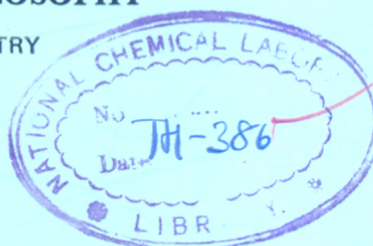


MICROBIAL ENZYMES :
STUDIES ON CITRATE LYASE (EC 4.1.3.6) OF
Klebsiella aerogenes and Escherichia coli

COMPUTERISED

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by Miss Subhalakshmi Subramanian was carried out by the
candidate under my supervision. Such material as has
been obtained from other sources has been duly
acknowledged in this thesis.


C.SivaRaman
Research Guide

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ABBREVIATIONS

List of abbreviations used other than the accepted ones.

ACP	Acyl-carrier protein
DTNB	5,5'-Dithiobis (2-nitrobenzoate)
DTT	Dithiothreitol
ϵ	Molar extinction coefficient
$E_{1\%}^{1\text{cm}}$	Absorbance index (1% w/v protein)
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MDH	Malate dehydrogenase (EC 1.1.1.37)
OAA	Oxaloacetic acid
SDS	Sodium dodecyl sulphate

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GENERAL INTRODUCTION

CITRATE ENZYMES

Three closely related groups of enzymes catalyse the synthesis/breakdown of citrate through a lyase reaction involving an equilibrium between citrate and its C_4 (oxaloacetate) and C_2 (acetyl) moieties. The close similarity in the stereochemistry of these reactions (Srere, 1975) and the cross-reaction of cofactors required for the activity of the three groups of enzymes (Robinson *et al.*, 1976) are suggestive of their evolution through divergence from a common ancestral gene. These enzymes are:

1. Citrate lyase (citrate oxaloacetate-lyase pro-3S-CH₂COO⁻ $\rightarrow$ acetate, EC 4.1.3.6) which catalyses the cleavage of citrate to oxaloacetate and acetate in presence of a divalent metal ion such as Mg^{2+} (Eq. 1).



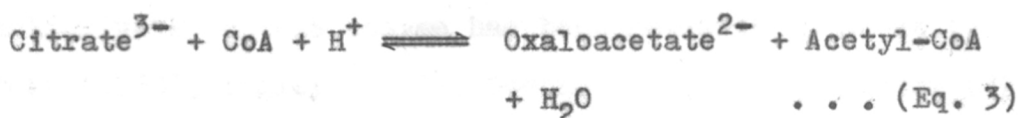
The enzyme has been found to occur only in procaryotes.

2. ATP-citrate lyase (ATP: citrate oxaloacetate-lyase pro-3S-CH₂COO⁻ $\rightarrow$ acetyl-CoA: ATP dephosphorylating, EC 4.1.3.8) which catalyses the breakdown of citrate to oxaloacetate and acetate in the presence of ATP, Coenzyme A and a divalent metal ion like Mg^{2+} (Eq. 2).



The enzyme is generally present only in eucaryotes in the cytosol. An exception is its occurrence in Chlorobium limicola (Ivanovsky et al., 1980; Antranikian et al., 1982a).

3. si-Citrate synthase (citrate oxaloacetate-lyase pro- $3S-CH_2COO^- \rightarrow$ acetyl-CoA (EC 4.1.3.7) which catalyses the synthesis of citrate and is ubiquitous in occurrence, being present both in procaryotes and eucaryotes. The reaction catalysed by this enzyme is shown in Eq. 3.



Besides these three citrate enzymes, an enzyme which has been little studied is:

re-Citrate synthase (citrate oxaloacetate-lyase pro- $3R-CH_2COO^- \rightarrow$ acetyl-CoA, EC number not assigned as yet) which has been found only in anaerobic microorganisms. The enzyme is the only one of the citrate enzymes which has an opposite stereochemistry compared to the other three.

The formation of citrate in vivo is catalysed by the citrate synthases while its dissimilation is catalysed by citrate lyase and ATP-citrate lyase. Among the citrate enzymes, citrate lyase has the simplest extraneous cofactor requirement and occurs only in the primitive forms of life. These observations have contributed to the postulate that citrate lyase may be the first of the citrate enzymes to have evolved, while ATP-citrate lyase which catalyses a more complex reaction and is present mainly in higher organisms

may be the most recent of the citrate enzymes (Srere, 1968).

The present study relates to the properties of citrate lyase (EC 4.1.3.6), an enzyme on which much interest has been focused in recent years mainly because of its multienzyme nature. The complex contains two enzymes and an acyl carrier protein with a covalently bound CoA like prosthetic group which oscillates between the two types of enzyme subunits. The rest of this Chapter reviews briefly the extensive literature on the citrate lyase multienzyme complex. The earlier work on this enzyme has been reviewed by Srere (1965), Srere (1968), Dagley (1969), Lowenstein (1969), Spector (1972), Srere (1972), Srere (1974), Srere and Singh (1975).

I. CITRATE LYASE (EC 4.1.3.6)

I.1 OCCURRENCE

The earliest reports on the anaerobic degradation of citrate date back to 1939 when Defner and Franke and Brewer and Werkman first presented evidence for oxaloacetate and acetate being the initial products of citrate fermentation by Klebsiella aerogenes and Aerobacter indologenes.

The enzyme which brings about the dissimilation, however, was characterized several years later by Dagley and Dawes (1953a) in K. aerogenes and in Escherichia coli and independently by Gunsalus and his coworkers in E. coli (Gillespie and Gunsalus, 1953) and in Streptococcus faecalis (Grunberg-Manago and Gunsalus, 1953). The enzyme is induced in the

presence of citrate in all sources except Streptococcus diacetilactis, where it is present constitutively. Harvey and Collins (1961) observed that citrate lyase is present in some strains of S. diacetilactis grown on a lactose medium, the enzyme level being enhanced almost two-fold when citrate is present in the medium. These authors also observed that some strains of Leuconostoc citrovorum and of Streptococcus liquefacians contain citrate lyase.

Among the phototropic bacteria, citrate lyase activity has been shown to be present in Rhodopseudomonas gelatinosa (Weckesser et al., 1969; Schaab et al., 1972) and in strains of Rhodopseudomonas palustris (Giffhorn and Kuhn, 1980). The enzyme levels in the R. palustris strains were found to be lower than in R. gelatinosa.

Salmonella typhimurium under aerobic conditions contained the enzymes of the citric acid cycle as well as of the citrate fermentation pathway (O'Brien et al., 1969). Citrate lyase has also been found to occur in Aerobacter cloacae (O'Brien and Geisler, 1974). Proteus rettgeri grown anaerobically on citrate has also been shown to have citrate lyase activity (Kroger, 1974).

Clostridium sphenoides, which is an anaerobic spore-former, can utilize citrate as carbon and energy source, the fermentation products being acetate, CO₂, ethanol and molecular hydrogen (Walther et al., 1977). Citrate lyase from this source has been shown to strictly require glutamate for activity (Antranikian et al., 1982).

I.2 ROLE IN METABOLISM

Citrate lyase is the key enzyme in the anaerobic metabolism of citrate by a number of bacteria, catalysing the initial degradation of citrate to oxaloacetate and acetate (Eq. 1). Further catabolism of oxaloacetate takes place either by decarboxylation or by reduction. In organisms such as K. aerogenes, S. faecalis and R. gelatinosa, the oxaloacetate is decarboxylated to pyruvate by oxaloacetate decarboxylase, an enzyme which is also induced in these sources in the presence of citrate. The two sequential enzymatic reactions constitute the "Citrate fermentation pathway" (Dagley and Dawes, 1953a; O'Brien and Stern, 1969). Subsequently, the pyruvate dehydrogenase complex brings about the oxidative decarboxylation of pyruvate to acetyl-CoA, which serves as the source for the generation of ATP and reducing power.

Organisms such as P. rettgeri (Kröger, 1974) and E. coli (Lutgens and Gottschalk, 1980) which metabolise citrate under anaerobic condition through the initial cleavage of citrate by citrate lyase do not contain oxaloacetate decarboxylase. In these malate dehydrogenase catalyses the conversion of the oxaloacetate formed from citrate to malate, which in turn forms fumarate in the presence of fumarase. Both P. rettgeri and E. coli have membrane bound fumarate reductases, which have been shown to produce ATP during the reduction of fumarate to succinate (Hirsch et al., 1963; Bernhard and Gottschalk, 1978). In P. rettgeri, the reducing

power required for succinate formation is generated by the oxidation of a portion of the available citrate through the tricarboxylic acid cycle, one mol ATP being produced per mol citrate oxidized in addition to the reducing power. In E. coli, however, the tricarboxylic acid cycle is repressed under anaerobic conditions (Amaransingham and Davis, 1965) and the presence of a cosubstrate like glucose is required for generation of reducing power. In contrast, K. aerogenes does not contain fumarate reductase and has to depend completely on oxaloacetate decarboxylase for production of acetyl-CoA (Kulla, 1976).

Evans et al. (1966) proposed the reductive carboxylic acid cycle for CO₂ assimilation by photosynthetic, anaerobic bacteria with citrate lyase functioning as a key enzyme of the cycle. Beuscher and Gottschalk (1972), however, could not detect the presence of citrate lyase in any of the green sulfur bacteria. Ivanovsky et al. (1980) and Antranikian et al. (1982a) have reported presence of ATP-citrate lyase activity in Chlorobium limicola.

1.3 REGULATION OF CITRATE LYASE

In microorganisms which can synthesize both citrate lyase and citrate synthase, simultaneous functioning of the two can lead to antagonistic reactions with loss of energy through futile cycles. Regulatory mechanisms which can switch off either of the two enzymes become vital and these have been detected in several microorganisms. Other modes of regulation like covalent or conformational changes have also been detected. The diverse regulatory mechanisms are

reviewed briefly below.

(1) Regulation in *K. aerogenes*: O'Brien and Stern (1969) observed that *K. aerogenes* NCTC 418 under anaerobic conditions grows on citrate as the sole source of energy only in the presence of Na^+ . The requirement was explained on the basis of the observation of Stern (1967) that the oxaloacetate decarboxylase of this organism is Na^+ -dependent. Sachan and Stern (1971a) have shown that the membrane-bound, Na^+ -requiring oxaloacetate decarboxylase also functions as the carrier protein for the transport of citrate. Under anaerobic growth conditions, the α -keto dehydrogenase of the citric acid cycle is repressed and citrate is catabolized only through the fermentation pathway. Under aerobic conditions, on the contrary, citrate lyase and oxaloacetate decarboxylase are not synthesized while all the enzymes of the citric acid cycle are active and cells grown either in presence of Na^+ or its absence metabolise citrate through the citric acid cycle (Wilkerson and Eagon, 1972; O'Brien, 1975a). Under aerated conditions, where no oxygen tension is detected, citrate is degraded by the fermentation pathway in presence of Na^+ and through the citric acid cycle in the absence of Na^+ (O'Brien et al., 1969).

Another regulatory mechanism, not involving repression of the enzymes of either the fermentation pathway or the citric acid cycle, is by chemical modification of the essential acetyl moiety in the citrate lyase complex (Buckel et al., 1971a). In vitro studies by Singh and Srere (1971) have shown that the enzyme from *K. aerogenes* undergoes reaction inactivation which was later shown by Srere et al.

(1972) to be as a result of deacetylation during the reaction catalysed by the enzyme. Kulla and Gottschalk (1977) have reported the absence of any deacetylase in K. aerogenes. Schmellenkamp and Eggerer (1974) have shown the presence of a distinct deacetyl citrate lyase ligase in this organism which can bring about the reactivation of any deacetyl enzyme in presence of ATP and acetate.

Kulla and Gottschalk (1977) studied citrate lyase regulation in K. aerogenes by growth shift experiments. When the organism was grown on citrate under anaerobic conditions, the specific activity of the citrate lyase slowly increased after an initial lag period. This increase was evident till the end of the growth phase (12 h) after which it slowly decreased. No deacetyl enzyme could be detected during the initial stages of growth. The rapid reaction inactivation of the K. aerogenes enzyme which occurs in vitro (Singh and Srere, 1971) through a process of deacetylation (Srere et al., 1972), apparently plays no role in the regulation of the citrate lyase activity. After 16 h of incubation, however, a small increase in the amount of deacetyl enzyme was observed, while after 24 h the increase was considerably more. To study the inactivation of the enzyme under aerobic and anaerobic conditions as well as with various substrates, growth shift experiments were carried out using a continuous culture with ammonia as the growth limiting substrate. Samples were withdrawn from the culture medium and tested for citrate synthase and citrate lyase activities before and after treatment with acetic anhydride. When the culture was shifted

from "anaerobic citrate" to "aerobic citrate", the specific activity of citrate lyase decreased slowly, active citrate lyase was gradually deacetylated and citrate synthase was induced. When the shift was from "anaerobic citrate" to "aerobic glucose", citrate lyase was rapidly inactivated while citrate synthase was induced. The steady-state concentration of citrate synthase was lower than during aerobic growth on citrate, this being attributed to the catabolite repression of the enzymes of the citric acid cycle by glucose (Gray et al., 1966; Magasanik, 1961). The culture when shifted from "anaerobic citrate" to "anaerobic glucose" surprisingly showed no inactivation of citrate lyase. However, there were problems in growth, probably as a result of a futile cycle taking place with a consequent depletion of glutamate in the cells, since this could be partially overcome by addition of 2-oxoglutarate to the culture medium. Citrate lyase was observed to be inactivated only under aerobic conditions. No enzyme responsible for inactivation could be detected in such cultures. Hence it was assumed that oxygen or the respiratory chain was involved in the inactivation. That oxygen is not directly involved in inactivation was shown by growth shift from "anaerobic citrate" to "anaerobic glucose plus nitrate", wherein the enzyme was inactivated indicating probably the involvement of the respiratory chain. When K. aerogenes cells grown in batch culture with citrate under anaerobic conditions were suspended in glucose medium and allowed to grow aerobically, citrate lyase was completely inactivated within 90 min. Chloramphenicol delayed the

inactivation considerably while 2,4-dinitrophenol prevented it. The inactivation was therefore assumed to be dependent on electron transport processes in the membrane. Citrate lyase was thus shown to behave like some other systems which are inactivated in presence of an energy source such as the nitrate reductase of K. aerogenes, Proteus mirabilis and E. coli (de Groot and Stouthamer, 1969; 1970a,b,c; Oltmann et al., 1976; Showe and Moss, 1968; Van'T Riet et al., 1968) and aspartate transcarbamylase in Bacillus subtilis (Waindle and Switzer, 1973).

SivaRaman and SivaRaman (1979) suggested that a mode of regulation of the enzyme from K. aerogenes may be through allosteric modifications. They reported evidence for a conformational change in the enzyme complex on binding of a divalent metal like Mn^{2+} . The metal-free enzyme does not catalyse the cleavage reaction. The energy-dependent in vivo inactivation was assumed to be through the formation or utilization of metabolite(s) which can directly act as modulators or help in complexing Mg^{2+} . The abstraction of Mg^{2+} from the metal-enzyme complex by ATP could result in regulation through conformational modulation. Blair et al. (1967) had speculated on a regulatory role of ATP from the observed in vitro inactivation of citrate lyase by ATP.

(11) Regulation in Rhodospirillaceae: R. gelatinosa readily utilizes citrate for growth under anaerobic conditions in the presence of light (Schaab et al., 1972). The mechanism of regulation of the enzyme activity in this source has been

shown by Gottschalk and his coworkers to differ from that of K. aerogenes and to be through covalent modifications. A citrate lyase deacetylase which had been shown to be present in this organism (Giffhorn et al., 1972) has been purified and characterized (Giffhorn et al., 1980). The deacetylase is activated only after citrate is exhausted from the medium (Giffhorn et al., 1972). When citrate is present as a substrate, the citrate lyase is converted from the inactive deacetyl form to active acetyl form by a citrate lyase ligase which is active only under anaerobic conditions and in presence of citrate and light (Giffhorn and Gottschalk, 1975a). On depletion of citrate, citrate lyase ligase is inactivated rapidly (Antranikian et al., 1978) and citrate lyase is deacetylated by the activated deacetylase (Giffhorn and Gottschalk, 1975a,b). The deacetylase is a small protein (M_r 14 300) and is highly specific with respect to the source of citrate lyase, not deacetylating citrate lyases from S. diacetilactis and K. aerogenes. R. gelatinosa citrate lyase deacetylase is synthesized along with citrate lyase. The deacetylase is strongly inhibited by L-glutamate and is thus regulated by the glutamate pool in the cells (Giffhorn and Gottschalk, 1975b). The intracellular concentration of L-glutamate which is dependent on the concentration of citrate acts as the signal which determines the availability of citrate for cleavage by the citrate lyase to oxaloacetate and acetate at high levels through inactivation of the deacetylase and the inactivation of the lyase by the

deacetylase at low levels rendering the citrate available for synthesis of L-glutamate via acetyl-CoA (Giffhorn et al., 1980). The citrate lyase ligase has been purified and crystallized along with citrate lyase by Antranikian and Gottschalk (1982). This enzyme has also been shown to be specific, acetylating the lyases only from R. gelatinosa and the closely related R. palustris (Giffhorn and Kuhn, 1980). Antranikian et al. (1978) have shown that cells of R. gelatinosa which have been grown on citrate, washed, suspended in buffer and incubated in light under anaerobic conditions contain both inactive citrate lyase as well as citrate lyase ligase. On addition of citrate, the ligase is rapidly activated which in turn activates the lyase.

The regulation of citrate lyase in R. palustris has been shown to follow the same pattern as in R. gelatinosa, viz. acetylation by citrate lyase ligase and deacetylation by citrate lyase deacetylase (Giffhorn and Kuhn, 1980; Giffhorn et al., 1981).

(iii) Regulation in C. sphenoides: Antranikian et al. (1982) have reported that the regulation of citrate lyase in C. sphenoides is by changes in the intracellular concentration of L-glutamate. The enzyme is active only in presence of glutamate. While in the Rhodospirillaceae, the regulatory action of glutamate is indirect through its effect on the deacetylase, in the Clostridium glutamate interacts directly with citrate lyase. The inactive lyase is not deacetylated in absence of glutamate but apparently is modulated conformationally. Electron microscopy of this citrate lyase

did indicate such an effect; the complex being present in undefined structures in the absence of glutamate and as star and ring forms in presence of the ligand.

(iv) Regulation in *P. rettgeri*: Under anaerobic conditions, citrate lyase has been observed to be induced, whereas citrate synthase is repressed. Oxaloacetate which is formed from citrate in the reaction catalysed by citrate lyase is reduced to succinate. It has been shown that oxaloacetate decarboxylase is not formed in this organism (Kroger et al., 1974).

(v) Regulation in other microorganisms: *S. diacetilactis* and *L. citrovorum* do not contain citrate synthase, consequently no antagonistic pathways are present and citrate lyase in these organisms are not regulated.

Aerobacter cloacae can be grown aerobically as well as anaerobically on citrate without the presence of Na^+ in the medium (O'Brien and Geisler, 1974). Under anaerobic conditions citrate lyase is induced, α -keto glutarate dehydrogenase being repressed and citrate is catabolised by the fermentation pathway. Citrate is metabolised aerobically via the citric acid cycle as α -keto glutarate dehydrogenase is induced and citrate lyase but not oxaloacetate decarboxylase is repressed. When the cells are grown under aerated conditions, only citrate lyase and not oxaloacetate decarboxylase is induced and hence the fermentation pathway is inoperational beyond the initial cleavage reaction (O'Brien, 1975). Both the fermentation pathway as well as the citric acid cycle is reported to be operative simultaneously under such conditions

causing considerable loss in energy resulting in poor cell yields.

Na^+ is required for the growth of S. typhimurium on citrate under aerobic conditions (O'Brien et al., 1969). The enzymes of the fermentation pathway as well as those of the citric acid cycle are present under these conditions. It is presumed that citrate is catabolised by the two different pathways, although the mode of regulation is not known yet. Kulla (1983) studied the regulatory mechanisms in S. typhimurium mutants. Citrate lyase could not be removed from this organism and the isolated mutants had an altered citrate regulatory system with the resulting phenotype resembling E. coli. According to Kulla (1983), citrate lyase in E. coli and S. typhimurium functions protectively rather than serving a catabolic purpose by probably reducing the high internal levels of citrate, which are likely to cause problems in certain vital cell functions by chelation of essential divalent cations.

I.4 CITRATE UPTAKE IN BACTERIA

(i) K. aerogenes: Dagley and Dawes (1953a,b) observed that in K. aerogenes, citrate was utilized after a marked lag period. This led Davis (1956) to suggest that the transport of citrate is induced only in the presence of the substrate. Villarreal-Moguel and Ruiz-Herrera (1969) and Wilkerson and Eagon (1972) showed that the induced carrier activity was associated with the cell membrane and was dependent upon protein synthesis.

Sachan and Stern (1971a) provided evidence for citrate transport in K. aerogenes NCTC 418 being mediated by the membrane bound, Na^+ -requiring oxaloacetate decarboxylase. Na^+ was shown to be essential for the anaerobic growth of the organism on citrate as sole carbon source. Strains of K. aerogenes have shown differences in the metal ion requirement for citrate transport. K. aerogenes UGa-I was shown by Eagon and Wilkerson (1972) to be K^+ dependent. It was later shown by Wilkerson and Eagon (1974) that Na^+ inhibited the uptake by K. aerogenes both in the UGa-I as well as in ATCC 12658 strains, while Na^+ was without effect in strain PRI-R3 of the organism.

(ii) S. faecalis: In the case of S. faecalis, citrate uptake is also apparently mediated by its oxaloacetate decarboxylase. The decarboxylation of oxaloacetate as well as citrate utilization have been shown to require Ca^{2+} and biotin (Sachan and Stern, 1971b).

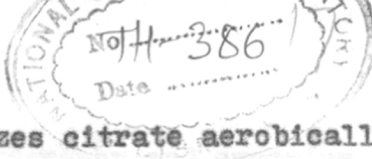
(iii) S. diacetylactis: Harvey and Collins (1962) reported that in S. diacetylactis, in which citrate lyase is constitutive, the transport system is induced in the presence of the substrate and by an energy requiring process. The appearance of spontaneous cryptic Cit^- -mutants of S. diacetylactis which had lost the ability for citrate uptake while retaining citrate lyase activity was reported by Collins and Harvey (1968). Kempler and McKay (1979) observed that citrate transport in this organism is plasmid linked as mutants which had lost the ability for citrate

uptake had also lost a 5.5 megadalton plasmid. One of the mutants (DRC₁-X) was believed to have undergone a point mutation as it showed the presence of the 5.5 megadalton plasmid even though it was Cit⁻.

(iv) E. coli: The inability of E. coli to utilize citrate as the sole source of carbon and energy is a criterion for its classification (Koser, 1923). Later work by Vaughn et al. (1950) showed that under anaerobic conditions, E. coli could degrade citrate if peptone and carbohydrates were also present in the medium. Under anaerobic conditions E. coli utilizes citrate in the presence of a cosubstrate such as glucose or lactose (Lütgens and Gottschalk, 1980). The nature and the specific requirements of the transport system are not yet known.

The failure of E. coli to utilize citrate under aerobic conditions has been attributed to the absence of a transport system (Lara and Stokes, 1952). There are several recent reports, however, of strains of E. coli isolated from a variety of birds and mammals which utilize citrate aerobically, an activity associated with the presence of Cit⁺ plasmids (Sato et al., 1978; Ishiguro et al., 1979; Ishiguro and Sato, 1980). The positive aerobic citrate marker has been located within a transposon of complex structure in the plasmid (Ishiguro et al., 1982).

Some thermosensitive H¹ plasmids from strains of chloramphenicol-resistant S. typhimurium transferred into a prototrophic E. coli K-12 strain have the ability to utilize citrate as sole energy source (Smith et al., 1978). Hall (1982) has isolated a spontaneous double mutant of E. coli



K-12 which utilizes citrate aerobically as sole source of carbon and energy. The data on citrate utilization indicated that the citrate uptake was not dependent on the presence of any plasmid but was the result of a mutation in two specific genes which probably constitute the citrate transport system.

(v) S. typhimurium: Citrate transport in S. typhimurium has been reported by Imai et al. (1973) and Kay and Cameron (1978) to involve three transport systems which are induced by citrate and its analogues. S. typhimurium strain LT2 which is well characterized genetically resembles K. aerogenes in its ability to utilize citrate both aerobically as well as anaerobically. Citrate lyase could not be deleted from mutants of the LT2 strain and this has led to the suggestion that the enzyme probably serves the vital role of scavenging citrate which might otherwise interfere with cell function by its metal chelating capacity (Kulla, 1983).

I.5 PURIFICATION OF CITRATE LYASE

The enzyme has been obtained pure from several sources such as K. aerogenes, S. diacetilactis, R. gelatinosa, S. faecalis, E. coli and C. sphenoides.

(1) Citrate lyase from K. aerogenes: The enzyme was obtained pure for the first time from this source (SivaRaman, 1961). Dagley and Dawes (1955) had described earlier a partial purification of the enzyme from this source. The pure enzyme is obtained from K. aerogenes NCTC 418 by a procedure involving streptomycin sulphate treatment, adsorption on alumina C gel and selective elution from the gel, DEAE-

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cellulose chromatography and ammonium sulphate fractionation. Some modifications of this procedure are gel filtration as an additional step (Mahadik and SivaRaman, 1968; Singh and Srere, 1975), treatment with salmine sulphate and CM-cellulose in place of alumina C_6 and DEAE-cellulose chromatography (Bowen and Mortimer, 1971). The preparations were homogeneous both ultracentrifugally (SivaRaman, 1961) and electrophoretically (Bowen and Rogers, 1963a; Singh and Srere, 1971). The purified enzyme has an absorbance index $E_{1\text{ cm}}^{1\%}$ of 6.2 at 278 nm and a specific activity of $73 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ at pH 7.4, 28°C and 10 mM MgCl_2 (Singh et al., 1976). Enzyme of a specific activity of about $90 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ protein was obtained by Tikare (1979) from this source by introducing an activation step with ATP and acetate initially.

Dimroth et al. (1977b) have described a procedure for the large scale isolation of the enzyme. This involves the precipitation of the alumina C_6 gel eluate with polymin HS (ethyleneimine polymer), dialysis and gel filtration through Sephadex G-200.

(ii) Citrate lyase from *S. diacetilactis*: Harvey and Collins (1963) and Ward and Srere (1965) reported a partial purification of the enzyme from this source. The pure enzyme was obtained later by Singh and Srere (1975) and Kümmel et al. (1975). The former used protamine sulphate treatment followed by fractionation with ammonium sulphate, gel filtration and DEAE-cellulose chromatography. The specific activity of the purified enzyme was reported to be $200 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ at pH 7.2 in presence of 2 mM MgCl_2 at 25°C . The enzyme was homogeneous in the ultracentrifuge. The method

of Kümmel et al. (1975) involved treatment with cetyltrimethylammonium bromide, alumina C_{γ} gel adsorption, ultrafiltration of the supernatant followed by gel filtration and DEAE-Sephadex A-50 chromatography. The purity of the enzyme was established by ultracentrifugation and polyacrylamide gel electrophoresis and the preparation had a specific activity of $125 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 7.2 in presence of 2 mM MgCl_2 . Citrate lyase ligase activity associated with this preparation (Kümmel et al., 1975) was shown to be an impurity which could be removed by alumina C_{γ} gel treatment (Bowien and Gottschalk, 1977).

(iii) Citrate lyase from *R. gelatinosa*: Beuscher et al. (1974) obtained homogeneous preparations of the enzyme from this source. Their procedure involves treatment of cell extracts with protamine sulphate, DEAE-cellulose chromatography and gel filtration. The preparation was homogeneous under conditions of sedimentation equilibrium and in polyacrylamide gel electrophoresis and had a specific activity of $139 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 7.2, 30°C and in presence of 3 mM MgCl_2 . The procedure has been modified by Giffhorn and Gottschalk (1978) to obtain the enzyme in a crystalline form. The purification steps include cetyltrimethylammonium bromide treatment followed by alumina C_{γ} gel adsorption and DEAE-cellulose chromatography. The enzyme fractions were concentrated by ultrafiltration. Crystals which were formed were recrystallized in presence of 0.1 M KCl. The recrystallized enzyme had a specific activity of $360 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$:

(iv) Citrate lyase from *S. faecalis*: The enzyme from this

source has been purified by Hiremath et al. (1976). The procedure of purification involves treatment of the sonic extracts with ATP and acetate to reactivate any deacetylated enzyme, followed by streptomycin sulphate treatment. Further steps include adsorption and elution of the enzyme from alumina C_{γ} gel, ammonium sulphate fractionation, DEAE-cellulose chromatography and gel filtration. Homogeneity of the preparation was checked by analytical ultracentrifugation and polyacrylamide gel electrophoresis. The pure enzyme had a specific activity of $90 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 8.0, 30°C and in presence of 20 mM MgSO_4 .

(v) Citrate lyase from E. coli: The enzyme from this source was partially purified by Dagley and Dawes (1955), Wheat and Aji (1955a) and Bowen and SivaRaman (1960). The enzyme was obtained pure by Nilekani and SivaRaman (1983). The purification process involves treatment with streptomycin sulphate followed by treatment with salmine sulphate, CM-cellulose and alumina C_{γ} gel, fractionation by ammonium sulphate, DEAE-cellulose chromatography and gel filtration. The homogeneity of the enzyme preparation was established by separation into its three biologically active subunits. The holoenzyme, however, showed multiple states of aggregation in the ultracentrifuge and was polydisperse in polyacrylamide gel electrophoresis. The purified enzyme has a specific activity of $120 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 8.0 and in presence of 10 mM MgSO_4 .

(vi) Citrate lyase from C. sphenoides: Antranikian et al. (1982) obtained a 72-fold purification of the enzyme from this

source. The enzyme was found to be active and stable only in presence of L-glutamate, which was therefore included in all the buffers used in the steps of purification. Cell extracts were treated with protamine sulphate, the activity fractionated with ammonium sulphate, followed by filtration through gel and concentration by ultrafiltration. The enzyme was then incubated with ATP and acetate to fully activate the enzyme. This was followed by differential sedimentation in a sucrose density gradient, concentration of the activity and gel filtration. The specific activity of the enzyme was $60 \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 7.2, 30°C and in presence of 10 mM L-glutamate and 3 mM MgCl_2 .

I.6 PROPERTIES OF CITRATE LYASE

(1) Stability:

A process of inactivation of citrate lyase is through loss of essential acetyl groups. When catalytically active, pure *K. aerogenes* [^{14}C]-acetyl citrate lyase was stored at 4°C for 4 days, nearly a third of its activity as well as an equivalent proportion of protein-bound radioactivity were lost, implicating spontaneous deacetylation as the cause of the inactivation (Buckel et al., 1971a). Complete inactivation results on deacetylation with neutralized hydroxylamine or dithiothreitol/dithioerythritol. The inactive enzyme is reactivated chemically with acetic anhydride (Buckel et al., 1971a) or enzymatically with citrate lyase ligase in presence of ATP and acetate (Schmellenkamp and Eggerer, 1974).

The stability of solutions of the pure enzyme was greater at higher protein concentrations (Bowen and Rogers, 1963a). Enzyme at a concentration of 0.04% rapidly lost all its activity at 20°C within 2 h, while a 0.3% solution lost 20% activity in 5 h at 20°C. The half life of a 0.3% enzyme solution at -14°C was 4 weeks. Crude extracts were found to be more stable than purified enzyme at 55°C. Cysteine does not stabilize the enzyme.

Eisenthal et al. (1966) have reported that in the presence of Mg^{2+} the extent of inactivation of the enzyme in dilute solutions is reduced. Blair et al. (1967) recognized two types of inactivation processes in dilute solution, a reversible and an irreversible process. Inactivation at low Mg^{2+} concentrations (0.025 - 1.0 mM) could partly be reversed by increasing the concentration of Mg^{2+} (2 mM). At low concentrations of Mg^{2+} , adenine nucleotides and EDTA increased the inactivation, while Ca^{2+} , which does not form an active complex with the enzyme, protected against inactivation. It was observed that both the reversible and the irreversible processes could be retarded by higher Mg^{2+} concentrations. Gruber and Moellering (1966) have reported that partially purified enzyme preparations lose only 10% of activity when freeze-dried with bovine serum albumin, sucrose and $MgSO_4$ and stored in the cold for a period of 1 month.

The E. coli enzyme is relatively unstable in crude extracts. Wheat and Ajl (1955a) have reported that partially purified E. coli enzyme is extremely unstable and loses its activity either in dilute solutions or on prolonged dialysis.

Nilekani (1980) has reported that the purified E. coli enzyme is stable in presence of saturated ammonium sulphate at 0°C in 0.05 M potassium phosphate buffer, pH 7.4 containing 1.6 mM MgSO_4 . Freezing and thawing of the enzyme preparations led to the loss of activity either in presence or absence of ammonium sulphate.

The enzyme from R. gelatinosa has been shown by Beuscher et al. (1974) to be present mainly in a deacetylated and oxidized form after the purification steps. Such preparations could be reduced with mercaptans and reactivated chemically. The crystalline enzyme from R. gelatinosa retained its activity for 8 months when stored at 4°C in the presence of 3 M ammonium sulphate (Giffhorn and Gottschalk, 1978).

Partially purified S. diacetilactis enzyme lost its activity rapidly if left overnight, while the purified enzyme retained most of its activity when stored for weeks at -90°C (Singh and Srere, 1975). It was also observed that the enzyme was stable at pH values upto 8.1, while at pH 8.3 - 8.7, dissociation of the complex occurred with loss in activity.

The purified enzyme from S. faecalis was stable when stored at 0°C or -20°C only in the presence of ammonium sulphate. Repeated freezing and thawing of the enzyme led to its inactivation with formation of artifacts (Hiremath et al., 1976).

The pure enzyme from C. sphenoides could be stored at 4°C for 4 days or at -20°C for 2 months, without appreciable

loss in activity (Antranikian et al., 1982). At 30°C, almost half the activity was lost within 4 h. The stability of this enzyme was shown to be markedly improved by the presence of L-glutamate. At low concentrations of glutamate (7 mM) and at 0°C, the enzyme was inactivated within 3 min. A glutamate concentration of 150 mM or more was required for stabilizing the enzyme. Loss of enzymatic activity in absence of L-glutamate could be reversed by the addition of L-glutamate. The presence of high concentrations of glycerol (30%), sucrose (40%) or bovine serum albumin (10 mg/ml) together with L-glutamate (7 mM) resulted in only a marginal loss in activity at 0°C. The reversible inactivation in the absence of glutamate was observed to follow a transition from ring and star shapes seen in electron micrographs to undefined shapes.

(ii) pH Optimum

The optimum pH for the reaction catalysed by citrate lyase is around 8.0 in case of K. aerogenes (Dagley and Dawes, 1955), E. coli (Dagley and Dawes, 1955; Wheat and Ajl, 1955a; Nilekani, 1980) and S. faecalis (Hiremath et al., 1976), while for the enzymes from S. diacetylactis (Harvey and Collins, 1963) and R. gelatinosa (Beuscher et al., 1974); The reported pH optimum is 7.0 - 7.3. The S. diacetylactis enzyme tends to dissociates at pH values of about 8.5 (Singh and Srere, 1975). The optimum pH for the C. sphenoides enzyme is around 6.5 - 7.0 (Antranikian et al., 1982).

(iii) Metal requirment

Requirement of divalent metal ions like Mg^{2+} , Mn^{2+} , Zn^{2+} ,

Fe^{2+} , Co^{2+} but not Ca^{2+} , Sr^{2+} , Ba^{2+} , Cu^{2+} and Hg^{2+} was shown by Dagley and Dawes (1955) in both K. aerogenes and E. coli citrate lyase preparations which had been partially purified. It was also shown that with the E. coli and K. aerogenes enzymes, Ca^{2+} acts as a competitive inhibitor of enzyme activation by other metal ions. Harvey and Collins (1963) have confirmed these results in the case of the enzyme from S. diacetylactis.

Observations of the activation by different metal ions led Dagley and Dawes (1955) to postulate that metals which activated the enzyme had a restricted ionic radius in the range of $0.72 \pm 0.08 \text{ \AA}$ while metals like Ca^{2+} , with an ionic radius of $0.99 \text{ \AA}$ were out of the range. They suggested that the enzyme would interact with the metal chelate complex of citrate. Harvey and Collins (1963) presented indirect evidence in favour of this suggestion by showing that the optimum pH range of partially purified S. diacetylactis enzyme corresponds to the range in which stable complexes of metal and substrate could be formed. It was also observed that maximum activity could be obtained only when both metal and substrate were present in equimolar ratios, higher concentrations of the metal ion causing inhibition.

Ward and Srere (1965) provided evidence against the view that a metal-substrate complex acts as the true substrate for the enzyme. Pulsed nuclear magnetic resonance studies made by them on partially purified enzyme from S. diacetylactis indicated the formation of a metal-enzyme binary complex which presumably acted on free citrate. The dissociation constant

K_d calculated for Mn^{2+} -enzyme was 4×10^{-5} M. No evidence could be obtained for the formation of a ternary metal-enzyme-citrate complex. However, the probability of a low steady-state concentration of the ternary complex being an alternative reason for the failure to detect the ternary complex was also recognized by them. The NMR studies confirmed the earlier observation of competition between Ca^{2+} and Mg^{2+} .

SivaRaman and SivaRaman (1979) have studied the metal binding properties of purified K. aerogenes citrate lyase. Sedimentation behaviour of metal-free enzyme as well as of the enzyme in presence of Mg^{2+} or Mn^{2+} were similar in the presence of the normal buffers, indicating no change in the state of aggregation. Equilibrium dialysis studies using radioactive $^{54}Mn^{2+}$ indicated positive cooperativity in metal binding. The Hill coefficient was 2.27 ± 0.05 , indicative of strong positive cooperativity. The Scatchard plot indicated total positive cooperativity in metal binding. The total number of Mn^{2+} binding sites per mol of enzyme complex was calculated to be 18. It was concluded that binding of a divalent metal ion results in a conformational change in the enzyme complex making it catalytically active in the cleavage reaction.

In R. gelatinosa, Mg^{2+} , Mn^{2+} and Co^{2+} have been shown to be the most effective activators of the enzyme, Zn^{2+} and Ni^{2+} being only slightly effective and Cu^{2+} and Fe^{2+} having no effect at all (Beuscher et al., 1974). The enzyme from

S. faecalis shows the highest activation with Mg^{2+} , followed by Mn^{2+} , Zn^{2+} and Fe^{2+} (Hiremath, 1977). Co^{2+} and Cu^{2+} show feeble activation while Ca^{2+} , Ba^{2+} , Hg^{2+} and Ni^{2+} are without any effect on the enzyme. In the case of the C. sphenoides enzyme, the highest activity is expressed in the presence of Mg^{2+} , while Mn^{2+} and Co^{2+} are less effective (Antranikian et al., 1982b). Zn^{2+} , Ni^{2+} , Cu^{2+} and Ca^{2+} do not activate the enzyme. The purified E. coli enzyme resembles the enzyme from S. faecalis in its activation by metals. Mg^{2+} is the most effective activator followed by Mn^{2+} , Zn^{2+} , Fe^{2+} and Co^{2+} in order of effectiveness; Cu^{2+} shows very low activity while Ca^{2+} , Ba^{2+} , Hg^{2+} and Ni^{2+} do not activate (Nilekani and SivaRaman, 1983).

Citrate lyase is a multienzyme complex with two distinct enzymes, an acyl-transferase which catalyses the formation of a citryl-enzyme intermediate and an acyl-lyase which catalyses the subsequent cleavage of the intermediate to oxaloacetate and the active acetyl-enzyme. Requirement of metal has been shown to be only for the lyase reaction and not for the transferase reaction (Buckel et al., 1973; Dimroth and Eggerer, 1975b).

(iv) Reaction kinetics

Early studies on the stoichiometry of the breakdown of citrate to oxaloacetate and acetate showed that with the enzymes from K. aerogenes (Dagley and Dawes, 1953a; b; Dagley and Dawes, 1955) and E. coli (Dagley and Dawes, 1955; Wheat and Ajl, 1955b), a mol of citrate is cleaved to a mol each of oxaloacetate and acetate. Dagley and Dawes (1955)

also showed that extracts of K. aerogenes and E. coli treated with Dowex-1, so as to remove coenzyme-A retained activity and that no enhancement in activity results on exogenous addition of CoA.

The oxaloacetate formed on cleavage of citrate by S. diacetilactis citrate lyase was characterized as the keto-form, by spectral analysis (Harvey and Collins, 1963; Ward and Srere, 1965). The enzyme from K. aerogenes was also shown to yield the keto-isomer in experiments carried out at low temperatures (2°C) and at pH 7.4 when spontaneous equilibration of the oxaloacetate tautomers is slowed down. The keto-isomer of oxaloacetate is also involved in the reaction catalysed by citrate synthase (Englard, 1959).

The overall reaction catalysed by citrate lyase is towards citrate cleavage. The reaction goes virtually to completion in presence of a large excess of the enzyme (Wheat and Ajl, 1955b; Bowen and Rogers, 1965). The reversibility of the reaction was demonstrated only through radioactive labelling of the citrate using $[^{14}\text{C}]$ -acetate, oxaloacetate and Mg^{2+} in presence of the enzymes from S. faecalis (Gillespie and Gunsalus, 1953) and from K. aerogenes (Bowen and Rogers, 1963b).

The equilibrium constant for the cleavage of citrate to oxaloacetate and acetate has been calculated to be around 1 M by Burton (1955). There have been many reports on the determination of the equilibrium constant of the reaction catalysed by citrate lyase (Smith et al., 1955; Harvey and Collins, 1963). These do not have much significance as the

reaction catalysed by citrate lyase is now known to be a sequence of two reactions and susceptible to reaction inactivation.

Tate and Datta (1965) had pointed out some of the other difficulties namely (1) Interactions of substrate and commonly used buffers with Mg^{2+} and oxaloacetate, (2) Formation of citrate and Mg^{2+} complexes, (3) Existence in aqueous solutions of both keto- and enol-isomers of oxaloacetate and formation of oxaloacetate- Mg^{2+} complexes.

Tate and Datta (1965) calculated the value of the equilibrium constant, K , $(\text{citrate}^{3-})/(\text{OAA}_{\text{keto}}^{2-}) \cdot (\text{acetate}^{1-})$ for citrate cleavage to be $3.08 \pm 0.72 \text{ M}^{-1}$ at 25°C using limiting concentrations of citrate (2 mM) and excess $MgCl_2$ in presence of triethanolamine-HCl buffer, pH 8.4, which does not complex Mg^{2+} .

The value of K , calculated from data for citrate synthesis showed variability due to inhibition of the enzyme by high concentrations of oxaloacetate and slowness of the reaction which resulted in the failure to reach equilibrium. Guyn et al. (1973) have reported the observed equilibrium constant (K_{obs}) determined under near physiological conditions (38°C , 0.25 M phosphate buffer, pH 7.0 and 10^{-3} M free Mg^{2+}) to be $2.22 \pm 0.16 \text{ M}^{-1}$. The value of K_{obs} varied with changes in magnesium concentrations. At free $(Mg^{2+}) = 0$, K_{obs} was $1.00 \pm 0.07 \text{ M}^{-1}$ and at free $(Mg^{2+}) = 10^{-2}$ M, K_{obs} was approximately 9. In these determinations, problems of inhibition were avoided by use of lower substrate concentrations and

there was good agreement between K_{obs} determined in the forward and backward directions.

At the time when the kinetics of the enzyme catalysed reaction were studied as described above, many of the factors given below were not known:

- (1) Multienzyme nature of citrate lyase having two distinct enzymatic activities (Dimroth and Eggerer, 1975^b).
- (2) The unusual phenomenon of reaction-inactivation (Singh and Srere, 1971), due to the spontaneous hydrolysis of the citryl-intermediate resulting in the formation of a deacetyl enzyme. The early studies on the kinetics of the enzyme catalysed reaction, therefore, do not have much significance.

(v) Stereochemistry

The stereochemical specificities of the citrate enzymes have been exclusively studied (Eggerer et al., 1970; Retey et al., 1970; Lenz et al., 1971; Wunderwald et al., 1971; Srere, 1975). To summarise the data: All the citrate enzymes except re-citrate synthase exhibit similar stereochemistry with regards the C-3 prochiral centre of citrate. In the reaction catalysed by si-citrate synthase, the attack of acetyl-CoA is at the si-face of oxaloacetate to form citrate having C-4 and C-5 derived from the acetyl moiety (Hanson and Rose, 1963). Both citrate lyase and ATP-citrate lyase show similar stereospecific behaviour. Citrate lyases from S. faecalis (Gillespie and Gunsalus, 1953) and E. coli (Wheat and Ajl, 1955^b) cleave citrate in such a manner that C-4 and C-5 of citrate, which are derived from acetyl-CoA in the reaction catalysed by si-citrate synthase, yield

acetate exclusively. Buckel et al. (1971a) have confirmed this by using radioactive labeled citrate and citrate lyase from K. aerogenes. When $[^{14}\text{C}]$ (3S)-citrate was used as the substrate, $[^{14}\text{C}]$ -acetyl-citrate lyase complex was formed, whereas $[1-^{14}\text{C}]$ (3S)-citrate resulted in the formation of unlabeled enzyme and $[^{14}\text{C}]$ -labeled oxaloacetate. Srere and Bhaduri (1964) have shown that ATP-citrate lyase also cleaves citrate so that the pro-3S-CH₂COOH is released as acetyl-CoA. The stereospecificity of re-citrate synthase is the reverse in that C-1 and C-2 of citrate (pro-3R-CH₂COOH) are derived from acetyl-CoA (Gottschalk and Barker, 1966; Stern and Bambers, 1966).

si-Citrate synthase, re-citrate synthase, citrate lyase and ATP-citrate lyase all show similar stereospecificity with respect to the two prochiral centres involving the methylene groups on C-2 and C-4 of citrate. Using synthetic chiral acetate, (R)-(²H, ³H)-acetate and (S)-(²H, ³H)-acetate, it has been shown that all the citrate enzymes cause inversion of the configuration at the methylene groups of citrate, which become the methyl group of acetate (Eggerer et al., 1970; Retey et al., 1970; Klinnan and Rose, 1971; Buckel et al., 1971b).

(vi) Product inhibition

Citrate lyases from E. coli and K. aerogenes were first shown by Dagley and Dawes (1955) to be irreversibly inhibited by oxaloacetate, a product of the reaction. Neither acetate nor pyruvate was found to inhibit the enzyme. Bowen and Rogers (1963a) observed that inhibition by oxaloacetate was

time and concentration dependent and there was no differences in the spectral or sedimentation profiles of the active and the inactivated enzymes. They also showed that while malate like oxaloacetate irreversibly inactivated the enzyme, oxalate, succinate, oxaloglutarate, tartarate, oxalosuccinate and isocitrate had no effect.

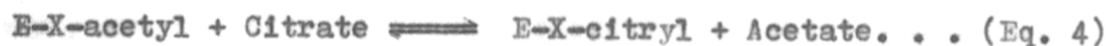
Eisenthal et al. (1966) studied the conditions under which oxaloacetate inhibits the enzyme. Oxaloacetate and its analogues were shown to require the presence of metal ions like Mg^{2+} and the inhibition was shown not to be reversed by malate dehydrogenase in presence of NADH. The profile of pH dependence of oxaloacetate inhibition indicated the involvement of the enolic form of oxaloacetate and it was postulated that two different Mg^{2+} -enolic oxaloacetate complexes are likely to be involved. This was supported by the finding that among the oxaloacetate analogues studied, only α,α -dimethyloxaloacetate which cannot enolise and ketomalonate did not inhibit the enzyme in presence of Mg^{2+} , while the analogues which could give enolic forms were inhibitory. The authors suggested that the structural requirement for inhibition was a C_4 straight chain, 1,4-dicarboxylic acid having an ionizable α -hydroxyl group. Inhibition by oxaloacetate was found to be dependent on the nature of the divalent metal ion; inhibition in presence of Co^{2+} was more severe than with Mg^{2+} , while Mg^{2+} was more effective than Ca^{2+} .

Buckel et al. (1971a) have shown that inhibition by oxaloacetate is due to the loss of essential acetyl groups of the enzyme with the formation of the inactive deacetyl citrate

lyase.

(vii) Essential acetyl group of citrate lyase

Buckel et al. (1971a) were the first to report the involvement of an essential acetyl group in the reaction mechanism of citrate lyase. They suggested that the acetyl group was linked covalently in this-ester linkage at the catalytically active site of the enzyme. The enzyme lost its activity on deacetylation. Stereospecifically labeled $[5-^{14}\text{C}]$ (3S) citrate was cleaved by citrate lyase to $[^{14}\text{C}]$ -labeled enzyme and unlabeled oxaloacetate, while $[1-^{14}\text{C}]$ (3R) citrate yielded unlabeled enzyme and $[^{14}\text{C}]$ -oxaloacetate. This could be explained as a result of the turnover of acetate during the course of the reaction catalyzed by the enzyme. Thus the overall reaction was recognized to be the sum total of two partial reactions, an acyl exchange reaction (Eq. 4) and an acyl lyase reaction (Eq. 5), with the cleavage at the si-face of oxaloacetate.



Buckel et al. (1971a) gave further evidences for the presence of the essential acetyl moiety at the active site: (a) When active $[^{14}\text{C}]$ -labeled enzyme was treated with hydroxylamine, the products obtained were $[^{14}\text{C}]$ -acetohydroxamic acid and inactive enzyme. Incubation of the $[^{14}\text{C}]$ -acetyl enzyme with 1 mM oxaloacetate and 1 mM MgCl_2 resulted in the loss of the label and of enzyme activity.

(b) Treatment of [^{14}C]-acetyl labeled enzyme with cold citrate followed by gel filtration through Sephadex resulted in the formation of low molecular weight radioactive products and unlabeled protein. This was an indication of an acyl exchange reaction taking place during the enzyme catalysed reaction.

(c) The kinetics of enzyme inactivation by hydroxylamine were similar to the hydroxylaminolysis of standard thio-esters like N-succinyl-S-acetylcysteamine. Citrate lyase could also be inactivated by mercaptans like dithiothreitol and dithioerythreitol. This was suggestive of acetate being present in thio-ester linkage with the enzyme.

(d) Citrate lyase which was inactivated either by hydroxylamine, mercaptans or oxaloacetate and Mg^{2+} could be reactivated chemically using acetic anhydride. Srere et al. (1972) later showed that citrate lyase which was inactivated by hydroxylamine or by reaction inactivation, in the presence of DTNB, could be reactivated by acetic anhydride only after incubation with DTT.

Kümmel et al. (1975) have shown that deacetyl citrate lyase from several sources like K. aerogenes, S. diacetilactis, R. gelatinosa and L. citrovorum can be chemically reactivated by 1-acetyl imidazole.

Schmellenkamp and Eggerer (1974) have been able to characterize an enzyme from K. aerogenes which can reactivate deacetyl citrate lyase. The enzyme, acetate:SH(acetyl carrier protein) citrate lyase ligase (AMP) was partially purified from K. aerogenes and shown to reactivate only in presence of

ATP and acetate or acetyl adenylate. This enzyme has been purified partially by Hiremath et al. (1976) from S. faecalis and shown to reactivate deacetyl citrate lyase both from S. faecalis and K. aerogenes. Bowien and Gottschalk (1977) obtained an electrophoretically pure preparation of citrate lyase ligase from S. diacetilactis which is active only towards deacetyl citrate lyase from S. diacetilactis, having no effect on the deacetyl R. gelatinosa or K. aerogenes enzymes.

Buckel et al. (1973) characterized two types of inactive citrate lyase (1) deacetyl citrate lyase which can be reactivated by acetic anhydride and (2) carboxymethylated citrate lyase obtained on treatment of the deacetylated enzyme with iodoacetate, which cannot be reactivated by acetic anhydride. Though both were inactive towards citrate, they catalytically cleaved citrate in presence of acyl-CoA derivatives such as acetyl-CoA and citryl-CoA. Citrate is cleaved to acetate and oxaloacetate in presence of acetyl-CoA, while (3S)-citryl-CoA is stereospecifically cleaved to oxaloacetate and acetyl-CoA. In the acetyl-CoA-dependent cleavage of citrate, the acetyl moiety of ^{14}C -labeled acetyl-CoA was liberated as radioactive acetate and the pro-(3S)- CH_2COOH group of citrate was transformed to acetyl-CoA. Kinetic data suggested the formation of citryl-CoA as an intermediate, which was confirmed by isolation of (3S)-citryl-CoA which accumulates when the reaction is performed in presence of EDTA (Buckel et al., 1973). Citrate lyase was thus shown to be a multienzyme complex catalysing initially an acyl exchange reaction which is not Mg^{2+} dependent and a subsequent cleavage of the citryl-

intermediate in presence of Mg^{2+} . Buckel et al. (1973) showed that cleavage of citrate by native citrate lyase as well as though the acetyl-CoA dependent reaction catalysed by deacetyl citrate lyase required acetylated acyl carrier groups, acetyl-CoA in the case of deacetyl enzyme and acetyl-enzyme in the case of the native enzyme. Several acyl-CoA derivatives were checked for their ability to substitute for acetyl-CoA in the reaction catalysed by deacetyl citrate lyase (Buckel et al., 1973). It was shown that malonyl-CoA, R,S-malyl-CoA as well as acetyl thio-esters such as N, S-diacetyl cysteamine, N-succinyl-S-acetyl cysteamine were inactive, whereas acetyl-dephospho-CoA and propionyl-CoA (reaction given below) were active.



Buckel et al. (1973) also showed that butyryl-CoA and valeryl-CoA neither activated nor inhibited the reaction.

Evidence has been obtained to show that citrate lyases from other sources such as S. diacetilactis (Singh and Srere, 1975), R. gelatinosa (Kümmel et al., 1975), S. faecalis (Hiremath et al., 1976), L. citrovorum (Kümmel et al., 1975) and E. coli (Nilekani and SivaRaman, 1983) are also acetyl-enzyme.

(viii) Reaction inactivation

Wheat and Ajl (1955b) while working on partially purified E. coli preparations observed that the reaction rate rapidly dropped to zero and citrate cleavage stopped. This

they thought was due to inhibition by oxaloacetate, a product of the reaction. Singh and Srere (1971) later observed in K. aerogenes that preincubating the enzyme with 1000-fold excess of oxaloacetate (both in the keto- and enolic-forms) over the amount that is formed during the reaction did not cause any appreciable inactivation. They also showed that there was no change in the reaction rates nor in citrate cleavage on constantly removing the oxaloacetate formed during the reaction by coupling with malate dehydrogenase and NADH.

The reaction inactivation followed first order kinetics and the rate of inactivation varied with the nature of the divalent metal ion. Amongst the divalent metal ions studied, the reaction with Zn^{2+} resulted in the slowest rate of inactivation while the rate was the highest with Mg^{2+} (10 mM). Singh and Srere (1971) described such reaction inactivation, which differed from product inhibition as enzyme "suicide".

Although the mechanism of reaction inactivation has not been fully understood, it is known to take place as a result of the deacetylation of the enzyme to HS-citrate lyase, as the inactivated enzyme has been shown to be reactivated chemically by acetic anhydride (Srere et al., 1972). Buckel et al. (1973) observed the labile nature of citryl-CoA at alkaline pH and this was suggested as one of the causes of reaction inactivation. Another mechanism to explain reaction inactivation was proposed by Dimroth and Eggerer (1975b). The isolated α -subunit of the K. aerogenes enzyme complex was shown by them to hydrolyze citryl-S-acyl carrier protein to deacylated ACP with liberation of citrate leading to reaction

inactivation of the enzyme.

On the basis of their reaction-inactivation behaviour, citrate lyases from various sources can be classed into two distinct groups; those that undergo severe reaction inactivation such as the enzymes from K. aerogenes (Singh and Srere, 1971) and R. gelatinosa (Kümmel et al., 1975) and those that undergo relatively weak reaction inactivation such as the enzymes from S. diacetylactis (Singh and Srere, 1975), L. citrovorum (Kümmel et al., 1975) and S. faecalis (Hiremath et al., 1976). The reaction inactivation follows first order kinetics and the rate constant (K) for reaction inactivation of the enzymes from K. aerogenes, S. diacetylactis (Singh and Srere, 1975), S. faecalis (Hiremath, 1977), and E. coli (Nilekani and SivaRaman, 1983) with Mg^{2+} at 25°C were determined to be 1.21, 0.06, 0.07 and 0.32 min^{-1} respectively. In presence of Zn^{2+} , the values for the K. aerogenes and S. diacetylactis enzymes were 0.17 min^{-1} and 0.014 min^{-1} respectively.

Kümmel et al. (1975) had suggested earlier that the enzymes from S. diacetylactis and L. citrovorum show weak reaction inactivation probably due to the association of an HS-citrate lyase acetylating activity as a component of the native enzyme complex. Such activity was not observed by them in the enzymes from K. aerogenes and R. gelatinosa. Hiremath et al. (1976) reported the absence of any ligase activity associated with citrate lyase from S. faecalis, even though it shows weak reaction inactivation. Bowien and Gottschalk (1977) later observed that in S. diacetylactis

also the ligase activity which was detected earlier was an impurity which could be separated from native citrate lyase by an alumina C_{γ} gel adsorption step.

(ix) Molecular weight

Purified citrate lyase from K. aerogenes was shown to have a $S_{20,w}^{\circ}$ value of about 16 - 18 S (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 have the same size as the K. aerogenes enzyme. The enzyme from S. diacetilactis has a $S_{20,w}^{\circ}$ value of 16.8 S and a molecular weight of 585 000 (Singh and Srere, 1975). The R. gelatinosa enzyme has a molecular weight of 530 000 - 560 000 (Beuscher et al., 1974). The $S_{20,w}^{\circ}$ value for S. faecalis enzyme has been reported to be 17.1 S and its molecular weight 600 000 (Hiremath et al., 1976). Antranikian et al. (1982) have reported a $S_{20,w}^{\circ}$ value of 16.3 S and a molecular weight of 515 000 for the enzyme from C. sphenoides both in presence and absence of L- glutamate. The purified E. coli enzyme exhibits multiple forms of aggregation in the ultracentrifuge which are independent of parameters such as presence of metal ions, ionic strength of buffers, buffer pH, presence of mercaptans (Nilekani and SivaRaman, 1983).

The enzyme from K. aerogenes has been shown by Mahadik and SivaRaman (1968) to dissociate reversibly in low ionic strength buffers in absence of divalent metal ions. A half molecule of molecular weight 273 000 dalton sedimenting at

10.3 S was observed in 2 mM EDTA + 1 mM potassium phosphate buffer, pH 7.4 while another component was observed sedimenting at 6.3 S in the presence of 1 mM EDTA + 1 mM potassium phosphate buffer, pH 7.4. Increasing the buffer concentrations (50 mM) or presence of 2 mM Mg^{2+} completely reversed any dissociation to species of lower molecular weight. These observations were confirmed by Bowen and Mortimer (1971) and Dimroth et al. (1973). Hiremath (1977) has reported that the S. faecalis enzyme does not dissociate in low ionic strength buffers even in absence of divalent metal ions.

(x) Subunit structure

The early sedimentation studies by Mahadik and SivaRaman (1968), Bowen and Mortimer (1971) and Dimroth et al. (1973) on K. aerogenes citrate lyase led to erroneous conclusions probably because of the complex interactions between the non-identifical subunits and incomplete dissociation under the denaturing conditions used. Dimroth et al. (1973) were the first to report the presence of a 10 000 dalton subunit, which functions as the acyl-carrier protein (ACP) in K. aerogenes. Singh et al. (1974) observed three protein bands of molecular weights of about 55 000, 33 800 and 11 900 daltons respectively on SDS-gel electrophoresis of citrate lyase from K. aerogenes. These components were separated on agarose columns in presence of 6 M urea. Carpenter et al., (1975) determined the molecular weight of the separated subunits more precisely by gel filtration through 6% agarose in 6 M guanidinium hydrochloride and by sedimentation equilibrium. The values of 54 500, 32 000 and 11 000 daltons

reported by these authors for the component subunits have been confirmed by Dimroth and Eggerer (1975a), ~~was~~ reported values of 54 000, 32 000 and 10 000 daltons.

Similar subunit structures have also been shown by SDS-polyacrylamide gel electrophoresis for citrate lyase from several other sources. The molecular weights of the subunits of the S. diacetilactis enzyme have been shown to be 54 000, 35 000 and 12 000 (Singh and Srere, 1975). The S. faecalis enzyme is composed of subunits of molecular weights 54 000, 37 000 and 14 000 (Hiremath et al., 1976). The purified enzyme from R. gelatinosa was thought at first to contain only two non-identical subunits (Beuscher et al., 1974), but drastic conditions of heating with 2% SDS, instead of the 1% which was used earlier, confirmed that the enzyme from this source also resembles other citrate lyases in having three non-identical subunits of molecular weights of 55 600, 31 600 and 11 400 (Giffhorn and Gottschalk, 1978).

The subunits of C. sphenoides citrate lyase have been shown by SDS-polyacrylamide gel electrophoresis to have molecular weights of 56 000, 32 000 and 11 700 (Antranikian et al., 1982b).

The purified E. coli enzyme has been reported recently to differ markedly from other citrate lyases, containing a large 85 000 dalton ACP subunit instead of the usual 10 000 - 14 000 dalton ACP subunit. The other two subunits of E. coli are of the usual size of 54 000 and 32 000 daltons (Nilekani and SivaRaman, 1983).

(xi) Subunit stoichiometry

The stoichiometric proportions of the three subunits in the citrate lyase complex have been determined through a

number of approaches. The subunits of K. aerogenes citrate lyase separated by SDS-polyacrylamide gel electrophoresis have been stained with Coomassie Brilliant Blue and estimated by scanning of the gels and the 54 000 and 32 000 subunits have been shown to be present in equimolar amounts with an estimated 6 copies of each in the enzyme complex (Dimroth and Eggerer, 1975a; Carpenter et al., 1975). The estimated number of copies of the ACP()-subunit determined by the same procedure varied according to the length of the development and the staining procedure (Dimroth and Eggerer, 1975a). This was explained as being caused by losses through diffusion of the small protein from the gel into the solution. It was assumed that the native enzyme complex has six copies each of the α - and the β -subunits and four copies of γ -subunit (Dimroth and Eggerer, 1975a; Carpenter et al., 1975).

Srere et al. (1972) estimated the pantothenate content in the K. aerogenes enzyme complex by chemical and microbiological assays. A value of about 3 mol of phospho-pantothenate per mol of enzyme was obtained. A similar value of 3-4 mol pantothenate per mol of enzyme, was obtained by Singh et al. (1975) from studies with $[^{14}\text{C}]$ -labeled pantothenate and pantothenate-requiring mutants of K. aerogenes. They also showed that the $[^{14}\text{C}]$ -pantothenate enzyme which was subsequently labeled to contain an $[^3\text{H}]$ -acetyl group had $[^3\text{H}]$ and $[^{14}\text{C}]$ associated only with the ACP(γ)-subunit of the complex.

A novel amino acid sequencing technique was employed

by Singh et al. (1976) to establish the presence of six copies each of the three different subunits in the holoenzyme from K. aerogenes. The enzyme was separated to give the subunits, each of which was sequenced to ten residues from the amino terminus. The intact holoenzyme was also analyzed and the ratios of amino acids released at various positions were estimated. From the amino acid sequence of the individual subunits, the ratios of the amino acids released at various positions were shown to be 1:1:1 indicating the presence of equimolar amounts of the three subunits in the enzyme complex.

Hiremath (1977) determined the stoichiometry of the citrate lyase subunits from S. faecalis by scanning the SDS-polyacrylamide gels stained with Coomassie Blue and obtained a molar ratio of 1:1:0.7 for the α -, β - and γ -subunits. Chemical analysis of the prosthetic group components indicated the presence of four prosthetic groups per mol of the holoenzyme in S. faecalis (Hiremath, 1977). Pantothenate assays in both S. faecalis (Hiremath, 1977) and S. diacetylactis (Singh and Srere, 1975) have shown the presence of four mol pantothenate per mol holoenzyme.

The C. sphenoides complex has also been shown to contain α : β : γ subunits in the ratio 0.9:1:0.7 from stained SDS-gel scans (Antranikian et al., 1982).

An exception to the general subunit stoichiometry of the enzyme complexes observed in K. aerogenes, S. faecalis, S. diacetylactis and H. gelatinosa, the enzyme from E. coli consists of six copies each of the α - and β - subunits and

a single copy of the γ -subunit as estimated from the SDS-gel scans (Nilekani and SivaRaman, 1983).

(xii) Subunit organization

The dimensions of the citrate lyase molecule and the subunit organization in the complex have been determined by Gottschalk and his coworkers from electron micrographs of negatively stained molecules of citrate lyases from different sources. Electron micrographs of purified R. gelatinosa enzyme showed the presence of six large and six small subunits arranged in two hexagonal rings lying face to face, each consisting of three large and three small subunits in alternating sequence; the complex had the dimensions of 160 Å diameter and 100 Å thickness. The large subunit showed up as an ellipse with axes of 58 Å and 49 Å, while the smaller subunit which was taken to be spherical had a calculated diameter of 43 Å (Beuscher et al., 1974). Dimroth and Eggerer (1975a) and Schramm (1981) have reported that the electron micrographs of K. aerogenes citrate lyase resemble those of the R. gelatinosa enzyme. More recently, Antranikian et al. (1982) have investigated by electron microscopy the citrate lyases from C. sphenoides, R. gelatinosa and S. diacetilactis. In addition to the ring forms, star and triangle forms were also observed in enzymes from all these sources. In samples from R. gelatinosa and S. diacetilactis, star and ring forms occurred in the ratio of about 1:9. With the S. diacetilactis enzyme the ratio could be altered in favour of the star form in presence of the substrate, citrate or its analogue tricarballoylate. The

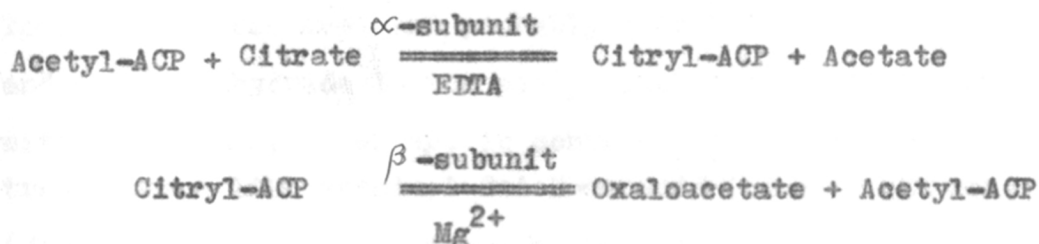
C. sphenoides enzyme which is active only in presence of L-glutamate showed up as stars and rings with the star form predominating in presence of glutamate and as undefined shapes in samples when the glutamate was removed by dialysis. The smallest (δ) subunit could not be observed in the electron micrographs of the native enzyme complexes, probably due to its relatively small size and the low resolution of the technique. The location of the δ -subunit was possible through immunoelectron microscopy (Zimmermann et al., 1982). Using subunit specific antibodies, the δ -subunit was shown to be attached closely to the α -subunit both in stars and rings with a symmetrical location in the star form and a polar attachment in the rings.

(xiii) Citrate as multienzyme complex and mechanism of action

That citrate lyase could be a multienzyme complex was apparent after the discovery of the two-step reaction mechanism, an acyl exchange and an acyl lyase reaction (Buckel et al. 1971a) and the detection of three non-identical subunits in citrate lyase (Singh et al., 1974). Dimroth and Eggerer (1975b) provided the necessary evidence for the multienzymic nature of citrate lyase by isolating the three component subunits in pure and biologically active states and characterizing their individual functions and by reconstituting the active enzyme from the isolated subunits. The 54 000 dalton α -subunit was shown to function as an acyl transferase catalysing the formation of $[1,5-^{14}\text{C}]$ citryl-ACP and acetate from $[1,5-^{14}\text{C}]$ citrate and the 10 000 dalton δ -subunit, which was earlier shown by Dimroth et al. (1973) to function as an

ACP. The transferase reaction required no metal ion for activity and the dimer of the 54 000 dalton polypeptide chain was reported to be the catalytically active form of the α -subunit. The 32 000 dalton β -subunit in presence of Mg^{2+} catalysed the breakdown of $[1,5-^{14}C]$ -citryl-ACP to $[4-^{14}C]$ -oxaloacetate and acetylated ACP, establishing the lyase function of the β -subunit in citryl-ACP cleavage.

The overall breakdown of citrate by citrate lyase was thus unequivocally shown to proceed as follows:



The active enzyme could be reconstituted only in the presence of all the three subunits and was found to be identical with the native enzyme complex in its sedimentation behaviour and specific activity.

The enzyme citramalate lyase from C. tetanomorphum shows striking resemblance with the citrate lyase from K. aerogenes even though they are present in different unrelated bacteria (Buckel and Bobi, 1976). The close relationships between the two enzyme complexes was further supported by the formation of hybrid enzymes. ACP of citramalate lyase incubated with the transferase and lyase subunits of citrate lyase forms a hybrid complex and the ACP of citrate lyase acts as a substrate for the transferase subunit of citramalate lyase although other combinations failed to produce an active

complex (Dimroth et al., 1977a).

The mechanism of action of the isolated acyl transferase subunit from K. aerogenes was shown by Dimroth et al. (1977b) to represent a CoA-transferase. The enzyme in the presence of acetate was found to catalysed the exchange between acetate and acetyl-ACP or acetyl-CoA; an incubation with acetyl-CoA and citrate, citryl-CoA was formed. Studies on affinity labeling with acetic anhydride and iodoacetate gave evidence for the transient formation of an anhydride as the intermediate. These anhydrides are most probably citric-acetic anhydride and acetic anhydride in the acetyl exchange reaction with and without citrate. However, in contrast to the classical CoA-transferases like succinyl-CoA:3-oxoacid CoA-transferase (Jencks, 1973), the CoA-transfer reaction from acetyl-CoA to citrate catalysed by the isolated transferase reaction, did not involve the formation of enzyme-CoA intermediate as studied by kinetic as well as by chemical assays. While the classical CoA-transferase generally follow ping-pong or double displacement mechanism, the reaction catalysed by the transferase subunit is a single displacement mechanism.

Srere and Singh (1974) and Dimroth and Eggerer (1975b) postulated that the cleavage of citrate by the multienzyme complex requires the oscillation of the acylated prosthetic group of the ACP subunit during reactions catalysed between the transferase (α) and lyase (β) subunits.

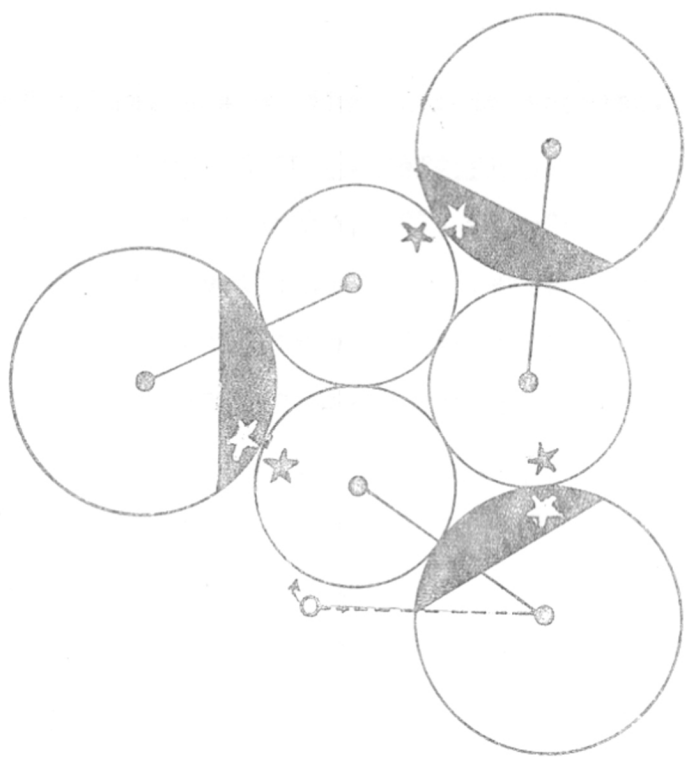
An alternate mechanism proposed by Zimmermann et al. (1982) from electron microscopic studies has been discussed in an earlier section of this Introduction. This involves the rotation of the α -subunit around its vertical axis causing transitions from the 'ring' form representing

the 'substrate' or citryl-enzyme to the 'star' form wherein the citryl residue on the ACP subunit is assumed to be brought close to the active site of the lyase (β) subunit. On cleavage of the citryl moiety to yield oxaloacetate and the acetyl-enzyme, the enzyme as postulated reverts to the 'ring' configuration.

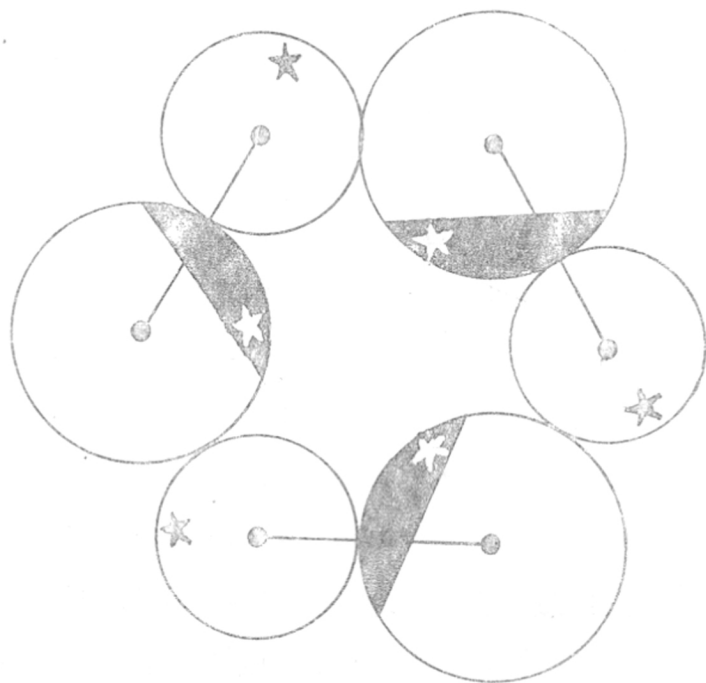
Based on electron microscopy and the results obtained with different bifunctional cross-linking reagents, a model has been postulated to explain the structure-function relationships in citrate lyase. According to the working model, two layers of subunit molecules are present one above the other. In a layer, a complex consisting of a α -subunit with a γ -subunit sitting as polar cap on α - and a β -subunit represents a structural unit. 'stars' and 'rings' can be transformed into one another by rotation around the vertical axis of the α -subunit and opening followed by closing of the contacts between α and β of neighbouring structural units. The 'triangle' would be an assumed transition form. In the 'star' form, areas on the surface of γ which probably comprise the active site are brought into contact with the corresponding areas on β ; while in the 'ring' form, these areas are far apart. The 'ring' configuration is postulated to represent the 'substrate' form and the 'star', the 'consumption' form. The model is illustrated in Figure 1.

(xiv) Substrate and inhibitor activities of citrate analogues

(a) Hydroxycitrates: Sullivan et al. (1977) have studied the effect of the four isomers of hydroxycitrate as substrates



STAR FORM



RING FORM

FIG. 1 Proposed structure-function model for citrate lyase from R. gelatinosa.

(Adapted from Zimmermann et al., 1982)

and inhibitors of the citrate enzymes. In the case of citrate lyase from K. aerogenes, the three isomers, (2R, 3R)-2-hydroxycitrate, (2R, 3S)-2-hydroxycitrate and (2S, 3S)-2-hydroxycitrate could serve as substrates, while no substrate activity was detected with (2S, 3R)-2-hydroxycitrate. (2R, 3R)-2-hydroxycitrate showed the highest reaction rate while the (2R, 3S)- and (2S, 3S)-isomers showed approximately 25% and 10%, respectively, of the reaction rate obtained with the (2R, 3R)-isomer. All the four isomers of hydroxycitrate were linear competitive inhibitors for citrate in the reaction catalysed by citrate lyases from K. aerogenes and S. diacetylactis. There was a difference in the calculated kinetic constants for the enzyme from the two sources. In the case of the K. aerogenes enzyme, (2S, 3S)-2-hydroxycitrate was the strongest inhibitor (K_i about 8 μM) and (2R, 3S)-isomer the weakest (K_i about 180 μM); while the determination of the K_i value for (2S, 3R)-isomer was not possible as it caused rapid inactivation of the enzyme. For the S. diacetylactis enzyme the (2R, 3R)-isomer was the strongest inhibitor (K_i about 170 μM) and the (2R, 3S)-isomer the weakest (K_i about 420 μM).

Citrate lyases from K. aerogenes and S. diacetylactis were shown to undergo reaction inactivation in presence of the hydroxycitrate isomers. The rate of inactivation was greater in the case of the K. aerogenes enzyme than of the S. diacetylactis enzyme and Zn^{2+} caused a slower rate of inactivation than Mg^{2+} . The most potent

inactivation was by the (2S, 3R)-isomer while the (2S, 3S)-isomer was the weakest. All the hydroxycitrate isomers caused complete deacetylation of [^{14}C]-acetyl citrate lyase to give free, labeled-acetate. This indicated that inactivation was caused by the loss of the essential acetyl moiety. This was confirmed by the reactivation of the inactivated enzyme with purified acetate:SH-(acyl carrier protein) enzyme ligase (AMP) in presence of acetate and ATP.

As the stereochemistry of citrate cleavage catalysed by citrate lyase is known, it was assumed that the (2R, 3R)- and the (2S, 3R)-isomers of hydroxycitrate yield acetate and hydroxyoxaloacetate; the latter being a substrate for the malate dehydrogenase reaction (Englard and Siegel, 1969), while the (2R, 3S)- and the (2S, 3S)-isomers give rise to oxaloacetate and glyoxalate.

Stallings et al. (1979) studied the activities of the hydroxycitrates towards citrate enzymes as well as the conformations of the metal chelates to obtain an insight into the geometry of the enzyme active sites. It was postulated that if hydroxycitrate binds in the same manner as citrate, C(3)-C(4) bond would be in the correct position to be cleaved. Alternate modes of binding, if they could be accommodated in the active sites of the enzymes were assumed, would be nonproductive and would compete with the citrate like mode, causing inhibition. It was possible that in the alternate modes of binding, side chains of the complex interacted with the active site causing severe inhibition.

(b) Fluorocitrate and tricarballylate: 3-Fluorocitrate has been shown by Rokita et al. (1982) to be a mechanism-based inhibitor, causing inhibition during the turnover of the acetyl enzyme. It was postulated that 3-fluorocitrate forms a mixed acetic-3-fluorocitric acid anhydride intermediate which might immediately diffuse from the active site of the enzyme leaving a deacetyl inactive enzyme. Alternatively, the 3-fluorocitryl-S-enzyme intermediate may be formed which could be hydrolyzed to form the deacetyl enzyme. To distinguish between deacetyl enzyme and 3-fluorocitryl enzyme reactivation studies with acetic anhydride and acetate were carried out. Buckel et al. (1971a) and Srere et al. (1972) have shown that acetic anhydride specifically activates the deacetyl enzyme. Acetate was thought might displace 3-fluorocitrate in the 3-fluorocitryl enzyme. Treatment of the inactive enzyme with either acetic anhydride or acetate restored about 50% activity indicating the presence of equal amounts of deacetyl and 3-fluorocitryl enzyme initially.

(xv) Amino acid composition

(a) Holoenzyme: The amino acid composition of the citrate lyase holoenzyme from K. aerogenes was estimated by Bowen and Rogers (1965), after separation of the amino acids in acid hydrolyzates by high voltage paper electrophoresis and assay as Cu^{2+} -complexes. Analysis by the conventional ion exchange chromatography and automatic amino acid analysis indicated marked differences in the values reported by Bowen and Rogers (1965) for several residues (Singh et al., 1976). All the three subunits of the enzyme complex were also shown

by Singh et al. (1976) to have methionine as the amino terminal residue and not lysine as reported earlier by Bowen and Mortimer (1971).

Hiremath (1977) determined the amino acid composition of citrate lyase from S. faecalis and showed marked differences between the amino acid make-up of the S. faecalis and K. aerogenes enzymes. The partial specific volume calculated for S. faecalis from the amino acid data was 0.742 ml.g^{-1} (Hiremath, 1977) and in case of K. aerogenes, 0.737 ml.g^{-1} (Singh et al., 1976). The amino acid composition of purified E. coli citrate lyase has been reported to resemble that of K. aerogenes (Nilekani, 1980).

(b) Subunits: The amino acid compositions of the α -(M_r 54 000) and β -(M_r 32 000) subunits of K. aerogenes citrate lyase were determined by Singh et al. (1976). The subunits were dissociated in 6 M guanidinium hydrochloride and separated by gel filtration through an agarose column. Amino acid sequences in all the three subunits of the enzyme upto ten residues from the amino terminus were also determined by them. They reported the presence of micro-heterogeneity in the β -subunits, equal amounts of proline and arginine being found to occupy the fourth position from the amino terminus.

The amino acid composition of the separated ACP(γ)-subunit of K. aerogenes citrate lyase has been reported by Dimroth (1975). Dimroth et al. (1973) had shown earlier the presence of an acylated cysteine residue in addition to the acetylated cysteamine residue of the prosthetic group on the

ACP subunit. This was established by treating the ACP subunit first with cold iodoacetate, then deacetylating with neutral hydroxylamine followed by differential labeling with iodo 2-¹⁴C -acetate. The labeled ACP subunit was then acid hydrolyzed and the labeled carboxymethylated derivatives separated and characterized as S-carboxymethyl cysteine and S-carboxymethyl cysteamine. Dimroth and Eggerer (1975a) showed indirectly by affinity labeling with low concentrations of iodoacetate in presence of an excess of dithioerythreitol that the cysteamine of the prosthetic group carries the biologically essential acetyl group and not the cysteine residues as the cysteamine-S-carboxymethyl-deacetyl citrate lyase which was formed could not be reactivated by treatment with acetic anhydride.

The amino acid sequence determination of the ACP subunit of K. aerogenes citrate lyase has been carried out by automated sequential Edman degradations (Beyreuther et al., 1978). The molecular weight of the ACP subunit from its amino acid sequence analysis has been calculated to be 9378.

There are marked differences in the amino acid compositions of the subunits of K. aerogenes and S. faecalis citrate lyase (Hiremath, 1977). The subunits of E. coli show some similarity in amino acid composition with the corresponding subunits of K. aerogenes and not of S. faecalis subunits (Nilekani, 1980). The ACP of the K. aerogenes enzyme contains no histidine residues while the S. faecalis enzyme contains four and the E. coli enzyme contains one for an equivalent molecular size of subunit. Tyrosine is present

in the ACP of E. coli, while K. aerogenes and S. faecalis do not contain any.

The ACP of citrate lyase from K. aerogenes resembles the ACP of the fatty acid synthetase from E. coli in its molecular weight (Dimroth et al., 1973; Vagelos, 1973) and function. They, however, differ markedly in their amino acid compositions and in the nature of their prosthetic groups. The fatty acid synthetase contains only 4-phosphopantetheine as its prosthetic group whereas the ACP of citrate lyase contains in addition adenine, phosphate and sugar residues (Dimroth, 1976; Robinson et al., 1976). Dimroth et al. (1973) have shown that an acyl-carrier protein from the crude extract of K. aerogenes but not the deacetylated purified ACP subunit of citrate lyase could substitute for the ACP of the fatty acid synthetase system in the malonyl-CoA/CO₂ exchange reaction. This clearly indicated the presence of two distinct ACPs in K. aerogenes, one for the fatty acid synthetase system and the other for the citrate lyase system. Tryptic peptide maps of the carboxymethylated ACPs of the citrate lyase from K. aerogenes and of the ACPs of the fatty acid synthetases from E. coli and K. aerogenes showed absence of any common peptide in the citrate lyase and fatty acid synthetase ACPs (Bayer and Eggerer, 1978). The ACPs of E. coli fatty acid synthetase and of K. aerogenes citrate lyase were also shown from amino acid sequence analysis to be completely different except for two very short regions (Beyreuther et al., 1978). Circular dichroism studies indicate that citrate lyase ACP as well as the fatty acid synthetase

ACP have the same α -helical content of about 40 - 50%.

(xvi) Nature of citrate lyase prosthetic group

Bucket et al. (1971a) had reported the presence of an essential acetyl group in thio-ester linkage in the enzyme. Srere et al. (1972) were able to demonstrate by microbiological experiments the presence in the holoenzyme of covalently bound pantothenate moieties. The enzyme had to be first treated with alkaline phosphatase and then subjected to alkaline hydrolysis for rendering the pantothenate available for microbial growth. It was therefore assumed, in analogy with the fatty acid synthetase system, that citrate lyase also contains covalently bound 4-phosphopantetheine moiety with the acetyl group attached to it. Dimroth et al. (1973) showed that the prosthetic group was present on the 10 000 dalton γ -subunit of K. aerogenes. Thus the ACP nature of this subunit was established.

Dimroth (1975; 1976) first recognized the more complex nature of the prosthetic group and showed that besides 4-phosphopantetheine, adenine, phosphate, sugar are also present as components of the ACP subunit of citrate lyase. Several chemical and enzymatic degradation products of the prosthetic group were isolated and analyzed and the prosthetic group was shown to be 3' (or 2') $\rightarrow$ 1''-(5''-phosphoribosyl) dephosphocoenzyme A (Robinson et al., 1976). Openheimer et al. (1979) studied the structure of the prosthetic group of K. aerogenes citrate lyase by nuclear magnetic resonance and mass spectrometry, confirming the 2' position as the site of attachment of the dephospho-CoA with the second ribose moiety.

The structure of the prosthetic group is ($1'' \rightarrow 2'$)- (5''-phosphoribosyl) dephospho-CoA. The attachment of the prosthetic group of citrate lyase to the ACP subunit has been shown to be through serine-14 (Beyreuther et al., 1978). Alkaline hydrolysis of the prosthetic group resulted in a β -elimination reaction and the dehydroalanine in the apo-ACP was characterized by NaB^3H_4 reduction to form ^3H -alanine.

Robinson et al. (1976) have shown that the prosthetic group of K. aerogenes citrate lyase obtained after pronase digestion of the enzyme can serve as a substrate for a crude preparation of acetate: SH-(acyl-carrier protein) enzyme ligase (AMP) from K. aerogenes, pure pig heart citrate synthase (EC 4.1.3.7) and pure rat liver ATP-citrate lyase (EC 4.1.3.8). Similarly acetyl-CoA and citryl-CoA can substitute for the citrate lyase prosthetic group in the reactions catalysed by the deacetyl enzyme (Buckel et al., 1971a; 1973) or the separated α - and β -subunits (Dimroth et al., 1977b).

Dimroth and Loyal (1977) have studied the prosthetic group of citramalate lyase from C. tetanomorphum and have shown that it resembles the K. aerogenes citrate lyase prosthetic group. The structural organization as well as the mechanism of action of the citramalate lyase are also similar to those of the K. aerogenes citrate lyase (Buckel and Bobl, 1976).

(xvii) Immunological behaviour

Singh and Srere (1975) have demonstrated that citrate lyases from K. aerogenes, E. coli and S. diacetylactis

are immunologically distinct and that the enzymes from E.coli and S. diacetilactis do not cross-react with rabbit antisera against purified K. aerogenes citrate lyase. Cross-reaction was observed with rabbit antisera against purified S. diacetilactis enzyme only with its own antigen.

SivaRaman (1980) has shown that S. faecalis citrate lyase cross-reacts with rabbit antisera against purified citrate lyase from K. aerogenes.

Antibodies to native citrate lyase from R. gelatinosa cross-reacted with R. palustris enzyme but not with the enzymes from K. aerogenes, S. diacetilactis or C. sphenoides (Giffhorn et al., 1981). The immunological experiments carried out to study cross-reactions indicated that the homology between the enzymes from R. gelatinosa and R. palustris was not complete. The antigenic determinants common to the citrate lyases from the two sources are located on the large α -subunit of the two enzymes and not on the β - or δ -subunits.

PRESENT INVESTIGATION

PRESENT INVESTIGATIONS

INTRODUCTION

Citrate lyase (EC 4.1.3.6) initiates the anaerobic metabolism of citrate by catalysing the breakdown of citrate to oxaloacetate and acetate in the presence of divalent metal ions such as Mg^{2+} and Mn^{2+} . The enzyme occurs only in the primitive procaryotic forms of life and represents probably the earliest of the citrate enzymes to evolve. The enzyme has been isolated from diverse bacterial sources and has been shown to exist as a multienzyme complex comprising three dissimilar polypeptide chains two with enzymatic activities and the third with the biological function of an acyl-carrier. The extensive literature on the properties, characterization and role of citrate lyase has been reviewed in the Introductory Chapter to the thesis.

Although the subunit structure of the citrate lyase complex has been relatively well studied, nothing is known about the amino acid residues which are involved at the active sites of the component subunits. Studies reported in the present thesis relate to the active site residues of the K. aerogenes and S. faecalis citrate lyases and to some unique structural features of the large ACP (γ) subunit of the E. coli enzyme. The investigations are described in two separate parts and the importance of the present contributions are discussed in these.

GENERAL SUMMARYPart I. Evidence for essential arginine residues at the active sites of the transferase and the lyase subunits of the citrate lyase complex

Arginine residues in enzymes are known to bind anionic substrates and cofactors. The presence of arginine residues and their involvement at the substrate binding sites both of the transferase and lyase subunits of K. aerogenes and S. faecalis citrate lyases have been studied using phenylglyoxal and 2,3-butanedione, which are known to specifically modify arginine residues.

Inactivation of the lyase activity of deacetyl citrate lyase by the arginine modifiers has been found to be concentration dependent and to follow pseudo-first order kinetics. The reaction order of about one was obtained which suggests that a single arginine residue is essential for the activity of the lyase subunit. To study whether the arginine is present at the substrate binding site, protection studies with different ligands were carried out. It was observed that (3S)-citryl-CoA, the normal substrate for the enzyme effectively protects against inhibition of the lyase subunit.

Similarly, inactivation of the transferase subunit by phenylglyoxal and 2,3-butanedione was found to follow pseudo-first order kinetics with the reaction order value of approximately one. Thus an essential arginine residue is also required for the activity of the transferase subunit.

When different ligands were studied for their protective abilities against inactivation of the transferase activity by the modifiers, it was observed that while citrate protects the enzyme, acetyl-CoA markedly enhances the inactivation. The data establish for the first time conformational changes at the citrate binding site in presence of acetyl-CoA, making an essential arginine residue more accessible to the modifiers.

Part II. Studies on the acyl-carrier protein of citrate lyase from Escherichia coli

The acyl-carrier protein (ACP) from E. coli citrate lyase has a molecular weight which is about six times larger than that of ACPs of citrate lyases from other sources. Evidence is presented for the first time for internal homologies in this large polypeptide chain. The number of peptide spots obtained by tryptic fingerprint analysis of the subunit was 25 which is far less than the 68 or 69 expected if the subunit was a single unique polypeptide chain without any homologous sequences. Although about 7 histidine residues are present in a mol of the ACP, only a single peptide fragment in the tryptic map reacts with Pauly reagent. Microbiological assay of pantothenate in the individual peptides in the tryptic fingerprint of the subunit showed that only a single peptide contains 86% of the total pantothenate with the remaining moieties of the prosthetic group being detected in a fragment closely located on the map.

These results are suggestive of intrapeptide homologies in the large ACP subunit of E. coli citrate lyase.

PART I

PRESENCE OF ESSENTIAL ARGININE RESIDUES AT THE ACTIVE SITES
OF THE CITRATE LYASE COMPLEX

PRESENCE OF ESSENTIAL ARGININE RESIDUES AT THE ACTIVE SITES
OF THE CITRATE LYASE COMPLEX

SUMMARY

The acyl-transferase activity both of the deacetyl enzyme as well as of the isolated α -subunit from K. aerogenes are inactivated by the arginine specific reagents, phenylglyoxal and 2,3-butanedione, the former reagent being the more potent inhibitor. Inactivation both by phenylglyoxal and 2,3-butanedione is found to be concentration dependent and the inactivation rates follow pseudo-first order kinetics. The ligands, citrate and (3S)-citryl-CoA, protect the transferase activity against inactivation, while acetyl-CoA markedly enhances the rate of the inactivation. Inactivation kinetics suggest the involvement of a single arginine residue at the active site of the transferase subunit, probably at the citrate binding site.

The acyl lyase activity of deacetyl citrate lyase is also inhibited by both the modifying reagents. Protection by the substrate citryl-CoA against inactivation of the lyase activity suggests that the arginine residue is present at the citryl-CoA binding site of the lyase subunit. Acetyl-CoA as well as citrate show very little protection. Kinetic studies indicate that one arginine residue is essential for forming an enzyme-inactivator complex.

The effect of phenylglyoxal on citrate lyase from S. faecalis has also been studied since the enzyme from this source is distinct from that in K. aerogenes in that it shows

a markedly slower rate of reaction-inactivation. As in the case of the K. aerogenes enzyme both the acyl-transferase and acyl-lyase activities are inactivated, inactivation rates following pseudo-first order kinetics. The influence of substrates on the inactivation of the S. faecalis enzymes the reagent was also similar to that observed with K. aerogenes deacetyl citrate lyase.

INTRODUCTION

Citrate lyase which catalyses the cleavage of citrate to oxaloacetate and acetate in presence of a divalent metal ion, has been shown to be present as a multienzyme complex consisting of three non-identical subunits α , β and γ (Carpenter et al., 1975). The most studied of the complexes is that from K. aerogenes in which the smallest of the subunits γ of molecular weight of 10 000 dalton acts as an acyl-carrier protein (ACP). The ACP has a covalently bound CoA-like prosthetic group (Oppenheimer et al., 1979) with an essential acetyl group in a thio-ester linkage (Buckel et al., 1971a). In presence of citrate and EDTA, the largest of the subunits, α of about 54 000 dalton functions as an acyl-transferase catalysing the formation of (3S)-citryl-ACP with liberation of acetate. The subsequent cleavage of the citryl-ACP intermediate is catalysed by the 32 000 dalton (β) lyase subunit in presence of a divalent metal ion to oxaloacetate with the regeneration of the acetyl-ACP (Dimroth and Eggerer, 1975b). The CoA derivatives, acetyl-CoA and propionyl-CoA serve as a substrates for the transferase reaction, while (3S)-citryl-CoA functions as a substrate for

the lyase reaction (Bucket et al., 1973; Dimroth et al., 1977b).

p-Azidobenzoyl-CoA has been shown recently by us to function as a photoaffinity reagent specifically inhibiting the lyase activity (Basu et al., 1982). Little is known, however, about the residues at the catalytic sites of the enzyme complex. Several arginine specific reagents are known which can modify the arginine residues at the active sites of the enzyme and cause inactivation. These include phenylglyoxal (Takahashi, 1968), 2,3-butanedione (Yankeelov et al., 1966; Yankeelov, 1970; Riordan, 1973), 1,2-cyclohexanedione (Patthy and Smith, 1975) and camphorquinone-10-sulfonic acid (Mautner et al., 1981). Studies on the involvement of arginine residues at the binding sites of citrate lyase subunits were undertaken, using phenylglyoxal and 2,3-butanedione as the modifying reagents.

MATERIALS

Phenylglyoxal monohydrate was obtained from Sigma Chemical Co., USA. Phenylglyoxal solution was prepared fresh in 50 mM potassium phosphate buffer, pH 7.6. 2,3-butanedione was obtained from Koch Light Laboratories, England. Streptomycin sulphate was obtained from Hindustan Antibiotics Ltd., Pune. Malate dehydrogenase from pig heart (specific activity $945 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$), NADH from yeast (disodium salt - $3\text{H}_2\text{O}$ grade III), citrate synthase from pig heart (specific activity $155 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$), adenosine triphosphate (disodium salt from Equine muscle), coenzyme A.

dithiothreitol (DTT), sodium dodecyl sulphate and Tris (Trizma base) were obtained from Sigma Chemical Co., USA. Acrylamide monomer, N,N'-methylene bisacrylamide and TEMED, N,N'-tetramethylethylenediamine (TEMED) were of electrophoresis grade and were obtained from Eastman Organic Chemicals, USA. Peptone powder was from Bengal Immunity Co. Ltd., Calcutta. Yeast extract was from Difco Laboratories, USA. Analytical grade hydroxylamine hydrochloride ($\text{NH}_2\text{OH} \cdot \text{HCl}$) was obtained from BDH (India) and a neutralized hydroxylamine solution, pH 7.0 was prepared by mixing equal volumes of 14% NaOH and 28% $\text{NH}_2\text{OH} \cdot \text{HCl}$ (Stadtman, 1957). Fresh solutions were prepared just before use. Analytical grade urea was also obtained from BDH (India). It was recrystallized from aqueous ethanol and the crystals stored dry over KOH in vacuum. 2-Mercapto-ethanol was obtained from Fluka, Switzerland. Lithium chloride was obtained from Loba Chemie, India. Sephadex G-10 and G-25 were from Pharmacia Fine Chemicals, Sweden. All the other chemicals were of the highest purity available.

Buffers

All the buffers were prepared in glass distilled water. The phosphate buffers were prepared by mixing and diluting 1 M stock solutions of K_2HPO_4 and KH_2PO_4 in the given proportions (Green and Hughes, 1955). All the buffers, except the 0.03 M potassium phosphate buffer, pH 7.0, which was used for extraction of the enzyme from sonicated cells, contained 1.6 mM Mg^{2+} . Tris-HCl buffer was prepared from Trizma base and the pH was adjusted by the addition of 1 N HCl.

Alumina C₆ gel

The gel (Grade A) was obtained aged from Calbiochem, USA, and had a solid content of 3.25%.

DEAE-cellulose

The anion exchanger DE-52 was obtained from Whatman Ltd., England. The exchanger was first thoroughly washed with water to remove the fines. Next, it was washed with 0.01 M potassium phosphate buffer, pH 7.5, containing 1.6 mM Mg^{2+} so as to equilibrate it and then packed in a column (30 cm x 1.5 cm) under pressure. During its use, the column was run under pressure at a flow rate of 18 - 20 ml/h. The gel could be reused by repeatedly washing the column with 0.5 M potassium phosphate buffer, pH 7.5, containing 1.6 mM Mg^{2+} followed by washing with glass distilled water and then again equilibrating the gel with 0.01 M potassium phosphate buffer, pH 7.5, containing 1.6 mM Mg^{2+} .

DEAE-cellulose (Cl⁻ form)

A suspension of DEAE-cellulose (DE-52 from Pharmacia, 20 g) was washed with water several times to remove the fine particles. It was then washed with 50 ml of 1 N HCl, followed by washing with distilled water, 50 ml of 1 N NaOH, distilled water and 50 ml of 1 N HCl. Except the acid washings which were carried out rapidly by filtering through a sintered Buchner funnel, all the washings were carried out by decantation. The gel was repeatedly washed with glass distilled water till the solution was neutral and then packed in a glass column (1 x 8 cm) under pressure.

Sephacrose CL-6B

The gel was obtained from Pharmacia Fine Chemicals, Sweden. The gel was thoroughly washed with water to remove the preservative and was equilibrated in 0.05 M potassium phosphate buffer, pH 7.5, containing 1.6 mM Mg^{2+} before packing in a column (110 x 2.0 cm) under gravitational pressure. Flow rate of the column was about 15 ml/h.

Ammonium sulphate fractionation

The amount of powdered ammonium sulphate which was required for fractionation was read off a nomogram where the concentrations were expressed in terms of saturation at 0 - 3°C. The enzyme after DEAE-cellulose chromatography and Sepharose gel filtration stages was precipitated with cold neutralized saturated ammonium sulphate, pH 7.0 (liq ammonia was used for neutralization).

METHODSProtein determination

During the purification of the enzyme, protein was determined by the method of Lowry et al. (1951), the colour was read at 500 nm. The protein was read off standard curves, using bovine serum albumin as the standard. Protein in samples containing streptomycin sulphate was determined after removal of the streptomycin sulphate by dialysis. The method of Singh et al. (1976) was also used, to estimate pure protein samples using the absorbance index $E_{1\%}^{1\text{cm}}$ of 6.2 at 278 nm.

Optical density

Optical density measurements were made on a Beckman DU,

Model-26 or a Shimadzu Model UV-210A spectrophotometer. Kinetic studies were carried out on the Shimadzu UV-210 spectrophotometer using a strip chart recorder.

pH measurements

A Philips Precision pH meter, Model PR-9405 or a Global digital pH meter Model DPH 500 with a combination glass electrode were used for pH measurements.

Conductivity measurement

Conductivity measurements were carried out using a Systronics Model 303 direct reading conductimeter.

Ultracentrifugation

The homogeneity of the enzyme preparations was observed in an analytical Spinco Ultracentrifuge Model E equipped with a phase plate Schlieren optics, a rotor temperature indicator and control device. All ultracentrifugations were carried out at 2 - 5°C and at 59 780 rpm.

Ultrafiltration

The isolated enzyme subunit preparations were concentrated by ultrafiltration in an Amicon Ultrafiltration Unit of 10 ml capacity and using a PM-10 membrane.

Polyacrylamide gel electrophoresis

Homogeneity of enzyme preparations were checked by disc electrophoresis according to the procedure of Davis (1964) using 5% polyacrylamide gels. Ammonium persulphate (0.07%) was used for polymerization of acrylamide gels. Sample and stacking gels were omitted. The separating gel contained Tris-HCl buffer, pH 8.9. A preliminary electrophoresis for one hour was carried out to remove the excess ammonium

persulphate. About 50 - 100 ug of the protein sample made dense with 30% sucrose was loaded on top of the gels. Bromophenol blue was used as the tracking dye. Tris-glycine buffer, pH 8.3 was used as the running buffer. Electrophoresis was carried out at 4°C and 4 mA/tube for about 4 h. Protein bands were visualized by staining the gels with 0.25% Coomassie Brilliant Blue in water containing 45.4% methanol and 9.2% glacial acetic acid followed by destaining of the gels by diffusion in a solution containing 87.5% glacial acetic acid and 5% methanol in water.

SDS-polyacrylamide gel electrophoresis

The procedure of Weber and Osborn (1969) was adopted. Polyacrylamide gels (10%) were prepared using 0.07% ammonium persulphate for polymerization. Native citrate lyase (approximately 100 ug) and isolated subunits (approximately 50 ug) were treated with SDS and 2-mercaptoethanol to final concentrations of 1% and 10 mM respectively. The samples were denatured by heating at 100°C for 3 min. The protein was loaded on the gel prepared in 0.1 M sodium phosphate buffer, pH 7.3, containing 0.1% SDS. The running buffer was 0.05 M sodium phosphate buffer, pH 7.3, containing 0.1% SDS. Electrophoresis was carried out at 8 mA/tube for 3 - 4 h. Gels were stained with 0.25% Coomassie Blue in 45.4% methanol and 9.2% glacial acetic acid overnight and destained with methanol (5%)-acetic acid (7.5%)-water mixture.

GROWTH AND EXTRACTION OF CITRATE LYASE FROM K. AEROGENESORGANISM

Klebsiella aerogenes (NCTC 418) was maintained routinely on nutrient agar slants and subcultured every month. The organism was first inoculated in nutrient broth containing beef extract (0.3%), NaCl (0.5%) and peptone (0.5%), adjusted to pH 7.0 with NaOH and then subcultured in citrate growth medium. Nutrient agar slants contained in addition 2% agar. The cultures were grown at 37°C for 16 - 20 h.

Growth

K. aerogenes (NCTC 418) was grown at 30°C without aeration in 10 L flasks filled with the medium described by Dagley and Dawes (1953a). The 10 L flasks contained trisodium citrate. $\cdot 2\text{H}_2\text{O}$, 90 g; KH_2PO_4 , 20 g; pH was adjusted to 7.0 with 40 ml of 2 N NaOH. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g and $(\text{NH}_4)_2\text{SO}_4$, 10 g were dissolved in 1 L flask and autoclaved separately to prevent precipitation of magnesium phosphate. An inoculum (10% final volume) which was adapted to the citrate growth medium was used. After 16 - 20 h growth, the cells were harvested in a Sharples Model A-12 Supercentrifuge at 12 000 g. From 10 L of medium, 18 - 20 g wet cells were obtained and these were stored at -20°C.

Extraction

About 120 g wet cells were processed for purification of the enzyme. Stored cells lost no activity during periods of over 2 weeks.

Extractions were carried out at 0 - 4°C. The frozen cells were thawed overnight and suspended in 0.03 M potassium

phosphate buffer, pH 7.0 (4 ml/g of cell paste). The cell suspension was disintegrated during two exposures of 3 min each at 20 Kc (300 W) in a Biosonik III sonicator (Bronwill Scientific Co., USA). The sonicate was centrifuged at 50 000 g for 60 min at 4°C to remove the cell debris.

Purification

The purification was carried out by the procedure of Mahadik and SivaRaman (1968) as modified by Tikare (1979). The purification steps were carried out at 0 - 4°C without storage. All the buffers contained 1.6 mM Mg^{2+} . A brief outline of the procedure is described below.

ATP and acetate treatment

The supernatant obtained after centrifuging the sonicated cell suspension was adjusted to a protein concentration of 0.5% with 0.03 M potassium phosphate buffer, pH 7.0 and then treated with 0.1 M ATP and 1 M acetate to give final concentrations of 0.5 mM and 1mM respectively. The solution was kept stirred for 45 min to reactivate deacetyl citrate lyase present in the cell extract. The activation has been shown by Tikare (1979) to yield a final enzyme preparation of higher specific activity.

Streptomycin sulphate treatment

To the above extract which was kept stirred, streptomycin sulphate was added slowly to a final concentration of 1.4%. After an hour, the precipitated nucleic acids were removed by centrifugation at 50 000 g for 45 min.

Alumina C gel adsorption

First Gel treatment: The supernatant from the streptomycin

sulphate treated extract was stirred constantly and alumina C gel was added till about 5% enzyme activity was adsorbed on the gel (10 ml gel suspension/600 ml supernatant). The solution was then spun at 3 000 g for 20 min and the gel discarded.

Second gel treatment: To the supernatant from the first gel treatment more alumina C gel was added (200 ml gel suspension/600 ml supernatant from streptomycin sulphate step). The solution was stirred for 15 min and then allowed to stand for another 15 min. About 90 - 95% of enzyme activity was adsorbed on the gel. The solution was centrifuged at 3 000 g for 20 min and the supernatant was discarded.

Elution of the enzyme from alumina C gel

The enzyme was eluted from the gel by homogenizing with 0.05 M potassium phosphate buffer, pH 7.0 (200 ml/600 ml streptomycin sulphate supernatant). After 15 min, the gel suspension was centrifuged at 3 000 g for 20 min. The gel was discarded and the supernatant processed further.

Ammonium sulphate fractionation

The gel eluate was treated with powdered ammonium sulphate to 0.2 saturation (20 g/200 ml). The precipitate formed was separated by centrifugation at 50 000 g for 30 min. The supernatant was taken to 0.5 ammonium sulphate saturation with addition of more of the powdered salt (38 g/200 ml of eluate volume) and stirred for 30 min. The precipitated enzyme was separated by centrifugation at 50 000 g for 30 min and dissolved in 0.05 M potassium phosphate buffer, pH 7.5 (4 - 5 ml). The enzyme solution was dialyzed overnight against

0.05 M potassium phosphate buffer, pH 7.5.

DEAE-Cellulose chromatography

The dialyzed protein solution (7 - 8 ml) was centrifuged at 10 000 g for 10 min to remove any denatured protein and the clear solution loaded on a DEAE-cellulose column (30 x 1.5 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. The enzyme was eluted from the column by using a continuous gradient of potassium phosphate buffer, pH 7.5 of increasing buffer strength from 0.01 M to 0.3 M. The gradient was obtained by taking 200 ml of 0.01 M buffer solution in a mixing chamber attached to the column and addition of 0.5 M buffer to this solution at the same flow rate as of the column. About 3 ml fractions were collected on an automatic fraction collector and a drop from each fraction was tested for enzyme activity. The enzyme eluted out between 90 - 120 ml and the fractions which contained the maximum activity (25 - 30 ml) were pooled and precipitated by addition of an equal volume of cold saturated neutralized ammonium sulphate solution to the stirred enzyme solution. After centrifugation at 50 000 g for 30 min, the precipitate was dissolved in 1 - 2 ml of 0.05 M potassium phosphate buffer, pH 7.5.

Gel filtration

The enzyme solution from the DEAE-cellulose step was loaded on a Sepharose CL-6B column (110 x 2.5 cm) previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The enzyme was eluted with buffer of the same composition. Fractions of 3 ml were collected on an automatic fraction

collector and a drop from each fraction was tested for enzyme activity. The enzyme eluted between 140 - 160 ml and the fractions containing high enzyme activity (15 - 20 ml) were pooled and precipitated with an equal volume of cold saturated neutralized ammonium sulphate. The precipitate was collected by centrifugation at 50 000 g for 30 min and dissolved in 1 - 1.5 ml of 0.05 M potassium phosphate buffer, pH 7.5.

GROWTH AND EXTRACTION OF CITRATE LYASE FROM S. FAECALIS

Organism

Streptococcus faecalis 10 Cl was a gift from Prof. I.C. Gunsalus, University of Illinois, USA. The organism was maintained routinely on nutrient agar slabs. 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 and the solution was adjusted to pH 7.6. The organism was grown at 37°C for 24 h and then transferred as slabs. Subculturing was done once in 2 - 3 weeks.

Growth

S. faecalis was grown without aeration in 10 L flasks. The growth medium contained sodium citrate, 100 g; KH_2PO_4 , 20 g; $(\text{NH}_4)_2\text{SO}_4$, 10 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 g; yeast extract, 50 g; peptone powder, 50 g and water to make the volume upto 10 L after the pH of the solution was adjusted to 7.2 with 2 N NaOH. MgSO_4 and $(\text{NH}_4)_2\text{SO}_4$ were dissolved in about 1 L water, autoclaved separately and mixed with the contains

in the 10 L flasks just before inoculation. An inoculum of about 10% of the final volume containing cells which were adapted to growth on citrate was added and the flasks incubated at 37°C for 24 - 26 h. The cells were harvested in a Sharples Model A-10 Supercentrifuge at 12 000 g. The cells were washed on the centrifuge with 2 L of 0.03 M potassium phosphate buffer, pH 7.0. About 15 - 20 g wet cells/10 L of citrate growth medium was obtained. The cells were frozen at -20°C and used within a week for extraction of the enzyme.

Extraction

Batches of 100 - 120 g cells were worked up for purifying the enzyme. Extraction as well as purification of the enzyme was carried out at 0 - 4°C. The cells were thawed out overnight at 0 - 4°C and were suspended in 0.03 M potassium phosphate buffer, pH 7.0 (about 3.5 ml/g of cell paste). Sonication of the cells was carried out at 20 kc (300 W) for 3 min on the Biosonic III (Bronwill Scientific Co., USA) sonicator. The sonic extract was centrifuged at 50 000 g for 45 min and the cell debris was discarded. The supernatant was checked for protein and suitably diluted to a protein concentration of 0.5% with 0.03 M potassium phosphate buffer, pH 7.0.

Purification

The purification procedure was that described by Hiremath et al. (1976). All the purification steps were carried out continuously without any storage of the enzyme. The temperature was maintained at 0 - 4°C and all the buffers contained 1.6 mM Mg^{2+} .

ATP and acetate treatment

The diluted protein solution (0.5% protein) was continuously stirred and to it was added 0.1 M ATP and 1 M acetate to give final concentrations of 0.5 mM and 1 mM, respectively. The enzyme was kept stirred for about 45 min.

Streptomycin sulphate treatment

To the ATP and acetate treated extract which was kept stirred, streptomycin sulphate was added slowly to give a final concentration of 1%. The nucleic acids which were precipitated were removed by centrifugation at 50 000 g for 45 min.

Alumina C gel treatment

First gel treatment: The gel was added to the supernatant of streptomycin sulphate treated extract with stirring till about 5 - 8% of the total activity was adsorbed (3 ml gel/200 ml streptomycin sulphate treated extract). The gel was removed by centrifugation at 3 000 g for 15 min and discarded.

Second gel treatment: More gel (33 ml/200 ml streptomycin sulphate treated extract) was added to the supernatant from the first gel treatment, till about 90 - 95% activity was adsorbed. The mixture was kept stirred for 15 min and then allowed to stand for another 15 min before centrifuging at 3 000 g for 20 min. The supernatant was discarded and the gel used further.

Washing of the gel

A gel suspension in 0.01 M potassium phosphate buffer, pH 7.0 (100 ml/200 ml streptomycin sulphate treated extract) was homogenized and then centrifuged at 3 000 g for 15 min.

The washings were discarded.

Elution of the enzyme

The enzyme was eluted from the gel by homogenizing with 0.05 M potassium phosphate buffer, pH 7.0 (100 ml/200 ml streptomycin sulphate treated extract). After 15 min the eluate was separated after centrifugation at 3 000 g for 15 min.

Ammonium sulphate fractionation

The eluate obtained in the above step was stirred and treated with powdered ammonium sulphate to 0.5 saturation (29 g/100 ml of eluate). The precipitate was removed by centrifugation at 50 000 g for 30 min. To the clear supernatant which was kept stirred, more powdered ammonium sulphate was added to 0.8 saturation (23 g) and the stirring continued for a further period of 30 min. The enzyme which precipitated was separated by centrifugation at 50 000 g for 30 min. The precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.5 (approximately 5 ml) and dialyzed for about 16 h against the buffer of the same composition.

DEAE-cellulose chromatography

The dialyzed solution was centrifuged at 10 000 g for 10 min to remove any turbidity. The clear solution was loaded on a DEAE-cellulose column (30 x 1 cm), equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The enzyme was eluted from the column by using a continuous gradient of buffer strength varying from 0.05 M to 0.75 M. The gradient was achieved by using 200 ml of 0.05 M buffer, pH 7.5 in the mixing chamber, which was connected to the column and by addition of 1 M buffer, pH 7.5 to the mixing chamber at the

same rate as the flow from the column. Fractions of about 3 ml were collected on an automatic fraction collector and a drop from each fraction was tested for enzyme activity. The fractions having high enzyme activity (25 - 30 ml) were pooled and precipitated with powdered ammonium sulphate to 0.8 saturation. After centrifugation at 50 000 g for 30 min, the precipitate was dissolved in 0.1 M potassium phosphate buffer, pH 7.5 (about 1 - 2 ml).

Gel filtration

The enzyme solution obtained after the DEAE-cellulose step was loaded on a Sepharose CL-6B column, equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. The enzyme was eluted from the column with buffer of the same composition. Fractions of 3 ml were collected on an automatic fraction collector and tested for enzyme activity. The fractions containing high enzyme activity were pooled (approximately 15 ml) and precipitated with powdered ammonium sulphate to 0.8 saturation. The precipitate was collected by centrifugation at 50 000 g for 30 min and dissolved in minimum amount of 0.1 M potassium phosphate buffer, pH 7.5.

Preparation of deacetyl citrate lyase

Deacetyl citrate lyase was prepared from K. aerogenes and S. faecalis citrate lyase holoenzyme as described by Buckel et al. (1973). Native citrate lyase (5 mg) was incubated at 25°C for 30 min in a final volume of 1 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 M neutralized hydroxylamine solution. The deacetyl enzyme was separated from excess hydroxylamine by filtration

through a Sephadex G-25 (1 x 40 cm) column, equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Fractions of 1 ml were collected and monitored for protein at 280 nm. Fractions containing high protein concentrations were pooled and showed no citrate lyase activity.

Preparation of citrate lyase subunits from *K. aerogenes*

The separation of subunits of *K. aerogenes* by gel filtration through Sepharose CL-6B under denaturing conditions using 6 M urea did not give good resolution nor high recoveries of protein. Citrate lyase subunits from *K. aerogenes* were therefore separated according to the procedure of Dimroth and Eggerer (1975b). Purified citrate lyase in 50 mM potassium phosphate buffer, pH 7.5 was dialyzed for 25 h against 10 mM potassium phosphate buffer, pH 7.0. The dialyzed protein (15 - 20 mg) was incubated at 0 - 4°C for 2 h in a total volume of 3 ml containing 5 M urea. The enzyme was loaded on a DEAE-cellulose column (0.8 x 6 cm), equilibrated with 10 mM potassium phosphate buffer, pH 7.0, containing 4 M urea. Elution with buffer of same composition yielded the first protein containing fraction. After 25 ml volume of solution was collected, the column was washed with 25 ml of 10 mM potassium phosphate buffer, pH 7.0, to remove urea. The column was then eluted with 25 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 0.4 M NaCl to obtain a second protein fraction. This was followed by eluting the column with 20 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 0.4 M NaCl and 4 M urea. wherein

a third protein fraction was obtained.

The first fraction was dialyzed for 16 h against 1 L of 0.5 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercapto-ethanol, followed by dialysis for 24 h against 1 L of 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM DTT. Before checking the activity, the enzyme fraction was dialyzed against 0.1 M Tris-HCl buffer, pH 8.0 from which DTT was omitted and concentrated by ultrafiltration.

The second fraction was dialyzed for 24 h against 1 L of 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercapto-ethanol and lyophilized.

The third fraction was dialyzed for 48 h with one change, against 1 L of 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 1 mM MgSO_4 . The enzyme fraction was concentrated by ultrafiltration.

All the three fractions were checked for purity by SDS-polyacrylamide gel electrophoresis.

Preparation of Coenzyme-A esters

Acetyl-CoA

Acetyl-CoA was prepared by acetylation of coenzyme-A with acetic anhydride. The procedure was similar to that described by Simon and Shamin (1953) for succinyl-CoA. 10 mg of CoA in 1 ml of distilled water was adjusted to pH 8.0 with potassium bicarbonate. Chilled acetic anhydride was added to the CoA solution to give a final concentration of 4.7 mM. The reaction mixture was stirred intermittently and maintained at 0°C. After 10 - 15 min, the reaction was complete as checked by disappearance of free thiol group

on estimation with DTNB. The solution was acidified with 1 N HCl to pH 4 - 5 and made upto 2 ml with distilled water.

Acetyl-CoA was estimated in the presence of citrate synthase and oxaloacetate by measuring the release of free CoA at 412 nm with DTNB (Srere et al., 1963). The reaction mixture contained in a total volume of 1 ml: 50 mM Tris-HCl buffer, pH 8.0; 1.1 U citrate synthase; 1 mM DTNB and 1 mM oxaloacetate. The reaction was initiated by addition of 10 μ l of acetyl-CoA.

(3S)-Citryl-CoA

(3S)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in presence of deacetyl citrate lyase and EDTA by a slight modification of the procedure of Dimroth et al. (1977). Deacetyl enzyme (100 μ g) was incubated at 30°C in a total volume of 1 ml containing 50 mM Tris-HCl buffer, pH 8.0; 10 mM EDTA; 10 mM acetyl-CoA and 100 mM citrate. After an hour, the solution was acidified with 1 N HCl to pH 4 - 5, diluted to 100 ml and loaded on a DEAE-cellulose (Cl^-)-column (1 x 8 cm). Citryl-CoA was separated from acetyl-CoA as described by Moffat and Khorana (1961). The DEAE-cellulose (Cl^-)-column on which citryl-CoA was loaded was washed with distilled water till the washings showed no absorption at 260 nm. Separation of the CoA esters was achieved by using a linear gradient of 0 to 0.2 M LiCl containing 0.003 M HCl. Fractions of 6 - 7 ml were collected at a flow rate of 60 ml/h. The fractions containing citryl-CoA were pooled and concentrated on a rotary evaporator, LiCl was removed by gel filtration

through a Sephadex G-10 column (1 x 50 cm). Citryl-CoA was estimated by measuring the increase in absorbance at 412 nm in presence of citrate synthase and DTNB (Buckel *et al.*, 1973; Srere *et al.*, 1963). The assay mixture at 25°C, contained in 1 ml total volume: 50 mM Tris-HCl buffer, pH 8.0; 1.1 U citrate synthase and 1 mM DTNB. The reaction was initiated by the addition of 10 ul of citryl-CoA.

Enzyme assays

Citrate lyase

The method which was routinely used in the purification procedure was that described by Mahadik and SivaRaman (1968). The reaction was carried out at 30°C and 3 ml of the test system contained: 100 mM sodium citrate; 10 mM MgSO_4 and 50 mM Tris-HCl buffer, pH 8.0. An aliquot containing about 5 - 10 ug of the enzyme was added to initiate the reaction and the keto acids formed were estimated by the method of Friedman and Haugen (1943) after arresting the reaction with 3 ml of 10% trichloroacetic acid.

A second method which was used for assaying purified enzyme preparations was a slight modification from that described by Singh and Srere (1971). The citrate lyase reaction was coupled with malate dehydrogenase and NADH and the change in optical density at 340 nm was recorded. The reaction was carried out at 30°C and the test system contained in a final volume of 1 ml: 5 mM sodium citrate; 3 mM MgSO_4 ; 50 mM Tris-HCl buffer, pH 8.0; 0.2 mM NADH and excess of malate dehydrogenase (about 15 ug). The

reaction was initiated by the addition of 0.1 - 0.5 ug of enzyme (0.01 ml) to the test system present in a 1 ml cuvette of 10 mm path length. A decrease of 6.22 in the optical density corresponds to 1 umol of keto acid formed.

1 U of enzyme is defined as the amount of enzyme required for catalysing the cleavage of 1 umol of citrate per min under the assay conditions. Specific activity is expressed as units. mg^{-1} protein.

Deacetyl citrate lyase

The overall cleavage activity, of deacetyl citrate lyase was assayed by coupling the citrate cleavage reaction with malate dehydrogenase and NADH in presence of citrate and acetyl-CoA (Bucket et al., 1973; Singh and Srere, 1971). The reaction was assayed at 25°C and 1 ml of assay mixture contained: 50 mM Tris-HCl buffer, pH 8.0; 3 mM MgSO_4 ; 0.2 mM NADH; about 15 ug malate dehydrogenase and 20 ug deacetyl citrate lyase. The reaction was initiated by adding citrate (5 mM final concentration) and acetyl-CoA (0.5 mM final concentration).

Transferase activity

The acyl transferase activity of the deacetyl enzyme as well as of the isolated α -subunit was determined from the initial velocity of CoA transfer from acetyl-CoA to citrate yielding citryl-CoA in presence of EDTA (Dimroth et al., 1977). Citryl-CoA which was formed was determined with DTNB at 412 nm by measuring CoA produced in presence of citrate synthase (Srere et al., 1963). The assay carried out at 25°C contained in a total volume of 1 ml: 50 mM

Tris-HCl buffer, pH 8.0; 1 mM DTNB; 1.1 U citrate synthase; 5 mM citrate; 10 mM EDTA and 0.5 mM acetyl-CoA. The reaction was initiated by the addition of the separated subunit (5 - 10 ug) or deacetyl citrate lyase (10 - 20 ug). Formation of citryl-CoA was monitored by the increase in absorbance at 412 nm. The concentration of citryl-CoA was calculated from the molar extinction coefficient $E_{412 \text{ nm}} = 13\,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ of the free thiol ion of DTNB (Ellman, 1959).

Lyase activity

The acyl lyase activity of the deacetyl citrate lyase complex was assayed from the initial rate of formation of oxaloacetate on cleavage of (3S)-citryl-CoA in presence of Mg^{2+} (Buckel *et al.*, 1973). Oxaloacetate formed was determined by the coupled assay procedure as described by Singh and Srere (1971). The reaction was carried out at 25°C. The reaction mixture contained in a total volume of 1 ml: 50 mM Tris-HCl buffer, pH 8.0; 3 mM MgSO_4 ; 0.2 mM NADH; 15 ug malate dehydrogenase and 100 uM citryl-CoA. The reaction was initiated by the addition of deacetyl citrate lyase (10 - 20 ug) and the decrease in absorbance at 340 nm was monitored.

Reaction with arginine modifying reagents

Deacetyl citrate lyase (0.5 mg/ml) or the isolated subunit (0.3 mg/ml) was incubated at 30°C in a final volume of 0.2 ml containing 50 mM potassium phosphate buffer, pH 7.6, either 10 mM EDTA or 3 mM MgSO_4 and various

Concentrations of phenylglyoxal or 2,3-butanedione. In experiments to determine the buffer which produces maximal effects, potassium phosphate buffer, pH 7.6 was replaced by 50 mM Tris-HCl buffer, pH 7.6 and 50 mM Hepes buffer, pH 7.6. Enzymes incubated with the buffer alone served as the control. The reaction mixture was shielded from light to avoid any photochemical effect when 2,3-butanedione was used as the probe, since among dicarbonyls, 2,3-butanedione is particularly prone to photosensitization (Riordan, 1973; Ryang and Wang, 1978; Rubin and Inbar, 1978; Makinen et al., 1982). Both the modifying reagents were prepared fresh. Unless otherwise stated, aliquots of 20 μ l were removed at different time intervals for assay of enzymatic activities.

It has been reported that 2,3-butanedione causes the inactivation of malate dehydrogenase (Foster and Harrison, 1974). Hence the effects of 2,3-butanedione as well as of phenylglyoxal on malate dehydrogenase and citrate synthase, the two coupling enzymes which are used in the assay systems for checking the lyase and transferase activities, were studied. Aliquots (20 μ l) of phenylglyoxal (10 mM) and 2,3-butanedione (30 mM) were added separately to 1 ml test systems for determination of malate dehydrogenase and citrate synthase activities. The assay systems contained 50 mM Tris-HCl buffer and the reactions were initiated by the addition of the enzymes. The former test system was similar to that described by Ochoa (1955), except that 50 mM Tris-HCl buffer was used. The latter test system was essentially that described by Srere et al., (1963), coenzyme A liberated being estimated with DTNB at 412 nm. No measurable loss in the

activities of malate dehydrogenase and citrate synthase was observed indicating that the modifiers had no apparent effect on the two enzymes under the experimental conditions used. Moreover, in the assay procedures malate dehydrogenase and citrate synthase were taken in excess.

The apparent first order rate constant depends upon the concentration of the modifier. This can be expressed by the following equation

$$K_{app} = K(M)^n$$

where K_{app} is the apparent first order rate constant for inactivation, K is the second order rate constant, M signifies the concentration of the modifier and n is a number equal to the average order of the reaction with respect to concentration of the modifier. Taking the logarithm of both sides leads to

$$\log K_{app} = \log K + n \log (M)$$

K_{app} can be calculated from a semi-logarithm plot of the enzyme activity remaining as a function of time. The order of the reaction (n) can be experimentally estimated by determining K_{app} at a number of different concentrations of the modifier. A plot of K_{app} against $\log (M)$ should give a straight line with a slope equal to n , where n is the number of molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme-inhibitor complex (Levy et al., 1963; Ramakrishna and Benjamin, 1981; Marcus et al., 1976).

Protection studies

The effect of various ligands on inactivation by phenylglyoxal or 2,3-butanedione was studied by preincubating deacetyl enzyme or the isolated subunit for 5 min at 30°C with the ligand followed by addition of the stated amounts of inhibitor.

Effect of ligands on transferase activity

Deacetyl citrate lyase (0.5 mg/ml) or isolated subunit (0.3 mg/ml) was incubated for 5 min in a final volume of 0.2 ml containing 50 mM potassium phosphate buffer, pH 7.6; 10 mM EDTA and various ligands: citrate (10 mM), (3S)-citryl-CoA (0.5 mM), acetyl-CoA (1 mM), citrate (10 mM) + acetyl-CoA (1 mM), followed by addition of phenylglyoxal or 2,3-butanedione to the desired concentrations. Aliquots of 20 μ l were removed thereafter at regular intervals of time and checked for residual transferase activity.

Effect of ligands on lyase activity

Deacetyl citrate lyase (0.5 mg/ml) was incubated for 5 min in a final volume of 0.2 ml containing 50 mM potassium phosphate buffer, pH 7.6; 3 mM MgSO_4 or 10 mM EDTA and various ligands: (3S)-citryl-CoA (0.5 mM), citrate (10 mM) and acetyl-CoA (1 mM), followed by addition of phenylglyoxal or 2,3-butanedione to the desired final concentrations. At regular intervals of time, aliquots of 20 μ l were removed and checked for residual lyase activity.

RESULTS

Purification of citrate lyase

The results of purification of citrate lyases from K. aerogenes and S. faecalis in typical experiments are briefly summarized in Tables I.1 and I.2.

As seen from the Tables, there is an increase in the total activity on addition of acetate and ATP to the sonic extracts, indicating the presence of some deacetyl citrate lyase which is reactivated by the enzyme acetate: SH (acyl-carrier protein) citrate lyase ligase in the extract.

The specific activity values and yields of citrate lyases from the two sources compared well with those reported earlier by Tikare (1979) and Hiremath et al. (1976) respectively.

The final enzyme preparation from K. aerogenes was homogeneous as seen in the ultracentrifuge (Fig. I.1) and polyacrylamide gel electrophoresis (Fig. I.2). Purity of the enzyme was also established by SDS-polyacrylamide gel electrophoresis. The electrophoretic patterns of the stained gels from K. aerogenes and S. faecalis are shown in Fig. I.3 and Fig. I.4. The enzymes dissociate into the three component non-identical subunits α , β and γ .

Separation of subunits of citrate lyase from K. aerogenes

Fractionation profile of the subunits from 15 - 20 mg of citrate lyase from K. aerogenes by DEAE-cellulose chromatography in presence of 5 M urea is shown in Fig. I.5. Fraction I which was not adsorbed on the column consisted of the α -chain. This subunit was enzymatically active. Fraction II which was

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 .	460	4.9	4733	2.1	-
Cell-free extract + acetate + ATP.	460	4.9	5860	2.6	100
Streptomycin sulphate treatment followed by centrifugation to remove precipitated nucleic acids.	450	3.9	5265	3.0	90
0.05 M buffer elutate of second alumina C gel.	200	2.75	3740	6.8	64
Ammonium sulphate fraction (0.2 - 0.5 saturation) dissolved in 0.05 M buffer, pH 7.5.	4.8	36	3456	20	59
DEAE-cellulose fraction pooled and fractionated with saturated $(\text{NH}_4)_2\text{SO}_4$ and the precipitate dissolved in 0.05 M buffer, pH 7.5.	1.4	31.7	2929	66	50
Sepharose CL-6B fractions pooled, precipitated with saturated $(\text{NH}_4)_2\text{SO}_4$ and the precipitate dissolved in 0.05 M buffer, pH 7.5.	1.2	11.2	1169	87	20

TABLE I.2 Purification of citrate lyase from *Streptococcus faecalis*

Wet weight of cells: 110 g

Fraction	Volume ml	Protein mg/ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract.	410	4.9	4420	2.2	-
Cell-free extract + acetate + ATP.	411	4.9	9667	4.8	100
Streptomycin sulphate fraction.	405	3.5	8222	5.8	85
0.05 M buffer eluate of second alumina C gel.	200	2.4	5760	12	60
Ammonium sulphate fraction (0.5 - 0.8 saturation) dissolved in 0.05 M buffer, pH 7.5.	5.2	20	4160	40	43
DEAE-cellulose fraction pooled and fractionated with saturated $(\text{NH}_4)_2\text{SO}_4$ and the precipitate dissolved in 0.05 M buffer, pH 7.5.	2.0	19.9	2308	58	24
Sepharose CL-6B fraction pooled, precipitated with saturated $(\text{NH}_4)_2\text{SO}_4$ and the precipitate dissolved in 0.05 M buffer, pH 7.5.	1.5	9.5	1254	88	13

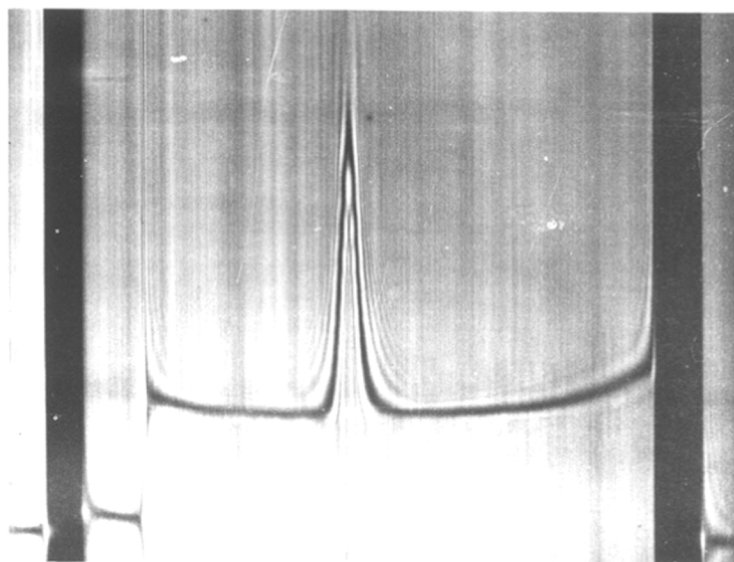


FIG. I.1 SEDIMENTATION PROFILE OF NATIVE CITRATE
LYASE FROM K. aerogenes

Protein concentration	3.5 mg/ml
Buffer	0.05 M potassium phosphate, pH 7.5 with 1.6 mM MgSO_4
Speed	59 780 rpm
Temperature	5°C
Phase plate	60°
Time	32 min

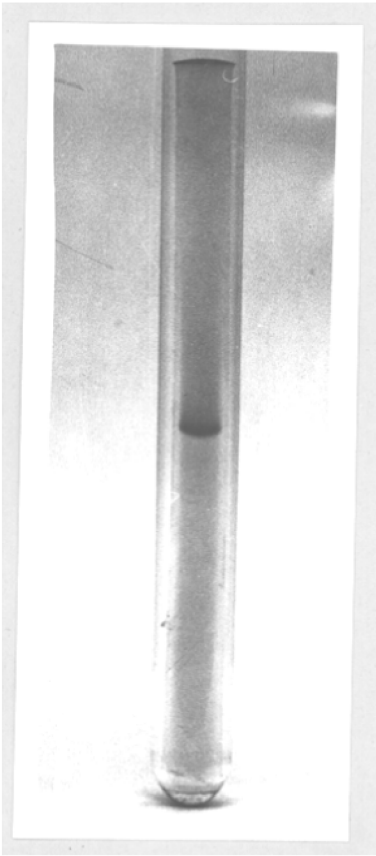


FIG. I. 2 POLYACRYLAMIDE GEL ELECTROPHORESIS OF
NATIVE CITRATE LYASE FROM K. aerogenes
5% gel; Tris-glycine buffer, pH 8.3;
4 mA/tube
Protein loaded 50 μ g

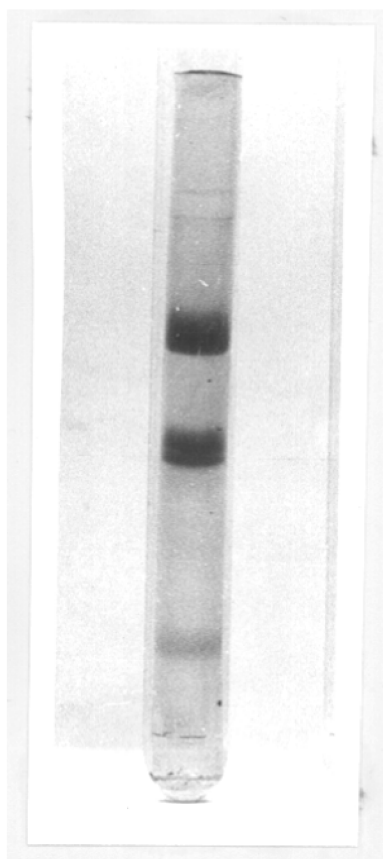


FIG. I.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
CITRATE LYASE FROM K. aerogenes

10% gel; 0.05 M sodium phosphate buffer,
pH 7.3, containing 0.1% SDS;

8 mA/tube

Protein loaded 100 μ g

FIG. I.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
CITRATE LYASE FROM S. faecalis

10% gel; 0.05 M sodium phosphate buffer,
pH 7.3, containing 0.1% SDS;

8 mA/tube

Protein loaded 100 ug

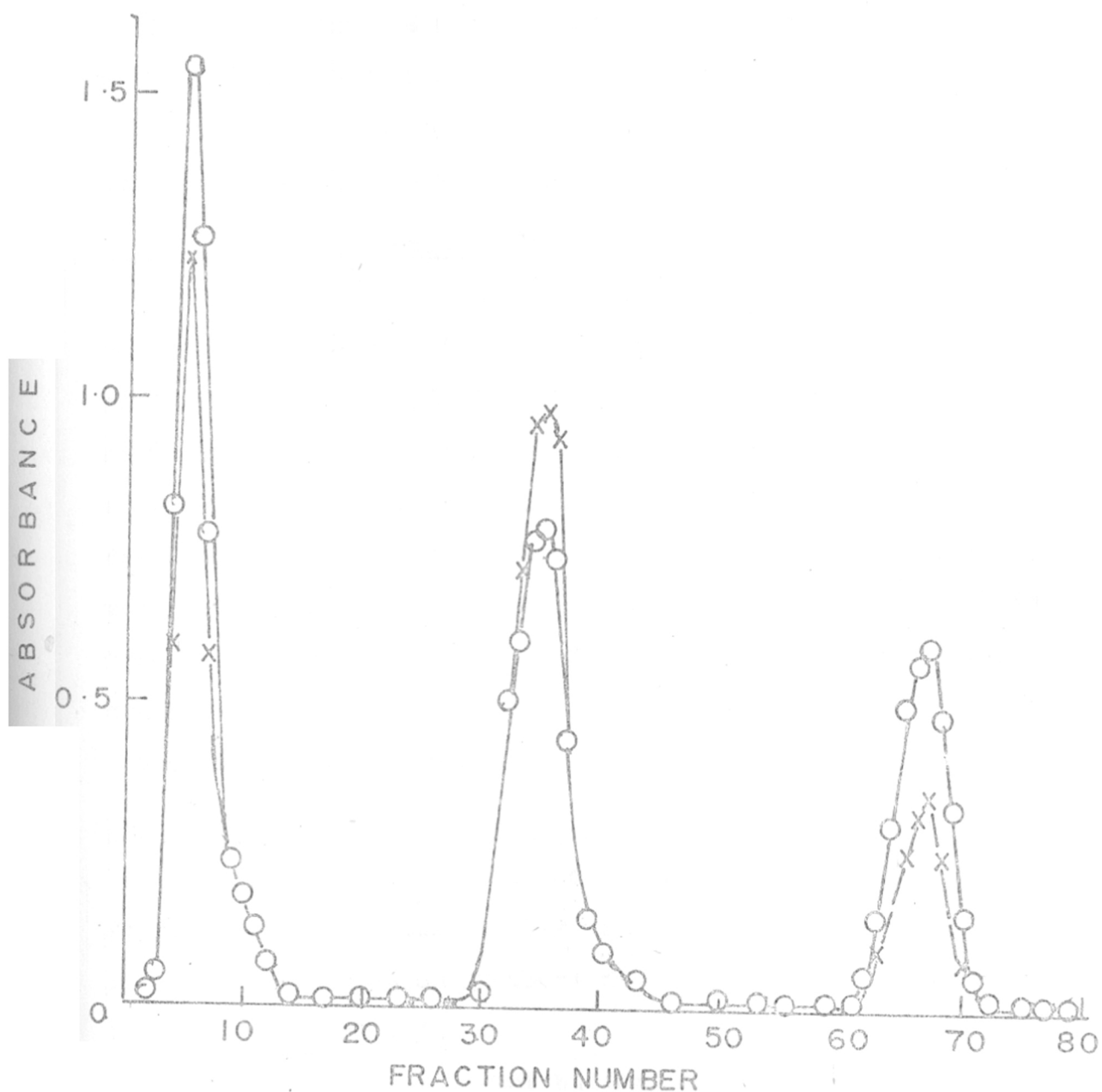


FIG. 1.5 Resolution of *K. aerogenes* citrate lyase subunits by DEAE-cellulose chromatography
 Absorbance at 280 nm
 Absorbance at 260 nm

eluted in presence of 0.4 M NaCl was shown to act as the acyl-carrier protein, while Fraction III which was eluted by buffer containing 4 M urea as well as 0.4 M NaCl contained the β -chain. The β -subunit was found to have lost its enzymic activity during the fractionation procedure. There was complete separation of all the three subunits.

Preparation of (3S)-citryl-CoA

The preparation of (3S)-citryl-CoA was carried out according to the method of Dimroth et al. (1977b). The purification of a typical preparation of (3S)-citryl-CoA is shown in Fig. I.6. All the fractions were checked for (3S)-citryl-CoA in presence of citrate synthase and DTNB as described in Materials and Methods. Three distinct peaks were obtained; the first was a very small peak not identified. The second and third peaks were characterized as acetyl-CoA and (3S)-citryl-CoA, respectively. Acetyl-CoA and (3S)-citryl-CoA eluted around 10 mmhos and 15 mmhos, respectively.

Inactivation of transferase activity

The acetyl-CoA mediated acyl-transferase activity both of deacetyl citrate lyase complex and of the isolated subunit was rapidly inactivated on incubation with phenylglyoxal or 2,3-butanedione at pH 7.6.

The effect of different buffers on the inactivation of K. aerogenes citrate lyase by phenylglyoxal is shown in Table I.3.

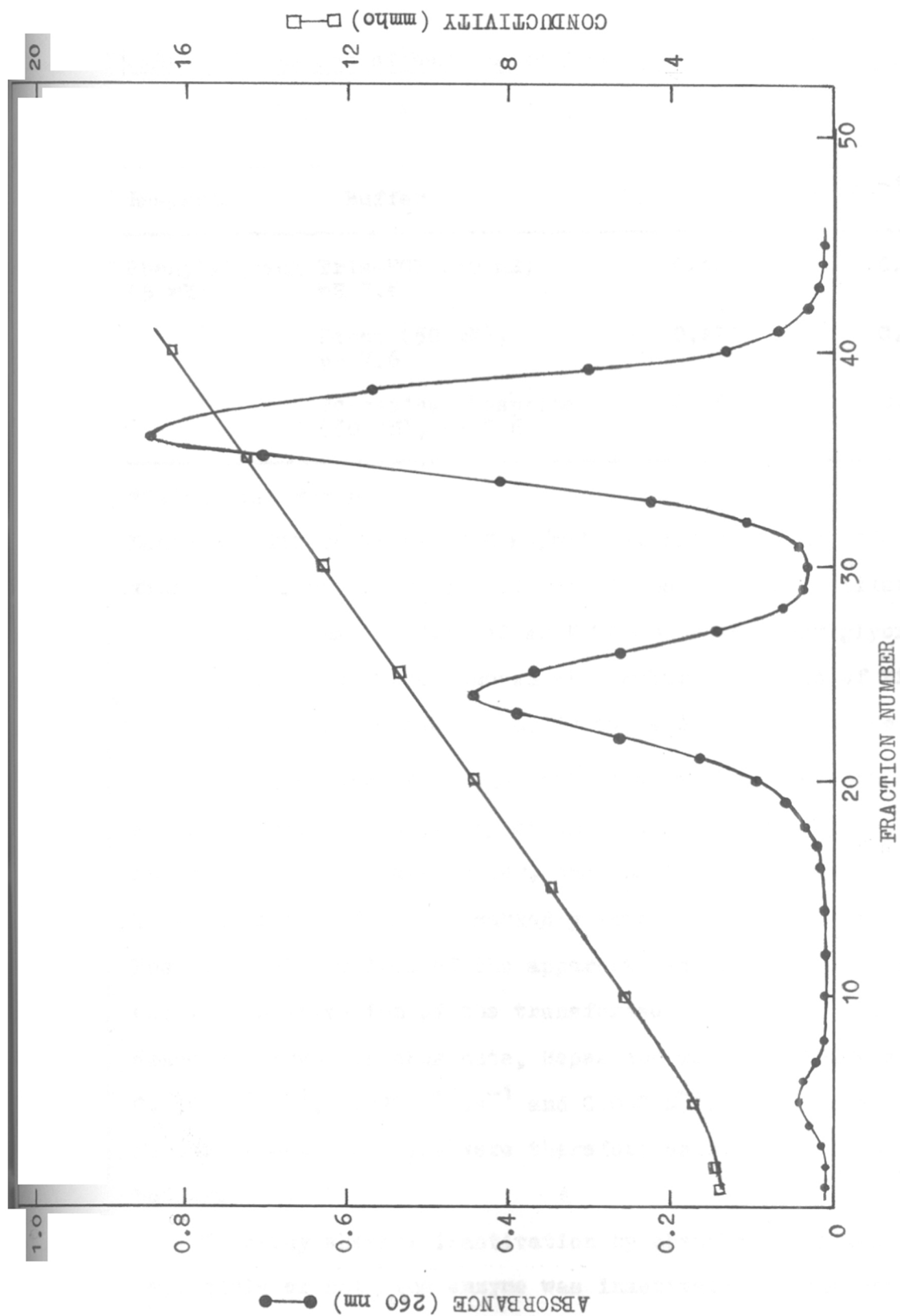


FIG. I.6 PURIFICATION OF (3S)-CITRYL-CoA BY DEAE-CELLULOSE (Cl⁻ - FORM) CHROMATOGRAPHY

TABLE I.3 Effect of buffers on inactivation of K. aerogenes citrate lyase by phenylglyoxal

Reagent	Buffer	$10^3 \times K_{app}(s^{-1})$	$K(M^{-1}.s^{-1})^*$
Phenylglyoxal (5 mM)	Tris-HCl (50 mM), pH 7.6	0.162	0.032
	Hepes (50 mM), pH 7.6	0.495	0.099
	Potassium phosphate (50 mM), pH 7.6	0.568	0.114

*Calculated for $n = 1$.

Deacetyl citrate lyase (0.5 mg/ml) was incubated at 30°C in a final volume of 0.2 ml containing 50 umoles of the different buffer solutions, pH 7.6; 10 mM EDTA and 5 mM phenylglyoxal. Aliquots of 20 μ l were removed at regular intervals of time and assayed for transferase activity as described in the text.

It can be seen from the Table that the maximum inactivation of the transferase activity of the deacetyl enzyme is obtained in potassium phosphate buffer, the inactivation being slightly less in Hepes buffer and markedly lower in Tris-HCl buffer. The calculated values of the apparent second order rate constant (K) for inactivation of the transferase activity of the deacetyl enzyme in phosphate, Hepes and Tris-HCl buffers are $0.114 M^{-1}.s^{-1}$, $0.099 M^{-1}.s^{-1}$ and $0.032 M^{-1}.s^{-1}$, respectively. Further kinetic studies were therefore carried out in phosphate buffer.

To study whether inactivation by phenylglyoxal was reversible or not, the enzyme was inactivated by the modifier

and excess amount of the modifier was removed from the incubation mixture. Deacetyl citrate lyase incubated with the reagent till about 10 - 20% of the original activity remained was immediately passed down a Sephadex G-25 column, equilibrated with 50 mM potassium phosphate buffer, pH 7.6. The fractions which contained high protein concentrations were pooled. Similar amounts of enzyme were tested for activity before and after passing down the column. The treated enzyme separated by filtration through the gel showed only 10 - 20% of the activity of the native enzyme indicating that the enzyme is irreversibly inactivated by phenylglyoxal.

Phenylglyoxal was found to be a more potent inhibitor than 2,3-butanedione. When K. aerogenes deacetyl citrate lyase was incubated separately with the inhibitors at the same final concentration of 10 mM and aliquots of the enzyme mixture were removed after 30 min of incubation and assayed for transferase activity, it was observed that the enzyme incubated with phenylglyoxal had lost 88% of its initial activity, the corresponding loss being only 51% with 2,3-butanedione.

The results obtained with deacetyl citrate lyase from K. aerogenes and various phenylglyoxal and 2,3-butanedione concentrations are presented in Fig. I.7 and Fig. I.8, respectively. The enzyme activity remained unchanged in the absence of the modifiers during the period of the experiment. The inactivation rates in presence of arginine reagents follow pseudo-first order kinetics, both plots being linear

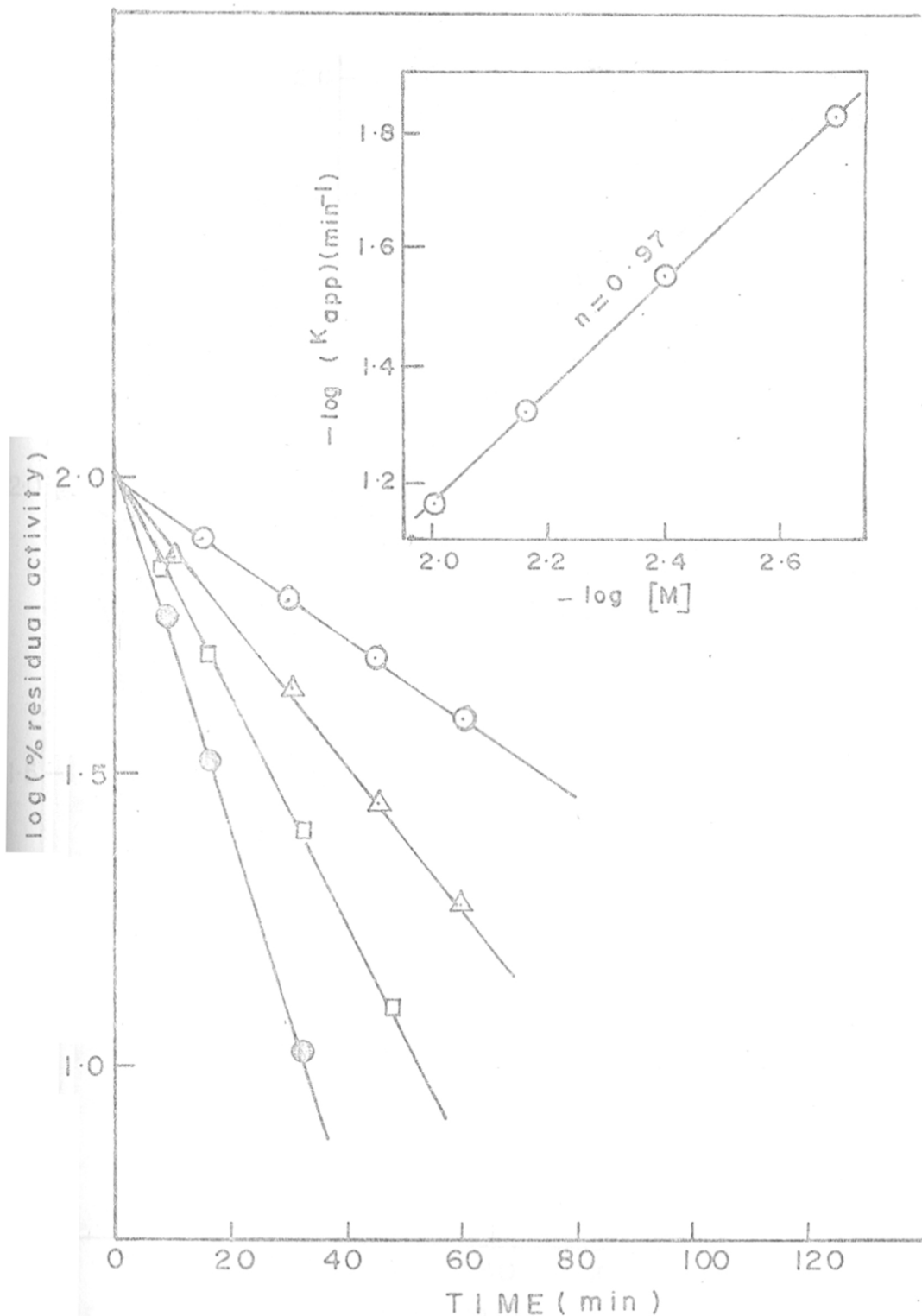


FIG. I.7 Inactivation of the acyl transferase activity of deacetyl citrate lyase from *K. aerogenes* by phenylglyoxal (PG).

2 mM PG (), 4 mM PG (), 7 mM PG (), 10 mM PG ().
 Inset: Determination of the order of the reaction with respect to phenylglyoxal.

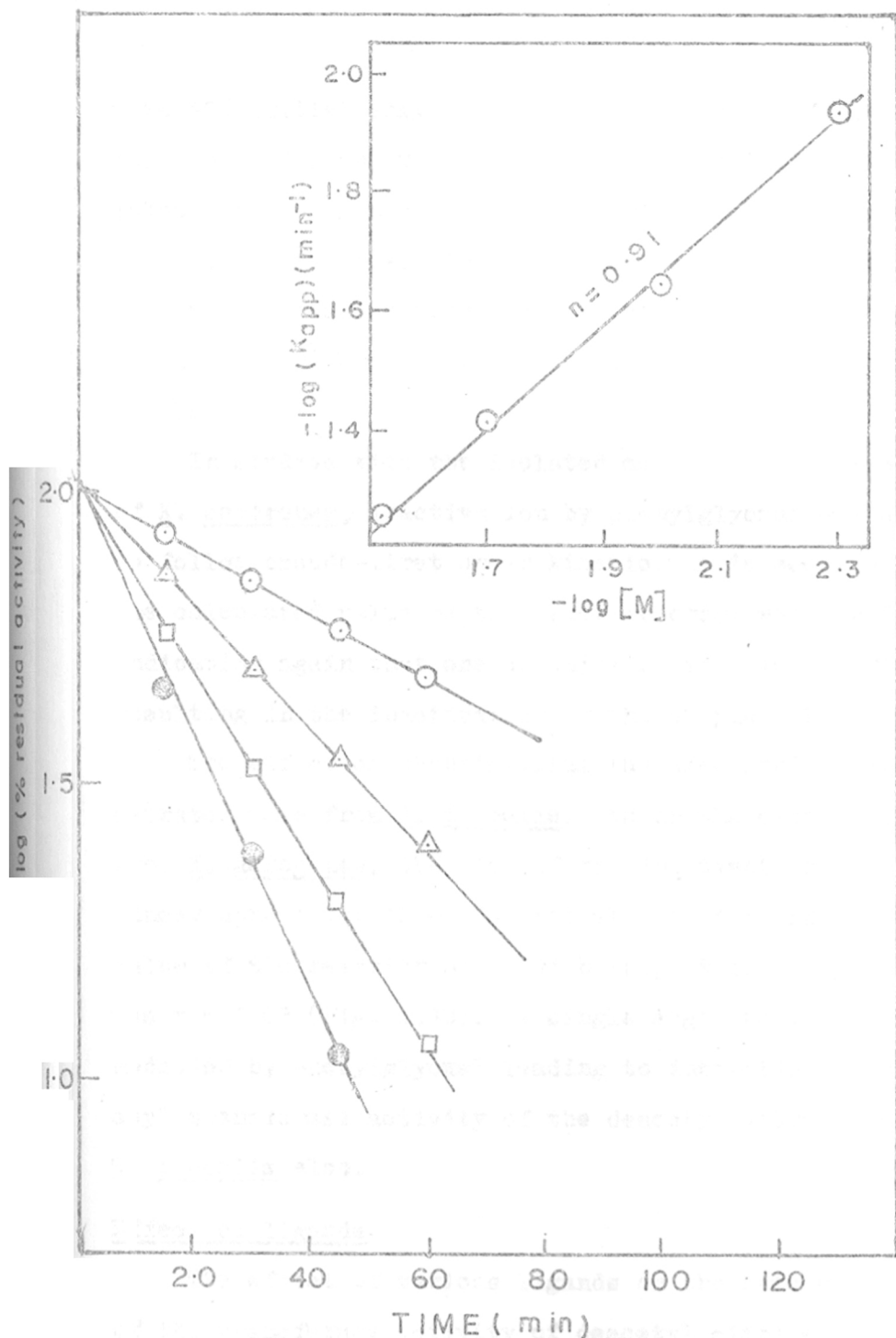


FIG. 1.3 Inactivation of the acyl transferase activity of deacetyl citrate lyase from *K. aerogenes* by 2,3-butanedione.

5 mM 2,3-butanedione (○), 10 mM 2,3-butanedione (□), 20 mM 2,3-butanedione (△), 30 mM 2,3-butanedione (●).

Inset: Determination of the order of the reaction with respect to 2,3-butanedione.

upto 10% initial enzyme activity. The values for the reaction order (n) with respect to the arginine reagent was calculated to be $n = 0.97$ for phenylglyoxal and $n = 0.91$ for 2,3-butanedione. These values would indicate that loss of activity results from reaction of one arginine residue per acyl transferase subunit in the deacetyl citrate lyase complex.

In studies with the isolated acyl transferase α -subunit of K. aerogenes, inactivation by phenylglyoxal was also found to follow pseudo-first order kinetics as is seen in Fig. I.9. The calculated value of the reaction order was $n = 0.93$, indicating again that one arginine residue is modified resulting in the inactivation of the enzyme subunit.

The effect of phenylglyoxal was also studied with deacetyl citrate lyase from S. faecalis. As in the case of the enzyme from K. aerogenes, the plot of the inactivation rate was linear upto about 10 - 20% initial activity and the calculated value of the reaction order with respect to phenylglyoxal was $n = 1.02$ (Fig. I.10). A single arginine residue is thus modified by phenylglyoxal leading to inhibition of the acyl transferase activity of the deacetyl citrate lyase from S. faecalis also.

Effect of ligands

The effect of various ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase by the modifying reagent was studied to determine whether the ligands protected the enzyme from inactivation by the modifier. The

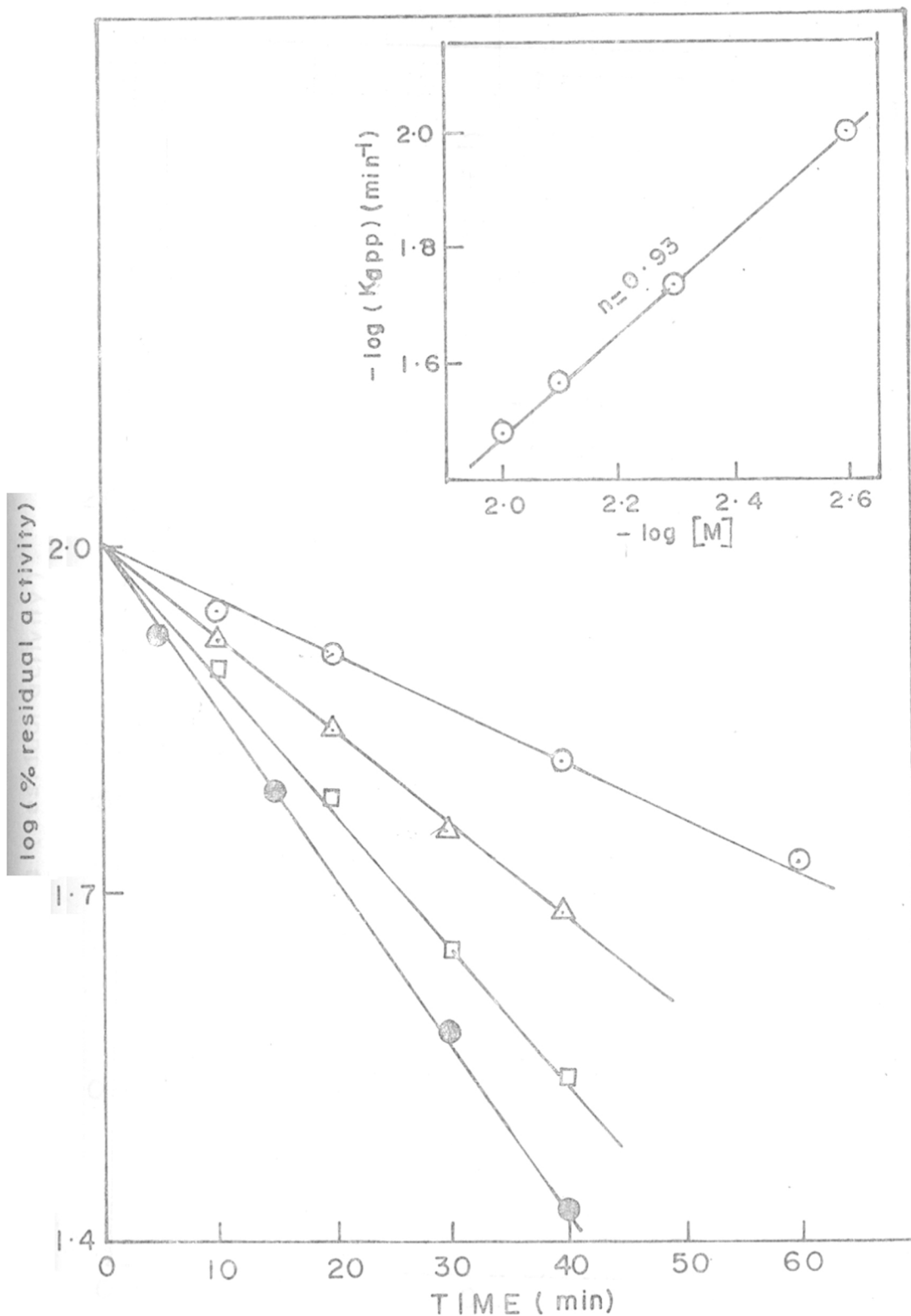


FIG. I.9 Inactivation of the isolated transferase -subunit of citrate lyase from *K. aerogenes* by phenylglyoxal (PG).

2.5 mM PG (○); 5 mM PG (△); 7.5 mM PG (□); 10 mM PG (○).

Inset : Determination of the order of the reaction with respect to phenylglyoxal.

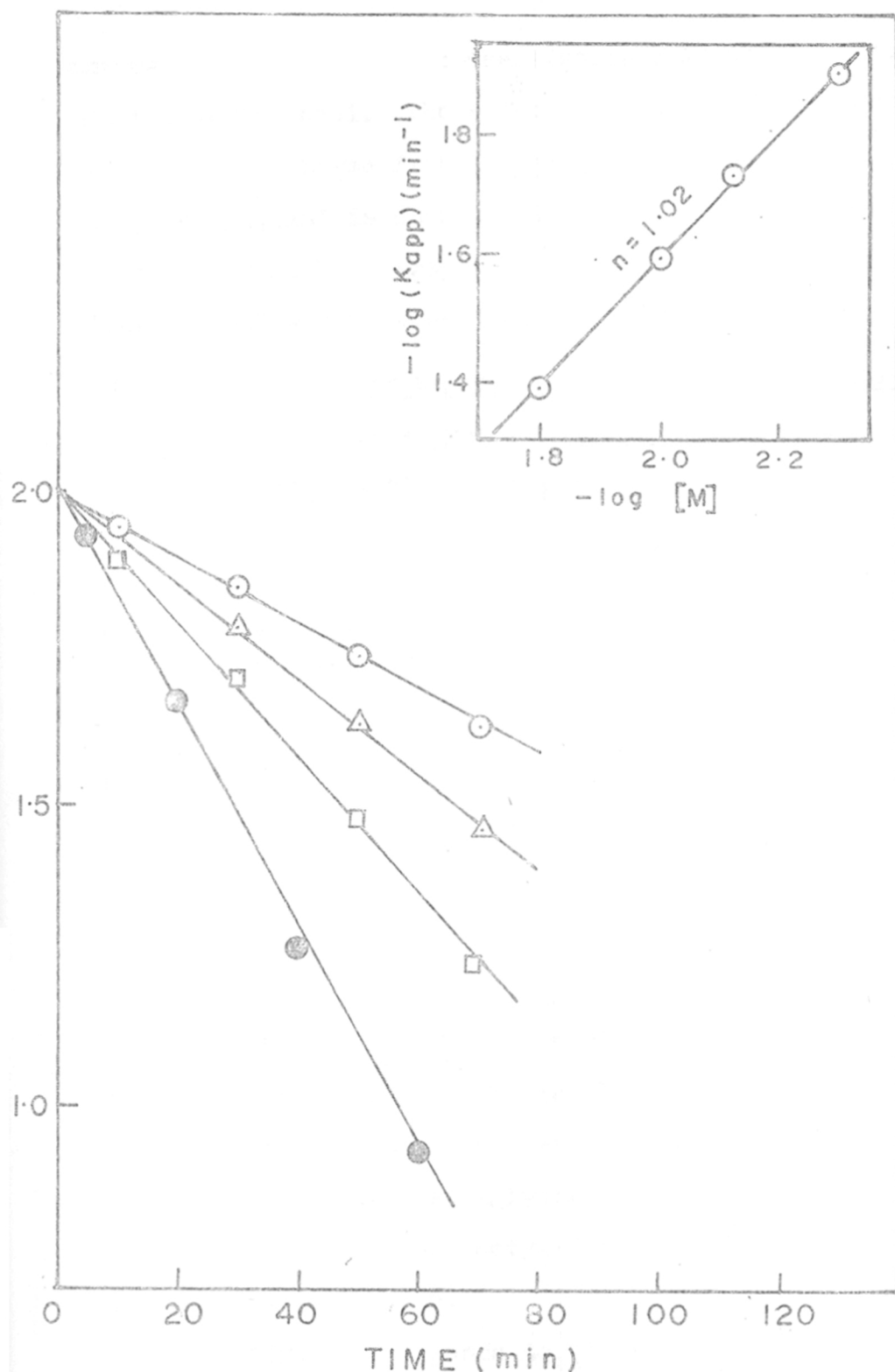


FIG. I.10 Inactivation of the acyl transferase activity of deacetyl citrate lyase from *S. faecalis* by phenylglyoxal (PG). 5 mM PG (○), 7.5 mM PG (□), 10 mM PG (△), 15 mM PG (●). Inset: Determination of the order of the reaction with respect to phenylglyoxal.

enzyme was incubated with the ligands followed by incubation with phenylglyoxal. The effect of the ligands in protecting the deacetyl enzyme from K. aerogenes against inactivation by phenylglyoxal is shown in Fig. I.11 and Table I.4. Inactivation of the enzyme in the presence of the added ligands followed pseudo-first order kinetics.

TABLE I.4 Effect of ligands on the transferase activity of deacetyl enzyme from K. aerogenes against inactivation by phenylglyoxal

Enzyme	Reagent	Ligand	$10^3 \times K_{app}$
Acyl transferase activity of deacetyl citrate lyase from <u>K. aerogenes</u>	Phenylglyoxal (5 mM)	-	34
		Citryl-CoA (0.5 mM)	14
		Citrate (10 mM)	18
		Citrate (10 mM) + Acetyl-CoA (1 mM)	28
		Acetyl-CoA (1 mM)	109

Citrate (10 mM) and (3S)-citryl CoA (0.5 mM) decreased the rate of inactivation by 45% and 55%, respectively. In contrast, acetyl-CoA (1 mM) was observed to enhance the rate of inactivation by phenylglyoxal almost 3-fold. A combination of citrate (10 mM) and acetyl-CoA, however, decreased the rate of inactivation by 20%.

The effect of ligands to protect deacetyl citrate lyase from K. aerogenes against inactivation by 2,3-butanedione

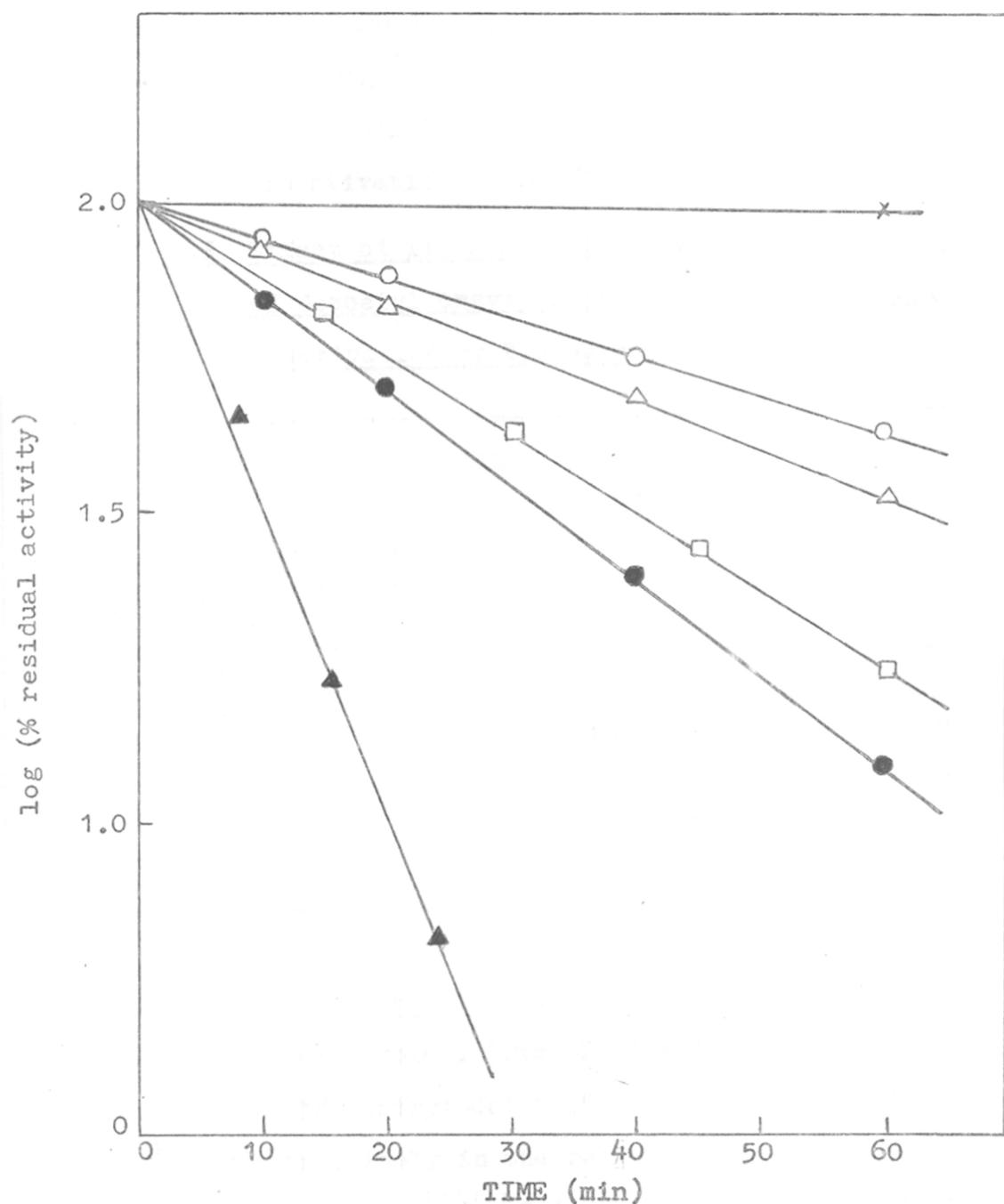


FIG. I.11. Effect of ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase from *K. aerogenes* by phenylglyoxal (PG). Control (x), 5 mM PG (●), 5 mM PG + 0.5 mM citryl-CoA (○), 5 mM PG + 10 mM citrate (Δ), 5 mM PG + 1 mM acetyl-CoA (▲), 5 mM PG + 10 mM citrate + 1 mM acetyl-CoA (□).

is shown in Fig. I.12 and Table I.5. Citrate (10 mM) and (3S)-citryl-CoA (0.5 mM) decreased the rate of inactivation by 40% and 50%, respectively, while acetyl-CoA (1 mM) increased the rate of inactivation almost 3-fold.

TABLE I.5 Effect of ligands on the transferase activity of deacetyl enzyme from *K. aerogenes* against inactivation by 2,3-butanedione

Enzyme	Reagent	Ligand	$10^3 \times K_{app}$ (min ⁻¹)
Acyl transferase activity of deacetyl citrate lyase from <i>K. aerogenes</i>	2,3-butanedione (5 mM)	-	12
		Acetyl-CoA (1 mM)	33
	2,3-butanedione (20 mM)	-	38
		Citryl-CoA (0.5 mM)	18
		Citrate (10 mM)	24

Protection studies with the isolated transferase subunit from *K. aerogenes* showed similar results as with the deacetyl enzyme complex, (Fig. I.13 and Table I.6). Citrate (10 mM) and (3S)-citryl-CoA (0.5 mM) caused 50% and 60% decreases respectively in the rates of inactivation, while acetyl-CoA (1 mM) caused a 5 to 6-fold enhancement in the rate of inactivation, when phenylglyoxal was used as the modifying reagent.

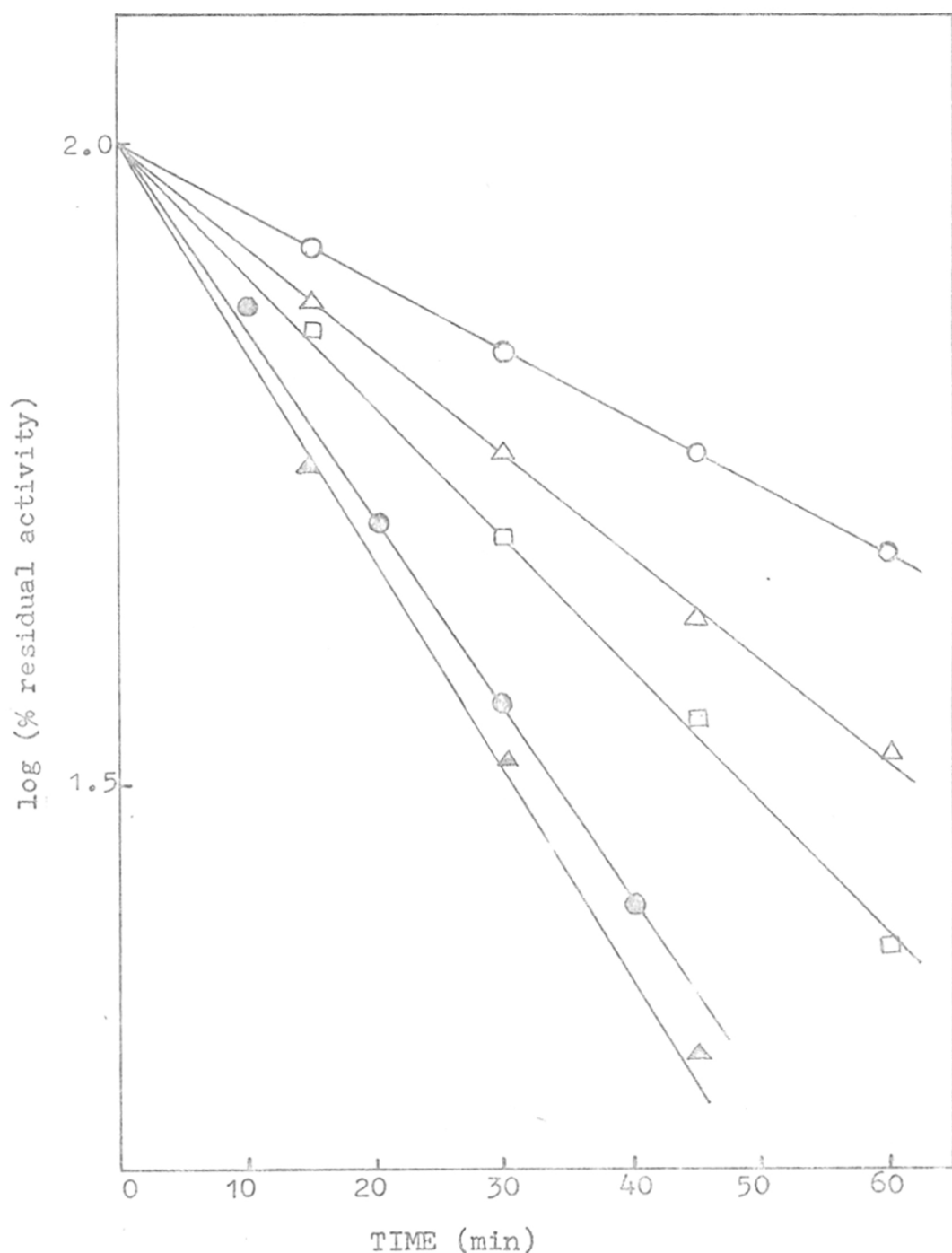


FIG. I.12. Effect of ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase from *K. aerogenes* by 2,3-butanediol.

5 mM Butanediol (O), 5 mM butanediol + 1 mM acetyl-CoA (●), 20 mM butanediol (Δ), 20 mM butanediol + 0.5 mM citryl-CoA (△), 20 mM butanediol + 10 mM citrate (□).

TABLE I.6 Effect of ligands on inactivation of the isolated transferase subunit from *K. aerogenes* by phenylglyoxal

Enzyme	Reagent	Ligand	$10^3 \times K_{app}$ (min ⁻¹)
<u>Isolated transferase subunit from <i>K. aerogenes</i></u>	Phenylglyoxal (5 mM)	-	18
		Acetyl-CoA (1 mM)	99
	Phenylglyoxal (7.5 mM)	-	25
		Citryl-CoA (0.5 mM)	9
		Citrate (10 mM)	14

The reaction order for inactivation was also determined in presence of acetyl-CoA (1 mM) (Fig. I.14). A value of $n = 1.15$ with phenylglyoxal obtained under these conditions would suggest that the rate of reaction of the essential arginine residue is enhanced without the involvement of any additional residues.

The effect of various ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase from *S. faecalis* by phenylglyoxal is shown in Fig. I.15 and Table I.7. Citrate (15 mM) protected the enzyme effectively by decreasing the rate of inactivation by 70%. Citryl-CoA (0.5 mM) decreased the rate by 50%. The effect of acetyl-CoA was similar to that shown in the case of *K. aerogenes*, the rate of inactivation being increased 3 to 4-fold.

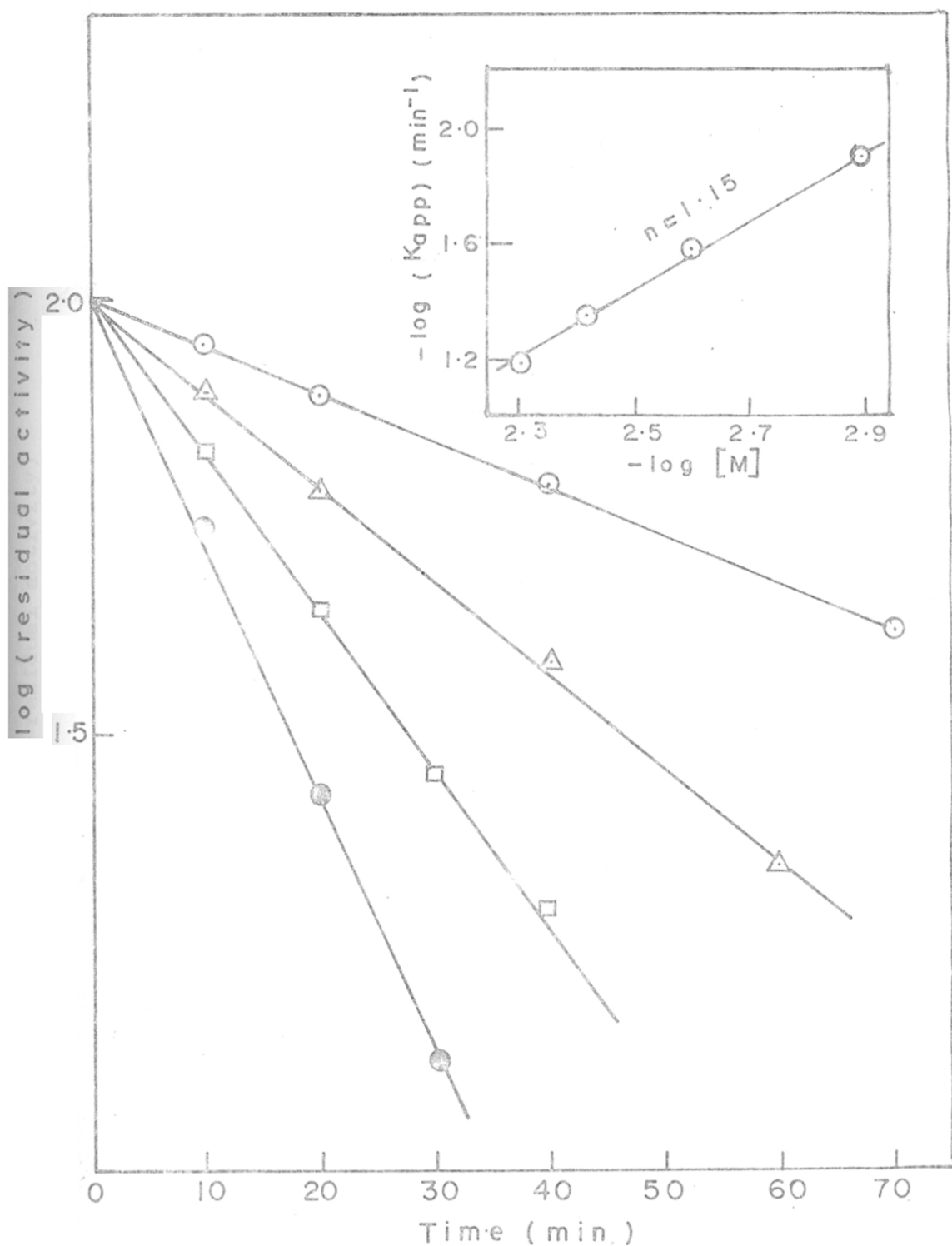


FIG. I.14 Inactivation of the acyl transferase activity of deacetyl citrate lyase from *K. aerogenes* by phenylglyoxal (PG) in presence of Acetyl-CoA (1 mM).

1.25 mM PG (○); 2.5 mM PG (□); 3.75 mM PG (△); 5 mM PG (●).

Inset : Determination of the order of the reaction with respect to phenylglyoxal.

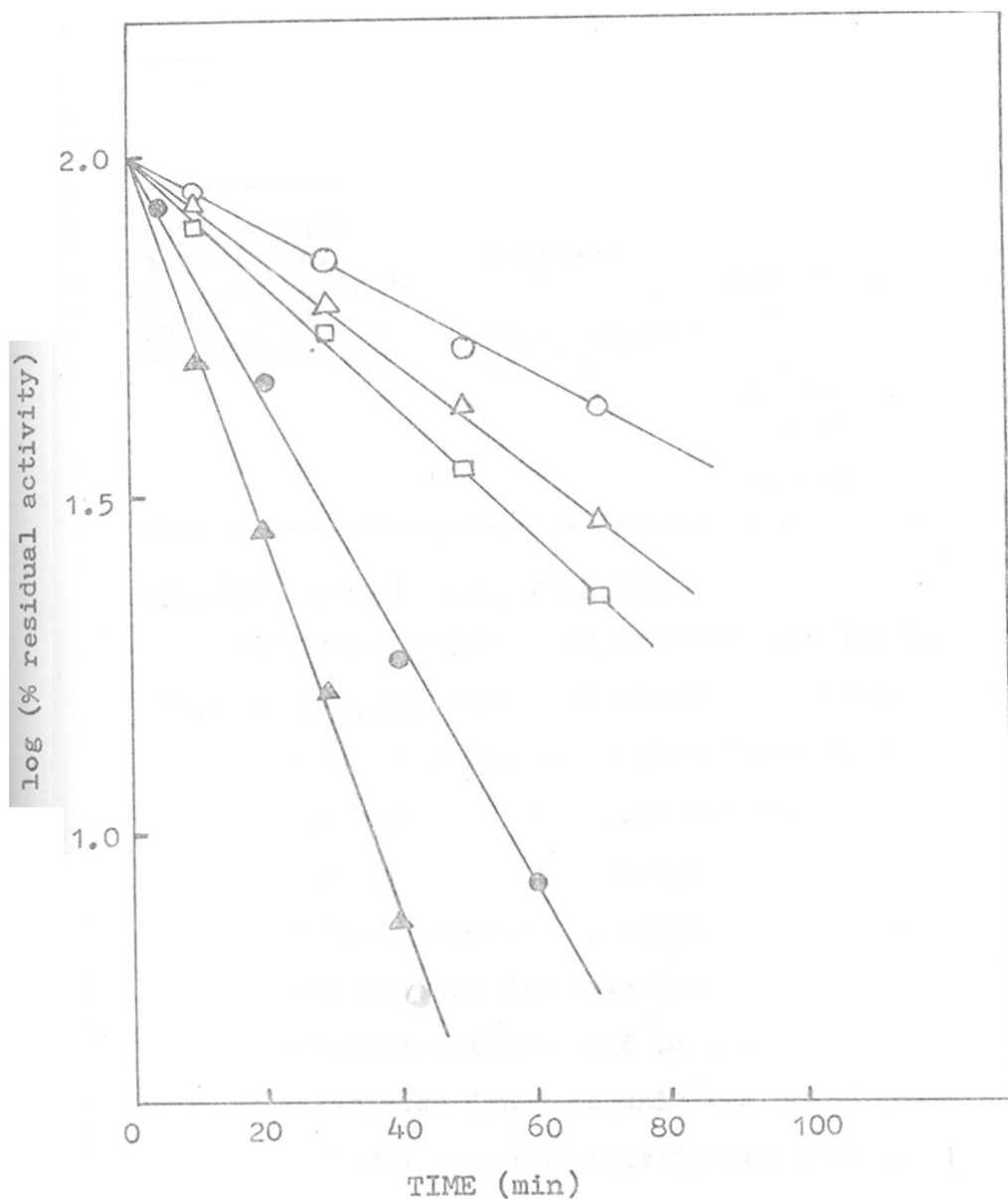


FIG. I.15. Effect of ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase from *S. faecalis* by phenylglyoxal.

7.5 mM PG (Δ), 7.5 mM PG + 1 mM acetyl-CoA (▲),
 15 mM PG (●), 15 mM PG + 0.5 mM citryl-CoA (□),
 15 mM PG + 15 mM citrate (○).

TABLE I.7 Effect of ligands on inactivation of transferase activity of deacetyl citrate lyase from *S. faecalis* by phenylglyoxal

Enzyme	Reagent	Ligand	$10^3 \times K_{app}$ (min ⁻¹)
Acyl transferase activity of deacetyl citrate lyase from <i>S. faecalis</i>	Phenylglyoxal (7.5 mM)	-	18
		Acetyl-CoA (1 mM)	61
	Phenylglyoxal (15 mM)	-	40
		Citryl-CoA (0.5 mM)	21
		Citrate (15 mM)	12

Inactivation of lyase activity

The lyase activity of deacetyl citrate lyase complex from *K. aerogenes* was also inactivated by both the modifiers. The rates of inactivation followed pseudo-first order kinetics as shown in Fig. I.16 and Fig. I.17. Values of $n = 0.92$ and $n = 0.91$ for phenylglyoxal and 2,3-butanedione, respectively, suggests the minimal requirement of one arginine residue per subunit for inactivation. Omission of $MgSO_4$ from incubation mixtures had no effect on the inactivation.

The isolated lyase subunit was not studied since it showed markedly lowered activity probably through denaturation during the isolation procedure (Dimroth and Eggerer, 1975b).

The inactivation of the lyase activity of deacetyl citrate lyase complex from *S. faecalis* by phenylglyoxal is shown in Fig. I.18. The value of the reaction order with

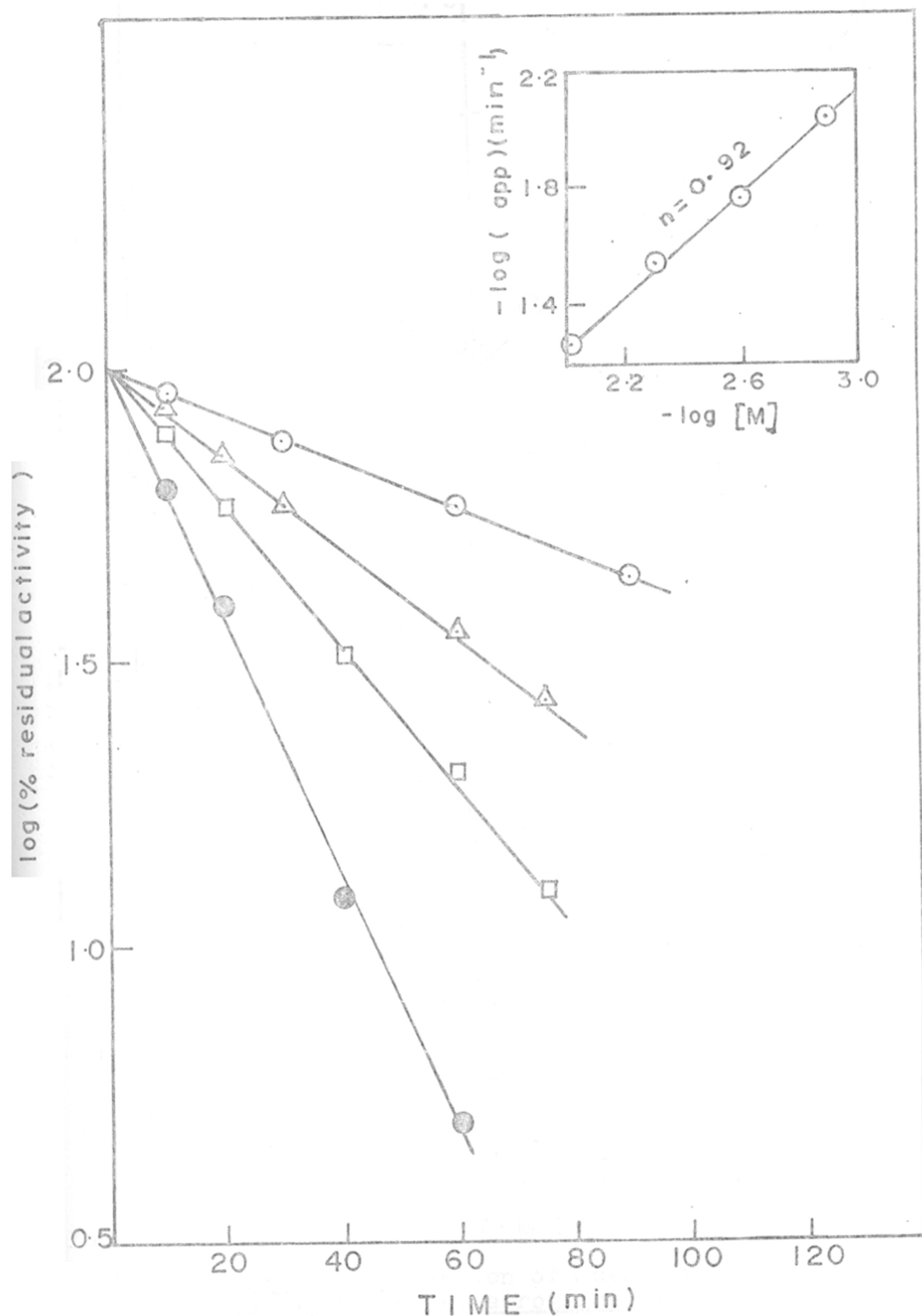


FIG. I.16 Inactivation of the acyl lyase activity of deacetyl citrate lyase from *K. aerogenes* by phenylglyoxal (PG). 1.25 mM PG (○), 2.5 mM PG (△), 5 mM PG (□), 10 mM PG (●). Inset: Determination of the order of the reaction with respect to phenylglyoxal.

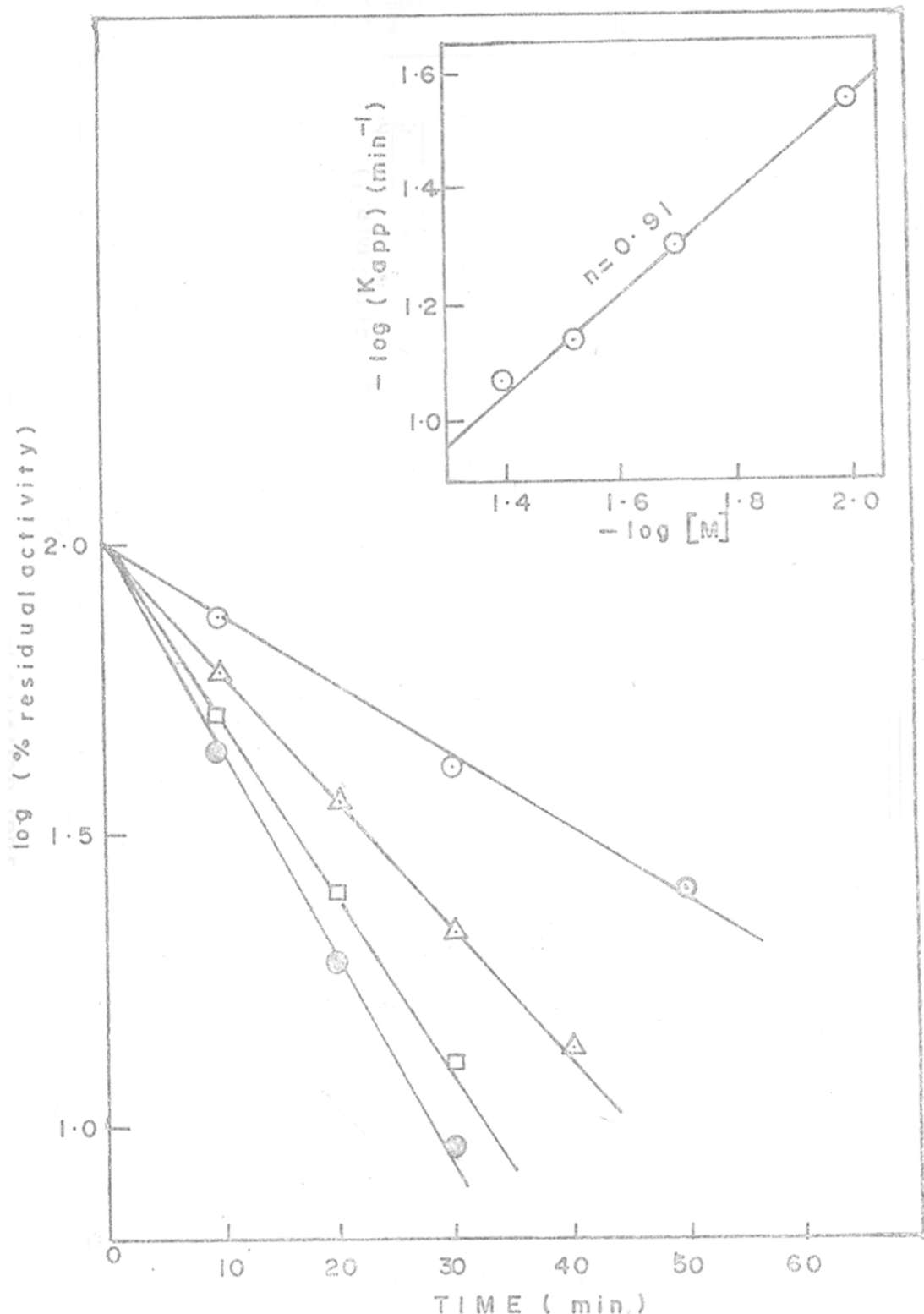


FIG. I.17 Inactivation of acyl lyase activity of deacetyl citrate lyase from *K. aerogenes* by 2,3-butanedione.

10 mM 2,3-butanedione (○); 20 mM 2,3-butanedione (△);
30 mM 2,3-butanedione (□); 40 mM 2,3-butanedione (●).

Inset : Determination of the order of the reaction with respect to 2,3-butanedione.

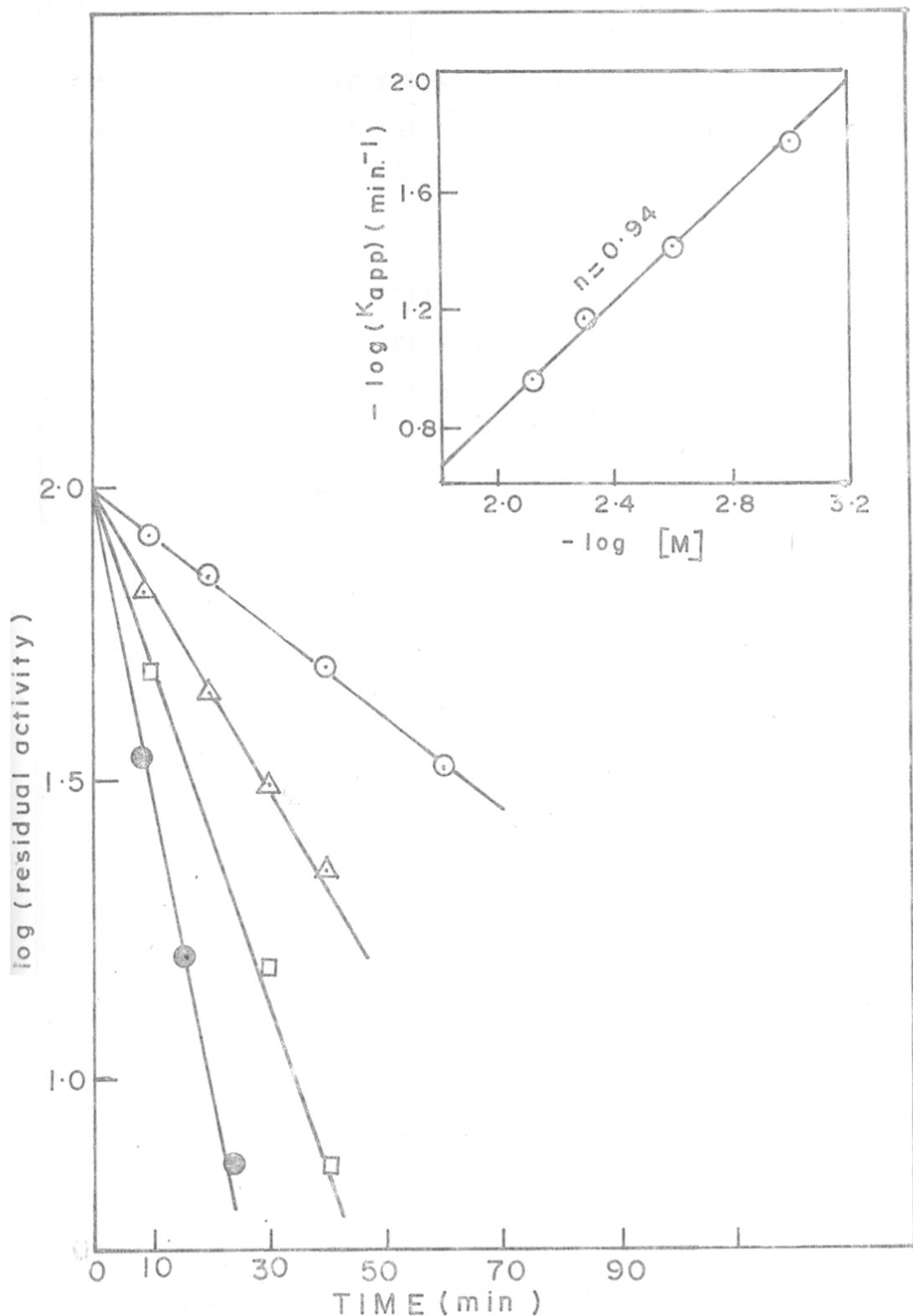


FIG. I.18 Inactivation of the acyl lyase activity of deacetyl citrate lyase from *S. faecalis* by phenylglyoxal (PG). 1.25 mM PG (○); 2.5 mM PG (△); 5 mM PG (□); 7.5 mM PG (●). Inset : Determination of the order of the reaction with respect to phenylglyoxal.

respect to phenylglyoxal $n = 0.94$ confirms that one arginine residue per subunit of the enzyme from this source also reacts with phenylglyoxal to form an inactive complex.

Effect of ligands

The effect of ligands on the rate of inactivation of the lyase activity of deacetyl citrate lyase from K. aerogenes by phenylglyoxal and 2,3-butanedione are shown in Fig. I.19, Table I.8 and Fig. I.20, Table I.9. Citrate (10 mM) and acetyl-CoA (1 mM) does not protect to any significant extent against inactivation, reducing the inactivation only slightly (15 - 20%), while the substrate (3S)-citryl-CoA (0.5 mM) was considerably more effective in protecting against inactivation by phenylglyoxal and 2,3-butanedione (55% and 60%, respectively).

TABLE I.8 Effect of ligands on inactivation of lyase activity of deacetyl citrate lyase from K. aerogenes by phenylglyoxal

Enzyme	Reagent	Ligands	$10^3 \times K_{app}$ (min^{-1})
Acyl lyase activity of deacetyl citrate lyase from <u>K. aerogenes</u>	Phenylglyoxal (5 mM)	-	29
		Citryl-CoA (0.5 mM)	12
		Citrate (10 mM)	27
		Acetyl-CoA (1 mM)	23

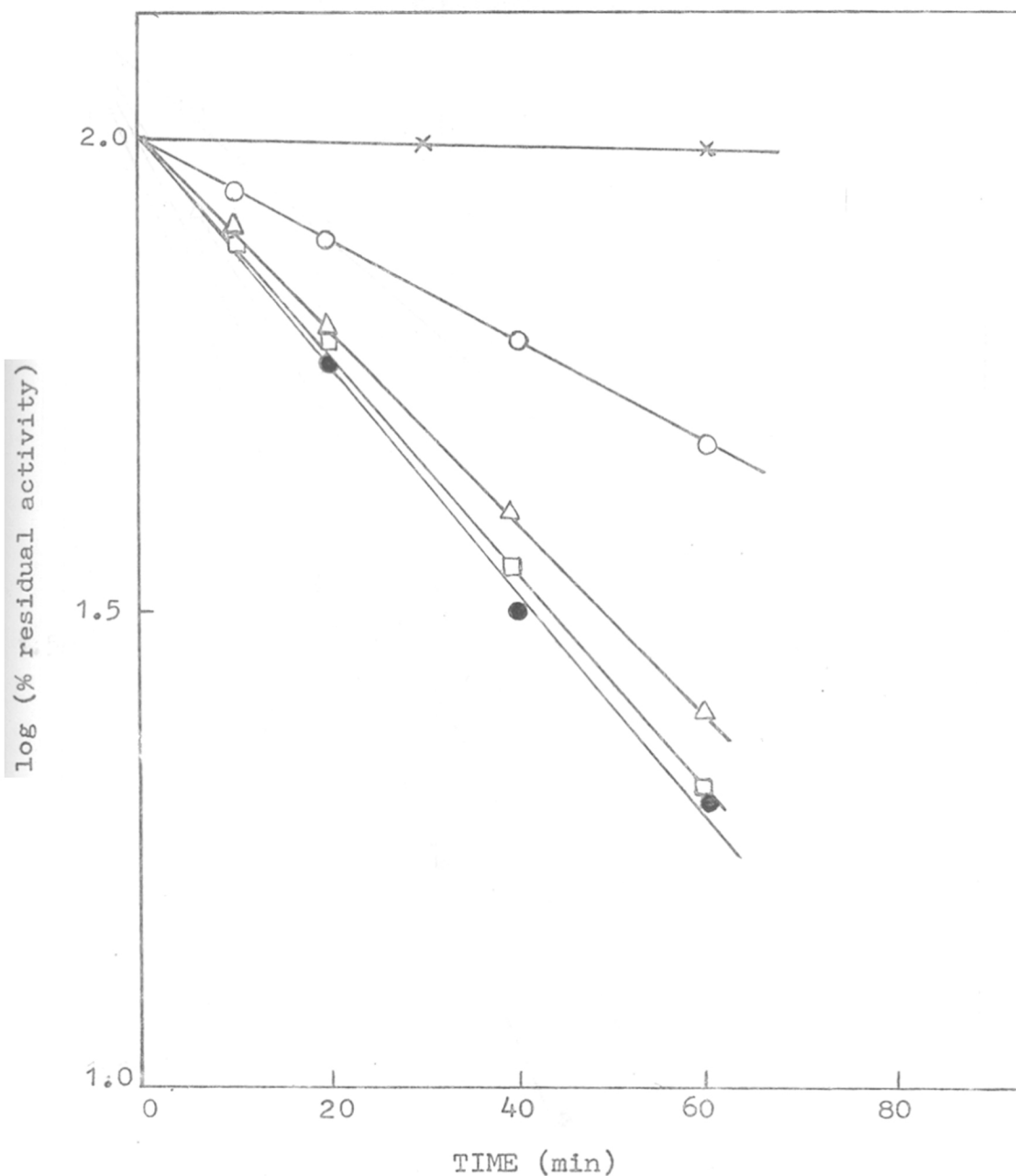


FIG. I.19. Effect of ligands on the rate of inactivation of the lyase activity of deacetyl citrate lyase from K. aerogenes by phenylglyoxal (PG).

Control (x), 5 mM PG (●), 5 mM PG + 0.5 mM citryl-CoA (○), 5 mM PG + 10 mM citrate (□), 5 mM PG + 1 mM acetyl-CoA (△).

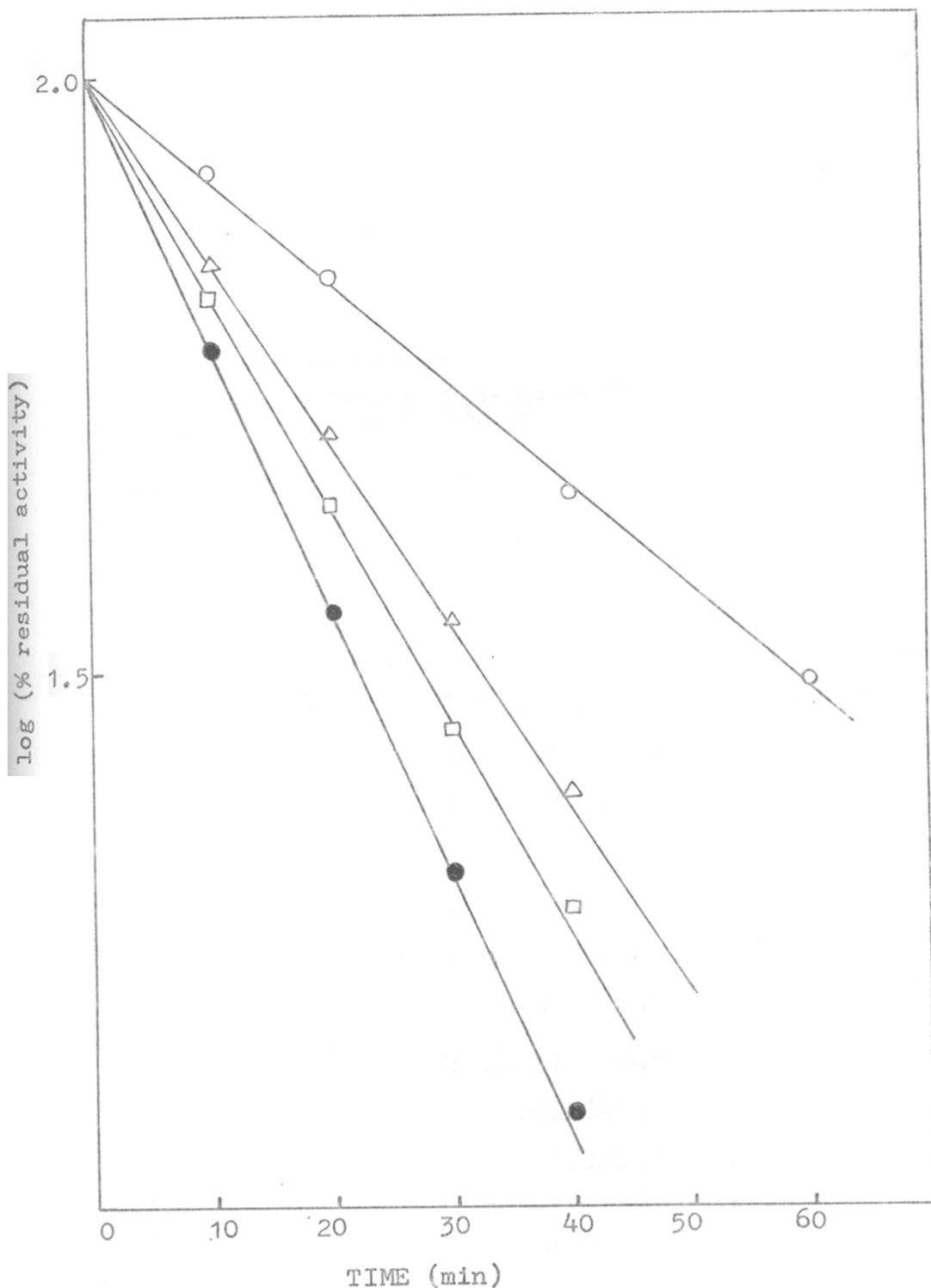


FIG. 1.20. Effect of ligands on the rate of inactivation of lyase activity deacetyl citrate lyase from *K. aerogenes* by 2,3-butanedione.

20 mM Butanedione (●), 20 mM butanedione + 0.5 mM citryl-CoA (○), 20 mM butanedione + 10 mM citrate (□), 20 mM butanedione + 1 mM acetyl-CoA (△),

TABLE I.9 Effect of ligands on inactivation of lyase
activity of deacetyl citrate lyase from
K. aerogenes by 2,3-butanedione

Enzyme	Reagent	Ligand	$10^3 \times K_{app}$ (min ⁻¹)
Acyl lyase activity of deacetyl citrate lyase from <u>K. aerogenes</u>	2,3-butanedione (20 mM)	-	26
		Citryl-CoA (0.5 mM)	10
		Citrate (10 mM)	21
		Acetyl-CoA (1 mM)	17

In case of the K. faecalis enzyme, too, as seen from Fig. I.21 and Table I.10, citryl-CoA (0.5 mM) markedly reduced the rate of inactivation of the lyase activity of the deacetyl citrate lyase, the rate of inactivation being decreased by about 80%. Citrate (10 mM) and acetyl-CoA (1 mM) showed only slight protection (15% and 30%, respectively), from inactivation by phenylglyoxal.

TABLE I.10 Effect of ligands on inactivation of lyase
activity of deacetyl citrate lyase from
S. faecalis by phenylglyoxal

Enzyme	Reagent	Ligand	$10^3 \times K_{app}$ (min ⁻¹)
Acyl lyase activity of deacetyl citrate lyase from <u>S. faecalis</u>	Phenylglyoxal (5 mM)	-	66
		Citryl-CoA (0.5 mM)	12
		Citrate (10 mM)	57
		Acetyl-CoA (1 mM)	46

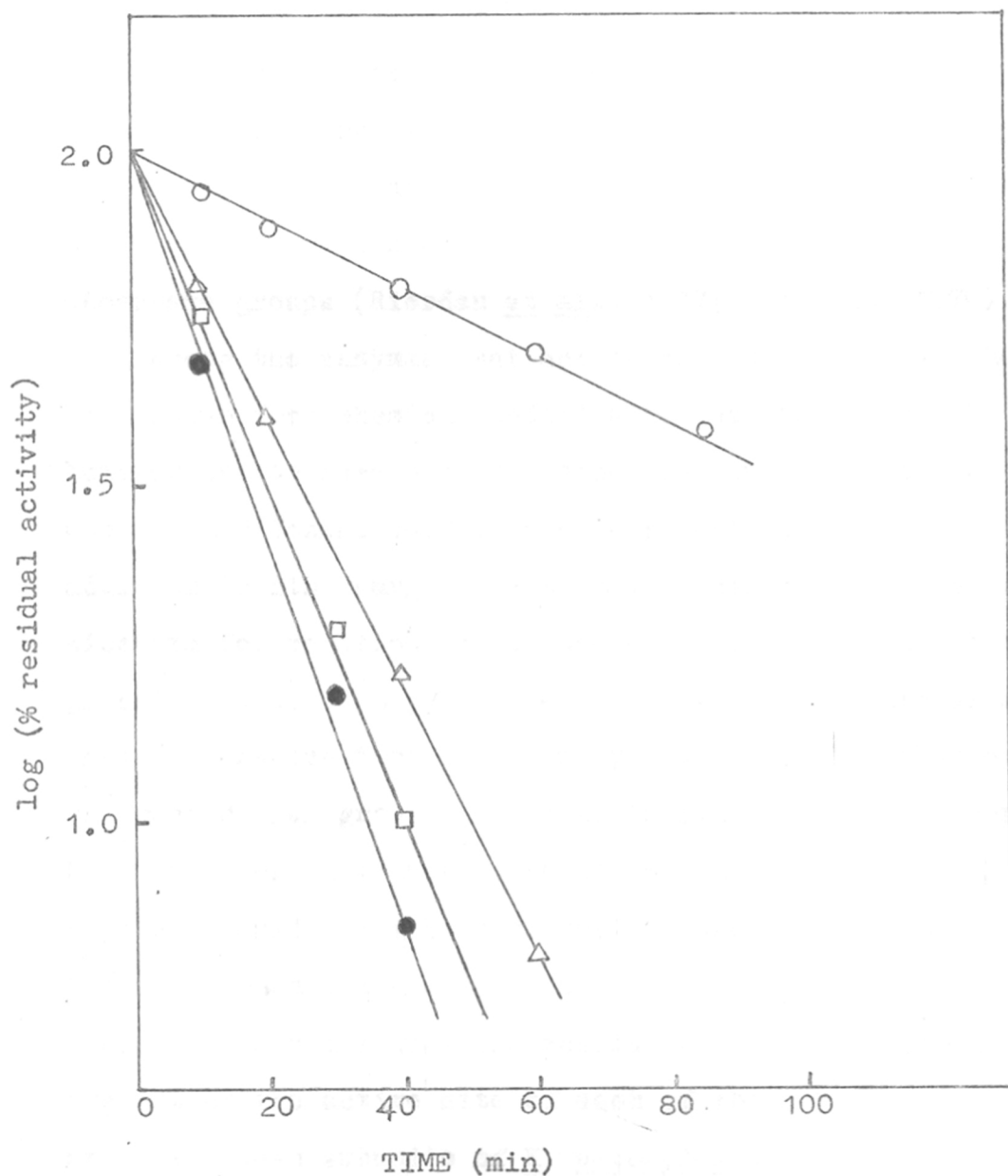


FIG. I.21. Effect of ligands on the rate of inactivation of lyase activity of deacetyl citrate lyase from *S. faecalis* by phenylglyoxal(PG).

5 mM PG (●), 5 mM PG + 0.5 mM citryl-CoA (○),
 5 mM PG + 10 mM citrate (Δ), 5 mM PG + 1 mM acetyl-CoA (□).

DISCUSSION

Positively charged arginine residues in general have been known to serve as anion binding sites, particularly for enzymes which catalyse the reaction of substrates and cofactors carrying negatively charged carboxylate and phosphate groups (Riordan et al., 1977; Riordan, 1979).

Among the enzymes that act on citrate, aconitase has been shown from chemical modification studies with 2,3-butanedione to have a single essential arginyl residue (Gawron and Jones, 1977). The functional arginine in addition to its charge effect is also assumed to provide a side-arm for rotation, since the conversion of citrate to isocitrate would only require a 180° rotation round an axis that is centered through the alkylated central carbon of the guanidinium group. ATP-citrate lyase from rat liver has also been shown more recently to carry an essential arginine residue which is present at the CoA-binding site (Ramakrishna and Benjamin, 1981). In the present work, a single essential arginine residue has been shown to be present at the active site of each of the acyl transferase and acyl lyase subunits of K. aerogenes and S. faecalis.

Ramakrishna and Benjamin (1981) have shown that phenylglyoxal has the maximum inhibitory effect on ATP-citrate lyase from rat liver in presence of Hepes buffer, followed by potassium phosphate and borate buffers. In the present studies on citrate lyase from K. aerogenes, phenylglyoxal in potassium phosphate buffer inactivated at

the fastest rate followed by the modifier in Heper buffer and in Tris-HCl buffer. In the case of 2,3-butanedione, complexation of the modifier with borate is known to enhance the irreversible inactivation rates (Riordan, 1973). However, since borate buffer caused marked lowering of citrate lyase activity even in the absence of 2,3-butanedione, this buffer was not used in the present studies.

Phenylglyoxal and 2,3-butanedione have been shown to inactivate citrate lyase from K. aerogenes. Phenylglyoxal inhibits the transferase activity of the deacetylated enzyme complex as well as of the isolated acyl transferase - subunit. The anionic substrates, citrate and (3S)-citryl-CoA protect the transferase activity against inactivation in both cases, whereas acetyl-CoA markedly increases the rates of inactivation of both the deacetyl enzyme complex and the isolated transferase -subunit. This would indicate that the essential arginine residue on the transferase subunit is present at the citrate binding site and not at the acetyl-CoA binding site of the transferase subunit. One of the points of attachment of citrate to the transferase subunit is presumably through the guanidino group at the active site.

Ligands have been shown to enhance inactivation in ATP-citrate lyase by arginine specific reagents (Ramakrishna and Benjamin, 1981). Mg-ATP complex at low concentrations (less than 0.5 mM) protected the enzyme against inactivation, but at higher concentrations the rates of inactivation were enhanced. In NADP-dependent isocitrate dehydrogenase, 2,3-

butanedione inactivated the enzyme and loss in activity was largely prevented by prior incubation with the substrate, isocitrate (Ehrlich and Colman, 1977). However, increase in the rate of inactivation by almost 5-fold is brought about on addition of the coenzyme, NADP. Such enhancements caused by ligands are probably the result of conformational changes in the enzyme complex brought about by ligand binding which renders the specific amino acid residue more reactive towards the modifying reagent.

In the case of the classical CoA-transferases, a conformational change at the carboxylate binding site of succinyl-CoA: 3-oxoacid CoA-transferase has been suggested to explain the enhanced reactivity of an essential thiol group on binding of acyl-CoA substrates (White et al., 1976). An alligator-type model has been postulated to explain this behaviour. Such a model could also explain the effects of ligands on the rates of inactivation of citrate lyase by arginine specific reagents. The two groups of enzymes also have similarities in some aspects of their mechanism of action, both acting through anhydride intermediates (Bimroth et al., 1977b). To extend the model to citrate lyase, the enzyme could be assumed normally to be present in a closed form in which access to the essential arginine residue at the citrate binding site is limited. On binding of acetyl-CoA at its binding site, the enzyme probably undergoes a conformational change to an open form in which the essential arginine at the citrate binding site is exposed to a greater

extent, making it more reactive towards phenylglyoxal and 2,3-butanedione as shown schematically in Fig. I.22. Binding of citrate or (3S)-citryl-CoA protects the inactivation of the transferase activity, presumably by blocking the essential arginine residue.

The lyase activity of K. aerogenes is also inactivated by the two arginine modifiers. (3S)-Citryl-CoA was effectively protective against the inactivation; while acetyl-CoA and citrate are significantly less effective. The essential arginine residue on the acyl lyase subunit is probably present at the (3S)-citryl-CoA binding site.

Citrate lyase from K. aerogenes and S. faecalis have been classed into two distinct categories on the basis of their reaction inactivation behaviour. The enzyme from K. aerogenes undergoes severe reaction inactivation with a half-life of only 30 sec. (Singh and Srere, 1971), whereas the enzyme from S. faecalis shows relatively weaker reaction inactivation with a half-life of about 9.4 min (Hiremath et al., 1976; Hiremath, 1977). A comparative study of the effect of arginine specific reagents on citrate lyases from K. aerogenes and S. faecalis indicates similarity in the inactivation behaviour. Inactivation in both cases by phenylglyoxal follows pseudo-first order kinetics. The reaction order with respect to phenylglyoxal is approximately one both for the transferase and lyase activities of the deacetyl enzymes from both sources, indicating that one arginine residue is

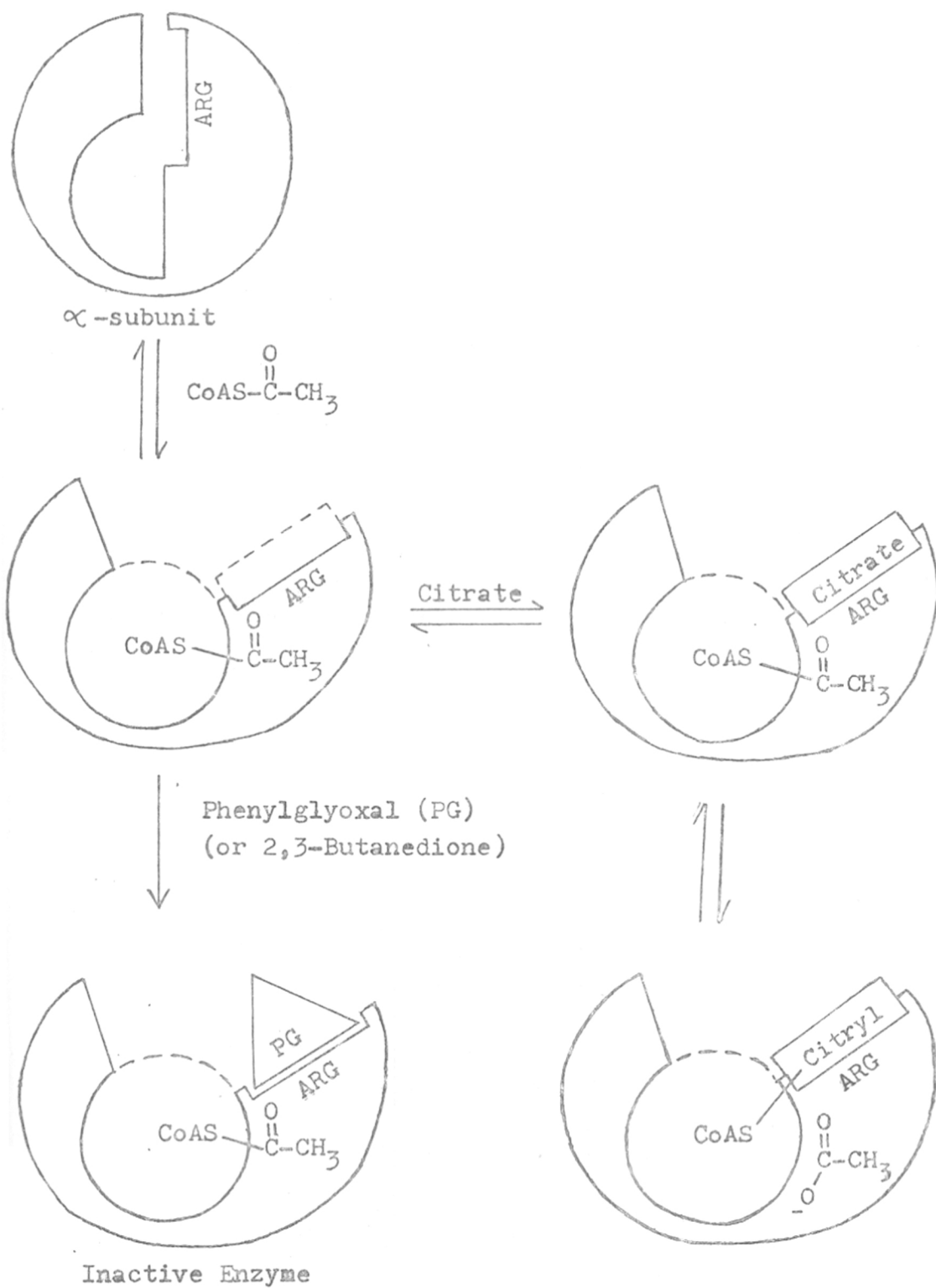


FIG. I.22. ALLIGATOR - TYPE MODEL

present at the active sites of each of the transferase and lyase subunits of citrate lyases from both sources. The effects of ligands on the action of modifiers are also similar in the enzymes from both the sources.

PART II

STUDIES OF THE ACYL-CARRIER PROTEIN OF CITRATE LYASE FROM Escherichia coli

STUDIES ON THE ACYL-CARRIER PROTEIN OF CITRATE LYASE FROMEscherichia coliSUMMARY

The large 85 000 dalton acyl-carrier protein subunit from Escherichia coli citrate lyase complex has been shown to have unique structural features.

The tryptic peptide map of the aminoethylated E. coli citrate lyase acyl-carrier protein subunit shows the presence of only 25 peptide fragments instead of the maximum of 69 expected from its amino acid composition. The polypeptide chain contains about 7 histidine residues, but only one peptide fragment in the tryptic map reacts with Pauly reagent. Microbiological assay of the pantothenate content of the 25 tryptic peptide fragments indicates that about 86% of the prosthetic group component is present in a single basic peptide fragment with the rest being present in a neighbouring one on the peptide map. The results are indicative of the presence of homologous regions in the polypeptide chain, particularly in domains carrying the prosthetic group.

INTRODUCTION

Citrate lyase (EC 4.1.3.6) from Escherichia coli has been shown recently to differ from the other enzymes of this group in several structural features (Nilekani and SivaRaman, 1983). The enzyme complex obtained pure hitherto from all other sources resemble the enzyme from K. aerogenes, a source from which the enzyme has been studied the most. The enzyme from K. aerogenes is built up of three non-identical subunits

of 54 000 (), 32 000 () and 10 000 () dalton. The smallest subunit of the enzyme from this source functions as an acyl-carrier protein (Dimroth et al., 1973) and carries a covalently attached phosphoribomyl dephospho CoA prosthetic group (Robinson et al., 1976; Oppenheimer et al., 1979). The active enzyme carries essential acetyl moieties in thio-ester linkage with the CoA-like prosthetic group (Buckel et al., 1971). The cleavage of citrate catalysed by the enzyme complex proceeds in two steps, the first being initiated by the acyl transferase ()-subunit resulting in the formation of (3S)-citryl-ACP intermediate with the release of acetate and the second being the cleavage of the intermediate to oxaloacetate and acetyl-ACP mediated by the acyl lyase () subunit. The estimates of subunit stoichiometry have shown the presence of six mol of each in the complex having the hexameric structure (, ,)₆. Citrate lyase from S. diacetilactis (Singh and Srere, 1975), R. argelatinosa (Giffhorn and Gottschalk, 1978), S. faecalis (Hiremath et al., 1976) and C. sphenoides (Antranikian et al., 1982b) have all been shown to resemble the enzyme from K. aerogenes in the subunit structure, size and composition. The aggregate in all the cases have been obtained as monodisperse entities. In marked contrast to the usual structural features of bacterial citrate lyase, the enzyme from E. coli has been shown to exist in multimeric forms even under non-denaturing conditions. While the acyl transferase (; M_r 54 000) and acyl lyase (; M_r 32 000) subunits of the complex from this source resemble the

enzyme subunits of citrate lyase from other sources, the acyl-carrier protein () subunit is a single large polypeptide chain of 85 000 dalton. The ACP subunit carries four covalently bound phosphopantetheine containing prosthetic groups. Six copies each of the - and -subunits have been shown to be associated with a single copy of the acyl-carrier protein (Nilekani and SivaRaman, 1983).

A central problem that arises out of the finding that a single ACP subunit in E. coli interacting with six copies of each of the acyl transferase and acyl lyase subunits is the possibility of intrapeptide homologies in the large acyl-carrier protein molecule. The present investigation reports on this aspect of the problem. Studies have been carried out on the tryptic peptide fragments obtained from the ACP subunit.

MATERIALS

Salmine sulphate was obtained from BDH, England. Carboxymethyl cellulose (CM-52) was from Whatman. Analytical grade urea was recrystallized as described in Part I of this thesis. Sulfanilic acid was from Aldrich Chemical Co. Trypsin from bovine pancreas (type XII, 2 x crystallized) was obtained from Sigma Chemical Co., USA. All other chemicals and reagents were similar to those described in Part I.

All the solvents for paper electrophoresis and chromatography were analytical grade reagents freshly redistilled. Pyridine was refluxed with 0.1% ninhydrin for 4 h and distilled. Calcium-D-pantothenate was from E. Merck, Darmstadt.

Crystalline alkaline phosphatase (type III from E. coli) was obtained from Sigma Chemical Co. (specific activity 22 U. mg^{-1}). High voltage electrophoresis was carried out using Whatman 3 MM paper and a Savant Instruments, Inc high voltage electrophoresis unit fitted with a cooling system.

Organisms

Escherichia coli (ATCC 8739) was obtained from the National Collection of Industrial Microorganisms, Pune and was maintained routinely on nutrient agar slants and subcultured once in 2 - 3 weeks. The organism was inoculated into nutrient broth containing beef extract (0.3%), ~~NaCl~~ NaCl (0.5%) and peptone (0.5%) adjusted to pH 7.0 with NaOH and then inoculated in citrate growth medium. Nutrient agar slants contained 2% agar. Cultures were grown at 30°C for 18 - 20 h.

Lactobacillus plantarum (ATCC 8014) was obtained from the National Collection of Industrial Microorganisms, Pune and subcultured in nutrient broth. The pantothenate - less basal medium for the microbiological assay of pantothenate was obtained from Bios Company, Bombay.

METHODS

PURIFICATION OF CITRATE LYASE FROM *Escherichia coli*

Growth of cells

E. coli (ATCC 8739) was grown without aeration at 30°C for 18 - 20 h in 10 L flasks containing the citrate medium, described by Dagley (1954). The medium contained KH_2PO_4 .

90 g; Na_3 citrate, 50 g; peptone, 56 g; glucose, 10 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g and water added to make up the volume to 10 L after the pH was adjusted to 7.2 with 5 M NaOH. Peptone, glucose and MgSO_4 were dissolved in water taken in 2 L flasks and autoclaved separately to prevent the precipitation of magnesium phosphate. The two solutions were mixed just before inoculation with an inoculum of about 10% of the final volume. The cells in the inoculum were previously adapted to the citrate growth medium. After an incubation period of 18 - 20 h at 30°C, 10 g glucose was added and left for 1 h. The cells were then harvested on a Sharples A-12 Supercentrifuge at 12 000 g. Yield of the cells were 18 - 20 g wet weight of cells per 10 L of the citrate growth medium. The cells were stored at -20°C and did not lose its activity for more than 1 week.

Extraction of cells

Batches of 110 - 120 g (wet weight) cells were worked up for purification of citrate lyase. During the extraction procedure, temperature was maintained at 0 - 4°C using an ice-salt mixture. The frozen cells after allowing to thaw overnight were suspended in 0.03 M potassium phosphate buffer, pH 7.0 (3.5 mg/g cell paste). The cell suspension was sonicated at 20 ke (300 W) in a Biosonik III (Bronwill Scientific Co., USA) sonicator, twice for 3 min each. The cell debris was separated by centrifugation at 50 000 g for 45 min at 4°C and discarded. The clear supernatant was used in the further steps of purification.

Purification of citrate lyase

The procedure used for the purification of citrate lyase from E. coli cells was that of Nilekani and SivaRaman (1983). Purification steps were carried out continuously without storage at any stage. The operations were performed in cold with the temperature maintained between 0 - 4°C. All the buffers contained 1.6 mM MgSO_4 for stabilizing the enzyme.

Streptomycin sulphate treatment

The clear supernatant obtained after sonication, was treated with streptomycin sulphate to a final concentration of 1.4% to remove nucleic acids. The solution was kept stirred for 45 min after which the precipitated nucleic acids were removed by centrifugation at 50 000 g for 45 min.

Salmine sulphate treatment

The supernatant from the streptomycin treatment step was kept continuously stirred and treated with 5 volume of freshly prepared cold solution of 0.5% salmine sulphate in 0.05 M potassium phosphate buffer, pH 7.4. After 30 min, the precipitated enzyme was separated by centrifugation at 50 000 g for 40 min. The supernatant showed no citrate lyase activity and was therefore discarded.

Elution of citrate lyase activity

The enzyme which was precipitated with salmine sulphate was obtained as a pellet. It was suspended in 0.05 M potassium phosphate buffer, pH 7.4, containing 1.6 mM MgSO_4 and 0.5 M NaCl (about 15 ml/100 ml streptomycin sulphate extract).

The enzyme was eluted by homogenizing for 20 min in a glass tube with a close fitting ground glass pestle and centrifuged at 50 000 g for 45 min to remove any undissolved material. 0.05 M Potassium phosphate buffer, pH 7.4 was added to the supernatant to dilute the sodium chloride to a concentration of 0.15 M and to precipitate out the enzyme.

Treatment with CM-cellulose

The eluate after dilution was stirred and treated with CM-cellulose (7 g wet weight of CM-cellulose per 100 ml supernatant) equilibrated with 0.05 M potassium phosphate buffer, pH 7.4, containing 1.6 mM $MgSO_4$ and 0.15 mM NaCl, till a clear solution was obtained. After a period of 20 min, CM-cellulose was removed after centrifugation of the suspension at 3 000 g for 20 min.

Alumina C Ugel treatment

The supernatant obtained after removal of CM-cellulose was treated with alumina C gel (20 ml/100 ml of CM-cellulose treated extract). The gel suspension was stirred for 15 min and then allowed to stand for another 15 min, after which it was centrifuged at 3 000 g for 20 min to separate the alumina C gel.

Ammonium sulphate fractionation

To the supernatant, which contained the enzyme activity, was added with continuous stirring finely powered ammonium sulphate till 0.6 saturation. The solution was stirred for 15 min and then allowed to stand for another 15 min. The protein with the enzyme activity which precipitated out was collected by centrifugation of 50 000 g for 30 min and the

supernatant which was free from activity was discarded. The precipitated protein was dissolved in 0.05 M potassium phosphate buffer, pH 7.4 (6 ml) and dialyzed overnight, against 0.01 M potassium phosphate buffer, pH 7.4.

DEAE-cellulose chromatography

The dialyzed solution (9 - 10 ml) was centrifuged at 10 000 g for 10 min to remove turbidity. The clear protein solution was loaded on a DEAE-cellulose column (1.5 x 35 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.4. The enzyme was eluted from the column using a continuous gradient of potassium phosphate buffer, pH 7.4 with a gradual increase in buffer strength from 0.01 M to 0.12 M. This concentration gradient was obtained by taking 200 ml of 0.01 M potassium phosphate buffer in the mixing chamber connected to the column and by adding 0.5 M potassium phosphate buffer, pH 7.4 to the solution in the chamber at the same rate as the effluent from the column. About 3.0 ml fractions were collected on an automatic fraction collector and a drop from each fraction was tested for enzyme activity. Fractions containing enzyme activity were pooled (25 - 30 ml) and the enzyme precipitated by addition of an equal volume of cold neutralized saturated ammonium sulphate solution. The precipitate was collected after centrifugation at 50 000 g for 30 min and then dissolved in the minimum volume of 0.05 M potassium phosphate buffer, pH 7.4 (1 - 2 ml).

Sepharose CL-6B chromatography

The final step in the purification procedure was gel filtration using Sepharose CL-6B. The enzyme obtained from the

DEAE-cellulose chromatography step was loaded on a Sepharose CL-6B column (2.5 x 110 cm) previously equilibrated with 0.05 M potassium phosphate buffer, pH 7.4. The enzyme was eluted with buffer of the same composition. Fractions of 3 ml were collected on an automatic fraction collector and tested for activity by sampling a drop from each fraction. The fraction which contained high enzyme activity were pooled (about 15 ml) and precipitated by addition of an equal volume of cold neutralized, saturated ammonium sulphate solution. The solution was centrifuged at 50 000 g for 20 min and the precipitate obtained was dissolved in 0.05 M potassium phosphate buffer, pH 7.4 (about 1.5 ml).

Preparation of E. coli citrate lyase subunits

Separation of the constituent subunits of E. coli citrate lyase under denaturing conditions using 6 M urea by gel filtration through Sepharose CL-6B has been described by Nilekani and SivaRaman (1983). The resolution of the three component subunits at high protein loads was poor as pointed out by these authors. The recovery of protein components when relatively lower loads were used was also low.

To obtain the subunits in high amounts for chemical and microbiological assays, Nilekani and SivaRaman (1983) have used the procedure of Hanoaka et al. (1979). This procedure involves separation of the component subunits on SDS-polyacrylamide gel electrophoresis, followed by cutting out of gel sections comprising protein bands and recovering the protein by reelectrophoresis through 6 M Urea-polyacrylamide gel columns.

The application of this technique resulted also in low recoveries of the component subunits. The following procedure was therefore standardized which resulted in recoveries better than 50% of electrophoretically homogeneous subunits of the E. coli enzyme: The enzyme subunits were initially separated on SDS-polyacrylamide gels containing 0.1% SDS and electrophoresis was carried out using 0.05 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS. Enzyme sample was initially denatured in the presence of 0.05 M sodium phosphate buffer, pH 7.2, containing 2% SDS and 1% 2-mercaptoethanol for 5 min in a boiling water-bath. The sample was cooled and to it was added sucrose to make the solution dense and bromophenol blue which was used as a tracking dye. The denatured enzyme preparation (about 1 mg) was then electrophoretically separated on a 7.5% polyacrylamide gel column (1×10 cm) at a current of 8 mA per tube. Sets of 4 gels were run at a time. After the electrophoretic run was complete, the gels were removed and cooled to 0 - 4°C. Gel regions containing protein could be differentiated visually by the appearance of three white bands against a translucent background. The gel regions corresponding to the three subunits (M_r 85 000, ; M_r 54 000, ; M_r 32 000,) were sliced out of the gel. Corresponding regions were pooled for recovery of protein.

Protein was recovered from the separately pooled gel slices using the method described by Stephens (1975). The gel slices containing individual subunits were cut into thin discs and placed in a tube (1×10 cm) with a slight constriction at one end. The gel discs were stacked one above the other using

0.01 M sodium phosphate buffer, pH 7.2, taking care that no air bubbles were trapped between the discs. A dialysis tubing (about 3 inches long) was tied off tightly at one end and filled with 0.01 M sodium phosphate buffer, pH 7.2. The constricted end of the tube containing the packed gel slices was inserted into the open end of the dialysis sac, again taking care not to include any air bubbles in the system. The tube was inserted till all but 1 ml of the buffer was displaced from the dialysis sac. The dialysis tubing was fixed by tying around the gel tube. Elution was carried out electrophoretically at 2 - 3 mA per tube and the protein solutions collected in the dialysis sacs. The solutions containing the subunits were dialyzed separately against 0.01 M sodium phosphate buffer, pH 7.2, containing 6 M urea for 12 h so as to remove SDS and then exhaustively dialyzed against distilled water to remove urea.

The subunits were separated from a number of batches of enzyme preparations and the individual components pooled and concentrated by lyophilization.

Homogeneity of separated subunits

The homogeneity of the isolated subunits was checked by SDS-polyacrylamide gel electrophoresis carried out as described by Osborn and Waber (1969) using 7.5% polyacrylamide. Electrophoresis was carried out at room temperature at 6 - 8 mA/tube till the tracking dye reached the bottom of the tube (4 - 5 h). Gels were stained with Coomassie Brilliant Blue and protein bands visualized by destaining the gels in a solution of 7.5% acetic acid and 5% methanol in water.

Amino acid analysis

The amino acid analysis of the E. coli citrate lyase (ACP () subunit has been reported by Nilekani (1980). The values of Threonine and Serine were not corrected in this work and no estimation was made of the tryptophan content. The corrections have now been made by extrapolation from values obtained after 24, 48 and 70 h periods of hydrolysis.

Estimation of tryptophan

The simultaneous determinations of tryptophan and tyrosine of the isolated α -subunit were carried out spectrophotometrically by the method described by Goodwin and Morton (1946). The principle of this method is described briefly: Most of the protein solutions show selective absorption in the ultraviolet region with the absorption maxima varying in its position depending on the pH. Only three amino acids are responsible for the observed absorption by protein solutions in the region 250 - 320 nm; these include phenylalanine, tyrosine and tryptophan. In 0.1 N NaOH, the absorption by tyrosine and tryptophan is much stronger than that by phenylalanine and hence the contribution by phenylalanine can be neglected. Under such conditions, the protein solution could be considered as a binary system for spectrophotometric analysis. The absorption at the point where the absorption curves for tryptophan and tyrosine intersect is a direct measure of the total molar solute (Trp + Tyr) concentration which will not vary with any change in the relative proportions of the two amino acids. At wavelengths other than where the

absorption curves intersect, the absorbance will change depending on the relative proportions of tyrosine and tryptophan. In 0.1 N NaOH, used as a solvent, the absorption curves for tyrosine and tryptophan intersect at 294.4 nm ($\epsilon = 2375 \text{ M}^{-1}.\text{cm}^{-1}$) and 257.15 nm ($\epsilon = 2748 \text{ M}^{-1}.\text{cm}^{-1}$). Determination of the absorbance at one of these two wavelengths would yield the sum of the molar concentrations of tyrosine and tryptophan. Measurements of the absorbance at any other suitable wavelength (e.g. at 280 nm) is sufficient for the determination of the relative proportions of tyrosine and tryptophan in the protein solution.

Thus if x = total gmol of tryptophan + tyrosine per litre and y = gmol of tyrosine, then $x - y$ = gmol of tryptophan.

At any wavelength other than a point of intersection, if

ϵ_{M} tyrosine be A and ϵ_{M} tryptophan be B and the observed absorbance for 10 mm light path be x ; then $x = yA + (x - y) B$

$$\text{or } y = \frac{x - xB}{A - B}$$

The value of x can be calculated from the observed absorbance at either point of intersection using the following relationship:

Absorbance at any point of intersection

$$x = \frac{\text{Absorbance at intersection}}{\epsilon_{\text{M}} \text{ tyrosine at the wavelength of intersection}}$$

The isolated α -subunit (μg) from E. coli citrate lyase was taken in 1 ml of 0.1 N NaOH and the absorption values measured at 280 nm and at 294.4 nm.

Tryptic peptide mapping

Principle

Trypsin specifically cleaves peptide bonds at the carboxyl groups that are contributed either by arginine or lysine residues. After aminoethylation, the sulphydryl residues of cysteine are modified forming S- -aminoethyl cysteine residues. These residues structurally resemble lysine and the peptide bonds which are contributed by the carboxyl group of the cysteine derivative are also susceptible to hydrolytic cleavage by trypsin (Raftery and Cole, 1963). The peptides obtained after digestion with trypsin are resolved usually by the two dimensional peptide mapping technique of Ingram (1958) using high voltage paper electrophoresis in the first direction followed by partition paper chromatography in the second direction. Each peptide spot occupies a position on the peptide map which is characteristic of its amino acid composition and to a lesser extent of its sequence.

Aminoethylation

The electrophoretically homogeneous 85 000 dalton acyl-carrier protein () subunit of E. coli citrate lyase (2 - 3 mg) was treated with 1.5 M Tris-HCl buffer, pH 8.6 (800 ul), 960 mg a urea, EDTA-Na₂ salt (80 ul of 5% solution). The mixture was made upto a final volume of 2 ml and was treated with 20 ul of 2-mercaptoethanol and left at room temperature for 30 min to ensure that the cysteine is in a reduced form. Ethyleneimine (20 ul) was then added to the solution and the

mixture allowed to stand for 2 h.

The aminoethylated protein was dialyzed against water to remove urea and the other reagents and then lyophilized.

Tryptic digestion

The lyophilized α -subunit preparation was dissolved in the minimum amount of distilled water and the pH adjusted to 8.1 with 50 mM NaOH. Salt free trypsin (2 x crystalline) was used without further purification and it was dissolved in 1 mM HCl just before its use. The protein solution was denatured by heating in a water-bath at 80°C for 10 min, cooled to 37°C and treated with trypsin solution at a substrate: enzyme ratio of 50:1 and the pH of the solution was adjusted and maintained at 8.1 with NaOH while the temperature was maintained at 37°C. Digestion was continued for 4 h till there was no more intake of alkali. The pH of the solution was lowered to 6.5 with 50 mM HCl to arrest the reaction. Enzyme activity was destroyed by heating the solution in a boiling water-bath for 1 min and the solution was then lyophilized.

Fingerprinting

The lyophilized sample (4.5 mg) was dissolved in distilled water (30 μ l) and was subjected to fingerprinting by two dimensional mapping of the tryptic peptide fragments.

Paper electrophoresis

Electrophoresis was carried in the first direction essentially according to the procedure of Ingram (1958) using Whatman 3 MM paper. The dimensions of the cut paper was 31 cm x 31 cm with a pair of sleeves 13 cm in length and 6 cm wide. The cut paper was dipped in Michl's volatile buffer,

pyridine-glacial acetic acid-water, pH 6.4, in the ratio of 10:0.4:90 by volume (Michl, 1951). Excess liquid was removed by placing the cut paper in between two filter papers. The moist filter paper was then placed immediately on a mylar sheet kept on the metal plate of the electrophoresis apparatus. The sample was applied on the paper 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 moist paper was covered with a glass plate and the sleeves dipped in the buffer vessels containing Michl's buffer. The electrodes were fixed in the buffer vessels. The buffer was allowed to diffuse into the paper for 15 min. Electrophoresis was then carried out at 1 000 V (about 18 V per cm) for 2 h.

Chromatography

At the end of the electrophoretic run, the sleeves were cut off and removed and the rest of the paper was dried at room temperature in a current of air (2 - 3 h). The dried paper was suspended in a chromatographic chamber over a trough containing the solvent system, pyridine-n-butanol-glacial acetic acid-water in the proportion of 20:30:6:24 by volume (Barnabas and Muller, (1962). After the paper was saturated, ascending chromatography was carried out for 14 h and the paper was again dried at room temperature in a current of air.

Staining of peptide maps

The specific colour reaction for detection of histidine-containing peptides and the ninhydrin (acidic) reaction for detection of all the ninhydrin-positive peptide spots were carried out in this sequence on the same paper. Such a

combination of tests has been shown to be compatible (Easley, 1965). The paper was sprayed from the back with Pauly reagent and was dried well before dipping through the acidic ninhydrin reagent.

Pauly reagent

Histidine-containing peptides were detected using the Pauly reagent.

Solution A contained sulfanilic acid (0.9 g), concentrated HCl (1 ml) and 100 ml of water which was warmed slightly to dissolve the reagent.

Solution B contained freshly prepared 5% aqueous NaNO_2 .

Solution C contained 10% aqueous Na_2CO_3 .

All three solutions were chilled separately in an ice-bath. Then one volume of Solution B was added to two volumes of Solution A and the paper was sprayed lightly with this mixture. After slight drying, the paper was then sprayed lightly with cold Solution C.

Acidic ninhydrin reagent

The paper was dipped in acidic ninhydrin solution prepared by adding 1 ml glacial acetic acid to 99 ml of 0.3% ninhydrin in acetone. The paper was dried in a current of air for about 30 min and then placed in a cool oven and the temperature raised gradually to 70 - 80°C to give a good colour development with minimum background. The peptide spots were marked after the development of the purple colour.

Detection of peptide fragments containing pantetheine moieties

Peptide fragments carrying the prosthetic group were

detected by microbiological assay of 4-phosphopantetheine. Individual fragments located as ninhydrin positive spots on the tryptic peptide map were numbered and cut out. The cut out portions were separately washed with ether to remove reagents and then treated separately with 5 ml of 0.1 M NaOH for 1 h at 60°C. The solution in each tube was neutralized with 1 N HCl and concentrated to about 1 ml in vacuo at room temperature. The pH of the solutions was then adjusted to 9.0 with 0.25 M Tris-HCl buffer and treated with E. coli, alkaline phosphatase (50 ug per test tube). The mixture was incubated at 37°C for 2 h after which it was neutralized with HCl and diluted to a final volume of 10 ml. Aliquots of 0.1 ml and 1.0 ml from each tube were taken separately in 5 ml of pantothenate-less medium and the total volume made upto 10 ml with distilled water and autoclaved.

Pantothenate was assayed using L. plantarum ATCC 8014 by the method described by Barton-Wright (1952).

Preparation of the inoculum

Cells of L. plantarum were transferred from a stab culture and grown in 10 ml of pantothenate-less basal medium supplemented with pantothenate (50 ug) for 18 - 20 h at 30°C. The cells were collected by centrifugation under aseptic conditions and washed three times with 10 ml of 0.9% saline solution. The cells after the last washing were suspended in 10 ml of 0.9% saline solution and 0.1 ml of this suspension was diluted to 10 ml with 0.9% saline solution; one drop of the final suspension was added to each of the tubes containing

test samples. The tubes were incubated at 30°C. After 18 h of incubation, growth was determined turbidometrically and at the end of 72 h, lactic acid produced was titrated against 0.1 N NaOH using bromothymol blue as internal indicator. Endpoint was determined by colour from yellow to light green.

A standard was run simultaneously. The standard curve relating the growth response of L. plantarum measured turbidometrically as well as titrimetrically as a function of pantothenate concentration using different concentrations of standard calcium-D-pantothenate is presented in Fig. II.1. All assays were made by replicate analysis. As can be seen from Fig. II.1, the growth response is linear over the range 0 - 50 nanograms pantothenate in the assay medium.

RESULTS

Purification of E. coli citrate lyase

Several batches of E. coli cell extracts were processed to obtain purified E. coli citrate lyase complex for the isolation of adequate amounts of the purified ACP () subunit for the characterization of this unusually large polypeptide chain. The results obtained in a representative batch are summarized in Table II.1.

Starting with about 120 g wet weight of cells, about 15 mg of the purified enzyme, with a specific activity of 110 - 120 U.mg⁻¹ was obtained in an yield of about 20%. The values of the specific activity as well as the final yield are comparable to those reported by Nilekani and SivaRaman (1983).

The purified enzyme showed multiple states of aggregation

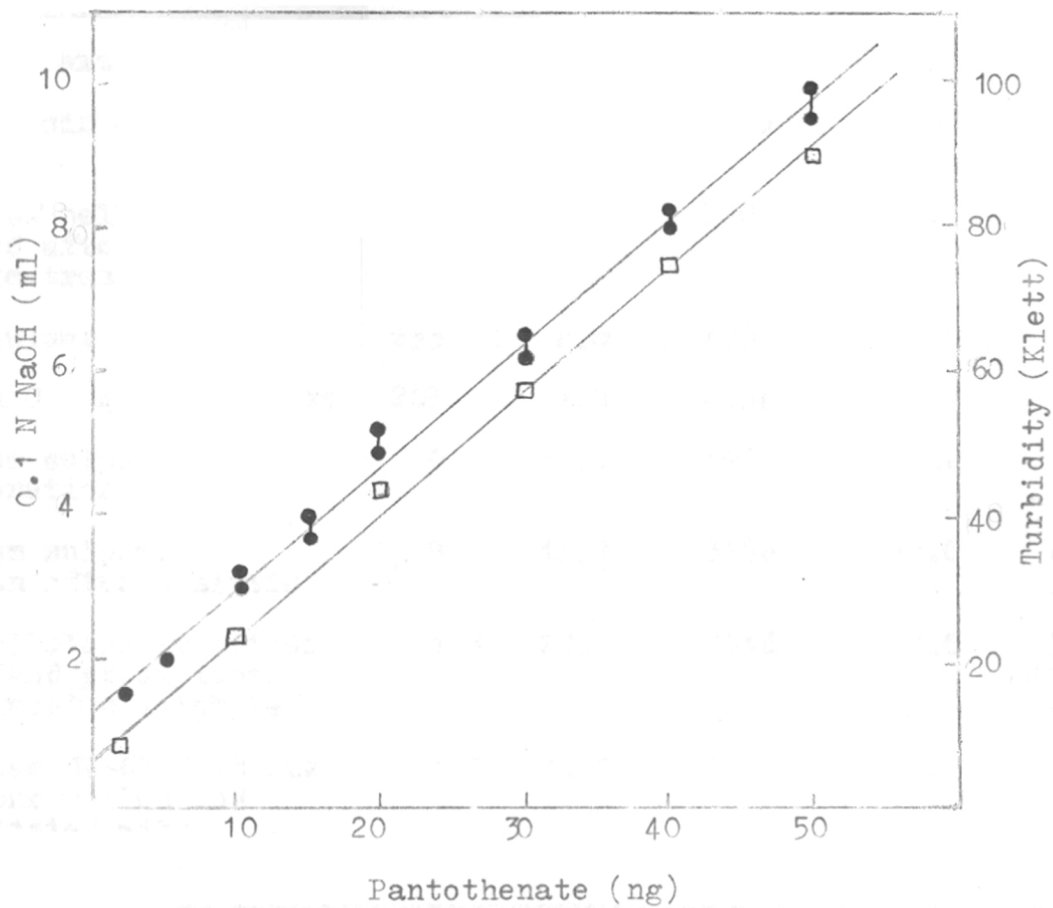


FIG. II.1 STANDARD CURVE FOR MICROBIOLOGICAL
ASSAY OF PANTOTHENATE USING
Lactobacillus plantarum

Table II.1 Isolation of citrate lyase from Escherichia coli

Wet weight of cells = 120 g.

Fraction	Volume ml	Protein mg/ml	Total activity U	Specific activity U/mg	Yield %
Cell-free extract	400	14.0	7840	1.4	100
Streptomycin sulphate treatment	390	9.35	7215	2.0	92
Eluate of pellet obtained after salmine sulphate treatment	70	14.3	5005	5.0	64
CM-cellulose treatment	233	2.0	4707	10.1	60
Alumina C gel treatment	265	1.2	4452	14.0	57
Ammonium sulphate fractionation	6	19.1	3920	34.2	50
Ammonium sulphate fraction after dialysis	9	12.8	3456	30.0	44
DEAE-cellulose fractions pooled and precipitated with ammonium sulphate	1.5	27.0	2916	72.0	37
Sepharose CL-6B fractions pooled and precipitated with ammonium sulphate.	1.3	12.0	1747	112.0	22

in the ultracentrifuge as can be seen from Fig. II.2. This has been shown by Nilekani and SivaRaman (1983) to be one of the unusual features of the E. coli enzyme compared to other bacterial citrate lyases. The SDS-polyacrylamide gel electrophoretic profile of the constituent subunits of E. coli citrate lyase is shown in Fig. II.3. As reported by Nilekani and SivaRaman (1983), the enzyme complex dissociates into three non-identical subunits of 85 000, 54 000 and 32 000 daltons. The function of the subunits have been characterized by isolation of the electrophoretically pure and biologically active polypeptides (Nilekani and SivaRaman, 1983). The 54 000 dalton subunit has been shown to function as the acyl-transferase () subunit catalyzing the formation of citryl-ACP (or citryl-CoA) in presence of citrate and the ACP subunit (or acetyl-CoA) with the release of acetate. The 32 000 dalton polypeptide has been characterized as the acyl lyase () subunit catalysing in presence of Mg^{2+} the cleavage of citryl-ACP (or citryl-CoA) to oxaloacetate with the regeneration of acetyl-ACP (or acetyl-CoA). The large 85 000 dalton subunit which carries the prosthetic group components has been shown to act as the ACP () subunit.

Separation of subunits

The procedure described by Nilekani and SivaRaman (1983) for the isolation of the individual subunits of E. coli citrate lyase gave low yields of the electrophoretically homogeneous components. The modified isolation procedure used in the present studies gave markedly higher yields of the

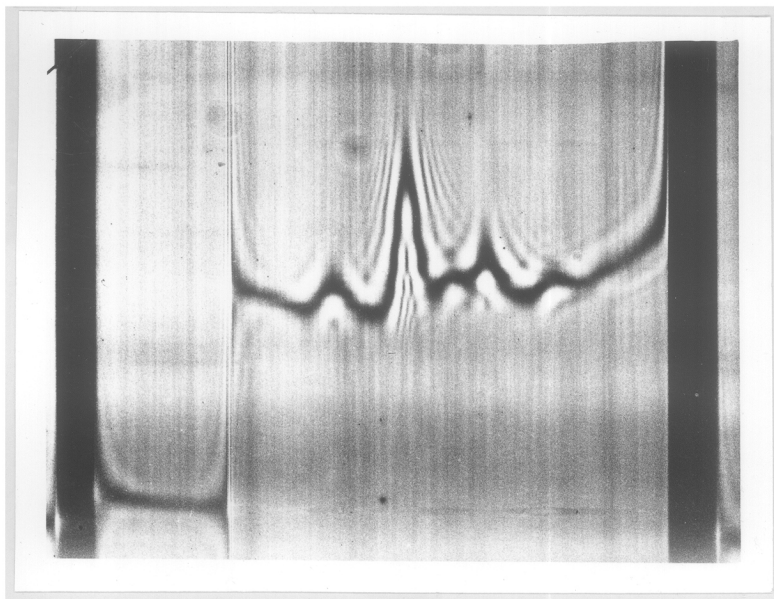


FIG. II.2 SEDIMENTATION PROFILE OF NATIVE CITRATE
LYASE FROM E. coli

Protein concentration	4 mg/ml
Buffer	0.05 M potassium phosphate, pH 7.5 with 1.6 mM MgSO_4
Speed	59 780 rpm
Temperature	4.2°C
Phase plate	45°
Time	26 min

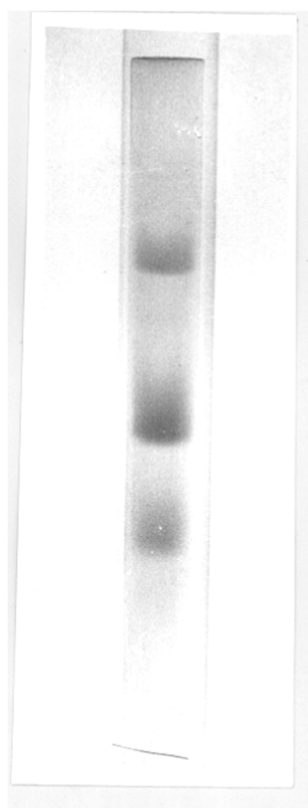


FIG. II.3 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF
CITRATE LYASE FROM E. coli
7.5% gel; 0.05 M sodium phosphate buffer,
pH 7.3, containing 0.1% SDS;
8 mA/tube
Protein loaded 100 μ g

pure component subunits. The electrophoretic homogeneity of the subunits isolated by the modified procedure will be apparent from Fig. II.4. SDS-Polyacrylamide gel electrophoretograms of the separated components showed the complete absence of the other component subunits in preparations obtained by the present procedure. Yields of the individual subunits were about 40 - 50% and recoveries were better than those obtained by the use of the Hancock (1978) procedure employed by Nilekani and SivaRaman (1983). Data from a typical batch is summarized in Table II.2.

Table II.2 Separation of subunits of E. coli citrate lyase

Citrate lyase complex from E. coli (1 mg) was dissociated by boiling with 2% SDS and the constituent subunits separated by SDS-polyacrylamide gel electrophoresis as described in Methods.

Subunit	M_r	% Recovery
	54 000	43
	32 000	52
	85 000	40

Amino acid analysis

The amino acid composition of the isolated 85 000 dalton ACP () subunit of E. coli citrate lyase is described in Table II.3. The earlier values reported by Nilekani (1980) were not corrected for degradation of serine and threonine during hydrolysis. The corrected values determined

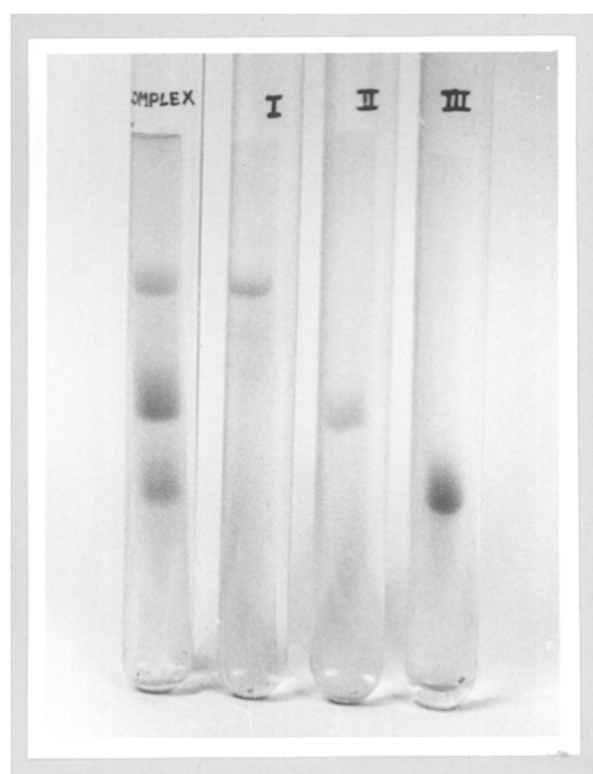


FIG. II.4 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein banding patterns of E. coli citrate lyase complex and of the separated subunits I, II, III.

Buffer, 0.05 M sodium phosphate, pH 7.3 with 0.1% SDS;

8 mA/tube

Protein loaded; Citrate lyase complex, 100 ug
Individual subunits, 50 ug

Table II.3 Amino acid composition of the ACP () subunit
of citrate lyase from Escherichia coli

Samples were subjected to acid hydrolysis as described by Moore and Stein (1963) and Hirs (1967) and analyzed on a Beckman Model 120 C automated amino acid analyser. Tryptophan was assayed spectrophotometrically by the method of Goodwin and Morton (1946)

Amino acid	Approximate No. of residue/mol M_r 85 000
Lysine	35
Histidine	7
Arginine	22
Aspartic acid	48
Threonine ^a	24
Serine ^a	79
Glutamic acid	90
Proline	28
Glycine	129
Alanine	59
1/2-Cystine ^b	11
Valine	39
Methionine	27
Isoleucine	29
Leucine	52
Tyrosine	4
Phenylalanine	19
Tryptophan ^c	7
-Alanine	4
Cysteamine ^b	4

A $\frac{3}{4}$

a - Corrected for the loss during acid hydrolysis

b - Determined after performic acid oxidation. Cysteamine was assayed as taurine

c - Determined spectrophotometrically

% Recovery based on assumption that protein loads as determined by the procedure of Lowry et al. (1953) represent actual dry weights of the polypeptide is 85%.

as described in Methods are included in Table II.3. Tryptophan which had also not been analyzed by Nilekani (1980) was estimated separately by the spectrophotometric method described in Methods. For the assay of tryptophan, 0.4 mg of the isolated ACP subunit was incubated with 0.1 N NaOH in a 2 ml cuvette at 30°C for 30 min. The absorbance was measured at 294.4 nm and 280 nm and found to be 0.130 and 0.204, respectively. From these values, the number of tryptophan residues was calculated to be 7 per mol of the α -subunit. The value for the number of tyrosine residues calculated from the spectrophotometric data was about 5 per mol of the α -subunit which compares reasonably well with the value of 4 determined by ion-exchange amino acid analysis.

Nilekani and SivaRaman (1983) have shown the presence of the prosthetic group components, α -alanine and cysteamine to be exclusively in the 85 000 dalton ACP (α) subunit, none being present in the acyl transferase (β) and acyl lyase (γ) subunits.

The ACP subunit from K. aerogenes has been reported not to contain histidine residues (Dimroth, 1975; Beyreuther et al., 1978). In contrast, E. coli citrate lyase has a total of about 7 histidine residues in M_r 85 000. Another characteristic feature of E. coli ACP subunit is the presence of 4 tyrosine residues/mol subunit, this amino acids also being absent in the K. aerogenes ACP subunit.

Tryptic peptide map

One of the objectives of the present study was to investigate the structural features of the single large

polypeptide chain which function as the acyl-carrier protine in citrate lyase from E. coli, containing 4 covalently bound pantothenate moieties (Nilekani and SivaRaman, 1983). An intriguing question is whether the pantothenate moieties are attached to stretches with homologous sequences within the polypeptide chain or not.

The basic approach in the present study was to use combinations of specific colour reactions and microbiological assay with the tryptic peptide fragments separated by the conventional two-dimensional mapping technique. Preliminary investigations indicated the compatibility of the following reaction combinations in the sequence indicated:

- (1) Spraying of tryptic fingerprints on paper with the specific Pauly reagent for detection of histidine - containing peptides
- (2) Acidic ninhydrin treatment for the identification of the ninhydrin - positive peptide fragments and
- (3) Microbiological assay for locating pantothenate - containing peptide fragments after excising ninhydrin - positive peptide spots from the tryptic map, removing the reagents used in steps (1) and (2) by washing of the excised paper pieces with ether, separately extracting the peptides out of the excised sections with glacial acetic acid, and the N NaOH pantothenic group components using hot aqueous 0.1 N NaOH solution followed by alkaline phosphatase treatment after pH adjustment of the alkaline extract to pH 9.0.

Although recoveries of pantothenate from standard compounds

were variable, the method gave uniform values for the relative contents of pantothenate under similar conditions of pretreatment.

The results obtained from the combination of these reactions are shown in Fig. II.5, Fig. II.6, Fig. II.7 and Table II.4.

Histidine-containing tryptic peptide fragment

The reaction observed on spraying the tryptic peptide map of the ACP () subunit of E. coli citrate lyase with Pauly reagent is shown in Fig. II.5. Amino acid analysis of the subunit indicates the presence of 6 - 7 histidine residues. Using the specific colour reaction for detection of histidine - containing peptides, only one fragment (Fragment No. 7 of Fig. II.7) showed up as a single pink coloured spot on the tryptic peptide map.

Amino acid analysis of the ACP subunit shows a content of approximately 6 - 7 histidine residues in a mol of the polypeptide chain. The identification of all the histidine residues in a single peptide fragment would suggest that either these residues are clustered within a single tryptic peptide or that homologous domains exist in the large polypeptide chain which yield identical peptide fragments on digestion with trypsin.

Ninhydrin-positive tryptic peptide fragments

The total number of Lys + Arg residues calculated from the amino acid analysis data is 57 and the number of half-cystine residues is 11 in the ACP polypeptide chain.



FIG. II.5 LOCATION OF HISTIDINE-CONTAINING PEPTIDE(S)
IN TWO-DIMENSIONAL TRYPTIC PEPTIDE MAP OF
ACP () SUBUNIT OF E. coli CITRATE LYASE

The presence of a single pink spot obtained
on spraying with Pauly reagent is shown in
the photograph.

Treatment of the ACP subunit with 2-mercaptoethanol in the presence of 8 M urea prior to treatment with ethyleneimine would result in any cystine and masked cysteine residues, in addition to unmasked cysteine residues being rendered susceptible to aminoethylation. The total number of residues susceptible to tryptic digestion would therefore be 68. A maximum of 69 tryptic peptide fragments is therefore expected on the peptide map of the digest, provided that none of these residues is present at the carboxyl terminus. The actual number of peptide fragments seen on the peptide map are only 25 (Fig. II.6).

The 85 000 dalton ACP subunit of E. coli citrate lyase has been shown to be a single polypeptide chain by Nilekani and SivaRaman (1983) from the estimate of a single amino terminal residue (isoleucine) in a mol of ACP and the failure of reduction with sodium borohydride in presence of 8 M urea to break the polypeptide into half or lower fractional fragments.

The presence of markedly lower number of tryptic peptide fragments in two-dimensional maps than that expected from the amino acid composition of the subunit is further evidence suggestive of the presence of internal homologies in the unusually large ACP subunit. All the 6 - 7 histidine residues in the ACP polypeptide chain are also accounted for in a single tryptic peptide fragment (No. 7 in Fig. II.7).

Pantothenate containing peptides

Detection of pantothenate in the tryptic peptide fragments

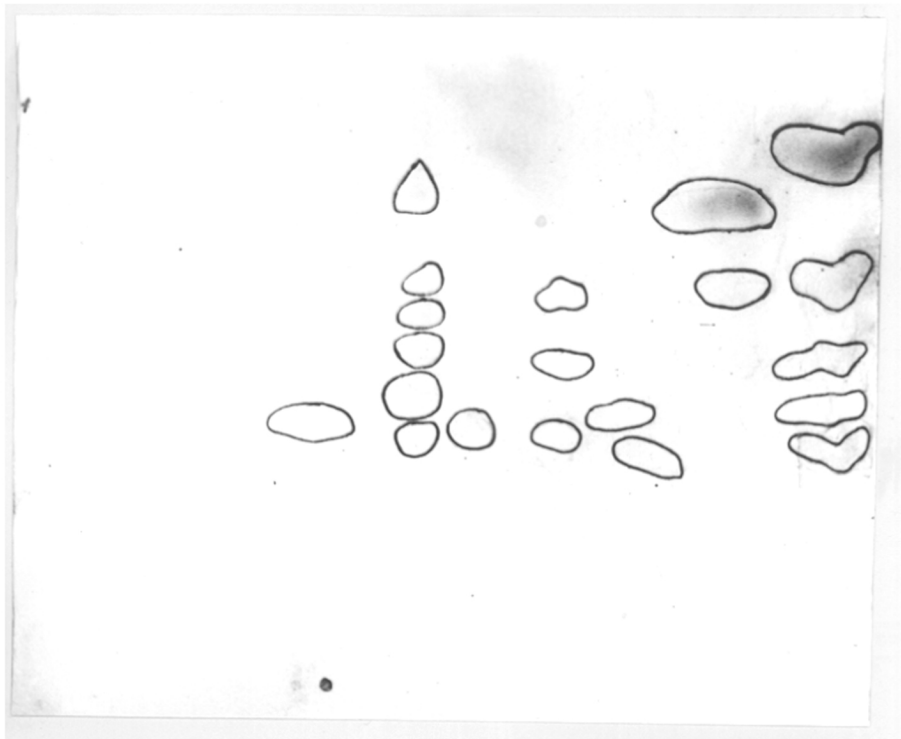


FIG. II.6 TWO-DIMENSIONAL TRYPTIC PEPTIDE MAP OF THE
ISOLATED ACP () SUBUNIT OF E. coli
CITRATE LYASE

Schematic reproduction is shown in FIG. II.6a
with numbering.

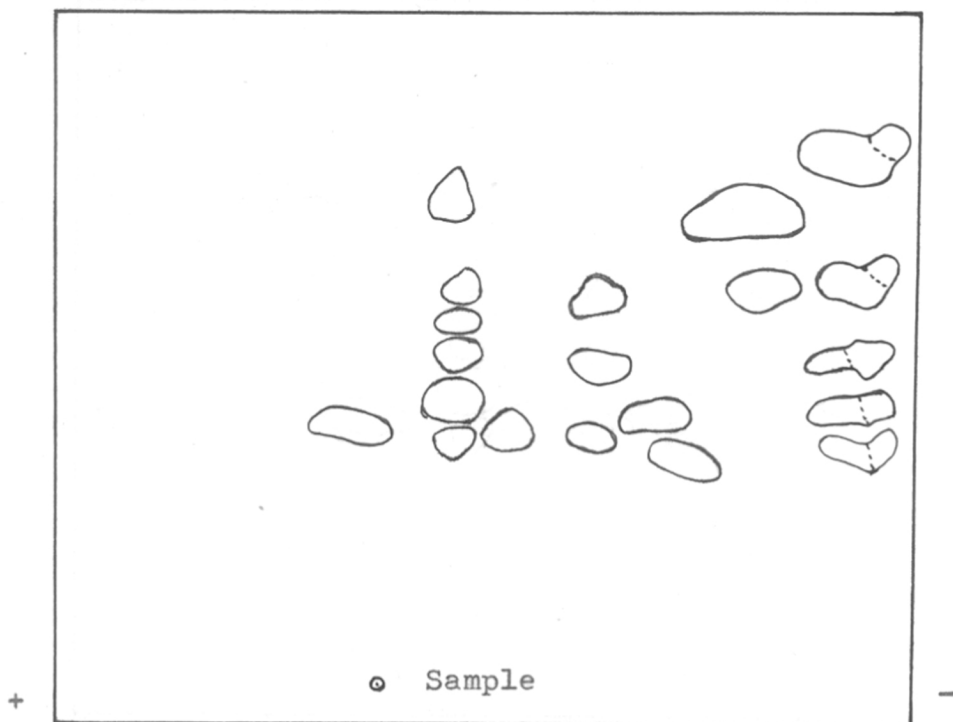


FIG. II.6a E. coli -subunit

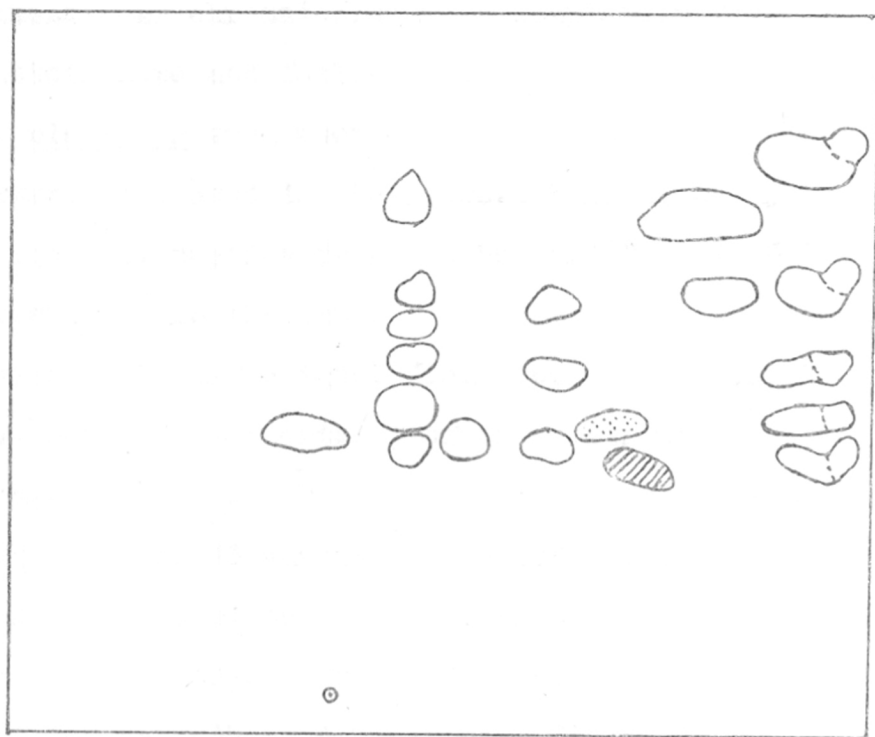


FIG. II.7 SCHEMATIC REPRESENTATION OF HISTIDINE-
AND PANTOTHENATE-CONTAINING PEPTIDES IN
THE TRYPTIC FINGERPRINT OF THE ISOLATED
ACP () SUBUNIT OF E. coli CITRATE LYASE

Peptide containing 86% of total
pantothenate

Peptide containing 14% of total
pantothenate

Histidine-containing peptide
detected by Pauly reagent

on the map was carried out on excised portions of ninhydrin-positive spots from the tryptic peptide map and estimating the pantothenate content microbiologically using L. plantarum ATCC 8014 as described in Methods.

Extracts of all except two of the ninhydrin-positive, tryptic peptide containing spots excised from the two-dimensional chromatogram were completely free from the pantothenate and failed totally to support growth of L. plantarum even when an aliquote size of one-tenth the extract was used in the microbiological assay. Such an aliquot of peptide fragment No. 12 (Fig. II.67) supported growth within the range of the quantitative assay procedure, while a similar aliquot from peptide fragment No. 13 (Fig. II. 7), however, supported profuse growth which was outside the range of assay. The aliquot size of the extract from Fragment No. 13 was therefore reduced to 0.1 ml representing one-hundredth of the total fragment to bring the values within the linear range. The results of the quantitative assay of the two pantothenate-containing fragments are shown in Table II.4.

It will be evident from the Table II.4 that the prosthetic group components are located mainly on a single tryptic fragment (peptide fragment No. 13). Since the paper had been subjected to previous spraying with the aqueous Pauly reagent, the possibility of slight spreading of the fragments with overlap between the two neighbouring fragments No. 12 and No. 13 on the chromatogram cannot be ruled out. The possibility of such overlap between the closely located

Table II.4 Pantothenate content in the tryptic peptide
map of the ACP () subunit of E. coli M_r 85 000

Tryptic digest of the ACP subunit (1.5 mg) was subjected to fingerprint analysis. Individual ninhydrin-positive peptide fragments were cut out, washed with ether, extracted and assayed microbiologically for pantothenate as described in Methods and Results. The values are average of replicate assays.

Peptide fragment No.	Pantothenate content (ng)
1 - 11	0
12	500
13	3150
14 - 25	0

fragments on the tryptic peptide map is indicated by the presence of only 14% of the total pantothenate in peptide fragment No. 12, while the remaining 86% is present in the closely located peptide fragment No. 13. The possibility of the 4 prosthetic groups of the ACP polypeptide chain being located in either a single tryptic peptide of the ACP subunit or in interspersed fragments with complete sequence homology is indicated from these results.

DISCUSSION

Although the general properties of citrate lyase from E. coli have been shown to be similar to those from K.aerogenes and other organisms previously studied, the E. coli enzyme

has been found by Nilekani and SivaRaman (1983) to have a much larger acyl-carrier protein subunit than the others and to exhibit a different stoichiometry for the component polypeptide chains, the E. coli enzyme having an 6×6 state of aggregation instead of the hexameric $6 \times 6 \times 6$ complex found in other sources. An intriguing problem which arises from the unusual structure of the E. coli citrate lyase complex is the abnormally large acyl-carrier protein, a single polypeptide chain of which has 4 attached pantothenate moieties functionally interacting with 6 of each of the acyl transferase () and acyl lyase () subunits.

The two-dimensional tryptic peptide map of the 85 000 dalton ACP subunit of E. coli citrate lyase shows a marked lower number of peptides (25) than the maximum expected (69) for a sequence without homologous regions comprising the residues of the tryptic fragments.

The two-dimensional tryptic peptide maps of the ACP subunits from K. aerogenes and S. faecalis citrate lyases have been determined in earlier work carried out in this Laboratory (SivaRaman, 1980). The ACP from the former source has also been mapped by Bayer and Eggerer (1978). The number of tryptic fragments obtained from the ACP subunit of K. aerogenes citrate lyase is 11 (SivaRaman, 1980), while the maximum expected number from the amino acid composition and sequence is 9. Such an anomaly has also been reported in the tryptic peptide maps of the ACP of E. coli fatty acid synthetase where 7 tryptic peptides have been observed instead of the 6 expected (Vanaman et al., 1968; Bayer and Eggerer,

1978). The appearance of the extra fragments has been attributed to incomplete (partial) or nonspecific fragmentation during processing. In the case of the ACP subunit of S. faeca faecalis citrate lyase, 16 tryptic fragments were obtained (SivaRaman, 1980), which corresponds to the maximum number expected from its amino acid composition (Hiremath, 1977). The ACP subunits of K. aerogenes and S. faecalis citrate lyases are 10 000 and 14 000 daltons in size, compared to the 85 000 daltons size of the corresponding E. coli citrate lyase subunit (Nilekani and SivaRaman, 1983). It can thus be seen that the number of peptide fragments obtained with the E. coli subunit is markedly lower than that expected for a polypeptide chain without internal homologous sequences.

A more positive evidence for intrapeptide homologues in the E. coli citrate lyase ACP subunit is the present finding that most (86%), if not all, of the pantothenate in the ACP polypeptide chain of E. coli citrate lyase is located in a single basic tryptic peptide fragment (fragment No. 13). Likewise the 6 - 7 histidine residues are also present in a single fragment (fragment No. 7). While the latter could be explained on the basis of clustering of the amino acid residues in an isolated region of a long polypeptide chain, such a clustering of the biologically active prosthetic groups at a restricted region of the subunit is unlikely since the acyl moieties attached to these groups in thio-ester linkage interact with multiple copies of the two enzymic subunits of the complex. These findings are

strongly suggestive of a remarkable sequence homology within the ACP subunit, atleast in the domains where the 4 prosthetic groups are located. The occurrence of only 25 tryptic peptide fragments in two-dimensional tryptic peptide maps in place of the expected 58 - 69 and the occurrence of all the 6 - 7 histidine residues in a single fragment would also support this hypothesis.

The possibilities of intrapeptide homologies have also been indicated in other systems. A well documented example is the "kringles" in the heavy chain of plasminogen which constitute a set of 5 homologous polypeptide domains, each built up of a chain of about 180 amino acid residues (Sottrup-Jensen et al., 1978). The 5 structural units exhibit identical 3-dimensional architecture and show remarkable sequence homologies. Based on these evidences it has been suggested that these domains of the polypeptide chain have resulted apparently from 5-fold internal repetition of the same gene segment (Trexler and Patthy, 1983; Young et al., 1979). The amino terminal part of prothrombin has also been shown to contain 2 "kringles", which bear close homology to those of plasminogen (Magnusson et al., 1975). Internal homology has also been reported in the amino acid sequence of calmodulin (Iida, 1982). The primary structure of calmodulin reveals 4 domains with similar amino acid sequences suggestive of intragenic duplication.

Computer analysis of the sequence of the 557 - residue long ribosomal protein S₁ from E. coli has revealed that this protein is probably made up of 6 homologous units each

of which is approximately 88 residues long (Doolittle et al. 1982).

Several examples of protein structures have been shown to be architecturally built up of pairs of very similar domains which have probably evolved by gene duplication within a polypeptide chain (McLachlan, 1979; Dixon, 1966; Ohno, 1970; Dayhoff, 1972). Examples of such molecular structures are yeast hexokinase (McLachlan, 1979), bacterial ferredoxin (Watenpaugh et al., 1973), muscle parvalbumin (Kretsinger and Nockolds, 1973), penicillopepsin (Tang et al., 1978), rhodanese (Ploegman et al., 1978) and chymotrypsin (Birktoft and Blow, 1972). In these examples, the active centres have been shown to lie between the two related halves of the molecule. However, sequence homologies have not been recognized in these polypeptides showing pairs of similar structural domains.

The present studies indicate strongly the possibility of intrapeptide homologies of amino acid sequences within the unusually large acyl-carrier protein subunit of citrate lyase from E. coli. This would support the speculation that the prosthetic group carrying domains of the large ACP subunit might have arisen from a sequence of gene duplications which probably resulted in 4 or more intrapeptide homologous regions in the large polypeptide chain.

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Photoaffinity Labeling of *Klebsiella aerogenes* Citrate Lyase by *p*-Azidobenzoyl Coenzyme A[†]

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ABSTRACT: *p*-Azidobenzoyl coenzyme A functions as a linear competitive inhibitor for (3*S*)-citryl-CoA in the citryl-CoA oxaloacetate-lyase reaction catalyzed by the *Klebsiella aerogenes* deacetylcytrate lyase complex ($K_i = 80 \mu\text{M}$; (3*S*)-citryl-CoA $K_m = 67 \mu\text{M}$). Inactivation is irreversible on photolysis of *p*-azidobenzoyl-CoA in the presence of the deacetylcytrate lyase complex. Mg^{2+} is not required for the inactivation. Inactivation is blocked by (3*S*)-citryl-CoA in

the presence of ethylenediaminetetraacetic acid. *p*-Azidobenzoyl-CoA has no effect on the acetyl-CoA: citrate CoA transferase activity of both the deacetylcytrate lyase complex and its isolated transferase subunit. The stoichiometry of the CoA ester binding has been investigated by the use of *p*-azido[¹⁴C]benzoyl-CoA as a photoaffinity reagent. The labeling is exclusively on the lyase β subunit of the citrate lyase complex.

The citrate lyase multienzyme complex (EC 4.1.3.6) from *Klebsiella aerogenes* is assembled from three dissimilar subunits α , β , and γ of molecular weights 54 000, 32 000, and 10 000, respectively (Carpenter et al., 1975; Dimroth & Eggerer, 1975a). The γ subunit functions as an acyl-carrier protein (ACP)¹ with an essential acetyl function in thioester linkage (Buckel et al., 1971) on a covalently bound CoA-like prosthetic group (Oppenheimer et al., 1979). The α subunit acts as an acyl transferase involved in the formation of (3*S*)-citryl-ACP¹ in the presence of citrate with the release of acetate, while the β subunit functions as an acyl lyase catalyzing the cleavage of the citryl-ACP intermediate to oxaloacetate and acetyl-ACP¹ (Dimroth & Eggerer, 1975b). Acyl coenzyme A (acyl-CoA) derivatives such as acetyl-CoA and propionyl-CoA have been shown to serve also as substrates for the transferase reaction, while (3*S*)-citryl-CoA acts as a substrate for the lyase reaction (Buckel et al., 1973; Dimroth et al., 1977). The mechanism of action of the citrate lyase complex requires the oscillation of the prosthetic group (or of acyl-CoA in the acetyl-CoA-mediated reaction) between the active sites on the α and β subunits (Srere & Singh, 1974).

The paper reports the use of *p*-azidobenzoyl-CoA as a photoaffinity reagent for probing the acyl-CoA binding sites of the complex.

Experimental Procedures

Products. NADH, *N*-hydroxysuccinimide, sodium dodecyl sulfate (NaDodSO_4),¹ citrate synthase, and malate dehydrogenase were from Sigma. DTNB¹ was from Calbiochem.

Sephacel CL-6B, Sephadex G-10, and Sephadex G-25 were from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was from Whatman. *p*-Amino[carboxyl-¹⁴C]benzoic acid (21 Ci/mol) was obtained from Radiochemicals Centre. All other products were of the highest purity obtainable commercially.

Preparation of CoA Esters. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (Simon & Shemin, 1953). (3*S*)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in the presence of *K. aerogenes* deacetylcytrate lyase and EDTA¹ (Dimroth et al., 1977) and assayed with citrate synthase in the presence of DTNB.

p-Azidobenzoyl-CoA was synthesized as described by Lau et al. (1977). *p*-Azido[¹⁴C]benzoyl-CoA was synthesized from *p*-amino[carboxyl-¹⁴C]benzoic acid without excessive dilution of the radioactivity. The concentration of the reagent was determined by using $\epsilon_{265\text{nm}} = 22.2 \text{ mM}^{-1}$ (Lau et al., 1977).

All acyl-CoA preparations were purified as described by Moffat & Khorana (1961) by chromatography on a DEAE-cellulose (Cl⁻ form) column using a linear gradient from 0 to 0.2 M LiCl containing 0.003 N HCl. Samples of the CoA esters were concentrated at 25 °C in a rotary evaporator and desalted by filtration through a Sephadex G-10 column (1.0 × 50 cm).

Purification of Citrate Lyase. Citrate lyase complex from *Klebsiella aerogenes* (NCTC 418) was purified by a modification of the procedure described earlier (Mahadik & SivaRaman, 1968), sonic extracts being treated with ATP and acetate at final concentrations of 0.3 and 1 mM, respectively, for reactivating any deacetylcytrate lyase (Leena, 1979).

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¹ Abbreviations: ACP, acyl-carrier protein; (3*S*)-citryl-ACP, (3*S*)-citrylacyl-carrier protein; acetyl-ACP, acetylacyl-carrier protein; Na-DodSO₄, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

Table I: Effect of Photoactivated *p*-Azidobenzoyl-CoA on Transferase and Lyase Activities of *Klebsiella aerogenes* Citrate Lyase^a

enzyme	buffer	<i>p</i> -azido-benzoyl-CoA (μ M)	% inhibition	
			trans-ferase act.	lyase act.
deacetyl citrate lyase	50 mM Tris-HCl (pH 8.0) + 3 mM Mg ²⁺	0	0	0
		80	0	25
		112	0	42
		160	0	50
		168	0	58
	50 mM Tris-HCl (pH 8.0) + EDTA	0	0	0
acyl transferase subunit	50 mM Tris-HCl (pH 8.0)	80	0	25
		160	0	48
		0	0	0
		80	0	0
		160	0	0

^a Samples contained, in a total volume of 0.3 mL, 150 μ g of deacetyl enzyme or about 25 μ g of isolated α subunit. Aliquots were withdrawn after a 5-min irradiation and activities assayed as described under Experimental Procedures. Controls in which *p*-azidobenzoyl-CoA was absent showed no loss in the enzymatic activities.

Preparations were homogeneous in the ultracentrifuge and in polyacrylamide gel electrophoresis and had a specific activity varying between 70 U/mg of protein and 90 U/mg of protein.

Enzyme protein in the purified complex was determined from absorbance measurements at 278 nm by using the absorption index $E_{1\text{cm}}^{1\%}$ of 6.2 (Singh et al., 1976).

Preparation of Deacetyl citrate Lyase. Deacetyl citrate lyase was obtained by treatment of the purified native enzyme with neutral hydroxylamine according to the procedure of Buckel et al. (1973).

Isolation of Citrate Lyase Subunits. Component subunits were isolated in the electrophoretically pure and catalytically active state after dissociation with urea by the procedure described by Dimroth & Eggerer (1975b). Protein measurements of the isolated subunit preparations were made by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Assay of Enzymatic Activities. Citrate cleavage activity of the native enzyme complex was determined by coupled assay with malate dehydrogenase (Singh & Srere, 1971).

Acyl transferase activity of the deacetyl citrate lyase complex and of the isolated α subunit was determined from the initial velocity of the CoA transfer from acetyl-CoA to citrate in the presence of EDTA by estimation of the citryl-CoA produced with citrate synthase in the presence of DTNB (Dimroth et al., 1977; Srere et al., 1963).

Acyl lyase activity of the deacetyl enzyme complex and of the isolated β subunit was determined from the initial rate of oxaloacetate formation on cleavage of (3S)-citryl-CoA in the presence of Mg²⁺, oxaloacetate being assayed with malate dehydrogenase (Buckel et al., 1973).

Kinetics. The K_m for citryl-CoA and K_i for *p*-azidobenzoyl-CoA were calculated from the double-reciprocal plots by least-squares analysis. *p*-Azidobenzoyl-CoA was tested at two different concentrations of 30 and 60 μ M with varied initial concentrations of (3S)-citryl-CoA.

Photoactivation of *p*-Azidobenzoyl-CoA. Photoactivation was carried out under a Philips black light lamp (125 W) with the sample placed in a glass well kept at a distance of 2 cm. Solutions were precooled and maintained at 0–4 °C in an ice bath. Mixing was achieved by using a magnetic stirrer.

Incorporation of radioactivity into protein on photolysis in the presence of the ¹⁴C-labeled reagent was measured by the filter paper disk method (Mans & Novelli, 1961). Dried disks were counted in 10 mL of dioxane-based scintillant on a Beckman Model LS-100 scintillation system.

NaDodSO₄-polyacrylamide gel electrophoresis of the enzyme photolyzed in the presence of the radioactive reagent was carried out according to the method of Weber & Osborn (1969). Samples were prepared by heating aliquots (50–100 μ L) containing about 200 μ g of protein with 1% NaDodSO₄ in a boiling water bath for 3 min. Gels were stained with Coomassie brilliant blue R-250 and destained by diffusion. Detection of radioactivity in the protein bands was carried out by the method described by Saitoh et al. (1980).

Results

Effect of *p*-Azidobenzoyl-CoA. (1) Acyl Transferase Activity. *p*-Azidobenzoyl-CoA had no effect on the acetyl-CoA-mediated acyl transferase activity, both of deacetyl citrate lyase from *K. aerogenes* and of the isolated α subunit. The reagent was tried up to 160 μ M final concentration.

(2) Lyase Activity. Kinetic studies were performed in the dark to see the effect of *p*-azidobenzoyl-CoA on the lyase activity of *K. aerogenes* deacetyl citrate lyase toward (3S)-citryl-CoA. Assay mixtures at 25 °C contained 12.5 μ g of deacetyl citrate lyase, varying concentrations of (3S)-citryl-CoA (up to 50 μ M), 0–60 μ M *p*-azidobenzoyl-CoA, 50 mM Tris-HCl buffer, pH 8.0, and 3 mM MgSO₄ in a final volume of 1.0 mL. Double-reciprocal plots indicated that *p*-azidobenzoyl-CoA was a competitive inhibitor with a K_i of 80 μ M. The K_m for (3S)-citryl-CoA determined in this experiment was 67 μ M.

Photoaffinity Labeling of Citrate Lyase with *p*-Azidobenzoyl-CoA. The data on the effect of photoactivated *p*-azidobenzoyl-CoA on *K. aerogenes* deacetyl citrate lyase are summarized in Table I. Deacetyl citrate lyase treated with *p*-azidobenzoyl-CoA (final concentrations 80–168 μ M) and photolyzed for 5 min showed no loss in acyl transferase activity. The photoactivated reagent was also without effect on the activity of the isolated transferase α subunit of *K. aerogenes* citrate lyase. Under these conditions the lyase activity of the deacetyl enzyme was inhibited 25%, 50%, and 58% in the presence of 80, 160, and 168 μ M concentrations of *p*-azidobenzoyl-CoA, respectively. The inactivation of lyase activity was similar both in the presence of EDTA (10 mM final concentration) and in the presence of Mg²⁺ (3 mM final concentration).

Another piece of evidence that *p*-azidobenzoyl-CoA is an active-site directed photoaffinity label is the protection from irreversible inactivation of the lyase activity by (3S)-citryl-CoA (Table II). Inactivation in the presence of 112 μ M reagent is completely blocked by 200 μ M (3S)-citryl-CoA.

Table II: Protection of Deacetylcytrate Lyase by (3*S*)-Citryl-CoA from Inhibition by Photoactivated *p*-Azidobenzoyl-CoA^a

<i>p</i> -azidobenzoyl-CoA (μM)	citryl-CoA (μM)	% inhibition
0	0	0
112	0	42
112	44	31.5
112	74	25
112	200	0

^a The reaction mixture contained, in a total volume of 0.3 mL, 150 μg of deacetyl enzyme; 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and the indicated amounts of the inhibitor and (3*S*)-citryl-CoA. The mixture was irradiated for 5 min, and 0.05-mL aliquots were assayed for residual lyase activity.

The isolated β subunit of *K. aerogenes* citrate lyase showed very low activity; thus it was probably being extensively and irreversibly denatured during the isolation procedure as reported by Dimroth & Eggerer (1975b). The effect of *p*-azidobenzoyl-CoA on the isolated subunit was therefore not studied.

Quantitative binding studies were carried out with *p*-azido[¹⁴C]benzoyl-CoA and deacetylcytrate lyase from *K. aerogenes*. A plot of the inactivation of the lyase activity toward (3*S*)-citryl-CoA as a function of the covalent incorporation of *p*-azido[¹⁴C]benzoyl-CoA after photolysis is shown in Figure 1. The activity of the enzyme which had been photolyzed in the presence of various concentrations of *p*-azidobenzoyl-CoA (0–100 μM) was determined by assaying aliquots from the irradiated mixture. Controls photolyzed without reagent showed no loss of activity and were taken as 100% activity. The stoichiometry of binding was calculated by using a value of 575 000 for the molecular weight of *K. aerogenes* citrate lyase complex (Mahadik & SivaRaman, 1968).

It will be seen from Figure 1 that the decrease in enzyme activity is linear with the irreversible incorporation of ¹⁴C-labeled reagent until about 30% inactivation. To check whether the incorporation of ¹⁴C-labeled reagent under limiting conditions was selectively at the active site or not, the photolysis with *p*-azido[¹⁴C]benzoyl-CoA (50 μM) was carried out in the presence of (3*S*)-citryl-CoA (200 μM) and EDTA (10 mM). This reaction was carried out in the absence of Mg²⁺ to prevent action of the enzyme on the substrate, the absence of the metal having been shown to have no effect on inactivation with photoactivated *p*-azidobenzoyl-CoA (Table I). Under these conditions the enzyme retained its full lyase activity, and the incorporation of radioactivity was completely blocked. In the presence of 50 μM of the radioactive aryl-CoA derivative, the enzyme was inactivated 25% in the absence of the substrate. This would suggest that the linearity up to at least 25% inactivation represents specific labeling of the active site by *p*-azidobenzoyl-CoA.

On the basis of this assumption an estimate of the total active sites per mole of enzyme was found by extrapolation of the linear part of the plot by fitting of the least-squares line for the data up to 25% inactivation. This extension was observed to include also the value for 30% inactivation. The stoichiometry of the number of active sites per mole of enzyme obtained in this manner was 7.4. The extended extrapolation would make this only an approximate estimate.

At concentrations of the reagent required for a higher degree of inactivation, photolysis apparently results in significant nonspecific incorporation even in the presence of Tris which functions as scavenger (Bayley & Knowles, 1977). Nonspecific binding of *p*-azidobenzoyl-CoA has also been reported for acylglycine *N*-acyltransferase of beef liver at values above

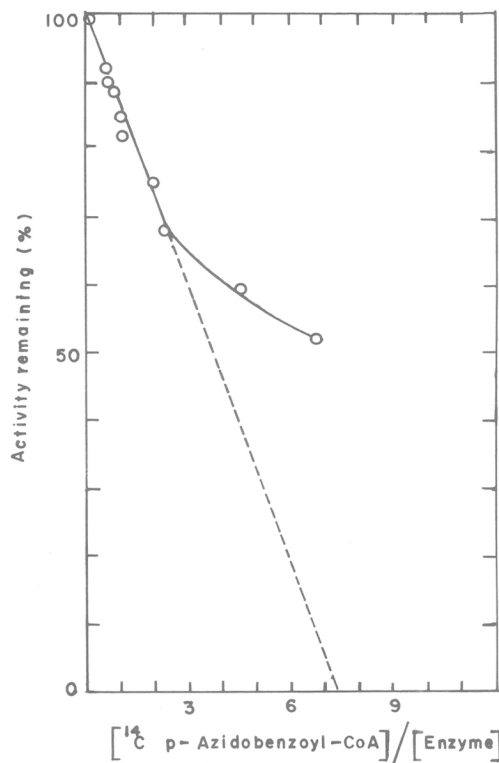


FIGURE 1: Determination of the number of active sites of lyase β subunits per mole of deacetylcytrate lyase complex accessible to *p*-azidobenzoyl-CoA. The irradiated mixture in 300 μL contained 75 μg of deacetylcytrate lyase, 50 mM Tris-HCl, pH 8.0, 3 mM MgSO₄, and varying amounts of *p*-azido[¹⁴C]benzoyl-CoA (0–100 μM). After 5-min photolysis, a 50-μL aliquot was withdrawn for the determination of lyase activity and a 200-μL sample withdrawn for estimation of ¹⁴C incorporation.

about 30% inhibition (Lau et al., 1977).

An enzyme sample photolyzed in the presence of the ¹⁴C-labeled *p*-azidobenzoyl-CoA was dissociated, and the constituent subunits were separated by NaDodSO₄-polyacrylamide gel electrophoresis. The electrophoretic profile of the separated subunits and the radioactivity distribution in the gel are represented in Figure 2. The presence of activity exclusively in the gel region containing the lyase subunit would confirm the evidence obtained from the kinetic data, Figure 1, and Table II that the active site of this subunit is specifically labeled on photolysis in the presence of the azidoaryl-CoA.

Discussion

p-Azidobenzoyl-CoA has been shown to have no inhibitory effect on the acyltransferase activity of *K. aerogenes* deacetylcytrate lyase. That the enzyme complex might have hindered the photoaffinity reagent from reaching the active site on the transferase α subunit has been ruled out, since the isolated α subunit is also not inhibited by *p*-azidobenzoyl-CoA on photoactivation. The active site of the transferase subunit itself apparently does not accept *p*-azidobenzoyl-CoA, possibly on account of the bulky aryl group.

The substrate binding site of the lyase subunit, however, accepts the aryl-CoA ester. The kinetic data establish that *p*-azidobenzoyl-CoA acts as a potent competitive inhibitor of the acyl lyase activity. The reagent presumably binds specifically to the CoA ester sites of the lyase subunits. Again during photolysis a linear relationship is observed between incorporation of the ¹⁴C-labeled reagent and loss in activity

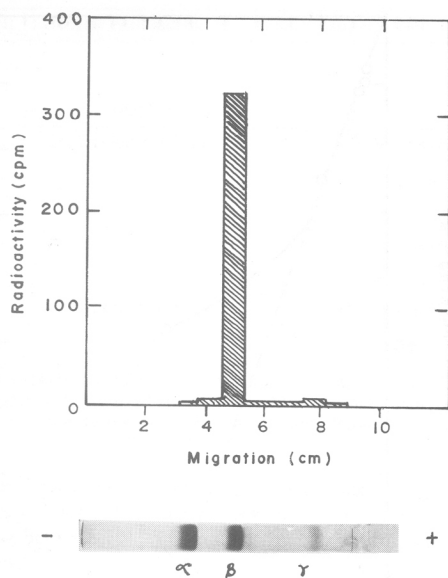


FIGURE 2: NaDodSO₄-polyacrylamide gel electrophoresis of deacetyl citrate lyase labeled by photolysis in the presence of *p*-azido-[¹⁴C]benzoyl-CoA. The irradiated mixture contained, in 0.5 mL, 400 μg of deacetyl citrate lyase, 50 mM Tris-HCl, pH 8.0, 3 mM MgSO₄, and 300 μM *p*-azido-[¹⁴C]benzoyl-CoA. An aliquot of about 0.2 mL was taken for electrophoresis. Gels were stained and sliced for determination of covalently bound radioactivity as described under Experimental Procedures. The histogram shows the profile of radioactivity in the separated subunits.

under limiting conditions of the reagent, the label being exclusively on the lyase β subunit. The reagent would thus be useful for selectively labeling the lyase active sites, having no action on the acetyl-CoA binding sites of the acyltransferase α subunits.

The lyase activity requires the presence of divalent metal ions such as Mg²⁺ (Dimroth & Eggerer, 1975b; Buckel et al., 1973). Divalent metal binding has been shown more recently to bring about a conformational change in the enzyme complex from *K. aerogenes* (SivaRaman & SivaRaman, 1979). In the present studies, the binding of *p*-azidobenzoyl-CoA to the lyase subunit and the subsequent irreversible inactivation on photolysis have been shown to occur both in the presence of Mg²⁺ and in the presence of an excess of EDTA. These results might suggest that (3*S*)-citryl-CoA binding is independent of divalent metals, while its cleavage requires the presence of Mg²⁺.

Although *K. aerogenes* citrate lyase has been shown to have a hexameric α₆β₆γ₆ structure (Carpenter et al., 1975; Dimroth & Eggerer, 1975a), careful estimation of the prosthetic group components have indicated that only four acyl-carrier proteins carry pantetheine moieties (Singh et al., 1976). This has posed the problem of the function of subunit combinations which are devoid of prosthetic groups. An earlier suggestion has been made that these subunits might function in citrate cleavage

mediated by acetyl-CoA (Robinson et al., 1976). The approximate estimate of about seven sites on the lyase subunits of the complex obtained in the present investigation is suggestive of the fact that all the lyase subunits are accessible to citryl-CoA. Thus in the intact organism the cleavage of citrate can proceed both via the built-in prosthetic group and through the acyl-CoA-mediated reaction.

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THE PRESENCE OF ESSENTIAL ARGININE RESIDUES AT THE ACTIVE SITES
OF CITRATE LYASE COMPLEX FROM KLEBSIELLA AEROGENES

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The acyl-transferase and acyl-lyase activities of Klebsiella aerogenes citrate lyase complex are inactivated by the arginine specific reagents phenylglyoxal and 2,3-butanedione, the former reagent being the more potent inhibitor. Citrate and (3S)-citryl-CoA protect the transferase activity, while acetyl-CoA markedly enhances the rate of the inactivation. (3S)-Citryl-CoA protects the lyase subunit in the complex from inactivation. The kinetics of inactivation suggest the involvement of a single arginine residue at each of the active sites of the transferase and of the lyase subunits.

Citrate lyase (EC 4.1.3.6) from Klebsiella aerogenes is a multienzyme complex comprising three non-identical subunits of about 54 000 (α), 32 000 (β) and 10 000 (γ) daltons respectively (1). The smallest subunit (γ) carries an essential acetyl group in thioester linkage with a CoA like prosthetic group and functions as an acyl-carrier protein (ACP) (2). In presence of citrate and EDTA, the α subunit functions as an acyl-transferase catalysing the formation of (3S)-citryl-ACP with liberation of acetate. The β subunit acts as an acyl-lyase involved in the cleavage of the (3S)-citryl-ACP intermediate in presence of Mg^{2+} (3). The CoA derivatives, acetyl-CoA and propionyl-CoA, serve as substrates for the transferase reaction, while (3S)-citryl-CoA functions as a substrate for the lyase reaction (4,5).

Abbreviations : ACP, acyl carrier protein; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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p-Azidobenzoyl-CoA has been shown recently by us to function as a photoaffinity reagent specifically inhibiting the lyase activity (6). Little is known, however, about the residues at the catalytic sites of the enzyme complex. The present report describes the involvement of arginine residues at the active sites of the transferase and lyase subunits.

MATERIALS AND METHODS

Citrate lyase. Citrate lyase enzyme complex was purified from K. aerogenes (NCTC 418) as described earlier (6,7). Protein in the purified enzyme complex was estimated by its absorbance at 278 nm (8). Deacetyl citrate lyase was obtained as described by Buckel et al. (2).

Citrate lyase subunits. The catalytically active transferase (α) and lyase (β) subunits were isolated by the procedure of Dimroth and Eggerer (3). Protein in the isolated subunits was estimated by the method of Lowry et al. (9) using bovine serum albumin as standard.

Enzyme assays. The overall citrate cleavage activity of the native enzyme complex and of the deacetyl citrate lyase as well as the individual acyl-transferase and acyl-lyase activities were determined as described earlier (6). Aliquots (10 to 20 μ l) of enzyme samples incubated with the arginine modifying reagent, phenylglyoxal or 2,3-butanedione, were taken in a total volume of 1 ml of the assay systems containing 50 mM Tris-HCl buffer. Under the assay conditions, the modifying reagents were without effect on the coupling enzymes, malate dehydrogenase and citrate synthase.

CoA esters. Acetyl-CoA was prepared by acetylation of CoA with acetic anhydride (10). (3S)-Citryl-CoA was prepared enzymatically from acetyl-CoA and citrate in presence of deacetyl citrate lyase and EDTA (5) and purified as described by Moffatt and Khorana (11).

Reaction with arginine modifying reagents. Citrate lyase (or the isolated subunit) was incubated at 30°C with either phenylglyoxal or 2,3-butanedione in 50 mM buffer solutions, pH 7.6. The buffers tested were potassium phosphate, Hepes and Tris-HCl. Borate buffer was not used as enzyme activities were markedly lowered in the presence of borate. The reaction mixture was shielded from light to avoid any photochemical effect when 2,3-butanedione was used as the probe (12). Enzyme incubated with the buffer alone served as the control. Aliquots were removed at different time intervals for assay of enzymatic activities. The effect of various ligands on inactivation by phenylglyoxal or 2,3-butanedione was studied after preincubating the enzyme with the compound for 5 min at 30°C followed by addition of the stated amounts of the inhibitor. Aliquots were removed thereafter at regular intervals of time and checked for loss in enzyme activity.

RESULTS

Inactivation of transferase activity. The acetyl-CoA mediated acyl-transferase activity, both of deacetyl citrate lyase complex and of the isolated transferase subunit, was rapidly inactivated on incubation with phenylglyoxal or 2,3-butanedione at pH 7.6. The maximum inactivation was in potassium phosphate buffer, the inactivation being slightly less in Hepes and markedly lower in Tris buffer. Kinetic studies were therefore carried out in phosphate buffer.

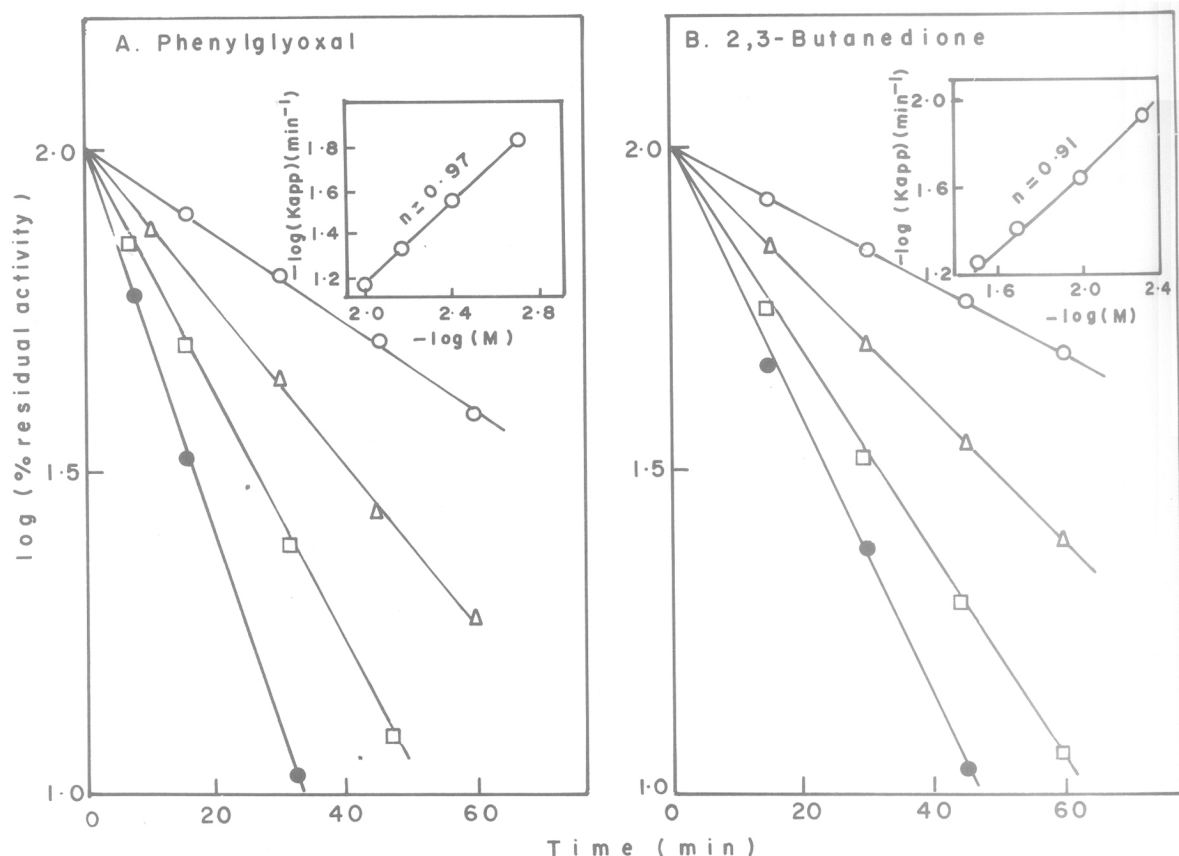


Figure 1 : Inactivation of the acyl-transferase activity of deacetyl citrate lyase by α -carbonyl reagents. The enzyme (0.5 mg/ml) was incubated at 30°C in 50 mM phosphate buffer (pH 7.6) containing 10 mM EDTA and various concentrations of the reagents. (A) Phenylglyoxal 2 mM ($\circ$), 4 mM (Δ), 7 mM ($\square$), and 10 mM ($\bullet$). (B) 2,3-butanedione 5 mM ($\circ$), 10 mM (Δ), 20 mM ($\square$), and 30 mM ($\bullet$). At the indicated times, aliquots were assayed for transferase activity as described in the text. Insets : Determination of the order of the reaction with respect to phenylglyoxal (A) and 2,3-butanedione (B).

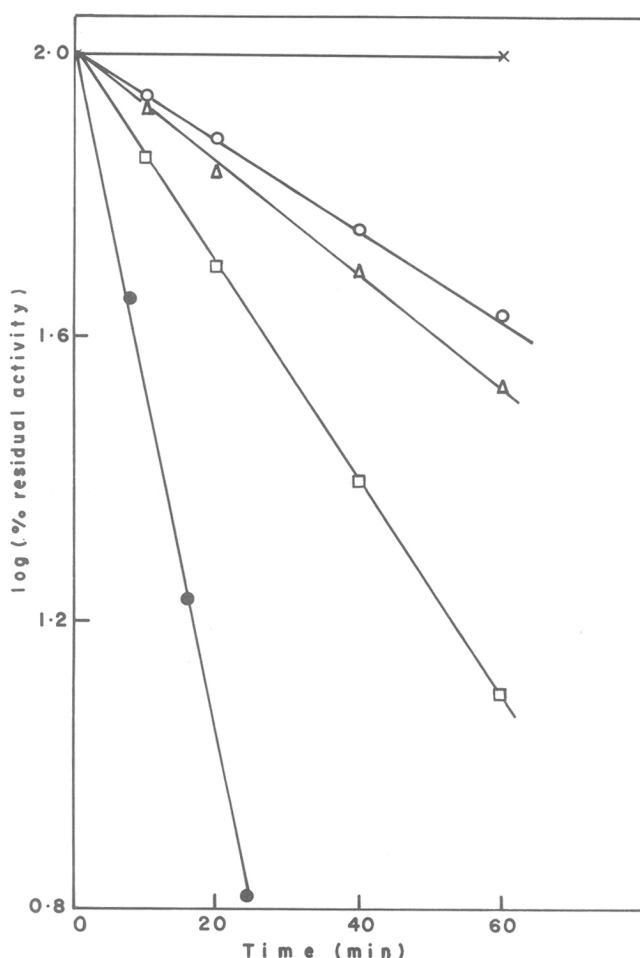


Figure 2 : Effect of ligands on the rate of inactivation of the acyl-transferase activity of deacetyl citrate lyase by phenylglyoxal. The enzyme (0.5 mg/ml) was preincubated with the ligands for 5 min at 30°C in 50 mM phosphate buffer (pH 7.6) containing 10 mM EDTA followed by incubation with 5 mM phenylglyoxal. Control without phenylglyoxal (x), no added ligand (□), 10 mM citrate (Δ), 0.5 mM (3S)-citryl-CoA (○), and 1 mM acetyl-CoA (●). Concentrations of ligands and modifier are final concentrations. Aliquots were assayed at specified time intervals for transferase activity.

The results obtained with deacetyl citrate lyase and various phenylglyoxal and 2,3-butanedione concentration are presented in Figure 1. The inactivation rates follow pseudo first-order kinetics, both plots being linear upto about 10% initial enzyme activity. The reaction order (n) with respect to the arginine reagent was determined from the plot of the logarithm of the

apparent first order rate constant, K_{app} , versus the logarithm of the reagent concentration (13) and the value of $n = 0.97$ for phenylglyoxal and of $n = 0.91$ for 2,3-butanedione suggest that loss of activity results from reaction of one arginine residue per acyl-transferase subunit (13 - 15). Similar results were obtained in case of the isolated α -subunit where a value of $n = 0.93$ was obtained with phenylglyoxal.

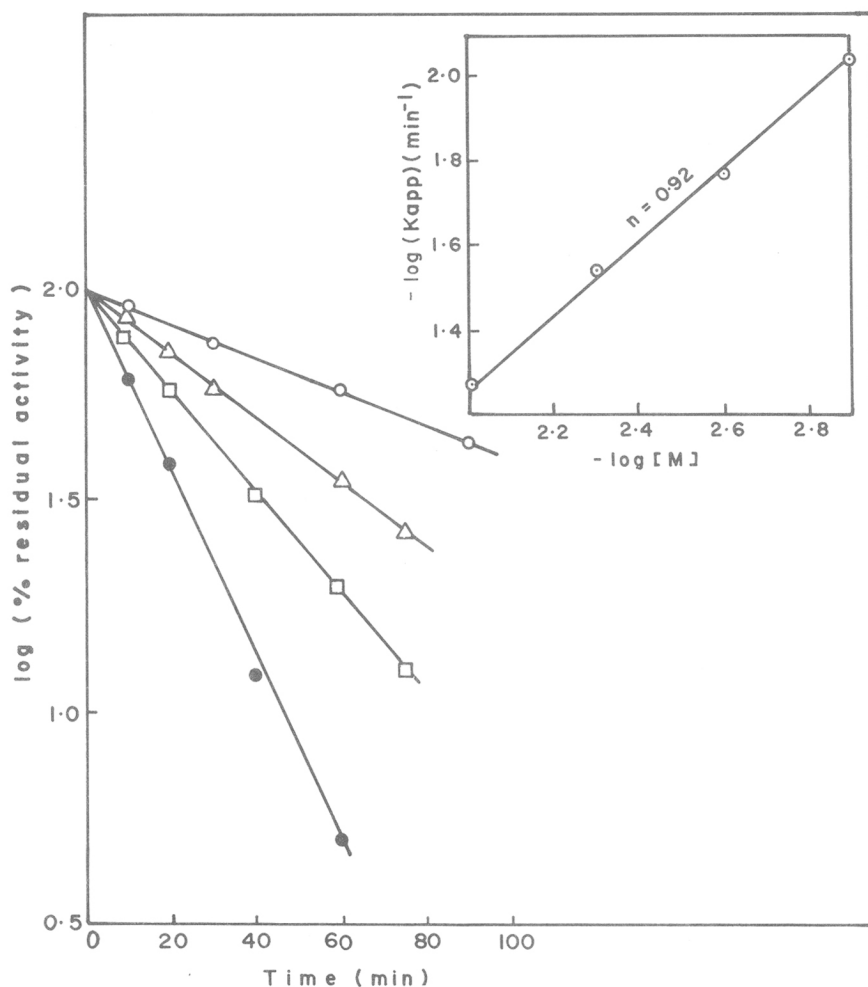


Figure 3 : Inactivation of the acyl-lyase activity of deacetyl citrate lyase by phenylglyoxal. The enzyme (0.5 mg/ml) was incubated at 30°C in 50 mM phosphate buffer (pH 7.6) containing 3 mM $MgSO_4$ and phenylglyoxal 1.25 mM (○), 2.5 mM (△), 5 mM (□) or 10 mM (●). Aliquots were assayed for lyase activity at indicated times. Omission of $MgSO_4$ from incubation mixtures had no effect on the inactivation. The inset of the figure shows the determination of the order of the reaction with respect to phenylglyoxal.

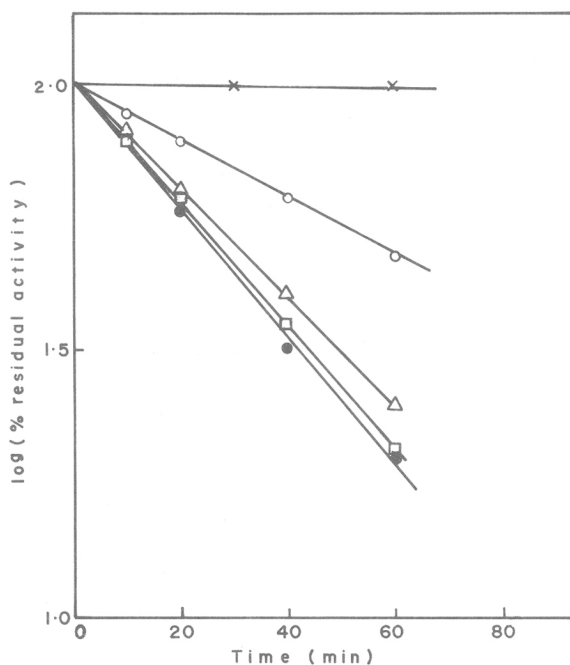


Figure 4 : Protection of lyase activity of deacetyl citrate lyase from inactivation by phenylglyoxal. The enzyme (0.5 mg/ml) was preincubated with various ligands in presence of EDTA. Conditions were the same as described in Figure 2. Control (X), no added ligand (●), 10 mM citrate (□), 0.5 mM citryl-CoA (○), and 1.0 mM acetyl CoA (Δ).

The effect of various ligands on the rate of inactivation of the transferase activity of deacetyl citrate lyase by phenylglyoxal is shown in Figure 2. Citrate (10 mM) and (3S)-citryl-CoA (0.5 mM) decreased the rate of inactivation by 45% and 55% respectively; while acetyl-CoA (1 mM) increased the rate of inactivation 3-fold. Similar effects were observed with the isolated transferase (α) subunit. The effects of 2,3-butanedione were similar to those of phenylglyoxal.

The reaction order for the inactivation was also determined in presence of acetyl-CoA (1 mM). A value of $n = 1.15$ with phenylglyoxal under these conditions would suggest that the rate of reaction of the essential arginine residue is enhanced without the involvement of any additional residues.

Inactivation of lyase activity. The lyase activity of deacetyl citrate lyase complex was also inactivated by the two reagents. The rate of inactivation followed pseudo first-order kinetics as shown in Figure 3. A value of $n = 0.92$ suggests the minimal requirement of one arginine residue per subunit. The isolated lyase subunit was not studied since it showed markedly lowered activity, probably due to denaturation during the process of isolation (3). The effect of ligands is shown in Figure 4, Citrate (10 mM) had no significant effect; acetyl-CoA (1 mM) reduced the inactivation slightly (15%); while the substrate (3S)-citryl-CoA (0.5 mM) more effectively protected against inactivation by phenylglyoxal.

DISCUSSION

The presence of essential arginine residues has been reported in several enzymes acting upon anionic cofactors and substrates (16). Among the citrate enzymes, ATP-citrate lyase from rat liver has been shown recently to carry an essential arginine residue (13). The citrate lyase complex from *K. aerogenes* has been shown in the present study to carry an essential arginine residue in each subunit with enzymatic activity. Protection studies against inactivation by arginine specific reagents indicate that the arginine residue is present at the citrate binding site of the transferase subunit, one of the points of attachment of citrate to the transferase subunit presumably being through the guanidino group at the active site. Acetyl-CoA markedly increases the rate of inactivation of the transferase subunit probably through a conformational change at the citrate binding site making the essential arginine residue more accessible to α -carbonyl reagents. In the case of the classical CoA transferases, a conformational change at the carboxylate binding site of succinyl-CoA: 3-oxoacid CoA-

transferase has been suggested to explain the enhanced reactivity of an essential thiol group on binding of acyl-CoA substrates (17).

The acyl-lyase activity of the deacetyl citrate lyase complex has also been shown in our studies to depend on an essential arginine residue; the acyl-CoA substrate, (3S)-citryl-CoA, in this case protecting against inactivation by arginine modifying reagents.

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S-ACYLATED RESIDUES OF THE ACYL-CARRIER PROTEIN SUBUNIT OF
Klebsiella aerogenes CITRATE LYASE

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SUMMARY Oxidation of the isolated deacetyl acyl-carrier protein subunit of citrate lyase from Klebsiella aerogenes with Cu^{2+} -o-phenanthroline complex leads exclusively to intrapeptide disulfide bridge formation indicating that the cysteamine and the cysteine residues are located in close proximity. The S-acetylation of the cysteine residue in deacetyl acyl-carrier protein subunit is catalysed by a citrate lyase ligase preparation in presence of acetate and ATP. Reaction-inactivation of citrate lyase results in deacetylation of the S-acetyl cysteamine residue of the prosthetic group but not of the S-acylated cysteine residue in the acyl-carrier protein.

Citrate lyase (EC 4.1.3.6) which catalyses the cleavage of citrate in a two step sequence of reactions (1) is a multienzyme complex containing two enzymes and an acyl-carrier protein (ACP)[†] (2). The most extensively studied of these complexes is the one from Klebsiella aerogenes, the ACP subunit of which has been shown to contain an S-acylated cysteine residue in addition to the essential S-acetyl moiety on the cysteamine residue of the CoA-like prosthetic group (3). Citrate lyase from K. aerogenes undergoes rapid inactivation in the course of the reaction it catalyzes with loss of the essential acetyl groups to give inactive deacetyl (HS-) citrate lyase (4). The HS-citrate lyase is re-

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[†]Abbreviations: ACP, acyl-carrier protein; SDS, sodium dodecyl sulfate; DTNB, 5,5'-(2-nitrobenzoic acid); DTE, dithioerythritol.

activated either chemically by acetic anhydride (4) or enzymatically by citrate lyase ligase in presence of acetate and ATP(5).

Some of the properties of the acylated thiol residues of the ACP subunit of *K. aerogenes* citrate lyase and the probable role of citrate lyase ligase in modification of the cysteine residue of the subunit are reported here.

MATERIALS AND METHODS

Iodo [$2-^{14}\text{C}$] acetic acid (57 Ci/mol) was obtained from Radiochemical Centre, Amersham and diluted with cold iodoacetate to a sp. act. of 20 Ci/mol. Sodium [$1-^{14}\text{C}$] acetate (47.6 Ci/mol) from the Bhabha Atomic Research Centre, Bombay, was diluted with cold acetate to about 3 Ci/mol before use.

S-Carboxymethyl derivatives of cysteine and cysteamine were prepared by standard procedures (6,7).

Citrate lyase The pure enzyme (sp. act. $\sim 70 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) was obtained from *K. aerogenes* and assayed as described earlier (8).

Deacetylated ACP subunit Citrate lyase was dissociated with 6 M urea in the presence of 10 mM dithiothreitol and the deacetylated ACP subunit isolated in electrophoretically pure state by the procedure described by Carpenter et al.(2), a Sepharose CL-6B column (1 x 100 cm) being used for the separation. Protein in the isolated subunit preparation was determined by the method of Lowry et al.(9) using bovine serum albumin as standard.

Oxidation with o-phenanthroline- Cu^{2+} complex The separated deacetyl ACP subunit (0.8 mg) in 0.5 ml of 0.05 M potassium phosphate buffer, pH 7.4 was treated with o-phenanthroline and copper at concentrations of 100 μM and 20 μM respectively (10). The mixture was incubated for 30 min at 25°C and the reaction quenched by adding 0.5 ml of 20% SDS[†] solution. Aliquots of the reaction system were separately taken for SDS-polyacrylamide gel electrophoresis and thiol group estimation. Sulfhydryl groups were estimated with DTNB[†] (11) before and after the oxidation of the subunit.

SDS-polyacrylamide gel electrophoresis SDS-gel electrophoresis was carried out by the method of Weber and Osborn (12) in the absence of 2-mercaptoethanol.

Characterization of the S-acylated residues The S-acetyl residue involved in the reaction mechanism was characterized by a modification of the method described for fatty acid ACP (13). Citrate lyase (1 mg) was reaction inactivated in a total volume of 0.2 ml containing 0.2 mmol, citrate (pH 8.0); 0.16 μmol , MgSO_4 by incubation for 15 min at 30°C. An aliquot was checked for citrate lyase activity by coupled assay with malate dehydrogenase (14) to confirm complete inactivation. The free sulfhydryl groups of the reaction-inactivated enzyme were blocked with cold iodoacetate. The thioester linkages were then cleaved with neutralized hydroxylamine and dithiothreitol and the liberated thiol groups reacted with iodo [^{14}C] acetate. The S- [^{14}C] carboxymethylated residue in the protein was characterized by paper electrophoresis of the acid hydrolysate. The experimental conditions used for the differential labeling and characterization of the

[^{14}C]carboxymethyl residue were similar to those described by Dimmoth et al. (3). A mixture of reference compounds, S-carboxymethyl cysteine and S-carboxymethyl cysteamine (2 μmol each), was also separated under identical conditions on a separate strip of paper and their positions after migration visualised by spraying with 0.1% ninhydrin in acetone. The strip with the hydrolysed S-carboxymethylated enzyme was cut into pieces at the end of the run and radioactivity located using a dioxane based cocktail and a Beckman LS-100 C counter.

Citrate lyase ligase The acetate: HS- acyl-carrier protein enzyme ligase (AMP) was partially purified from *K. aerogenes* as described earlier (15). The ligase preparation was free from citrate lyase activity.

[^{14}C]-acetyl citrate lyase The [^{14}C]-acetyl labeled enzyme was prepared by acetylation of HS-citrate lyase with citrate lyase ligase in presence of [^{14}C]-acetate and ATP. Citrate lyase from *K. aerogenes* was deacetylated with neutralized hydroxylamine (16) before the DEAE-cellulose step in the purification procedure (17) and excess reagent separated by filtration through Sephadex G-25 (1.5 x 40 cm) using 0.05 M potassium phosphate buffer, pH 7.6 containing 1.6 mM MgSO_4 . An aliquot withdrawn from the protein fraction showed complete loss of activity. The deacetyl-enzyme (250 mg) in 5 to 6 ml of the phosphate buffer was treated with citrate lyase ligase in presence of ATP and acetate (8 uCi), final concentrations of 6 mM and 0.5 mM, respectively; and incubated at 25°C for 30 min. Adequate citrate lyase ligase was used to reactivate to about 50% of the initial activity. The protein was then precipitated by addition of an equal volume of cold saturated ammonium sulfate solution and collected by centrifugation for 30 min at 50 000 g and 4°C, dissolved in a minimum volume of 0.05 M potassium phosphate buffer, pH 7.6 and subjected to DEAE-cellulose chromatography and gel filtration through Sepharose CL-6B essentially as described earlier (17).

The radioactivity distribution in the subunits was determined after dissociation of [^{14}C]-acetyl citrate lyase (200 μg) with 1% SDS in the absence of 2-mercaptoethanol followed by SDS-gel electrophoresis. The gel was stained, destained and the regions corresponding to the subunits were cut and checked for radioactivity as described by Saitoh et al. (18).

The extent of deacetylation of [^{14}C]-acetyl citrate lyase on reaction-inactivation was estimated by exhaustive dialysis of the completely inactivated enzyme against 0.05 M potassium phosphate buffer, pH 7.5 and determination of the remaining protein-bound radioactivity.

RESULTS

Oxidation of deacetyl ACP The effect of *o*-phenanthroline- Cu^{2+} on the content of -SH groups of the isolated deacetyl ACP subunit is shown in Table 1. The content of 2-SH/mol in deacetyl ACP is in agreement with the presence of a cysteine and a cysteamine residue in the ACP molecule (19). Oxidation with the reagent

Table 1. Oxidation of sulfhydryl groups of deacetyl ACP by o-phenanthroline- Cu^{2+} complex.

System	-SH/mol of ACP ^a
Deacetyl ACP	1.9
Deacetyl ACP + <u>o</u> -phenanthroline- Cu^{2+} complex	0.3

The isolated deacetyl ACP was oxidized with o-phenanthroline- Cu^{2+} complex (as described under Materials and Methods) and the reduction in thiol groups estimated. The values given are an average of three separate estimations.

^a M_r = 9378 (20).

results in a decrease to about 0.3 -SH/mol. SDS-gel electrophoresis of aliquots from the reaction mixture showed that the molecular weight of the oxidized subunit was unchanged. This was established by electrophoresis along with the unoxidized ACP. Interpeptide cross-linking is apparently excluded and oxidation results exclusively in intrapeptide disulfide bridge formation between the thiol groups of the cystamine residue on the prosthetic group and of the cysteine residue in the polypeptide chain of the ACP subunit.

Acetylation of HS-citrate lyase by citrate lyase ligase The distribution of the radioactive label in the subunits of [^{14}C]-acetyl citrate lyase obtained on acetylation of HS-citrate lyase by citrate lyase ligase in presence of [^{14}C]-acetate and ATP is shown in Figure 1. Acetylation by the ligase is specifically of the ACP subunit, radioactivity being associated exclusively with the region in the gel containing the 10 000 daltons ACP (γ) subunit, the 54 000 daltons acyl transferase (α) and the 32 000 daltons acyl lyase (β) subunits carrying no radioactive label.

S-Acylated residues of reaction-inactivated citrate lyase The characterization of the S-acylated residues present in reaction-inactivated citrate lyase is shown in Figure 2. It can be seen from Figure 2 that total reaction-inactivation of citrate lyase

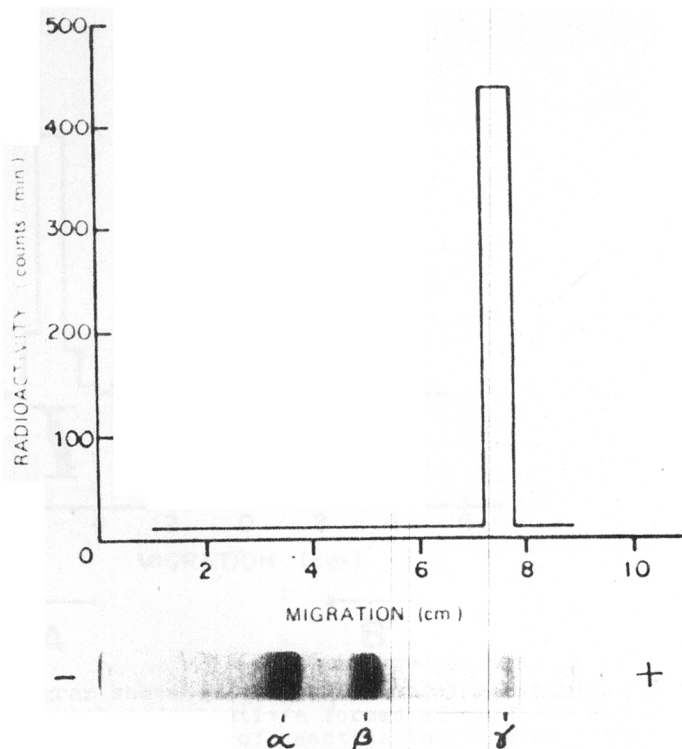


Figure 1. SDS-polyacrylamide gel electrophoresis of $[^{14}\text{C}]$ -acetyl citrate lyase prepared as described under Materials and Methods. The gel was stained and sliced for determination of protein bound radioactivity. The histogram shows the profile of radioactivity in the separated acyl transferase (α), acyl lyase (β) and ACP (γ) subunits.

results in the complete loss of acetyl groups on the cysteamine moiety of the prosthetic group, while S-acetylated cysteine residues are still present in the inactivated enzyme. Reaction-inactivation results in the selective deacetylation of the acetyl-S-cysteamine residues of the prosthetic groups.

Partial deacetylation was also observed on reaction-inactivation of $[^{14}\text{C}]$ -acetyl citrate lyase. The specific radioactivity of the active enzyme and of the reaction-inactivated complex are shown in Table 2. The presence of about 30% of the initial radioactivity in protein bound form after total reaction-inactivation and the finding that the cysteamine residues of the prosthetic

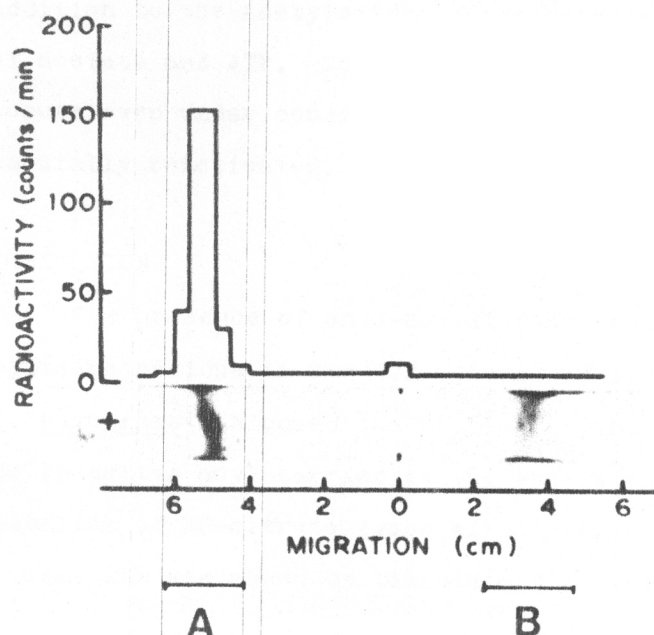


Figure 2. The histogram shows the distribution of radioactivity between the S-carboxymethyl derivatives formed after differential labeling of S-acetylated residues of reaction-inactivated citrate lyase with iodo[^{14}C]-acetate. See text for details. A, S-carboxymethyl cysteine; B, S-carboxymethyl cysteamine.

groups in the inactivated complex are fully deacetylated would indicate that the [^{14}C]-acetyl moieties are retained only on the cysteine residues of the ACP subunits. The results are suggestive of the role of citrate lyase ligase in the modification of the

Table 2. Effect of reaction-inactivation on protein-bound radioactivity of [^{14}C]-acetyl citrate lyase.

Enzyme	cpm/mg
[^{14}C]-acetyl citrate lyase	13070
[^{14}C]-acetyl citrate lyase after reaction-inactivation	4120

[^{14}C]-Acetyl citrate lyase (430 μg) was incubated in a total volume of 1 ml of the reaction mixture containing 1 mmol citrate; 3 μmol , MgSO_4 and 50 μmol , Tris-HCl buffer, pH 8.0. Aliquots tested after 15 min incubation at 30°C showed complete loss of citrate lyase activity. Radioactivity was measured as described in the text.

cysteine residues of the ACP subunits to acetyl-S-cysteines in addition to the acetylation of the prosthetic groups in presence of acetate and ATP. The acetylation of the cysteine residue occurs even under conditions where HS-citrate lyase is only partially reactivated.

DISCUSSION

The presence of an S-acylated cysteamine and an S-acylated cysteine residue in the ACP subunit of citrate lyase from K. aerogenes has posed the problem of the nature of the biologically active acyl-carrier in the enzyme complex (3). Affinity labeling of HS-citrate lyase with iodoacetate in presence of excess DTE was shown by Dimroth and Eggerer (21) to result exclusively in the carboxymethylation of the cysteamine residue to yield a carboxymethylated complex inaccessible to reactivation either chemically with acetic anhydride or enzymatically by citrate lyase ligase. The present studies provide direct evidence that the cysteamine residue functions as the biologically active acyl-carrier, the reaction-inactivated citrate lyase being devoid of acetyl-S-cysteamine while still retaining S-acetylated cysteine residues.

Numerous instances of the presence of amino acid residues with modified side chains as constituents of proteins have been reported in the literature (22). The in vivo mechanisms of such modifications are not often understood. The data presented here suggest the involvement of citrate lyase ligase in the acetylation of cysteine residues in the ACP subunits of the K. aerogenes citrate lyase complex. The possible function of this modification might be to protect against intrapeptide disulfide bridge formation with the juxtaposed sulfhydryl residue on the prosthetic group of the enzyme in the deacetylated form.

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