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An Immobilized Three-Enzyme System: A Model for Microenvironmental Compartmentation in Mitochondria

(malate dehydrogenase/citrate synthase/lactate dehydrogenase/Krebs cycle)

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ABSTRACT **An immobilized three-enzyme system, malate dehydrogenase (EC 1.1.1.37)–citrate synthase (EC 4.1.3.7)–lactate dehydrogenase (EC 1.1.1.27), was investigated as a model for the rate of oxalacetate production and utilization in mitochondria. Lactate dehydrogenase is included to mimic the NADH-utilizing system of mitochondria. This three-enzyme system was immobilized in three different ways (*1*) on Sephadex G-50 (surface coupling), (*2*) on Sepharose 4B (internal–external coupling), and (*3*) entrapped in polyacrylamide gel. The rate of citrate production from malate, NAD^+, and acetyl CoA was determined continuously in a flow system. Up to about 100% rate enhancements were observed when the immobilized system was compared to identical systems of free enzyme. An even more pronounced increase of rate of up to about 400% compared to the soluble system was measured after addition of pyruvate (to reoxidize formed NADH). These results are interpreted in relation to microenvironmental changes of oxalacetate production and the possible organization of enzymes of the Krebs cycle.**

Recent reports (1, 2) have supported the older idea (3) that the concentration of oxalacetate is primarily responsible for regulation of the rate of oxidation in the Krebs cycle. One of the problems in understanding this regulatory mechanism is that the apparent free concentration of oxalacetate in the mitochondrial matrix is very low, about 40 nM (4). This concentration is so low that the rate of its reaction in the citrate synthase (EC 4.1.3.7) reaction would not be commensurate with the known rate of the cycle in mitochondria, as estimated from O_2 utilization.

If one proposes, however, an organization of Krebs cycle enzymes within the matrix (4), then one can imagine segregated metabolic pools in which locally high substrate concentrations could be maintained in the region of each enzyme's active site in spite of the measured or calculated low substrate concentration, which would represent an average concentration of the whole matrix. We have attempted to demonstrate the kinetic advantage of such a microenvironmental effect by the use of an immobilized three-enzyme system, malate dehydrogenase (EC 1.1.1.37), citrate synthase, and lactate dehydrogenase (EC 1.1.1.27). Inherent kinetic advantages in immobilized enzyme systems over the corresponding free enzyme systems have already been demonstrated for the two-enzyme sequence hexokinase–glucose-6-phosphate dehydrogenase (5) and for the three-enzyme sequence β-galactosidase–hexokinase–glucose-6-phosphate dehydrogenase (6).

In the present study the three enzymes, malate dehydrogenase, citrate synthase, and lactate dehydrogenase, are immobilized together in various ratios by covalent coupling to three different types of matrices, Sephadex G-50, Sepharose 4B, and a glutaraldehyde-derivatized Sepharose 4B, or by entrapment together in a polyacrylamide gel. The rate of CoASH production from malate and acetyl CoA of the bound-enzyme system is measured and compared to a free-enzyme system that contains identical activities of enzymes.

(*1*) Malate + NAD^+ = OAA + NADH + H^+

(*2*) H_2O + OAA + AcSCoA = Citrate + CoASH + H^+

(*3*) (sum) H_2O + Malate + NAD^+ + AcSCoA = Citrate + CoASH + NADH + $2H^+$

Then the effect of removal of NADH on this rate is also measured by the use of pyruvate and lactate dehydrogenase. This enzyme is used to mimic the reoxidation of NADH, which occurs in the mitochondria.

(*4*) H^+ + NADH + Pyruvate = NAD^+ + Lactate

(*3* + *4*) H_2O + Malate + AcSCoA + Pyruvate = Citrate + Lactate + CoASH + H^+

The change in rate on addition of pyruvate is compared also to the change that occurs in a comparable free enzyme system when pyruvate is added.

MATERIALS AND METHODS

Acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and *N,N'*-methylene-bis-acrylamide (BIS) were purchased from Eastman (Rochester, N.Y.); Sepharose 4B and Sephadex G-50 were obtained from Pharmacia, Uppsala, Sweden.

Oxalacetic acid, pyruvic acid, *l*-malic acid, dithiobis(2-nitrobenzoic acid), and lactate dehydrogenase from beef heart were obtained from Sigma (St. Louis, Mo.); malate dehydrogenase (from pig heart), citrate synthase (from pig heart), coenzyme A, NAD^+, and NADH were purchased from Boehringer (Tutzing, Germany). Acetyl coenzyme A was prepared from coenzyme A and acetic anhydride (7).

Simultaneous Coupling of Malate Dehydrogenase, Citrate Synthase, and Lactate Dehydrogenase to Sephadex G-50. The cyanogen bromide method (8) was modified as follows: 100 mg of Sephadex G-50 was activated at pH 11 with 4.0 ml of BrCN (25 mg/ml of water). The pH was kept constant by addition of 4 M NaOH. After 8 min, the activated gel was washed on a glass filter with 200 ml of cold 0.1 M $NaHCO_3$. The enzymes were added to the activated gel, and coupling proceeded for 14 hr at 4° in a rotating test tube. The reaction mixture consisted of 2.0 ml of 0.1 M $NaHCO_3$, activated gel, and various quantities of the ammonium sulfate suspensions of the en-

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zymes. The enzyme–gel preparation was then washed 30 min with each of the following cold solutions: 0.1 M $NaHCO_3$ that was 50 mM in mercaptoethanol, 0.5 M NaCl that was 50 mM in mercaptoethanol, and finally 0.05 M potassium phosphate buffer (pH 7.5). Later preparations were washed without addition of mercaptoethanol.

Simultaneous Coupling of the Enzymes to Sepharose 4B. 1 g of well-packed and thoroughly washed Sepharose 4B was suspended in 8.0 ml of BrCN solution (25 mg/ml) and activated at pH 11.0. Activation proceeded at pH 11 for 10 min; the coupling of enzymes to the gel and the washing was then done as described above for the activated Sephadex G-50.

Simultaneous Coupling of the Enzymes to 1,6-Diaminohexamethylene-Sepharose. 1.0 g of well-washed and packed derivatized gel (BrCN-activated Sepharose 4B reacted with excess of 1,6-diaminohexane at pH 8.5) was treated for 4 hr at room temperature (25°) with 20 ml of 0.1 M potassium phosphate buffer (pH 6.9) containing 4% (v/v) glutaraldehyde. One aldehyde group of glutaraldehyde reacts with the amino group of the derivatized Sepharose, the other terminal aldehyde group remains free. This new gel material was carefully washed with water and could then be used for enzyme coupling. 0.5 g of the aldehyde-gel was suspended in 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.5) together with the enzymes. Coupling of the enzyme to the gel and washing of the final product was done under the same conditions as described above for the Sephadex system.

Simultaneous Trapping of the Enzymes within Crosslinked Polyacrylamide Gels. The enzymes in potassium phosphate buffer (pH 7.5) and crosslinking monomer (BIS) dissolved in the same buffer were mixed to a total volume of 0.50 ml. 300 μl of the acrylamide solution (340 mg/ml of buffer) and the catalyst system, 100 μl of TEMED and 150 μl of ammonium persulfate solution (10 mg/ml of water), were mixed separately in a small test tube. About 1 min after addition of the catalyst system to the acrylamide solution, the enzyme–BIS mixture was also added and the solution was carefully mixed. All solutions were kept cold during the whole procedure (5). A gel was formed within 1–3 min, but the polymerization was allowed to proceed for 4 hr at 4°. The gel was then cut into 1-mm slices and each slice was pressed through a nylon net (30 mesh). The granula obtained were washed 30 min in each of the following cold solutions: 0.25 M potassium phosphate buffer (pH 7.5), 0.1 M potassium phosphate buffer (pH 7.5), 0.25 M potassium phosphate buffer (pH 7.5), and 0.1 M potassium phosphate buffer (pH 7.5).

Assay Procedure for Immobilized Enzymes. Incubations were done in a 25-ml thermostated (25°) Erlenmeyer flask with the enzyme–matrix suspended in a total volume of 12.0 ml. The incubation solution was stirred (120 rpm, dimensions of Teflon bar used 0.4 × 1.5 cm) and continuously pumped through a flow cuvette placed in a spectrophotometer and back to the reaction vessel (5) using a nylon net to keep the gel in the reaction vessel.

Citrate synthase activity was assayed in 0.1 M Tris·HCl (pH 8.1) containing 4.8 μmol of dithiobis(2-nitrobenzoic acid) and 1.44 μmol of acetyl coenzyme A. The reaction was initiated by addition of 60 μmol of oxalacetate. Activity was recorded at 412 nm, the absorption maximum of the thiophenol chromophore produced.

Malate dehydrogenase was determined by the rate of oxidation of NADH to NAD^+ at 340 nm in the presence of oxalacetate. The measurements were done in 0.1 M potassium phosphate buffer (pH 7.5) containing 2.12 μmol of NADH and 2.40 μmol of oxalacetate. Lactate dehydrogenase activity was measured in a similar manner at 340 nm by measurement of the oxidation of NADH to NAD^+ in the presence of pyruvate. The incubation solution used was 0.1 M potassium phosphate (pH 7.5) containing 2.12 μmol of NADH and 12.0 μmol of pyruvate.

Determinations of the Activities of the Immobilized Enzyme Systems. The gel was suspended in 0.1 M Tris·HCl (pH 8.1) containing 4.80 μmol of dithiobis(2-nitrobenzoic acid), 3.6 μmol of NAD^+, 1.44 μmol of acetyl coenzyme A. After the reaction mixture had reached a constant temperature, the reaction was initiated by addition of 6.0 μmol of malate. The amount of citrate produced (equivalent to coenzyme A produced) was continuously recorded at 412 nm as the chromophore was formed. The influence on the rate of citrate production of lowering the NADH concentration (formed in the malate dehydrogenase reaction) was investigated by addition of 12.0 μmol of pyruvate; the lactate dehydrogenase also present in the gel caused a reoxidation of NADH.

After measurements on the two- and three-enzyme systems the incubation solution was removed, the enzyme–gel particles were thoroughly washed with 0.1 M Tris·HCl buffer (pH 8.1), and the separate enzyme activities were determined. Citrate synthase activity was measured as described above. After this determination the gel was washed again, but in 0.1 M potassium phosphate buffer (pH 7.5), and malate dehydrogenase activity was determined. After a final wash of the gel, the activity of lactate dehydrogenase was assayed. We have not observed any loss of enzyme activities in these assays.

Determinations of the Activities of Free Enzyme Systems. Activities of the free enzymes were determined with the same concentrations of reagents as described for the bound enzymes, but in a total volume of 1.0 ml. The two dehydrogenases were measured at 340 nm as described above, but they were also assayed under the same conditions used for the coupled enzyme assay, so that activity ratios could represent activities under the same conditions rather than V_{max} ratios. Thus malate dehydrogenase was also measured when working in the forward reaction (i.e., converting malate to oxalacetate) in the presence of excess of both acetyl coenzyme A and citrate synthase and either in the absence of dithiobis(2-nitrobenzoic acid), at 340 nm as NADH formed, or in its presence at 412 nm, as the amount of chromophore formed.

Lactate dehydrogenase was measured, except as already described, with excess malate dehydrogenase and citrate synthase as auxiliary enzymes. The incubation solution was 0.1 M Tris buffer (pH 8.1) containing 0.4 μmol of dithiobis(2-nitrobenzoic acid), 0.5 μmol of malate, 1.0 μmol of pyruvate, 0.12 μmol of acetyl coenzyme A, 0.17 μmol of NADH, 3.6 units of citrate synthase, and 16 units of malate dehydrogenase.

In order that valid comparisons could be made between the matrix-bound and the free systems, the activities on the bound system were determined and then the identical ratios of free enzymes were carefully reconstructed. Routinely, the same total number of units per volume of citrate synthase, malate dehydrogenase, and lactate dehydrogenase as measured on the matrix-bound systems were mixed in a 1-ml cuvette, and

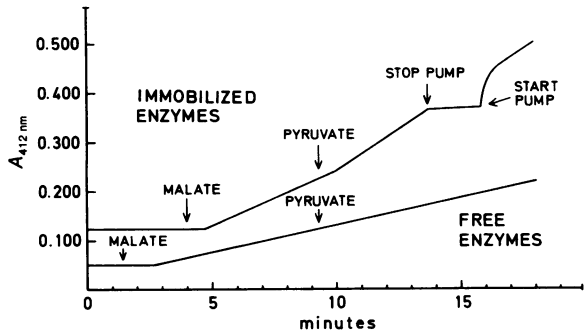

FIG. 1. Time-course of the coupled reactions of an immobilized three-enzyme system, malate dehydrogenase–citrate synthase–lactate dehydrogenase, and its corresponding free enzyme system. The enzymes immobilized in polyacrylamide showed 2.0 mU of malate dehydrogenase, 4.6 mU of citrate synthase, and 8.4 mU of lactate dehydrogenase. Incubation was done in 12 ml of 0.1 M Tris·HCl buffer (pH 8.1) containing 3.6 μmol of NAD^+, 1.44 μmol of acetyl coenzyme A, and 4.8 μmol of dithiobis(2-nitrobenzoic acid). At *arrows* indicated, 6.0 μmol of malate and 12.0 μmol of pyruvate were added. The continuous flow system was interrupted by stopping the peristaltic pump of the system, as indicated.

the overall reaction was measured. After the system had reached constant rate, lactate dehydrogenase activity was initiated by addition of 1.0 μmol of pyruvate.

K_m Determination of Citrate Synthase. K_m for oxalacetate for immobilized citrate synthase was measured by the method of Walker and Schmidt (9), which is a simple technique suitable for more-or-less irreversible enzyme reactions with low K_m values and previously applied to citrate synthase by Kosicki and Srere (10). The data were obtained from complete progress curves of the citrate synthase reaction with a computer program.

RESULTS

The three-enzyme system studied, malate dehydrogenase–citrate synthase–lactate dehydrogenase, was immobilized by four different matrices. For Sephadex G-50, Sepharose 4B, and glutaraldehyde-derivatized Sepharose 4B, the enzymes were covalently bound to these matrices; for polyacrylamide, the technique of entrapment was used.

For each preparation the three enzymic activities were determined separately. The activities of the coupled reactions were determined as coenzyme A produced [thiophenol chromophore from dithiobis(2-nitrobenzoic acid)] for both the malate dehydrogenase–citrate synthase and the malate dehydrogenase–citrate synthase–lactate dehydrogenase systems. The corresponding soluble systems were made to contain the same amount of enzyme units each per volume as that determined for the bound systems. The course of the coupled reactions, as measured by the increase in absorbancy at 412 nm of the immobilized system as compared to that of a corresponding soluble enzyme system, is shown in Fig. 1. In the two-enzyme reaction, catalyzed by malate dehydrogenase–citrate synthase, a higher rate of the immobilized system over that of the free system was observed. A further rate increase was obtained on initiation of lactate dehydrogenase activity by addition of pyruvate (leading to reoxidation of the NADH formed). The rate of the corresponding soluble system, however, was not effected by addition of pyruvate. To ensure that no enzyme leakage from the immobilized enzyme preparation into the system had occurred, the flow was stopped. No increase in absorbance at 412 nm was registered.

In all cases the rate of the immobilized systems was faster than the rate of the corresponding soluble systems (Table 1). Rate enhancements of the immobilized over the corresponding free system of up to about 100% were observed for the two-enzyme sequence, malate dehydrogenase–citrate synthase, and even larger rate increases of up to about 400% were found with the three-enzyme sequence, malate dehydrogenase citrate synthase–lactate dehydrogenase.

To ensure that the different matrices used did not preferentially adsorb the participating dicarboxylic acids, thus *per se* creating a favorable microenvironment, binding studies were done with [^{14}C]malate. No specific binding of the radioactive compound to any of the matrices was found. Any enrichment in the vicinity of the bound enzymes of cofactors participating in the enzymic reactions could also be neglected based on previous studies (5). On the contrary, in all cases, a higher K_m of citrate synthase for oxalacetate than observed for free enzyme was obtained (Table 2). A low malate concentration was used in these studies, giving a low concentration of oxalacetate (below that of the K_m of citrate synthase); the rate of the coupled enzyme system was thus sensitive to changes in the concentration of this intermediate. The conditions chosen also reflect *in vivo* situations with their low concentration of Krebs cycle intermediates found in mitochondria.

As to be expected, when the malate concentration was increased so that saturating concentrations were present, the rate of the free system was the same as the immobilized system due to the fact that now citrate synthase in the free system has the same oxalacetate concentration as that formed in the microenvironment of immobilized citrate synthase. We tested the effect of an increase in malate concentration in a reconstituted free-enzyme system corresponding to a Sephadex preparation (exp. no. 3a, Table 1). To increase the rate of reaction by 70% (as was observed with the immobilized system), the malate concentration had to be increased by 160%.

DISCUSSION

Four different immobilized malate dehydrogenase–citrate synthase–lactate dehydrogenase systems were used. In all faster rates of citrate formation were seen with the immobilized malate dehydrogenase–citrate synthase systems than with their free enzyme counterparts. In addition, when pyruvate was added so that lactate dehydrogenase was activated, stimulation of the rate was seen in the fixed enzyme systems while little or none was seen in the corresponding free systems. Let us consider the factors that could account for the observed faster rates of the immobilized systems as compared to their free enzyme controls.

(*1*) In immobilized enzymes, microenvironmental effects of pH will cause changes in the rates of the system compared to the free system (11, 12). With the two-enzyme couple, malate dehydrogenase–citrate synthase, $2H^+$ are produced per mol of reaction so one expects a decrease of the pH in the microenvironment of the enzymes. We, however, used 0.1 M buffer, which is sufficient to eliminate pronounced microenvironmental pH effects seen with an immobilized glucose oxidase–trypsin enzyme system (Gestrelius, Mattiasson, and Mosbach, unpublished). Increases in H^+ concentration would

TABLE 1. *Separate enzymic activities and activities in the coupled reactions of the three enzymes, malate dehydrogenase (MDH), citrate synthase (CS), and lactate dehydrogenase (LDH), immobilized together with four different matrices*

Matrix	Exp. no.	Ratio of enzymic activities nmol/min per ml MDH/CS/LDH	Activity in the coupled reaction in % of maximal CS activity*		Ratio of activity in the coupled reactions: bound/free (%)	
			Before addition of pyruvate	After addition of pyruvate	MDH-CS system	MDH-CS-LDH
Polyacrylamide						
Immobilized	(1)	0.90/2.2/7.0	27	54	230	460
Free	(1*a*)	0.90/2.2/7.0	12	12		
Immobilized	(2)	1.7/3.7/12	24	57	200	480
Free	(2*a*)	1.7/3.7/12	12	12		
Immobilized	(3)	1.9/0.59/18	43	82	110	200
Free	(3*a*)	1.9/0.59/18	39	41		
Sephadex G-50						
Immobilized	(4)	3.5/2.1/12	22	42	145	250
Free	(4*a*)	3.5/2.1/12	15	17		
Immobilized	(5)	4.7/1.1/13	62	78	125	135
Free	(5*a*)	4.7/1.1/13	50	58		
Immobilized	(6)	2.8/0.42/6.8	83	96	170	185
Free	(6*a*)	2.8/0.42/6.8	49	52		
Sepharose 4B						
Immobilized	(7)	5.1/8.9/29	17	42	110	215
Free	(7*a*)	5.1/8.9/29	16	19		
Immobilized	(8)	4.8/2.7/31	44	78	115	150
Free	(8*a*)	4.8/2.7/31	39	51		
Immobilized	(9)	5.4/2.1/19	44	100	110	200
Free	(9*a*)	5.4/2.1/19	39	51		
Aldehyde-Sepharose 4B						
Immobilized	(10)	11/20/75	17	44	100	215
Free	(10*a*)	11/20/75	17	20		

* Activities are expressed as % of the total V_{max} of citrate synthase possible in each system. V_{max} is measured with saturating acetyl CoA and oxalacetate concentrations. The velocity in each system represents one obtained with saturating acetyl CoA and generated oxalacetate. Thus, the higher the steady-state oxalacetate concentration, the closer these numbers will be to 100.

tend to decrease the rate of citrate synthase, when operating at or below pH optimum, rather than to cause an increase in its reaction rate, as was observed in these experiments.

(*2*) Changes in apparent K_m values of immobilized enzymes with uncharged matrices have been reported in several systems (13). In most cases, K_m values have increased, as we have observed here for the K_m with externally added oxalacetate for immobilized citrate synthase. Diffusion limitations, such as caused by unstirred layers (Nernst diffusion layer), have been interpreted to cause these changes. This diffusion limitation effect is illustrated in Table 2. The "surface"-bound enzyme on Sephadex G-50 has a much lower K_m than the entrapped enzyme, and, as expected, the Sepharose-bound enzyme has an intermediate K_m value. An increased K_m value for acetyl coenzyme A, malate, pyruvate, etc. would tend to reduce rather than increase the rate of citrate production. We used saturating amounts of acetyl coenzyme A [dithiobis(2-nitrobenzoic acid) was also in excess so that its reaction with coenzyme A cannot be rate limiting] and pyruvate.

(*3*) Increased local concentration of substrate due to ionic or hydrogen binding to the matrices alone might give the enhanced rate effects. However, at the rather high ionic strengths used, such preferential binding is not likely to occur; furthermore, one would not expect the observed similar enhanced rate effects for all of the four different matrices. For NAD^+, analogous experiments (5) have shown that no specific binding occurs to matrices of these types, and we have shown here that malate does not bind to these matrices.

(*4*) The simplest explanation for these results is that locally increased concentrations of oxalacetate occur in the microenvironment of citrate synthase in the immobilized enzyme system. A concentration gradient of the intermediate within the unstirred layer will be created, going from high (matrix vicinity) to low (bulk solution).

When pyruvate was added, an enhancement of rate occurred in the immobilized system that was not seen in the free system. Again the microenvironment for citrate synthase was changed due to a higher concentration of oxalacetate formed when the NAD^+/NADH ratio was increased within the mi-

TABLE 2. *K_m values for free and immobilized citrate synthase*

	K_m of oxalacetate (μM)
Free citrate synthase	2.1
Sephadex G-50-citrate synthase	8.7
Sepharose 4B-citrate synthase	13.0
Polyacrylamide-citrate synthase	31.8

croenvironment of malate dehydrogenase. The effectiveness of lowering NADH concentration in the matrix where lactate dehydrogenase is fixed in the vicinity of malate dehydrogenase shows an additional microenvironmental effect, whereas no similar increase in rate was observed in the free enzyme system. Similar results have been observed in immobilized coupled enzyme systems (5, 6).

In these earlier systems, all separate enzymic reactions were energetically favorable. In the present study, however, the malate dehydrogenase step is unfavorable ($\Delta G = +6.7$ kcal) while the citrate synthase step is favorable ($\Delta G = -9.1$ kcal). The equilibrium over the first step creates a constant concentration of the intermediate (oxalacetate) in the microenvironment of malate dehydrogenase and a decreasing concentration gradient of oxalacetate depending on the distance from the site of production. When they are immobilized together, the statistical mean distance between a malate dehydrogenase molecule and a citrate dehydrogenase molecule is shorter than when they are in free solution. This may result in a steeper concentration gradient and a higher diffusion rate of oxalacetate in the immobilized system, due to the fact that while citrate synthase is lowering the oxalacetate concentration, a constant concentration of oxalacetate is maintained in the microenvironment around malate dehydrogenase. This, in turn, will lead to a higher rate of production in the malate dehydrogenase step and, therefore, to an increased rate in the overall reaction. The earlier systems studied showed pronounced lag phases during which enrichment of intermediates within the microenvironment created high local concentrations, whereas in this system no such pronounced effects can occur due to the equilibrium over the first step.

The immobilized malate dehydrogenase–citrate synthase system is more efficient compared to the corresponding free system, not only during an initial stage, but as a consequence of the equilibrium conditions in the first step during the whole reaction.

If one assumed an unstructured, random distribution of components in the mitochondrial matrix, the following data set the conditions for the malate dehydrogenase–citrate synthase couple in rat-liver mitochondria. (*1*) pH = 7.0, (*2*) malate = 0.23 mM (14), (*3*) $NAD^+/NADH = 7$ (14), (*4*) a viscous medium, (*5*) acetyl coenzyme A = 0.1 μM (15), and (*6*) oxalacetate (calculated from *1*, *2*, and *3*) = 45 nM. Thus the rate of citrate synthase must be reduced by some factor to account for the difference between these conditions and V_{max} conditions for citrate synthase. Some factors must also be introduced for increased diffusion coefficients in the gel-like matrix. The fact that substrate concentrations are each less than 0.1 K_m is responsible for the reduced maximal rate of citrate synthase by a factor of about 400. From this factor and the V_{max} citrate synthase capacity of rat liver of about 14 μmol/min per g (16), it is apparent that the value obtained is much less than the rate of oxidation of acetate by rat liver of 1 μmol/min per g (17). It is unlikely that an error in the estimation of the quantities of enzyme was made since similar figures were obtained by several groups with various extraction techniques (4, 18, 19). The estimation of oxalacetate concentration may not be reliable, but in isolated mitochondria the *total* oxalacetate present is 1 μM (19, 20). When this value is corrected for the total binding sites of oxalacetate in mitochondria, then a value of 10 nM for *free* oxalacetate is not at all unreasonable. Recent data proposed the possible compartmentation of oxalacetate within rat-liver mitochondria, based on studies with labeled substrate indicating two different pools of oxalacetate (21).

The enhancement shows some dependence on the ratio of enzymes used (Table 1). However, effects are seen with malate dehydrogenase/citrate synthase ratios that are within physiological range (22).

It is probable that in multienzyme systems such as fatty-acid synthetase and ketoacid dehydrogenases, the complex of enzymes allows the concentration of substrate intermediates to be high in the microenvironment of each active site of the complex. Although no such complex of Krebs cycle enzyme has been demonstrated, a clustering or arrangement of these enzymes would confer a kinetic advantage on the system and the apparent discrepancy would be resolved. If a similar situation existed in the matrix of a mitochondrion, i.e., fixed positions of the enzymes, in a way that is more specific than can be achieved by the chemical methods used here, it is possible that even greater kinetic efficiency could be achieved.

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