MICROBIAL ENZYMES:

studies on citrate lyase (E.C. 4.1.3.6)

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BY

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No part of this thesis has been submitted for a degree or diploma or other academic award. The literature concerning the problems investigated had been surveyed, and all the necessary references are given in the thesis. The present work has been clearly indicated separately. The experimental work has been carried out entirely by me. In accordance with the usual practice, due acknowledgement has been made whereever the work presented is based on the results of other workers.

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ABBREVIATIONS

List of abbreviations used other than the accepted ones.

ACP	-	Acyl carrier protein
DSP	-	Dithiobis(succinimidyl propionate)
DTNB	-	5,5'-Dithiobis(2-nitrobenzoate)
DTT	-	Dithiothreitol
OAA	-	Oxaloacetic acid
SDS	-	Sodium dodecyl sulfate
$E^{1\%}_{1\ cm}$	-	Absorbance index (1% w/v protein)
ϵ_m	-	Molar extinction coefficient
2-ME	-	2 mercaptoethanol
MDH	-	malate dehydrogenase (EC 1.1.1.37)

CONTENTS

GENERAL INTRODUCTION

CITRATE LYASES

Citrate lyase (citrate oxaloacetate-lyase pro-3S-$CH_2COO^- \rightarrow$ acetate, (EC 4.1.3.6)), an enzyme found only in procaryotes, catalyses the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metals such as Mg^{2+} and Mn^{2+} by the overall reaction shown in Eq. 1.

$$\text{Citrate}^{-3} \xrightleftharpoons{M^{2+}} \text{Oxaloacetate}^{-2} + \text{Acetate}^{-1} \qquad \text{(Eq. 1)}$$

Three other groups of enzymes are also known to catalyse the breakdown/synthesis of citrate by a lyase reaction involving an equilibrium between citrate on the one hand and oxaloacetate and an acetyl moiety on the other. These are:

(1) ATP-citrate lyase (ATP: citrate oxaloacetate-lyase (pro-3S-$CH_2COO^- \rightarrow$ acetyl-CoA: ATP dephosphorylating), EC 4.1.3.8). The enzyme, found in the cytosol and only in eucaryotic cells, catalyses the overall dissimilation reaction shown in Eq. 2.

$$\text{Citrate}^{-3} + \text{CoA} + \text{ATP}^{-4} \xrightleftharpoons{M^{2+}} \text{Acetyl-CoA} + \text{Oxaloacetate}^{-2} + \text{ADP}^{-3} + P_i^{-2} \qquad \text{(Eq. 2)}$$

(2) si-citrate synthase (citrate oxaloacetate-lyase (pro-3S-$CH_2COO^- \rightarrow$ acetyl-CoA), EC 4.1.3.7) of the citric acid cycle. This enzyme is involved in the synthesis of citrate according to the reaction shown in Eq. 3.

$$\text{Citrate}^{-3} + \text{CoA} + H^+ \rightleftharpoons \text{Acetyl-CoA} + \text{oxaloacetate}^{-2} + H_2O \qquad \text{(Eq. 3)}$$

(3) re-citrate synthase (citrate oxaloacetate-lyase(pro-3R-CH_2COO^- ⟶ acetyl-CoA) EC number not assigned yet), present in some anaerobic microorganisms. This is the only enzyme of the family which has the opposite stereochemistry as compared with the other three enzymes of the group.

Citrate is a central intermediate in a number of divergent metabolic pathways and the involvement of several groups of enzymes in the synthesis/dissimilation of citrate is to be expected. Close similarities, however, exist in the mechanism of action of most of the enzymes that catalyse a lyase reaction leading to the cleavage or formation of citrate. These are dealt with in greater detail in the later section of the INTRODUCTION. The existence of such resemblances between the different groups of enzymes might suggest divergence in evolution from a common ancestral or primordial enzyme. It has been conjectured that citrate lyase which occurs only in the most primitive forms of life and has the simplest supplementary requirement amongst this group of enzymes might be the earliest evolutionarily (Srere, 1968). ATP-citrate lyase, occurring only in higher organisms and catalysing the most complex reaction involving three substrates and a divalent metal ion probably represents the last of the group to evolve.

I.1. CITRATE LYASE (EC 4.1.3.6)

Interest has been focussed on this enzyme in recent years with the discovery of it's multienzymic nature. The enzyme complex has been the subject of several reviews

(Dagley, 1969; Lowenstein, 1969; Spector, 1972; Srere, 1965; 1968; 1972; 1974; 1975; Srere and Singh, 1974). The extensive literature on this enzyme is summarized briefly in this Chapter. Related topics of bacterial citrate transport and the role and regulation of citrate lyase activity *in vivo* are also discussed.

I.1 (i) OCCURRENCE: The enzyme has been found only in bacterial sources. Table 1 includes the sources in which the activity has been detected till now.

TABLE 1: SOURCE OF CITRATE LYASE

Nature of activity	Bacteria	Reference
1. Induced	*Klebsiella aerogenes**	Dagley and Dawes (1953b)
	Escherichia coli	Grunberg-Manago and Gunsalus (1953); Dagley (1954)
	Streptococcus faecalis	Gillespie and Gunsalus (1953)
	Aerobacter cloacae	O'Brien (1975c)
	Leuconostoc citrovorum	Harvey and Collins (1961)
	Streptococcus liquefaciens	Harvey and Collins (1961)
	Salmonella typhimurium	O'Brien *et al.* (1969)
	Rhodopseudomonas gelatinosa	Weckesser *et al.* (1969); Schaab *et al.* (1972)
2. Constitutive	*Streptococcus diacetilactis*	Harvey and Collins (1961)

*Known earlier as *Aerobacter aerogenes*; also known as *Klebsiella pneumoniae* and *Enterobacter aerogenes*.

The enzyme occurs as a substrate induced activity in all sources except Streptococcus diacetilactis in which it occurs constitutively. Historically the fermentation of citrate by K. aerogenes (Deffner and Franke, 1939) and Aerobacter indologenes (Brewer and Werkman, 1939) to oxaloacetate and acetate was known more than 4 decades ago. However, the first characterization of the enzyme was made only 14 years later by Dagley and Dawes (1953a) in K.aerogenes and E.coli and by Gunsalus and his group in E.coli (Gillespie and Gunsalus, 1953) and S.faecalis (Grunberg-Manago and Gunsalus, 1953).

The occurrence of citrate lyase as a constitutive enzyme in S.diacetilactis was reported by Harvey and Collins (1961). In this source activity was detected in cultures grown on lactose without citrate; supplementation of the medium with citrate enhancing enzyme levels in the cell.

R.gelatinosa is amongst the few phototropic bacteria which grows readily on citrate. The presence of citrate lyase activity in the source was first described by Weckesser et al. (1969)

In Clostridium sphenoides, an anaerobic spore former characterized by it's ability to grow on citrate as the carbon and energy source, the use of stereospecifically labelled ^{14}C-citrate has suggested the possible involvement of citrate lyase in the utilisation of citrate by this organism (Walther et al., 1977).

A pathway in which citrate lyase has been postulated as a key enzyme is the reductive carboxylic acid cycle (Evans et al., 1966). However in the anaerobic phototrophic bacteria Chlorobium thiosulphatophilum and Rhodospirullum rubrum which are known to synthesize ∝-keto acids from acyl-CoA esters by reductive carboxylation, no citrate lyase activity could be detected (Beucher and Gottschalk, 1972).

I.1 (ii) PURIFICATION OF CITRATE LYASES: The enzyme has been obtained pure from K.aerogenes, S.diacetilactis, R.gelatinosa and S.faecalis. The various procedures used for purification of the enzyme from these sources are outlined below.

(a) Citrate lyase from K. aerogenes: Citrate lyase was obtained pure for the first time from this source (SivaRaman, 1961). Enzyme from the organism grown on citrate as carbon and energy source was purified from extracts of sonicated cells by a procedure involving preliminary removal of nucleic acids with streptomycin sulphate, adsorption of the enzyme on alumina C_γ gel followed by elution from the gel with higher concentrations of buffer, chromatography on DEAE-cellulose followed by ammonium sulphate precipitation. Later modifications of the original procedure include final filtration through Sephadex G-200 gel (Mahadik and SivaRaman, 1968), or Biogel A 1.5 m (Singh and Srere, 1971). Other changes have been the replacement of alumina C_γ gel adsorption and DEAE-cellulose chromatography steps by salmine sulphate treatment and CM-cellulose chromatography (Bowen and Mortimer, 1971). A recent modification for purification

of the enzyme which is reported to be suitable for large scale preparations involves precipitation of the alumina C_γ gel eluate with polymin HS (ethyleneimine polymer) followed by extraction of activity from the polymer, dialysis and filtration through Sephadex G-200 (Dimroth et al., 1977b).

The homogeneity of the purified enzyme preparation has been established through analytical ultracentrifugation (SivaRaman, 1961) electrophoresis in free solution (Bowen and Rogers, 1963a) and by polyacrylamide gel electrophoresis (Singh and Srere, 1971). The purified enzyme has been reported to have an adsorption index $E^{1\%}_{1\ cm}$ of 6.2 at 278 nm and a specific activity of 73 $\mu mol.min^{-1}.mg^{-1}$ at pH 7.4, 28°C and 10 mM $MgCl_2$ (Singh et al., 1976).

(b) Citrate lyase of S. diacetilactis: Citrate lyase from this source has been purified by Singh and Srere (1975) and Kummel et al. (1975). The former authors have purified the enzyme from supernates of centrifuged sonicates of citrate-lactose broth grown cells of the organism by a procedure involving protamine sulphate treatment, ammonium sulphate fractionation, gel filtration through BioGel A - 0.5 m and DEAE-cellulose chromatography. Final fractions concentrated by ultrafiltration were shown to be homogeneous on ultracentrifugal analysis and had a specific activity of 125 $\mu mol.min^{-1}.mg^{-1}$ at pH 7.2 in presence of 2 mM $MgCl_2$.

In the procedure employed in Kummel et al. (1975), sonicates of the organism were first treated with cetyltrimethylammonium bromide and then with alumina C_γ gel followed by ultrafiltration of supernatant and two

successive gel-filtrations through Sepharose 6B columns followed by chromatography on DEAE-Sephadex A-50. The homogeneity of the final preparations was established by analytical ultracentrifugation and by polyacrylamide gel electrophoresis. The pure enzyme showed a specific activity of 125 $\mu mol.min^{-1}.mg^{-1}$ at pH 7.2. The preparation, however, showed citrate lyase ligase activity. This activity was believed at the time to be associated as a subunit of the citrate lyase complex from this organism (Kummel et al., 1975). However, Bowien and Gottschalk (1977) established that the ligase activity was present as an impurity and could be separated from the citrate lyase complex by alumina C_γ gel treatment.

(c) R. gelatinosa citrate lyase: The enzyme was purified from citrate-grown cells of the organism. Extracts of the cells disintegrated by passing through a French press were fractionated with protamine sulphate, chromatography through DEAE-cellulose and two separate steps of gel-filtration through Sepharose 6B. The preparation was homogeneous in sedimentation equilibrium runs as well as in polyacrylamide gel electrophoresis. A specific activity of 139 $\mu mol.min^{-1}$, mg^{-1} at pH 7.2, 30°C and in the presence of 3 mM $MgCl_2$ was reported for this preparation.

The enzyme from this source has been obtained crystalline by a modified purification procedure in which cell extracts were treated first with cetyltrimethylammonium bromide followed by alumina C_γ gel treatment and DEAE-cellulose chromatography (Giffhorn and Gottschalk, 1978).

Active fractions from the ion-exchange chromatography step were concentrated by ultrafiltration till the appearance of turbidity. The first crystals so obtained were recrystallised from appropriate buffer solutions by the addition of KCl and reconcentration by ultrafiltration. The recrystallised enzyme had a specific activity of 360 μmol. $min^{-1}.mg^{-1}$, approximately three times that of the homogeneous preparation obtained earlier.

(d) S.faecalis enzyme: Citrate lyase has been obtained pure from this organism grown in presence of citrate by Hiremath et al. (1976). Sonicates of the cells were centrifuged and the supernatant incubated with ATP and acetate to reactivate any deacetylated enzyme. The purification procedure involved removal of nucleic acids by streptomycin sulphate treatment, adsorption and subsequent elution of the activity from alumina C_γ gel, ammonium sulphate fractionation, column chromatography through DEAE-cellulose and a final step of gel filtration through Sepharose CL-6B. The enzyme was shown to be homogeneous both by analytical ultracentrifugation and polyacrylamide gel electrophoresis. The specific activity of the pure enzyme was reported to be 90 μmol.$min^{-1}.mg^{-1}$ at pH 8 and 30°C and in the presence of 20 mM $MgSO_4$.

(e) E.coli enzyme: Enzyme from this source has only been partially purified (Dagley and Dawes, 1955; Wheat and Ajl, 1955a; Bowen and SivaRaman, 1960).

I.1 (iii) PROPERTIES OF CITRATE LYASE

(a) Metal requirement: The absolute requirement for a divalent metal ion such as Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} or Co^{2+} for the cleavage of citrate catalysed by citrate lyase was reported even in the early work (Dagley and Dawes, 1955). It was also shown that Ca^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+} and Hg^{2+} could not substitute for the active metal ions in the case of enzymes from E.coli and K.aerogenes. Further, Ca^{2+} was shown to be a linear competitive inhibitor of enzyme activation by Mg^{2+}, Mn^{2+} and other metal ions in the case of citrate lyase from E.coli and K.aerogenes (Dagley and Dawes, 1955) and S.diacetilactis (Harvey and Collins, 1963). These authors concluded that citrate lyase probably acts on a metal-citrate complex since the pH optimum of the enzymes from E.coli and K.aerogenes was also the same as the optimum pH for the stability of the metal-citrate complex. It was further reported by Harvey and Collins (1963) that the enzyme from S.diacetilactis is optimally active when citrate and Mg^{2+} are present in equimolar amounts, higher levels of the metal causing inhibition of enzyme activity.

On the basis of the results obtained with various divalent metal ions suplementation, Dagley and Dawes (1955) postulated that the activating metals have a restricted ionic radius of 0.72 ± 0.08 Å while those that fail to do so like Ca^{2+} ion (0.99 Å) have ionic radii outside this specific range.

Pulsed nuclear magnetic resonance studies were made by Ward and Srere (1965) with a partially purified enzyme

preparation from S.diacetilactis in presence of Mn^{2+}. Results obtained in these studies were contrary to the earlier hypothesis and were indicative of a metal-enzyme binary complex acting on free citrate. The Mn^{2+}-enzyme complex had a calculated dissociation constant (K_d) value of 4×10^{-5}M. In this study further addition of citrate to the system gave no indication of ternary enzyme-metal-substrate complex formation. The authors concluded that either metal-enzyme acts on citrate or a ternary complex exists, if at all, at steady state concentrations too low to be detected experimentally. The earlier observation that Ca^{2+} competes with Mn^{2+} for the metal binding site on the enzyme was confirmed in the NMR studies.

The effectiveness of divalent metal ion activation of citrate lyase from R.gelatinosa has been reported to be in the following order Mg^{2+}, Mn^{2+} and Co^{2+} (Beucher et al., 1974). Zn^{2+} and Ni^{2+} were only slightly effective while Cu^{2+} and Fe^{2+} were without effect. Hiremath (1977) reported maximum activation of the enzyme from S.faecalis with Mg^{2+} followed in diminishing order of effectiveness by Mn^{2+}, Zn^{2+} and Fe^{2+}. Co^{2+} and Cu^{2+} showed feeble activation and Ca^{2+}, Ba^{2+}, Hg^{2+} and Ni^{2+} showed none at all.

More recent studies, discussed later in this Chapter, have established that citrate lyase is a multienzyme complex with two distinct enzymatic activities, one catalysing a transferase reaction leading to the formation of a citryl-enzyme intermediate and the other a lyase reaction resulting in cleavage of the citryl-intermediate to yield oxaloacetate.

The divalent metal ion has been shown to be required exclusively for the lyase reaction and not for the transferase activity (Buckel et al., 1973; Dimroth and Eggerer, 1975b).

(b) pH optimum: The pH optimum for the cleavage of citrate is around 8 for citrate lyases from E.coli (Dagley and Dawes, 1955; Wheat and Ajl, 1955a), K.aerogenes (Wheat and Ajl, 1955b) and S.faecalis (Hiremath et al., 1976), an optimum pH of about 7 - 7.3 has been reported for the citrate lyases from S.diacetilactis (Harvey and Collin, 1963) and R.gelatinosa (Beucher et al., 1974). In the case of the S.diacetilactis enzyme, Singh and Srere (1975) have reported dissociation at pH values of above 8.1.

(c) Reaction kinetics: Kinetics of the reaction catalysed by citrate lyase are complex for various reasons which are discussed later in detail. In outline these are:

(1) Multienzymic nature of the complex with two distinct enzymatic activities (Dimroth and Eggerer, 1975b);

(2) A further complication is the phenomenon of reaction-inactivation (Singh and Srere, 1971) presumably through the spontaneous hydrolysis of the labile citryl-intermediate to citrate and inactive HS-enzyme;

(3) Existence of the various forms of citrate-divalent metal chelates as well as the presence of one of the products namely oxaloacetate in keto and enol forms, both again capable of chelating divalent metals.

Many of the factors were not recognised at the time of the earlier studies on the kinetics of the enzyme

catalysed reaction and these findings are of doubtful significance. Some of the earlier data are mentioned here only in their broad outline.

That a mol of citrate is cleaved to a mol each of oxaloacetate and acetate was shown in the early stoichiometric studies with the enzymes from K.aerogenes (Dagley and Dawes, 1953a and b; Dagley and Dawes, 1955) and E.coli (Dagley and Dawes, 1955, Wheat and Ajl, 1955b). That CoA was not required in the reaction was established by Dagley and Dawes (1955) in experiments in which extracts from K.aerogenes and E.coli were treated with Dowex-1 for removal of the coenzyme and shown to retain activity which was also not enhanced on CoA supplementation.

Harvey and Collins (1963) showed for the first time that the keto isomer of oxaloacetate was formed in the reaction catalysed by the enzyme from S.diacetilactis and the findings were later confirmed by Ward and Srere (1965). The enzyme from K.aerogenes was also shown to yield the keto isomer of oxaloacetate in experiments in which the reaction was carried out at pH 7.4 and 2°C, conditions under which the spontaneous equilibration of oxaloacetate isomers is considerably slowed down (Tate and Datta, 1964). In this respect citrate lyase resembles citrate synthase (Englard, 1959), since both enzymes involve the keto form of oxaloacetate in the reactions they catalyse.

The overall reaction catalysed by citrate lyase under optimal conditions is almost exclusively in favour of citrate cleavage. That the reaction goes virtually to

completion when the enzyme is taken in large excess has been shown by Wheat and Ajl (1955b) in the case of the E.coli enzyme and by Bowen and Rogers (1963b) with the K.aerogenes enzyme. The reversibility of the overall cleavage reaction has been shown only through incorporation of ^{14}C-labelled acetate into citrate in the presence of oxaloacetate and Mg^{2+} and the enzyme from S.faecalis (Gillespie and Gunsalus, 1953) or from K.aerogenes (Bowen and Rogers, 1963b).

The calculated equilibrium constant for the cleavage of citrate to oxaloacetate and acetate is 1 M (Burton, 1955). Several studies have been made to determine the equilibrium constant of the citrate lyase catalysed reaction. The early studies of Smith et al. (1956) and of Harvey and Collins (1963) did not take into account the existence of keto and enol forms of oxaloacetate and their complexes with Mg^{2+} as well as the interaction between the metal and phosphate buffer systems used. Tate and Datta (1965) showed that the determination of the equilibrium constant K, $(citrate^{-3})/(OAA^{-2}_{keto})$. (acetate), is markedly effected by the concentrations of the reactants and metal; a value of $3.08 \pm 0.72\ M^{-1}$ was obtained by these authors at limiting concentrations of citrate (2 mM) in presence of excess $MgCl_2$ at 25°C and triethanolamine HCl buffer pH 8.4 which has no complexing effect on Mg^{2+}. Guynn et al. (1973) obtained a value for K_{obs} of $2.22 \pm 0.16\ M^{-1}$ at pH 7.0 in 0.025 M potassium phosphate buffer at 38°C and 10^{-3}M free Mg^{2+}. The value of K_{obs} was found to vary with Mg^{2+}

concentration. At $(Mg^{2+})_{free}$ = 0 the K_{obs} was 1.00 ± 0.07 M^{-1} and at $(Mg^{2+})_{free}$ = 10^{-2}M the K_{obs} was 9. Here the problem of OAA-inhibition was reduced by use of low concentration of substrate and the values obtained for the forward and reverse directions were in agreement.

(d) Stereospecificity: Before discussing the stereospecificity of the citrate enzymes in general and of citrate lyases in particular, a brief description of the prochiral centres of the citrate molecule is outlined here. That citric acid behaves asymmetrically in reactions involving chiral reagents like enzyme molecules was recognised in the classical studies of Ogston (1948). Thus the two $-CH_2COOH$ groups of citrate are non-equivalent and discriminated in enzyme catalysed reactions. An extension of the standard R (rectus)/S(sinister) convention (Cohn et al., 1966) is conveniently used to distinguish between such groups. This mode of representation is pictorially explained in Fig. I. Fig. Ia. represents citric acid as a pentane derivative with the carbon atoms in the backbone of the structure numbered 1-5. Here C-3 is one of the pro-chiral centres. Depiction according to the pro R/pro S nomenclature is shown in perspective in Fig. Ib where the central carboxyl group is above the plane of the paper and the hydroxyl group is below the plane and both are to the right of the backbone. The two $-CH_2COOH$ groups are then distinguished by the pro R ("upper" group) greater than pro S ("lower" group) convention (Hirschmann and Hanson, 1971); the pro 3R ("upper") $-CH_2COOH$ group carbon atoms being numbered 1 and 2 and the pro 3S

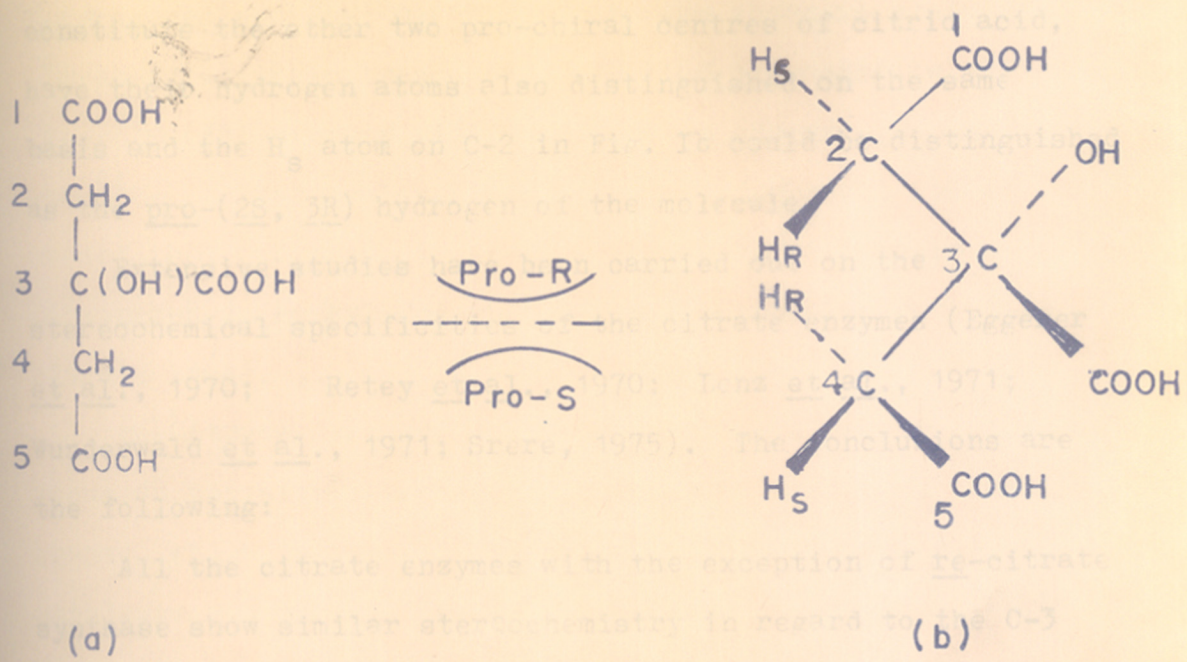


FIG I DEPICTIONS OF CITRIC ACID

(a) As a pentane derivative

(b) Perspective representation according to (pro-R / pro-S) nomenclature

(Adapted from Glusker and Srere, 1973)

("lower") $-CH_2COOH$ group being numbered as 4 and 5. In like manner the methylene groups at C-2 and C-4, which constitute the other two pro-chiral centres of citric acid, have their hydrogen atoms also distinguished on the same basis and the H_s atom on C-2 in Fig. Ib could be distinguished as the pro-(2S, 3R) hydrogen of the molecule.

Extensive studies have been carried out on the stereochemical specificities of the citrate enzymes (Eggerer et al., 1970; Retey et al., 1970; Lenz et al., 1971; Wunderwald et al., 1971; Srere, 1975). The conclusions are the following:

All the citrate enzymes with the exception of re-citrate synthase show similar stereochemistry in regard to the C-3 prochiral centre. Thus si-citrate synthase catalyses a reaction in which the acetyl moiety of acetyl-CoA attacks the si-face of oxaloacetate and the resulting citrate has C-4 and C-5 derived from this acetyl moiety (Hanson and Rose, 1963).

Both citrate lyase and ATP-citrate lyase have been shown to behave like si-citrate synthase stereochemically. Gillespie and Gunsalus (1953) using a crude preparation of citrate lyase from S.faecalis have demonstrated that the enzyme catalyses cleavage of citrate with the elimination of the pro 3S $-CH_2COOH$ exclusively as acetate. The acetate formed in this reaction is that derived by the substrate from acetyl-CoA in the si-citrate synthase reaction. A similar pattern of cleavage has been demonstrated by Wheat and Ajl (1955b) using a partially purified preparation of

citrate lyase from E.coli. The findings have been extended by Buckel et al.(1971a) to the enzyme purified from K.aerogenes and (5-^{14}C)(3S) citrate was shown to yield ^{14}C-acetyl-citrate lyase and unlabelled oxaloacetate while (1 - ^{14}C)(3R)-citrate yields unlabelled enzyme complex and ^{14}C-labelled oxaloacetate. Srere and Bhaduri (1964) showed that the pro(3S) $-CH_2COOH$ group of citrate is eliminated as acetyl-CoA in the reaction catalysed by ATP-citrate lyase.

re-Citrate synthase is the only citrate enzyme which shows an opposite stereospecificity in this regard. This enzyme, which is present in certain anaerobic bacteria, catalyses synthesis of citrate in which the pro (3R) $-CH_2COOH$ group of citrate (C-1 and C-2) is derived exclusively from the acetyl-moiety of acetyl-CoA (Gottschalk and Barker, 1966; Stern and Bambers, 1966).

With regard to the two pro-chiral centres of citrate involving the methylene groups on C-2 and C-4, all citrate enzymes including re-citrate synthase show similar stereospecificity. Chiral acetates (R) (^{2}H, ^{3}H) acetate and (S) (^{2}H, ^{3}H) acetate have been used in these studies in reactions catalysed by si-citrate synthase, re-citrate synthase, citrate lyase and ATP-citrate lyase and shown to produce inversion of configuration at the methyl group of the acetyl moiety (Eggerer et al., 1970; Klinman and Rose, 1971; Retey et al., 1970; Buckel et al., 1971b),

(e) Role of essential acetyl group of citrate lyase:

The earliest evidence that an essential acetyl group is involved in the reaction mechanism of citrate lyase was presented by Buckel et al.(1971a). Results obtained in their experiments were suggestive of this acetyl group being linked covalently through a thio-ester linkage at the catalytically active site of citrate lyase. Thus the enzyme was found to be inactivated on deacetylation. When stereospecifically labelled (5-^{14}C)-(3S) citrate was used as substrate for the citrate lyase catalysed reaction, the product was non-radioactive oxaloacetate and a ^{14}C-labelled enzyme, However, with (1-^{14}C)-(3R) citrate the products were ^{14}C-oxaloacetate and unlabelled enzyme; these results could be explained only on the basis of turnover of acetate during the course of the reaction catalysed by the enzyme, the overall reaction being the sum total of two separate steps viz. an acyl exchange reaction resulting in a citryl-enzyme intermediate (Eq. 4) followed by an acyl lyase reaction (Eq. 5) with cleavage of the citryl moiety at the si-face of oxaloacetate

$$\text{E-X-acetyl} + \text{citrate} \rightleftharpoons \text{E-X-citryl} + \text{acetate} \qquad \text{(Eq. 4)}$$

$$\text{E-X-citryl} \rightleftharpoons \text{E-X-acetyl} + \text{OAA} \qquad \text{(Eq. 5)}$$

Additional evidence obtained by these authors for the existence of an essential acetyl group at the active site of the enzyme were the following:

(1) Treatment of the active enzyme with hydroxylamine

resulted in both deacetylation and inactivation, the ^{14}C-labelled enzyme yielding ^{14}C-acetohydroxamic acid.

(2) Inactivation of ^{14}C-labelled enzyme on incubation with 1 mM oxaloacetate and 1 mM $MgCl_2$ resulted in loss of the labelled acetyl group. Reaction of ^{14}C-acetyl labelled citrate lyase with cold citrate resulted in the formation of unlabelled enzyme and ^{14}C-acetate which could be separated by filtration through Sephadex indicating an acyl exchange during the course of the reaction.

(3) Kinetics of hydroxylamine inactivation of enzyme were similar to hydroxyaminolysis of standard thioester compounds like N-succinyl-S-acetylcysteamine . Citrate lyase could also be inactivated by mercaptans like DTT and DTE. These findings were suggestive of the essential acetyl moiety being present in thioester linkage in the enzyme.

(4) Citrate lyase inactivated by treatment either with hydroxylamine or mercaptans could be chemically reactivated on treatment with acetic anhydride. Enzyme inactivated by oxaloacetate in presence of Mg^{2+} was also reactivated chemically with acetic anhydride (Buckel *et al.*, 1971a).

These findings were confirmed and extended by other groups. Srere *et al.* (1972) observed that enzyme inactivated in presence of DTNB either by hydroxylamine or by reaction inactivation could be reactivated by acetic anhydride only after preliminary incubation with DTT. Kummel *et al.* (1975) showed that deacetylated citrate lyases from several sources such as *S.diacetilactis*, *K.aerogenes*, *R.geletinosa* and *E.citrovorum* could be chemically reactivated

with 1-acetyl imidazole.

A specific enzyme which reactivates inactive deacetylated citrate lyase in K.aerogenes has been characterized by Schmellenkamp and Eggerer (1974). This enzyme acetate: SH (acyl carrier protein) citrate lyase ligase(AMP) was partially purified from K.aerogenes and shown to catalyse acetylation of deacetylcitrate lyase in presence of acetate and ATP or acetyl adenylate. Hiremath et al. (1976) partially purified the ligase from S.faecalis and separated it from the citrate lyase activity. The enzyme from this source was shown to be active towards deacetyl citrate lyases from both S.faecalis and K.acrogenes.

Bowien and Gottschalk (1977) obtained the ligase in electrophoretically pure state from S.diacetilactis and showed that the enzyme was specific for the lyase from this source, having no action on the deacetylated enzyme either from R.gelatinosa or K.aerogenes.

Buckel et al. (1973) demonstrated that while deacetyl citrate lyase can be reactivated with acetic anhydride, the carboxymethylated derivative of the enzyme obtained by treatment of the deacetylated enzyme with iodoacetate cannot be reactivated chemically. An interesting finding was that while deacetylated lyase and its carboxymethylated derivatives were inactive towards citrate, both were catalytically active in presence of acyl-CoA derivatives like acetyl-CoA or citryl-CoA. Thus citrate is cleaved to acetate and oxaloacetate in presence of acetyl-CoA while (3S)-citryl CoA is stereospecifically cleaved to

acetyl-CoA and oxaloacetate under these conditions. The use of ^{14}C-labelled citrate established that the acetyl group of acetyl-CoA is liberated as acetate and the pro(3S)-CH_2COOH group of citrate is transformed to acetyl-CoA in the course of the acetyl-CoA dependent cleavage of citrate. Kinetic data was suggestive of the formation of a citryl-intermediate and this was established by isolation of citryl -CoA which accumulated in presence of EDTA. These results were the first evidence for the multienzymic nature of citrate lyase involving an acyl exchange reaction which is not Mg^{2+}- dependent and a lyase reaction which is Mg^{2+}-dependent in which the citryl-intermediate is cleaved. Buckel et al. (1973) rightly concluded that both acetyl-CoA dependent cleavage of citrate by the deacetyl enzyme as well as the cleavage of citrate by native citrate lyase requires acetylated acyl group carriers, acetyl-CoA in the case of deacetyl enzyme and acetyl enzyme in the case of the native enzyme. Buckel et al. (1973) further checked the ability of several acyl-CoA derivatives to substitute for acetyl CoA in the cleavage reaction catalysed by HS-citrate lyase. Malonyl CoA, (R, S)-malyl-CoA as well as acetyl thioesters like N, S-diacetyl cysteamine, N-succinyl -S-acetyl cysteamine were inactive. Acetyl-dephospho CoA and propionyl-CoA, however, were active; the reaction sequence in the latter case being

Propionyl-CoA + citrate $\rightleftharpoons$ citryl-CoA + propionate (Eq. 6)

Citryl-CoA $\rightleftharpoons$ acetyl-CoA + OAA (Eq. 7)

Butyryl-CoA and valeryl-CoA, however, were inactive and also not inhibitory (Buckel et al., 1973).

Evidence has been obtained to show that all citrate lyases obtained till now from other sources such as S.diacetilactis (Singh and Srere, 1975), R.gelatinosa (Kummel et al., 1975), S.faecalis (Hiremath et al., 1976) and L.citrovorum (Kummel et al., 1975) are also acetyl enzymes.

(f) Product inhibition by oxaloacetate:

Inhibition of citrate lyases from E.coli and K.aerogenes by oxaloacetate, a product of the reaction, was first shown by Dagley and Dawes (1955). Neither acetate nor pyruvate was found to have any inhibitory effect. Bowen and Rogers (1963a) observed that inhibition by oxaloacetate was time and concentration dependent and that the native and inactivated enzymes had similar adsorption spectra and sedimentation behaviour. These authors also reported that malate, like oxaloacetate, irreversibly inactivated the enzyme while oxalate, succinate, oxaloglutarate, tartrate, oxalosuccinate and isocitrate were without effect.

The conditions under which oxaloacetate exerts inhibitory effect on the enzyme were investigated by Eisenthal et al.,(1966). Inhibition of the enzyme from K.aerogenes by oxaloacetate and its analogues was shown to require the presence of divalent metal ions like Mg^{2+} and inhibition could not be reversed by malate dehydrogenase in presence of NADH. The profile of the pH dependence of oxaloacetate inhibition was indicative of the

involvement of the enolic form of oxaloacetate and the authors suggested that probably two different Mg^{2+}-enolic oxaloacetate complexes are involved. In support of this hypothesis was their finding that amongst the oxaloacetate analogues tested, only α,α-dimethyloxaloacetate which cannot enolise and ketomalonate failed to be inhibitory in presence of Mg^{2+}, while derivatives of the two acids which could give enolic forms were inhibitory. The structural requirement for inhibition was observed to be a C_4 straight chain, 1,4-dicarboxylic acid with an ionisable α-hydroxyl group. Oxaloacetate inhibition was also found to be dependent on the nature of the divalent metal. Thus inhibition in presence of Co^{2+} was more severe than that with Mg^{2+}, while Ca^{2+} was less effective than Mg^{2+}.

The mechanism of oxaloacetate inactivation was shown by Buckel et al. (1971a) to be through the loss of the essential acetyl group of the enzyme to give inactive deacetyl citrate lyase.

(g) Reaction inactivation:

Citrate lyase undergoes irreversible inactivation in vitro during the course of the reaction it catalyses. The reaction-inactivation is rapid in the case of the enzyme from K.aerogenes and the phenomenon has been classed as enzyme "suicide" (Singh and Srere, 1971).

In earlier work on partially purified citrate lyase from E.coli, Wheat and Ajl (1955b) had observed that the reaction rate drops rapidly and the cleavage ceases during the course of the reaction. It was believed at that time

that the inhibition was due to the oxaloacetate formed as a product of the cleavage. It was much later that Singh and Srere (1971) recognized that the rapid irreversible reaction-inactivation could not be due to oxaloacetate. It was observed that addition of oxaloacetate (both keto- and enol-forms) at levels more than one thousand-fold in excess of that produced during the cleavage reaction failed to inactivate the enzyme completely. Further, these authors observed that continuous removal of the oxaloacetate formed by coupling the reaction with malate dehydrogenase and NADH had no effect on the rate of inactivation or the extent of citrate cleavage. The study of the kinetics of the reaction-inactivation showed this to be a simple first order process. The rate of reaction-inactivation was also found to depend on the nature of the divalent metal cofactor. Zn^{2+} was found to give the slowest rate amongst several metals tested (Singh and Srere, 1971; Singh and Srere, 1975). Although the mechanism of reaction-inactivation is not fully understood, this is known to be through a process of deacetylation of the enzyme to HS-citrate lyase since the reaction-inactivated enzyme is reactivated chemically with acetic anhydride (Srere et al., 1972). The mechanism could possibly be explained on the basis of the labile nature of the citryl-intermediate from analogy with the spontaneous hydrolysis of citryl-CoA at an alkaline pH (Buckel et al., 1973). Another possible mechanism suggested by Dimroth and Eggerer (1975b) is that the hydrolysis of the citryl-intermediate to citrate and

HS-enzyme is catalysed by the ∝ subunit of the citrate lyase complex (transferase subunit) since the isolated subunit has been shown to have such an effect on the citryl-ACP subunit.

In their reaction-inactivation behaviour, citrate lyases from various sources fall under two distinct groups; types that undergo severe reaction-inactivation such as the enzymes from K.aerogenes (Singh and Srere, 1971) and R.gelatinosa (Kummel et al., 1975) and those that show only a weak reaction-inactivation like the enzymes from S.diacetilactis (Singh and Srere, 1975), L.citrovorum (Kummel et al., 1975) and S.faecalis (Hiremath et al., 1976). The values of the rate constants (K) for reaction-inactivation of the enzymes from K.aerogenes, S.diacetilactis (Singh and Srere, 1975), S.faecalis (Hiremath, 1977) with Mg^{2+} at 25°C were determined to be 1.21, 0.06, 0.07 min^{-1}, respectively. The corresponding values for the K.aerogenes and S.diacetilactis enzymes with Zn^{2+} were 0.17 min^{-1} and 0.014 min^{-1}, respectively.

Kummel et al. (1975) suggested that the weak reaction-inactivation observed with enzymes from S.diacetilactis and L.citrovorum may be due to the association of a HS-citrate lyase acetylating activity as a component of the enzyme complex. Such activity was detected in preparations of citrate lyase obtained from S.diacetilactis and L.citrovorum and not in enzymes isolated from K.aerogenes and R.gelatinosa. That this was not the case in the enzyme from S.faecalis was established by

Hiremath et al. (1976) since the enzyme from this source has no associated ligase activity although it undergoes only a weak reaction-inactivation. Later work by Bowien and Gottschalk (1977) confirmed that even in the S.diacetilactis enzyme the ligase activity detected earlier was present as an impurity and the ligase could be separated from the citrate lyase complex by including an alumina C_γ gel adsorption step.

(h) Substrate and inhibitor activities of hydroxycitrates and other citrate analogues:

All the four possible isomers of hydroxycitrate have been studied by Sullivan et al.(1977) and Stallings et al. (1979) for their substrate and inhibitor activities on citrate enzymes (Sullivan et al., 1977) and interesting speculations on the geometry of the active sites of these enzymes have been put forward based on the correlation between the conformational structures of the hydroxycitrates and their effects on the enzymes (Stallings et al., 1979). The results are briefly summarised below:

In the case of bacterial citrate lyase, (2R, 3S)-2-hydroxycitrate is the only isomer which is not a substrate. The other isomers show varying substrate activities towards this enzyme. (2R, 3S)-2-hydroxycitrate showed the highest reaction rates. The relative rates of cleavage with the (2R, 3R)- and the (2S, 3R)- isomers of 2-hydroxycitrate were approximately 25 and 10%, respectively, of the reaction rates observed with the (2R, 3S)-isomer. In presence of excess enzyme, complete cleavage was obtained with the

active substrates. In these studies the cleavage reaction was followed by NADH oxidation with the malate dehydrogenase coupled system. The products probably were oxaloacetate and glyoxalate in the case of the (2R, 3S)- and the (2S, 3S)-2-hydroxycitrate and acetate and hydroxyoxaloacetate in the case of the (2R, 3R)-isomer, hydroxyoxaloacetate presumably being a substrate for the malate dehydrogenase reaction as indicated in earlier work by Englard and Seigel (1969).

As in the case of citrate, 2-hydroxycitrates caused "reaction-inactivation" through deacetylation of the active enzyme in studies in which ^{14}C-acetyl labelled citrate lyases were incubated in the presence of the 2-hydroxycitrate and a divalent metal like Mg^{2+} or Zn^{2+}. As with citrate, the hydroxycitrates under suitable conditions also caused complete deacetylation of the enzyme to give free-labelled acetate and the deacetylated-enzyme. This was confirmed by the ability of citrate lyase ligase to reactivate the inactivated enzyme in the presence of acetate and ATP. In studies in which tricarballylate was used in place of the hydroxycitrate isomers, only partial deacetylation was observed. All four isomers of hydroxycitrate were shown to act as linear competitive inhibitors for citrate in the cleavage reaction catalysed by citrate lyases from S.diacetilactis and K.aerogenes, some differences being observed in the behaviour of the enzymes from the two sources.

None of the hydroxycitrates was shown to act as a substrate for si-citrate synthase and in general the isomers were only very weak inhibitors of this enzyme. However, atleast one of the analogous fluorocitrate is known to function as a substrate of citrate synthase (Brady, 1955). In the case of ATP-citrate lyase, (2S, 3R)-2-hydroxycitrate did not serve as substrate while the other three isomers of hydroxycitrate exhibited substrate activities.

It was proposed on the basis of the observed biochemical activities of the hydroxycitrates and the postulated conformation of the metal chelates of hydroxycitrates that different modes of binding might result in the interaction with the citrate enzymes. One of these modes in the case of hydroxycitrate is apparently in the same manner as citrate itself. In the alternate modes of binding some might compete with or replace the citrate-like binding mode resulting in inhibition without cleavage of the hydroxycitrate. An interesting finding with far reaching application in human nutrition is the behaviour of (2S, 3R)-2-hydroxycitrate, which occurs in rinds of fruits of Garcinia species (Lewis and Neelakantan, 1965), as one of the most potent linear competitive inhibitors of ATP-citrate lyase (Watson et al., 1969) resulting in an inhibition of triglyceride and cholesterol synthesis in vivo (Sullivan et al., 1977).

(i) Molecular weight:

Citrate lyase from K.aerogenes has been shown to have a $s^{\circ}_{20,w}$ value of about 16 - 17.6S (SivaRaman, 1961; Bowen

and Mortimer, 1971) and a molecular weight of 534 000 - 575 000 (Mahadik and SivaRaman, 1968; Bowen and Mortimer, 1971; Carpenter et al., 1975; Singh et al., 1976). Citrate lyases purified from other sources have been shown to resemble the K.aerogenes enzyme in their molecular weights. The S.diacetilactis enzyme has a $s^{\circ}_{20,w}$ value of 16.8S and a molecular weight of 585 000 (Singh and Srere, 1975). The R.gelatinosa enzyme has been reported to have a molecular weight of 530 000 - 560 000 (Beuscher et al., 1974). The enzyme from S.faecalis has a $s^{\circ}_{20,w}$ value of 17.1S and a molecular weight of 600 000 (Hiremath et al., 1976; Hiremath, 1977). The enzyme from K.aerogenes has been shown to dissociate reversibly in buffers of low ionic strength and in the absence of divalent metal ions. Complete dissociation to a half molecule of 10.3S and 273 000 daltons was observed in 2 mM EDTA + 1 mM potassium phosphate buffer, pH 7.4 while a further component sedimenting at 6.3 S was observed in the presence of 1 mM EDTA + 1 mM potassium phosphate buffer, pH 7.4 (Mahadik and SivaRaman, 1968). The dissociation to species of lower molecular weights was completely reversed with Mg^{2+} at 2 mM concentration or buffers of higher concentration (50 mM). These observations have been confirmed by Bowen and Mortimer (1971) and Dimroth et al.(1973).

The enzyme from S.faecalis has been reported not to dissociate in buffers of low ionic strength even in the absence of divalent metal ions (Hiremath, 1977).

(j) Subunit structure:

Citrate lyases obtained hitherto from various bacterial sources have been shown to be complexes of three non-identical subunits of about 55 000 (α); 30 000 (β) and 10 000 (γ) daltons. The subunit composition and function have been studied extensively in the enzyme from K.aerogenes. The presence of the 10 000 daltons (γ) subunit which functions as an acyl carrier protein (ACP) was first reported by Dimroth et al.(1973) in the enzyme complex from K.aerogenes. The presence of two further subunits (α and β) was detected by Singh et al. (1974) in K.aerogenes enzyme complex by electrophoretic separation in SDS-polyacrylamide gel. The molecular weights of the three subunits were characterized by Carpenter et al. (1975) by several precise analytical approaches including sedimentation equilibrium in 6 M guanidinium hydrochloride, SDS-polyacrylamide gel electrophoresis and filtration through 6% agarose beads in presence of 6 M guanidine HCl and values of 54 000 (α), 32 000 (β) and 11 000 (γ) were obtained for the three subunits for the enzyme from this source. Values in close agreement to these were obtained by Dimroth and Eggerer (1975a). More recently the precise molecular weight of the γ- subunit (ACP) has been characterized by its amino acid sequence analysis and a value of 9 378 has been reported (Beyreuther et al., 1978).

Subunits of citrate lyases from other sources have also been found by SDS-polyacrylamide gel electrophoresis to resemble those of the enzyme from K.aerogenes in their

molecular sizes. In the case of the enzyme from S.diacetilactis, an organism in which the activity is constitutive, the molecular weights of the three subunits have been shown to be 54 000, 32 000 and 12 000 (Singh and Srere, 1975). Subunits of the enzyme from S.faecalis have been shown to be of 54 000 daltons, 37 000 daltons and 14 000 daltons (Hiremath et al., 1976). The enzyme from R.gelatinosa which was earlier regarded as being built up of only two non-identical subunits (Beuscher et al., 1974), has been shown in later work to dissociate into subunits of 55 600, 31 600 and 11 400 daltons when treated with 2% SDS (instead of 1% which was tried earlier). In the enzyme complexes from all these sources the subunit with the smallest size has been shown to contain the covalently bound prosthetic group and to function as ACP.

(k) Subunit stoicheiometry:

The determination of the precise stoicheiometric proportions of the three subunits in citrate lyase complexes has been beset with some intrinsic difficulties, mainly on account of the low molecular weight of the γ-subunit (ACP). Earlier attempts to determine the subunit composition through scanning of Coomassie blue stained bands obtained after electrophoretic separation of the component subunits in SDS-polyacrylamide gels showed equimolar amounts of α and β subunits in the enzyme from K.aerogenes calculated as 6 copies of each in a mole of the complex while the estimates of the γ-subunit indicated only around 4 copies in the complex (Dimroth and Eggerer, 1975a; Carpenter

et al., 1975). It was realised in these studies that the lower value for γ-subunit was probably due to diffusional losses of the small γ-chains from the gel into the destaining medium. A novel amino acid sequence technique was used by Singh et al. (1976) to establish the presence of a total of 6 copies of the γ-subunit in the holo enzyme from K.aerogenes. In their studies, the separated individual subunits were preliminarily sequenced to ten residues from the amino terminal. This was followed by a sequence analysis of the intact holo enzyme and estimation of the ratios of amino acids released at various positions. The knowledge of the amino acid sequence of the individual subunits enabled them to determine the ratio of the three subunits present in the holo enzyme. The ratio of amino acids released at various positions on quantitation was shown to be 1:1:1 indicating a similar molecular ratio of the three subunits in the complex.

Subunit stoicheiometry of citrate lyase from S.faecalis was determined by gel scanning of Coomassie blue stained components after electrophoretic separation on SDS-polyacrylamide gels and molar ratio of α, β - and γ -subunits was found to be 1:1:0.7, as in the case of the K.aerogenes enzyme (Hiremath, 1977).

Preliminary electron microscopic studies have been carried out with the enzyme from R.gelatinosa by Beuscher et al. (1974) and a model proposed from the molecular profiles. Two distinctive projections were evident, one having the appearance of a ring-shaped doughnut-like structure

and the other a sandwich pattern consisting of two paralel rows of density. Results obtained in analysis using the rotation technique of Markham et al. (1963) were suggestive of an arrangement of three large ellipses (axes of 58 Å and 49 Å) and three smaller spheres (diameter 43 Å) in alternate sequence in the ring structure. Based on the assumption that the ring-like structures were face-on views of the holo enzyme molecule and the sandwich pattern that of a side-on view, the authors proposed a hexameric structure with alternate dimers of the large and small subunits at the corners of hexagon in which the two rings are stacked one above the other. The enzyme from R.gelatinosa was thought at the time this model was proposed to consist of only two types of subunits. It is likely that the elliptical shaped subunit consists of the largest (α) together with the smallest (γ) subunit present side by side. The profiles obtained with the enzyme complex from K.aerogenes in electronmicrographs have been reported to resemble the R.gelatinosa enzyme in its gross structural features (Dimroth and Eggerer, 1975a).

(1) Amino acid composition:

(1) Holo enzyme: Bowen and Rogers (1965) determined the amino acid composition of citrate lyase from K.aerogenes as Cu^{2+}-complexes of the components separated after acid hydrolysis followed by high voltage paper electrophoresis. The more reliable procedure of automatic amino acid analysis by ion-exchange chromatography was used by Singh et al. (1976) to determine the amino acid composition

of the enzyme from the same source and the reported values for several residues were at variance with the earlier data. The latter authors also demonstrated that methionine was the only residue at the amino terminus of the three subunit components contrary to the earlier finding of Bowen and Mortimer (1971) that lysine was the only amino terminal residue.

Hiremath (1977) reported the amino acid composition of the holo enzyme from S.faecalis and showed marked differences in the composition of the enzyme from this source as compared to that from K.aerogenes.

(2) Subunits: Amino acid composition of the α-(M_r = 54 000) and β-(M_r = 32 000) subunits of the K.aerogenes enzyme were determined by Singh et al. (1976). These authors separated the subunits after dissociation in 6 M guanidinium hydrochloride by filtration through a column of agarose beads. The partial amino acid sequence of all the three component subunits upto 10 residues from the amino terminus was also determined by them. An interesting report was the micro-heterogeneity observed in the β-subunit sequence at position four from the amino-end, equal amounts of proline and arginine being present here.

Dimroth et al. (1973) established the presence of one cysteine and one cysteamine residue in the γ-(ACP) subunit chain of the K.aerogenes enzyme by amino acid analysis of the separated subunit. These authors also showed qualitatively that the cysteine and cysteamine residues in the ACP subunit were both acylated. The

strategy used was treatment of the ACP subunit first with cold iodoacetate followed by deacylation with hydroxylamine and subsequent treatment with iodo-(2-^{14}C)-acetate. The labelled ACP was finally submitted to acid hydrolysis and the ^{14}C-labelled carboxymethylated residues were separated and characterized as S-carboxymethyl-cysteine and S-carboxymethyl-cysteamine. Evidence that the cysteamine residue of the prosthetic group and not the cysteine residue of the ACP carries the biologically essential acetyl group was obtained by the specific alkylation of the prosthetic cysteamine and not of cysteine in the deacetyl enzyme by affinity labelling with a low concentration of iodoacetate in the presence of excess DTT (Dimroth and Eggerer, 1975a). The cysteamine S-carboxymethyl-deacetyl citrate lyase so obtained could not be reactivated by treatment with acetic anhydride.

The complete amino acid composition of the ACP subunit of citrate lyase from K.aerogenes was reported by Dimroth et al.(1975). The analysis showed the presence of one residue each of cysteamine and β-alanine contributed by a single prosthetic group. The complete amino acid sequence of the subunit has been reported by Beyreuther et al. (1978) using automated sequential Edman degradations. The ACP was shown to be composed of 78 residues and its amino acid composition was marked by the absence of both Tyr and His residues. A high degree of α-helical content in the ACP structure was indicated in circular dichroism studies.

Although the ACP of citrate lyase from K.aerogenes and of fatty synthetase from E.coli resemble each other in both function and molecular weight (Dimroth et al., 1973; Vagelos, 1973), these differ markedly in their amino acid composition as well as in their prosthetic groups. The prosthetic group of the fatty acid synthetase (ACP) consists of 4'-phosphopantetheine while the citrate lyase ACP contains in addition to 4'-phosphopantetheine also adenine, phosphate and sugar residues. The isolated deacetylated ACP of citrate lyase cannot substitute for the ACP of E.coli fatty acid synthetase (Dimroth et al., 1973) in the malonyl-CoA/CO_2 exchange reaction. However a crude extract of K.aerogenes sonicate could replace the ACP of fatty acid synthetase system. It was concluded therefore that atleast two distinct acyl carrier proteins are present in K.aerogenes, one in the citrate lyase complex and the other in the fatty acid synthetase system. That the ACP of citrate lyase from K.aerogenes is distinct from the fatty acid synthetase from E.coli was further established by fingerprint analysis, no common peptides being observed in their tryptic digests (Dimroth, 1975; Bayer and Eggerer,1978).

Differences in the amino acid compositionsof the separated subunits of enzymes from S.faecalis and K.aerogenes have been reported by Hiremath (1977). Marked differences in the amino acid contents of all three component subunits were observed, one of the features of differences being the presence of four histidine residues in the ACP subunit of S.faecalis enzyme while that from K.aerogenes has none.

The ACP of the S.faecalis enzyme however resembles the K.aerogenes enzyme in containing a single cysteine residue as an S-acylated residue.

(m) Prosthetic group of citrate lyase:

Evidence to show that citrate lyase from K.aerogenes contains a covalently attached pantothenate moiety was reported for the first time by Srere et al. (1972) Alkaline hydrolysates of the enzyme preliminarily treated with alkaline phosphatase were shown to contain bound pantothenate microbiologically. Since pantothenate could be assayed only after alkaline phosphatase treatment, it was concluded from analogy with the fatty acid synthetase system, that citrate lyase also contains a covalently bound 4-phosphopantetheine moiety. Since earlier work of Buckel et al. (1971a) had established the presence of an essential acetyl group in thioester linkage in the enzyme it was assumed that the 4-phosphopantetheine carried this biologically active acyl residue. The evidence for the presence of the prosthetic group on the 10 000 dalton γ-subunit in K.aerogenes enzyme was provided by Dimroth et al. (1973), thus establishing the ACP nature of this subunit.

Subsequent work indicated that the prosthetic group of citrate lyase is more complex than the 4-phosphopantetheine of the fatty acid synthetase system. Dimroth (1975; 1976) found the presence of adenine, phosphate, sugar, cysteamine, β-alanine and pantoic acid as components of the citrate lyase prosthetic group. Robinson et al. (1976) obtained

evidence from analyses of chemical and enzymic degradation products in support of the structure 3'(or 2') → 2" (5"-phosphoribosyl)dephospho-CoA for the prosthetic group. NMR and Mass spectrometry data have been presented more recently by Oppenheimer et al. (1979) in support of the structure (1" → 2')-(5"-phosphoribosyl)dephospho-CoA (Fig. 2).

The prosthetic group of citrate lyase from K.aerogenes has been shown to be linked through the 5"-phosphoryl moiety to serine - 14 of the γ- (ACP) subunit from characterization of the products obtained after β-elimination of the prosthetic group in 0.1 N NaOH at 65°C and subsequent NaB^3H_4 reduction of the dehydroalanine in the apo-ACP to (^{3}H)-alanine (Beyreuther et al., 1978).

An interesting observation has been that the citrate lyase prosthetic group could substitute for CoA in the citrate si-synthase reaction as well as in the ATP-citrate lyase catalysed reaction (Robinson et al., 1976). In these studies the prosthetic group was obtained after pronase digestion of citrate lyase from K.aerogenes and was used as a substrate for the other two citrate enzymes. The isolated prosthetic group was also shown to act as a substrate for acetate: HS-citrate lyase ligase (AMP) from K.aerogenes. The isolated prosthetic group is the only compound reported hitherto to substitute for CoA in the si-citrate synthease and ATP-citrate lyase catalysed reactions. That acetyl-CoA and citryl-CoA could substitute for its prosthetic group in reactions catalysed by citrate

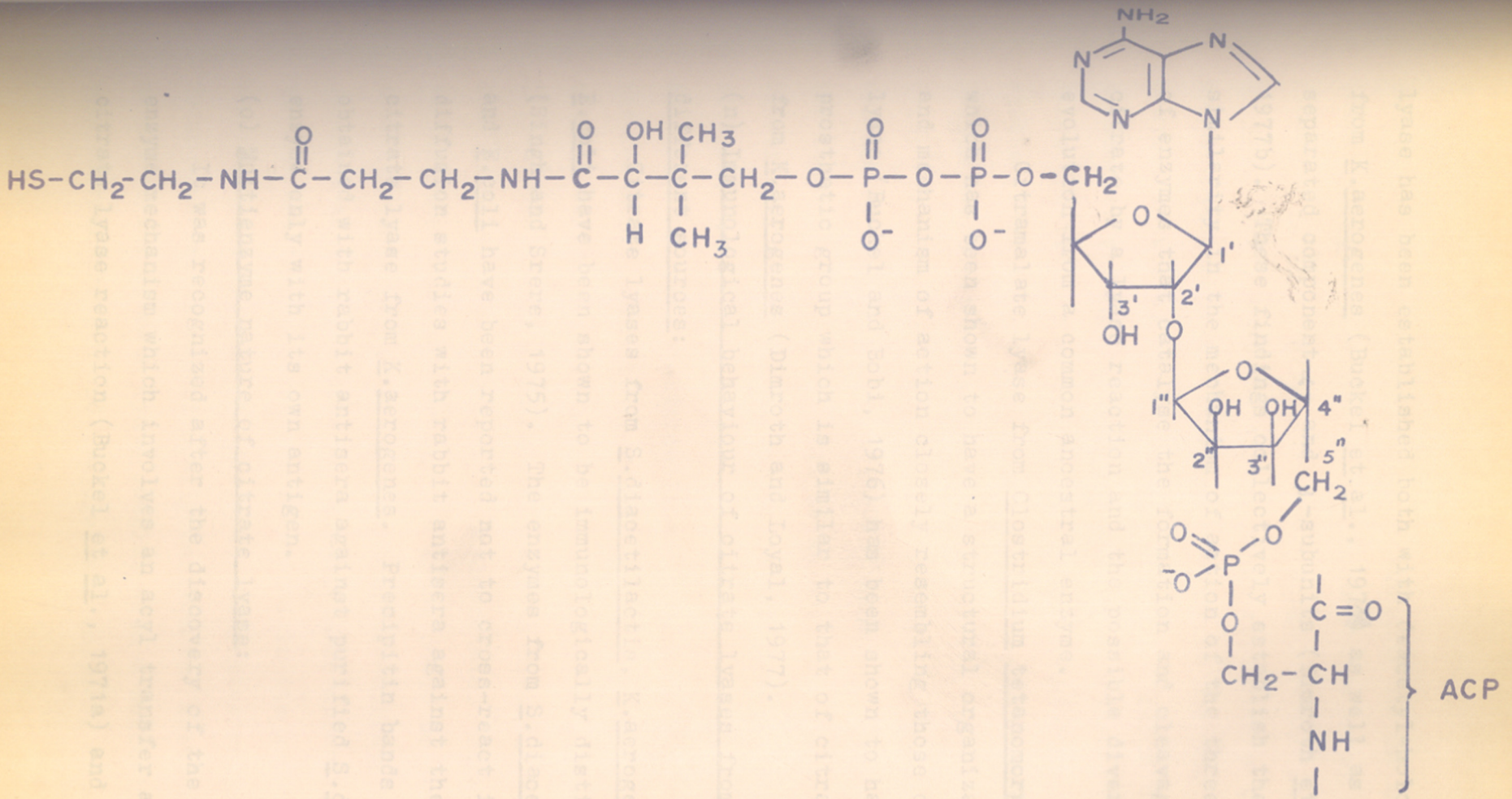


FIG. 2 PROPOSED STRUCTURE OF THE PROSTHETIC GROUP OF *K. aerogenes* CITRATE LYASE
(Reproduced from Oppenheimer *et al.*, 1979)

lyase has been established both with deacetyl-holo enzyme from K.aerogenes (Buckel et al., 1973) as well as the separated component α- and β-subunits (Dimroth et al., 1977b). These findings collectively establish the striking similarity in the mechanism of action of the three groups of enzymes that catalyse the formation and cleavage of citrate by a lyase reaction and the possible divergent evolution from a common ancestral enzyme.

Citramalate lyase from Clostridium tetanomorphum which has been shown to have a structural organization and mechanism of action closely resembling those of citrate lyase (Buckel and Bobi, 1976) has been shown to have a prosthetic group which is similar to that of citrate lyase from K.aerogenes (Dimroth and Loyal, 1977).

(n) Immunological behaviour of citrate lyases from different sources:

Citrate lyases from S.diacetilactis, K.aerogenes and E.coli have been shown to be immunologically distinct (Singh and Srere, 1975). The enzymes from S.diacetilactis and E.coli have been reported not to cross-react in double diffusion studies with rabbit antisera against the purified citrate lyase from K.aerogenes. Precipitin bands were obtained with rabbit antisera against purified S.diacetilactis enzyme only with its own antigen.

(o) Multienzyme nature of citrate lyase:

It was recognized after the discovery of the two step enzyme mechanism which involves an acyl transfer and a citrate lyase reaction (Buckel et al., 1971a) and the

detection of three non-identical subunits in the complex (Singh et al., 1974) one of which functions as an ACP (Dimroth et al., 1973; Srere and Singh, 1974; Buckel et al., 1973), that the system might represent a built-in multienzyme complex. Conclusive evidence for the multienzymic nature of citrate lyase from K.aerogenes was provided by Dimroth and Eggerer (1975b) by the isolation of the three constituent subunits in pure and catalytically active states (Dimroth and Eggerer, 1975b). The isolated 54 000 dalton α-subunit was shown to catalyse the formation of (1,5-^{14}C)-citryl ACP and acetate from (1,5-^{14}C)-citrate and the 10 000 dalton acetyl ACP γ-subunit. This established the function of the α-subunit as an acyl transferase, a reaction which was shown not to require the presence of divalent metals such as Mg^{2+}. It was also shown in these studies that the catalytically active form was a dimer of the α-subunit. The lyase function of the 32 000 dalton (β) subunit was demonstrated by its catalysing the cleavage of (1,5-^{14}C)-citryl ACP in presence of the divalent metal ions to acetyl-ACP and (4-^{14}C) oxaloacetate. It was also demonstrated that active citrate lyase could be reconstituted from its isolated α, β- and γ-subunits. Citrate cleavage occurred only in the presence of all three subunits and the reconstituted enzyme was identical in its sedimentation behaviour and specific activity to the native enzyme complex. These experiments unequivocally confirm the multienzyme nature of the citrate lyase complex with a built-in acyl carrier protein and two distinct enzymes, an acyl

transferase and a lyase; the overall mechanism of action of the enzyme involving consecutive reactions of acyl exchange (Eq. 8) and acyl cleavage (Eq. 9).

$$\text{Acetyl-ACP} + \text{citrate} \xrightleftharpoons{\alpha\text{- subunit}} \text{Citryl-ACP} + \text{Acetate} \quad \text{(Eq. 8)}$$

$$\text{Citryl-ACP} \xrightleftharpoons{\beta\text{- subunit} + Mg^{2+}} \text{Acetyl-ACP} + \text{OAA} \quad \text{(Eq. 9)}$$

Such a mechanism of action requires the oscillation of the acyl function on the ACP component between the active sites on the transferase and lyase subunits during the course of the citrate cleavage reaction (Srere and Singh, 1974; Dimroth and Eggerer, 1975b).

A closely related built-in enzyme complex is the citramalate lyase of C.tetanomorphum. The similarity in molecular weight, subunit composition and function and mechanism of action between the two enzyme complexes from the two distinct microorganisms have been established by Buckel and Bobi (1976). Even more striking is the cross-reaction reported between the isolated subunits of the two enzyme complexes (Dimroth et al., 1977a). Thus the formation of an active hybrid enzyme complex has been reported with acetyl transferase (α) and the lyase (β) subunits contributed by citrate lyase of K.aerogenes and the ACP contributed by the citramalate lyase of C.tetranomorphum (Dimroth et al., 1977a). Other hybrid combinations of heterologous subunits were without catalytic activity.

The mechanism of action of the isolated acyl transferase (α) subunit from K.aerogenes citrate lyase has been studied by Dimroth et al. (1977b). The isolated subunit has been shown to function as an acetyl CoA-citrate CoA transferase. The subunit further catalyses the exchange of acetyl residues between acetyl-ACP or acetyl-CoA and acetate in a citrate-independent reaction. The isolated subunit resembled the deacetyl-holo-enzyme in catalysing the formation of 3S-citryl CoA from acetyl CoA and citrate. Kinetic data supported the hypothesis that an anhydride intermediate is generated on the transferase subunit of citrate lyase, the anhydride probably being citric-acetic anhydride in the citrate dependent acyl-exchange reaction and acetic anhydride in the acetate reaction in the absence of citrate. While in these respects the transferase subunit of citrate lyase resembles classical CoA transferases like succinyl-CoA: 3-oxo acid CoA-transferase (Jencks, 1973), the CoA transfer from acetyl-CoA to citrate catalysed by the isolated transferase subunit of citrate lyase apparently does not involve an enzyme-CoA intermediate (Dimroth et al., 1977b).

I.2 BACTERIAL CITRATE TRANSPORT:

Since the results obtained in the present studies throw light on the regulation of bacterial citrate metabolism in vivo, citrate transport and the regulation of citrate metabolism are reviewed here.

Indirect evidence has been obtained by various investigators that citrate transport into bacterial systems occurs

probably through the mediation of membrane-bound oxaloacetate decarboxylase.

That citrate transport is induced only in the presence of the substrate was suggested by Davis (1956) on the basis of the earlier observation that a marked lag period precedes utilisation of citrate by both K.aerogenes and E.coli (Dagley and Dawes, 1953a, b). Villarreal-Moguel and Ruiz-Herrara (1969) investigated the mediated citrate transport system in K.aerogenes (mutants obtained from strain 1143). Evidence was obtained for the induced carrier activity being associated with the cell membrane and being dependent upon protein synthesis. The carrier system was demonstrated to act specifically for citrate, related compounds such as isocitrate and cis-aconitate not being transported. N-Ethylmaleimide, dinitrofluorobenzene, uranylnitrate and cyanide blocked transport. Further evidence for the membrane associated nature of the carrier system was the finding of Wilkerson and Eagon (1972) that citrate uptake activity was lowered in osmotically-shocked, citrate-grown cells of K.aerogenes; uptake activity being restored on continued incubation with citrate in the medium but not when chloramphenicol was also present.

The uptake of citrate into washed citrate grown cells of K.aerogenes NCTC 418 was shown by Stern and Sachan (1970) to require Na^+ specifically. These authors have proposed a model for citrate transport in the organism wherein citrate transport across the membrane is mediated through the membrane-bound Na^+-requiring oxaloacetate decarboxylase

functioning as a carrier (Sachan and Stern, 1971a). Evidences for this were the specific Na^+ requirement for citrate uptake and the ability of citrate to bind to oxaloacetate decarboxylase and to competitively inhibit the decarboxylase reaction. Membrane vesicles prepared from K.aerogenes NCTC 418 have been used to study transport of citrate by Johnson et al.(1975). Vesicles of cells grown anaerobically on citrate contained no citrate lyase activity and permitted study of citrate uptake without appreciable citrate catabolism subsequently. Studies with such vesicles which were shown to contain the Na^+-requiring oxaloacetate decarboxylase confirmed the earlier findings that citrate transport was Na^+-dependent. Citrate uptake was also shown to be inhibited by cyanide, azide, dinitrophenol, hydroxy-citrate, fluorocitrate and sulphydryl reagents.

Differences have been observed in the citrate transport phenomenon in some strains of K.aerogenes. In the case of K.aerogenes strains UGa-I and ACTC 12658, Na^+ has been shown to actually inhibit the uptake, while in strain PRI-R3, Na^+ had no effect on citrate uptake (Wilkerson and Eagon, 1974). Earlier work of Eagon and Wikerson (1972) had shown that citrate transport into K.aerogenes UGa-I is K^+-dependent.

The uptake of citrate by S.faecalis has also been postulated to be mediated by oxaloacetate decarboxylase, both uptake and decarboxylation requiring Ca^{2+} and biotin (Sachan and Stern, 1971b). Citrate uptake by S.diacetilactis, an organism in which citrate lyase activity is constitutive, has been shown to be through a transport system induced in

the presence of the substrate and by a process which is energy-requiring (Harvey and Collins, 1962). The appearance of spontaneous mutants of S.diacetilactis which had lost the ability to ferment citrate was reported by Collins and Harvey (1968). These were found to be cryptic cit⁻mutants which had lost the ability to transport citrate while retaining citrate lyase activity. Evidence has recently been presented to support the hypothesis that citrate transport in this microorganism is probably plasmid-linked (Kempler and McKay, 1979). Treatment of the organism with acridine orange resulted amongst other mutants to some which had lost the ability to utilise citrate. These cit⁻ mutants when examined for plasmid profiles were found to have lost a 5.5 mega dalton plasmid. Cell-free extracts of the cit⁻mutants as well as cit⁻cells treated with toluene to destroy the permeability barrier showed the presence of citrate lyase activity though lower than in the parent strain. These results were suggestive of citrate transport being plasmid-dependent. However one of the mutants (DRC_1-X) was cit⁻ while retaining the 5.5 mega dalton plasmid. This mutant was presumed to have undergone a point mutation on the plasmid. E.coli is generally characterized by its inability to grow on citrate as the sole carbon source. Dagley and Dawes (1953a,b) observed that E.coli NCTC 5928 utilises citrate when cells are actively dividing in a medium which also includes glucose, while the utilisation of citrate by resting cells with induced citrate lyase activity is negligible despite high levels of citrate lyase activity

in the cytosol. It has, therefore, been suggested that the organism has no active carrier for citrate transport (Gunsalus, 1958; Davis, 1956). More recently evidence has been presented that citrate transport could occur in E.coli as a plasmid-dependent activity (Sato et al., 1978; Ishiguro et al., 1979; Ishiguro and Sato, 1980). Several strains of E.coli have been isolated from pigeons and farm animals, which have the ability to grow on Simmons citrate agar. Citrate-utilising ability in the cit^+- strain was transmissible to recipient E.coli strains through conjugation. Trans-conjugants had citrate-utilizing activity generally associated with drug resistance. In a few instances Cit^+ R^--transconjugants were obtained. Smith et al. (1978) had reported the transmissibility of thermosensitive plasmids from strains of chloramphenicol-resistant Salmonella typhimuruim to a prototrophic E.coli K 12 strain. The transformed E.coli utilised citrate as sole energy source.

Pseudomonas aeruginosa (Eagon and Asbell, 1966) and an aconitase-mutant of B.subtilis (Willecke and Pardee, 1971), organisms that lack citrate lyase activity, have both been shown to transport citrate though substrate induced carrier systems. Citrate transport in S.typhimurium (Imai et al., 1973; Kay and Cameron, 1978) and in Proteus microbilis (Imai, 1978) is through complex systems, three distinct routes being induced by citrate and its analogues for the transport of tricarboxylic acids.

3. METABOLIC ROLE AND REGULATION OF CITRATE LYASE

(i) Role in citrate metabolism:

Citrate lyase initiates the anaerobic metabolism of citrate in several bacteria. The oxaloacetate formed in the lyase reaction is then converted to pyruvate through the action of oxaloacetate decarboxylase. The sequence of reactions catalysed by these two enzymes has been termed the citrate fermentation pathway (Dagley and Dawes, 1953a; O'Brien and Stern, 1969).

Since the simultaneous functioning of the fermentation pathway and the citric acid cycle would lead to futile cycles of antagonistic reactions, diverse regulatory mechanisms operate in such microorganisms capable of synthesising both citrate lyase and citrate synthase.

(ii)(a) Regulation in K.aerogenes:

Under anaerobic conditions, the growth of K.aerogenes NCTC 418 on citrate as sole energy source specifically requires the presence of Na^+, a requirement essential for oxaloacetate decarboxylase activity in the organism (Stern, 1967; O'Brien and Stern, 1969). Under such anaerobic growth conditions, the citric acid cycle is turned off because of the repression of α-ketoglutarate dehydrogenase activity. Citrate break down then proceeds only through the fermentation pathway. However, under aerobic growth conditions on citrate, Na^+ is not required by this organism and cells grown with or without Na^+ utilise citrate through the citric acid cycle with all enzymes of this pathway becoming operative under the aerobic conditions. Antagonism by by the fermentative pathway is prevented as citrate lyase

and oxaloacetate decarboxylase are not synthesised under the aerobic environment (O'Brien, 1975a; Wilkerson and Eagon, 1972). Under controlled aerated conditions wherein no oxygen tension is detected in the cultures, metabolism of citrate proceeds through the citric acid cycle in the absence of Na^+, but mainly through the fermentation pathway in the presence of Na^+ (O'Brien *et al.*, 1969).

The gross regulation under such growth conditions is through the control of the synthesis of constituent enzymes of the two divergent pathways. However, a mode of regulation recognised more recently is through the conversion of citrate lyase itself from an active acetyl enzyme to an inactive deacetylated form (Buckel *et al.*, 1971a). Under *in vitro* conditions, the enzyme from *K.aerogenes* rapidly undergoes inactivation through such deacetylation during the course of the reaction it catalyses (Singh and Srere, 1971; Srere *et al.*, 1972). The organism contains no citrate lyase deacetylase that could inactivate the enzyme (Kulla and Gottschalk, 1977). The microorganism however contains a distinct deacetyl citrate lyase ligase which catalyses the reactivation of the deacetyl enzyme to the active acetyl form in the presence of acetate and ATP (Schmellenkamp and Eggerer, 1974).

Kulla and Gottschalk (1977) carried out several growth shift experiments to study the mode of regulation of citrate lyase and citrate synthase activities in *K.aerogenes* grown in continuous culture. In cells grown anaerobically on

citrate, citrate lyase activity appeared after an initial lag period and increased until the end of growth phase (12 h). The enzyme was present mainly in the active acetyl form with little or no deacetyl enzyme being present under the in vivo conditions in the initial growth phase. The inactive form was detected after 15 h period and considerable proportions were apparent only after 24 h of incubation. The growth shift experiments were carried out in continuous cultures with ammonia as the growth limiting substrate and changes in citrate lyase and citrate synthase activities were monitored following growth shifts from anaerobic growth on citrate to aerobic growth on citrate, aerobic growth on glucose to anaerobic growth on glucose + nitrate. Cultures shifted from "anaerobic citrate" to "aerobic citrate" showed very low rates of synthesis of citrate lyase and slow inactivation of the active enzyme while the synthesis of citrate synthase was induced. Citrate lyase was inactivated rapidly on shift from "anaerobic citrate" to "aerobic glucose", while citrate synthase formation was induced but to levels significantly lower than in aerobic growth on citrate. Growth difficulties were observed during shift from "anaerobic citrate" to "anaerobic glucose" with a marked but transient drop in cell population. This was apparently caused by the persistence of citrate lyase activity during induction of citrate synthase activity after the shift leading to antagonistic and futile cycles between the fermentation and citric acid pathways. An evident cause for the stress was the depletion of glutamate

since the cultures after the shift showed a less marked decrease in cell counts on supplementation with α-keto-glutarate.

In these studies, citrate lyase inactivation was observed to occur only under aerobic conditions. No detectable citrate lyase deacetylase was present even in cells grown aerobically on citrate. Oxygen was also found to play no role in the inactivation phenomenon since cells grown anaerobically on glucose in presence of nitrate also showed marked citrate lyase inactivation. In batch cultures, cells initially grown anaerobically on citrate when shifted to aerobic glucose conditions showed complete inactivation of citrate lyase activity in 90 min. This inactivation was completely prevented by 2,4-dinitrophenol and delayed by chloramphenicol. The results indicated that the inactivation was dependent on electron transport processes in the membrane and was energy dependent as observed in the case of other enzymes e.g. nitrate reductases of K.aerogenes, Proteus mirabilis and E.coli (de Groot and Stouthamer, 1969; 1970a, b, c; Oltmann et al., 1976; Showe and De Moss, 1968, van' T Riet et al., 1968) and asparatate transcarbamylase of B.subtilis (Waindle and Switzer, 1973).

Regulation in R.gelatinosa:

Regulation of the two antagonistic pathways in R.gelatinosa follows a pattern distinctly different from that in K.aerogenes. R.gelatinosa which unlike many other phototropic bacteria, readily utilises citrate and

the regulation of citrate lyase activity in this bacterium is through covalent modifications. A citrate lyase deacetylase is present in this organism (Giffhorn et al., 1972). The deacetylase is activated only after citrate is depleted from the culture medium and at this point deacetylase inactivates the citrate lyase to a deacetyl form. The deacetylase is specific for R.gelatinosa citrate lyase and has no action on the K.aerogenes and S.diacetilactis enzymes. L-Glutamate strongly inhibits the R.gelatinosa deacetylase and in this manner the glutamate pool is involved in regulation of citrate lyase activity in the microorganism (Giffhorn and Gottschalk, 1975b). Glutamate levels in turn are directly related to the levels of its precursor, citrate, in the medium. The depletion of citrate from the medium leads to a drop in glutamate levels and the deacetylase is activated in the absence of its inhibition by L-glutamate. Citrate lyase thus is inactivated only after depletion of citrate from the growth medium. A citrate lyase ligase required for the activation of the deacetyl citrate lyase is also present in the bacterium. The ligase is active only in the presence of citrate, light and anaerobic conditions (Giffhorn and Gottschalk, 1975a). This ligase is rapidly inactivated after the depletion of citrate from the medium (Antranikian et al., 1978). The regulation of citrate lyase in the organism is thus through the chemical modifications brought about by the two enzymes, citrate lyase deacetylase and citrate lyase ligase, the deacetylase being rendered inactive during citrate utilisation by the L-glutamate

derived from the substrate and the ligase being inactivated after the depletion of citrate.

(c) Regulation in other bacteria:

The lactic bacteria S.diacetilactis and L.citrovorum contain no citrate synthase activity and citrate is metabolised only through the fermentation pathway (Giffhorn and Gottschalk, 1975a). No mechanism for regulation is therefore required in these microorganisms. In the case of Aerobacter cloacae, citrate utilisation under both aerobic and anaerobic conditions is unaffected either by the presence or by the absence of Na^+. Na^+ does not cause repression of α-ketoglutarate dehydrogenase as in the case of K.aerogenes (O'Brien and Geisler, 1974). Under anaerobic conditions of growth however the synthesis of α-ketoglutarate dehydrogenase is repressed and citrate metabolism proceeds through the fermentation pathway. Aerobic conditions result in induction of α-ketoglutarate dehydrogenase and repression of citrate lyase. Under conditions of controlled aeration wherein oxygen tension in the medium is zero, citrate lyase activity is induced but oxaloacetate decarboxylase activity is low rendering the fermentation pathway unoperative beyond the initial cleavage reaction. Under ~~such controlled~~ aeration conditions the organism was apparently under stress and showed low growth yields, obviously due to energy loss through the futile cycles.

S.typhimurium specifically requires Na^+ for aerobic growth on citrate and the cells contain enzymes of both the

fermentation and citric acid pathways. The mode of regulation in this instance is not understood (O'Brien et al., 1969).

PRESENT INVESTIGATION

PRESENT INVESTIGATION

INTRODUCTION

Bacterial citrate lyase (EC 4.1.3.6) catalyses the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metal ions such as Mg^{2+} and Mn^{2+}. The enzyme has been shown to be present only in procaryotes and has been obtained pure from Klebsiella aerogenes (SivaRaman, 1961), Rhodopseudomonas gelatinosa (Beuscher, et al., 1974). Streptococcus diacetilactis (Singh and Srere, 1975; Kummel et al., 1975) and Streptococcus faecalis (Hiremath et al., 1976). The enzymes from these sources have been shown to be complexes of three nonidentical subunits. The enzyme complex from K.aerogenes, which has been investigated the most, has equimolar amounts of three subunit polypeptide chains of about 54 000, 32 000 and 10 000 daltons (Carpenter et al., 1975; Dimroth et al., 1973; Singh et al., 1976). The smallest subunit functions as an acyl carrier protein and carries an essential acetyl moiety in thioester linkage with a CoA-like prosthetic group (Dimroth, 1975; Oppenheimer et al., 1979). The 54 000 daltons subunit functions as an acyl transferase involved in citryl-ACP formation with release of acetate; and the 32 000 dalton subunit catalyses the subsequent cleavage of the citryl-ACP intermediate with liberation of oxaloacetate and regeneration of acetyl-ACP (Dimroth and Eggerer, 1975b). Acetyl-CoA also serves as substrate for the transferase and lyase activities (Buckel et al., 1973; Dimroth et al., 1977b).

The enzyme undergoes irreversible reaction inactivation in the course of the reaction it catalyses presumably through the loss of acyl group from the unstable citryl-ACP intermediate to the inactive deacetylated form.

Two distinct types of citrate lyases are known dependent on their reaction-inactivation behaviour. The enzyme complexes from sources such as K.aerogenes (Singh and Srere, 1971) and R.gelatinosa (Kummel et al., 1975) undergo severe reaction-inactivation while those from S.diacetilactis (Singh and Srere, 1975) Leuconostoc citrovorum (Kummel et al., 1975) and S.faecalis (Hiremath et al., 1976) show only a weak reaction-inactivation with rate constants lower by more than an order of magnitude.

GENERAL SUMMARY OF PRESENT INVESTIGATION

The present studies add new knowledge to the structure and mechanism of action of this enzyme system. Comparative studies have also been carried out on the immunological behaviour and on the structural differences between enzymes representative of the class that undergoes severe reaction-inactivation and the one that shows only weak reaction-inactivation.

The salient features and significant results of the present investigations are summarised under the following relevant sections.

I. Metal Binding studies on citrate lyase from K.aerogenes: Although extensive studies have been reported on the subunit composition of the citrate lyase multienzyme complex,

relatively little is known about the nature of the interaction between the enzyme and divalent metal cofactors. In K.aerogenes the acyl exchange reaction involved in the formation of a citryl-CoA or of the citryl-enzyme intermediate has been shown to occur in the presence of EDTA, the subsequent lyase reaction leading to the cleavage of the citryl-intermediate requires the presence of a metal co-factor such as Mg^{2+}.

In the present investigations the divalent metal binding properties of pure citrate lyase from K.aerogenes have been studied by equilibrium dialysis assays using radioactive $^{54}MnCl_2$. The data obtained in these studies show for the first time the cooperative binding of divalent metal cofactor by citrate lyase. The enzyme complex from K.aerogenes has been shown to have 18 Mn^{2+}-binding sites/mol enzyme, the sites showing total positive cooperativity. Binding of the divalent metal evidently involves a conformational change in the enzyme complex, probably to a form which is catalytically active in the cleavage reaction. The allosteric behaviour of the enzyme could be a regulatory mechanism of biological significance.

II. Comparative studies on citrate lyases from K.aerogenes and S.faecalis:

The enzymes from these two sources represent distinctive classes of citrate lyases; the enzyme from the former source undergoing severe reaction-inactivation and that from the latter only a weak inactivation during the course of the cleavage reaction it catalyses.

The present studies were designed to investigate other differences in the behaviour and structure of these distinctive types of the enzyme. Enzymes from both sources were obtained pure by known procedures and the purity of the preparation established by ultracentrifugal as well as polyacrylamide gel electrophoretic analysis.

(i) Immunological behaviour

The immunological behaviour of the purified enzymes indicated cross-reactions. Rabbit anti-sera against purified K.aerogenes enzyme cross-reacts with S.faecalis enzyme in double diffusion studies. Similar experiments carried out with enzymes from K.aerogenes, S.diacetilactis and E.coli have been reported to show no cross-reactions between these three enzymes (Singh and Srere, 1975).

(ii) Kinetic behaviour:

Reaction-inactivation of citrate lyase has been shown to be caused by a deacetylation process leading to the formation of the deacetylated SH-citrate lyase (Srere et al., 1972). The weak reaction-inactivation observed in the case of S.diacetilactis and L.citrovorum was attributed to the association of a SH-citrate lyase acetylating activity with an enzyme complex (Kummel et al., 1975). However this was shown not to be the case in the enzyme from S.faecalis since the enzyme from this source has no associated SH-citrate lyase acetylating activity and still undergoes only a weak reaction-inactivation (Hiremath et al., 1976). Later work showed that even in S.diacetilactis the acetylating activity was present as a contaminant (Bowien and

Gottschalk, 1977). In the present study the kinetic behaviour of the enzymes from K.aerogenes and S.faecalis were studied to determine whether the relative rates of transferase and lyase activities could throw any light on the differences in the reaction-inactivation behaviour of the two enzymes. The rates of the individual enzymic reactions were determined using the deacetylated enzymes and determining the rates of citryl-CoA formation and citryl-CoA cleavage using acetyl-CoA to substitute for the corresponding acyl carrier protein derivatives. These studies indicated that the relative rate of the lyase to transferase reaction was actually lower in the S.faecalis complex than in the K.aerogenes enzyme. This would indicate that the accumulation of the unstable citryl intermediate which is known to hydrolyse spontaneously is not the cause for the rapid reaction-inactivation behaviour of the K.aerogenes enzyme.

(iii) Comparative studies on the chemical composition of citrate lyases from K.aerogenes and S.faecalis:

Structural differences in the primary structure of the non-identical subunits of citrate lyases from K.aerogenes and S.faecalis were studied by fingerprint analysis.

Interesting differences were observed in fingerprints of all three subunits separated from the two sources. The corresponding subunits also show several homologous peptide fragments. Subunit homologous relationships in the tryptic peptide maps would suggest evolutionary relatedness. This would indicate subunit divergence from an ancestral gene leading to present day bacteria.

Definitive proof however must await the determination of the entire amino acid sequences in subunits of citrate lyase from different sources.

The present studies are the first reports on such detailed comparative studies between citrate lyases from different sources.

PART I

STUDIES ON INTERACTION BETWEEN CITRATE LYASE FROM *Klebsiella aerogenes* AND DIVALENT METAL COFACTOR

PART I

STUDIES ON INTERACTION BETWEEN CITRATE LYASE FROM KLEBSIELLA AEROGENES AND DIVALENT METAL COFACTOR:

SUMMARY

Pure citrate lyase isolated from Klebsiella aerogenes was preliminarily obtained Mg^{2+}-free by extensive dialysis against EDTA containing buffer solutions. The absence of the metal was established by atomic absorption spectrophotometry. The enzyme complex was also shown to retain its quaternary structure in 0.05 M Tris-HCl buffer even in the absence of the divalent metal.

The Mg^{2+}-free enzyme was used for studies on Mn^{2+}-binding by equilibrium dialysis technique using radioactive Mn^{2+} as probe. These studies have indicated for the first time the cooperative binding of Mn^{2+} to the pure enzyme. The enzyme has been shown to have 18 Mn^{2+}-binding sites per mol enzyme, the sites showing total positive cooperativity with a Hill coefficient of 2.27 $\pm$ 0.05 and a dissociation constant of 4.5×10^{-5}M at $(Mn)_{free} \longrightarrow \infty$. These are the first reports of the allosteric behaviour of the enzyme. The biological significance of such behaviour in the regulation of citrate lyase activity *in vivo* is discussed.

I.1. INTRODUCTION

It will be apparent from the foregoing survey of the literature that little is known about the nature of the interaction between citrate lyase and divalent metal cofactor. Aspects dealt with in greater detail in the GENERAL INTRODUCTION but relevant to the present Chapter on metal-binding studies on citrate lyase are briefly summarised here.

Bacterial citrate lyase (EC 4.1.3.6) catalyses the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metal ions, such as Mg^{2+} and Mn^{2+} (Dagley and Dawes, 1955).

The enzyme has been obtained pure from several sources (SivaRaman, 1961; Singh and Srere, 1975; Hiremath *et al.*, 1976; Giffhorn and Gottschalk, 1978) and shown in all cases to be a complex of three non-identical subunits of about 55 000 (α), 30 000 (β) and 10 000 (γ) daltons. The subunit composition and function have been studied extensively in the enzyme from *K.aerogenes* (Carpenter *et al.*, 1975; Dimroth *et al.*, 1973; Singh *et al.*, 1976). The γ-subunit acts as an acyl carrier protein (ACP) and carries an essential acetyl moiety (Dimroth *et al.*, 1973). The α-subunit functions as an acyl transferase involved in citryl-ACP formation with release of acetate and the β-subunit catalyses the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP (Dimroth and Eggerer, 1975b). Acetyl-CoA also serves as substrate for the transferase and lyase activities (Buckel *et al.*, 1973;

Dimroth et al., 1977b). The transferase reaction proceeds in the presence of EDTA, while the lyase reaction requires the presence of Mg^{2+} (Dimroth and Eggerer, 1975b; Buckel et al., 1973). While extensive studies have been reported on the quaternary structure and the mode of action of the citrate lyase complex, relatively little is known about the nature of the interaction between the enzyme and divalent metal cofactors. The formation of a binary Mn^{2+}-protein complex has been suggested from pulsed NMR studies on partially purified citrate lyase from Streptoccoccus diacetilactis (Ward and Srere, 1965), although the impure nature of the preparation precludes unambiguous conclusions.

I.2. MATERIALS AND METHODS

MATERIALS:

Carrier-free $^{54}MnCl_2$ was obtained from the Bhabha Atomic Research Centre, India. Streptomycin sulfate was obtained from Hindustan Antibiotics Ltd., Poona. Acrylamide and N-N'methylene biscrylamide were of electrophoresis grade and were obtained from Eastman Organic Chemicals, USA. Adenosine 5'-triphosphate (disodium salt) from Equine muscle, Malate dehydrogenase from pig heart (specific activity 945 $umol.min^{-1}.mg^{-1}$), NADH from yeast (disodium salt-$3H_2O$ grade III), crystalline bovine serum albumin and primary standard grade Tris were obtained from Sigma Chemicals Co., USA. All other chemicals were of analytical grade.

I.2 (i) Buffers:

All the buffers used in purification were prepared in glass-distilled water by mixing and diluting 1 M stock solution of dipotassium hydrogen phosphate and potassium dihydrogen phosphate in the given proportion (Green and Hughes, 1955). All the buffers excepting the 30 mM potassium phosphate buffer, pH 7.0 used in the extraction of the enzyme from sonicated cells, contained 1.6 mM $MgSO_4$. The tris-HCl buffer was prepared from Trizma base.

I.2 (ii) Alumina C$_\gamma$ gel:

The gel (Grade A) was obtained aged from Calbiochem., USA and had a solid content of 3.25%. The gel was also prepared according to the method of Willstater and Kraut (1923) and aged at least 3 months before use.

I.2 (iii) DEAE-cellulose:

The anion exchanger DE-52 was obtained from Whatman Ltd., England, and could be used several times after washing with 0.5 M potassium phosphate buffer, pH 7.5. The exchanger was first washed with water and then equilibrated by washing three times with 100 ml lots each of 0.5 M potassium phosphate buffer, pH 7.5 containing 1.6 mM $MgSO_4$. This is followed by washing three times with 200 ml lots each of 0.01 M potassium phosphate buffer, pH 7.5 containing 1.6 mM $MgSO_4$. The exchanger was packed in a column (30 cm x 1.5 cm) and the packing of the column was done under pressure. The column was equilibrated by passing the buffer with the lower molarity overnight through the column. When in use the column was run under pressure at flow rates of 18-20 ml/h.

I.2 (iv) Sepharose CL-6B:

The gel was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The sodium azide which is used as a preservative is washed off with water, and the gel is then thoroughly washed with 0.05M potassium phosphate buffer. The Sepharose CL-6B is packed into a column (110 cm x 2.5 cm) under gravitational pressure. The column flow rates were 14-16 ml/h.

I.2 (v) Ammonium sulfate fractionation:

In the early steps of the purification procedure, powdered ammonium sulfate was used. The quantities of solid ammonium sulfate required for the fractionation was read off a nomogram where the concentrations are expressed in terms of saturation at 0-3°C. After the DEAE-cellulose and Sepharose chromatography steps nuetralised, cold saturated ammonium sulfate solution was used for precipitating the enzyme.

I.2 (vi) Equilibrium dialysis cells:

Cells used for equilibrating with $^{54}Mn^{2+}$-containing buffer were of the type descibed by Myer and Schellman (1962) and were fabricated from lucite rods. Each assembly was constructed containing two separate sets of cells for economy of space as shown schematically in Fig. I.1. Each set of cells consisted of two cylindrical cavities (A, B) separated by a circular Visking membrane (M) which was cut with the precision cutting tool used generally for Beckman ultracentrifuge cells. The cell size was designed to accept such cut membranes and the compartments were sealed

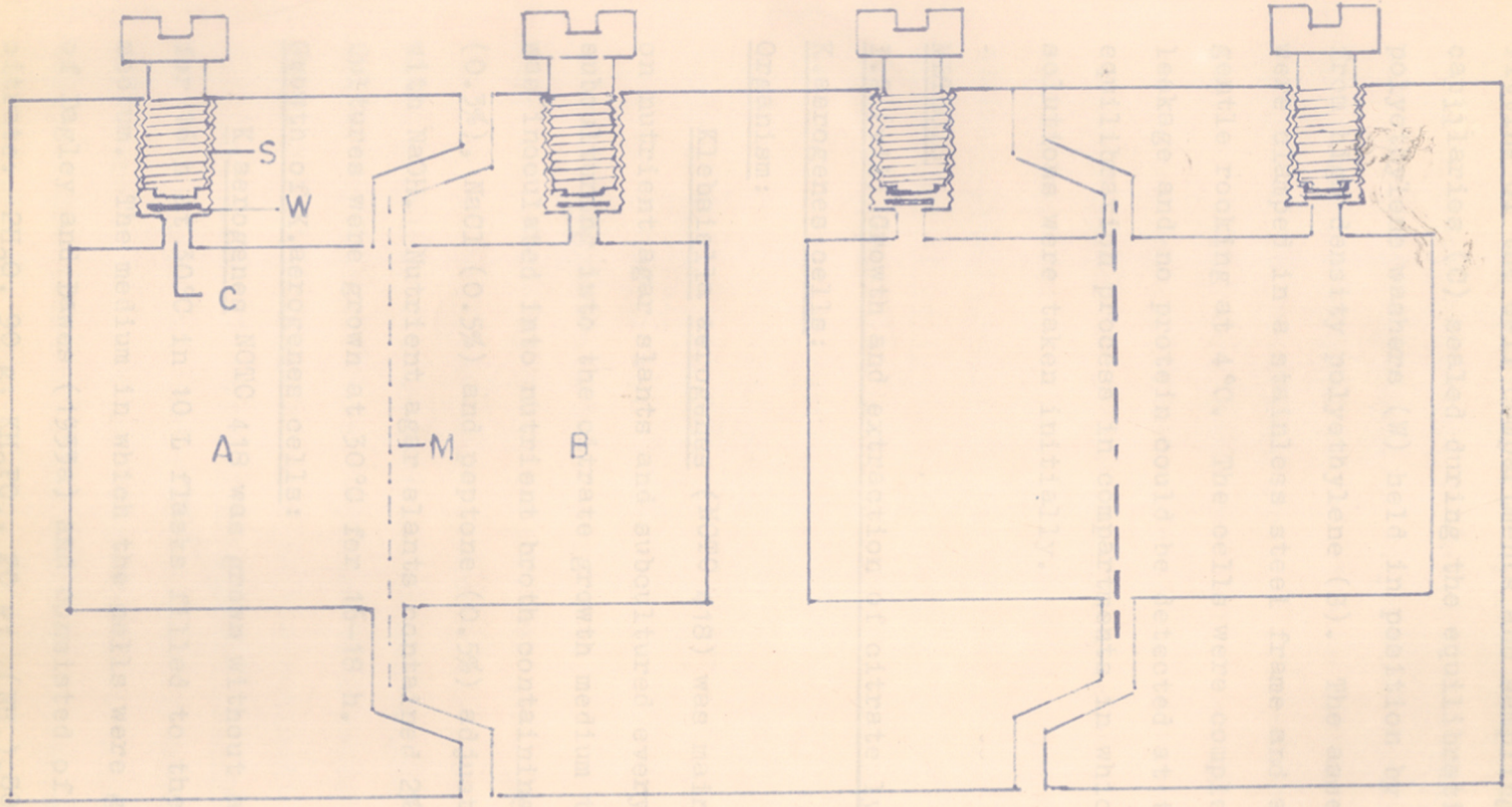


FIG 1 : 1 SCHEMATIC DIAGRAM OF TWIN CELLS USED FOR DIALYSIS EQUILIBRIUM

by compression, the capacity of each compartment was 1 ml. Fitting of the cell compartments and sampling were through capillaries (C) sealed during the equilibration with polyethylene washers (W) held in position by screws made from high density polyethylene (S). The assembled cells were clamped in a stainless steel frame and agitated by gentle rocking at 4°C. The cells were completely free from leakage and no protein could be detected at the end of the equilibration process in compartments in which only buffer solutions were taken initially.

METHODS

I.2 (vii) Growth and extraction of citrate lyase from K.aerogenes cells:

Organism:

Klebsiella aerogenes (NCTC 418) was maintained routinely on nutrient agar slants and subcultured every month. Before subculturing into the citrate growth medium the organism was inoculated into nutrient broth containing beef extract (0.3%), NaCl (0.5%) and peptone (0.5%) adjusted to pH 7.0 with NaOH. Nutrient agar slants contained 2% agar. Cultures were grown at 30°C for 16-18 h.

Growth of K.aerogenes cells:

K.aerogenes NCTC 418 was grown without aeration for 16 h at 30°C in 10 L flasks filled to the neck with the medium. The medium in which the cells were grown was that of Dagley and Dawes (1953a) and consisted of tri-sodium citrate. $2H_2O$, 90 g; KH_2PO_4, 20 g; $(NH_4)_2SO_4$, 10 g;

$MgSO_4.7H_2O$, 4 g, pH adjusted to 7 with 40 ml of 2 N NaOH. The latter two chemicals namely $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$ were autoclaved separately in 1.5 L of distilled water and then mixed with the rest of the medium. Cells were preliminarily adapted to the citrate medium by two successive subcultures. Inoculum size was 10% of the final volume. Cells were grown for 16 h after which they were harvested in a Model A-12 Sharples supercentrifuge at 12 000 g. Each 10 L of medium yielded about 18-20 g of wet cells. Wet cells could be stored about a week at -20°C, without loss of enzyme activity.

Extraction of cells:

The enzyme was purified from batches of 110 - 120 g of cells. All operations were carried out at 0 - 4°C. Thawed cells were suspended in 0.03 M potassium phosphate buffer, pH 7 in the proportion of 1 g wet weight of cells to 4 ml of buffer and disintegrated in a Biosonik III sonicator (Brownwill Scientific Co., USA). Sonications were done twice at 20 Kc (300 W) for three minutes each time. Cell debris was removed by spinning the suspension in the Model L Spinco ultracentrifuge at 50 000 x g for 60 min, the clear supernate was adjusted to a protein concentration of 0.5% with the buffer used in the sonication step.

I.2.(viii) Purification of K.aerogenes citrate lyase:

The enzyme was purified by a modification of the procedure of Mahadik and SivaRaman (1968). The modification involved treatment of crude sonicate extracts with ATP and acetate for reactivation of any HS- citrate lyase

present in the extracts.

The enzyme was purified without storage, all buffers contained 1.6 mM $MgSO_4$ to stabilise the enzyme. Batches of 400 ml of crude enzyme were processed at one time. An outline of the purification steps is described below. All steps were carried out at 0 - 4°C.

ATP and acetate treatment:

The crude, sonicate extract was adjusted to a protein concentration of 0.5% and was treated with ATP and acetate to give a final concentration of 0.5 mM and 1 mM of ATP and acetate, respectively. The extract was kept stirred for 15 min. This step is necessary to reactivate any HS-citrate lyase which may be present in the sonicate.

Streptomycin sulfate treatment:

1.4% streptomycin sulfate was added to the enzyme from the previous step to precipitate out nucleic acids. The solution is kept stirred for another 30 min after which it is left aside for one h. The solution is then spun for 40 min at 50 000 x g and the precipitate is discarded.

Adsorption on alumina C_γ gel:

First gel treatment: 8 ml of alumina C gel (4.7% solids) is added to the above supernatant with constant and gentle stirring. This treatment absorbs about 5% of the enzyme activity. The solution was then spun at 3 000 x g for 20 min and the gel was discarded.

Second gel treatment: The supernatant from the first gel treatment is now treated with 92 ml of the same concentration of alumina C_γ gel as used above. The gel is

added with gentle stirring and after addition, the solution is stirred for another 30 min. About 90 - 95% of the remaining enzyme activity was absorbed on the gel in this manner. The solution was again spun at 3 000 x g for 20 min and the supernatant is discarded.

Elution of the enzyme from alumina C_γ gel: The buffer used was 0.05 M potassium phosphate buffer, pH 7. The gel was homogenised with 200 ml of this buffer for 15 min, after which the suspension was again spun at 3 000 x g for 20 min. The gel was discarded and the supernatant subjected to ammonium sulfate precipitation.

Ammonium sulfate fraction: The eluate was treated with ammonium sulfate to 0.2 saturation (20 g/200 ml). After addition, the solution is spun at 50 000 x g for 30 min and the precipitate was discarded. The supernatant was again treated with ammonium sulfate to 0.5 saturation (38 g/200 ml/ original vol.) and after addition, the solution was kept stirred for a further 15 min period. The solution was then centrifuged at 50 000 x g for 30 min and the supernatant discarded.

The precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.5 (about 5 ml) and left for dialysis overnight against 0.01 M potassium phosphate buffer, pH 7.5.

DEAE-cellulose chromatography: The dialysed solution (about 7 ml) is centrifuged at 10 000 x g for 5 min to remove any turbidity and the clear solution is loaded on a DEAE-cellulose column (30 x 1.5 cm) which is previously equilibrated with 0.01 M potassium phosphate, pH 7.5.

The enzyme was obtained by ion-exchange chromatography with the help of a continuous gradient with increasing ionic strength of potassium phosphate buffer in the range of 0.01 M to 0.3 M. This concentration gradient was achieved by adding 200 ml of 0.01 M potassium phosphate buffer, pH 7.5 to the lower chamber attached to column and addition of 0.5 M potassium phosphate buffer, pH 7.5 to this buffer solution at the same rate as it is flowing into the column. Fractions of 3 ml were collected on an automatic fraction collector and enzyme activity was detected by withdrawing a drop of the fraction and testing for enzyme activity. The enzyme usually eluted out between 90 - 120 ml. Fractions with the highest enzyme activity were pooled (25 - 30 ml) and precipitated with the continuous addition under stirring of an equal volume of cold saturated ammonium sulfate solution previously neutralized with aqueous ammonia to pH 7.0. After 15 min, precipitate was collected by centrifugation at 50 000 x g for 30 min. The supernatant was discarded and the precipitate dissolved in the minimum volume of 0.05 M potassium phosphate buffer, pH 7.5 (1 - 2 ml).

Sepharose CL-6B gel filtration: The enzyme solution from the previous step was loaded on a Sepharose CL-6B column (110 cm x 2.5 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5, and eluted with the 0.05 M buffer. Fractions of 2 ml were collected on an automatic fraction collector and analysed for enzyme activity by withdrawing a drop as sample from each fraction. The enzyme eluted around 180 ml eluate volume and fractions with high enzyme

activity were pooled and the volume reduced from about 20 ml to 2-3 ml by ultrafiltration through an Amicon Diaflo PM10 membrane.

I.2 (ix) Equilibrium dialysis:

Principle: Tris-HCl buffer, 0.05 M (pH 7.4) was used in the equilibrium dialysis experiments. Tris under these conditions has been shown to have no detectable interaction with Mn^{2+} in EPR and NMR studies (Mildvan and Cohn, 1963).

The protocol used for the preliminary preparation of samples for equilibrium dialysis involved the following steps:

(1) Obtaining Mg^{2+}-free enzyme by dialysis against 2 mM EDTA + 50 mM potassium phosphate buffer, pH 7.5.

(2) Removal of the phosphate buffer by dialysis against 2 mM EDTA + 50 mM Tris-HCl buffer, pH 7.5. This is required to prevent precipitation of Mn^{2+} in the presence of the phosphate buffer, pH 7.5, when subsequent equilibration is done directly against Mn^{2+}-containing Tris-HCl buffer, pH 7.5.

(3) Exhaustive dialysis of Mg^{2+}-free enzyme in Tris-HCl buffer, pH 7.5, against Tris-HCl buffer, pH 7.5, containing non-radioactive $MnCl_2$ of the desired concentration of free Mn^{2+}. This adjusts the Mn^{2+}-free concentration inside the enzyme solution to that of the dialysing buffer.

(4) Equilibrium dialysis was carried out in analytical dialysis cells using on one side of the membrane enzyme solutions adjusted previously to known Mn^{2+}-free concentration by the exhaustive dialysis procedure outlined

in step (3) above and the final Mn^{2+}-containing buffer used for exhaustive dialysis treated with carrier-free $^{54}MnCl_2$ on the other side of the membrane. The volume of carrier-free $^{54}MnCl_2$ taken was negligible and dilution corrections were insignificantly low and made no difference to the determined values. Because of prior equilibration to the desired free-Mn^{2+} concentration, no volume change was observed in the protein solutions through electrokinetic transport of water (Myer and Schellman, 1962).

Experimental procedures: All dialyses were done at 0-4 °C. Preparation of Mg^{2+}-free citrate lyase: Enzyme solutions (4-8 mg/ml) were dialysed 24 h against 50 mM potassium phosphate-2mM EDTA (Ph 7.5) with 3 changes, 250 vol. each. Equilibrium dialysis: Mg^{2+}-free enzyme solution, prepared as above, was dialysed 16 h against 500 vol. 50 mM Tris-2mM EDTA (pH 7.5), then equilibrated by dialysis for 36 h against 50 mM Tris (pH 7.5) containing non-radioactive $MnCl_2$ at desired concentration, with 3 changes, 250 vol. each.

Of the final buffer against which the enzyme solution had been equilibrated, 20 ml was treated 0.01 ml carrier-free $^{54}MnCl_2$ solution. The concentration of the buffer was adequate to neutralise the HCl present in the $^{54}MnCl_2$ with a resulting pH change of ≤ 0.1 unit. Addition of the small volume of $^{54}MnCl_2$ was assumed not to alter the total Mn^{2+} concentration in the buffer, being carrier-free. The trace quantities of non-radioactive ^{54}Cr, the decay product of ^{54}Mn, was assumed not to compete with Mn^{2+} binding.

Dialysis cells were of the type described by Myer and Schellman (1962) with 1 ml capacity compartments. Dialysis

membranes were cut from Visking dialysis tubing pretreated by boiling successively with 100 ml volume of 100 mM $NaHCO_3$ - 10 mM EDTA and 100 ml volume of 100 mM acetic acid and then again with the previous solution: the tubing is then rinsed with several changes of water double-distilled in glass and finally with the equilibration buffer before use. Cell compartments were filled with the enzyme solution on one side and an equal volume of the equilibration buffer with added $^{54}MnCl_2$ on the other. The cell-assembly was gently rocked for 24 h at 0 - 4°C until equilibrium is reached as determined from preliminary trials. Samples were withdrawn separately from the compartments for both ^{54}Mn and protein determinations. The free-Mn^{2+} concentration i.e. $(Mn^{2+})_f$ was taken to be that in the buffer against which the enzyme had been dialysed extensively. The concentration of bound Mn^{2+} i.e. $(Mn^{2+})_b$ was calculated from the difference in radioactivity counts between the enzyme solution and the corresponding equilibration buffer. The stoichiometry of binding was calculated for molecular weight 575 000 of the enzyme (Mahadik and SivaRaman, 1968).

Protein determinations: The method used for the determination of the protein during purification of the enzyme was of Lowry et al. (1951). The procedure was standardised against pure citrate lyase from K.aerogenes. Samples containing streptomycin sulfate were first dialysed exhaustively against water to remove the streptomycin sulfate before protein estimation as the antibiotic

interferes with the estimation. The color was read either at 500 nm or 750 nm depending on the color intensity and protein was read off standard curves. Pure protein samples were also estimated by the method of Singh et al. (1976) using the absorbance index $E^{1\%}_{1\ cm}$ of 6.2 at 278 nm. The values obtained by both procedures were completely in agreement.

Enzyme assay: Two different methods were used for estimating the enzyme activity, the one routinely used in the preliminary steps of fractionation was that described by Mahadik and SivaRaman (1968). The test system (3 ml) containing sodium citrate, 100 mM; $MgSO_4$, 10 mM, Tris-HCl buffer, 50 mM, pH 8. The reaction was initiated with an aliquot containing an equivalent of 5 - 10 µg of the enzyme. The temperature at which the reaction was carried out was 28°C. The keto acid formed was estimated by the method of Friedmann and Haugen (1943) after stopping the reaction with addition of 3 ml of 10% trichloroacetic acid. Initial rates of the reaction were determined by values obtained at 15 secs.

The second procedure used for pure enzyme samples was a slight variation of the coupled assay method described by Singh and Srere (1971). The oxaloacetate produced in the citrate lyase reaction was assayed with malate dehydrogenase in the presence of NADH. The change in optical density was recorded at 340 nm. The test system contained in 1 ml final volume, sodium citrate, 100 µmol; $MgSO_4$, 10 µmol; Tris-HCl buffer (pH 8), 50 µmol; excess

of malate dehydrogenase (approximately 15 µg); NADH, 0.1 - 0.2 µmol; 0.1 - 0.5 µg citrate lyase contained in 0.01 ml to initiate the reaction which was carried out in a 1 ml capacity cuvette of 10 mm light path. The temperature of the reaction was maintained at 28°C. A decrease of 6.22 in the optical density corresponded to 0.1 umol of keto acid formed.

1 U of the enzyme is defined as the amount of enzyme required for catalysing cleavage of 1 µmol of citrate per min under assay conditions.

Optical density: Beckman DU or Model 26 spectrophotometer was used for taking all optical density measurements. All estimations by the coupled assay method were carried out on the Beckman Model 26 spectrophotometer equipped with strip chart recorder.

pH measurements: All pH measurements were carried out on a Phillips precision pH meter Model P.H. 9405 with a combination glass electrode.

I.2 (x) Analytical ultracentrifugation:

Analytical ultracentrifugal runs were carried out on a Spinco Model E ultracentrifuge equipped with a phase plate schlieren optical system and a rotor temperature control device. A standard cell of 12 mm light path and with 4°-sector duralium centrepiece was used together with a counterbalance with the usual drilled reference holes to obtain reference points for the determination of radial distances from the axis of rotation. All determinations were made at 2 - 5°C. Corrections for the stretching of

the AnD analytical rotor at 59780 rpm was 0.02 cm determined according to the procedure of Kegeles and Gutter (1951). Photographic plates of sedimentation profiles were read on a model M-2060 microcomparator of Gaertner Scientific Corporation, USA.

Sedimentation coefficients were calculated in the usual manner from the plots of the logarithm of distance of sedimenting boundary from the axis of rotation versus time (Schachman, 1957). The observed sedimentation coefficient (s_{obs}) were normalised to water at 20° ($s_{20,w}$) after applying density and viscosity corrections according to the equation:

$$s_{20,w} = s_{obs} \left(\frac{\eta_t}{\eta_{20}}\right) \left(\frac{\eta}{\eta_o}\right) \left(\frac{1 - \bar{v}\,\rho_{20,w}}{1 - \bar{v}\,\rho_t}\right)$$

where η_t/η_{20} is the viscosity of water at t° relative to that at 20°C; (η/η_o) is the relative viscosity of the solvent to that of water; $\rho_{20,w}$ is the density of water at 20° and the ρ_t the density of solvent at t°. The term ρ_t was calculated from the relationship $\rho_t = (\rho/\rho_o)\,\rho_{t,w}$ where (ρ/ρ_o) is the relative density of solvent at any temperature and $\rho_{t,w}$ the density of water at t°. The partial specific volume ($\bar{v}$) of citrate lyase from K.aerogenes has been computed to be 0.737 $ml.g^{-1}$ by Singh and Srere (1976) from amino acid composition data, and this value was used in the calculations.

I.2.(xi) Polyacrylamide gel electrophoresis:

The method used was that of Davis (1964). Concentration of gel used was 5% and polymerisation was achieved with 0.07% ammonium persulfate; the sample and stacking gels were omitted. Buffer used with the separating gel was that of Davis containing Tris-HCl buffer, pH 8.3. Unreacted ammonium persulfate was removed by a preliminary electrophoresis of 90 min. About 50 µg of protein saturated with 30% sucrose was carefully layered on the top of the gels in a volume of about 200 µl and then the Tris-glycine electrode buffer, pH 8.3 was carefully layered on top. Bromophenol blue was used as the tracking dye. Electrophoretic run was carried out at 4 °C and 4 mA/tube for about 6 h. After the run was complete, the gels were stained overnight with 0.25% Coomassie Brilliant Blue prepared in water containing 45.4% methanol and 9.2% glacial acetic acid. Destaining was achieved by diffusion in a solution containing 7.5% glacial acetic acid and 5% methanol in water.

I.2 (xii) Ultrafiltration:

The enzyme was concentrated to the desired volume in a Amicon ultrafiltration cell of 10 ml capacity using PM-10 membrane.

I.2 (xiii) Metal determinations

Manganese in stock solutions of $MnCl_2$ (200 mM) was determined chemically (Cook, 1941). ^{54}Mn was determined by γ-ray spectrometry using a model SC 603 Instrument of the Electronics Corporation of India. Magnesium was determined by atomic absorption spectrophotometry using a Perkin Elmer Model 303 instrument.

Magnesium was determined after wet combustion of lyophilized protein samples with concentrated HCl/HNO_3, 3:1 (v/v).

I.3. RESULTS

I.3 (i) Purification of citrate lyase from K.aerogenes:

The results obtained in a representative batch for the purification of citrate lyase from K.aerogenes are summarized in Table I.1. It will be seen from Table I.1 that the inclusion of the activation step with acetate and ATP results in approximately 25% enhancement of the citrate lyase activity in the cell free extracts of the organism. This increase would indicate the presence of some inactive deacetylated enzyme in the sonicate. The presence of the enzyme acetate: SH (acyl carrier protein) citrate lyase ligase (AMP) in K.aerogenes has been established earlier (Schmellenkamp and Eggerer, 1974) and the reactivation observed in the present procedure would be through the action of this enzyme. The inclusion of this step resulted in a final enzyme preparation with uniformly high specific activity in the range of 70 - 90 U per mg of protein. The final recovery of about 26% of initial activity compares with the yields of about 28% reported originally by Mahadik and SivaRaman (1968).

The final enzyme preparation was homogeneous both ultracentrifugally and electrophoretically. The sedimentation profile of a representative preparation is shown in Fig. I.2. The enzyme sediments as a single symmetrical peak characteristic of a homogeneous preparation. Polyacrylamide gel electrophoretic pattern of the stained protein is shown in Fig. I.3. A single band confirms the homogeneity of the preparation.

TABLE I. 1

Purification of citrate lyase from Klebsiella aerogenes

Wet weight of cells: 120 g

Fraction	Volume ml	Protein mg/ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract	437	5.0	3822	1.75	-
Cell-free extract + acetate + ATP	436	5.0	4796	2.2	100
Streptomycin sulfate treatment followed by centrifugation to remove precipitated nucleic acids.	430	3.8	4085	2.5	85
0.05 M buffer eluate of second alumina C_γ gel	200	2.52	2520	5.0	52.5
Ammonium sulfate fraction (0.2 - 0.5 Sat.) dissolved in 0.05 M buffer, pH 7.5.	5	30.0	2280	15.2	47.5
DEAE-cellulose fraction pooled and fractionated with saturated $(NH_4)_2SO_4$ and the precipitate dissolved in 0.05 M buffer, pH 7.5	2	14.5	2009	69.0	41.8
Sepharose CL-6B fractions after concentration over PM-10 membrane by ultrafiltration	2	6.9	1242	90.0	26.

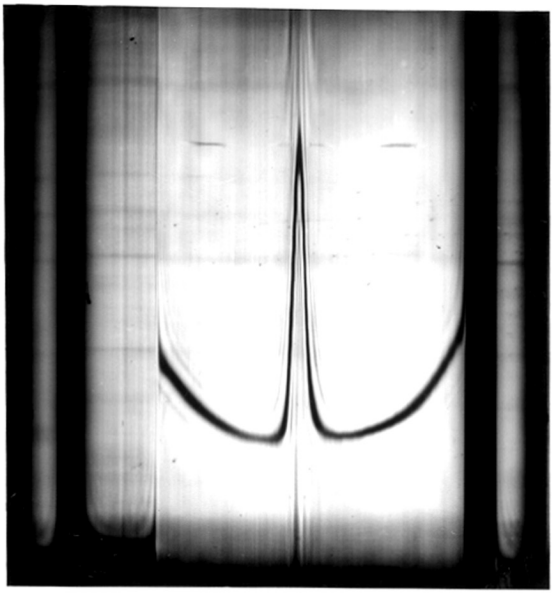


FIG. I. 2 SEDIMENTATION PROFILE OF CITRATE LYASE FROM K. aerogenes

Protein concentration	- 3.5 mg/ml
Buffer	- 0.05 M potassium phosphate buffer, pH 7.5 with 1.6 mM $MgSO_4$.
Speed	- 59 780 rpm
Temperature	- 5°C
Phase plate	- 60°
Time	- 40 min.

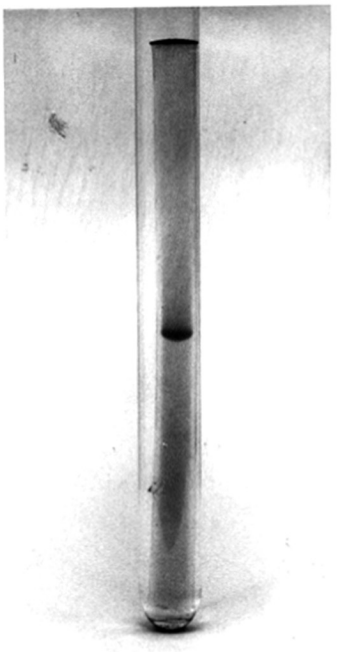


FIG. I.3 POLYACRYLAMIDE GEL ELECTROPHORESIS OF NATIVE CITRATE LYASE FROM K.AEROGENES

5% gel; Tris-glycine buffer, pH 8.3; 4 mA/tube 3 h.

Protein loaded 30 ug.

I.3 (ii) Preparation of Mg^{2+}-free citrate lyase:

Enzyme solution which had been exhaustively dialysed against 50 mM potassium phosphate - 2 mM EDTA (pH 7.5) were wet combusted and assayed for Mg^{2+}-content by atomic absorption spectrophotometry as described under MATERIALS AND METHODS. The results are summarized in Table I.2.

TABLE I.2

K.aerogenes citrate lyase solutions (4 - 8 mg/ml) were dialysed 24 h at 0 - 4 °C against 50 mM potassium phosphate - 2 mM EDTA (pH 7.5), with 3 changes, 250 vol. each. Samples containing about 10 mg protein were wet combusted and analysed for Mg^{2+} by atomic absorption spectrophotometry.

Conditions	$/Mg^{2+}/$/mol enzyme
50 mM potassium phosphate - 2 mM EDTA (pH 7.5)	0.0

I. 3 (iii) Sedimentation behaviour of metal-free and Mn^{2+}-citrate lyase from K.aerogenes:

For the unequivocal interpretation of protein-ligand binding data it becomes necessary to determine whether the macromolecule retains its state of aggregation or not in the presence of the ligand species. In the present study this was examined by determining the sedimentation coefficient which in turn is an indication of the molecular size, shape and density of the protein. The sedimentation behaviour

of citrate lyase from K.aerogenes was therefore determined both in the absence and presence of Mg^{2+} and under conditions where Mg^{2+} is replaced by Mn^{2+}. The sedimentation behaviour of the enzyme in the absence and in the presence of Mg^{2+} is described in Fig. I.4 and Fig. I. 5 respectively and the results are summarized in Table I.3.

TABLE I.3

Sedimentation behaviour of citrate lyase from K.aerogenes. Speed 59 780 rev./min; temp. 2.2 - 4.5°C.

Enzyme	Buffer solution (pH 7.4 - 7.5)	$s^{\circ}_{20,w}$ (S)
Mg^{2+}-free	2 mM EDTA-50 mM Tris-HCl	17.6
Native	2 mM $MgSO_4$-50 mM Tris-HCl	17.8
Mn^{2+}	2 mM $MnCl_2$-50 mM Tris-HCl	($s^{0.4\%}_{20,w}$ = 16.7)

It will be seen from Table I.3 that the Mg^{2+}-free enzyme and the enzyme in the presence of the metal have almost similar $s^{\circ}_{20,w}$ values of 17.6 S and 17.8 S, respectively. The sedimentation value of the Mn^{2+}-enzyme obtained by equilibrating the Mg^{2+}-free enzyme with 2 mM $MnCl_2$-50 mM Tris HCl (pH 7.4) was 16.7 at 4 mg/ml protein concentration which is similar to that of corresponding concentration of the enzyme in presence of Mg^{2+}. The absence of Mg^{2+} as well as the replacement of Mg^{2+} with Mn^{2+} evidently causes no change in the quaternary structure of the enzyme under the conditions used.

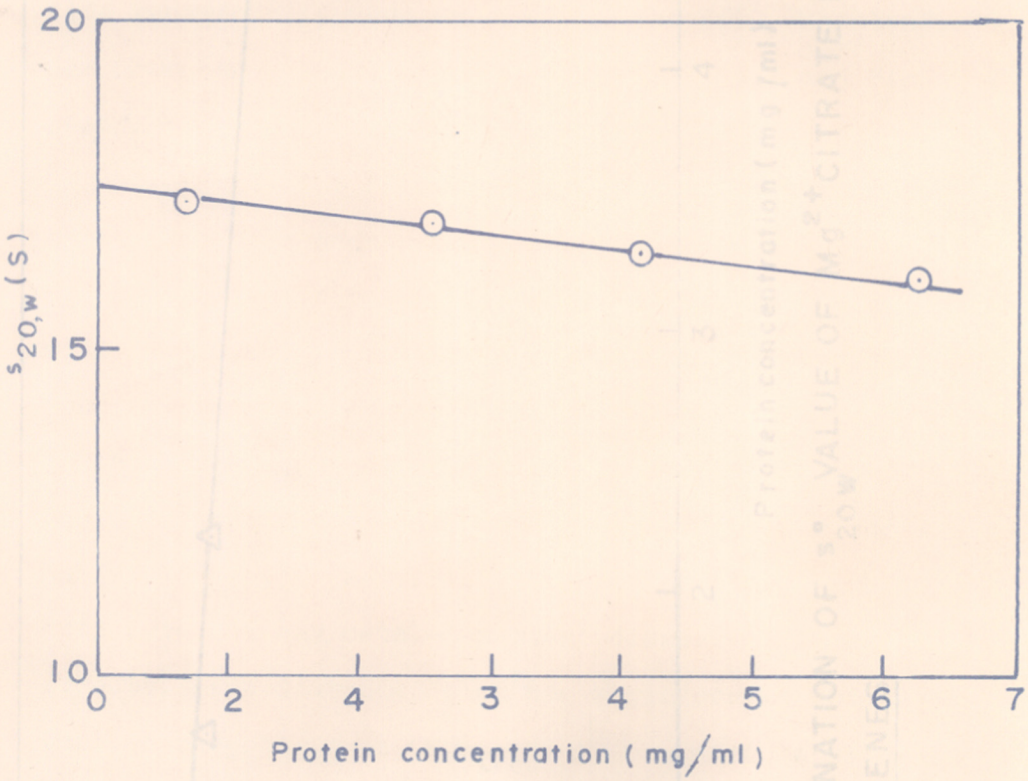


FIG.I:4. DETERMINATION OF $s^{\circ}_{20,w}$ VALUE OF Mg^{2+} - FREE CITRATE LYASE COMPLEX FROM K. AEROGENES.

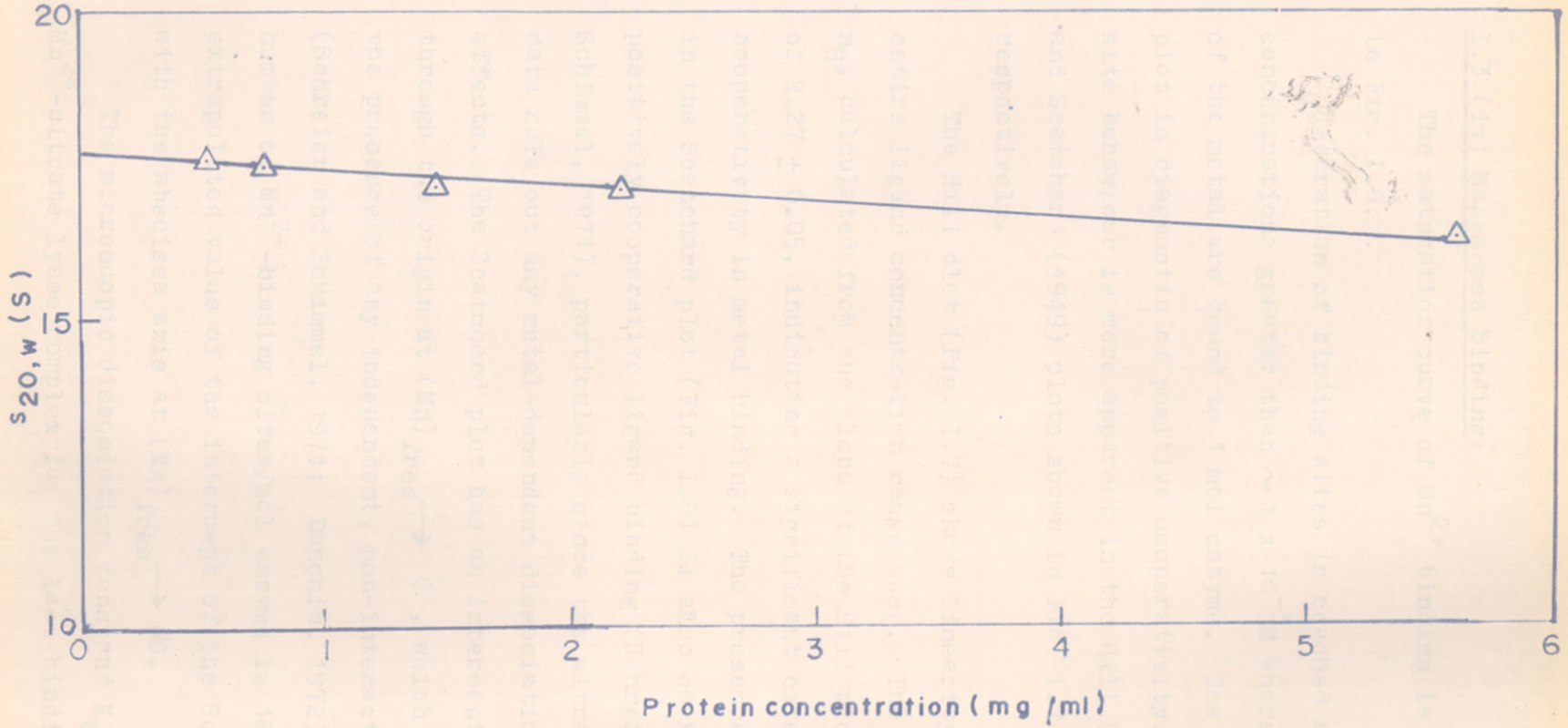


FIG.I:5. DETERMINATION OF $s^{\circ}_{20,w}$ VALUE OF Mg^{2+} CITRATE LYASE COMPLEX FROM K AEROGENES

I.3 (iv) Manganese binding:

The saturation curve of Mn^{2+} binding is presented in Fig. I.6.

Saturation of binding sites is reached at free-Mn^{2+} concentrations greater than $\sim 1 \times 10^{-3}$M where 18 g atoms of the metal are bound to 1 mol enzyme. The sigmoidal plot is diagnostic of positive cooperativity. The interacting site behaviour is more apparent in the Hill (Koshland, 1970) and Scatchard (1949) plots shown in Fig. I.7 and Fig. I.8, respectively.

The Hill plot (Fig. I.7) shows linearity over the entire ligand concentration range used. The Hill coefficient, n_H, calculated from the slope of the Hill plot has a value of 2.27 ± 0.05, indicating a significant extent of positive cooperativity in metal binding. The presence of a maximum in the Scatchard plot (Fig. I.8) is also characteristic of positively cooperative ligand binding (Schreier and Schimmel, 1974), particularly since the ultracentrifuge data rule out any metal-dependent dissociation-association effects. The Scatchard plot has an intercept which passes through the origin at $(Mn)_{free} \longrightarrow 0$, which would rule out the presence of any independent, non-interacting sites (Schreier and Schimmel, 1974; Danchin, 1972). The total number of Mn^{2+}-binding sites/mol enzyme is 18 from the extrapolated value of the intercept of the Scatchard plot with the abscissa axis at $(Mn)_{free} \longrightarrow \infty$.

The microscopic dissociation constand $K_{d,n}$ of the Mn^{2+}-citrate lyase complex for the last binding step was

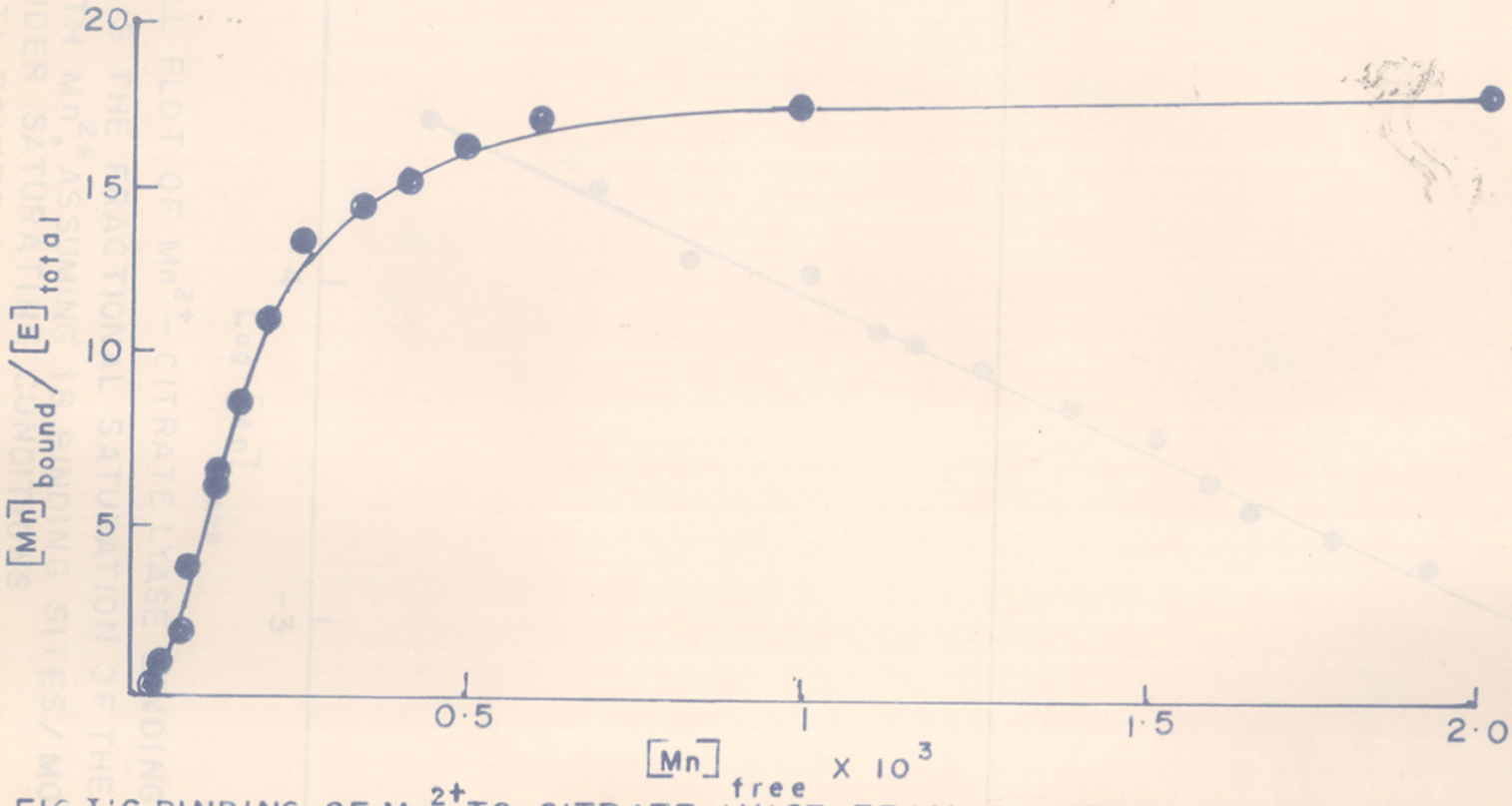


FIG. 1.6. BINDING OF Mn^{2+} TO CITRATE LYASE FROM K. AEROGENES. EQUILIBRIUM DIALYSIS PERFORMED IN 50mM TRIS-HCL BUFFER (pH 7·4) CONTAINING VARYING CONCENTRATIONS OF $MnCl_2$. PROTEIN 4–8 mg/ml; TEMP 0–4°C.

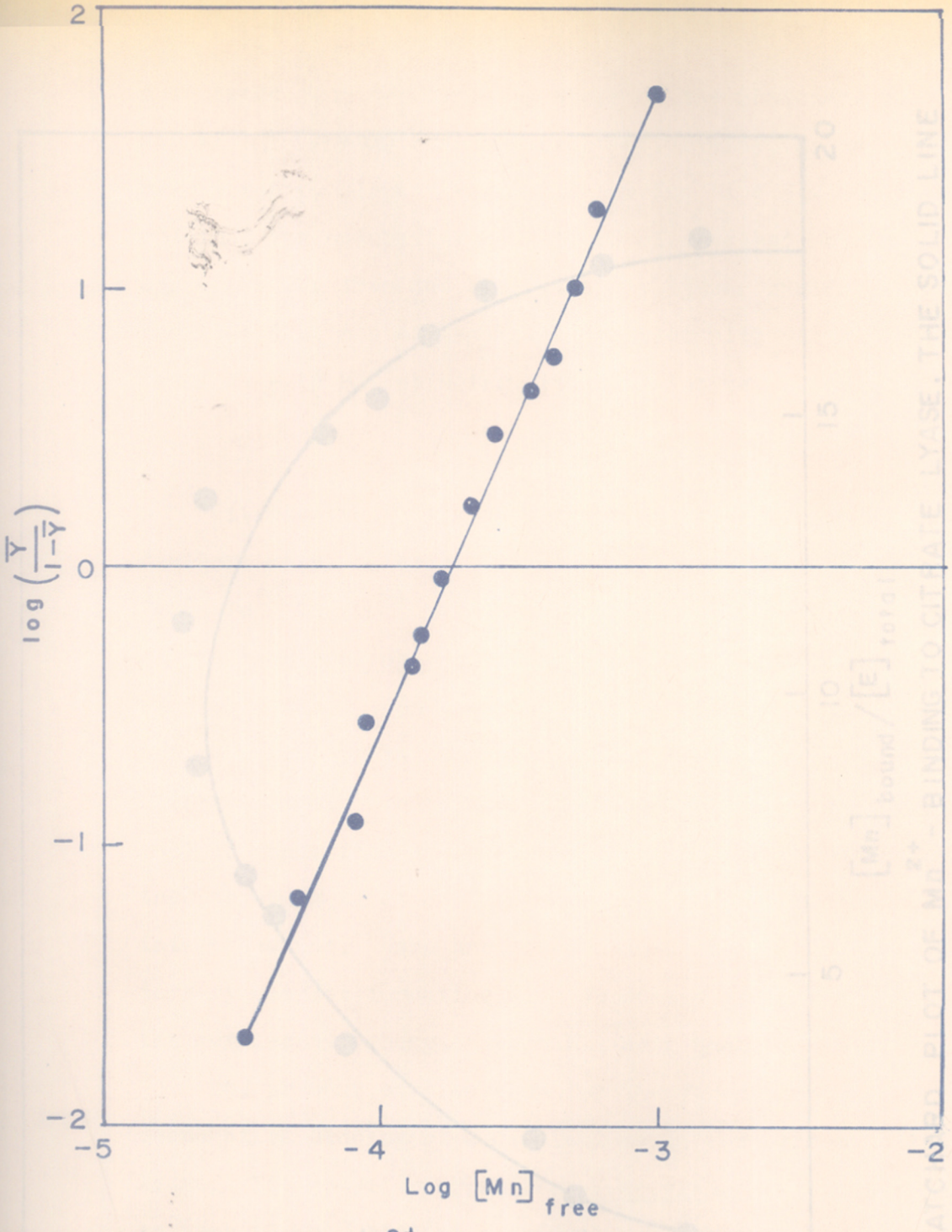


FIG I:7. HILL PLOT OF Mn^{2+} – CITRATE LYASE BINDING DATA.
$\bar{Y}$ IS THE FRACTIONAL SATURATION OF THE ENZYME WITH Mn^{2+}, ASSUMING 18 BINDING SITES / MOL ENZYME UNDER SATURATING CONDITIONS.
DATA POINTS PLOTTED ARE FOR $\bar{Y}$ VALUES IN THE RANGE 0·02 – 0·98
THE STRAIGHT LINE IS THE LEAST SQUARES FIT OF EXPERIMENTAL DATA.

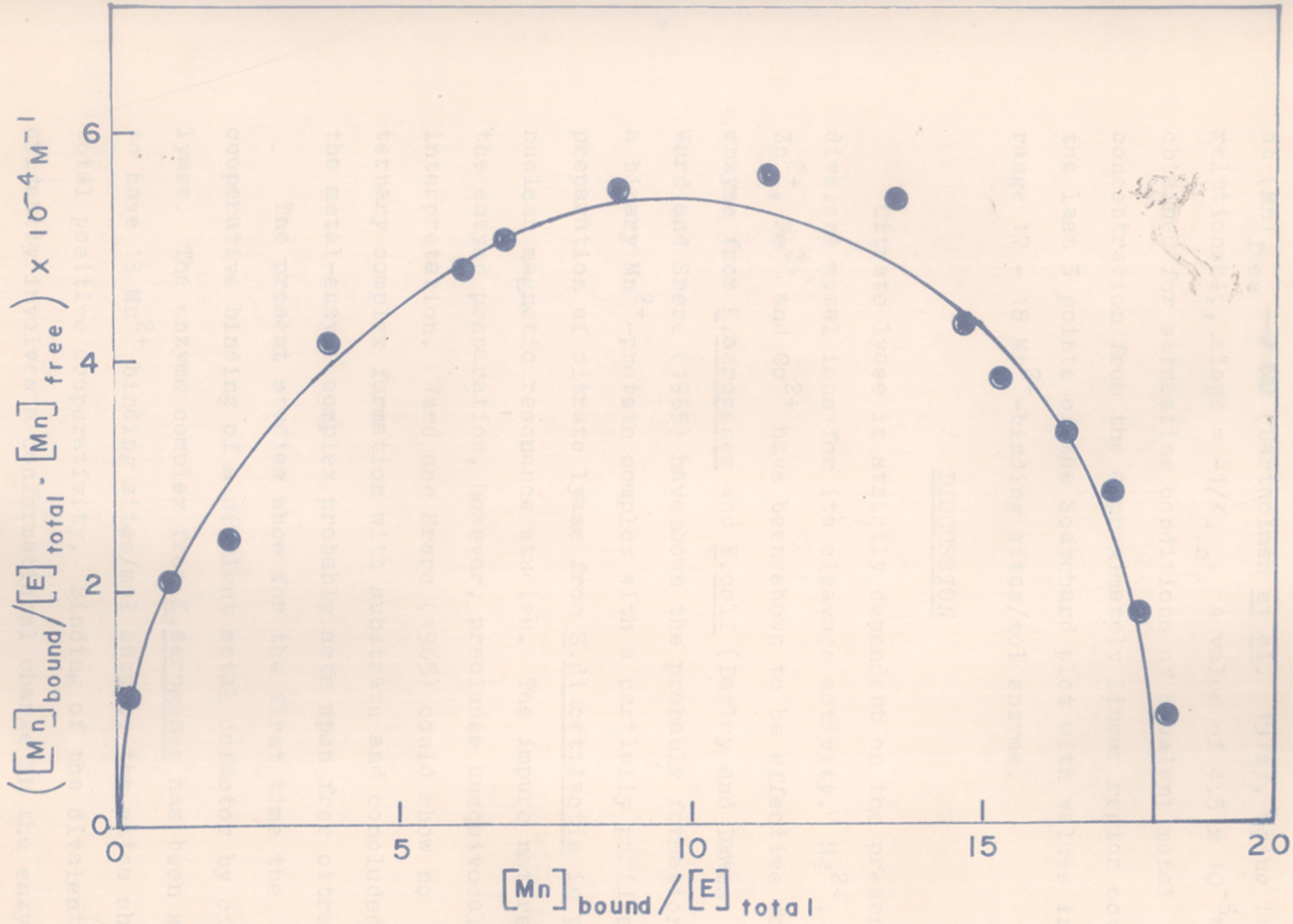


FIG. I:8. SCATCHARD PLOT OF Mn^{2+} - BINDING TO CITRATE LYASE. THE SOLID LINE IS CONSTRUCTED FROM VALUES CALCULATED FROM THE HILL PLOT AND OVERLAPS APPROXIMATELY CURVE DRAWN BY EYE.

calculated from the limiting slope of the Scatchard plot at $(Mn)_{free} \rightarrow \infty$ (Bartholmes et al. 1976), using the relationship, slope = $-1/K_{d,n}$. A value of 4.5×10^{-5}M was obtained for saturating conditions of divalent metal concentration from the approximately linear region covering the last 3 points of the Scatchard plot with values in the range 17 - 18 Mn^{2+}-binding sites/mol enzyme.

DISCUSSION

Citrate lyase is strictly dependent on the presence of divalent metal ions for its cleavage activity. Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+} have been shown to be effective for the enzyme from K.aerogenes and E.coli (Dagley and Dawes, 1955). Ward and Srere (1965) have shown the probable formation of a binary Mn^{2+}-protein complex with a partially purified preparation of citrate lyase from S.diacetilactis in pulsed nuclear magnetic resonance studies. The impure nature of the enzyme preparation, however, precludes unequivocal interpretation. Ward and Srere (1965) could show no ternary complex formation with substrate and concluded that the metal-enzyme complex probably acts upon free citrate.

The present studies show for the first time the cooperative binding of a divalent metal cofactor by citrate lyase. The enzyme complex from K.aerogenes has been shown to have 18 Mn^{2+} binding sites/mol enzyme, the sites showing total positive cooperativity. Binding of the divalent metal evidently involves a conformational change in the enzyme complex, probably to a form which is catalytically active

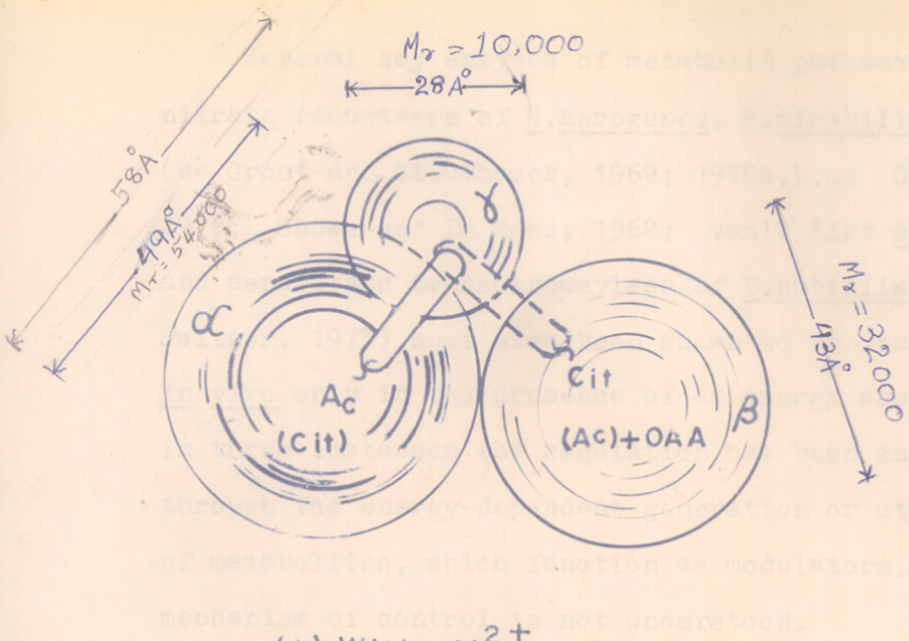
in the cleavage reaction.

This finding has been confirmed in recent data obtained in this Laboratory by an entirely different approach (Tikare, 1979). In these confirmatory studies, cross-linking experiments were carried out using the reactive bifunctional reagent, dithiobis-(succinimidyl propionate) (DSP), a reagent first described by Lomant and Fairbanks (1976) which has an extended span of 12 Å (Bragg and Hou, 1976). Such bifunctional reagents are useful for the study of subunit spatial arrangement in oligomeric and multicomponent proteins (Davis and Stark, 1970). The use of the bifunctional reactive ester, DSP, with citrate lyase from K.aerogenes in the presence of Mg^{2+}, yielded a cross-linked species of 178 000 daltons. This molecular weight corresponds to a dimer of cross-linked α - and β-subunits which was confirmed after cleavage of the cross-links. However, cross-linking with DSP in the absence of the divalent metal yielded significantly lower amounts of the 178 000 daltons aggregate together with almost equal amounts of a 200 000 dalton species, probably corresponding to a $\alpha_2 \beta_2 \gamma_2$ cross-linked aggregate. These results confirm directly the present finding that divalent metal cofactor binding to citrate lyase leads to a conformational change in the enzyme complex. The slight difference in sedimentation coefficient $s^{\circ}_{20,w}$ values of 17.6 for the Mg^{2+}-free enzyme and 17.8 for the Mg^{2+} enzyme might also probably be due to conformationally distinct states of the enzyme complex.

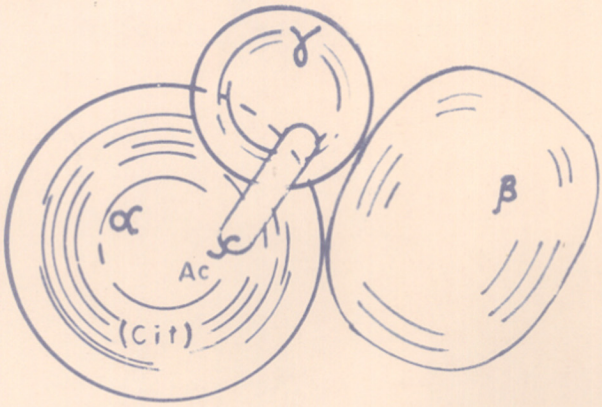
The present observations contribute the first evidence for the allosteric behaviour of the citrate lyase complex. Allosteric modulations play an important role in the regulation of metabolic activity at the enzyme level. The mechanism of action of citrate lyase from K.aerogenes is known to involve two consecutive reactions. The initial acyl transferase reaction is catalysed by the 54 000 dalton α-subunit resulting in the formation of citryl-ACP (citryl γ-subunit) intermediate in presence of the substrate with displacement of the essential acetyl group on the enzyme as acetate. The subsequent cleavage of the citryl-intermediate is catalysed by the 32 000 dalton lyase (β-) subunit with formation of oxaloacetate and the regeneration of acetyl-ACP (Dimroth and Eggerer, 1975b). The former reaction proceeds even in the presence of EDTA in excess, while the latter requires the presence of divalent metal ions as cofactor. The mechanism of action of the citrate lyase complex requires the oscillation of the acyl function on the ACP (γ-subunit) component between the active sites on the transferase (α-) subunit and the lyase (β-) subunit during the course of the citrate cleavage reaction (Srere and Singh, 1974; Dimroth and Eggerer, 1975b). A model of subunit interaction suggested for the coupled reaction by Srere and Singh (1974) is represented in Fig. I.9 (i). The evidence obtained in the present study establishes that the binding of metal cofactor to the enzyme leads to a conformational change

in the structure of the complex. The conformation of the divalent metal cofactor-enzyme complex is a prerequisite for the cleavage (lyase) reaction. In the absence of the metal cofactor, only the transferase reaction proceeds with accumulation of the labile citryl-ACP intermediate. A speculative model in which the conformation of the lyase (β-) subunit is presumed to be modulated by metal cofactor binding is shown in Fig. I.9 (ii).

The allosteric behaviour of the enzyme could be a regulatory mechanism of biological significance. Citrate lyase from K.aerogenes undergoes rapid inactivation in vitro during the course of the reaction it catalyses through a process of deacetylation (Singh and Srere, 1971; Singh and Srere, 1975). However such reaction-inactivation has been shown to play no role in citrate lyase regulation in vivo (Kulla and Gottschalk, 1977). The inactivation in vivo has been shown to be energy-dependent (Kulla and Gottschalk, 1977). Conformational modulations in citrate lyase complex could be a possible mechanism of regulation, if the requirement of energy is assumed to be for the formation or utilization of metabolite(s) acting either directly as modulator(s) or indirectly through complexing of Mg^{2+}. The earlier speculation (Blair et al., 1967) that a mechanism for regulation of citrate lyase activity in the cell could be through the ability of ATP to chelate metal ions, could indeed be so through modulation in the conformation of citrate lyase when Mg^{2+} is abstracted from the complex.



(i) With M^{2+}



(ii) Without M^{2+}

FIG. I.9. SPECULATIVE MODEL OF METAL COFACTOR INTERACTION WITH CITRATE LYASE FROM K. aerogenes.

(Dimensions from Beuscher et al. 1974; Srere & Singh, 1974)

Several key enzymes of metabolic pathways, such as nitrate reductases of K.aerogenes, P.mirabilis and E.coli (de Groot and Stouthamer, 1969; 1970a,b,c; Oltmann et al., 1976; Showe and De Moss, 1968; van'T Riet et al., 1968) and asparatate transcarbamylase of B.subtilis (Waindle and Switzer, 1973) have also been shown to be inactivated in vivo only in the presence of an energy source. Although in these instances the regulation has been assumed to be through the energy-dependent generation or utilization of metabolites, which function as modulators, the mechanism of control is not understood.

PART II

COMPARATIVE STUDIES BETWEEN CITRATE LYASES FROM *K. aerogenes* and *S. faecalis*

PART II

COMPARATIVE STUDIES BETWEEN CITRATE LYASES FROM K.aerogenes AND S.faecalis

SUMMARY

Comparative studies on citrate lyase complexes from the two sources, K.aerogenes and S.faecalis have been carried out. The enzymes from these sources are representative of the two classes of citrate lyases, ones that undergo severe reaction-inactivation and ones that show markedly weaker reaction-inactivation behaviour.

The enzyme complexes from K.aerogenes and S.faecalis have been shown to cross-react immunologically. Enzymes from other sources have hitherto been shown to be immunologically distinct.

The steady-state concentrations of citryl-CoA, the intermediate in the acetyl-CoA dependent cleavage of citrate in presence of deacetylated citrate lyases from K.aerogenes and S.faecalis have been estimated. In the reaction catalysed by the enzyme from K.aerogenes the concentration has been shown to be markedly lower than in the reaction catalysed by the S.faecalis enzyme. These results are indirect evidence against the hypothesis that reaction-inactivation behaviour is determined solely by the spontaneous degradation of a labile citryl-intermediate.

Homologous relationships were detected in the tryptic peptide maps of corresponding subunits of citrate lyase complexes from K.aerogenes and S.faecalis. These are the first reports on the detailed structural similarities between citrate lyases from diverse sources. The data might indicate divergent evolution from a common ancestral protein.

II.1 INTRODUCTION

Considerable interest has been focussed on the citrate lyase complex on account of its unusual multienzymic nature with a built-in acyl carrier protein (ACP) which contains a covalently attached CoA-like prosthetic group. The enzyme from K.aerogenes which has been studied the most has been shown to contain three non-identical subunits of 54 000 (α), 32 000 (β) and 10 000 (γ) daltons; with equimolar amounts of each viz. 6 copies of each type (Singh et al., 1974; Carpenter et al., 1975; Dimroth and Eggerer, 1975a). The smallest subunit carries an essential acetyl group and functions as an ACP (Dimroth et al., 1973). The 54 000 dalton (α) subunit functions as an acyl transferase and catalyses the first of a two step reaction in presence of citrate leading to the formation of citryl-ACP with elimination of the essential acetyl group as acetate. The subsequent cleavage of the citryl-ACP intermediate is catalysed by the 32 000 dalton (β) subunit which functions as a lyase to regenerate acetyl-ACP with release of oxaloacetate (Dimroth and Eggerer, 1975b). The enzyme undergoes severe reaction-inactivation with loss of essential acetyl residues to give the deacetyl enzyme in the course of the reaction it catalyses (Singh and Srere, 1971; Singh and Srere, 1975).

Citrate lyases obtained pure from diverse bacterial sources such as Streptococcus diacetilactis (Singh and Srere, 1975; Kummel et al., 1975), Rhodopseudomonas

gelatinosa (Giffhorn and Gottschalk, 1978), Streptococcus faecalis (Hiremath et al., 1976) have all been shown to be closely related in subunit structure and stoichiometry as well as in subunit function to the enzyme from K.aerogenes. All the enzymes also undergo reaction-inactivation with the loss of essential acetyl residues. However some like the enzymes from K.aerogenes and R.gelatinosa undergo severe reaction-inactivation while those from S.diacetilactis and S.faecalis are inactivated at markedly slower rates with rate constants for reaction-inactivation 10 - 20 fold lower than of the former group (Singh and Srere, 1975; Hiremath, 1977). The weak reaction-inactivation in the latter group of the enzymes was believed to be due to the association of a HS-citrate lyase acetylating activity with the native citrate lyase complex (Kummel et al., 1975). This was shown not to be so by Hiremath et al. (1976) and later by Bowien and Gottschalk (1977), the ligase activity reported earlier (Kummel et al., 1975) being due to a contaminating ligase activity which could be separated from the citrate lyase complex.

Despite the striking similarities in the quaternary structures and subunit functions of citrate lyases from various sources, the enzymes from difference sources have hitherto been shown to be immunologically distinct. In a comparative study of the immunological behaviour of citrate lyases from K.aerogenes, S.diacetilactis and E.coli, Singh and Srere (1975) showed that the enzymes from S.diacetilactis and E.coli do not cross react with the

rabbit sera against pure citrate lyase from K.aerogenes in double diffusion experiments. Likewise, rabbit antisera against purified S.diacetilactis enzyme was shown to give precipitin bands only with the antisera against enzyme from the same source.

The present study was mainly directed towards understanding of the structural homologies between citrate lyases from the two sources, K.aerogenes and S.faecalis. Studies on the immunological behaviour of the enzyme from the two sources showed that citrate lyase from S.faecalis cross-reacts with rabbit antisera against pure citrate lyase from K.aerogenes. In similar studies the immunological differences between the K.aerogenes and S.diacetilactis enzymeswas confirmed.

As pointed out earlier, citrate lyase from K.aerogenes and from S.faecalis represent two distinct types in so far as reaction-inactivation behaviour is concerned; the former undergoing severe reaction-inactivation and the latter only weakly so. The differences observed in the reaction-inactivation behaviour of these two distinct types of citrate lyase complexes could be attributed either to an enhanced stability of the citryl-intermediate towards hydrolytic cleavage or to inherent differences in the relative rates of formation of the citryl-intermediate and its subsequent cleavage. The latter aspect was studied in the present investigation using deacetylated enzymes from K.aerogenes and S.faecalis and determining the comparative rate constants of the formation of the intermediate

citryl-CoA in presence of acetyl-CoA and of the subsequent cleavage of the citryl-CoA to yield acetyl-CoA and oxaloacetate. These estimates were made from kinetic analysis of the transient phase and the steady-state of the reaction.

While citrate lyases from different sources have been shown to contain three non-identical subunits of approximately similar sizes, no information is available on subunit homology. Citrate lyase functions as a key enzyme in the anaerobic metabolism of citrate. The first anaerobic bacteria to evolve under the primitive atmosphere presumably developed primordeal citrate fermentation ability. From such a beginning it is likely that other lyase activities towards citrate, such as citrate synthase and ATP-citrate-lyase developed evolutionarily (Srere, 1968).

As a first step towards the study of evolutionary relatedness between citrate lyases from different bacterial sources, K.aerogenes and S.faecalis homologous relationships were studied in their tryptic peptide maps of the separated subunits of the pure enzymes from the two sources.

II.2 MATERIALS AND METHODS

MATERIALS:

All chemicals used were of the highest purity available. Sodium dodecyl sulfate (SDS) was a preparation of Hico Products,Pvt. Ltd., Bombay. Analytical grade hydroxylamine hydrochloride ($NH_2OH.HCl$) was obtained from

BDH Ltd., and a neutralised hydroxylamine solution, pH 7.0 was prepared by mixing equal volumes of 28% $NH_2OH.HCl$ and 14% NaOH (Stadtman, 1957). Fresh solutions were prepared just before use. 5,5'-Dithiobis(2-nitrobenzoate) (DTNB) was from Calbiochem, USA; Trizma base and acetyl-CoA were obtained from Sigma Chemical Co., USA. Dithiothreitol (DTT) was also from Sigma Chemical Co., USA. Urea was of analytical grade (BDH) chemical. It was recrystallised from aqueous methanol and the crystals stored dry over P_2O_5 in vacuum. Solutions of about 10 M concentrations were made fresh just before use and passed through a column of Amberlite monobed resin MB-1 prior to use to remove any cyanate ions (Freedman, Slobin, Robbins and Sela, 1966). Trypsin (type XII, 2 x crystallised) was obtained from Sigma Chemical Co., USA. 2-Mercaptoethanol was from Fluka AG. Buchs. SG. Citrate synthase was from Sigma Chemical Co. Ltd. (specific activity 155 $\mu mol.min^{-1}.mg^{-1}$). Agar used for double diffusion and for immunoelectrophoresis was Noble agar of Difco Chemical Co., USA. BACTO ADJUVANT COMPLETE FREUND (containing paraffin and *Mycobacterium butyricum*) was from Difco Laboratories. These chemicals are besides those described in Part I of this thesis.

All solvents used in paper electrophoresis and chromatography were freshly distilled analytical grade reagents.

Buffers and all other reagents used in the purification steps were similar to those in Part I.

METHODS

II.2 (i) Growth and extraction of citrate lyase from K.aerogenes cells:

The enzyme from K.aerogenes was prepared as described in Part I of this thesis. The enzyme was assayed as described earlier (Part I of this thesis). Enzyme used had a specific activity of 70 - 90 U/mg and was ultracentrifugally and electrophoretically pure.

II.2 (ii) Growth and extraction of citrate lyase from S.faecalis cells:

Organism:

Streptococcus faecalis 10CI was a gift from Professor T.C. Gunsalus, University of Illinois, USA. The organism was maintained routinely on nutrient agar slants. Before subculturing it was inoculated into the following enrichment medium: 100 ml of the medium contained glucose, 0.5 g; lactose, 0.5 g; liver extract, 0.6 g; yeast extract, 0.5 g; sodium acetate, 0.6 g; and the usual minerals, pH 7.6. Organism was grown for 24 h at 37°C and subcultured every 2 - 3 weeks.

Growth of S.faecalis cells:

S.faecalis 10CI was grown without aeration in 10 L flask filled till the neck with the medium. The growth medium had the following composition: Na_3-citrate.$2H_2O$, 100 g; KH_2PO_4, 20 g; $(NH_4)_2SO_4$, 10 g; $MgSO_4.7H_2O$, 4 g; yeast extract, 50 g; peptone powder, 50 g and water to make upto 10 L after the pH had been adjusted to 7.2 with 2 N NaOH. $MgSO_4.7H_2O$ and $(NH_4)_2SO_4$ were autoclaved

separately in 1.5 L of water and then added to the rest of the medium to avoid precipitation. An inoculum of 10% final volume comprising of cells adapted to growth on citrate is finally added to the medium and the flasks incubated at 37°C for 24 - 30 h. The cells were harvested in a Sharples Model A-12 supercentrifuge and washed once on the centrifuge with 2 L of 0.03 M potassium phosphate buffer, pH 7. Each 10 L batch of medium yielded about 10 - 20 g of cells. The cells were not very stable to storage and were stored frozen for not more than a week.

The enzyme was purified by the method described by Hiremath et al. (1976).

Extraction of cells:

The enzyme was purified from batches of 80 - 100 g of cells. Temperature of sonication was maintained at 0-4°C. The cells were suspended in 0.03 M potassium phosphate buffer, pH 7, to a cell concentration of 1 g/3.5 ml buffer. The sonication was carried out at 20 Kc (300 W) for 3 min in the Biosonik III (Bronwill Scientific Co., USA). sonicator. The sonicate extract was centrifuged at 50 000 x g for 45 min at 4°C and the cell debris was discarded. The supernatant was adjusted to a protein concentration of 0.5% with the same buffer as that used for sonication.

II.2 (iii) Purification of S.faecalis citrate lyase:

As the crude enzyme is unstable on storage, all the purification steps were carried out continuously. All operations were carried out at 0 - 4°C and all buffers used included 1.6 mM $MgSO_4$. Batches of 300 ml were run at a time.

ATP and acetate treatment:

The enzyme solution containing 0.5% protein was kept stirred and treated with acetate and ATP to give final concentrations of 1 mM and 0.5 mM respectively. This step was done to ensure that all the citrate lyase is in the active form. The solution was kept stirred for about half an hour.

Streptomycin sulfate treatment:

The crude extract was then treated with streptomycin sulfate (1.4%) which was added gradually and with vigorous stirring. The solution was kept stirred for a further period of one hour after which it was spun at 50 000 x g for 45 min. This step was to remove the nucleic acids which are precipitated out with the antibiotic.

Alumina C_γ gel:

First gel treatment: Alumina C_γ gel (4.7% solids) was added to the supernate from the streptomycin sulfate step till about 5-8% of the activity was absorbed (5-8 ml gel). The gel was added with stirring after which it was spun off at 2 000 x g for 10 min and the supernatant subjected to a second gel absorption step.

Second gel treatment: 92 ml of alumina C_γ gel (4.7% solids) was now added to the first gel supernate fraction till 95% of the enzyme activity is absorbed. The solution was kept stirred for another half an hour and then spun at 2 000 x g for 20 min. The gel was processed further and the supernatant was discarded.

Washing of the gel: The gel was washed with 0.01 M potassium phosphate buffer, pH 7 in a homogenizer. The suspension was spun at 2 000 x g for 20 min and the supernatant discarded.

Elution of activity from the gel: The washed gel was now homogenized for 15 min with 0.05 M potassium phosphate buffer, pH 7. The suspension was spun at 2 000 x g for 20 min, the gel discarded and the eluate processed further.

Ammonium sulfate fractionation: The eluate from the previous step was kept stirred and powdered ammonium sulfate added to a 0.5 saturation (29 g/100 ml). The fraction was spun at 5 000 x g for 10 min and the precipitate discarded. The supernatant was then treated further with powdered ammonium sulfate to a 0.8 saturation (23 g/100 ml original vol.) and was kept stirred for a further 15 min, after which it was spun at 50 000 x g for 20 min. The supernatant was discarded and the precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.5 (2 - 3 ml) and dialysed against the same buffer for 16 h.

DEAE-cellulose chromatography: The dialysed enzyme solution was spun at 10 000 x g for 5 - 10 min to remove the faint turbidity. The clear solution was applied to a DEAE-cellulose column (30 x 1.5 cm) which has been previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The enzyme was eluted out of the column by means of a gradual increase in the ionic strength of the buffers over a range 0.05 - 0.5 M. This is achieved by placing the lower strength buffer namely 0.05 M in the chamber

attached to the column and dropwise addition of 1 M potassium phosphate buffer at the rate of flow of buffer out of the column. Fractions (3 ml) are collected on an automatic fraction collector and tested for enzyme activity by withdrawing a drop from each tube. The enzyme elutes at a 0.34 M concentration of the buffer. The fractions having maximum activity were pooled together (30 ml) and treated with powdered ammonium sulfate to 0.8 saturation (15.6 g) with stirring. The solution was spun at 50 000 x g for 20 min, the supernatant was discarded and the precipitate dissolved in 0.1 M potassium phosphate buffer, pH 7.5 (1 ml).

Sepharose CL-6B gel filtration: The enzyme solution from the above step was carefully layered on a Sepharose CL-6B column (110 x 1.5 cm) which had already been equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The column was run with the same buffer and fractions (1 ml) were collected on an automated fraction collector. The enzyme usually eluted between 30 - 40 ml. The fractions were tested for activity by withdrawing a drop from each tube and testing for the enzyme. The fractions having the maximum enzyme activity were pooled together (8-10 ml) and treated with stirring with powdered ammonium sulfate to 0.8 saturation. The precipitate was collected by spinning at 50 000 x g for 20 min and dissolved in 0.05 M potassium phosphate buffer, pH 7.5.

Enzyme and protein assays were carried out as described in Part I of this thesis.

II.2 (iv) Growth and partial purification of citrate lyase from S.diacetilactis:

Organism:

Streptococcus diacetilactis DRCI was propagated routinely in sterile skim milk fortified with 0.75% nonfat milk solids.

Growth of S.diacetilactis cells:

S.diacetilactis DRCI was grown without aeration in 10 L flasks filled till the neck with a citrate-lactose medium of Harvey and Collins (1963). The medium consisted of the following components: Bactopeptone, 100 g; Lactose, 200 g; tri-sodium citrate.$2H_2O$, 50 g; KH_2PO_4, 5 g; $MgSO_4.7H_2O$, 2 g; sodium acetate, 20 g; yeast extract, 150 g; pH adjusted to 7 - 7.2 with 2 N NaOH. The growth was initiated with 10% of inoculum and kept at 30°C for 40 h. The cells were harvested in a Sharples Model A-12 supercentrifuge. Each 10 L batch of medium yielded about 15 - 20 g of cells. The cells could be stored frozen for not more than a week.

Extraction of cells:

All operations were carried out at 0 - 4°C. Thawed cells were suspended in 0.03 M potassium phosphate buffer, pH 7 in the proportion of 1 g wet weight of cells to 4 ml of buffer and disintegrated in a Biosonik III sonicator (Brownwill Scientific Co., USA). Sonications were done once at 20 Kc (300 W) for three minutes. Cell debris was removed by spinning the suspension in the Model L Spinco ultracentrifuge at 50 000 x g for 45 min, the clear

supernatant was adjusted to a protein concentration of 0.5% with the buffer used in the sonication step.

II.2 (v) Purification of S.diacetilactis citrate lyase:

Streptomycin sulfate treatment:

1.4% streptomycin sulfate was added to the sonicate to precipitate out nucleic acids. The solution was kept stirred for another 30 min after which it was left aside for one h. The solution was then spun for 40 min at 50 000 x g and the precipate was discarded.

Ammonium sulfate fractionation: The steptomycin sulfate fraction was treated with ammonium sulfate 0.6 saturation (35 g/100 ml) after addition the solution is stirred for another 10 min and then spun at 50 000 x g for 30 min. The supernatant was discarded and the precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.5 (2-5 ml).

II.2 (vi) Preparation of rabbit antisera against K.aerogenes citrate lyase:

Immunisation schedule: Male adult rabbits were pre-bled (15 - 25 ml every time) at one week's interval from the ear vein and immunised immediately after the second bleeding by intramuscular injection of pure citrate lyase from K.aerogenes emulsified with an equal volume of Complete Freunds Adjuvant. The enzyme before use was preliminarily dialysed exhaustively against 0.8% saline for 18-24 h at 0-4 °C. 2 ml of the enzyme in 0.8% saline containing 3 mg protein was mixed with 2 ml of the adjuvant with the help of two syringes connected together through a short length

of polythene tubing, the enzyme solution being taken in the barrel of one syringe and the adjuvant in the other. The solutions were homogenized by vigorous passage between the syringes. Four injections of 1 ml each of the enzyme-adjuvant suspension were administered into each of the thigh and shoulder muscles; this was done immediately after the second bleeding. The animal was again bled through the ear vein twice at one week intervals after the first injection. After two weeks, the animal was again prebled (15 - 25 ml) and a booster of the same dosage as before was given as in the previous manner. The animals were then bled regularly every week from alternate ear veins (15 - 25 ml each time). Booster doses were administered thereafter at three and six months intervals.

Preparation of antiserum: Antisera was collected one week after the booster dose. Blood was collected in glass conical centrifuge tubes and stood at 20 - 25°C for one to two hours and then left at 0 - 4°C for 16 h. The clot that is formed was cut and the straw colored serum was collected by spinning the tube at 3 000 x g for 10 min. The serum was decanted off and stored at 0 - 4°C with 0.08% azide as preservative.

II.2. (vii) Ouchterlony's double diffusion technique:

The method used was that of Ouchterlony (1949). Microscopic slides were used and these were pretreated in the following manner before use. The slides were boiled in 0.1% sodium lauryl sulfate for 10 min and then thoroughly washed with glass distilled water and dried.

The slide was then coated with 0.3% hot agar prepared in 0.05 M potassium phosphate buffer, pH 7.5, and kept in a slanting position till completely dry. The slide was then left flat and about 3 - 5 ml of 1.5% hot agar solution was layered rapidly but with care to prevent formation of any air bubbles. The agar was allowed to set for 30 min and the slide was then left in a moist atmosphere inside a covered petri dish containing filter paper moistened with glass distilled water. The slide with the solidified agar is now placed on a prepared pattern of the positions of the wells to be cut. Using a wide capillary (tip width about 1.5 - 2 mm) which was attached to a water pump, the agar was slowly sucked out. The samples were now added with the antisera in the central well and the enzymes in circumferential wells. Amounts of sample were about 0.1 ml adequate to just fill the well without spilling out on the agar.

The plates were again left in a humid atmosphere at about 18°C for 16 h for the development of the precipitin lines.

II.2 (viii) Immunoelectrophoresis:

The method used was that of Scheidegger (1955). The microscopic slides used for immunoelectrophoresis were pretreated in the same way as for the Ouchterlony double diffusion technique. They were dipped in a hot solution of 0.3% agar and left in a slanting position till completely dry. A 3% agar solution was prepared and diluted with equal volumes of 0.1% barbital buffer to give a final concentration of 1.5% agar. The amount of agar used per slide was 3 ml.

With the help of a glass capillary (1.5 - 2 mm tip) and water pump two holes equidistant from the edge of the slide were punched; these were filled with the enzymes from K.aerogenes and S.faecalis (0.1 ml each). The slide was then placed between two buffer vessels containing 0.1% barbital buffer, pH 8.5, and Whatman 3 MM paper strips soaked in the same buffer were dipped in the troughs and aligned on 1/2 cm. of the slide edge on either side. The whole contraption was then connected to the power supply and a current of 4 - 6 volts/cm was applied for 180 min. At the termination of the period, the current was disconnected and the slide removed. Then a longtitudinal slit measuring 5 - 6 cm in length and about 1 mm in width was cut in the centre of the slide with the help of a cutter fitted with razor blades at 1 mm spacing, and the agar was carefully removed from the cut slit with the help of a narrow, steel spatula. The trough was now filled with the antiserum against pure citrate lyase from K.aerogenes and the slide was left in a moist atmosphere at 18°C for about 1 - 2 days.

II.2 (ix) Preparation of the HS-citrate lyase from K.aerogenes and S.faecalis:

Pure citrate lyases from both sources were separately deacetylated according to the procedure of Buckel et al. (1971a). The enzyme (about 13 mg) in 0.8 ml of 0.05 M potassium phosphate buffer, pH 7.5 was treated with 0.4 ml of neutralised hydroxylamine (2 M). The solution was incubated at 30°C for 30 min. The deacetylated enzyme was separated from excess hydroxylamine by passing through

Sephadex G-50 (40 cm x 1.5 cm) column equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. Fractions were tested for protein by measuring absorbance at 280 nm and peak tubes were pooled and tested and showed complete loss of enzymic activity towards citrate.

II.2 (x) Reactivation of HS-citrate lyases:

0.05 ml of the inactive enzyme solution containing about 0.7 mg protein was made up to 1 ml with 0.05 M potassium phosphate buffer, pH 7.5 and treated with 0.01 ml of DTT (4 mM) and incubated at 30°C for fifteen min. A 0.5 ml aliquot of this enzyme solution is then treated with 0.05 ml of acetic anhydride (final concentration 4.7 mM). After thorough mixing a 0.2 ml aliquot is tested immediately for activity. Reactivation was also checked with addition of thiolacetic acid in place of acetic anhydride (Thiolacetic acid diluted thus 0.01 ml to 2 ml and 0.005 ml used for activation of 0.05 ml of enzyme, at 7 mM final concentration)

II.2 (xi) Kinetic analysis of transferase and lyase reactions during acetyl-CoA mediated cleavage of citrate by HS-citrate lyase:

The general rate equation derived by Laidler (1958) for the steady state approach was used for calculating reaction rate constants as suggested by Buckel et al.(1973). The outline of the basic principles of this approach have been enumerated by Buckel et al.(1973) and are set out briefly below:

An equation describing the transient and steady state phases of a one-substrate reaction with an enzyme through the transient enzyme-substrate complex formation has been

derived by Laidler (1958) and its analogy to the cleavage of citrate by deacetylated citrate lyase in the presence of acetyl-CoA via the intermediary formation of citryl-CoA will be evident from the corresponding reactions described by equations II.1 and II.2 respectively.

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} E.S \xrightarrow{K_2} E + P \qquad \text{Eq.II.1}$$

$$\text{Acetyl-CoA} + C \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} \text{Citryl-CoA} + \text{Acetate} \xrightarrow{K_2} \text{Acetyl-CoA} + \underset{\text{oxaloacetate}}{} \qquad \text{Eq.II.2}$$

The outlines of the treatment of the rate equation which describes the transient and steady state phases are briefly enumerated and are taken from the earlier models (Laidler, 1958; Buckel et al., 1973).

The experimental conditions are set out below:

1. The concentration of citrate can be assumed to be unchanged during the short interval of time of the experiment (cit) $\gg$ (citryl-CoA), (OAA) and is much greater than the K_m of the inactive enzyme for citrate (Buckel et al., 1973). Citrate becomes saturating with respect to the rate of formation of the intermediate citryl-CoA and therefore the reaction becomes independent of citrate concentration and K_{+1}(citrate) can be replaced by the constant K_1.

2. The concentration of acetyl-CoA is much smaller than K_m of the inactive enzyme for acetyl-CoA ((ACoA) $\ll K_m \sim$ 5 mM

3. The reverse reaction does not take place: K_{-1} is a bimolecular rate constant, but the concentrations of acetate are low during the initial period of reaction and K_m for acetate is presumed to be high (Buckel et al., 1973). The reverse of the cleavage reaction does not take place because the oxaloacetate formed is reduced immediately by NADH in the presence of malate dehydrogenase. The rate equation II.2 then approximates to:

$$\frac{d(\text{citryl-CoA})}{dt} = K_1' \left((\text{Acetyl-CoA})_o - (\text{Citryl-CoA})\right) - K_2 (\text{Citryl-CoA}) \qquad \text{Eq. II.3}$$

Integration ((Citryl-CoA) = 0, t = 0) of Eq. II.3 gives rise to Eq. II.4.

$$(\text{Citryl-CoA}) = \frac{K_1'}{K_1' + K_2} (\text{Acetyl-CoA})_o \left(1 - e^{-(K_1' + K_2)t}\right) \qquad \text{Eq. II.4}$$

which by insertion of the rate of product formation, $d(\text{oxaloacetate})/dt = K_2(\text{citryl-CoA})$ and integration yields equation II.5.

$$(\text{oxaloacetate}) = \frac{K_1' \cdot K_2}{K_1' + K_2} (\text{acetyl-CoA})_o \cdot \left(t + \frac{1}{K_1' + K_2} \left(e^{-(K_1' + K_2)t} - 1\right)\right) \qquad \text{Eq. II.5}$$

Eq. II.5 describes both the transient and steady state phases (Laidler, 1958). In the steady state phase at an

adequate period of time the expenential term vanishes and the formation of oxaloacetate becomes linear with time.

$$(\text{oxaloacetate}) = \frac{K_1' \cdot K_2}{K_1' + K_2} (\text{acetyl-CoA})_o \left(t - \frac{1}{K_1' + K_2}\right) \quad \text{Eq.II.6}$$

Extrapolation of this linear part of the curve to the time axis ((oxaloacetate) = 0) yields an intercept (Eq.II.7), the initial lag period of the reaction.

$$\tau = \frac{1}{K_1' + K_2} \quad \text{Eq.II.7}$$

Experimental determination of τ values have been shown to be independent of acetyl-CoA concentrations and depend only on the magnitude of the rate constants K_1' and K_2 which in turn take different positive values at different enzyme concentrations. The slope v_s of oxaloacetate formation under steady state conditions is dependent however on both acetyl-CoA and enzyme concentrations.

$$v_s = \frac{d(\text{oxaloacetate})}{dt} = \frac{K_1' \cdot K_2}{K_1' + K_2} \cdot (\text{Acetyl-CoA})_o \quad \text{Eq.II.8}$$

Equation II.8 is valid only at $(\text{Acetyl-CoA})_o \ll K_m$

II.2 (xii) Experimental procedure:

The cleavage of citrate catalysed by inactivated citrate lyase and acetyl-CoA was followed by the coupled enzyme assay procedure using malate dehydrogenase and NADH. Determinations were carried out at 25°C. The spectrophotometer cuvette contained in a final volume of 1 ml: 100 µmol Tris-HCl buffer, pH 8.0; 1.6 µmol $MgSO_4$; 0.3 µmol NADH; 10 µg of malate dehydrogenase and 1 U HS-citrate

lyase (in terms of the active enzyme). Citrate (2μmol) was added initially followed by 0.75 μmol acetyl-CoA. Formation of oxaloacetate was measured by monitoring NADH oxidation at 340 nm (as described in Part I of the thesis).

Determination of steady state concentration of acetyl CoA: CoASH, citryl-CoA and acetyl-CoA were all determined in a single assay as described by Buckel et al. (1973). The spectrophotometer cuvette contained in a final volume of 1 ml: 100 μmol Tris-HCl buffer, pH 8 and 1 μmol DTNB (5,5'-Dithiobis(2-nitrobenzoate)) and an aliquot test sample (30 μl) withdrawn from the reaction mixture used for determination of cleavage of citrate catalysed by HS-citrate lyase and acetyl-CoA. The slight increase in absorbance at 405 nm was due to free CoASH. A further increase in $A_{405\ nm}$ observed after addition of 10 μg citrate synthase in 5 μl volume was due to citryl-CoA (Srere et al., 1963). An additional increase of $A_{405\ nm}$ on the final addition of oxaloacetate (5 μl containing 1μmol) was due to acetyl-CoA present in the sample (Srere et al., 1963). A value of $14.1 \times 10^{3} M^{-1} cm^{-1}$ at 405 nm for the molar extinction coeffecient of the 2 mercapto-4 nitrobenzoate was used.

II.2 (xiii) Preparation of subunits:

Pure citrate lyase (about 13 mg) in 1 ml of 0.05 M potassium phosphate buffer, pH 7.5, was treated with urea (recrystallised) to give a final concentration of 8 M. The enzyme was kept at 13°C for 16 h. The treated enzyme was then loaded on a Sepharose CL-6B column (70 cm x 1.5 cm) which was previously equilibrated with 0.05 M potassium

phosphate buffer, pH 7.5, containing 6 M urea and 10 mM 2-mercaptoethanol. The fractions were collected at 15 min interval (2 ml) and tested for protein at 280 nm; the fractions comprising the subunits (α-, β-, γ-) are pooled separately and immediately dialysed exhaustively against the following buffers: α-subunit was dialysed against 0.1 M Tris-HCl buffer, pH 7.5; β-subunit was dialysed against 0.05 M potassium phosphate buffer, pH 7 containing 1 mM $MgCl_2$ and the γ-subunit was dialysed against 0.01 M potassium phosphate buffer, pH 7. After the dialysis is over, the fractions are dialysed against glass-distilled water, lyophilised and stored frozen.

SDS-polyacrylamide gel electrophoresis: SDS-gel electrophoresis was carried out according to the procedure of Weber and Osborne (1969) using 0.07% ammonium persulfate for polymerization. Protein samples (approx. 50 μg) were treated with SDS to a final concentration of 1% and heated at 100°C for 3 min. The samples were then cooled and loaded on 7% polyacrylamide gel prepared in 0.1 M sodium phosphate buffer, pH 7.3 containing 0.1% SDS. After the electrophoresis, the gels were stained with 0.25% Coomassie Blue for 6 h and destained with methanol-acetic acid-water mixture.

II.2 (xiv) Fingerprinting:

Principle: Homologous relationships between proteins which are derived by divergent evolution from an ancestral protein are conveniently detected in tryptic peptide maps. Trypsin specifically cleaves peptide bonds where the carboxyl

groups are contributed either by arginine or lysine residues. With aminoethylation, the sulphydryl of cysteine residues react with the reagent to form S-β-aminoethyl cysteine residues which on account of its structural resemblance to lysine renders peptide bonds contributed by the carboxyl group of the cysteine derivative also susceptible to hydrolytic cleavage by trypsin (Raftery and Cole, 1963). Peptides obtained by tryptic digestion are conveniently resolved by the two dimensional peptide mapping technique using high voltage paper electrophoresis in one direction and partition paper chromatography in the other (Ingram, 1958).

Experimental procedures:

(1) Aminoethylation: Separated subunits (1-5 mg each) were indivitually reduced before aminoethylation. The subunit was treated with Tris-HCl buffer, pH 8.6, (300 µl of 1.5 M buffer), urea (360 mg), Na_2.EDTA (30 µl of 5% solution) and the mixture diluted with glass-distilled water (to approx. 750 µl). The solution was treated with 10 µl of 2-mercaptoethanol and left at room temperature for 30 min in an atmosphere of nitrogen. Ethyleneimine (20 µl) was then added to the solution and the mixture stood for another 2 h.

The aminoethylated protein was freed from urea and other reagents by filtration through a column of Sephadex G-25 (55 cm x 1 cm) equilibrated previously with 0.2 N acetic acid. The column was eluted with acetic acid solution and the eluate monitored for protein by determination of

absorbance at 280 nm. Fractions of the eluate comprising the protein peak were collected, pooled and lyophilised.

(2) Tryptic digestion: Salt free trypsin (2 x crystalline) was used without further purification. The trypsin was dissolved in 1 mM HCl (1 mg/200µl) immediately before use. The lyophilised subunit was dissolved in the minimum amount of distilled water with adjustment of pH to 8.1 using 50 mM NaOH. The solution was denatured by heating at 80°C for 7 min, cooled to 37°C in a thermostat and treated with trypsin solution at a substrate-enzyme ratio of 50; the pH was adjusted and maintained at 8.1 with the alkali. The digestion was continued for 3 h by which time alkali intake ceased. The enzyme reaction was stopped by lowering the pH to 6.5 with 50 mM HCl. The tryptic activity was destroyed by heating in a boiling water bath for one min. The clear solution was preserved at -20°C till needed for fingerprint analysis.

(3) Fingerprinting: Fingerprinting was done by two dimensional mapping of the tryptic fragments.

The electrophoresis was carried out essentially as described by Ingram (1958) on Whatman No. 3 MM paper which had been dipped into Michl's volatile buffer (Michl, 1951) pyridine - glacial acetic acid-water, pH 6.4, in the proportion of 10:0.4:90 by volume. Fig. II.1 shows the manner in which the paper was cut to T shapes of identical size and from a single sheet with similar grain directions. The dimension of the paper are described in Fig. II.1 and were 31 cm x 31 cm with a pair

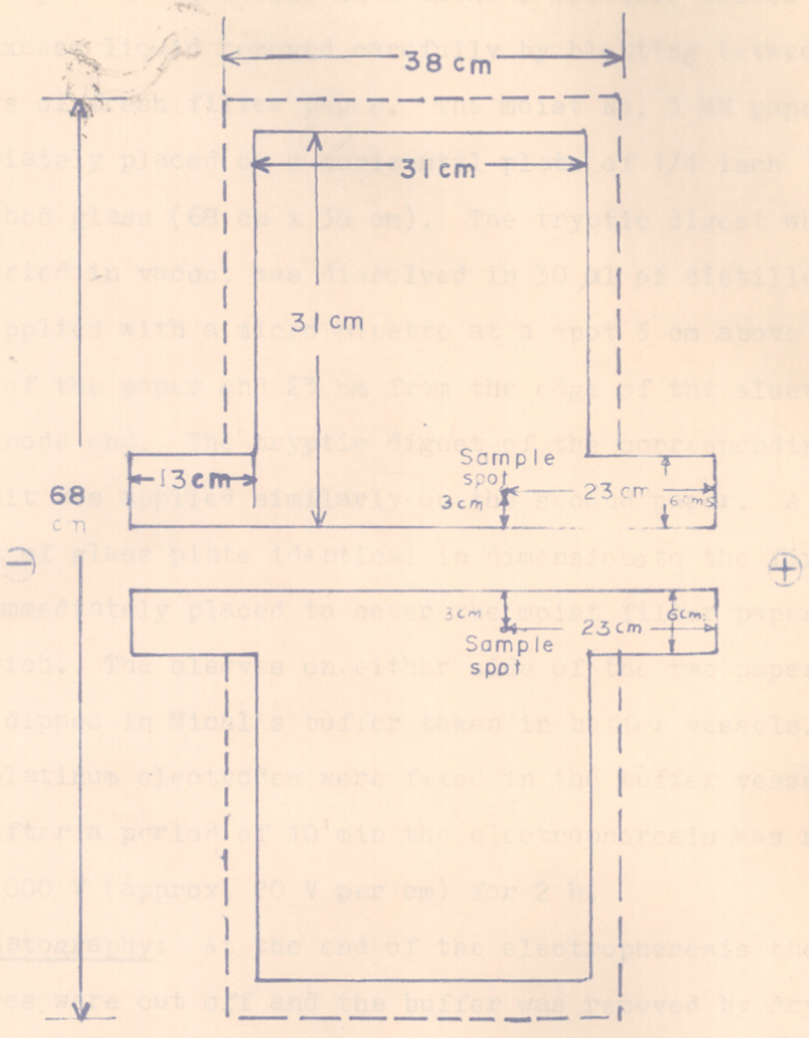


FIG. II-1 FILTER PAPER DIMENSIONS IN FINGER PRINTING

of sleeves of 13 cm length and 6 cm width. The cut paper was dipped one at a time into Michl's volatile buffer and the excess liquid removed carefully by blotting between two sheets of fresh filter paper. The moist No. 3 MM paper was immediately placed on a horizontal plate of 1/4 inch polished glass (68 cm x 38 cm). The tryptic digest which was dried in vacuo, was dissolved in 30 µl of distilled water and applied with a micro pipette at a spot 3 cm above the edge of the paper and 23 cm from the edge of the sleeve at the anode end. The tryptic digest of the corresponding subunit was applied similarly on the second paper. A second sheet of glass plate identical in dimensions to the first was immediately placed to cover the moist filter paper as a sandwich. The sleeves on either side of the two papers were dipped in Michl's buffer taken in buffer vessels. Two platinum electrodes were fixed in the buffer vessels and after a period of 10 min the electrophoresis was run at 1 000 V (approx. 20 V per cm) for 2 h.

Chromatography: At the end of the electrophoresis the sleeves were cut off and the buffer was removed by drying (room temp.) in a current of air (minimum 2 h). The dried papers were hung for two h in the air space of a chromatography chamber containing the chromatographic solvent, pyridine-n-butanol-glacial acetic acid-water in the proportion of 20:30:6:24 by volume respectively (Barnabus and Muller, 1962). After saturation in the vapour, ascending chromatography was carried out for 12 h. The papers were again dried in a current of air at room temperature.

(4) Staining of peptide maps: The peptide spots were revealed by dipping the paper into ninhydrin in acetone. The reagent consisted of 2.5 g ninhydrin, 475 ml acetone, 25 ml phosphate buffer (0.05 M pH 7.0 prepared by dissolving 1.98 g KH_2PO_4 and 5.45 g $Na_2HPO_4.2H_2O$ in 1 L of distilled water). Paper soaked in the reagent was dried in air for 15 min followed by suspension in water vapour-saturated atmosphere at 80°C for 10 min for intensifying the colored spots. Photographic records were made immediately. Peptide maps were preserved after fixing with a copper reagent of the following compositions: Cupric nitrate, 1 g; acetone, 100 ml; nitric acid, 20%, 0.05 ml.

II.3 RESULTS

II.3 (i) Purification of S.faecalis citrate lyase:

The results obtained in a representative batch are summarized in Table II.1.

Treatment of the cell-free extract with ATP and acetate results in a 2-fold increase in the total activity indicating that half of the total citrate lyase originally present in the extract is in the inactive deacetylated HS-citrate lyase form. A similar finding has been reported earlier by Hiremath et al. (1976). The reactivation is evidently through the action of the acetate: HS-citrate lyase ligase present in the extract of the organism. The earlier report of Hiremath et al. (1976) that the lyase activity is separated from the ligase activity at the

TABLE II.1

Purification of citrate lyase from S. faecalis.

Wet weight of cells 80 g.

Fraction	Volume (ml)	Protein (mg/ml)	Total activity U	Specific activity U/mg	Yield %
Sonicate extract	300	5.5	4620	2.8	-
Sonicate + acetate + ATP	300	5.5	9240	5.6	100
Streptomycin sulfate fraction	290	4.0	7420	6.4	80
0.05 M buffer eluate of second alumina C gel	150	2.1	4030	12.8	44
Ammonium sulfate fraction	5	14.0	2740	39.0	30
DEAE-cellulose fraction pooled and fractionated with ammonium sulfate and the precipitate dissolved in 0.05 M buffer, pH 7.5	1.5	10.5	900	57.0	10
Sepharose CL-6B fraction after concentrating over PM-10 membrane by ultrafiltration	1.5	6.0	810	90.0	9

second alumina C_γ step, the lyase being adsorbed on the gel at this stage and the ligase remaining in the supernatant, was also confirmed in the present investigation.

The final step yields enzyme of specific activity 90 U/mg corresponding to the value reported earlier. The yields are also approximately similar to those reported by Hiremath et al. (1976).

The final enzyme preparation was homogeneous ultracentrifugally and electrophoretically. The sedimentation profile of the purified citrate lyase from S.faecalis is presented in Fig. II.2. The enzyme sediments as a single symmetrical peak. $S^{0.4\%}_{20,w}$ value was 16.3 S. The $s_{20,w}$ value reported for the enzyme is 17.1S (Hiremath, 1977). The polyacrylamide gel electrophoretic pattern of the native enzyme is shown in Fig. II. 3. The single band obtained on staining establishes the electrophoretic homogeneity of the preparation.

II.3 (ii) Partial purification of S.diacetilactis citrate lyase:

The enzyme from this source was purified only partially as this was required only to check its immunological behaviour against rabbit antiserum obtained for citrate lyase from K.aerogenes.

The partial purification data with a typical batch of S.diacetilactis cells are summarized in Table II.2.

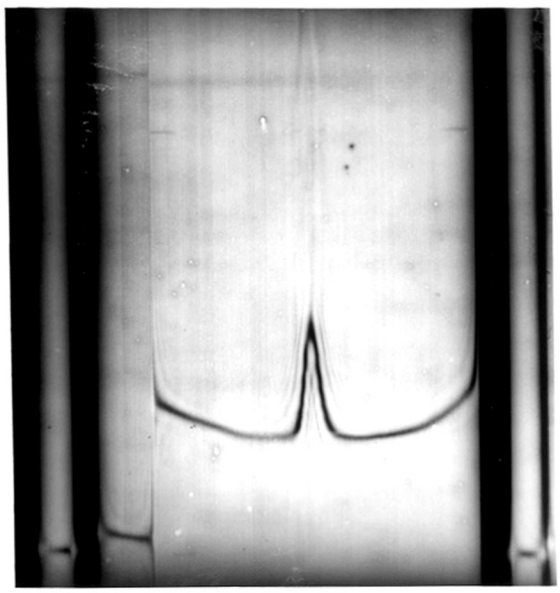


FIG. II. 2 SEDIMENTATION PROFILE OF NATIVE CITRATE LYASE FROM S.faecalis

Protein concentration	- 2 - 3 mg/ml
Buffer	- 0.05 M potassium phosphate buffer, pH 7.5 with 1.6 mM $MgSO_4$.
Speed	- 59 780 rpm
Temperature	- 5.2°C
Phase plate	- 60°
Time	- 40 min.

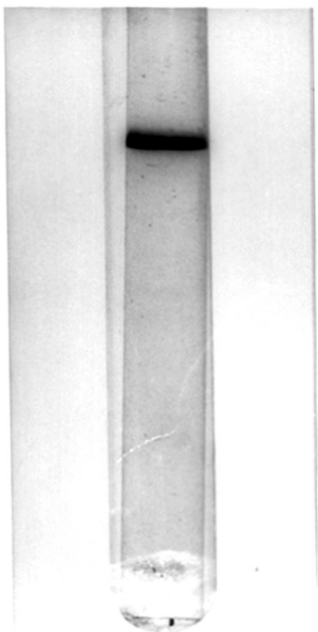


FIG. II.3 POLYACRYLAMIDE GEL ELECTROPHORESIS OF NATIVE CITRATE LYASE FROM S. faecalis
5% gel; Tris-glycine buffer, pH 8.3;
4 mA/tube, 5 h
Protein loaded 60 ug.

TABLE II.2

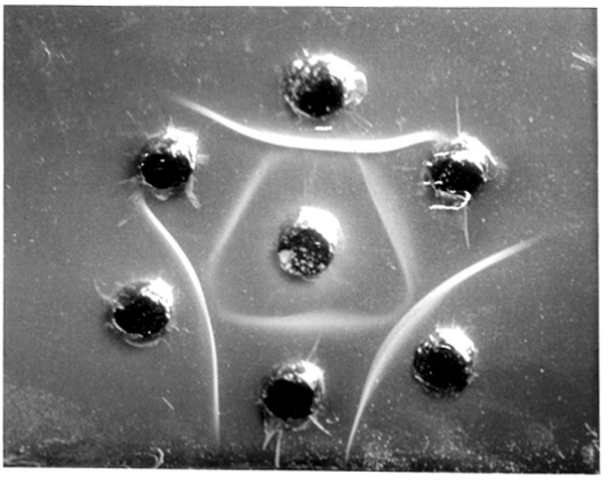
Partial purification of S.diacetilactis citrate lyase.

Wet cells, 16 g.

Fraction	Volume (ml)	Protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	60	5	300	1.0	-
Streptomycin sulfate treatment followed by centrifugation to remove precipitated nucleic acids	58	5	319	1.1	100
Ammonium sulfate (0.6S) fraction	2	25	250	5.0	78

II.3.3 Immunological behaviour:

The results of gel double-diffusion tests on an Ouchterlony's plate are shown in Fig. II.4. It will be seen from Fig. II.4 that the tests show the spur of a cross-reaction; the enzyme from K.aerogenes showing more antigenic sites than the heterologous enzyme from S.faecalis. Earlier studies by Singh et al. (1975) had shown that specific rabbit antiserum towards K.aerogenes or S.diacetilactis does not cross-react with the E.coli enzyme. Similarly the specific rabbit antiserum to the K.aerogenes has been shown to have no cross-reactions with citrate lyase from S.diacetilactis on Ouchterlony's plates. The present report is the first evidence for the immunological



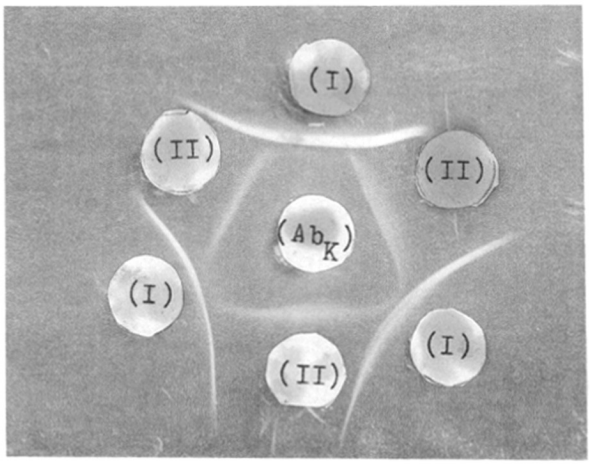

(I)
(II)
(II)
(Ab$_K$)
(I)
(II)
(I)

FIG. II.4 Ouchterlony double - immunodiffusion of anti *K. aerogenes* citrate lyase (Ab_K) versus the following antigen *K. aerogenes* citrate lyase (I) and *S. faecalis* citrate lyase (II).

relatedness of citrate lyases from two different sources, namely K.aerogenes and S.faecalis.

S.diacetilactis enzyme is shown by us not to give precipitin lines with the antisera from K.aerogenes. This confirms the earlier observation of Singh et al. (1975).

The immunoelectrophoretic data with the pure citrate lyase from K.aerogenes and S.faecalis and the specific rabbit serum against the former enzyme are diagrammatically shown in Fig. II.5. The immunoelectrophoretic pattern indicates single arcs of precipitin between the antiserum and antigen with both enzymes from K.aerogenes and S.faecalis. The results establish the homogeneity of enzyme from K.aerogenes as checked by immunological methods in addition to that obtained from ultracentrifugal and electrophoretic analyses.

II.3.4 Preparation of HS-citrate lyases from K.aerogenes and S.faecalis:

The deacetylated enzyme preparations were obtained by hydroxylaminolysis as described in MATERIALS AND METHODS.

The separation of the deacetylated K.aerogenes enzyme from hydroxylamine and acetohydroxamate by filtration through Sephadex G-50 is shown in Fig. II.6. The protein eluted between 11 - 14 ml while the hydroxylamine eluted between 22 - 33 ml. The fractions containing protein were pooled and concentrated by ultrafiltration. The elution profile with hydroxylamine treated citrate lyase from S.faecalis was similar to that of the enzyme from K.aerogenes.

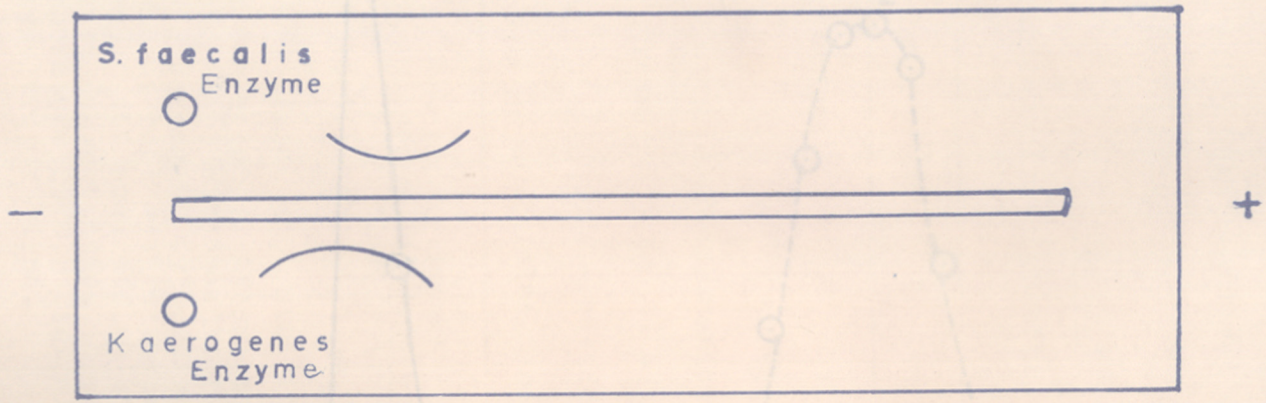


FIG. II·5 IMMUNOELECTROPHORESIS
DIAGRAMMATIC REPRESENTATION OF PRECIPITIN ARCS, CONDITIONS AS DESCRIBED IN TEXT. ENZYME SAMPLES IN SIDE WELLS AS SHOWN AND RABBIT ANTI-k. aerogenes CITRATE LYASE ANTISERIUM IN CENTRAL TRENCH.

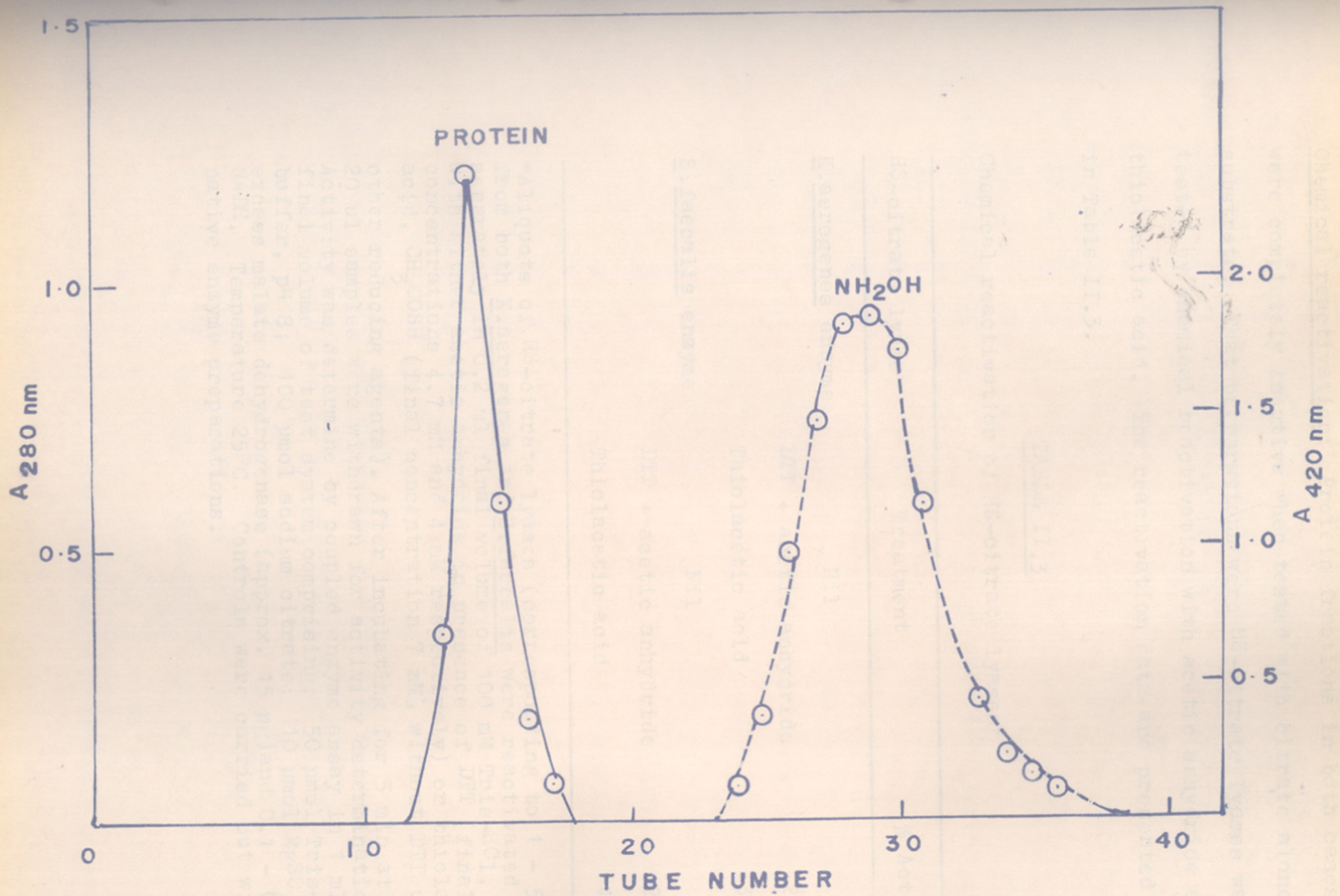


FIG II·6 SEPARATION OF HS-CITRATELYASE FROM HYDROXYL AMINE

O—O A_{280nm} O-·-O A_{420nm} WITH 2, 4, 6 – TRINITRO BENZENE SULFONATE

Chemical reactivation: Protein fractions in both cases were completely inactive when tested with citrate alone as substrate. That preparations were HS-citrate lyases was tested by chemical reactivation with acetic anhydride or thiolacetic acid. The reactivation data are presented in Table II.3.

TABLE II.3

Chemical reactivation of HS-citrate lyases*

HS-citrate lyase	Treatment	% Activity
K.aerogenes enzyme	Nil	0
	DTT + acetic anhydride	24
	Thiolacetic acid	38
S.faecalis enzyme	Nil	0
	DTT + acetic anhydride	21
	Thiolacetic acid	40

*Aliquots of HS-citrate lyases (corresponding to 1 - 5 U) from both K.aerogenes and S.faecalis were reactivated separately in 0.2 ml final volume of 100 mM Tris-HCl, pH 8, with either acetic anhydride in presence of DTT (final concentrations 4.7 mM and 4 mM respectively) or thiolacetic acid, CH_3COSH (final concentration 7 mM), without DTT or other reducing agents). After incubating for 5 min at 30°C, 20 ul samples were withdrawn for activity determinations. Activity was determine by coupled enzyme assay in 1 ml final volume of test system comprising; 50 µmol Tris-HCl buffer, pH 8; 100 µmol sodium citrate; 10 µmol $MgSO_4$; excess malate dehydrogenase (approx. 15 µg)and 0.1 - 0.2 µmol NADH. Temperature 25°C. Controls were carried out with native enzyme preparations.

II.3.5 Determination of steady-state concentration of citryl-CoA during the cleavage of citrate catalysed by HS-citrate lyases from K.aerogenes and S.faecalis in presence of acetyl-CoA:

The cleavage of citrate catalysed by the deacetylated K.aerogenes citrate lyase in presence of acetyl-CoA is shown in Fig. II.7. The corresponding reaction catalysed by HS-citrate lyase from S.faecalis is shown in Fig. II.8. It will be seen from Fig. II.7 and II.8 that the rates of citrate cleavage in the presence of acetyl-CoA increase initially and thereafter reach a constant value. In the graphical representations the constant phase in rate is characterised by $\tan\alpha = v_s$. The extrapolation of the linear regions of oxaloacetate formation to the time axis yields the time interval τ, the lag period which characterises the transient phase (Buckel et al., 1973; Laidler, 1958). Determination of initial and final concentrations of acetyl-CoA in the reacting systems were made in separate experiments under identical conditions during the initial and steady state phases. These values are shown in Figs. II.7 and II.8. It will be seen that the acetyl-CoA concentration remains virtually constant during the citrate cleavage both in the transient and in the steady-state phases.

The kinetic analysis of the reactions were done as described in MATERIALS AND METHODS. The values of τ and v_s were used for calculation of K_1' and K_2 by the use of the equations II.7 and II.8 and the applied concentration

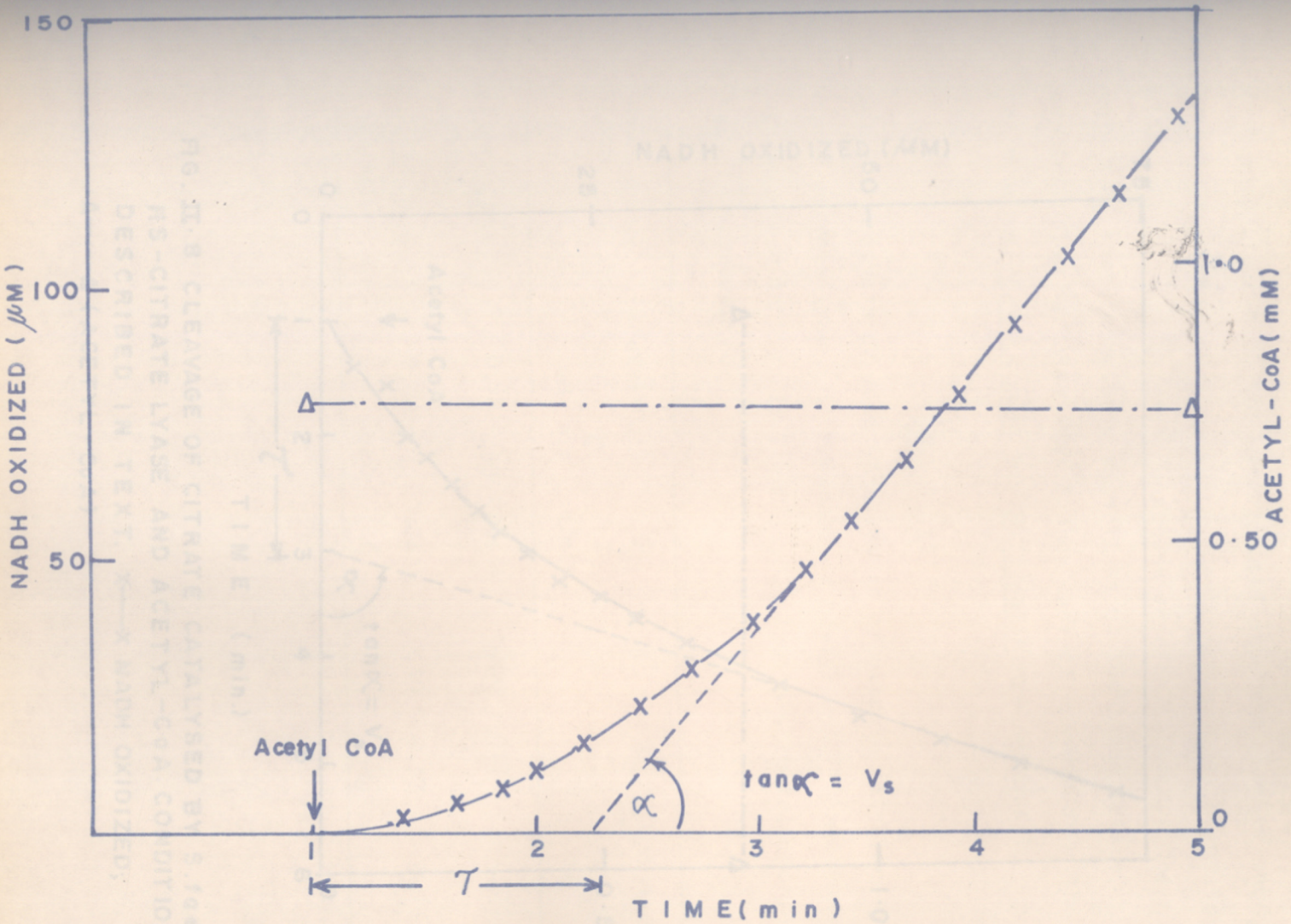


FIG II·7 CLEAVAGE OF CITRATE CATALYSED BY K. aerogenes HS-CITRATE LYASE AND ACETYL-CoA. CONDITIONS DESCRIBED IN TEXT.
X——X NADH OXIDIZED; Δ-·-·Δ ACETYL-CoA

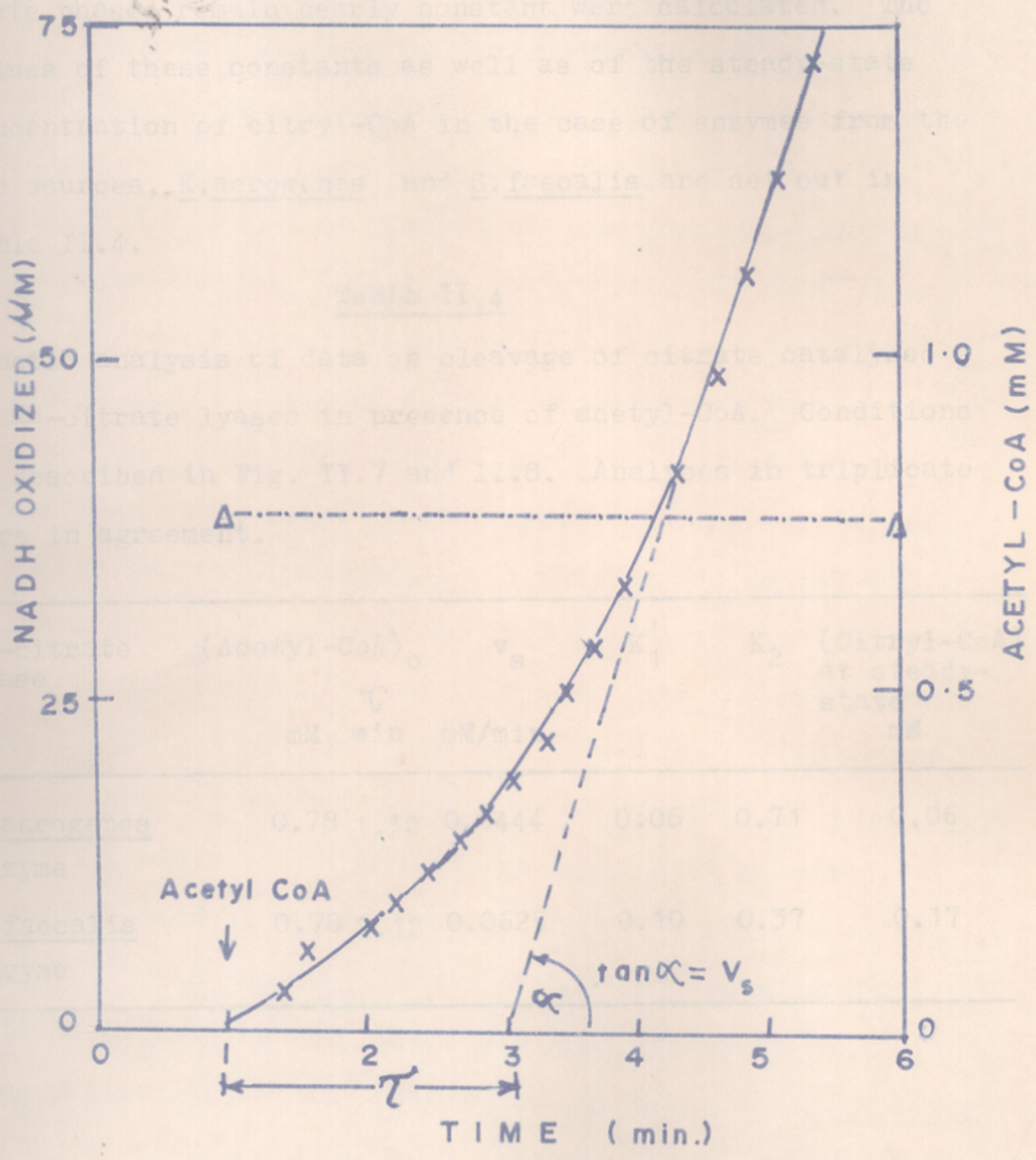


FIG. II·8 CLEAVAGE OF CITRATE CATALYSED BY S.faecalis HS-CITRATE LYASE AND ACETYL-CoA. CONDITIONS DESCRIBED IN TEXT. X——X NADH OXIDIZED; Δ—·—Δ (ACETYL-CoA)

of acetyl-CoA. The values of the terms K_1' and K_2 which were compatible with the experimental observation that the acetyl-CoA concentration during the transient and the steady-state phases remain nearly constant were calculated. The values of these constants as well as of the steady-state concentration of citryl-CoA in the case of enzymes from the two sources, K.aerogenes and S.faecalis are set out in Table II.4.

TABLE II.4

Kinetic analysis of data on cleavage of citrate catalysed by HS-citrate lyases in presence of acetyl-CoA. Conditions as described in Fig. II.7 and II.8. Analyses in triplicate were in agreement.

HS-citrate lyase	$(Acetyl\text{-}CoA)_o$	τ	v_s	K_1'	K_2	(Citryl-CoA) at steady-state
	mM	min	mM/min			mM
K.aerogenes enzyme	0.78	1.30	0.0444	0.06	0.71	0.06
S.faecalis enzyme	0.78	2.12	0.0625	0.10	0.37	0.17

It will be seen from Table II.4 that the formation of citryl-CoA is the rate limiting step of the overall reaction. The values obtained in the present investigation with K.aerogenes HS-citrate lyase are in close agreement with the values reported earlier by Buckel et al. (1973). The present studies extend these findings further to the HS-citrate lyase from S.faecalis, a source in which the citrate lyase differs from the K.aerogenes enzyme in undergoing only a weak reaction-inactivation. The values obtained for the S.faecalis enzyme indicate that under similar conditions the steady-state concentration of the citryl intermediate is actually higher than that observed with the K.aerogenes enzyme. If the kinetic behaviour observed with acetyl-CoA-dependent cleavage of citrate is assumed to be comparable to that of the acetyl-ACP (γ-subunit) dependent breakdown of citrate catalysed by the native enzymes, it would appear that the steady-state concentration of the labile citryl-ACP is higher in the case of citrate lyase from S.faecalis as compared to K.aerogenes enzyme. This would indicate that the weak reaction-inactivation of the S.faecalis enzyme is not due to any lower concentration of the labile citryl-intermediate. It is surprising that despite higher steady-state concentrations of the citryl intermediate, the S.faecalis enzyme undergoes a markedly lower reaction-inactivation. The reported rate constants for reaction-inactivation in the case of the enzymes from K.aerogenes and S.faecalis with Mg^{2+} at 25°C are 1.21 min^{-1} (Singh and Srere, 1975) and

0.07 min^{-1} (Hiremath, 1977) respectively.

II.3.6 Separation of subunits:

The presence of 3 non-identical subunits in the citrate lyase complexes have been well established in earlier studies. The separation of these in SDS-polyacrylamide gel electrophoresis in the case of the enzyme from K.aerogenes is shown in Fig. II.9 and of the enzyme from S.faecalis in Fig. II.10. The separation of the component subunits by gel filtration through Sepharose CL-6B under denaturing conditions has also been standardized earlier and the separated components shown to be homogeneous in SDS-gel electrophoresis both with the S.faecalis enzyme (Hiremath, 1977) and with the K.aerogenes enzyme (Tikare, 1979). These procedures were used in the present study.

The separation of subunits of citrate lyase from K.aerogenes by filtration through a Sepharose CL-6B column in presence of 6 M urea is shown in a representative instance in Fig. II.11 and the corresponding separation of subunits of citrate lyase from S.faecalis in Fig. II.12. The former Fig. II.11 indicates overlap of α- and β-subunits when higher loads are used. In such cases the fractions on the leading edge of the α-subunit peak and the fractions on the trailing end of the β-subunit component alone were pooled separately. When loads were lower as shown in Fig. II.12, the α- and β-components were completely separated. The γ-subunit in both cases emerged distinct from the other subunits. Separations were done in replicate samples and corresponding subunits of enzymes from each source were separately pooled for

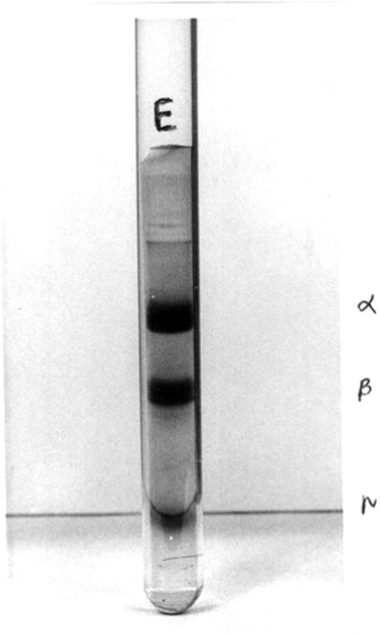

E
α
β
IV

FIG. II.9 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CITRATE LYASE FROM K.aerogenes

7% gels; sodium phosphate buffer, pH 7.3, containing 0.1% SDS; 8 mA/tube; 28°C; 4 h. Protein loaded 100 ug.

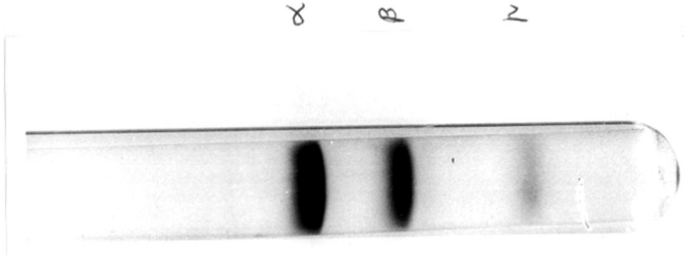

α
β
γ

FIG. II. 10 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

7% gel; sodium phosphate buffer, pH 7.3;
containing 0.1% SDS, 8 mA/tube, 4.5 h
Protein loaded 100 ug, *S. faecalis* citrate lyase

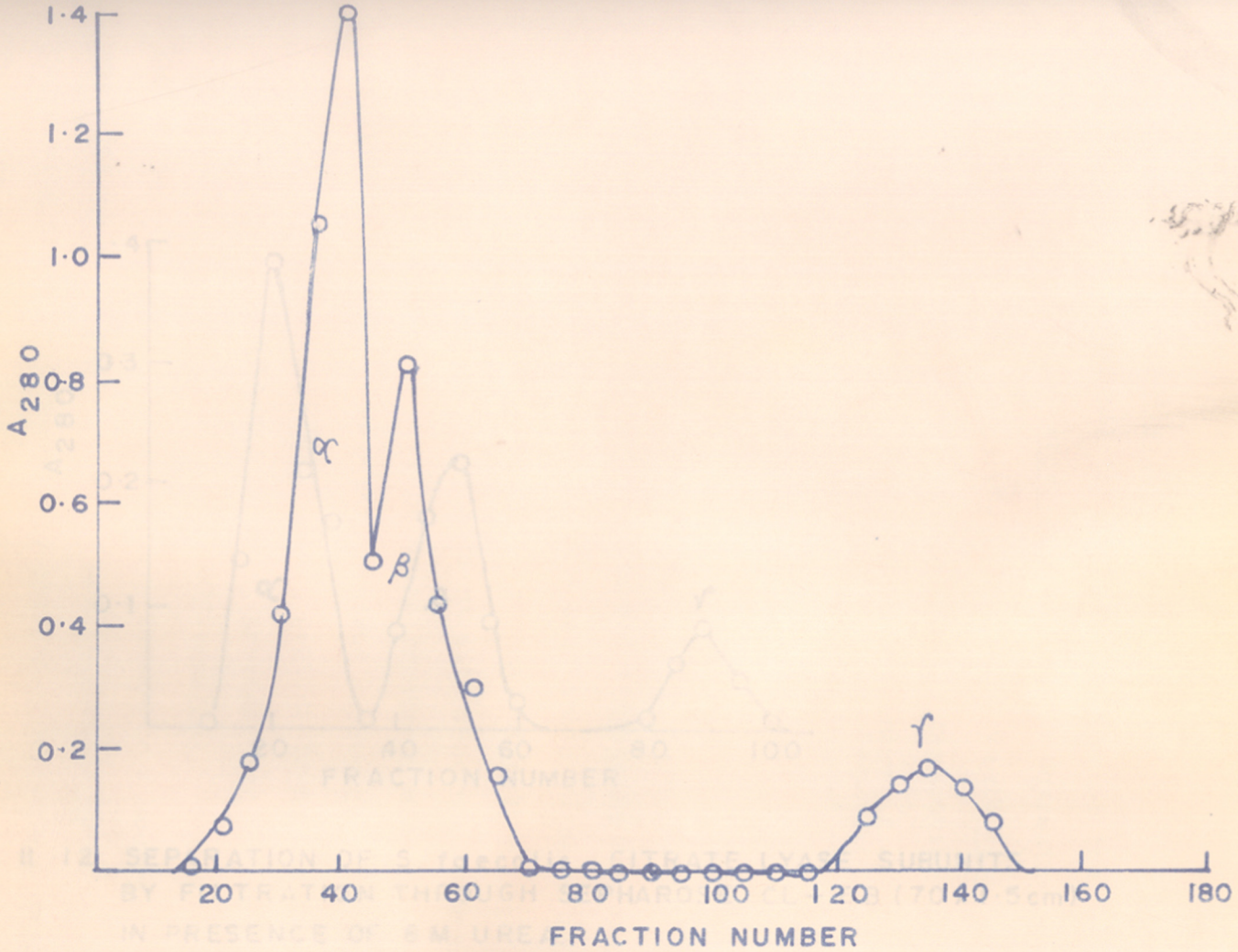


FIG. II·11 SEPARATION OF K. aerogenes CITRATE LYASE SUBUNITS BY FILTRATION THROUGH SEPHAROSE CL-6B (70 x 1·5 cm) IN PRESENCE OF 6M UREA.

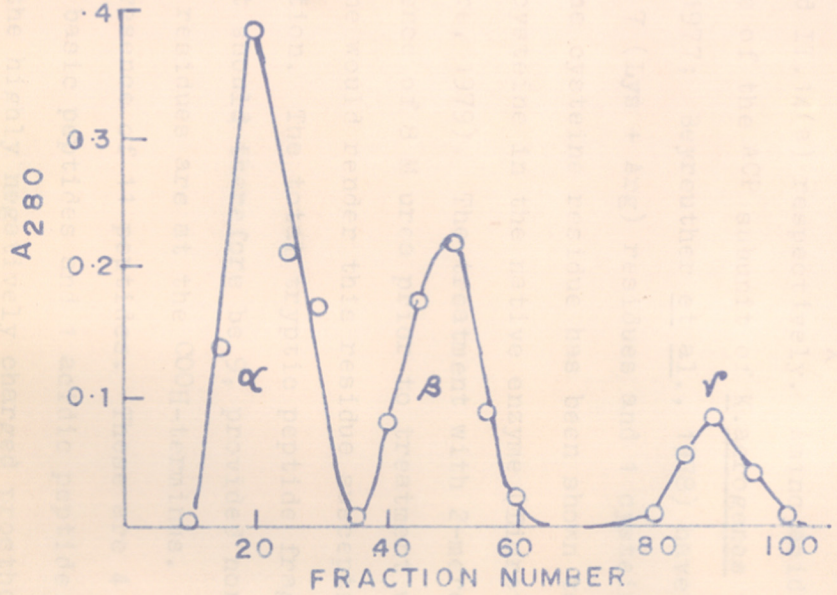


FIG. II ·12 SEPARATION OF S. *faecalis* CITRATE LYASE SUBUNITS BY FILTRATION THROUGH SEPHAROSE CL – 6B (70 x 1·5 cm) IN PRESENCE OF 6 M UREA.

fingerprint analysis. Fractions were freed from urea by dialysis as described in MATERIALS AND METHODS and samples lyophilized.

II.3.7 Fingerprint analysis:

The two dimensional tryptic peptide maps of the Υ-(ACP) subunits from K.aerogenes (M_r = 9378; Beyreuther et al., 1978) and from S.faecalis (M_r = 14 000; Hiremath et al., 1976) are shown in Figs. II. 13 and II.14 respectively. The photographs were printed alike for thesake of comparison. Schematic representations of maps including faint and yellow spots not registered in photographs are reproduced in Figs. II.13(a) and II.14(a) respectively. Amino acid analysis and sequence of the ACP subunit of K.aerogenes (Dimroth and Loyal, 1977; Beyreuther et al., 1978) have shown the presence of 7 (Lys + Arg) residues and 1 cysteine residue per mol. The cysteine residue has been shown to be as an S-acylated cysteine in the native enzyme (Dimroth et al., 1973; Tikare, 1979). The treatment with 2-mercaptoethanol in the presence of 8 M urea prior to treatment with ethyleneimine would render this residue susceptible to aminoethylation. The total tryptic peptide fragments from this subunit should therefore be 9, provided none of the susceptible residues are at the COOH-terminus. Fig. II.13 shows the presence of 11 peptides. These are 4 neutral peptides, 6 basic peptides and 1 acidic peptide (possibly containing the highly negatively charged prosthetic group). The extra peptide probably may have arisen from incomplete (partial)or nonspecific fragmentation during processing.

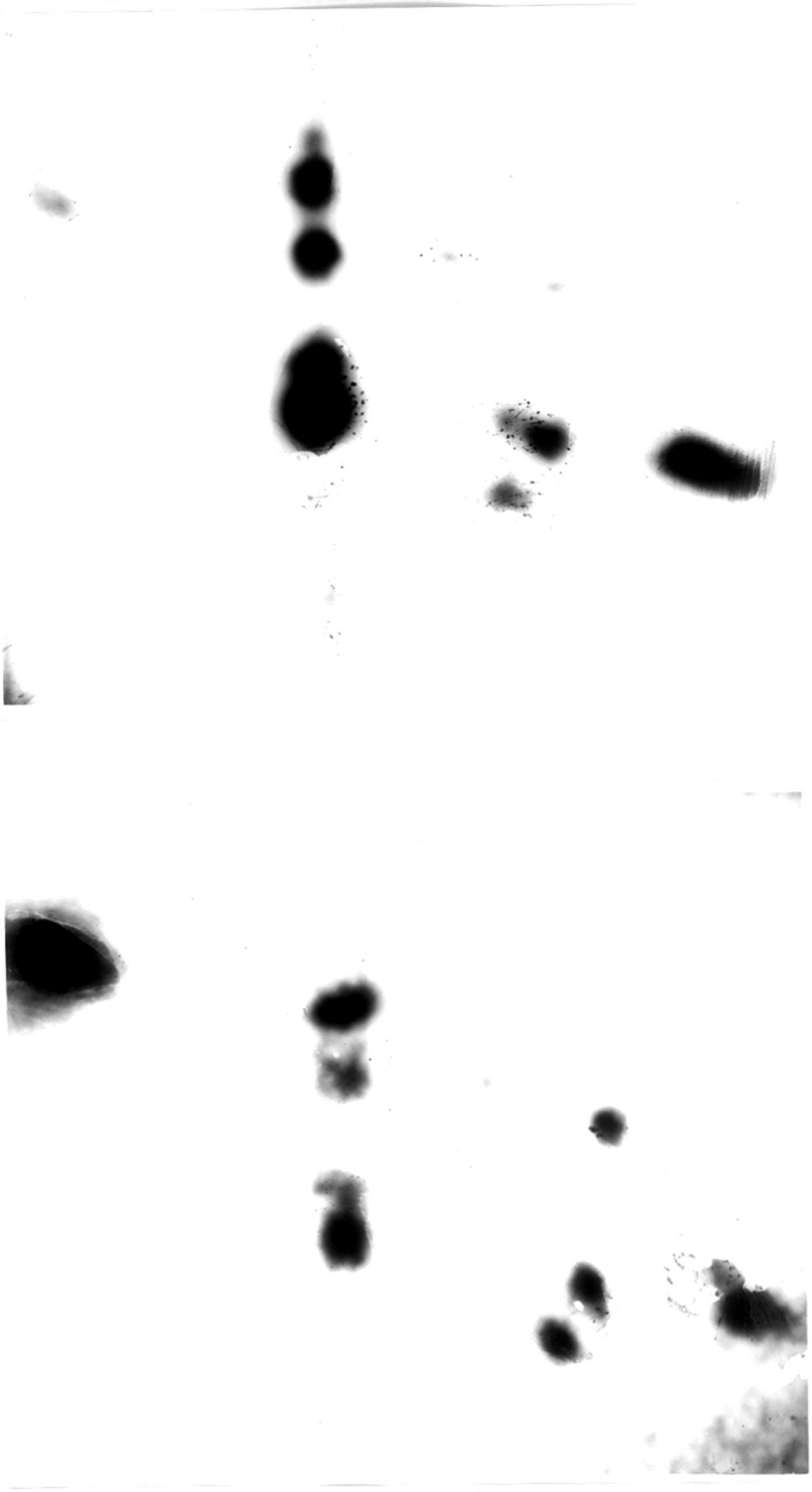


FIG. II. 13 Fingerprint of ACP (γ-) subunit of K.aerogenes. Schematic reproduction including faint as well as yellow coloured peptide spots not apparent in photograph is shown in Fig. II. 13(a).

FIG. II. 14 Fingerprint of ACP (γ-) subunit of S.faecalis. Schematic reproduction is shown in Fig. II. 14(a)

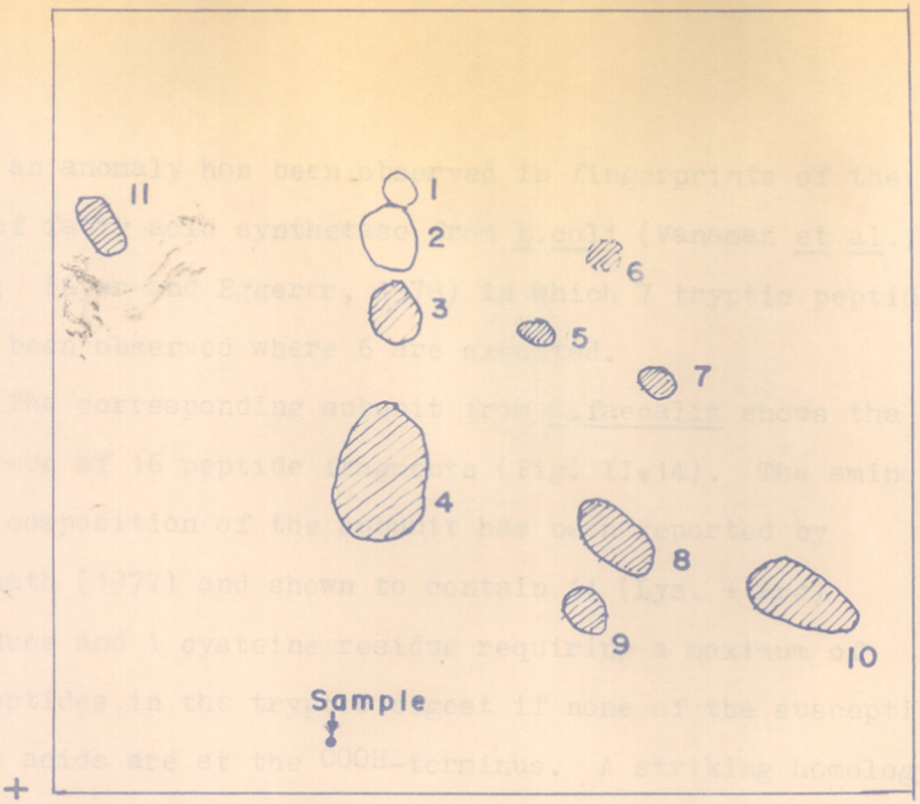


FIG II·13a : γ — SUBUNIT (k. aerogenes)
◌ Faint spot

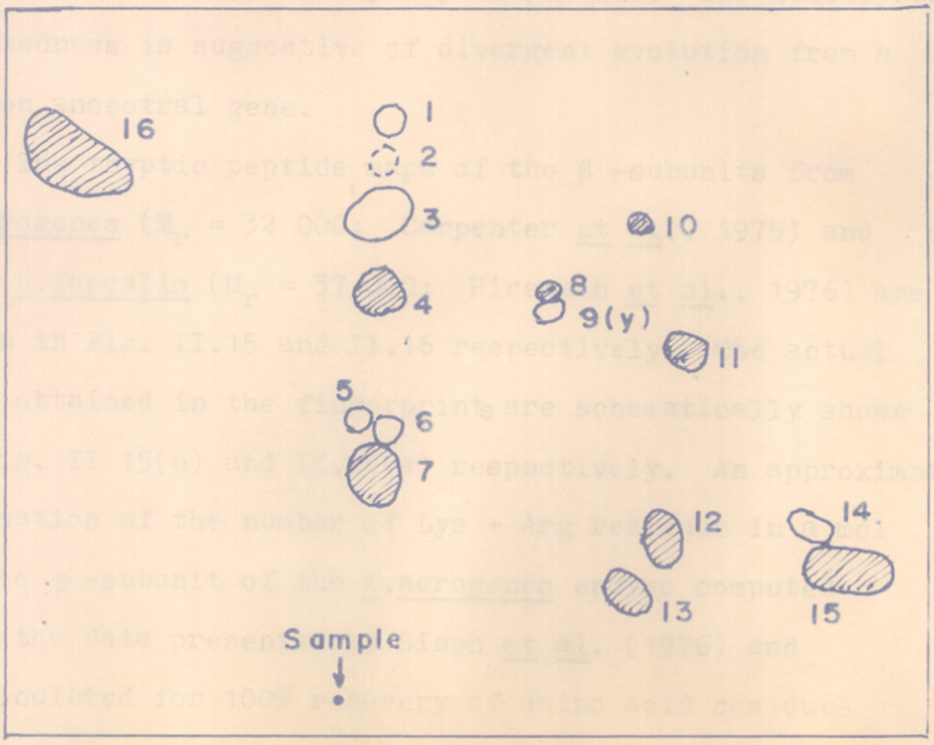


FIG. II 14a γ — SUBUNIT (S. faecalis)
(y) yellow spot, ◌ faint spot

Such an anomaly has been observed in fingerprints of the ACP of fatty acid synthetase from E.coli (Vanaman et al., 1968; Bayer and Eggerer, 1978) in which 7 tryptic peptides have been observed where 6 are expected.

The corresponding subunit from S.faecalis shows the presence of 16 peptide fragments (Fig. II-14). The amino acid composition of the subunit has been reported by Hiremath (1977) and shown to contain 14 (Lys. + Arg) residues and 1 cysteine residue requiring a maximum of 16 peptides in the tryptic digest if none of the susceptible amino acids are at the COOH-terminus. A striking homology in the two corresponding subunits are shown in 9 shaded spots which are apparently superimposable. The homologous relatedness is suggestive of divergent evolution from a common ancestral gene.

The tryptic peptide maps of the β -subunits from K.aerogenes (M_r = 32 000; Carpenter et al., 1975) and from S.faecalis (M_r = 37 000; Hiremath et al., 1976) are shown in Fig. II.15 and II.16 respectively. The actual maps obtained in the fingerprints are schematically shown in Fig. II 15(a) and II.16(a) respectively. An approximate estimation of the number of Lys + Arg residues in a mol of the β -subunit of the K.aerogenes enzyme computed from the data presented by Singh et al. (1976) and recalculated for 100% recovery of amino acid residues indicates a value of about 50 Lys + Arg residues. The content of cysteine residues is 2 (Tikare, 1979). The number of tryptic peptides would then be 53 assuming the

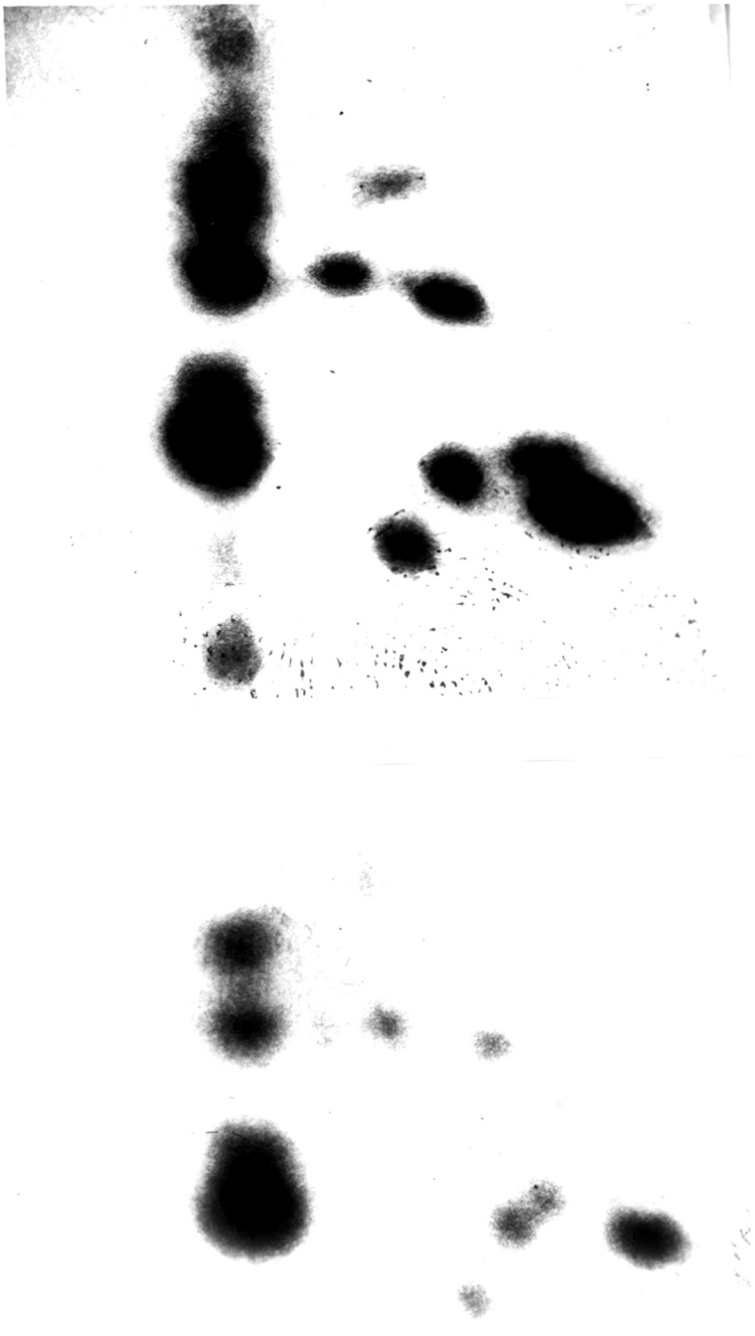


FIG.II. 15 Fingerprint of lyase (β-) subunit of K.aerogenes. Schematic reproduction is shown in Fig. II. 15(a).

FIG. II. 16 Fingerprint of lyase (β-) subunit of S.faecalis. Schematic representation is shown in Fig. II. 16(a).

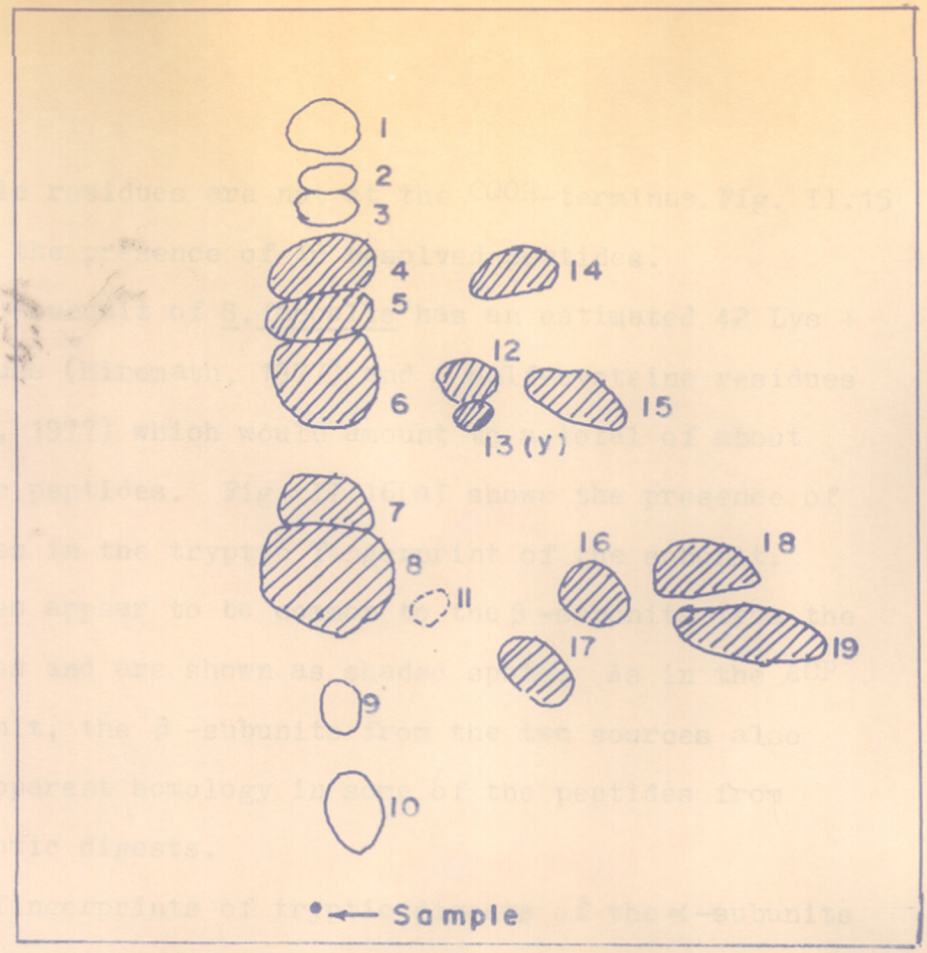


FIG. II· 15a β-SUBUNIT (k. aerogenes)
(y) Yellow spot ◌ Faint spot

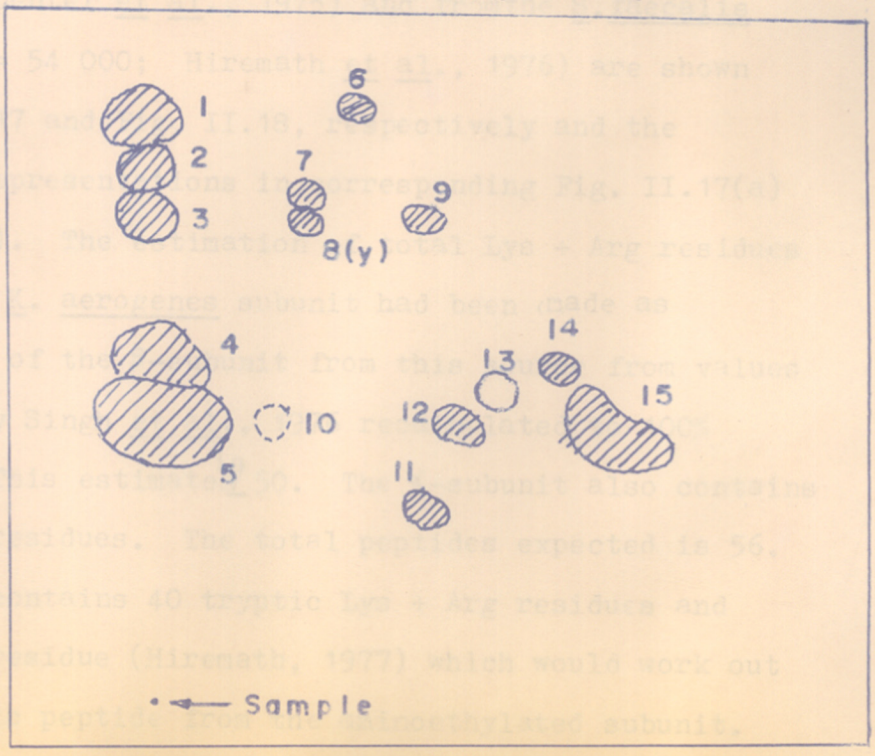


FIG II 16a β-SUBUNIT (S. faecalis)
(y) Yellow spot ; ◌ Faint spot

susceptible residues are not at the COOH-terminus. Fig. II.15 (a) shows the presence of 19 resolved peptides.

The β-subunit of S.faecalis has an estimated 42 Lys + Arg residues (Hiremath, 1977) and 4 half-cysteine residues (Hiremath, 1977) which would amount to a total of about 47 tryptic peptides. Fig. II.16(a) shows the presence of 15 peptides in the tryptic fingerprint of the subunit. 14 peptides appear to be common to the β-subunits from the two sources and are shown as shaded spots. As in the ACP (γ-) subunit, the β-subunits from the two sources also exhibit apparent homology in some of the peptides from their tryptic digests.

The fingerprints of tryptic digests of the α-subunits from K.aerogenes enzyme (M_r = 54 000; Dimroth and Eggerer, 1975a; Carpenter et al., 1975) and fromthe S.faecalis enzyme (M_r = 54 000; Hiremath et al., 1976) are shown in Fig. II.17 and Fig. II.18, respectively and the schematic representations in corresponding Fig. II.17(a) and II.18(a). The estimation of total Lys + Arg residues in a mol of K. aerogenes subunit had been made as in the case of the β-subunit from this source from values presented by Singh et al., 1976 recalculated to 100% recovery. This estimates is 50. The α-subunit also contains 5 cysteine residues. The total peptides expected is 56. S.faecalis contains 40 tryptic Lys + Arg residues and 1 cysteine residue (Hiremath, 1977) which would work out to 42 tryptic peptide from the aminoethylated subunit.

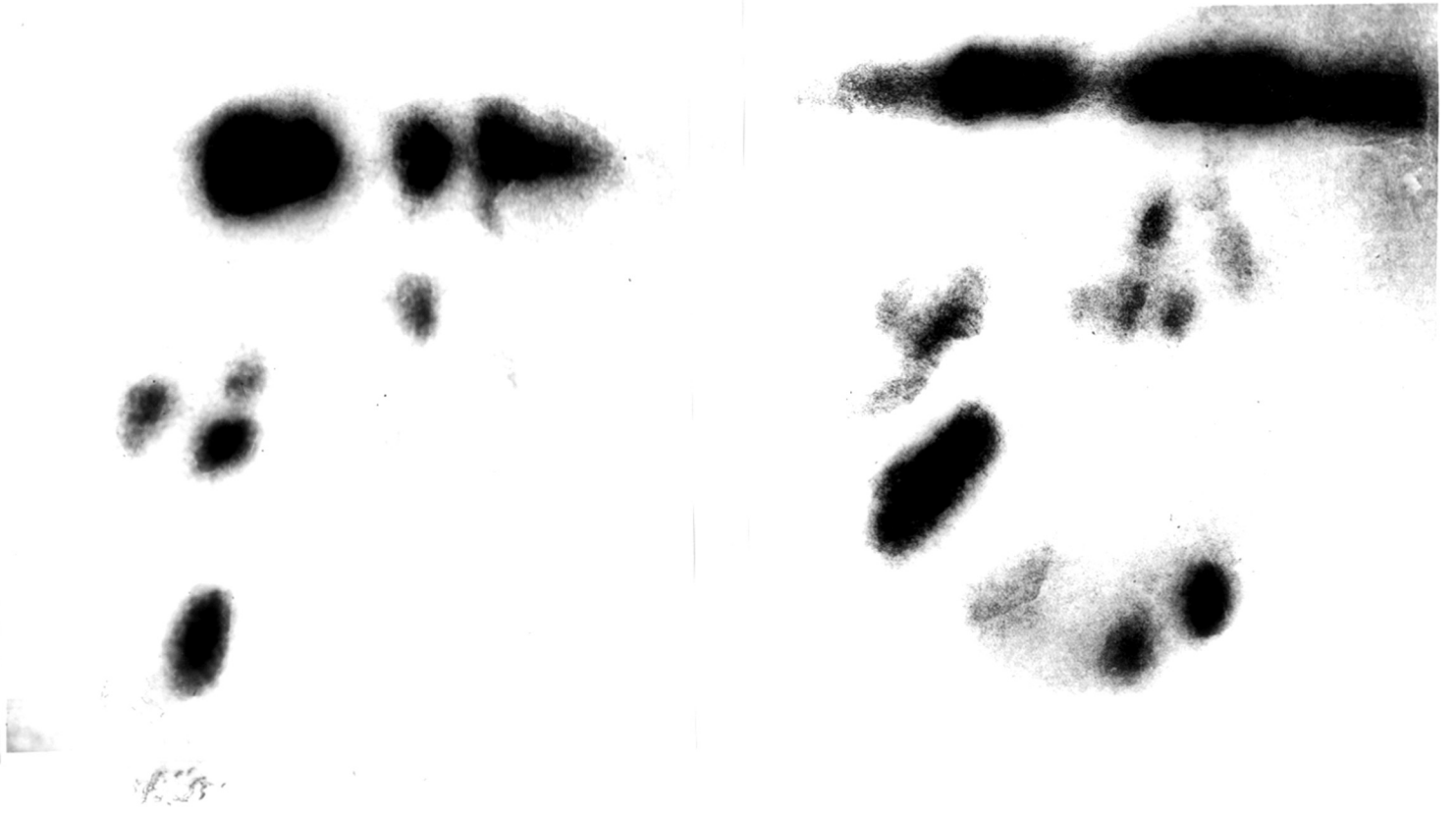


FIG. II. 17 Fingerprint of transferase (α-) subunit of K.aerogenes. Schematic representation is shown in Fig. II. 17(a).

FIG. II. 18 Fingerprint of transferase (α-) subunit of S.faecalis. Schematic representation is shown in Fig. II.18 (a).

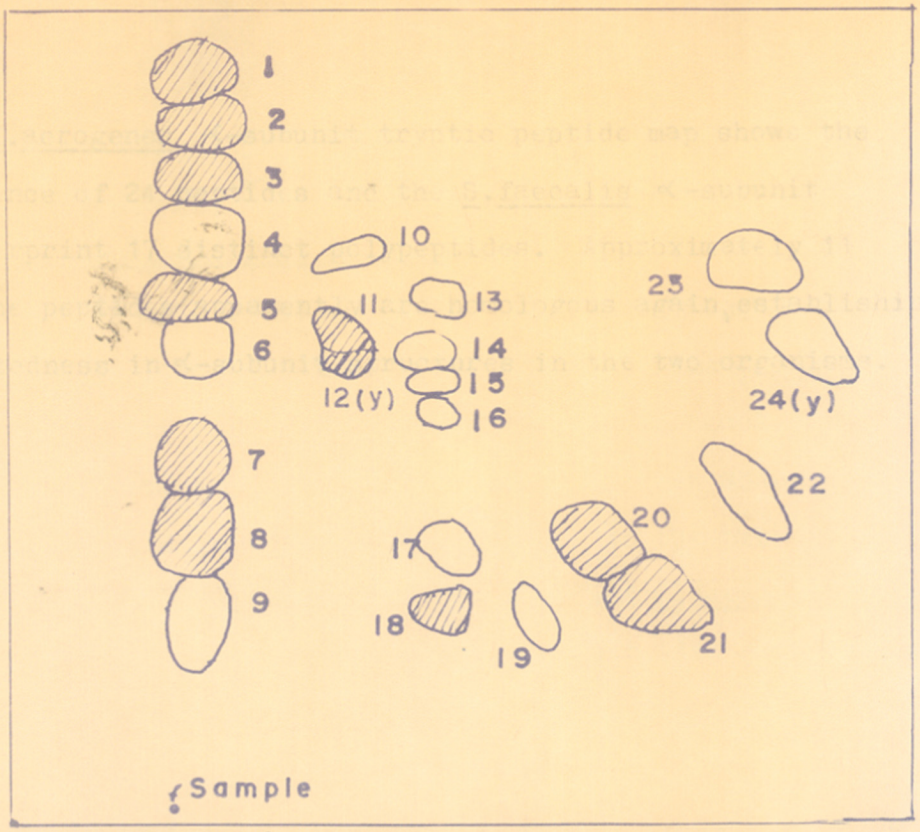


FIG. II·17a ∝ − SUBUNIT (K. aerogenes)
(y) yellow spot

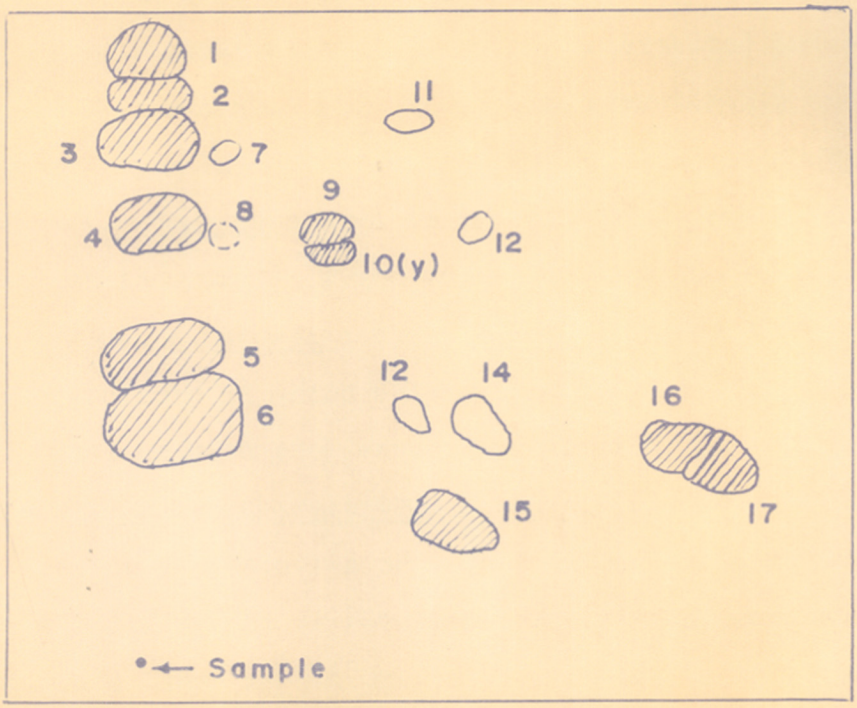


FIG. II·18a ∝ − SUBUNIT (S. faecalis)
(y) yellow spot ◌ faint spot

The K.aerogenes ∝-subunit tryptic peptide map shows the presence of 24 peptides and the S.faecalis ∝-subunit fingerprint 17 distinct polypeptides. Approximately 11 of the peptides apparently are homologous again, establishing relatedness in ∝-subunit structures in the two organisms.

II.4. DISCUSSION

II.4. (i) Immunological behaviour:

Earlier work of Singh et al. (1975) and Singh and Srere (1975) had shown that citrate lyases from E.coli, K.aerogenes and S.diacetilactis do not cross-react except with homologous antiserum. This would suggest absence of common antigenic surface determinants. In the present work, rabbit antiserum specific towards purified K.aerogenes enzyme complex has been shown not to react with crude enzyme preparation from S.diacetilactis, confirming the earlier observation. However the enzymes from K.aerogenes and S.faecalis have been shown in the present studies to cross-react both in double diffusion and immunoelectrophoresis. Such immunological relatedness would be expected between enzymes that resemble each other both structurally and functionally. In the present work the rabbit antiserum against K.aerogenes enzyme complex has also been used to establish the purity of the enzyme preparations from the two sources, K.aerogenes and S.faecalis, using immuno-electrophoresis in which only single and well-defined arcs were obtained with each enzyme preparation.

II.4 (ii) Reaction-inactivation behaviour:

The present studies provide indirect evidence to support that the difference in reaction-inactivation behaviour of citrate lyase is not due to differences in the steady state concentration of the citryl-intermediate which in the case of citryl-CoA is known to be unstable at alkaline pH

values and to undergo spontaneous cleavage to citrate and the deacetylated coenzyme (Buckel et al., 1973). The present studies have been carried out with acetyl-CoA mediated cleavage of citrate in presence of the deacetylated enzyme, assuming that the behaviour of acetylated-ACP resembles that of acetyl-CoA in catalysing citrate cleavage. Assuming such a resemblance, the steady state concentration of citryl-ACP would be higher in S.faecalis than in K.aerogenes. The weak reaction-inactivation of the S.faecalis enzyme might therefore suggest an inherent stability of the citryl-intermediate or its slower hydrolysis catalysed by the transferase (α-) subunit of the complex. The latter possibility of the transferase subunit catalysed cleavage of citrate from citryl-ACP has been shown to occur in studies with the isolated subunits and has also been suggested as a possible cause for the reaction-inactivation of citrate lyase (Dimroth and Eggerer, 1977b). The possible cause of the differences in the reaction-inactivation behaviour maybe in the differences in the microenvironment of the active sites of the enzymes from the two sources.

II.4 (iii) Fringerprint analysis

The fingerprint analysis of separated subunits of the citrate lyases from K.aerogenes and S.faecalis reveal an apparent close homology between the corresponding subunits of the two enzyme complexes. While this evidence is only circumstantial in the absence of the complete subunit sequence data, the immunological cross-reactions would support the hypothesis of apparent

structural relationships between the two enzyme complexes. The present data provides the first preliminary evidence of homologous relationships between corresponding subunits of the citrate lyase complexes from two different sources.

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COOPERATIVE BINDING OF MANGANESE TO CITRATE LYASE FROM *KLEBSIELLA AEROGENES*

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1. Introduction

Bacterial citrate lyase (EC 4.1.3.6) catalyses the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metal ions such as Mg^{2+} and Mn^{2+} [1].

The enzyme has been obtained pure from several sources [2–5] and shown to be a complex of three non-identical subunits of ~55 000 (α), 30 000 (β) and 10 000 (γ) daltons. The subunit composition and function has been studied extensively in the enzyme from *K. aerogenes* [6–8]. The γ subunit acts as acyl carrier protein (ACP) and carries an essential acetyl moiety [7]. The α subunit functions as an acyl transferase involved in citryl-ACP formation with release of acetate; and the β subunit catalyses the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP [9]. Acetyl-CoA also serves as substrate for the transferase and lyase activities [10,11]. The transferase reaction proceeds in the presence of EDTA, while the lyase reaction requires the presence of Mg^{2+} [9,10].

Relatively little is known about the nature of the interaction between the enzyme and divalent metal cofactors. The formation of a binary Mn^{2+}–protein complex has been suggested from pulsed NMR studies on partially purified citrate lyase from *Streptococcus diacetilactis* [12], although the impure nature of the preparation precludes unambiguous conclusions.

This paper reports studies on the Mn^{2+}-binding properties of pure citrate lyase from *K. aerogenes* by equilibrium dialysis assays using $^{54}MnCl_2$.

2. Materials and methods

Carrier-free $^{54}MnCl_2$ was obtained from the Bhabha Atomic Research Centre, India. Tris was primary standard grade from Sigma Chemical Co., USA. All other reagents were of analytical grade.

Citrate lyase was purified from *K. aerogenes* NCTC 418 as in [13] and preparations were homogeneous in the ultracentrifuge and in polyacrylamide gel electrophoresis. The enzyme assayed by coupling with malate dehydrogenase [14] had spec. act. 70 μmol .min^{-1} at 30°C.

Protein was determined by the method in [15] standardised with pure enzyme.

Sedimentation profiles were recorded on a Spinco Model E ultracentrifuge equipped with phase plate.

2.1. *Metal determinations*

Manganese in stock solutions of $MnCl_2$ (200 mM) was determined chemically [16]. ^{54}Mn was determined on an ECIL Model SC 603 Gamma counter. Magnesium was determined after wet combustion of lyophilized protein samples with conc. HCl/HNO_3, 3:1 (v/v) on a Perkin Elmer Model 303 atomic absorption spectrophotometer.

2.2. *Preparation of Mg^{2+}-free citrate lyase*

Enzyme solutions (4–8 mg/ml) were dialysed 24 h at 0–4°C against 50 mM potassium phosphate–2 mM EDTA (pH 7.5), with 3 changes, 250 vol. each. Enzyme solutions treated in this manner contained no detectable Mg^{2+}.

2.3. *Equilibrium dialysis*

Tris/HCl buffer, 50 mM (pH 7.4), was used in the equilibrium dialysis experiments. Tris under these conditions has been shown to have no detectable interaction with Mn^{2+} in EPR and NMR studies [17]. The absence of Mg^{2+} and Mn^{2+} as impurities in the buffer was established by metal determinations.

All dialysis operations were carried out at 0–4°C. Mg^{2+}-free enzyme solution, prepared as above, was dialysed 16 h against 500 vol. 50 mM Tris–2 mM EDTA (pH 7.5) then equilibrated by dialysis for 36 h against 50 mM Tris (pH 7.5) containing non-radioactive $MnCl_2$ at the desired concentration, with 3 changes, 250 vol. each.

Of the final buffer against which the enzyme solution had been equilibrated 20 ml was treated with 0.01 ml carrier-free $^{54}MnCl_2$ solution. The concentration of the buffer was adequate to neutralise the HCl present in the $^{54}MnCl_2$ with a resulting pH change of $\leqslant$0.1 unit. Addition of the small volume of $^{54}MnCl_2$ was assumed not to alter the total Mn^{2+} concentration in the buffer, being carrier-free. The trace quantities of non-radioactive ^{54}Cr, the decay product of ^{54}Mn, was assumed not to compete with Mn^{2+}-binding.

Dialysis cells were of the type described in [18] with 1 ml capacity compartments. Dialysis membranes were cut from Visking dialysis tubing pretreated by repeated boiling, successively, in 100 mM $NaHCO_3$–10 mM EDTA; 100 mM acetic acid; and several changes of water double-distilled in glass. The membranes were rinsed in the equilibration buffer before use. Cell compartments were filled with enzyme solution on one side and an equal volume of equilibration buffer with added $^{54}MnCl_2$ on the other. The cell-assembly was gently rocked for 24 h at 0–4°C until equilibrium was reached, as determined from preliminary trials. Samples were withdrawn separately from the compartments for both ^{54}Mn and protein determinations. The free-Mn^{2+} concentration was taken to be that in the buffer against which the enzyme had been dialysed extensively. The concentration of bound Mn^{2+} was calculated from the difference in radioactivity counts between the enzyme solution and the corresponding equilibration buffer. The stoicheiometry of binding was calculated for mol. wt 575 000 of the enzyme [13].

Table 1
Sedimentation behaviour of citrate lyase from *K. aerogenes*

Enzyme	Buffer solution (pH 7.4)	$s^\circ_{20,w}$ (S)
Mg^{2+}-free	2 mM EDTA–50 mM Tris–HCl	17.6
Native	2 mM $MgSO_4$–50 mM Tris–HCl	17.8

Speed 59 780 rev./min; temp., 2.2–4.5°C

3. Results

3.1. *Sedimentation behaviour of metal-free and Mn^{2+}-citrate lyase*

Table 1 describes the sedimentation behaviour of Mg^{2+}-free enzyme as well as that of the enzyme in presence of 2 mM $MgSO_4$.

The Mg^{2+}-free enzyme and the enzyme in presence of the metal have almost similar $s^\circ_{20,w}$ values of 17.6 S and 17.8 S, respectively. The sedimentation behaviour of the Mn^{2+}-enzyme obtained by equilibrating the Mg^{2+}-free enzyme with 50 mM Tris–2 mM $MnCl_2$ (pH 7.4), was similar to that of corresponding concentrations of the enzyme in presence of $MgSO_4$. The absence of Mg^{2+} as well as the replacement of Mg^{2+} with Mn^{2+} evidently causes no change in the quaternary structure of the enzyme under the conditions used.

3.2. *Manganese binding*

The saturation curve of Mn^{2+} binding is presented in fig.1a.

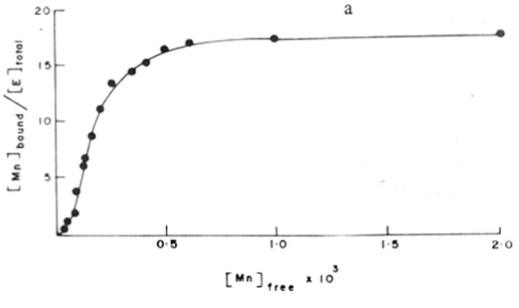

Fig.1a. Binding of Mn^{2+} to citrate lyase from *K. aerogenes*. Equilibrium dialysis performed in 50 mM Tris–HCl buffer (pH 7.4) containing varying concentrations of $MnCl_2$. Protein, 4–8 mg/ml; temp. 0–4°C.

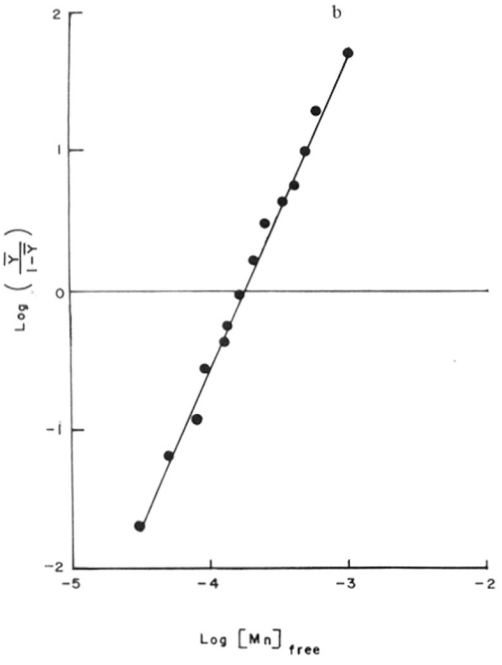

Fig.1b. Hill plot of Mn^{2+}-citrate lyase binding data. $\bar{Y}$ is the fractional saturation of the enzyme with Mn^{2+}, assuming 18 binding sites/mol enzyme under saturating conditions. Data points plotted are for $\bar{Y}$ values in the range 0.02–0.98. The straight line is the least squares fit of experimental data.

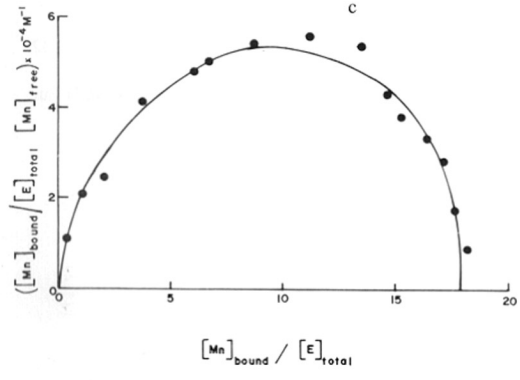

Fig.1c. Scatchard plot of Mn^{2+}-binding to citrate lyase. The solid line is constructed from values calculated from the Hill plot and overlaps approximately curve drawn by eye.

Saturation of binding sites is reached at free-Mn^{2+} concentrations greater than $\sim 1 \times 10^{-3}$ M where 18 g atoms of the metal are bound to 1 mol enzyme. The sigmoidal plot is diagnostic of positive cooperativity. The interacting site behaviour is more apparent in the Hill [19] and Scatchard [20] plots shown in fig.1b and fig.1c, respectively.

The Hill plot (fig.1b) shows linearity over the entire ligand concentration range used. The Hill coefficient, n_H, calculated from the slope of the Hill plot has a value of 2.27 ± 0.05, indicating a significant extent of positive cooperativity in metal binding. The presence of a maximum in the Scatchard plot (fig.1c) is also characteristic of positively cooperative ligand binding [21], particularly since the ultracentrifuge data rule out any metal-dependent dissociation–association effects. The Scatchard plot has an intercept which passes through the origin at $(Mn)_{free} \rightarrow 0$, which would rule out the presence of any independent, non-interacting sites [21,22]. The total number of Mn^{2+}-binding sites/mol enzyme is 18 from the extrapolated value of the intercept of the Scatchard plot with the abscissa axis at $(Mn)_{free} \rightarrow \infty$.

The microscopic dissociation constant $K_{d,n}$ of the Mn^{2+}-citrate lyase complex for the last binding step was calculated from the limiting slope of the Scatchard plot at $(Mn)_{free} \rightarrow \infty$ [23], using the relationship, slope $= -1/K_{d,n}$. A value of 4.5×10^{-5} M was obtained for saturating conditions of divalent metal concentration from the approximately linear region covering the last 3 points of the Scatchard plot with values in the range 17–18 Mn^{2+}-binding sites/mol enzyme.

4. Discussion

These studies show for the first time the cooperative binding of a divalent metal cofactor by citrate lyase. Binding of the divalent metal evidently involves a conformational change in the enzyme complex, probably to a form which is catalytically active in the cleavage reaction.

The allosteric behaviour of the enzyme could be a regulatory mechanism of biological significance. Citrate lyase from *K. aerogenes* undergoes rapid inactivation in vitro during the course of the reaction it catalyses through a process of deacetylation

[14,24]. However such reaction inactivation has been shown to play no role in citrate lyase regulation in vivo [25]. The inactivation in vivo has been shown to be energy dependent [25]. Conformational modulations in citrate lyase complex could be a possible mechanism of regulation, if the requirement of energy is assumed to be for the formation or utilization of metabolite(s) acting either directly as modulator(s) or indirectly through complexing of Mg^{2+}. The earlier speculation [26] that a mechanism for regulation of citrate lyase activity in the cell could be through the ability of ATP to chelate metal ions, could indeed be so through modulation in the conformation of citrate lyase when Mg^{2+} is abstracted from the complex.

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