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J. Biol. Chem. 1978, 253:8697-8701.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 253, No. 24, Issue of December 25, pp. 8697-8701, 1978
Printed in U.S.A.

Substrate Inhibition of the Mitochondrial and Cytoplasmic Malate Dehydrogenases*

(Received for publication, March 10, 1978)

Larry H. Bernstein,‡ Matthew B. Grisham, Kenneth D. Cole, and Johannes Everse§

From the Department of Pathology, University of South Alabama College of Medicine, Mobile, Alabama 36618 and the Department of Biochemistry, Texas Tech University School of Medicine, Lubbock, Texas 79430

The mechanism that leads to an inhibition of enzyme activity in the presence of high concentrations of substrate was investigated with the two malate dehydrogenase isoenzymes obtained from pig heart. The inhibition is promoted by an abortive binary complex formed by the enzymes and the *enol* form of oxalacetate. Neither the oxidized coenzyme nor the reduced coenzyme appears to be involved in the formation of this complex. These results suggest that the mechanism of substrate inhibition that occurs with the pig heart malate dehydrogenases is different from that observed with the lactate dehydrogenases from chicken hearts.

The inhibition constants for oxalacetate are 2.0 mM with the mitochondrial enzyme and 4.5 mM with the cytoplasmic enzyme. Since the *in vivo* concentration of oxalacetate is reported to be about 10 μM, these data suggest that the substrate inhibition that is exhibited by the malate dehydrogenases may not be of any significance *in vivo*.

Several years ago, we demonstrated that the inhibition of lactate dehydrogenase by high concentrations of pyruvate is caused by the formation of an abortive complex consisting of the enzyme, pyruvate, and NAD^+ (1-5). An investigation of the structural properties of the ternary complex revealed that the complex possesses an absorption maximum at 335 nm and that a covalent bond was formed between the nicotinamide ring of the NAD^+ and the pyruvate moiety (3, 4). Further investigations by Sugrobova *et al.* (6-8) and by Burgner and Ray (9) demonstrated that the *enol* form of pyruvate is responsible for the complex formation.

The study described in this paper was initiated under the assumption that similar abortive ternary complexes may be formed by the malate dehydrogenases. Such a hypothesis appeared reasonable because previous experiments have shown that the malate dehydrogenases are also subject to a significant inhibition when these enzymes are assayed in the presence of high concentrations of substrate (10-12). Kitto and Kaplan (10) demonstrated that the mitochondrial malate dehydrogenase from chicken hearts is more sensitive to inhibition by high concentrations of oxalacetate than the cytoplasmic enzyme. On the other hand, the cytoplasmic enzyme shows a stronger inhibition in the presence of high concentrations of L-malate than does the mitochondrial enzyme.

Since the two forms of malate dehydrogenase serve as part of a shuttle for the transport of reducing equivalents across the mitochondrial membrane, Kaplan (13, 14) proposed that in this function, the activities of the two enzymes could be regulated by the formation and dissociation of abortive ternary complexes. Such regulation of the activities of the two malate dehydrogenases could play an important role in the maintenance of the oxidation-reduction state of the pyridine nucleotides inside as well as outside the mitochondrion.

It appeared, therefore, appropriate to conduct a more detailed investigation of the substrate inhibition of the two malate dehydrogenases in order to establish the mechanism by which this inhibition occurs. This paper deals with the results of such a study.

MATERIALS AND METHODS

Sodium pyruvate, oxalacetic acid, and pig heart mitochondrial and cytoplasmic malate dehydrogenase were purchased from Miles Laboratories. NADH and NAD^+ were obtained from P-L Biochemicals. All other reagents were obtained from commercial sources at the highest available purity.

The reduction of oxalacetate was routinely carried out in 0.1 M potassium phosphate buffer, pH 7.5, in a 3-ml cuvette. NADH concentration was 0.14 mM and oxalacetate concentration was 0.34 mM unless otherwise stated. Absorbance changes at 340 nm were measured at 25°C either with a Gilford 2400 recording spectrophotometer or with a Beckman model 24 recording spectrophotometer. Oxalacetate and NADH solutions were prepared fresh daily.

For the preparation of *enol*-oxalacetate solutions, a 100 mM oxalacetic acid solution was prepared in absolute ethyl ether. The required amount of this solution (usually 10 to 100 μl) was deposited on the tip of a stainless steel spatula, and the ether was evaporated in a stream of dry air. The remaining *enol*-oxalacetic acid was then introduced into the cuvette by rapid stirring.

The stopped flow experiments were carried out with an Aminco-Morrow stopped flow apparatus, equipped with a DASAR (data aquisition, storage, and retrieval) system. Data were retrieved with an MFE model 705 X-Y recorder.

RESULTS

Substrate Inhibition of Mitochondrial Malate Dehydrogenase—The activity of the mitochondrial malate dehydrogenase from pig hearts at increasing concentrations of oxalacetate is shown in Fig. 1. Optimal activity is obtained at about 0.3 mM oxalacetate and the activity decreases rapidly with increasing substrate concentrations. Only 35% of the optimal activity remains at 5 mM oxalacetate. Optimal activity of the mitochondrial enzyme from chicken hearts was obtained at 0.1 mM oxalacetate, whereas 30% activity remained at 2.3 mM oxalacetate (10). These two enzymes are thus quite similar in their properties with regard to substrate inhibition.

When the substrate inhibition of the pig heart mitochon-

* This work was supported in part by Grant-in-Aid 75-979 from the American Heart Association and with funds contributed in part by the Texas Heart Association and by Grant 76-17-9551 from the Milheim Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of United States Public Health Service Training Grant 5-T01-HL-05858 from the National Institutes of Health.

§ To whom to address correspondence at the Department of Biochemistry, Texas Tech University School of Medicine, P. O. Box 4569, Lubbock, Tex. 79430.

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drial malate dehydrogenase is determined as a function of pH, one obtains a curve such as the one shown in Fig. 2. A much larger inhibition of the enzyme is found at acidic pH values than one observes in the alkaline pH region. A similar pH dependence of the substrate inhibition has been observed with various malate dehydrogenases from plants (11) as well as with the chicken heart lactate dehydrogenase (1).

Inhibition Constant of Oxalacetate—Fig. 3 represents a Lineweaver-Burk plot of the pig heart enzyme, with oxalacetate as the variable substrate. The K_m for oxalacetate, obtained from this graph, is 40 μM. This value is comparable to the value of 38 μM that was obtained for the chicken heart enzyme (10).

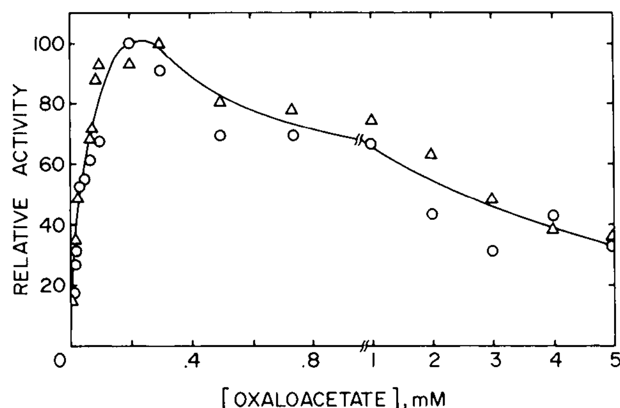

FIG. 1. Activity of pig heart mitochondrial malate dehydrogenase as a function of oxalacetate concentration. Assays were performed in 0.1 M phosphate buffer, pH 7.4. NADH concentration, 0.14 mM. The two symbols (○, △) indicate two independent, identical experiments.

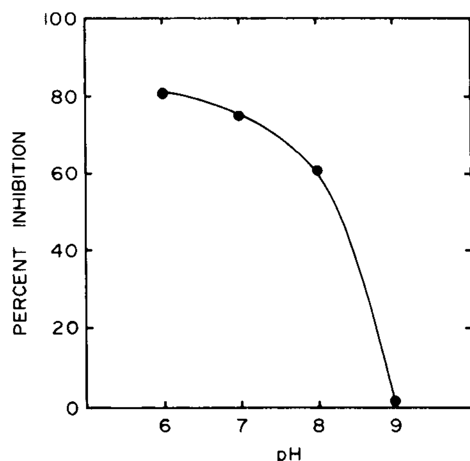

FIG. 2. Substrate inhibition of pig heart mitochondrial malate dehydrogenase as a function of pH. The inhibition was determined by the activity obtained with 3.4 mM oxalacetate as a function of the activity obtained with 0.34 mM oxalacetate. Assay conditions: 0.13 mM NADH, 0.1 M phosphate buffer, pH 7.4.

The inhibition constant of oxalacetate cannot be readily obtained since oxalacetate is also a substrate. However, a theoretical activity curve may be generated using the equation:

$$V = \frac{V_{max}\,[\text{oxalacetate}]}{K_m + [\text{oxalacetate}]}$$

Such a theoretical curve, calculated from the K_m and V_{max} obtained from Fig. 3, is shown in Fig. 4 together with the curve that is obtained experimentally. Assuming that the theoretical curve represents the activity that would be found if no substrate inhibition occurred, one may calculate the percentage inhibition that occurs as a function of the oxalacetate concentration. The result of such a calculation is shown in Fig. 5. According to this calculation, the concentration of oxalacetate that produces 50% inhibition of its own reduction is about 2 mM.

Stopped Flow Kinetics—When mitochondrial malate dehydrogenase is assayed at various oxalacetate concentrations and the initial rates are obtained with a stopped flow apparatus, results are obtained as shown in Fig. 6. The data clearly indicate that the inhibition at high oxalacetate concentrations is present at the very onset of the reaction, suggesting that no product of the reaction is involved in the formation of the inhibiting species. No difference was found whether the enzyme was premixed with NADH and the reaction was started by the addition of oxalacetate, or the enzyme was premixed with oxalacetate and the reaction was initiated with NADH.

Inhibition Constant of NAD$^+$—Fig. 7 represents the percentage inhibition of the reaction upon the addition of various

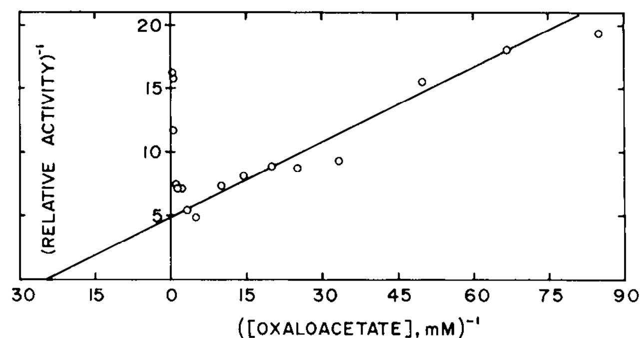

FIG. 3. Determination of the K_m for oxalacetate at pH 7.4 in 0.1 M phosphate buffer. NADH concentration, 0.14 mM.

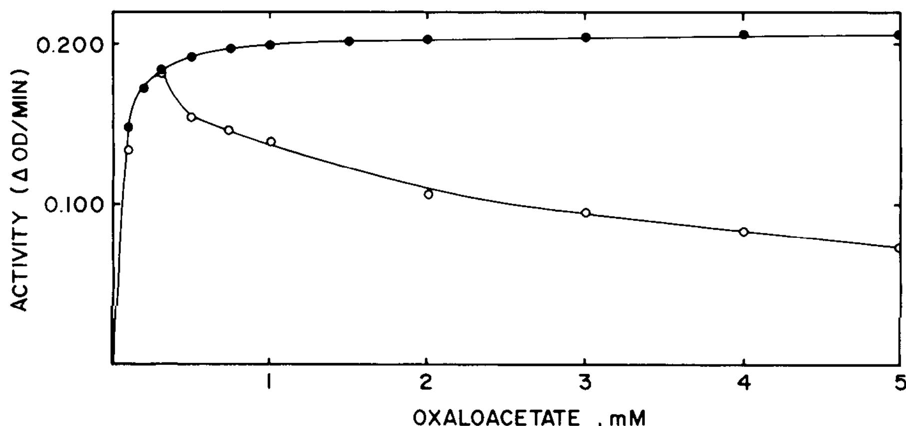

FIG. 4. Activity of pig heart mitochondrial malate dehydrogenase as a function of oxalacetate concentration. ○——○, calculated from the K_m and V_{max}, obtained from the data in Fig. 3. △——△, experimental data; conditions were identical with those in Fig. 3.

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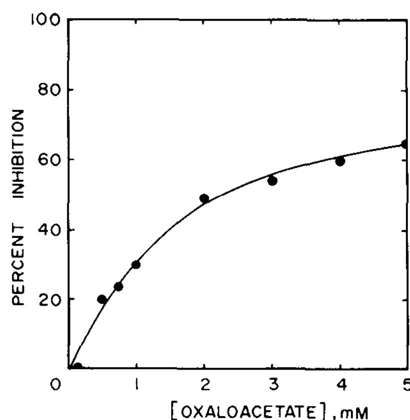

FIG. 5. Inhibition of pig heart mitochondrial malate dehydrogenase by increasing concentrations of oxalacetate. The percentage inhibition is determined from the data shown in Fig. 4.

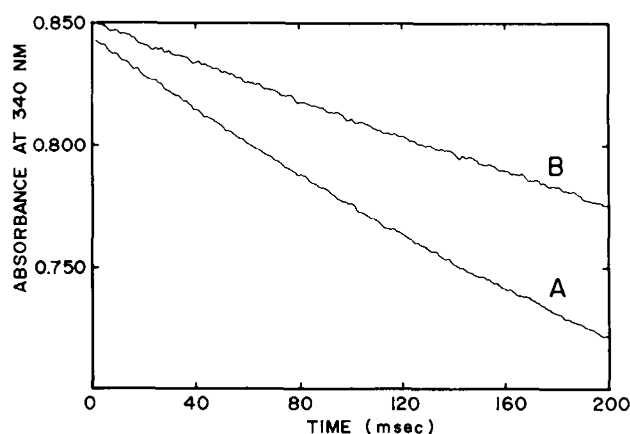

FIG. 6. Initial rates of pig heart mitochondrial malate dehydrogenase with 0.3 mM (*Curve A*) and 3 mM (*Curve B*) oxalacetate. NADH, 0.14 mM; 0.1 M phosphate buffer, pH 7.4.

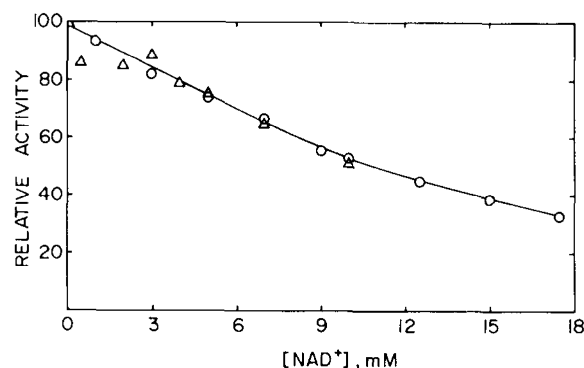

FIG. 7. Inhibition of pig heart mitochondrial malate dehydrogenase by increasing concentrations of NAD^+ using 0.1 mM oxalacetate (○——○) and 0.5 mM oxalacetate (△——△). NADH, 0.14 mM; 0.1 M phosphate buffer, pH 7.4.

amounts of NAD^+ prior to the initiation of the reaction with NADH. The experiment was done with two concentrations of oxalacetate, one (0.1 mM) being in the region where the reaction displays normal Michaelis-Menten kinetics and the other (0.5 mM) being in the region where the reaction shows significant substrate inhibition. As illustrated in Fig. 7, identical results were obtained with both oxalacetate concentrations. The concentration of NAD^+ which yielded 50% inhibition was about 10.3 mM. When the enzyme was incubated with NAD^+ and oxalacetate at room temperature for 15 min prior to the addition of NADH, we obtained essentially the same results as without preincubation.

Inhibition by Enol-oxalacetate—It has been established that the formation of the abortive ternary complex consisting of lactate dehydrogenase, NAD^+, and pyruvate involves the *enol* form of pyruvate (6-9), whereas the *keto* form is the actual substrate of the enzyme (15, 16). It appeared, therefore, important to establish whether or not the *enol* form of oxalacetate plays a role in the phenomenon of substrate inhibition.

The *enol* form of oxalacetate may be prepared by dissolving oxalacetic acid in absolute ethanol, ether, or acetone (17-19). The solid form of the acid may then be obtained by evaporating the solvent. Using this principle, we did some preliminary investigations to evaluate the effect of *enol*-oxalacetate on the malate dehydrogenase reaction.

The tautomerization of oxalacetate is normally quite rapid. Furthermore, the reaction is catalyzed by a large variety of ions, including phosphate. The half-time of the tautomerization reaction in 0.1 M phosphate buffer, pH 8.3, at different temperatures is presented in Table I. This table also lists the half-times at 15°C as a function of pH.

TABLE I

Rate of tautomerization of enol-oxalacetate as a function of temperature and pH

Data taken from Ref. 17.

At pH 8.3		At 15°C	
Temperature	$t_{1/2}$	pH	$t_{1/2}$
°C	s		s
5	228	8.3	90
15	90	7.5	58
25	40	7.0	39
35	15	6.5	27
		6.0	18

The tautomerization rate of oxalacetate is relatively slow at low temperatures. Under those conditions, it is therefore possible to evaluate the inhibitory effect of *enol*-oxalacetate on the malate dehydrogenase reaction. Oxalacetate was dissolved in absolute ethanol to a concentration of 10 mM and small aliquots were added to a malate dehydrogenase assay at 5°C. The activity was determined within 15 s after the addition of the *enol*-oxalacetate, thus minimizing the degree of tautomerization that took place during the assay. In this manner, 50% inhibition of the malate dehydrogenase reaction is obtained when the final concentration of *enol*-oxalacetate in the assay mixture reaches 0.06 mM.[1] These results support the hypothesis that the substrate inhibition of malate dehydrogenase is promoted by the *enol* form of oxalacetate.

We also investigated the question whether or not the binding of *enol*-oxalacetate is competitive with respect to the binding of *keto*-oxalacetate. Our results indicate that the inhibition of the pig heart malate dehydrogenase by *enol*-oxalacetate does not display ordinary Michaelis-Menten kinetics, but is somewhat more complex than one would anticipate. The details of the interaction between *enol*-oxalacetate and the malate dehydrogenase subunit are presently under investigation in our laboratory and these results will be presented in a separate communication.

Effect of NADH Concentration on Substrate Inhibition—When the inhibition of chicken heart lactate dehydrogenase by pyruvate is determined in the presence of various concentrations of NADH, one observes an inverse relationship between the NADH concentration and the degree of inhibition that is observed (1). Little inhibition (21%) is observed in the

[1] It should be noted that the inhibition is more pronounced at lower temperatures.

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presence of 0.30 mM NADH, whereas as much as 80% inhibition occurs in the presence of 0.06 mM NADH. These results indicate that there is a competition between the formation of the lactate dehydrogenase·NAD·pyruvate complex and the binding of NADH to the enzyme (1).

When a similar experiment was done with the pig heart mitochondrial malate dehydrogenase, we found no change in the degree of substrate inhibition when the NADH concentration was varied between 0.5 and 250 μM. This indicates that NADH does not participate in the formation of the inhibitory complex and that the reduced coenzyme is not capable of dissociating the complex.

Inhibition of the Cytoplasmic Malate Dehydrogenase by Oxalacetate—The inhibition of the pig heart cytoplasmic malate dehydrogenase by high concentrations of oxalacetate was also investigated. The K_m value of this enzyme for oxalacetate was found to be 30 μM. From this value, a theoretical Michaelis-Menten curve was constructed in the same manner as previously done for the mitochondrial enzyme (Fig. 5). The inhibition of the enzyme as a function of oxalacetate concentration was then determined from the difference between the theoretical curve and the experimentally obtained values. The inhibition constant, obtained in this manner for the cytoplasmic enzyme, was 4.5 mM.

Varying the NADH concentration did not affect the inhibition of the cytoplasmic enzyme, and the inhibition found by the addition of NAD^+ could be explained on the basis of product inhibition. This suggests that the substrate inhibition of the cytoplasmic enzyme is similar in nature to that of the mitochondrial enzyme.

DISCUSSION

The phenomenon of substrate inhibition has been observed with a number of pyridine nucleotide-linked dehydrogenases. These include the malate dehydrogenases (10–12), the lactate dehydrogenases (1–9, 20, 21), liver alcohol dehydrogenase (22), and glutamate dehydrogenase (23). Kaplan and his colleagues (3–5) have shown that the inhibition of the lactate dehydrogenases by high concentrations of pyruvate is associated with the formation of an abortive ternary complex consisting of the enzyme, NAD^+, and pyruvate, and a mechanism for the formation of such a complex was proposed (5, 21).

The ability of the pig heart mitochondrial malate dehydrogenase to form an abortive complex consisting of enzyme, reduced coenzyme, and L-malate was demonstrated by Thorne and Kaplan (24) as well as by Silverstein and Sulebele (25). Evidence has also been presented indicating that malate dehydrogenases are able to form an abortive complex with oxalacetate in the presence of the acetylpyridine analog of NAD^+ (26, 27). On the basis of these observations, we postulated that the substrate inhibition observed with the malate dehydrogenases may be due to the formation of an abortive complex among the enzyme, NAD^+, and oxalacetate. The results that we obtained, however, clearly indicate that this hypothesis is incorrect, and that the mechanism of substrate inhibition of the malate dehydrogenases is due to the formation of an abortive binary complex, consisting of the enzyme and *enol*-oxalacetate.

The data presented in Figs. 6 and 7 indicate that NAD^+ plays an insignificant role in the inhibition of mitochondrial malate dehydrogenase at high oxalacetate concentrations. The dissociation constant of the enzyme·NAD^+ complex is about 750 μM, whereas that of the enzyme·NADH complex is about 5 μM (28). The concentrations of the coenzymes at which 50% inhibition was observed (Fig. 7) were 0.14 mM NADH and 10.3 mM NAD^+. From the equations

$$K = \frac{[E][\text{NADH}]}{[E\text{-NADH}]} = 5\ \mu\text{M}$$

and

$$K = \frac{[E][\text{NAD}^+]}{[E\text{-NAD}^+]} = 750\ \mu\text{M},$$

it follows that

$$\frac{[E]}{[E\text{-NADH}]} = 3.57 \times 10^{-2}$$

and

$$\frac{[E]}{[E\text{-NAD}]} = 7.5 \times 10^{-2}$$

under the conditions of our experiment. Assuming that the free enzyme concentration equals unity, the concentration of the enzyme·NADH complex equals 28 and that of the enzyme·NAD^+ complex equals 13.5. Hence, 31% of the total amount of enzyme is bound in the form of the inactive enzyme·NAD^+ complex. In view of the uncertainty in the values for the dissociation constants (28, 29), these data strongly suggest that the inhibition by NAD^+, as shown in Fig. 7, is largely if not completely a result of product inhibition and presents no evidence for the formation of an abortive ternary complex.

This conclusion is further substantiated by the observation that substrate inhibition is observed at the onset of the reaction. Using a stopped flow apparatus, inhibition is clearly observable at 20 ms after the reaction started. At that time, the concentration of NAD^+ that has been generated is about 1.6 μM. These data suggest either that the affinity of the enzyme for NAD^+ to form an abortive complex must be extremely high or that no complex involving NAD^+ is formed.

Our observations, therefore, suggest that the formation of an enzyme NAD·oxalacetate complex as an explanation of substrate inhibition is not tenable. It should be noted that Silverstein and Sulebele (25) arrived at the same conclusion on the basis of equilibrium kinetic experiments.

There appears to be little doubt that the *enol* form of oxalacetate is involved in the substrate inhibition of the malate dehydrogenases. The K_I for oxalacetate was found to be about 2 mM at pH 7.4 and 25°C. At this pH, the equilibrium mixture of the tautomeric forms of oxalacetate contains about 15% of the *enol* form (19). Hence, if the *enol* form of oxalacetate is the inhibitory species, one would expect a K_I for *enol*-oxalacetate of about 0.3 mM at 25°C. The observed K_I for *enol*-oxalacetate at 5°C was 60 μM.

In vitro, the tautomerization rate of oxalacetate is quite rapid (see Table I) and the reaction is catalyzed in the presence of various ions, including phosphate and chloride ions (17). From these as well as other data, one would expect that, *in vivo*, the tautomerization of oxalacetate would be complete in only a few seconds. Moreover, Annett and Kosicki (18) have purified a tautomerase from hog kidney that catalyzes the tautomerization of oxalacetate. Although the physiological significance of this enzyme is still unknown, it appears certain from the above data that at any given time, a significant portion of the oxalacetate present *in vivo* may be present as *enol*-oxalacetate which could serve as an inhibitor of the malate dehydrogenase. However, since the concentration of oxalacetate in liver cells has been estimated to be in the order of 10 μM in the mitochondria as well as in the cytoplasm (30, 31), the *in vivo* concentration of *enol*-oxalacetate in the liver is not expected to exceed 1 μM. Our data indicate an approximate K_I for *enol*-oxalacetate of 0.2 mM for the mitochondrial malate dehydrogenase and of 0.45 mM for the cytoplasmic

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enzyme. These data appear to rule out the possibility that the inhibition of the malate dehydrogenases by *enol*-oxalacetate has any physiological significance.

An abortive complex of the composition enzyme·NADH·*enol*-oxalacetate could also be postulated. Our results, however, appear to rule out such a possibility since the substrate inhibition is independent of the NADH concentration between 0.5 and 250 μM.

Abortive complexes consisting of enzyme, NADH, and L-malate are readily formed by various malate dehydrogenases (24, 25). Moreover, Kitto and Kaplan (10) found that both forms of chicken heart malate dehydrogenase are inhibited by high concentrations of L-malate. The question as to whether or not the formation of such complexes may be of physiological importance, however, remains to be investigated.

Acknowledgments—The skillful technical assistance of Mr. George R. Raschbaum and Ms. Sherell Guichard is gratefully acknowledged.

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