Pig heart M-MDH (3.4 mμmoles) was activated in 0.1 M K phosphate buffer. A control enzyme solution was similarly prepared and treated without PMB. The assay mixture for determination of net initial velocity contained 320 μmoles of Tris-NO_3 (pH8.0), 209 mμmoles of NADH, 0.365 μg of enzyme, and oxalacetate as indicated in a total volume of 1.635 ml at 5°. (A) Substrate–velocity plot. (B) Double reciprocal plot (different experiment); (○) native enzyme, (●) PMB-activated enzyme.

concentration by increasing its rate of conversion into malate when its concentration is high and decreasing the rate of conversion when concentration is low. Substrate inhibition by oxalacetate in excess of 0.12 mM (native enzyme) found in these experiments is characteristic of mitochondrial malate dehydrogenase and has been described by others (Siegel and Englard, 1961).

PMB activation was specific for physiological substrate; activation was absent with the unphysiologic substrate mesoxalate (Figure 4). At all mesoxalate concentrations studied PMB-reacted enzyme was less active than native. The reason for this is indicated in Table I. In contrast to results with oxalacetate, K_m was increased by only 4%, while V_{max} was decreased by 73% instead of increased.

Effect of NADH Concentration on Initial Velocity of Oxalacetate Reduction. The role of NADH in determining activation of oxalacetate reduction was next investigated. It was found that activation was present at all NADH levels studied with oxalacetate at saturating levels (0.5 mM) (Figure 5). V_{max} was increased by 68% while the K_m was increased by 91% (Table I). Substrate inhibition by NADH above 200 μM was less marked for PMB-activated enzyme than for native

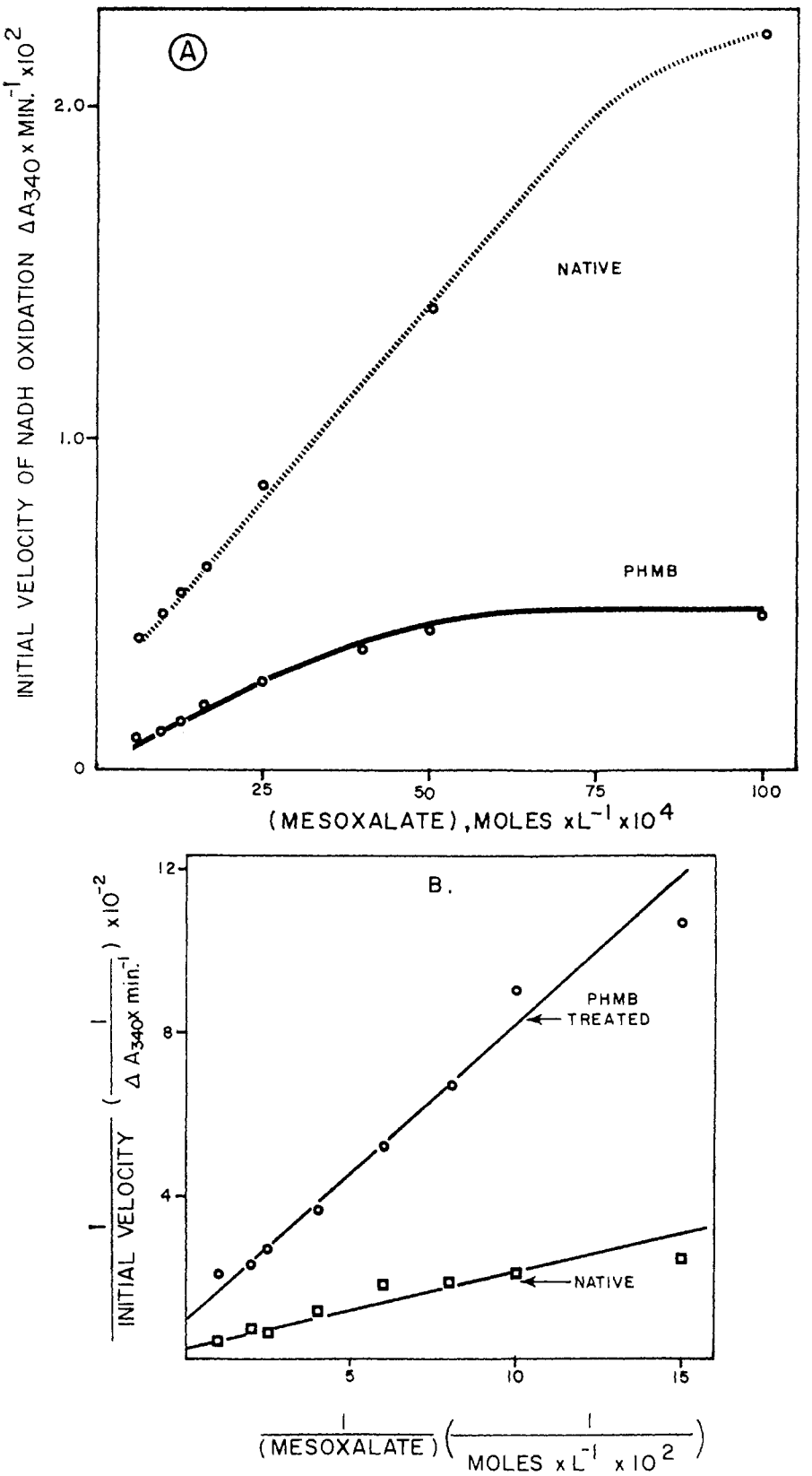

FIGURE 4: Effect of mesoxalate concentration on native and *p*-mercuribenzoate-activated pig heart mitochondrial malate dehydrogenase catalyzed initial rate of NADH oxidation. Conditions were identical with those in Figure 3 except for replacement of oxalacetate by mesoxalate. (A) Substrate-velocity plot. (B) Double-reciprocal plot.

TABLE I: Kinetic Parameters of Native and *p*-Mercuribenzoate-Activated Pig Heart Mitochondrial Malate Dehydrogenase.

Reactant	V_{max}, ΔA_{340}/min $\times 10^2$		K_m (μM)	
	Native	PMB Activated	Native	PMB Activated
Oxalacetate	15	21	30	70
Mesoxalate	4.2	1.1	80×10^2	83×10^2
Malate	5.8	7.2	28×10	77×10
NADH	3.5	5.9	14	26
NAD	7.4	11	79	73

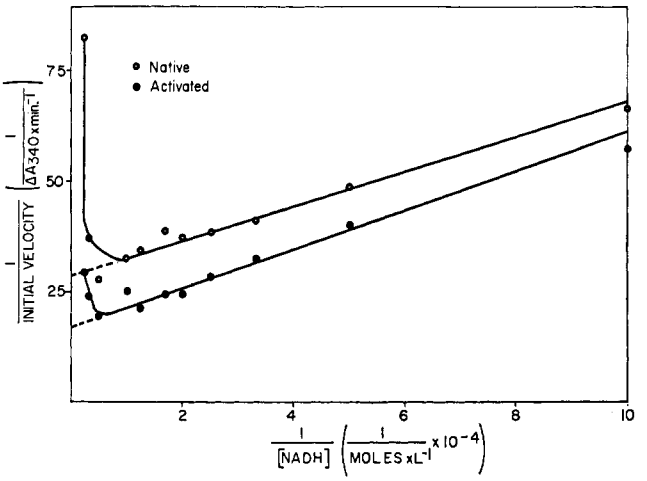

FIGURE 5: Effect of NADH concentration on the initial rate of oxalacetate reduction with native and *p*-mercuribenzoate-activated pig heart mitochondrial malate dehydrogenase. M-MDH was activated and assayed as in Figure 3. Oxalacetate concentration was 0.5 mM and NADH as indicated. Double-reciprocal plot.

enzyme, resulting in the most marked activation at the highest NADH concentrations studied.

The critical role of oxalacetate in determining activation was shown by the observation that at lower nonsaturating levels of oxalacetate (0.12 mM) at which equality of PMB-treated and native MDH initial velocities were observed with 128 μM NADH, equality of rates were also observed at varying levels of NADH. Thus activation is governed by substrate rather than coenzyme concentration.

Effect of Malate Concentration on Initial Velocity of NAD Reduction. Catalysis of malate oxidation by PMB-activated M-MDH was similarly altered. Activation of malate oxidation, just as with oxalacetate reduction, was dependent on its concentration. Activation was observed above 2 mM malate and inhibition below (Figure 6). With NAD at 500 μM V_{max} was increased by 26.5% while K_m was increased by 175% (Table I). For both native and activated enzyme malate concentration above 100 mM caused inhibition.

Effect of NAD Concentration on Initial Velocity of Malate Oxidation. NAD reduction at 61 mM malate catalyzed by PMB-treated enzyme was activated as compared with native enzyme at all NAD concentrations (Figure 7). In a manner analogous to that found with oxalacetate reduction, the concentration of malate rather than NAD governed the presence of activation or inhibition. V_{max} for PMB-activated enzyme was increased by 45% as compared with native MDH while K_m decreased by 8% (Table I).

Kinetics at Equilibrium. In order to determine whether any features not observable by initial rate kinetics were occurring with PMB-activated and -inactivated heart M-MDH and to gain more complete understanding of the mechanism by which PMB–mercaptide formation affects the catalysis, the effect of PMB multiplicity on kinetics at equilibrium was investigated and compared with net initial reaction velocities (Silverstein, 1968, 1970). An equilibrium mixture near saturation with respect to all reactants was prepared, mitochondrial MDH solutions previously reacted with various equivalents of PMB/mole of enzyme were added, and the reaction rates, NAD $\rightleftarrows$ NADH and oxalacetate $\rightleftarrows$ malate, were determined.

The NAD $\rightleftarrows$ NADH and oxalacetate $\rightleftarrows$ malate rates at equilibrium generally followed a pattern of activation and inactivation which was similar to that seen with initial velocity measurements (Figure 8). The marked disparity between NAD $\rightleftarrows$ NADH and oxalacetate $\rightleftarrows$ malate rates at all but the most elevated PMB levels indicates that under these conditions coenzyme dissociation rather than chemical transformation is rate limiting in the overall catalysis (Boyer, 1959; Silverstein, 1963; Boyer and Silverstein, 1963; Silverstein and Sulebele, 1969a). The increased maximum velocity of initial net reaction (V_{max}) of PMB-activated M-MDH is thus due to increased rate of dissociation of coenzyme. The simultaneous activation of the oxalacetate $\rightleftarrows$ malate rate by PMB, coupled with increased K_m for both oxalacetate and malate by initial net velocity kinetics, is best explained as being due to increased rate of substrate dissociation which is likely limiting under these conditions in the oxalacetate $\rightleftarrows$ malate rate.

At greater than activating levels of PMB the oxalacetate $\rightleftarrows$ malate rate fell steadily to almost equality with the NAD $\rightleftarrows$ NADH rate at the highest inhibitor level studied (Figure 8). This result suggests the interesting possibility that PMB–mercaptide formation with the more slowly reacting sulfhydryls results in decreasing chemical transformation rate which may be limiting under these conditions in the oxalacetate $\rightleftarrows$ malate rate. Equality of oxalacetate $\rightleftarrows$ malate and NAD $\rightleftarrows$ NADH rates would be expected if the chemical transformation rate became much smaller than dissociation rates (Boyer, 1959; Silverstein, 1963; Boyer and Silverstein, 1963; Silverstein and Boyer, 1964).

Sucrose Density Gradient Centrifugation. In order to detect any subunit structure alteration associated with PMB activation and inactivation as has been seen with *Escherichia coli* asparate transcarbamylase (Gerhart and Schachman, 1965), sucrose density gradients were run with activated and inactivated pig heart M-MDH. No difference in the sedimentation of native, activated, and inactivated enzyme was observed. No evidence was thus obtained for any subunit dissociation or association phenomena on reaction with PMB which could be correlated with activation or inactivation of mitochondrial MDH.

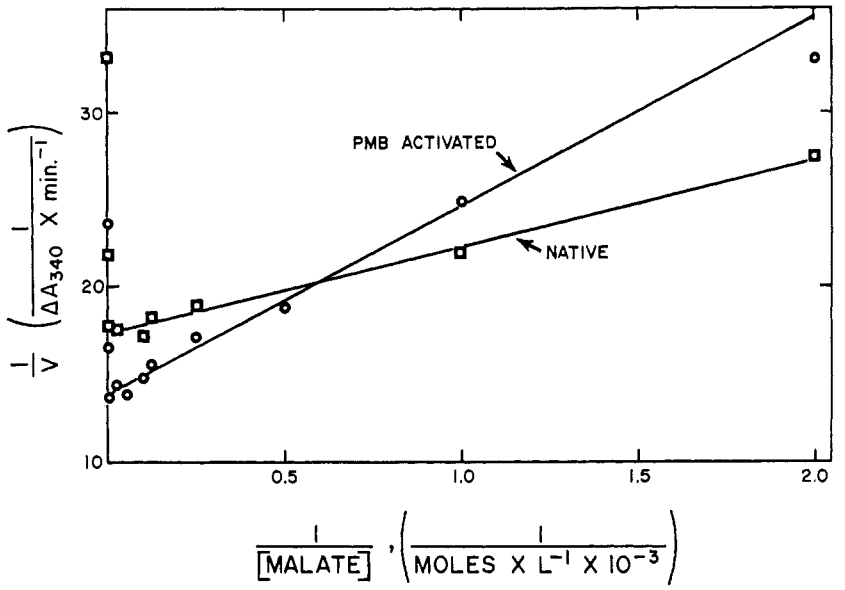

FIGURE 6: Effect of malate concentration on the initial rate of NAD reduction with native and *p*-mercuribenzoate-activated pig heart mitochondrial malate dehydrogenase. Activation was in K phosphate (2.04 mμmoles of MDH/0.4 ml; see Materials and Methods). The assay mixture contained 55 mM glycine-Na, 500 μM NAD, 420 μμM MDH, and malate as indicated at 5°. Double-reciprocal plot.

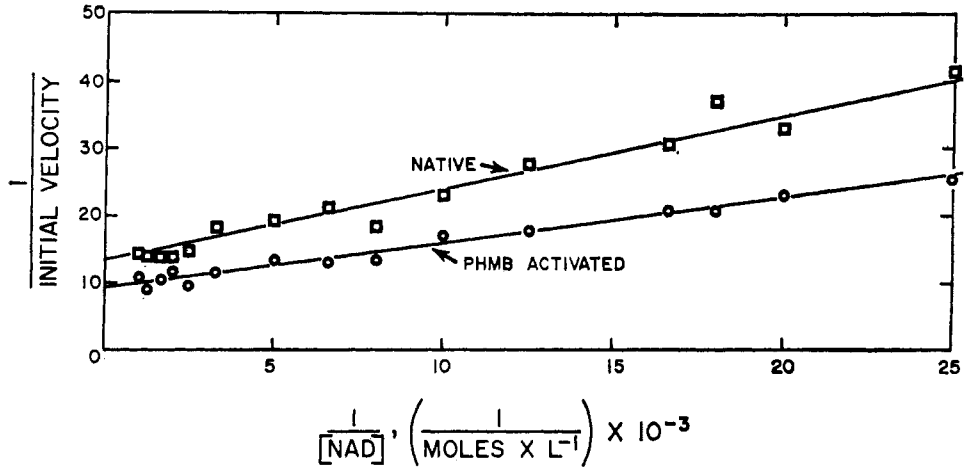

FIGURE 7: Effect of NAD concentration on the initial rate of malate oxidation with native and *p*-mercuribenzoate-activated pig heart mitochondrial malate dehydrogenase. Activation and assay were performed as in Figure 6 except for the presence in the assays of 61 mM malate and NAD as indicated.

Discussion

The most important aspect of these results is the kinetic elucidation of the mechanism of *p*-mercuribenzoate activation of mitochondrial malate dehydrogenase and the potential allosteric control mechanism for intramitochondrial substrate concentration which this mechanism suggests. The maximum initial velocity and Michaelis constant for both oxalacetate and malate were increased. This dual effect has the interesting result of causing an increased velocity of reaction when substrate concentration is at or near saturation and a decreased velocity when substrate is substantially less than saturating. Such a change in M-MDH activity would tend to maintain substrate concentration at a more constant intermediate level (neither as low or as high) than would result with the same concentration of native M-MDH molecules. Such a phenomenon may conceivably become operative under conditions requiring substrate homeostasis and be reversed under conditions necessitating rapid reaction of substrate at low concentration or build-up of high substrate levels.

The rapidly reversible PMB activation of M-MDH thus suggests a possible mechanism for control of enzyme activity and intramitochondrial homeostasis by a naturally occurring reactant having an effect similar to PMB by reaction with two sulfhydryl groups per M-MDH subunit at an allosteric site (Silverstein and Sulebele, 1970). The possibility of a naturally occurring sulfhydryl allosteric effector has been suggested on the basis of the fluorodinitrobenzene and PMB activation of rabbit muscle aldolase (Cremona *et al.*, 1965).

The possible physiologic significance of PMB activation is further suggested by its absence with the unnatural substrate mesoxalate (ketomalonate) with which inhibition is seen at all concentrations due to reduction instead of increase in V_{max}. This result further suggests the possibility of a reaction mechanism for ketomalonate different from that for oxalacetate in which substrate dissociation or chemical transformation

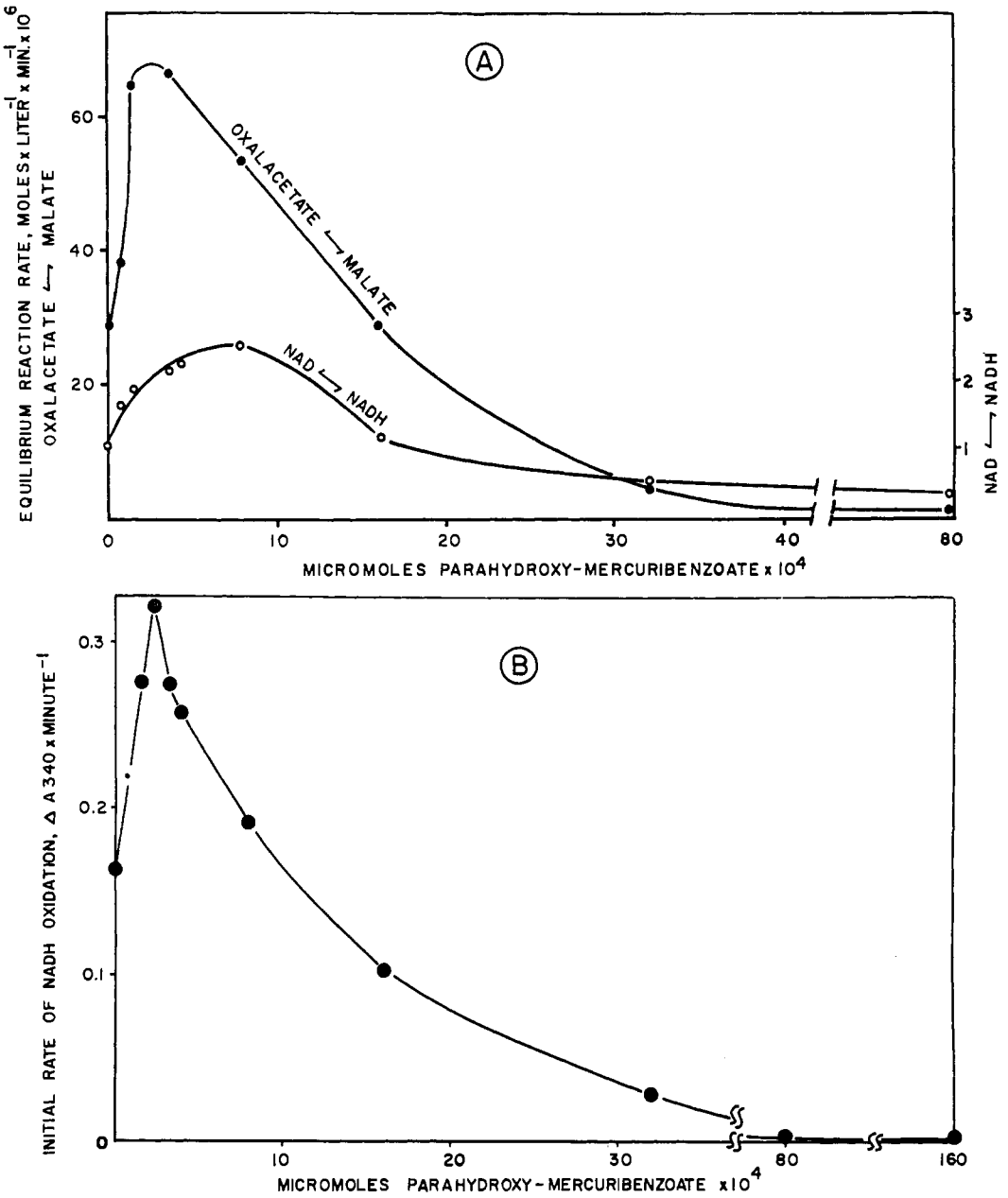

FIGURE 8: Effect of *p*-mercuribenzoate on reaction velocity catalyzed by mitochondrial malate dehydrogenase at 1°. Bovine heart M-MDH (98 $\mu\mu$moles) was reacted in 0.3 ml of 0.2 M Tris-NO_3 (pH 8.0) with PMB as indicated for 20 hr at 0°. (A) Reaction rates at equilibrium. Reactant concentrations were 6.14 mM NAD, 61.8 μM NADH, 29 mM malate, 290 μM oxalacetate, and 43 mμM MDH in 109 mM Tris-NO_3 (pH 8.0). (B) Initial rate of oxalacetate reduction.

rather than coenzyme dissociation may be rate limiting. If such a difference exists, it may perhaps offer an alternative explanation for recent interesting kinetic results with ketomalonate obtained by Harada and Wolfe (1968) other than the reciprocating compulsory order mechanism proposed (Silverstein and Sulebele, 1969a).

Study of kinetics at equilibrium reveals the basic mechanism of activation, increase in rate-limiting coenzyme dissociation rate which results in increase in V_{max}. This is indicated by the increase in the NAD $\rightleftarrows$ NADH rate, which is only about 3% of the oxalacetate $\rightleftarrows$ malate rate. Increase in K_m for oxalacetate and malate is compatible with increased dissociation rates for both malate and oxalacetate and with increase in the oxalacetate $\rightleftarrows$ malate equilibrium rate. This analysis suggests that under these conditions substrate dissociation rather than chemical transformation is normally limiting in the oxalacetate $\rightleftarrows$ malate rate and that the rate of chemical transformation is still greater (Silverstein and Sulebele, 1969a). PMB activation with its dual increase in V_{max} and K_m thus likely results from increases in the rates of dissociation of both substrate and coenzyme.

PMB inactivation by reaction of increasing numbers of sulfhydryls beyond about seven–eight/mole results in a marked decrease in both the oxalacetate $\rightleftarrows$ malate equilibrium rate as

well as in the marked disparity between oxalacetate $\rightleftarrows$ malate and NAD $\rightleftarrows$ NADH rates, suggesting that chemical transformation may be markedly reduced to near rate limiting in PMB inactivation. These as well as other studies with horse liver alcohol dehydrogenase (Silverstein and Sulebele, 1967; Silverstein, 1968) and glutamate dehydrogenase (Silverstein and Sulebele, 1969b), suggest that investigation of equilibrium reaction rate may be useful in elucidating the mechanism of enzyme modifier action (Silverstein, 1970).

Gross subunit structure as determined by sucrose density gradient centrifugation was similar for native and PMB-activated enzyme as well as inactivated enzyme. This finding apparently rules out the suggestion of Devenyi *et al.* (1966) that activation may be an association phenomenon, since no aggregate larger than native was discerned. The pH optimum of native and activated enzyme were also similar, while activated enzyme activity was more heat labile than native until about 80% inactivation had occurred. From these results it appears probable that activation does not qualitatively alter the basic mechanism by which catalysis occurs, but likely effects change to an enzyme conformation which is more heat labile, generally binds reactant ligands less tightly, and presumably has a less compact structure than native enzyme.

While the precise mechanism by which activation is induced by PMB–mercaptide formation is not definitely proven, the weight of evidence suggests that the mercaptide does not participate directly in enhanced catalysis at the catalytic center (Silverstein and Sulebele, 1970) but rather induces conformation change which results in more rapid dissociation of substrate and coenzyme at the catalytic site.

It is of interest that activity changes suggestive of those found in these experiments with PMB activation were reported by Theorell and McKinley-McKee (1961) for liver alcohol dehydrogenase. The ligand imidazole stimulated the reaction at high substrate concentration due to increase in coenzyme dissociation rate and was inhibitory at low substrate concentration due to an effect on the initial phase of reaction. These authors also pointed out that substances of this kind would have an homeostatic function in maintaining substrates at a suitable concentration.

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