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CITRATE LYASE - CATALYZED  
PROTON TRANSFERS

BY

CARL V. BRAUN

A Thesis

Submitted to the Faculty of Graduate Studies through the  
Department of Chemistry in Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science at the  
University of Windsor

Windsor, Ontario

1969

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259864

## ABSTRACT

Deuterium was used as a tracer to detect proton transfers catalyzed by citrate lyase (EC 4.1.3.6) from Aerobacter aerogenes. The cleavage of citrate was shown to proceed by an internal elimination mechanism involving the direct proton transfer from one methylene group of citrate to the other. The enzyme-catalyzed exchange of the methyl protons of the acetate so produced was also demonstrated.

A tentative reaction pathway is proposed. Our experimental results, as well as the data of other workers, are considered in terms of this model.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

BSA	bovine serum albumin
ca.	about
CL	citrate lyase
DSS	sodium 2,2-dimethyl-2-silapentane-5-sulphonate
EDTA	ethylene diamine tetra-acetic acid
K	equilibrium constant
$K_{app.}$	apparent equilibrium constant
$M^{++}$	divalent metal cation
MDH	malate dehydrogenase
mg	milligram
ml	milliliter
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NMR	nuclear magnetic resonance
°	degree Centigrade
OAA	oxaloacetate
TEA.HCl	triethanolamine.hydrochloride
Tris	tris (hydroxymethyl) aminomethane
$V_o$	observed initial velocity

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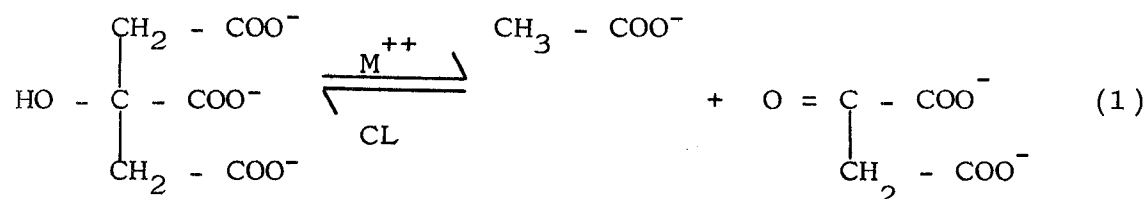
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## CHAPTER 1

### INTRODUCTION

Citrate lyase (citrate-oxaloacetate) (EC 4.1.3.6) catalyzes the reaction by which citrate is cleaved at the same point at which it is synthesized by citrate synthase (EC 4.1.3.7) (Wheat and Ajl, 1955b) (eq. 1):



This substrate-induced enzyme, formerly known as citritase, citridesmolase, citrase, and citrate aldolase, has been obtained from Escherichia coli (Wheat and Ajl, 1955a; Bowen and Siva Raman, 1960); Aerobacter aerogenes (Dagley and Dawes, 1953b; Siva Raman, 1961), Streptococcus diacetilactis (Harvey and Collins, 1962), Leuconostoc citrovorum (Harvey and Collins, 1961), and Streptococcus faecalis (Gillespie and Gunsalus, 1953; Smith et al., 1956). Regardless of its source, it has a pH optimum around pH 8.0 (Daron and Gunsalus, 1962). It shows an

absolute requirement for a divalent metal cation such as  $Mg^{++}$ ,  $Mn^{++}$ ,  $Fe^{++}$ , or  $Zn^{++}$  (Dagley and Dawes, 1955). In contrast to citrate synthase (Lipmann, 1945; Nachmansohn and Berman, 1946), citrate lyase functions in complete absence of CoA (Gillespie and Gunsalus, 1953; Wheat and Ajl, 1955b).

Bowen and Rogers (1962, 1963a, 1965) have reported various physical properties in the literature for the enzyme from Aerobacter aerogenes whose purity was established by analytical ultracentrifugation and electrophoresis at pH 7.1 and 6.0. The diffusion coefficient was found to be  $4.16 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$ . A value of  $S_{20,w}^0 = 16.2$  Svedberg units was obtained for the sedimentation coefficient. From their amino acid analyses, a partial specific volume of 0.735 was determined. Employing the sedimentation-diffusion method, a molecular weight of  $3.18 \times 10^5$  was calculated. Approach-to-equilibrium studies led to a molecular weight of  $3.14 \times 10^5$ . In contrast, Mahadik and Siva Raman (1968) reported a markedly higher value of  $5.75 \times 10^5$  from approach-to-equilibrium runs. Their  $S_{20,w}^0$  values, however, were in agreement. Evidence in favor of this higher value is the gel filtration behavior of the enzyme. Data have also been

presented which suggest that the enzyme is composed of eight subunits of identical size. The octameric form of the enzyme has been shown to dissociate reversibly in two stages, first to a tetramer and then to a dimer when dialyzed against EDTA solutions of low ionic strengths at pH 7.4 (Mahadik and Siva Raman, 1968).

The nature of the active complex has been studied by various groups. It was suggested by Dagley and Dawes (1955) and Harvey and Collins (1963) that citrate lyase acts on a metal-citrate complex. In contradiction to this theory, Ward and Srere (1965) deduced from nuclear magnetic resonance studies on the enzyme from Streptococcus diacetilactis that a metal-enzyme complex acts on free citrate. The citrate lyase from Aerobacter aerogenes rapidly loses activity in dilute solutions and the presence of magnesium largely prevents this inactivation (Eisenthal et al., 1966), suggesting the formation of a magnesium-enzyme complex. Blair et al. (1967) showed that the processes occurring on dilution of citrate lyase in the presence of low magnesium ion concentration are complex. At least two inactivation processes take place - one reversible by incubation with excess magnesium and the other one apparently irreversible. Increasing concentrations of magnesium

retard both these processes showing that the active species of the enzyme is a magnesium-enzyme complex (Blair et al., 1967). The metal-binding site may be identical with the active site, which would require a compulsory order mechanism for the formation of the enzyme-metal-substrate complex such as suggested by Ward and Srere (1965). It is possible, however, that the metal binds at a site or sites distinct from the active site thus causing a conformational change to give the active species of the enzyme. In such a case, a random order mechanism in which the enzyme-metal-substrate complex may form either from enzyme-metal and citrate or from metal citrate and enzyme cannot be ruled out.

Dagley and Dawes (1955) found that with high concentrations of citrate, zinc ions at low concentrations are nearly as good activators for citrate lyase as magnesium ions. At higher  $Zn^{++}$  concentrations activation is still better, so that a greater amount of citrate can be decomposed by a given amount of enzyme before inactivation occurs. Gruber and Moellering (1966) found that the higher activity of citrate lyase in the presence of zinc ions was due to the stabilization of the enzyme.

Since the most inhibitory substance is a complex of the enol trianion (Eisenthal et al., 1966) of oxaloacetate with  $Mg^{++}$  (Dagley and Dawes, 1955; Wheat and Ajl, 1955b; Bowen and Rogers, 1963; Tate and Datta, 1965), Moellering and Gruber (1966) assumed that zinc ions react with oxaloacetate differently than magnesium ions and that the zinc complex is less inhibitory. This assumption is supported by differences in the spectral shifts caused by zinc ions on oxaloacetate compared with those caused by magnesium ions (Gruber and Moellering, 1966).

The irreversible inactivation of citrate lyase may be due to conformational changes, dissociation of the enzyme into subunits, or both. Dissociation into subunits is likely since less inactivation occurs with increasing enzyme concentrations (Blair et al., 1967). Bowen and Rogers (1963a) obtained evidence for the formation of a lower molecular weight species on inactivation of the enzyme on storage. Blair et al. (1967) suggest that dissociation of  $Mg^{++}$  from a metal-enzyme complex precedes the irreversible inactivation and that the enhanced inactivation of citrate lyase by the adenine nucleotides appears to be due to the chelation of  $Mg^{++}$ .



The protection of citrate lyase in dilute solutions by calcium is interesting since calcium does not form a catalytically-active complex with the enzyme; in fact, it competitively inhibits the cleavage of citrate in the presence of magnesium (Dagley and Dawes, 1955; Harvey and Collins, 1963). Dagley and Dawes (1955) attribute the non-reactivity of calcium to the fact that it has a greater ionic radius than those metals which are able to activate the enzyme. On the other hand, Ward and Srere (1965) present evidence indicating that  $\text{Ca}^{++}$  competes for the binding site of  $\text{Mn}^{++}$  on the enzyme, and Blair et al. (1967) report that  $\text{Ca}^{++}$  can form a complex with citrate lyase, although they state that this may not be at the catalytic site.

Product inhibition, well known for many enzyme systems (Frieden and Walter, 1963a, 1963b), has been observed in citrate lyase preparations from Escherichia coli, Aerobacter aerogenes and Streptococcus faecalis (Dagley and Dawes, 1955; Harvey and Collins, 1961; Bowen and Rogers, 1963b). In contrast to this, Harvey and Collins (1961) reported that crude citrate lyase preparations from Leuconostoc citrovorum and Streptococcus diacetylactis were not inhibited by reaction products.

Harvey and Collins (1963), using citrate lyase purified from Streptococcus diacetilactis, and Tate and Datta (1964), using enzyme preparations from Aerobacter aerogenes, showed that it is the keto isomer which is produced by this enzymatic cleavage of citrate at pH 7.4. Tate and Datta (1964) postulated that the reverse reaction, the citrate lyase-catalyzed synthesis of citrate (Smith et al., 1956), may require ketonic oxaloacetate as the reactive species. In this specificity, citrate lyase would resemble citrate synthase for which ketonic oxaloacetate is known to be the substrate (Englard, 1959; Annett and Kosicki, 1969). Based on the postulate that keto-oxaloacetate is the active species, it has been suggested that the enol isomer is the cause of the observed inhibition of citrate lyase by oxaloacetate (Dagley and Dawes, 1955; Bowen and Rogers, 1963b; Tate and Datta, 1965). This suggestion is further strengthened by the observation that both oxaloacetate and malate completely inhibited the enzyme but acetate, pyruvate, oxalate, succinate, oxoglutarate, tartarate, oxalosuccinate and isocitrate had no effect on citrate lyase activity (Bowen and Rogers, 1963b).

On account of this inhibition, true equilibrium can be reached in the direction of citrate cleavage only with a limited initial concentration of citrate and when enzyme, metal ions, and acetate are provided in excess (Tate and Datta, 1965). Apparent equilibrium constants expressed in terms of total citrate, oxaloacetate, and acetate concentrations are markedly effected by the initial concentrations of the reactants (Tate and Datta, 1965). This is due to the ability of  $Mg^{++}$  ions, added as the cofactor, to complex with citrate and the isomers of oxaloacetate. Early measurements of the equilibrium constant have been of the apparent constant defined as follows:

$$K_{app.} = \frac{\boxed{\text{Citrate}}_{\text{total}}}{\boxed{\text{OAA}}_{\text{total}} \boxed{\text{Acetate}}_{\text{total}}} \quad (2)$$

Smith et al. (1956) using an enzyme from Streptococcus faecalis at pH 7.6 with  $Mn^{++}$  as the activating cation, found  $K_{app.}$  to be  $1.56 \text{ l. mole}^{-1}$  at  $27^{\circ}$ , whereas Harvey and Collins (1963) found  $K_{app.}$  to be  $15.7 \text{ l. mole}^{-1}$  with an enzyme from Streptococcus diacetylactis at pH 7.0 and  $30^{\circ}$ , presumably with  $Mg^{++}$  as the cofactor. Smith et al. (1956) used a tris buffer, which reacts with oxaloacetate

(Mahler, 1961). Harvey and Collins (1963) used a phosphate buffer which forms complexes with  $\text{Mg}^{++}$  (Clarke et al., 1954). For these reasons their reported equilibrium constant values (Smith et al., 1956; Daron and Gunsalus, 1961; Harvey and Collins, 1963; Williamson and Corkey, 1969) appear to be in error (Dagley, 1969a). Performing the reaction in triethanolamine - hydrochloric acid buffer, pH 8.4 (which does not complex with  $\text{Mg}^{++}$ ) and allowing for the equilibria existing between  $\text{Mg}^{++}$  and the substrates, Tate and Datta (1965) calculated the equilibrium constant defined by the equation:

$$K = \frac{[\text{Citrate}^{3-}]}{[\text{OAA}^{2-}_{\text{Keto}}] [\text{Acetate}^{-}]} \quad (3)$$

to be  $3.08 \pm 0.72$  which is constant over a range of concentrations of  $\text{Mg}^{++}$  and substrates.

In this work we determined the various sets of optimum conditions which we then used to study the effect of citrate lyase on both the exchange of the methyl protons of acetate and the proton transfer to acetate during the enzymatic cleavage of citrate.

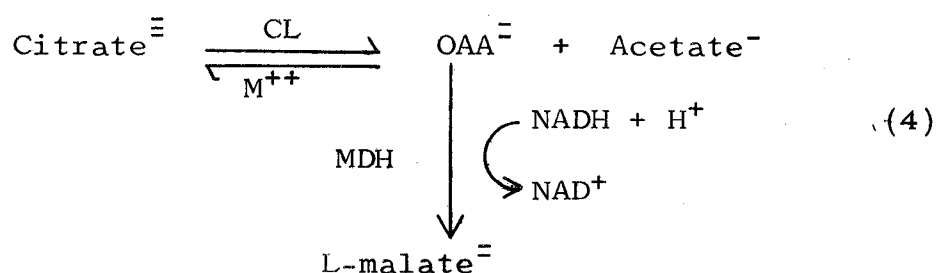
## CHAPTER 2

### EXPERIMENTAL

#### A. METHODS

##### 1) Ultraviolet Spectrometric Assay Method.

Citrate lyase was assayed under the conditions given in the legends to the figures and tables by coupling the reaction with MDH and NADH (eq. 4):



and following the oxidation of NADH spectrophotometrically at 340 mμ (Moellering and Gruber, 1966; Dagley, 1969b). Under the experimental conditions, the breakdown of one mole of citrate results in the oxidation of exactly one mole of NADH (Dagley, 1963). The molar absorbancy of NADH at 340 mμ is 6220 (Horecker and Kornberg, 1948).

Citrate, MDH, buffer, metal ion, NADH, and distilled, deionized water were incubated at 25°C for 2 to 3 minutes. The CL reaction was then initiated by the

addition of the CL preparation and the rate was recorded on a Gilford Model 2000 Absorbance Recorder attached to a Beckman DU monochromator. The temperature of the reaction chamber was kept constant at  $25.0 \pm 0.2^{\circ}\text{C}$  by a circulating water bath. The rate was never linear for more than thirty seconds and therefore the initial rates ( $V_0$ ), expressed as  $\mu\text{moles}$  of OAA formed per minute, were determined by drawing tangents to the origin.

2) Nuclear Magnetic Resonance Spectrometric Assay Method.

The extent of the hydrogen-deuterium exchange in the methyl protons of acetate as well as the extent of the enzymatic reaction in the forward direction were monitored by nuclear magnetic resonance spectroscopy with a Jeol C-60 Spectrometer. For each run, all components were mixed together and then split into two equal portions. To one, the citrate lyase was added and to the other, the control, an equivalent volume of  $\text{D}_2\text{O}$  was added. The NMR spectra were run and triplicate integrations were performed periodically for a minimum of three hours.

a) Hydrogen-Deuterium Exchange in Acetate.

The signal for the methyl group of acetate appears in  $\text{D}_2\text{O}$  at  $\delta 1.92$  with respect to the methyl peak of

the internal standards sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) ( $\delta 0.00$  in  $D_2O$ ) or t-butyl alcohol ( $\delta 1.26$  in  $D_2O$ ) (Kosicki, 1968).

If citrate lyase catalyzes an exchange of acetate's methyl protons with the medium's deuterons, the integrated area of the peak at  $\delta 1.92$  would diminish relative to that of t-butyl alcohol.

b) Enzymatic Cleavage of Citrate to Acetate and Oxaloacetate.

The chemical shift of the strong methylene doublet (Lowenstein and Roberts, 1960) of citrate under the experimental conditions was  $\delta 2.65$  with respect to the internal standards DSS and t-butyl alcohol. As the enzymatic cleavage of citrate proceeds, the integrated area of the doublet at  $\delta 2.65$  should diminish relative to that of t-butyl alcohol and be replaced by a singlet at  $\delta 1.92$ .

3) Infrared Spectrometric Assay Method.

Since replacement of active hydrogen by deuterium in alcohols causes a great shift of the infrared absorption band of the O-H stretching mode (Fales and Robertson, 1963), the extent of spontaneous exchange of the active alcohol proton in citrate for deuterium can be approximated.

Thus, infrared spectra were used as an indication of the extent of this exchange under acidic and neutral conditions. For the acid-catalyzed experiments, citric acid was dissolved in  $\text{H}_2\text{O}$  ( $\text{D}_2\text{O}$  for the deuteronic system), acidified to a pH less than 1.0, incubated 15 minutes, alkalized to a pH in the range 6.5 to 7.5 with NaOH, evaporated to dryness under vacuum on a Buchi rotary evaporator, and finally dried in an oven at  $156^\circ\text{C}$  for two days. In the uncatalyzed experiments, sodium citrate was dissolved both in  $\text{H}_2\text{O}$  and in  $\text{D}_2\text{O}$ . Immediately after dissolution the contents of the flasks were evaporated under vacuum on a rotary evaporator and then placed in a drying oven at  $156^\circ\text{C}$  for four days.

All dried samples were pressed into potassium bromide discs. Infrared spectra were measured using a Beckman-IR 10 Infrared Spectrophotometer.

#### 4) Mass Spectrometric Assay Method.

A 111 ml reaction mixture containing ca. 600  $\mu\text{moles}$  TEA.HCl -NaOH buffer, pH (pD) 7.6; 589  $\mu\text{molar}$  units MDH; 666  $\mu\text{moles}$   $\text{MgSO}_4$ ; 266.4  $\mu\text{moles}$  NADH and 15 mg citrate lyase were incubated at room temperature for three minutes. During this time the absorbance of a 3 ml aliquot was determined with a Beckman DU Monochromator. The appropriate form of sodium citrate was added to the incubation mixture



and stirred for two minutes. The absorbance change of a 3 ml aliquot of this reaction mixture was determined and the enzymes in the remainder were denatured by acidification to less than pH 1 with  $\text{H}_2\text{SO}_4$ .

The acidified reaction mixture was distilled under vacuum (ca. 90 mm,  $49^\circ$ ). The distillate was titrated to pH 8.2 - 8.8 with standardized NaOH. The solution was evaporated to dryness under vacuum. The sample was then dissolved in water and evaporated to dryness under vacuum three times. (The third time in the sample vial.)

All samples thus obtained were dried at  $126^\circ$ , 4 mm for 30 hours prior to infrared and mass spectrometric analysis. All mass spectra were obtained using a Hitachi-Perkin-Elmer RMU-6D. Mass Spectrometer. Morgan Schaffer Corporation, Montreal, performed all isotopic abundance measurements and calculations.

##### 5) Materials.

The following materials were commercial preparations: CL, MDH, NADH, TEA.HCl (C. F. Boehringer & Soehne GmbH, Mannheim); L-malic acid, MDH (Sigma Chemical Co.); OAA, BSA (Cal-biochem); deuterium oxide, DSS (Merck Sharp & Dohme of Canada Limited).  $\text{MgSO}_4$ ,  $\text{MgCl}_2$ ,  $\text{MnCl}_2$ ,  $\text{KHCO}_3$ ,  $\text{CH}_3\text{COONa}$  (Fisher Scientific Co., Ltd.); sodium citrate,

$\text{H}_2\text{SO}_4$ ,  $\text{HCl}$ ,  $\text{ZnCl}_2$  (Allied Chemical Canada, Ltd.);  $(\text{CH}_3)_3\text{COH}$  (Mallinckrodt Chemical Works, Ltd.) and ascarite (Arthur H. Thomas Company) were reagent grade.

The lyophilized citrate lyase preparation from Aerobacter aerogenes (24% citrate lyase, 24% albumin, 48% saccharose, and 4%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) was dissolved in a CL-diluent composed of 0.01 M TEA.HCl-NaOH buffer, 0.3 mM  $\text{ZnCl}_2$  and 0.454 M  $(\text{NH}_4)_2\text{SO}_4$ .

The NADH-diluent was 0.01 M TEA.HCl-NaOH buffer. The MDH-diluent was 0.01 M TEA.HCl-NaOH buffer containing 1.0 mg BSA per ml. All dilution buffers were adjusted to the pH of the system in which the preparations were used.

$\text{ZnCl}_2$  was dissolved in deionized water acidified with concentrated HCl.

Oxaloacetate was dissolved in  $\text{D}_2\text{O}$ , stirred for a minimum of fifteen minutes to equilibrate the tautomeric mixture and then alkalized (to pH 7.5 - 8.5) with solid  $\text{K}_2\text{CO}_3$ . By following this procedure, the keto form of the tautomeric equilibrium mixture is 84.7% (Kosicki, 1962).

The NaOH used for the titrations of acetate was prepared daily with boiled, deionized water and standardized against primary standard grade potassium hydrogen phthalate (Fisher Scientific Co., Ltd.).

In deuteronic systems, all components in the reaction vessel during the enzymatic reaction were prepared in D<sub>2</sub>O (99.7 Atom % D). In these systems, the pH reading from a glass-electrode pH meter was converted to pD by adding 0.4 units, as described by Lumry et al. (1951) and experimentally confirmed by Glascoe and Long (1960).

## B. RESULTS

### 1) Nature of the Enzymatic Catalysis.

The rate of citrate cleavage increases linearly with increasing enzyme concentration (Fig. 1). Increasing amounts of NADH greatly reduce the initial velocity of the enzymatic reaction (Fig. 2).

Divalent metal ions at concentrations below the concentration of the substrate used show an activation effect on the CL in the order  $Mg^{++} > Mn^{++} > Zn^{++}$ .  $Ca^{++}$  was unable to activate citrate lyase.  $Zn^{++}$  and  $Mn^{++}$  show no optimum concentration, whereas  $Mg^{++}$  produces the greatest activation effect at concentrations approximately one-half those of citrate (Fig. 3).

Citrate lyase is spontaneously inactivated by incubation in the CL diluent (Fig. 4).

Fig. 1. Catalysis of citrate cleavage as a function of enzyme concentration.

The standard assay procedure was used. Each cuvette (1.0 cm light path, 3.00 ml volume) contained 6.67 mM sodium citrate; ca. 160  $\mu$ molar units MDH; 95.3 mM TEA.HCl-NaOH buffer, pH 7.6; 5.0 mM  $\text{ZnCl}_2$ ; 0.235 mM NADH and CL protein with a specific activity of 9.9 as indicated.

FIGURE 1

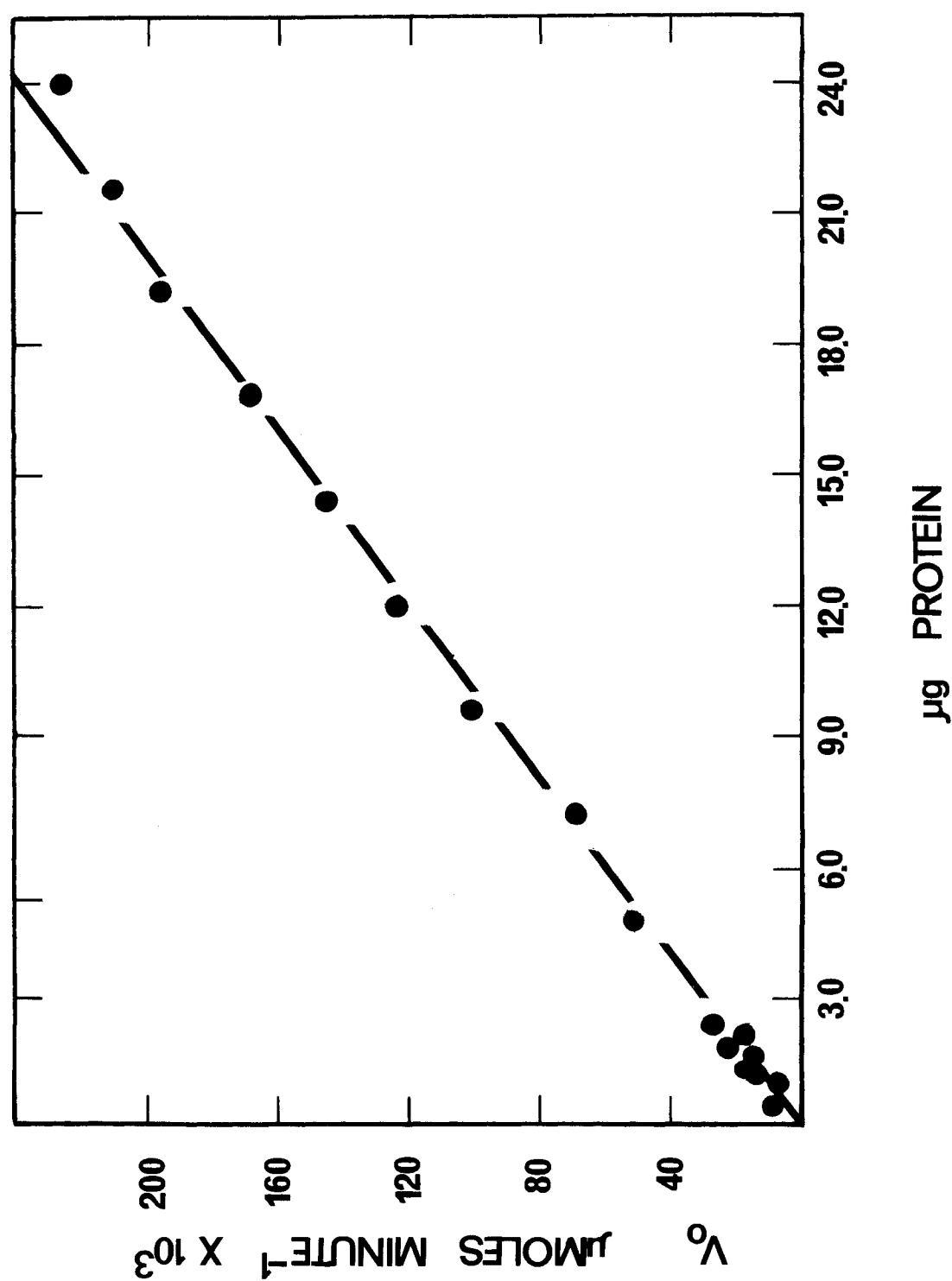


Fig. 2. Effect of NADH concentration on the activity of citrate lyase.

The standard assay procedure was used. Each cuvette (1.0 cm light path, 1.00 ml volume) contained 10 mM sodium citrate; ca. 16 uM  $\mu$ units MDH; 50 mM Tris-HCl buffer, pH 7.6; 10 mM  $\text{MgCl}_2$ ; NADH as indicated and 2.4  $\mu$ g CL protein.

FIGURE 2

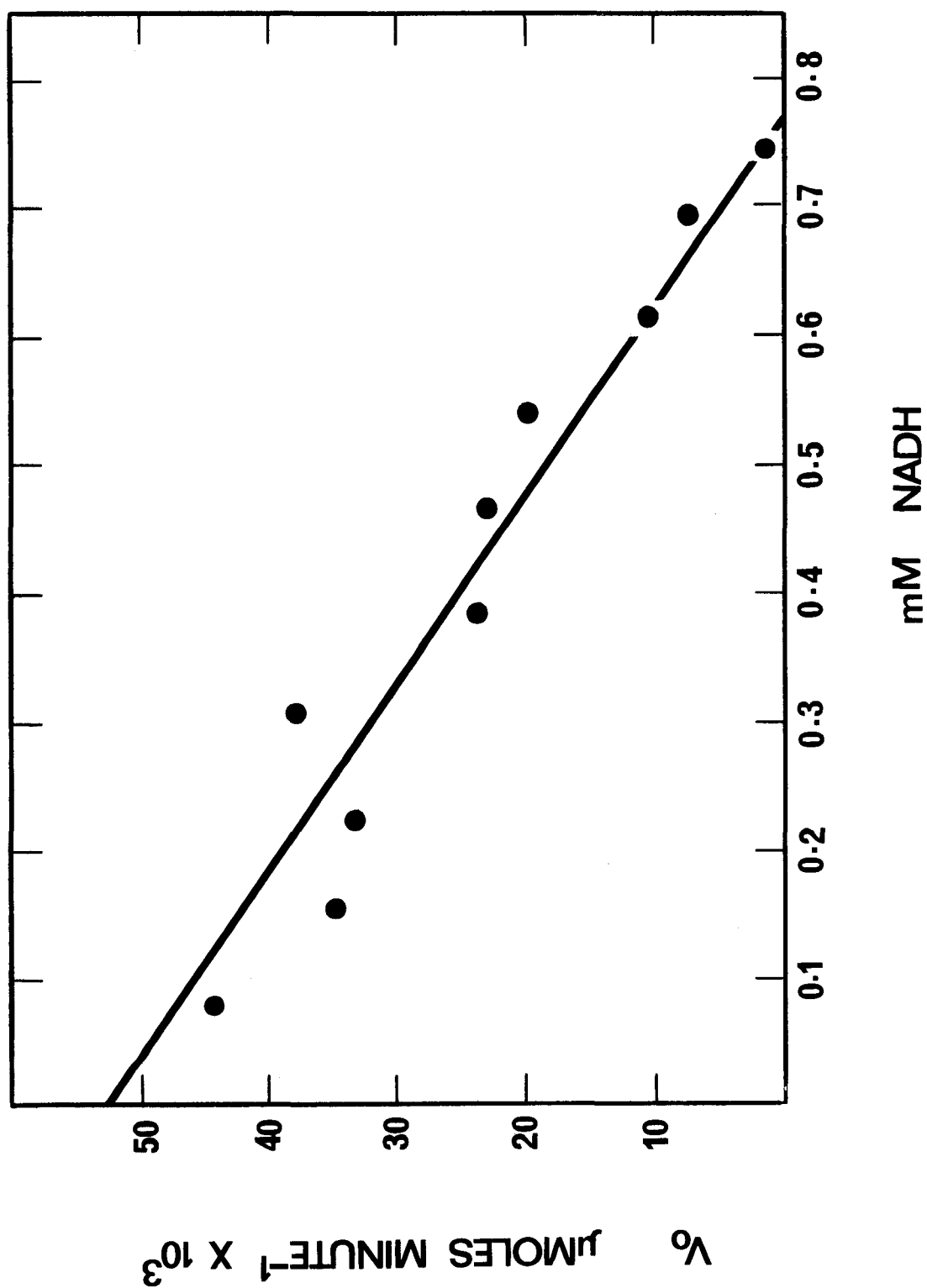




Fig. 3. Effect of metal ions ( $\text{Mg}^{++}$ , ●;  $\text{Mn}^{++}$ , ▲;  $\text{Zn}^{++}$ , ■; and  $\text{Ca}^{++}$ , ▼) on the activity of citrate lyase. The standard assay procedure was used. Each cuvette (1.0 cm light path, 1.00 ml volume) contained 10 mM sodium citrate; ca. 16  $\mu\text{M}$  units MDH; 50 mM Tris-HCl buffer, pH 7.6; 0.327 mM NADH; metal chlorides as indicated and 2.4  $\mu\text{g}$  CL protein.

FIGURE 3

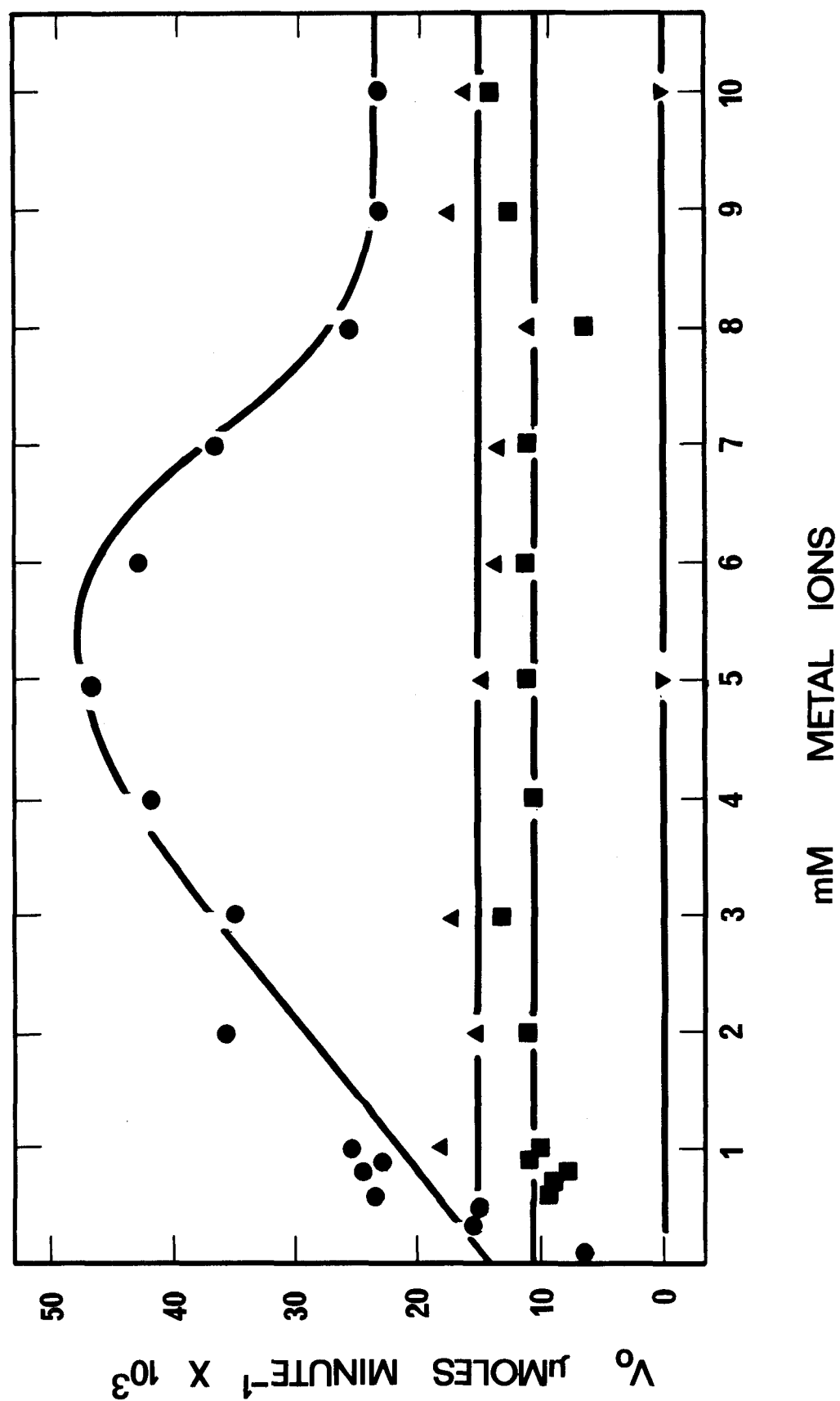
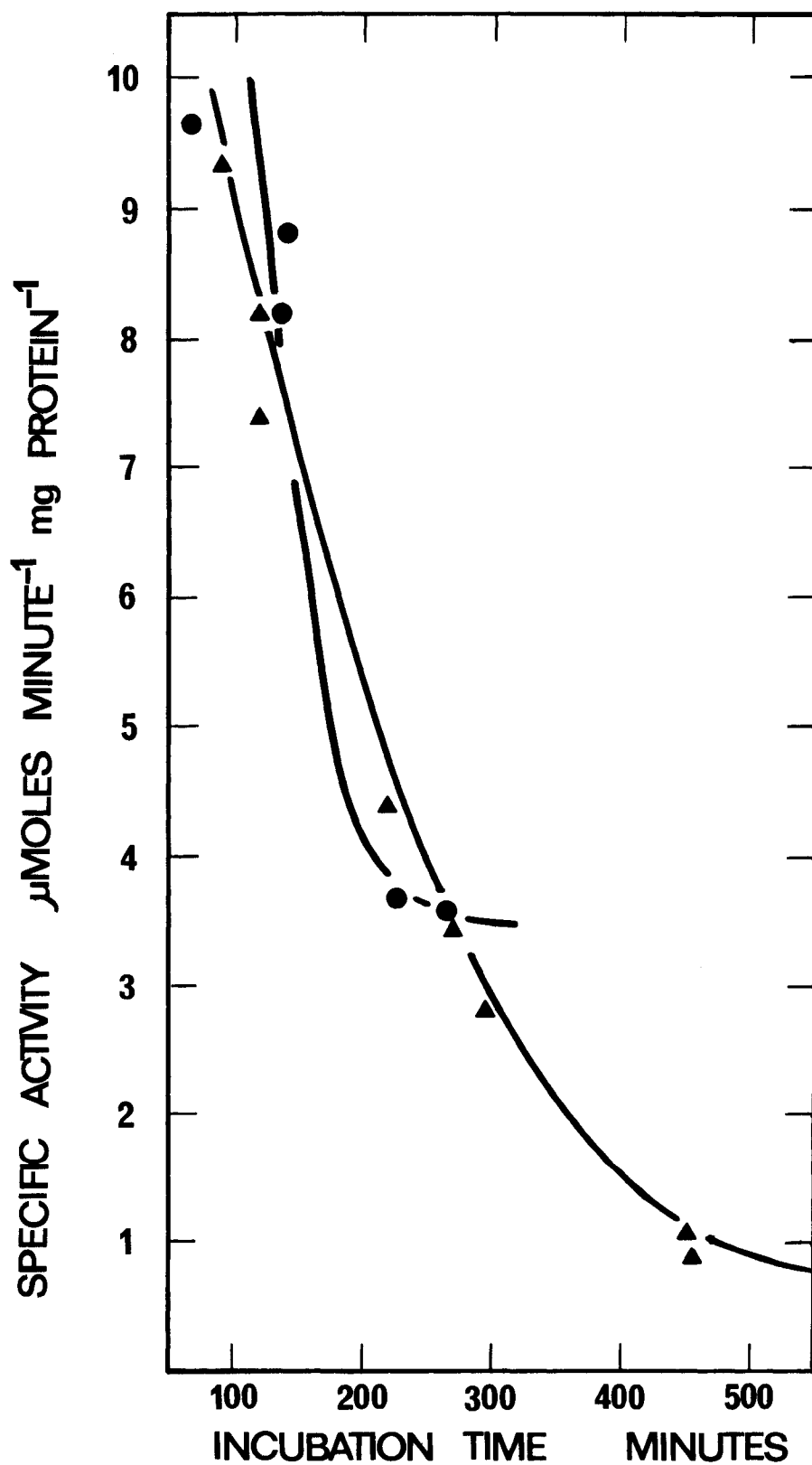


Fig. 4. Inactivation of citrate lyase with time.

The standard assay procedure was used. Each cuvette (1.0 cm light path, 3.00 ml volume) contained 6.67 mM sodium citrate; ca. 16  $\mu$ M units MDH; 95.3 mM TEA.HCl-NaOH buffer, pH 7.6; 0.235 mM NADH and 7.0 mM  $\text{ZnCl}_2$ . The reaction was initiated by the addition of 6.0  $\mu$ g CL protein with the exception of the 90 minute assay in which case 5.0  $\mu$ g CL protein was added. For the standard control assay ( $\bullet$ ), 0.3 mg CL protein was dissolved in 10.0 ml CL diluent. For the UV activity assay of the NMR assay system in which all solutions were prepared in  $\text{D}_2\text{O}$  ( $\blacktriangle$ ), 0.3 mg CL protein dissolved in 0.1 ml CL diluent was added to 0.9 ml containing 0.2 M TEA.HCl-NaOH buffer, pH 8.0; 0.4 M sodium acetate; 0.1 M t-butyl alcohol; 0.4 M  $\text{MgSO}_4$  and 0.4 M L-malic acid. This mixture was then diluted to 10.0 ml with CL diluent.

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FIGURE 4



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## 2) Nuclear Magnetic Resonance Spectrometric Assays.

Acetate- $\text{h}_3$ , even after a three hour incubation with  $\text{D}_2\text{O}$  in buffered solution in the presence of enzyme and divalent metal ions, failed to show any loss of methyl protons from acetate by a decrease in the appropriate nuclear magnetic resonance signal (Table I). In contrast to the citrate synthase reaction in which both OAA and L-malate induce hydrogen-deuterium exchange between the acetyl group of acetyl CoA and the deuterons of the medium (Srere, 1967), neither keto-oxaloacetate nor its structural analogue L-malic acid were able to induce the exchange of protons between the methyl carbon of acetate and the deuterons of the medium (Table II and Table III).

No enzymatic activity was detected when citrate lyase was mixed into a buffered system in  $\text{H}_2\text{O}$  or  $\text{D}_2\text{O}$  containing citrate and  $\text{MgSO}_4$  (Table IV). The addition of MDH and NADH to this buffered system prior to the addition of citrate lyase did not result in the observance of any enzymatic activity (Table IV).

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TABLE I: Nuclear Magnetic Resonance Spectrometric Assay  
for Hydrogen - Deuterium Exchange in Acetate.

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Components of Reaction Mixture				Extent
NaAc	MgSO <sub>4</sub>	pD	CL Protein	of
(M)	(M)	of System	(mg)	Exchange
0.4	0.38	7.7	0.1	0
0.32	0.30	7.7	0.3	0
0.40	0.40	8.0	0.9	0
0.38	0.38	7.7	1.0	0

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The standard NMR assay procedure was used. In addition to the components indicated, the reaction mixtures contained 0.3 - 0.4 M TEA.HCl-NaOH buffer and 0.10 - 0.15 M t-butyl alcohol.

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TABLE II: Effect of L-Malic Acid on the Citrate Lyase - Catalyzed Hydrogen - Deuterium Exchange in Acetate.

Components of Reaction Mixture				Extent
NaAc	MgSO <sub>4</sub>	L-Malic Acid	CL Protein	of
(M)	(M)	(M)	(mg)	Exchange
0.4	0.4	0.4	0.3	0
0.4	0.4	0.4	0.6	0
0.4	0.4	0.4	0.9	0
0.4	0.4	0.2	0.9	0

The standard NMR assay procedure was used. In addition to the components indicated, the reaction mixtures contained 0.2 M TEA.HCl-NaOH buffer, pD 8.0 and 0.1 M t-butyl alcohol.

TABLE III: Effect of Keto - Oxaloacetate on the Citrate Lyase - Catalyzed Hydrogen - Deuterium Exchange in Acetate.

Components of Reaction Mixture				Extent
NaAc	MgSO <sub>4</sub>	Keto-OAA	CL Protein	of
(M)	(M)	(M)	(mg)	Exchange
0.3	0.2	0.01	0.75	0
0.3	0.2	0.02	1.05	0
0.3	0.2	0.01	1.20	0

The standard NMR assay procedure was used. In addition to the components indicated, the reaction mixtures contained 0.2 M TEA.HCl-NaOH buffer, pD 8.0 and 0.2 M t-butyl alcohol. In the second run the MgSO<sub>4</sub> was incubated with the CL protein for ten minutes prior to the initiation of the reaction. In the third run the MgSO<sub>4</sub> was incubated with all reaction components except the CL protein for ten minutes prior to the initiation of the reaction.



TABLE IV: Nuclear Magnetic Resonance Spectrometric Assay for Citrate Lyase Activity.

Sodium Citrate	MgSO <sub>4</sub>	pH	pD	MDH	NADH	CL Protein	Enzymatic Activity
(M)	(M)			( $\mu$ M Units)	(mM)	(mg)	
0.40	0.05		8.0			0.6	0
0.40	0.30		8.0			0.3	0
0.40	0.30	7.6				0.3	0
0.40	0.30		8.0			0.6	0
0.40	0.30	7.6				0.6	0
0.40	0.30		8.0	160	7.51	0.6	0

The standard assay procedure was used. In addition to the components indicated, the reaction mixtures contained TEA.HCl-NaOH buffer and t-butyl alcohol.

3) Spontaneous Exchange of the Active Alcohol Proton in Citrate for Deuterium.

Three sets of experiments were carried out as outlined in Methods. The infrared spectra of the isolated sodium citrate (Fig. 5; Fig. 6; and Fig. 7) give an indication of the extent of the spontaneous hydrogen-deuterium exchange of the hydroxyl proton in citrate.

The values reported for the extent of exchange (Table V) were calculated after assuming no exchange when sodium citrate is dissolved in deionized  $H_2O$  (Fig. 6a) and 100% exchange when sodium citrate is incubated for four weeks in  $D_2O$  (Fig. 7).

Fig. 5. The infrared spectra showing the acid-catalyzed hydrogen-deuterium exchange in citrate. The details of the composition of the reaction mixtures are given in Methods. Solutions of citric acid were incubated at pH 1 for fifteen minutes prior to neutralization and drying.

- a) Protonic system
- b) Deuteronic system

FIGURE 5

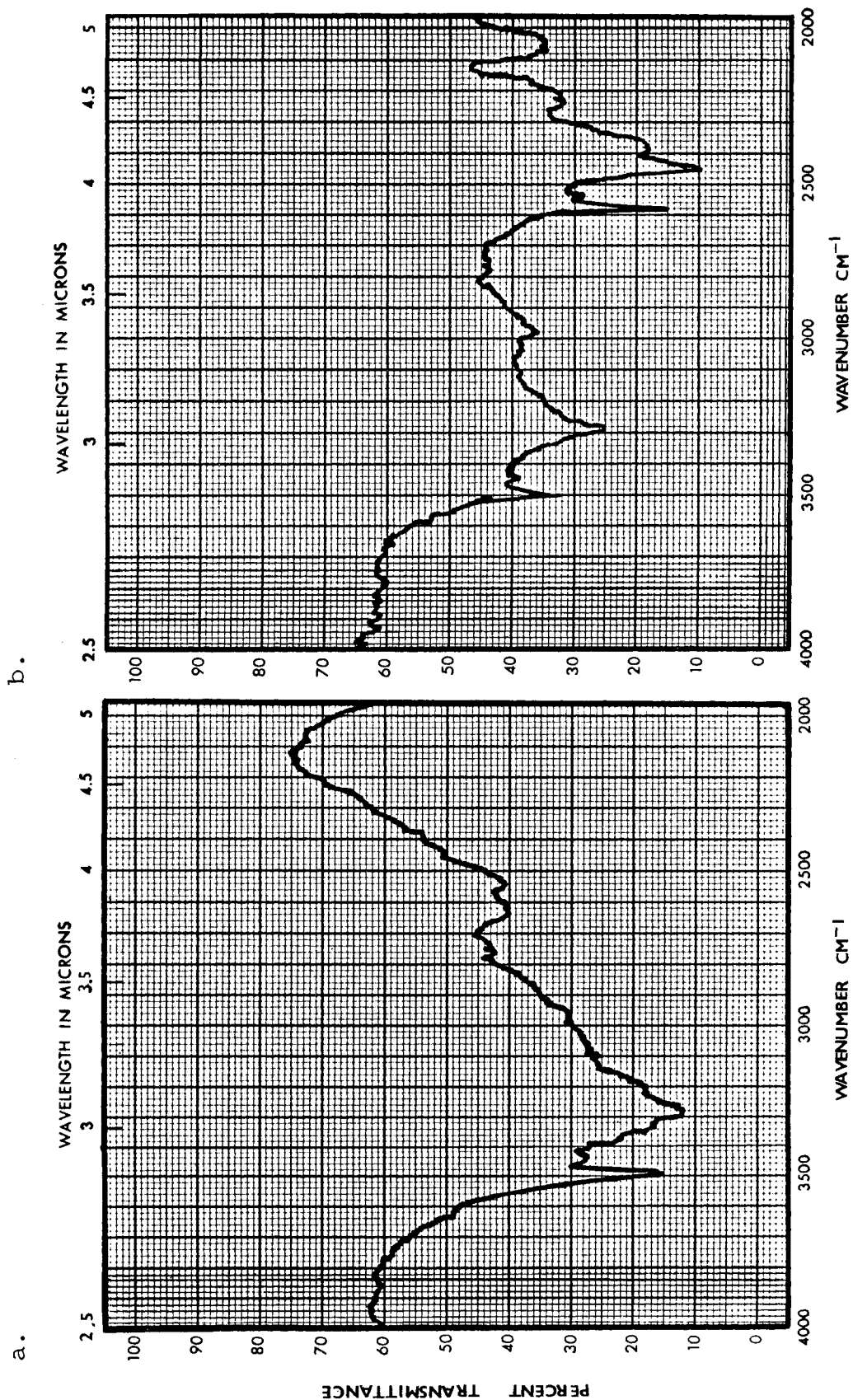


Fig. 6. The infrared spectra showing the uncatalyzed hydrogen-deuterium exchange in citrate in fifteen minutes.

The details of the composition of the reaction mixtures are given in the Methods. Solutions of sodium citrate were stripped to dryness (approximately fifteen minutes) as soon as the sodium citrate was dissolved.

- a) Protonic system
- b) Deuteronic system

FIGURE 6

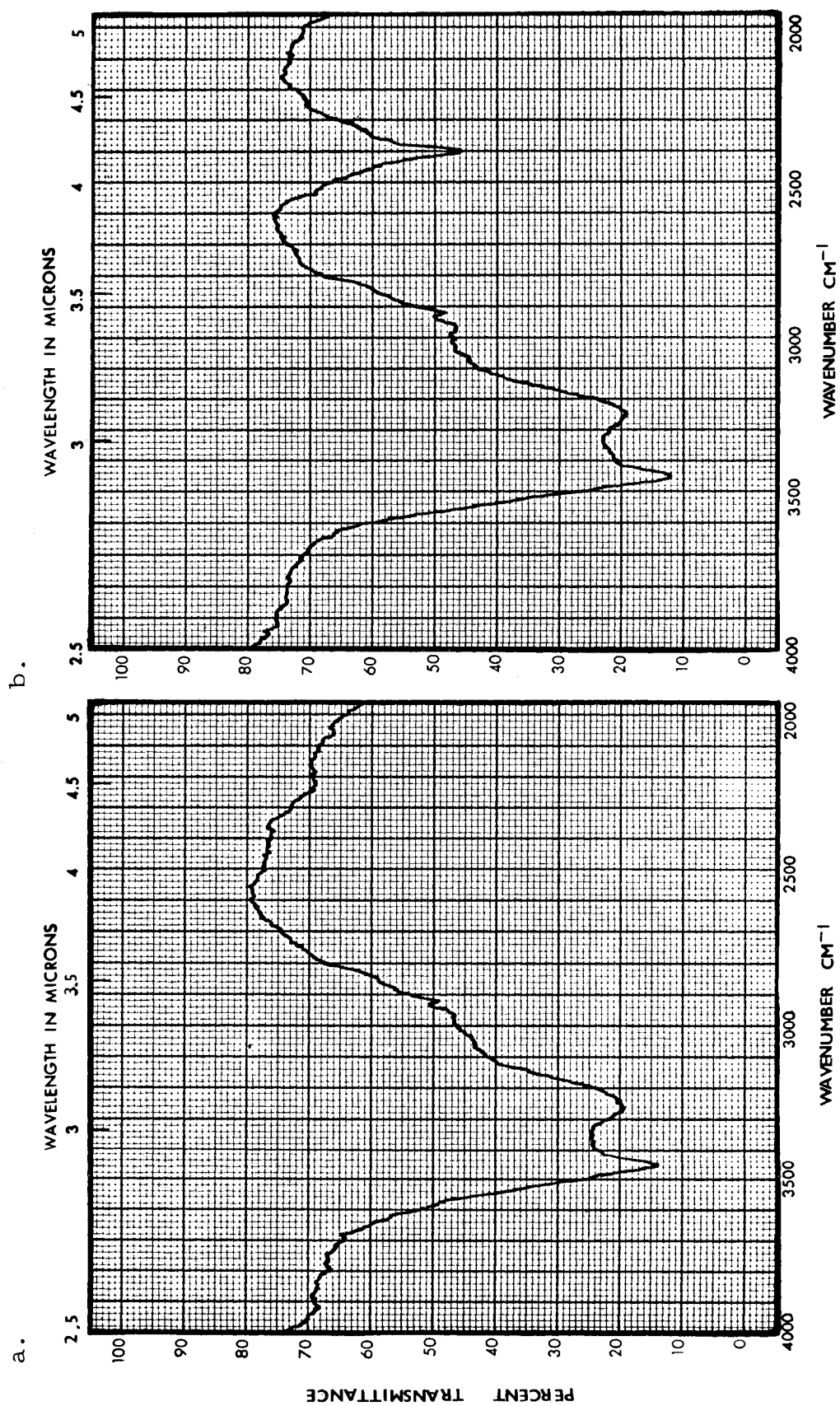


Fig. 7. The infrared spectra showing the uncatalyzed hydrogen-deuterium exchange in citrate in four weeks. A 1.0 M solution of sodium citrate in  $D_2O$  was incubated for four weeks. It was then evaporated to dryness under vacuum and dried in an oven at  $156^{\circ}$  for four days before being pressed into a potassium bromide disc.

FIGURE 7

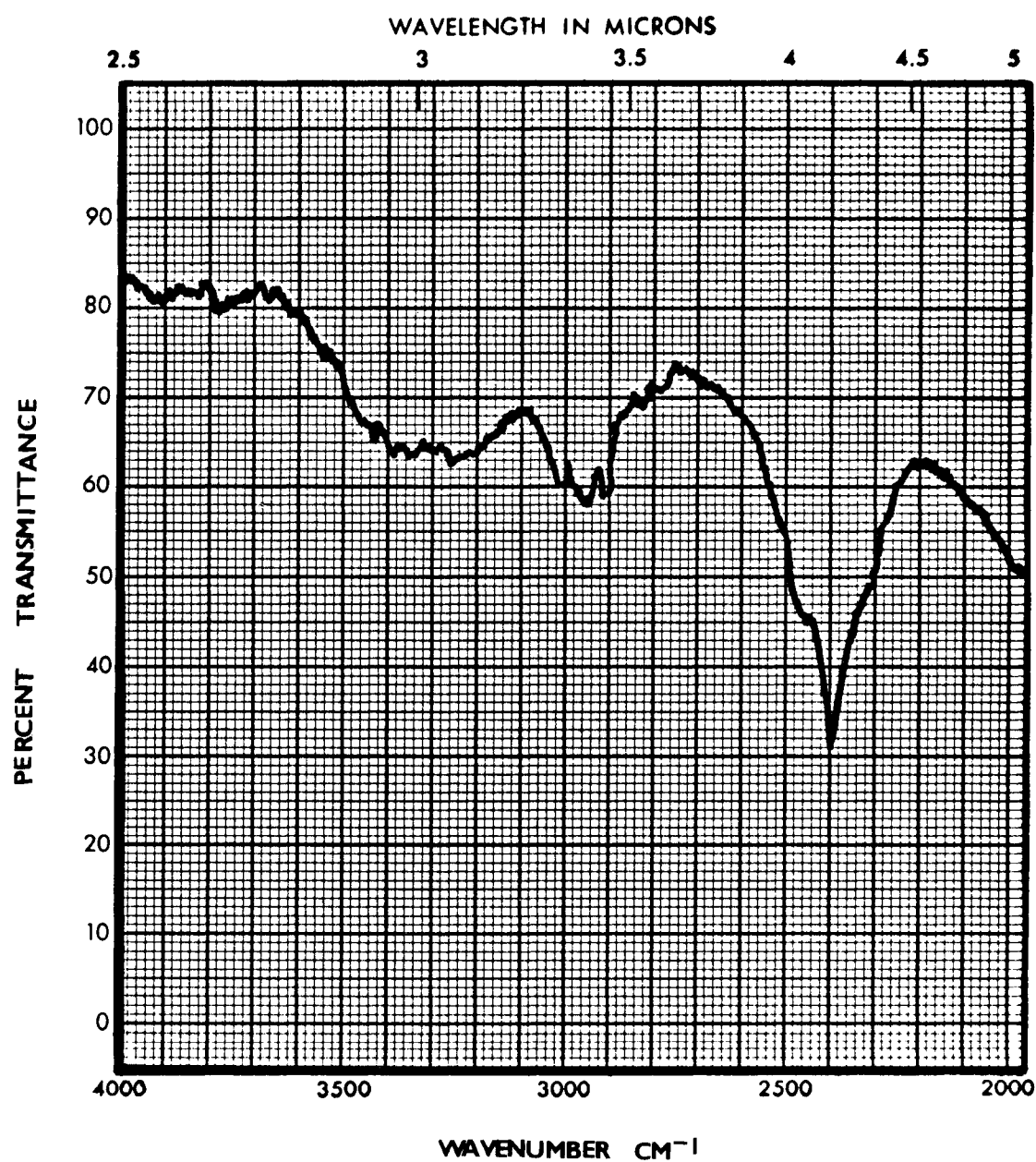




TABLE V: Spontaneous Exchange of the Active Alcohol Proton in Citrate for Deuterium.

Figure	Incubation Conditions		Time of Incubation	Percent Transmittance in the O-D Region	Percent Exchange
7	Neutral	D <sub>2</sub> O	4 weeks	0.0	100.0
5b	Acidic	D <sub>2</sub> O	15 minutes	0.5	99.2
6b	Neutral	D <sub>2</sub> O	15 minutes	24.0	61.3
6a	Neutral	H <sub>2</sub> O	15 minutes	63.5	0.0

Each sample was prepared as described in Methods. The percent transmittance in the O-D region are reported relative to a 27% transmittance at 2980 cm<sup>-1</sup> (2930 cm<sup>-1</sup> for 5b).

#### 4) Incorporation of Deuterium into Acetate.

Two reactions were carried out in  $D_2O$  medium to follow the incorporation of protons into acetate. The reaction mixtures were identical except for the type of citrate introduced (see Table VI). Undeuterated citrate was added as the solid trisodium salt of citric acid. The citrate containing a deuterated hydroxyl group was introduced into the system as an equilibrated solution in  $D_2O$ .

The deuterium content of the acetate isolated from the reaction mixture was from zero to three deuterons per molecule. Up to 46% of the acetate molecules isolated contained three carbon-bound deuterons (Table VI). Tracings of the mass spectra used for the isotopic abundance calculations are given in Fig. 9.

Table VI: Mass spectrometric assay for incorporation of deuterium into enzymatically produced acetate. Each reaction mixture was treated as described in Methods. The amounts of keto-OAA formed were calculated from the change in absorbance of 3 ml aliquots of the complete reaction mixtures. The amounts of acetate formed were determined by titration of the distilled reaction mixtures. The minimum isotopic purity of the  $D_2O$  reaction mixtures was calculated to be 99.0 Atom % D.

TABLE VI: Mass Spectrometric Assay for the Incorporation of Deuterium into Enzymatically Produced Acetate.

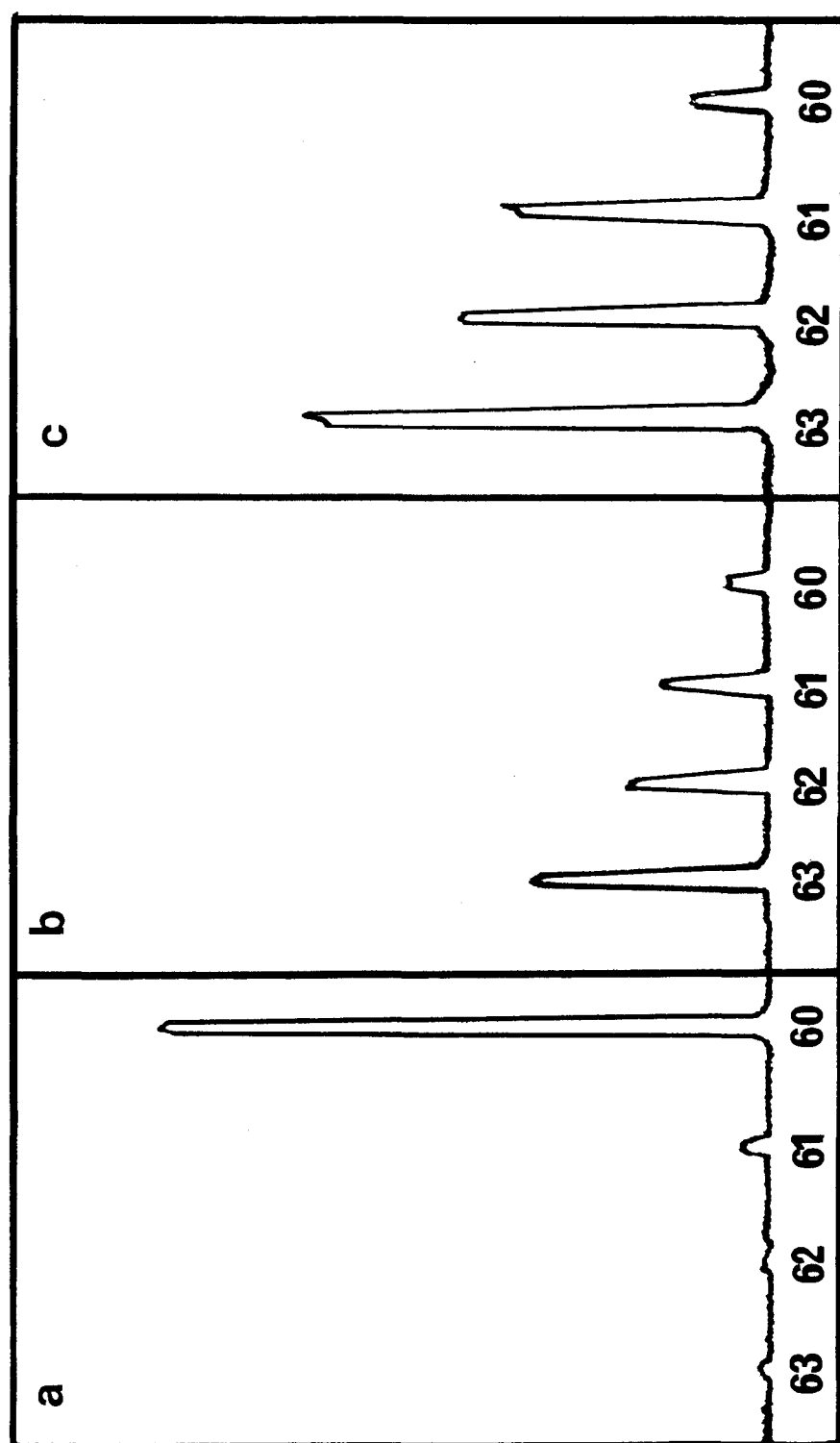
No.	Substrate	Citrate added ( $\mu$ moles)	Keto-OAA formed ( $\mu$ moles)	Acetate formed ( $\mu$ moles)	Isotopic Abundance Calculations (Mass)			
					$\text{CH}_3\text{COOH}$ (60)	$\text{CH}_2\text{DCOOH}$ (61)	$\text{CHD}_2\text{COOH}$ (62)	$\text{CD}_3\text{COOH}$ (63)
1.	$\begin{array}{c} \text{CH}_2\text{COONa} \\   \\ \text{HO}-\text{C}-\text{COONa} \\   \\ \text{CH}_2\text{COONa} \end{array}$	923	37.26	121.7	6.7%	20.3%	26.2%	46.8%
2.	$\begin{array}{c} \text{CH}_2\text{COONa} \\   \\ \text{DO}-\text{C}-\text{COONa} \\   \\ \text{CH}_2\text{COONa} \end{array}$	930	35.85	144.5	6.7%	23.2%	28.2%	41.9%

Fig. 9. The mass spectra of enzymatically produced acetic acid.

Solid samples of sodium acetate were acidified with concentrated sulfuric acid to convert the acetate to free acetic acid.

- a) Control. Acidified reagent grade sodium acetate.
- b) Experiment 1. Acidified sodium acetate isolated from reaction mixture No. 1.
- c) Experiment 2. Acidified sodium acetate isolated from reaction mixture No. 2.

FIGURE 9



MASS

## CHAPTER 3

### DISCUSSION

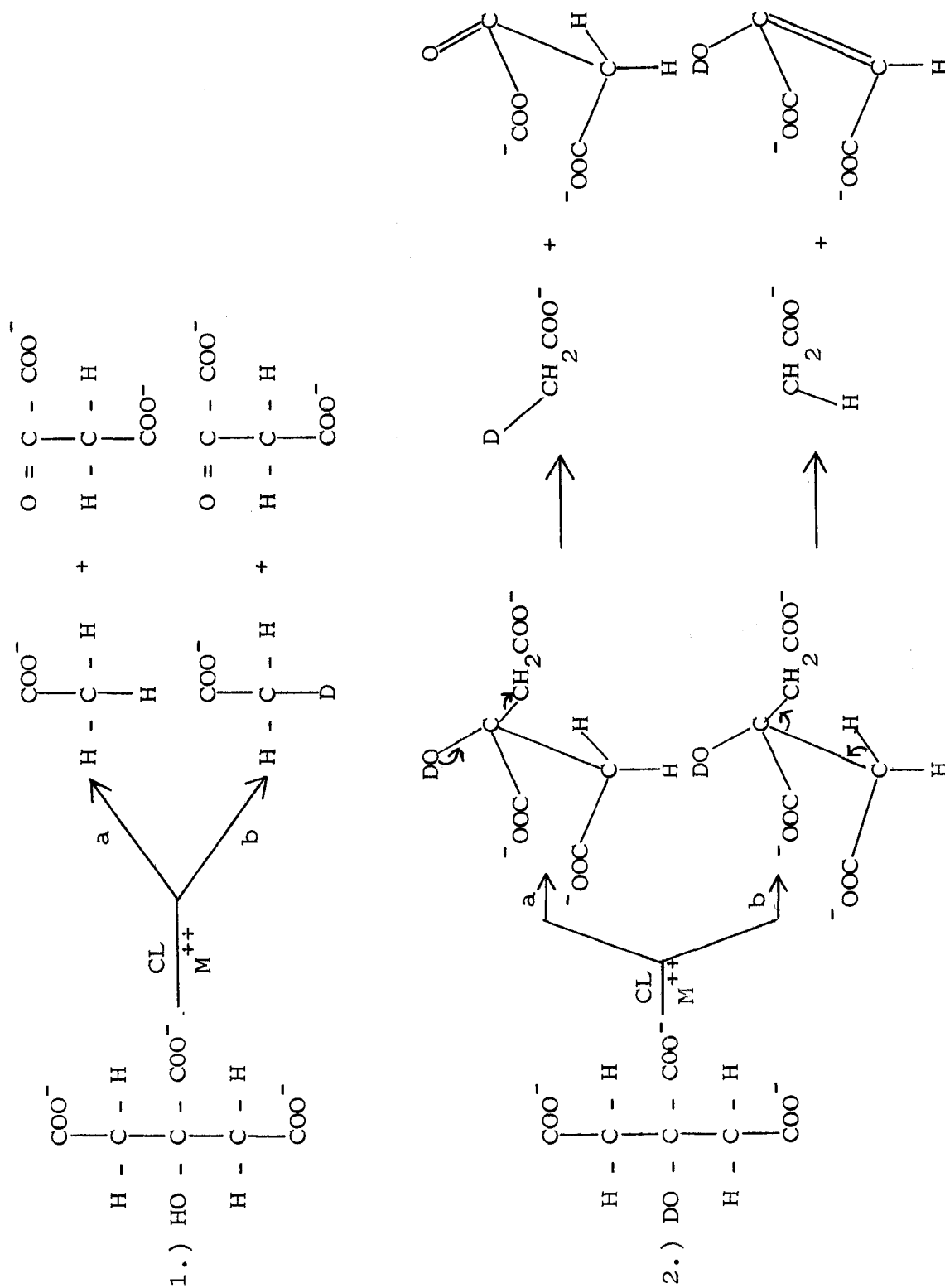
Elucidation of the mode of action of citrate lyase is complicated by the various difficulties indicated in the Introduction, that is, metal ion inhibition, product inhibition and partial reaction. In light of this, the experimental approaches are limited. And yet certain questions can be asked.

#### A. MECHANISM OF CITRATE CLEAVAGE

Our approach to the problem may be visualized by the reactions formulated in Fig. 9.

Equation 9:1 illustrates the possibilities arising when a reaction starting with undeuterated citrate and the other components necessary to achieve citrate cleavage is carried out in  $D_2O$ . If the enzyme catalyzes a direct proton transfer to the methyl group of acetate, some unlabeled acetic acid should be obtained (Equation 9:1a). Some deuterium may be incorporated due to the spontaneous exchange of the hydroxyl protons of citrate with the deuterons of the medium. It was, therefore, necessary to complete the reaction as quickly as possible with the

FIGURE 9: Mechanism of Citrate Lyase-Catalyzed Citrate Cleavage in D<sub>2</sub>O.





use of excess citrate lyase. On the other hand, a reaction involving indirect transfer of hydrogen through the medium to the methyl group of acetate must result in the formation of acetic acid containing at least one atom of nonexchangeable, carbon-bound deuterium per molecule (Equation 9:1b).

The data used to decide which of these transfers is correct are given in Table VI. A direct hydrogen transfer is compatible with the production of 6.7%  $\text{CH}_3\text{COOH}$  in Experiment 1. A mechanism involving indirect hydrogen transfer through the medium can be ruled out by the presence of this unlabeled acetic acid.

Two possible interpretations are consistent with the results of Experiment 1. Either a retrograde aldol condensation or an internal elimination mechanism is feasible. Equation 9:2 shows the possibilities arising when the citrate cleavage is carried out in  $\text{D}_2\text{O}$  starting with citrate previously equilibrated so that the hydroxyl protons were exchanged for deuterium. If the mechanism is a retrograde aldol condensation (Equation 9:2a) as suggested by Dagley and Dawes (1955), Smith et al. (1956), and Eisenthal et al. (1966), at least one carbon-bound deuterium must be present per molecule of acetic acid. If an internal elimination reaction mechanism is followed

(Equation 9:2b), some unlabeled acetic acid should be obtained. The experimental results rule out a retrograde aldol condensation mechanism. From the above considerations, the tentative conclusion is reached that the citrate lyase-catalyzed cleavage of citrate proceeds by an internal elimination mechanism.

In the preceeding discussion, no mention was made of the possibility of an exchange between the methyl protons of acetate and the deuterons of the medium. If no exchange occurs, the maximum number of deuterium atoms incorporated per molecule would be one. Our results show that in over 40% of the acetate molecules produced three deuterons were incorporated per molecule. This indicates that citrate lyase is also able to catalyze a hydrogen-deuterium exchange in the methyl group of acetate. The pattern of increasing proportions of  $\text{CH}_3\text{COOH}$ ,  $\text{CH}_2\text{DCOOH}$ ,  $\text{CHD}_2\text{COOH}$  and  $\text{CD}_3\text{COOH}$  formed (Table VI) further supports a consecutive exchange reaction. Table I shows that the methyl protons of acetate do not exchange spontaneously with the medium.

Our nuclear magnetic resonance spectrometric results gave no indication of such an enzymatically catalyzed exchange (Table I, Table II, and Table III). There are two possible explanations for this inconsistency.

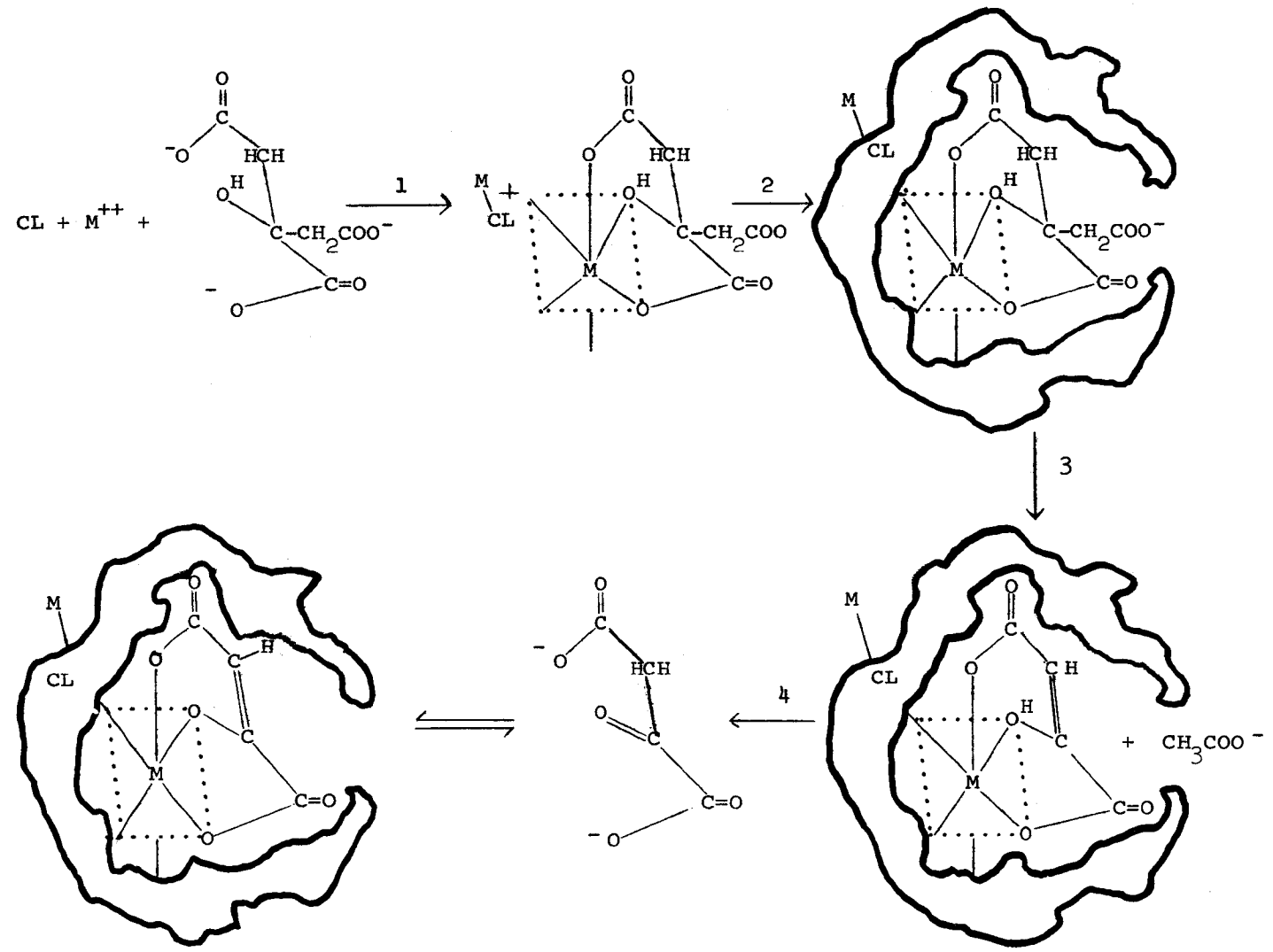
First, the other reaction product, enol-oxaloacetate (see Section B following), still bound to the enzyme or metal ion, may be required for the exchange. Second, the high ionic strength of the reaction mixtures used in the nuclear magnetic resonance spectrometric assay systems may have completely inhibited the enzyme. This second possibility is supported by our inability to observe any enzymatic activity even when citrate lyase was added to a buffered solution of citrate and  $\text{MgSO}_4$  (Table IV). Further experimentation is required before any conclusions can be drawn concerning the nature of this exchange.

## B. PROPOSED REACTION PATHWAY

We will propose a tentative mode of action for the citrate lyase reaction (Fig. 10). With this as a basis, our data, as well as that of other workers, will be interpreted and discussed.

According to our model, the enzyme requires the binding of a metal at a site or sites distinct from the active site. This binding would cause a conformational change to yield the active species of the enzyme. A compulsory ordered mechanism is then followed. The first step requires the chelation of the metal with citrate to form the citrate-metal active complex (Reaction 10:1). The metal portion of the active complex then chelates with the enzyme to form the ternary complex (Reaction 10:2). By an internal elimination reaction, acetate is split off (Reaction 10:3). Enolic oxaloacetate remains bound to the enzyme through the metal. Upon dissociation of the oxaloacetate from the metal-enzyme complex, it is converted to its keto tautomer (Reaction 10:4). This dissociation proceeds until the equilibrium conditions defined by the stability constants of the various forms of the metal-oxaloacetate chelates are satisfied. The feasibility of this model system will now be discussed.

FIGURE 10: Proposed Reaction Pathway for the Citrate Lyase-Catalyzed Cleavage of Citrate.



Any mechanism for citrate lyase action must take account of five salient observations, namely: activation and inactivation by  $Mg^{++}$ ; protection and competitive inhibition by metal ions; activation strength of various divalent metal ions; product inhibition; and incomplete reactions.

As the magnesium ion concentration increases, the enzymatic activity is progressively increased, reaches a maximum, and then decreases (Harvey and Collins, 1963) (Fig. 3). Using our model, this can be interpreted in two ways. As the low  $Mg^{++}$  concentration is raised, the formation of the  $Mg^{++}$ -citrate active species is enhanced by the equilibrium defined by the stability coefficient for the complex. A point is reached after which the chelation of  $Mg^{++}$  at the active site of the enzyme is favored. This may be the cause of the observed inhibition in the presence of excess  $Mg^{++}$ . It seems more likely though, that the excess  $Mg^{++}$  inhibits by maintaining a high concentration of the inhibitory  $Mg^{++}$ -enol-oxaloacetate complex.

Calcium and magnesium both protect the enzyme from inactivation in dilute solutions (Dagley and Dawes, 1955; Harvey and Collins, 1963). This may be due to the

binding of the metal at an allosteric site, thus causing a conformation change which in turn prevents the dissociation into subunits.

The stability coefficients for magnesium-citrate and calcium-citrate chelation complexes are about the same (Martell and Calvin, 1952). Therefore, there exists a competition between the  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  for the citrate ion. It appears that perhaps the calcium-citrate complex is unable to form an active ternary complex with the enzyme. Dagley and Dawes (1955) attributed the non-reactivity of calcium to its greater ionic radius than those metals which are able to activate the enzyme. Eisinger et al. (1966) offered an alternate explanation. Calcium may be unable to form a catalytically-active complex because of the different structures of the magnesium and calcium chelates.

In terms of initial velocity, magnesium is seen to be a more efficient activator than zinc (Fig. 3). The rate of inactivation, however, is three times faster with  $\text{Mg}^{++}$  than with  $\text{Zn}^{++}$  (Moellering and Gruber, 1966). This would be consistent with a higher stability of the magnesium complexes than those of zinc. Thus  $\text{Mg}^{++}$  could form a more stable ternary complex than  $\text{Zn}^{++}$  and therefore a greater initial velocity would be observed. By the same reasoning,

$Mg^{++}$  would cause a faster inactivation than the  $Zn^{++}$  since the magnesium-oxaloacetate complex produced would be bound more tightly to the active site of the enzyme. It is interesting to note that the chelate complexes of zinc generally have rather high stability constants on the basis of ionic radius compared to magnesium ions. Thus, the radius of  $Mg^{++}$  is actually less than that of  $Zn^{++}$ , whereas the stability constants of the latter are generally much higher. It seems that zinc chelates involve bonds of a different type than those of magnesium (Martell and Calvin, 1952). It is possible, therefore, that the citrate lyase ternary complexes containing magnesium involve different bond types than those containing zinc. Thus, the former may be rendered more stable in spite of the fact that zinc chelates are generally thought to have higher stability constants.

Eisenthal et al. (1966) presented evidence which revealed the highly specific structural requirements for inhibition; namely, a straight chain,  $C_4$ , dicarboxylic acid with an ionizable  $\alpha$ -hydroxy group, and the presence of a divalent metal cation. This structural specificity is consistent with our proposal. The inactivated species postulated is an enzyme—metal-ion—enol-oxaloacetate



ternary complex. The formation of such a complex would require an ionizable  $\alpha$ -hydroxy group and two carboxylic acid groups to participate in chelate formation with the metal ion.

There is no doubt that product inhibition is partially responsible for the incompleteness of the reaction. It also appears that the coupled ultraviolet spectrometric assay at 340  $\mu\text{m}$  is not a true indication of the extent of the reaction. Titration of the distilled reaction mixture suggests that more than three times as much citrate may be cleaved than the coupled assay indicated (Table VI). It is possible that the oxaloacetate produced remains complexed with magnesium in a form which is not assayed.

The model suggested is speculative but reasonable. It suggests many interesting experiments for further study as well as offering some explanation of the present data.

## CHAPTER 4

### SUMMARY

- 1) Suitable methods and conditions were developed for the various spectrometric assay systems.
- 2) Using a nuclear magnetic spectrometric assay method no citrate lyase-catalyzed exchange between the methyl protons of acetate and the deuterons of the medium could be observed.
- 3) Infrared spectrometric studies showed that the exchange of the hydroxyl proton of citrate is not instantaneous.
- 4) Mass spectrometry was used to show that the citrate lyase-catalyzed cleavage of citrate proceeds by an internal elimination mechanism. This was shown to involve a direct proton transfer from the methylene group of citrate to the methyl group of acetate.
- 5) A citrate lyase-catalyzed exchange of the methyl protons of acetate was also demonstrated by the mass spectrometric assay method.

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