

**MICROBIAL ENZYMES :
STUDIES ON CITRATE
OXALOACETATE - LYASE (EC 4.1.3.6)
from
Klebsiella aerogenes**

COMPUTERISED

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ABBREVIATIONS

List of abbreviations used other than the accepted ones:

ACP	-	Acyl carrier protein
CFA	-	Citroyl formic acid
DMA	-	Dimethyl adipimidate
DMS	-	Dimethyl suberimidate
DSP	-	Dithiobis (succinimidyl propionate)
DTNB	-	5,5'-Dithiobis (2-nitrobenzoate)
DTE	-	Dithioerythritol
DTT	-	Dithiothreitol
OAA	-	Oxaloacetic acid
PPO	-	2,5-Diphenyloxazole
SDS	-	Sodium dodecyl sulfate
$E_{1\text{ cm}}^{1\%}$	-	Absorbance index (1% w/v protein)
ϵ_m	-	Molar extinction coefficient
2-ME	-	2-mercaptoethanol
MDH	-	Malate dehydrogenase (EC 1.1.1.37)

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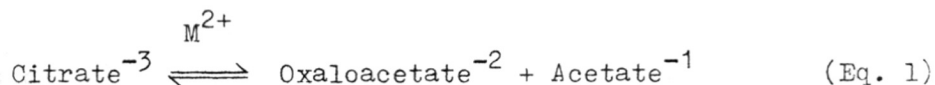
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GENERAL INTRODUCTION

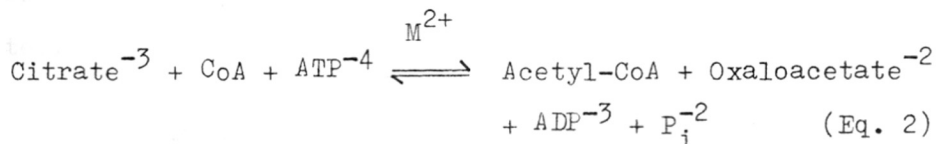
CITRATE ENZYMES

Citrate is a key intermediate in a number of metabolic pathways. Three groups of enzymes are known to catalyse the dissimilation or synthesis of citrate by a lyase reaction involving an equilibrium between citrate and oxaloacetate and an acetyl moiety. The enzymes are:

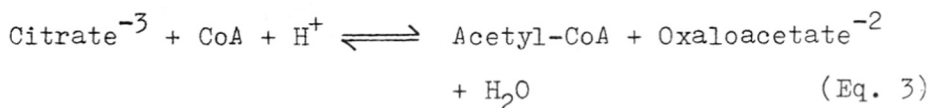
1. Citrate lyase (citrate oxaloacetate-lyase (pro-3S-CH₂COO⁻ → acetate) EC 4.1.3.6) which has been found only in procaryotes and catalyses the cleavage of citrate to oxaloacetate and acetate in the presence of divalent metal by the reaction shown in Eq. 1.



2. ATP-citrate lyase (ATP: citrate oxaloacetate-lyase (pro-3S-CH₂COO⁻ → acetyl-CoA: ATP dephosphorylating) EC 4.1.3.8), a cytosolic enzyme found only in eukaryotic cells which catalyses the reaction shown in Eq. 2.



3. Citrate synthase which functions in the synthesis of citrate according to the overall reaction (Eq. 3):



Two distinct types of citrate synthases are known based on the stereochemistry of the reactions they catalyse. They are,

- (i) si-citrate synthase, citrate oxaloacetate-lyase (pro-3S- $\text{CH}_2\text{COO}^- \longrightarrow \text{acetyl-CoA}$) EC 4.1.3.7, of the citric acid cycle, and
- (ii) re-citrate synthase, citrate oxaloacetate-lyase (pro-3R- $\text{CH}_2\text{COO}^- \longrightarrow \text{acetyl-CoA}$) (EC number not assigned yet), present in some anaerobic microorganisms.

The equilibrium of the reactions catalysed in vivo by citrate lyase and ATP-citrate lyase almost exclusively favours citrate cleavage, whereas that catalysed by citrate synthase favours citrate synthesis. On the basis of its occurrence in the most primitive organisms and its simple supplementary requirements, it has been suggested that citrate lyase might be evolutionarily the earliest of this group of enzymes (Srere, 1968). ATP-citrate lyase, which occurs only in higher organisms and catalyses the most complex reaction requiring three substrates and a divalent metal ion, was probably the last to evolve.

A number of reviews on these enzymes have been published recently (Dagley, 1969; Lowenstein, 1969; Spector, 1972; Srere, 1965; 1968; 1972; 1974; 1975; Srere and Singh, 1974). The extensive work on citrate lyase is summarised in this Chapter.

CITRATE LYASE (EC 4.1.3.6)

Occurrence

The bacterial sources in which citrate lyase (EC 4.1.3.6) has been detected are given in Table 1.

TABLE 1: SOURCES OF CITRATE LYASE

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

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

Although the fermentation of citrate by K.aerogenes (Deffner and Franke, 1939) and by Aerobacter indologenes (Brewer & Werkman, 1939) to oxaloacetate (OAA) and acetate was observed much earlier, the first characterisation of the enzyme was made by Dagley and Dawes (1953a) and by Gunsalus and his group (Gillespie and Gunsalus, 1953; Grunberg-Manago and Gunsalus, 1953). In all the sources reported hitherto, with the

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exception of S.diacetilactis, the enzyme occurs as a substrate-induced activity. Citrate lyase however is a constitutive enzyme in S.diacetilactis and is produced in this organism grown on a lactose medium without citrate. However, supplementation with citrate has been shown to enhance enzyme levels in the cell.

Amongst the anaerobic spore-formers, Clostridium sphenoides is characterised by its ability to grow on a medium containing citrate as the carbon and energy source and experiments with stereospecifically ^{14}C -labelled citrate indicated the involvement of citrate lyase in citrate degradation in this organism (Walther et al. 1977).

The anaerobic phototropic bacteria, Chlorobium thiosulfatophilum and Rhodospirillum rubrum, are known to synthesise α -keto acids from acyl-S-CoA esters by reductive carboxylation (Evans and Buchanan, 1965). Although citrate lyase is a key enzyme of the reductive carboxylic acid cycle postulated by Evans et al. (1966), no citrate lyase (or ATP-citrate lyase) activity has been found in extracts of these microorganisms (Beuscher and Gottschalk, 1972).

Citrate transport in bacteria

Evidence has been presented for the involvement of a membrane-bound OAA-decarboxylase as the citrate transport protein in K.aerogenes. That the citrate transport activity is induced only in the presence of substrate was first suggested by Davis (1956) from the earlier observation of a marked lag period before cells can utilise citrate (Dagley and Dawes, 1953a,b). The mediated transport system was first

investigated by Villarreal-Moguel and Ruiz-Herrera (1969) and evidence was presented for the membrane-associated nature of the induced carrier activity. Protein synthesis was shown to be necessary for induction of citrate transport and the carrier system was shown to be specific for citrate, not acting on the related compounds isocitrate and *cis*-aconitate. The transport was blocked by N-ethylmaleimide, dinitrofluorobenzene and uranyl nitrate. Cyanide inhibited citrate but not glucose uptake. Osmotically-shocked citrate-grown cells of K.aerogenes have been shown to have diminished citrate uptake activity which is restored on continued incubation with citrate in the medium but not in the presence of chloramphenicol (Wilkerson and Eagon, 1972).

The specific Na^+ requirement for citrate uptake by washed citrate-grown cells of K.aerogenes NCTC 418 was shown by Stern and Sachan (1970). Sachan and Stern (1971a) later proposed a model for citrate transport in the organism in which the membrane-bound Na^+ -requiring OAA-decarboxylase functions also as a carrier protein for citrate transport across the membrane. Citrate was shown to bind to OAA-decarboxylase and also to act as a competitive inhibitor in the decarboxylation reaction. Membrane vesicles prepared from K.aerogenes NCTC 418 which had been grown anaerobically on citrate as sole carbon source were used by Johnson et al. (1975) to study the transport phenomenon. These vesicles were free of citrate lyase activity and permitted citrate uptake to be studied without appreciable citrate catabolism subsequently. The

vesicles were shown to contain the Na^+ requiring OAA-decarboxylase and entry of citrate into them was also shown to require Na^+ specifically. (2R, 3R)-2-Fluorocitrate was not transported into the vesicles while (2S, 3S)-2-hydroxycitrate was. Citrate uptake was inhibited by all the four isomers of hydroxycitrate, fluorocitrate, cyanide, azide, dinitrophenol and sulphydryl reagents.

Marked physiological differences have been observed in the citrate uptake by different strains of K.aerogenes (Wilkerson and Eagon, 1974). While Na^+ was required by strain NCTC 418, Na^+ actually inhibited uptake of citrate by K.aerogenes UGa-1 and ATCC 12658 and this ion was without effect on citrate uptake by strain PRL-R3. Citrate uptake in K.aerogenes UGa-1 had earlier been shown to be through K^+ -dependent transport (Eagon and Wilkerson, 1972).

The transport of citrate in S.faecalis has been postulated to take place also via the OAA-decarboxylase since both the uptake and decarboxylation in this organism require Ca^{2+} and biotin (Sachan and Stern, 1971b).

Uptake of citrate by S.diacetilactis has also been shown to be mediated through an inducible transport system and to be an energy-requiring process (Harvey and Collins, 1962). Mutants of S.diacetilactis which had spontaneously lost the ability to ferment citrate were isolated by Collins and Harvey (1968). These were shown to be cryptic cit^- variants defective in transporting citrate across the cell membrane while still retaining citrate lyase activity. In recent work evidence has been presented to indicate that citrate utilisation is

probably plasmid-linked in this organism. The ability to utilise citrate was cured by the use of acridine orange and a 5.5 megadalton plasmid was found to be missing in the cit^- mutants. Cell-free extracts of cit^- mutants as well as toluene-treated whole cells showed citrate lyase activity, though lower than in the cit^+ cells. These results suggest that citrate transport is plasmid-dependent in S.diacetilactis (Kempler and McKay, 1979).

Citrate utilisation by E.coli occurs only when glucose is present in the medium and the cells are actively dividing, and the utilisation of citrate by resting cells with induced citrate lyase activity is negligible although cell extracts are highly active (Dagley and Dawes, 1953a,b). It has been suggested therefore that an active carrier for transport is not present in this organism (Gunsalus, 1958; Davis, 1956). Evidence has been presented recently that the ability to grow on citrate is plasmid-dependent in E.coli (Sata et al., 1978).

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

Role in citrate metabolism and Regulation of activity

In several bacteria the anaerobic metabolism of citrate is initiated by citrate lyase and the oxaloacetate formed in the lyase reaction is subsequently converted to pyruvate through the mediation of OAA-decarboxylase. This pathway, constituted by these two enzymes, has been termed as the "citrate fermentation pathway" (Dagley and Dawes, 1953a; O'Brien and Stern, 1969). Since citrate lyase and citrate synthase catalyse antagonistic reactions, diverse regulatory mechanisms have been detected in such organisms capable of synthesising both enzymes.

Regulation in K.aerogenas: K.aerogenes NCTC 418 grown under anaerobic conditions shows a specific Na^+ requirement for growth on citrate as energy source (O'Brien and Stern, 1969), which has been explained by the Na^+ -requirement of the OAA-decarboxylase of this organism (Stern, 1967). The citric acid cycle is inoperative in this microorganism under such anaerobic growth conditions due to repression of α -ketoglutarate dehydrogenase activity and citrate breakdown proceeds only through the fermentation pathway. For aerobic growth of this organism on citrate, Na^+ is not required and cells grown with or without Na^+ metabolise citrate through the citric acid cycle. All the enzymes of this pathway are operative under aerobic conditions while the enzymes of the fermentation pathway are absent (O'Brien, 1975a; Wilkerson and Eagon, 1972). Under intermediate conditions of controlled aeration, in which no oxygen tension is detected in the cultures, the metabolism

of citrate by the organism takes place through the citric acid cycle in the absence of Na^+ but mainly by the fermentation pathway in presence of Na^+ (O'Brien et al., 1969).

Besides the gross effects of growth conditions on the synthesis of the constituent enzymes of the two pathways, a more subtle regulation of citrate lyase activity has been recognised in more recent work. Such a mode of regulation was recognised only after the discovery that citrate lyase can exist in both an active form which carries essential, covalently-bound acetyl groups and an inactive deacetylated form (Buckel et al., 1971a). The enzyme undergoes inactivation through a process of deacetylation during the course of the reaction it catalyses (Singh and Srere, 1971; Srere et al., 1972). K.aerogenes has no detectable, distinct citrate lyase deacetylase (Kulla and Gottschalk, 1977). The organism however has a deacetyl citrate lyase ligase which catalyses reactivation of the inactive deacetyl enzyme to the active acetyl form in the presence of ATP and acetate (Schmellenkamp and Eggerer, 1974).

A series of interesting growth shift experiments have been carried out by Kulla and Gottschalk (1977) in order to understand the mode of regulation of citrate lyase and citrate synthase activities in K.aerogenes. In cells grown anaerobically on citrate the expected increase in citrate lyase activity was observed, after an initial lag period, until 12 h marking the end of the growth phase. Under in vivo conditions little or no inactive deacetyl enzyme could be detected. The inactive form was apparent only after a 15 h

period and was considerable only after about 24 h of incubation. Growth shift experiments were carried out in continuous culture, with ammonia as the limiting substrate, for studying the inactivation process. When cultures were shifted from "anaerobic citrate" to "aerobic citrate", citrate lyase synthesis was at very low rates and the active enzyme was slowly inactivated by deacetylation while citrate synthase synthesis was induced. During the shift from "anaerobic citrate" to "aerobic glucose", rapid inactivation of citrate lyase was observed and citrate synthase formation induced but at significantly lower levels than in aerobic growth on citrate. The organism had growth difficulties during transition from "anaerobic citrate" to "anaerobic glucose", presumably due to futile cycles between the fermentation pathway and citric acid cycle caused by the persistence of the citrate lyase activity and the induction of citrate synthase after the shift. This was apparent from a marked, but transient, drop in cell-count. The stress was evidently caused by depletion of glutamate since supplementation with 2-oxoglutarate to cultures shifted from citrate to glucose, both under anaerobic conditions, showed less pronounced decrease in the cell population.

It was also apparent that citrate lyase inactivation occurs only under aerobic conditions. No deacetylating enzyme could be detected however in cells initially grown aerobically with citrate. It was also shown that oxygen was not directly involved in inactivation since cells grown anaerobically on glucose in presence of nitrate also showed appreciable citrate lyase inactivation. Cells grown in batch cultures

anaerobically with citrate when transferred to "aerobic glucose" conditions showed complete inactivation of citrate lyase activity in 90 min. This inactivation could be delayed with chloramphenicol and completely prevented by 2,4-dinitrophenol. It was concluded from these experiments that in K.aerogenes citrate lyase inactivation is energy dependent as observed in the case of some other enzyme systems, such as, nitrate reductases of E.aerogenes, Proteus mirabilis and E.coli (de Groot and Stouthamer, 1969; 1970a,b,c; Oltmann et al., 1976; Showe and De Moss, 1968; van'T Riet et al., 1968) and aspartate transcarbamylase of B.subtilis (Waindle and Switzer, 1973).

Regulation in R.gelatinosa: R.gelatinosa is amongst the few phototropic bacteria which grow readily on citrate. Regulation of the two antagonistic pathways in this organism is through covalent modification. Unlike in K.aerogenes, a distinct enzyme citrate lyase deacetylase has been reported in this organism (Giffhorn et al., 1972). The deacetylase is activated after exhaustion of citrate in the culture medium and inactivates citrate lyase to a deacetyl form. The deacetylase is specific for the citrate lyase from R.gelatinosa and is inactive towards the enzymes from K.aerogenes and S.diacetilactis. The deacetylase is strongly inhibited by L-glutamate, evidently being regulated by the glutamate pool (Giffhorn and Gottschalk, 1975b). The deacetylase is rendered inactive until the glutamate levels drop after citrate, its precursor in the medium, is used up. With the depletion of citrate, the deacetylase is activated and, in turn, this enzyme inactivates citrate lyase. A citrate lyase ligase for the activation of

deacetyl citrate lyase is also present in this organism. The ligase is active only in the presence of light, citrate and anaerobic conditions (Giffhorn and Gottschalk, 1975a). With depletion of citrate the ligase is rapidly inactivated (Antranikian et al., 1978). Thus the regulation of citrate lyase in this organism involves two enzymes, citrate lyase deacetylase and citrate lyase ligase. The former enzyme is regulated by L-glutamate levels. This leads to the inactivation of citrate lyase after depletion of citrate. The ligase is also regulated by citrate levels but in this case being inactivated when citrate levels are depleted.

Regulation in other organisms: The regulation of citrate lyase in other organisms has not been studied in much detail.

In Aerobacter cloacae, growth on citrate under both aerobic and anaerobic conditions is unaffected by the presence or absence of Na^+ . Under anaerobic conditions of growth, the fermentation pathway was utilised for metabolism of citrate and α -ketoglutarate dehydrogenase was repressed. Aerobic conditions resulted in a repression of citrate lyase and the induction of α -ketoglutarate dehydrogenase. Na^+ did not cause repression of α -ketoglutarate dehydrogenase as in case of K.aerogenes (O'Brien and Geisler, 1974). Under controlled aeration maintaining a zero oxygen tension, citrate lyase was induced but the OAA-decarboxylase activity was low and therefore the fermentation pathway was mainly inoperative beyond the citrate lyase reaction. This resulted in considerable energy loss and consequent lowering in growth yield (O'Brien, 1975b). Part of the citrate was metabolised

through the citric acid cycle and both pathways were functional under such conditions of controlled aeration.

Na^+ is specifically required for the aerobic growth of S.typhimurium on citrate and these cells possess the enzymes of both the citric acid cycle and the fermentation pathway. It is probable that both pathways operate but their mode of regulation is not known (O'Brien et al., 1969). In case of the lactic acid bacteria, S.diacetilactis and L.citrovorum, citrate synthase is absent and there is no necessity for regulation of the pathways open for citrate metabolism which proceeds via the fermentation pathway alone (Giffhorn and Gottschalk, 1975a).

PURIFICATION

Citrate lyase has been obtained in a homogeneous form from the following sources: K.aerogenes, S.diacetilactis, R.gelatinosa and S.faecalis. The purification procedures for the enzyme from these sources are described briefly.

(i) Citrate lyase from K.aerogenes:

The enzyme from K.aerogenes was the first to be purified and was obtained homogeneous by SivaRaman (1961). The purification procedure involved precipitation of nucleic acids from the cell-free extracts by addition of streptomycin sulfate, adsorption of enzyme on alumina- C_γ gel, elution from the gel, ion-exchange chromatography on DEAE-cellulose and ammonium sulfate fractionation. Modifications of this procedure in later work make use of a final gel-filtration through Sephadex G-200 (Mahadik and SivaRaman, 1968) or Bio-gel A 1.5m (Singh and Srere, 1971) or replacement of the alumina C_γ gel and

DEAE-cellulose steps by salmine sulfate treatment and CM-cellulose chromatography (Bowen and Mortimer, 1971). Analytical ultracentrifugation (SivaRaman, 1961) and electrophoresis in free solutions (Bowen and Rogers, 1963a) as well as in polyacrylamide gels (Singh and Srere, 1971) was used to establish homogeneity. The pure preparation has an absorbance index $E_1^{1\%}$ 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).

A recent modification of the purification procedure reported to be suitable for large scale isolation involves precipitation of the alumina C_1 gel eluate with ethyleneimine polymer (polymin HS), extraction of the precipitate, dialysis and filtration through a Sephadex G-200 column (Dimroth et al., 1977b).

(ii) Citrate lyase from *S.diacetilactis*:

The enzyme has been obtained pure from this source by Singh and Srere (1975) and Kummel et al. (1975). The purification procedure of Singh and Srere involves treatment with protamine sulfate, fractionation with ammonium sulfate followed by gel-filtration through Bio-gel A 0.5m and ion-exchange chromatography on DEAE-cellulose. The enzyme concentrated by ultrafiltration had a specific activity of $200 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at pH 7.4, 25°C and 10 mM MgCl_2 . The preparation was homogeneous when studied by sedimentation velocity and sedimentation equilibrium experiments. Kummel et al. employed cetyltrimethylammonium bromide treatment, alumina C_1 gel addition, followed by ultrafiltration of the

supernatant and two successive gel-filtration steps on Sepharose 6B and DEAE-Sephadex A-50 chromatography. Homogeneity was established by analytical ultracentrifugation and polyacrylamide gel electrophoresis. The enzyme 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 . The citrate lyase ligase activity, which was earlier believed to be part of the S.diacetilactis enzyme complex (Kümmel et al., 1975) was separated from the lyase activity by Bowien and Gottschalk (1977).

(iii) Citrate lyase from R.gelatinosa:

The purification of citrate lyase from this organism has been reported by Beuscher et al. (1974) and the procedure involves protamine sulfate treatment, DEAE-cellulose chromatography and two gel-filtrations through Sepharose 6B columns. The homogeneity of the preparation was established by sedimentation equilibrium studies and by polyacrylamide gel electrophoresis. The preparation so obtained 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 enzyme has recently been crystallised by Giffhorn and Gottschalk (1978) by a procedure involving cetyltrimethylammonium bromide treatment followed by addition of alumina C γ gel and DEAE-cellulose chromatography. The fractions obtained from the column were concentrated by ultrafiltration. The crystals of citrate lyase so obtained were dissolved and recrystallised in presence of 0.1 M KCl. The crystals were dissolved in 3 M ammonium sulfate and the citrate lyase enzyme had a specific activity of $360 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, three times higher than that of the preparation obtained

by the earlier procedure.

(iv) Citrate lyase from *S.faecalis*:

Hiremath et al. (1976) have purified the enzyme from this source by a procedure which involves treatment of the cell-free extracts with ATP and acetate followed by removal of nucleic acids by streptomycin sulfate addition, adsorption and elution of the enzyme from alumina C_Y gel, ammonium sulfate fractionation, DEAE-cellulose chromatography and a final gel-filtration through Sepharose CL-6B. The homogeneity of the enzyme was checked by 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 .

Citrate lyase from *E.coli*: Citrate lyase from *E.coli* has been purified only partially (Dagley and Dawes, 1955; Wheat and Ajl, 1955a; Bowen and SivaRaman, 1960).

PROPERTIES

Stability:

Partially purified citrate lyase from *E.coli* is highly unstable in dilute solutions (Wheat and Ajl, 1955a) and loss in enzyme activity occurs on exhaustive dialysis. In crude extracts, the enzyme from *K.aerogenes* is comparatively unstable and inactivation occurs as a result of deacetylation of the enzyme to the HS- form. The crude extracts lose 70% of their activity in 4 days at -20°C (Dimroth and Eggerer, 1975a). Bowen and Rogers (1963a) reported that the pure enzyme is less stable in dilute solutions. 20% activity is lost at 20°C in

5.5 h at 0.3% protein concentration. At 0.04% protein concentration the enzyme loses all activity in 2 h at 20°C. The pure enzyme undergoes rapid inactivation at 55°C but the crude extract is more stable at this temperature. Cysteine does not protect the enzyme from the inactivation.

It was observed that presence of Mg^{2+} decreased inactivation in dilute solutions (Eisenthal et al., 1966). Two types of processes were shown to occur during the inactivation, one a reversible and the other an irreversible one (Blair et al., 1967). Increase in metal concentration (2.5 mM) can partly reverse the inactivation which occurs at low Mg^{2+} concentrations (0.025 - 1.0 mM). At higher protein concentrations the inactivation is mainly by the reversible process and higher Mg^{2+} concentrations retard both types of inactivation processes. Adenine nucleotides and EDTA chelate Mg^{2+} and consequently increase inactivation. Blair et al. (1967) have suggested that the ability of ATP to chelate metal ions and thus to inactivate citrate lyase may form the basis for regulation of citrate lyase activity in the cell. Ca^{2+} , although unable to activate citrate lyase, can protect the enzyme from inactivation in dilute solutions (Blair et al., 1967). When the partially purified enzyme from K.aerogenes was stored freeze-dried in presence of bovine serum albumin, sucrose and $MgSO_4$, the loss in activity after a month was only 10% (Gruber and Moellering, 1966).

It was shown that an acetyl group, linked by a thioester linkage to a sulfhydryl group in the enzyme, was essential for enzyme activity (Buckel et al., 1971a). The irreversible

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loss in enzyme activity on storage was shown to be a direct result of spontaneous removal of acetate from the active acetyl enzyme. Thus loss in radioactivity of ^{14}C -acetyl labelled enzyme on storage was accompanied by concomitant loss in enzymic activity. Chemical reactivation of the deacetylated enzyme could be achieved by treatment with acetic anhydride (Buckel et al., 1971a) or enzymically by citrate lyase ligase in presence of acetate and ATP (Schmellenkamp and Eggerer, 1974).

During the purification of citrate lyase from R.gelatinosa, the enzyme was present mainly in the deacetylated and oxidised form. Enzyme was activated in such preparations by reduction with DTT followed by acetylation with acetic anhydride. The crystalline enzyme obtained by Giffhorn and Gottschalk (1978) was stable at 4°C for 8 months in presence of 3 M ammonium sulfate, showing no detectable loss in activity during such storage.

The partially purified enzyme from S.diacetilactis after ammonium sulfate fractionation rapidly lost activity overnight, but the pure enzyme was stable to storage at -90°C for several weeks. The enzyme lost activity above pH 8.1 and at pH 8.7 dissociation of the enzyme was shown to occur (Singh and Srere, 1975). Pure citrate lyase from S.faecalis was stable to storage at 0°C or -20°C in presence of ammonium sulfate (Hiremath et al., 1976). In absence of ammonium sulfate, stability was decreased. Repeated freezing and thawing of the enzyme resulted in complete inactivation with formation of artifacts representing a half molecule as well as dimeric and trimeric species.

Metal requirement :

Citrate lyases show an absolute requirement for a divalent metal ion such as Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+} or Co^{2+} for the cleavage of citrate (Dagley and Dawes, 1955a). Ca^{2+} , Sr^{2+} , Ba^{2+} , Cu^{2+} and Hg^{2+} cannot substitute for these in E.coli and K.aerogenes. The E.coli enzyme activation in presence of Mn^{2+} is greater than for the K.aerogenes enzyme. Excess of Mg^{2+} causes inhibition of enzyme activity in S.diacetilactis (Harvey and Collins, 1963). It has also been shown that Ca^{2+} is a competitive inhibitor of the enzyme activation by Mg^{2+} , Mn^{2+} and other metals in case of citrate lyases from E.coli, K.aerogenes and S.diacetilactis (Dagley and Dawes, 1955; Harvey and Collins, 1963). It was suggested that citrate lyase acts on a metal-citrate complex (Dagley and Dawes, 1955; Harvey and Collins, 1963) since the pH optimum of the enzymes from K.aerogenes and E.coli corresponds to the stability range for the metal-citrate complex. Harvey and Collins (1963) further observed that in the S.diacetilactis enzyme, Mg^{2+} and citrate in equimolar amounts gave maximum activity. On the contrary, nuclear magnetic resonance studies on partially purified S.diacetilactis enzyme were indicative of a metal-enzyme acting on free citrate (Ward and Srere, 1965). The K_d of the Mn^{2+} -enzyme complex was calculated to be $4 \times 10^{-5}M$. On the addition of citrate to this system no ternary complex formation was observed and this was suggestive of a metal-enzyme acting on citrate. It was recognised, however, that the failure to detect the ternary complex could also be due to the low steady-state concentration of the complex. In

these studies Ca^{2+} was shown to compete with Mn^{2+} for the metal-binding site of the enzyme from S.diacetilactis.

It has been observed by Dagley and Dawes (1955) that the activating divalent metal ions have a restricted ionic radius of $0.72 \pm 0.08 \text{ \AA}$ while those which do not activate the enzyme have ionic radii outside this range, for e.g. Ca^{2+} has an ionic radius of $0.99 \text{ \AA}$. In case of R.gelatinosa enzyme, divalent metal activation was in the following order of effectiveness, Mg^{2+} , Mn^{2+} and Co^{2+} . Zn^{2+} and Ni^{2+} showed slight activation while Cu^{2+} and Fe^{2+} did not activate the enzyme (Beuscher et al., 1974). Citrate lyase from S.faecalis showed maximum activity with Mg^{2+} followed by Mn^{2+} , Zn^{2+} and Fe^{2+} in decreasing order of effectiveness. Only feeble activity was shown in presence of Co^{2+} and Cu^{2+} and no activity was detected in presence of Ca^{2+} , Ba^{2+} , Hg^{2+} and Ni^{2+} (Hiremath, 1977). The mechanism of action of the enzyme, which is discussed in detail later, involves two separate steps; a transferase reaction which results in the formation of a citryl-enzyme intermediate and release of acetate followed by a lyase reaction resulting in the cleavage of the citryl intermediate to OAA and regeneration of the acetyl enzyme. The requirement of metal has been shown to be only for the lyase and not for the transferase reaction (Buckel et al., 1973; Dimroth and Eggerer, 1975b).

pH optimum:

The enzymes from E.coli (Dagley and Dawes, 1955; Wheat and Ajl, 1955a), K.aerogenes (Wheat and Ajl, 1955b) and S.faecalis (Hiremath et al., 1976) show a pH optimum around 8.0,

while the citrate lyases from S.diacetilactis and R.gelatinosa (Harvey and Collins, 1963; Beuscher et al., 1974) have a pH optimum of 7.0 to 7.3. The enzyme from S.diacetilactis has been reported to dissociate at pH values above 8.1 (Singh and Srere, 1975).

Reaction kinetics:

Stoichiometric studies 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) indicated that one mole of citrate is cleaved to one mole each of oxaloacetate and acetate. Non-participation of CoA in the reaction was established by carrying out the reaction with extracts treated with Dowex-1 to remove any CoA present. Citrate lyase is now known to be a multienzyme complex with 2 distinct enzyme activities and built in acetyl-ACP subunits (Dimroth and Eggerer, 1975b). In the overall reaction an initial acyl exchange is catalyzed by the transferase subunit to give a citryl-ACP intermediate with release of acetate and the intermediate is subsequently cleaved to yield OAA and the acetyl-enzyme in the presence of the lyase subunit. Further, the enzyme undergoes reaction-inactivation (Singh and Srere, 1971) presumably through the spontaneous hydrolysis of the unstable citryl-intermediate to citrate and the inactive HS-enzyme. Since these aspects were not evident at the time of the earlier studies on the kinetics of the enzyme catalysed reaction, the findings are of limited significance. Harvey and Collins (1963) first showed that the keto-isomer of OAA was formed during the course of the reaction in case of the

S.diacetilactis enzyme and these results were confirmed by Ward and Srere (1965). Tate and Datta (1964) extended these findings to the enzyme from K.aerogenes in studies of the reaction carried out at 2°C and pH 7.4 to slow down spontaneous equilibration of the isomers. Citrate lyase thus was shown to resemble citrate synthase (Englard, 1959) in that both the enzymes involve the keto-form of OAA in the reactions they catalyse.

The citrate lyase catalysed reaction under optimal conditions is almost exclusively in the direction of citrate cleavage and in presence of excess enzyme goes virtually to completion (Wheat and Ajl, 1955b; Bowen and Rogers, 1963b). The reversibility of the cleavage reaction has been demonstrated with citrate lyase from S.faecalis (Gillespie and Gunsalus, 1953) and K.aerogenes (Bowen and Rogers, 1963b) only through incorporation of ^{14}C -labelled acetate into citrate in the presence of OAA and Mg^{2+} .

Burton (1955) calculated the equilibrium constant for citrate cleavage to OAA and acetate to be 1 M. Although a number of studies have been made to determine the equilibrium constant of the enzyme-catalysed reaction (Smith et al., 1956; Harvey and Collins, 1963) they are all of doubtful significance due to a number of factors. Tate and Datta (1965) have discussed some of these difficulties and have pointed out that OAA in aqueous solution exists in the keto and enol forms, both of which complex with Mg^{2+} . Also, the commonly used phosphate and tris buffer systems interact with Mg^{2+} . Citrate also chelates to Mg^{2+} and the inhibition by OAA and reaction-

inactivation of the enzyme further complicates the determination of the equilibrium constant. The equilibrium constant K , $[\text{citrate}^{-3}]/[\text{OAA}_{\text{keto}}^{-2}].[acetate]$, is markedly affected by the concentration of the reactants and metal. The equilibrium constant obtained by Tate and Datta for citrate cleavage was $3.08 \pm 0.72 \text{ M}^{-1}$ at a limiting concentration of citrate (2 mM) and excess MgCl_2 at 25°C in presence of triethanolamine-HCl buffer, pH 8.4, which does not complex with Mg^{2+} .

More recently Guynn et al. (1973) have determined the equilibrium constant, K_{obs} , under physiological conditions and have obtained a value of $2.22 \pm 0.16 \text{ M}^{-1}$ at pH 7.0 in 0.025 M potassium phosphate buffer at 38°C and 10^{-3}M free Mg^{2+} . The K_{obs} value varied with Mg^{2+} concentration. At $(\text{Mg}^{2+})_{\text{free}} = 0$, the K_{obs} was $1.00 \pm 0.07 \text{ M}^{-1}$ and at $(\text{Mg}^{2+})_{\text{free}} = 10^{-2}\text{M}$, the K_{obs} was 9. Here the problem of OAA-inhibition was reduced by use of low concentration of substrate and the values obtained for the forward and reverse direction were in agreement.

Stereochemistry:

Although citric acid does not possess an asymmetric carbon atom, it behaves asymmetrically in reactions with chiral reagents like enzyme molecules (Ogston, 1948). In consequence the two $-\text{CH}_2\text{COOH}$ groups of citrate are non-equivalent and are distinguished by an enzyme molecule.

Citric acid is depicted as a pentane derivative in Fig. 1 a with the C-atoms in the backbone of the structure numbered 1 to 5. In this representation, C-3 constitutes a prochiral centre of citrate. In the perspective depiction in Fig. 1 b where the central $-\text{COOH}$ group is above the plane of

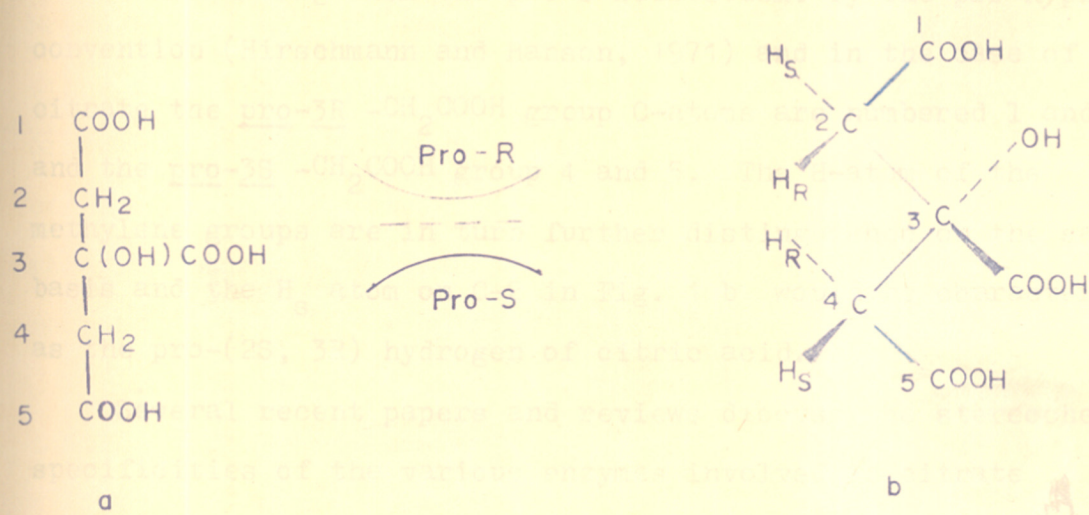


FIG. 1: DEPICTIONS OF CITRIC ACID

a - As a pentane derivative

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

(Adapted from Glusker and Srere, 1973)

the paper and the -OH group is below this plane and both are to the right of the backbone, the two $-\text{CH}_2\text{COOH}$ groups are distinguished as pro-3R ("upper" group) and pro-3S ("lower" group) by an extension of the R(rectus)/S(sinister) convention (Cahn et al., 1966). The pro-R substituent is distinguished by a lower numbering than the pro-S substituent by the pro-R>pro-S convention (Hirschmann and Hanson, 1971) and in the case of citrate the pro-3R $-\text{CH}_2\text{COOH}$ group C-atoms are numbered 1 and 2 and the pro-3S $-\text{CH}_2\text{COOH}$ group 4 and 5. The H-atom of the methylene groups are in turn further distinguished on the same basis and the H_S atom on C-2 in Fig. 1b would be characterised as the pro-(2S, 3R) hydrogen of citric acid.

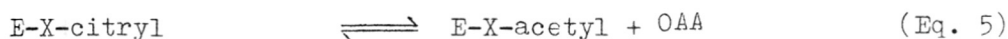
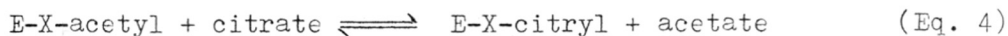
Several recent papers and reviews discuss the stereochemical specificities of the various enzymes involved in citrate synthesis and cleavage reactions (Eggerer et al., 1970; Retey et al., 1970; Lenz et al., 1971; Wunderwald et al., 1971; Srere, 1975). The main stereochemical features of these enzymes are described briefly here. In the reaction catalysed by si-citrate synthase, the acetyl moiety of acetyl-S-CoA attacks the si-face of OAA (Hanson and Rose, 1963) and the C-4 and C-5 of the product citrate are derived from the acetyl moiety. The stereochemistry of citrate lyases has been shown to resemble that of si-citrate synthase. Partially purified citrate lyase preparations from S.faecalis (Gillespie and Gunsalus, 1953) and E.coli (Wheat and Ajl, 1955b) have been shown to cleave citrate in a manner that the pro-3S $-\text{CH}_2\text{COOH}$ (C-4 and C-5) of the substrate which are derived from acetyl-CoA in the si-citrate synthase reaction, yield acetate

exclusively. A similar behaviour has been shown with purified citrate lyase from K.aerogenes in which only the (5-¹⁴C) (3S) citrate gives ¹⁴C-acetyl-citrate lyase complex while (1-¹⁴C) (3R)-citrate yields unlabelled citrate lyase (Buckel et al., 1971a). The pro-3S -CH₂COO⁻ group of citrate is eliminated as acetyl-CoA in case of ATP-citrate lyase also (Srere and Bhaduri, 1964). The only citrate enzyme which shows an opposite stereospecificity is the re-citrate synthase present in certain anaerobic bacteria. In this case the pro-3R -CH₂COO⁻ group of citrate, i.e. the C-1 and C-2, is contributed by the acetyl moiety of acetyl-CoA (Gottschalk and Barker, 1966; Stern and Bambers, 1966).

The stereospecificity of the citrate enzymes in regard to the two prochiral centres of citrate involving C-H bonds on C-2 and C-4 have been elegantly studied using chiral acetates, (R)-(²H, ³H)-acetate and (S)-(²H, ³H)-acetate. All the citrate enzymes, si-citrate synthase, re-citrate synthase, citrate lyase and ATP-citrate lyase were shown to produce inversion of configuration at the methyl group of the acetyl moiety (Eggerer et al., 1970; Klimman and Rose, 1971; Retey et al., 1970). Citrate lyase thus cleaves citrate with inversion of configuration at the methylene group (Buckel et al., 1971b).
Acetyl group of citrate lyase:

Labelling experiments carried out by Buckel et al. (1971a) showed the presence of an acetyl group at the catalytically active site of citrate lyase. This acetyl group was found to be linked covalently to the enzyme through a thioester linkage and the enzyme was found to be inactivated by deacetylation.

(5-¹⁴C)-(3S)-citrate when used as the substrate for citrate lyase resulted in the formation of non-radioactive OAA and a ¹⁴C-labelled enzyme. However reaction with (1-¹⁴C)-(3R)-citrate gave radioactive OAA and unlabelled enzyme. This was explained on the basis of turn-over of acetate during the course of the enzyme-catalysed reaction which is the sum total of two reactions, namely, an acyl exchange reaction (Eq. 4) and an acyl lyase reaction (Eq. 5), with the cleavage at the si-face of OAA.



The presence of an essential acetyl group in the enzyme was confirmed by further evidence. On treatment with hydroxylamine the enzyme was deacetylated and inactivated as indicated by the fact that ¹⁴C-labelled enzyme yields ¹⁴C-acetohydroxamic acid on such treatment. Inactivation of the labelled-enzyme on incubation with 1 mM OAA and 1 mM MgCl₂ also resulted in loss of the labelled acetyl group. Reaction of ¹⁴C-acetyl labelled citrate lyase with cold citrate, followed by separation of the products on a Sephadex column, resulted in a radioactive low molecular weight product and the unlabelled enzyme. This could be explained only on the basis of an acyl exchange during the course of the reaction.

Kinetics of hydroxylamine inactivation of the enzyme resembled the hydroxylaminolysis of standard thioesters like S-acetyl N-succinyl cysteamine. Mercaptans also caused inactivation of the enzyme. These findings indicated that

the acetyl moiety was present in thioester linkage in the enzyme.

Citrate lyase inactivated either by hydroxylamine treatment or by mercaptans could be chemically reactivated (Buckel et al., 1971a; Srere et al., 1972). A 65-70% recovery of enzyme activity has been reported on acetic anhydride treatment (4.7 mM) of citrate lyase inactivated by OAA in presence of Mg^{2+} (Buckel et al., 1971a). Srere et al. (1972) reported that the enzyme inactivated in presence of DTNB either by hydroxylamine or by reaction-inactivation could be reactivated by acetic anhydride only after prior incubation with DTT. Kummel et al. (1975) have also used 1-acetyl imidazole for chemical reactivation of the deacetylated citrate lyases obtained from several sources, viz. S.diacetilactis, K.aerogenes, R.gelatinosa and L.citrovorum.

The enzymic reactivation of the inactive HS-citrate lyase has been described by Schmellenkamp and Eggerer (1974). The enzyme, acetate: SH (acyl carrier protein) citrate lyase ligase (AMP) was partially purified from K.aerogenes and was shown to catalyse the acetylation of HS-citrate lyase in presence of acetate and ATP or acetyl adenylate. The ligase from S.faecalis was partially purified and separated from citrate lyase activity by Hiremath et al. (1976) and the enzyme was shown to be active with the HS-citrate lyases from both S.faecalis and K.aerogenes. The ligase was obtained electrophoretically pure from S.diacetilactis by Bowien and Gottschalk (1977) and in this case shown to be specific for the lyase from the same source, not acetylating the HS-citrate lyases from R.gelatinosa and K.aerogenes.

While HS-citrate lyase can be reactivated with acetic anhydride, the deacetyl enzyme after treatment with iodoacetate yields a carboxymethylated derivative which cannot be reactivated chemically. Both these species which are inactive towards citrate are, however, catalytically active in presence of acyl-CoA derivatives like acetyl-CoA or citryl-CoA (Buckel et al., 1973). Thus (3S)-citryl-CoA is cleaved to acetate and OAA in the presence of acetyl-CoA. Using ^{14}C -labelled substrate it was shown that the acetyl group of acetyl-CoA is liberated as acetate and the pro-(3S) $-\text{CH}_2\text{COOH}$ group of citrate is transformed to acetyl-CoA in the course of the acetyl-CoA dependent cleavage of citrate. Kinetic analysis of the reaction indicated the formation of a citryl-intermediate and this was substantiated by the isolation of citryl-CoA which accumulated in the presence of EDTA. The multienzyme nature of citrate lyase was evident from these studies involving an acyl exchange reaction which is not dependent on Mg^{2+} and a divalent metal dependent lyase reaction in which the citryl-intermediate is cleaved. Comparing the acetyl-CoA dependent cleavage of citrate by the deacetyl enzyme with the mode of action of native citrate lyase, Buckel et al. (1973) concluded that both require acetylated acyl group carriers (acetyl-enzyme in citrate lyase and acetyl-CoA in deacetyl citrate lyase).

Citrate lyases from S.diacetilactis (Singh and Srere, 1975), R.gelatinosa (Kümmel et al., 1975), S.faecalis (Hiremath et al., 1976), L.citrovorum (Kümmel et al., 1975) have also been shown to be acetyl-enzymes.

Product inhibition and reaction inactivation:

Dagley and Dawes (1955) first reported that the enzymes from E.coli and K.aerogenes are inhibited by OAA, a product of the reaction. Acetate and pyruvate were found not to inhibit the enzyme. Bowen and Rogers (1963a) showed that oxalate, succinate, oxaloglutarate, tartarate, oxalosuccinate and isocitrate also did not inactivate the enzyme, while malate like OAA caused irreversible inactivation of the enzyme. Inhibition by OAA was shown to be time and concentration dependent. No difference could be observed in the sedimentation behaviour and the absorption spectrum of the inactivated and native enzyme.

Eisenthal et al. (1966) reported a requirement of Mg^{2+} for inhibition of the enzyme from K.aerogenes by OAA (or its analogues) and this inhibition could not be reversed by NADH and MDH. The OAA inhibition was shown to be pH dependent and the profile of this dependence was suggestive of the involvement of the enolic-form of OAA, probably as two Mg^{2+} -enolic OAA complexes. This was supported by the fact that α,α -dimethyl-OAA, which cannot enolise, as well as ketomalonate do not inhibit the enzyme in presence of Mg^{2+} . However the derivatives of OAA and malonate which can yield enolic forms, could inhibit the enzyme. The inhibition by OAA was more pronounced in presence of Co^{2+} than with Mg^{2+} while Ca^{2+} was less effective than Mg^{2+} . On the basis of these findings, the authors postulated that the inhibitor acts at the active site and that the structural requirement for inhibition was a C_4 straight chain, 1,4-dicarboxylic acid with an ionisable α -hydroxyl group. As stated earlier, Buckel et al. (1971a) have shown

that the mechanism of OAA-inactivation is through the loss of the essential acetyl group of the enzyme to give inactive HS-citrate lyase.

Citrate lyase undergoes inactivation during the reaction it catalyses. The rapid, irreversible reaction-inactivation of the enzyme from K.aerogenes was termed as enzyme "suicide" by Singh and Srere (1971). Wheat and Ajl (1955b) had shown earlier that with partially purified enzyme from E.coli the rate of reaction drops rapidly to zero during the course of the cleavage. This was attributed to inhibition by OAA formed in the reaction. However, Singh and Srere (1971) observed that prior incubation with even a 1000-fold excess of OAA, (both keto and enol forms), above that produced during the reaction does not lead to the complete inactivation of the enzyme. No change in the rate of inactivation or the extent of reaction was observed even on constant removal of OAA formed by coupling the reaction with malate dehydrogenase and NADH. The reaction-inactivation was shown to be a simple first order process. The rate of inactivation was found to vary with the nature of the divalent metal. Amongst the divalent metals tested Zn^{2+} was shown to result in the slowest rate of inactivation (Singh and Srere, 1971; Singh and Srere, 1975). That reaction-inactivation was through a deacetylation process leading to the formation of HS-citrate lyase was established by chemical reactivation of the inactivated enzyme with acetic anhydride (Srere et al., 1972). Studies with the isolated subunits have indicated that the transferase (α -) subunit causes slow release of citrate through a hydrolysis of citryl-ACP and this

has been suggested as a possible cause for the observed reaction-inactivation of citrate lyase (Dimroth and Eggerer, 1975b).

Citrate lyases can be grouped under two types on the basis of their reaction-inactivation behaviour. The enzymes from K.aerogenes (Singh and Srere, 1971) and R.gelatinosa (Kümmel et al., 1975) undergo severe reaction-inactivation while the enzymes from S.diacetilactis (Singh and Srere, 1975), L.citrovorum (Kümmel et al., 1975) and S.faecalis (Hiremath et al., 1976) show only a weak reaction-inactivation. The inactivation of the enzymes from K.aerogenes, S.diacetilactis and S.faecalis was shown to follow first order reaction kinetics. The rate constant (K) for reaction-inactivation in case of the enzymes from K.aerogenes, S.diacetilactis (Singh and Srere, 1975) and S.faecalis (Hiremath, 1977) with Mg^{2+} at 25°C was found to be 1.21, 0.06 and 0.07 min^{-1} , respectively. In presence of Zn^{2+} the values of K were 0.17 min^{-1} and 0.014 min^{-1} for the K.aerogenes and S.diacetilactis enzymes, respectively.

The weak reaction-inactivation observed in case of the enzyme from S.diacetilactis and L.citrovorum was believed to be due to the association of a HS-citrate lyase acetylating activity with the native citrate lyase enzyme complex (Kümmel et al., 1975). Hiremath et al. (1976) have shown that the enzyme from S.faecalis undergoes only a weak reaction-inactivation although it contains no ligase activity associated as part of the enzyme complex. That this was also true of the S.diacetilactis enzyme was shown later by the separation of the contaminating ligase activity from the citrate lyase (Bowien and Gottschalk, 1977).

Inhibitor and substrate activities of citrate analogues:

An interesting study of the four isomers of hydroxycitrate as substrates and inhibitors of the three citrate enzymes has been reported recently (Sullivan et al., 1977). All four hydroxycitrates were shown to be linear competitive inhibitors for citrate in the cleavage reaction catalysed by citrate lyases from S.diacetilactis and K.aerogenes and in this the two enzymes showed differences in their behaviour. In case of the S.diacetilactis enzyme, (2R, 3R)-2-hydroxycitrate was the strongest inhibitor and the (2R, 3S)-isomer the weakest, while for the K.aerogenes enzyme the (2S, 3S)-isomer was the strongest and (2R, 3S)- the weakest. Determination of rate constants of inactivation by the process of deacetylation in the presence of citrate analogues indicated that in the presence of either Mg^{2+} or Zn^{2+} the (2S, 3R)-hydroxycitrate caused the fastest rate of inactivation while the (2S, 3S)-isomer caused the slowest in enzymes from both sources. A study of the mechanism of this inactivation was made using ^{14}C -acetyl-citrate lyases from both the organisms and incubating the labelled enzymes separately with citrate, tricarballoylate or the hydroxycitrates. Both citrate and the hydroxycitrate isomers caused a complete deacetylation of the enzymes to give free labelled acetate. Tricarballoylate caused only a partial deacetylation. These results indicated that inactivation with the tricarboxylic acids is due to loss of the essential acetyl groups of the enzyme. This was confirmed by the observation that the inactivated enzyme could be reactivated by citrate lyase ligase in the presence of acetate and ATP.

It was further observed that all the hydroxycitrate isomers, except (2S, 3R)-2-hydroxycitrate, could serve as substrates for citrate lyase. In presence of excess enzyme, complete cleavage occurred with the active substrates, probably to acetate and hydroxyoxaloacetate in the case of the (2R, 3R)-isomer and to oxaloacetate and glyoxalate in the case of the (2S, 3S)- and the (2R, 3S)-isomers. In these studies the cleavage was assayed by the malate dehydrogenase-coupled system, hydroxy-oxaloacetate presumably being a substrate for the malate dehydrogenase reaction as indicated by England and Siegel (1969). None of the isomers of hydroxycitrate were found to serve as substrate for citrate synthase. All the isomers except (2S, 3S)-2-hydroxycitrate could act as substrates for ATP-citrate lyase. (2S, 3S)-2-hydroxycitrate is a potent linear competitive inhibitor of ATP-citrate lyase (Watson et al., 1969).

Molecular weight of citrate lyase complex:

The pure enzyme from K.aerogenes was shown to have a $s_{20,w}^{\circ}$ value of 16 S (SivaRaman, 1961) and a molecular weight of 534 000 - 575 000 (Mahadik and SivaRaman, 1968; Bowen and Rogers, 1963a; Bowen and Mortimer, 1971; Carpenter et al., 1975; Singh et al., 1976). Pure citrate lyases from other sources have been shown to be of approximately the same size. The enzyme from S.diacetilactis has an $s_{20,w}^{\circ}$ value of 16.8 S and molecular weight of 585 000 (Singh and Srere, 1975). The enzyme from R.gelatinosa has been reported to have a molecular weight between 530 000 - 560 000 (Beuscher et al., 1974). The pure enzyme from S.faecalis has a $s_{20,w}^{\circ}$ value of 17.1 S and

a molecular weight of 600 000 (Hiremath, 1977).

Mahadik and SivaRaman (1968) reported that in presence of buffers of low ionic strength and in the absence of divalent metal ions, the K.aerogenes enzyme dissociates reversibly. In 2 mM EDTA + 1 mM potassium phosphate buffer, pH 7.4, a half molecule of 273 000 dalton sedimenting at 10.3 S was obtained while in presence of 1 mM EDTA + 1 mM potassium phosphate buffer, pH 7.4, a component sedimenting at 6.3 S was observed. Mg^{2+} (2mM) or buffer of higher ionic strength completely reversed this dissociation. These observations were confirmed by Bowen and Mortimer (1971) and Dimroth et al. (1973). In contrast the S.faecalis enzyme does not dissociate in buffers of low ionic strength even in the absence of Mg^{2+} (Hiremath, 1977).

Amino acid composition of holoenzyme:

The amino acid analysis of citrate lyase complex was reported first by Bowen and Rogers (1965) using high voltage paper electrophoresis for separation of the amino acids of the acid hydrolysate of the K.aerogenes enzyme. More recently Singh et al. (1976) have determined the composition of the enzyme from this source by the more precise ion exchange chromatography technique in an amino acid analyser and have shown significant differences in several residues as compared to the values reported earlier. They showed by end group analysis that methionine was the only residue at the NH_2 -terminus of the three subunits. This is ~~an~~ variance with the finding of Bowen and Mortimer (1971) who reported lysine to be the only NH_2 -terminal residue.

The amino acid composition of the holoenzyme from S.faecalis has been reported by Hiremath (1977). The enzyme from this source was shown to differ markedly from the K.aerogenes enzyme in amino acid composition. The calculated partial specific volume of the S.faecalis enzyme from the amino acid analysis data has been reported to be 0.742 ml.g^{-1} .

Immunological behaviour:

Singh and Srere (1975) reported that the S.diacetilactis enzyme showed immunological differences from the enzyme of K.aerogenes and E.coli. The enzymes from S.diacetilactis and E.coli did not cross-react with rabbit antisera against pure citrate lyase from K.aerogenes in double diffusion experiments. The rabbit antiserum against purified S.diacetilactis enzyme gave precipitin bands only with its own antigen.

Subunits of citrate lyase:

All citrate lyases isolated till now have been shown to contain three non-identical subunits. The enzyme from K.aerogenes was earlier reported to be composed of 8 identical subunits of molecular weight 74 000 (Mahadik and SivaRaman, 1968) from ultracentrifugal analysis under denaturing conditions but this was shown to be due to the omission of β -mercapto-ethanol from the denaturing system (Bowen and Mortimer, 1971). Bowen and Mortimer (1971) also using ultracentrifugal technique concluded that the enzyme consists of 16 identical subunits of 34 000 daltons each, although SDS gel electrophoresis indicated the presence of components of about 55 000 and 35 000 daltons. The presence of a small subunit of 10 000 daltons functioning as an acyl carrier protein (ACP) was first reported by

Dimroth et al. (1973) for the enzyme from K.aerogenes. Using SDS-polyacrylamide gel electrophoretic techniques, Singh et al. (1974) showed the presence of two more subunits of 55 800 and 33 800 daltons in addition to the ACP (11 900 daltons). More precise determinations of the molecular weights of the separated subunits was carried out by Carpenter et al. (1975) who obtained values of 54 500 (α), 32 000 (β) and 11 000 (γ) for the three subunits by sedimentation equilibrium in 6 M guanidine. HCl, SDS-polyacrylamide gel electrophoresis and gel-filtration through 6% agarose in 6 M guanidine.HCl. These results were confirmed by Dimroth and Eggerer (1975a) who obtained values of 54 000, 32 000 and 10 000. The molecular weight of γ -subunit (ACP) determined from its amino acid sequence analysis has been found to be 9378 (Beyreuther et al., 1978).

The citrate lyases from other sources also contain three non-identical subunits of approximately similar sizes. The subunits of the enzyme from S.diacetilactis have been shown to have molecular weights of 54 000, 35 000 and 12 000 (Singh and Srere, 1975). The enzyme from S.faecalis has subunits of 54 000, 37 000 and 14 000 daltons (Hiremath, 1977). The enzyme from R.gelatinosa which was earlier believed to have only two non-identical subunits (Beuscher et al., 1974) has now been shown to conform with the general structure (Giffhorn and Gottschalk, 1978), having subunits of 55 600, 31 600 and 11 400 when dissociated under more drastic conditions (2% SDS in place of 1% SDS). All subunit molecular weights in these enzymes were determined by SDS-polyacrylamide gel electrophoresis. The smallest subunit in all cases have been shown to carry the

prosthetic group and to function as ACPs.

Subunit stoichiometry:

Several approaches have been used for determining subunit stoichiometry in citrate lyases. Data obtained from SDS-polyacrylamide gel electrophoresis in which the separated subunits of citrate lyase from K.aerogenes were stained with coomassie blue and estimated by gel scans, showed that the α - and β -subunits are present in equimolar amounts with six copies of each in the complex (Dimroth and Eggerer, 1975a; Carpenter et al., 1975). The estimation of the number of copies of the γ -subunit from such data posed problems since varying values were obtained depending on the length of development and the staining procedure (Dimroth and Eggerer, 1975a). The difficulty was due, apparently, to loss of the small polypeptide chain by diffusion from the gel into the medium. Gel scans indicated molar composition of approximately 1:1:0.8 for α -, β - and γ -subunits (Dimroth and Eggerer, 1975a, Carpenter et al., 1975). All three subunits were assumed to be in equimolar quantities when allowances are made for losses of the γ -subunit estimated by gel scanning. Attempts to estimate γ -subunit content by chemical and microbiological assays of the pantothenate component of the prosthetic group which is associated with this subunit indicated values of 3-5 ACP's (γ -subunit)/mol of the complex (Srere et al., 1972; Dimroth, 1975). Singh et al. (1975) estimated 3-4 mol pantothenate/mol enzyme from studies with ^{14}C -labelled pantothenate and a pantothenate requiring mutant of K.aerogenes. These authors also established that the pantothenate and the acetate are associated solely with the ACP

of the citrate lyase by analysis of double-labelled enzyme in which ^3H -acetate was incorporated into the ^{14}C -pantothenate-containing enzyme. That the holoenzyme consists of six copies of each of the subunits was established by a novel amino acid sequencing technique (Singh et al., 1976). After sequencing the individual subunits to 10 residues from the NH_2 -terminus, the holoenzyme was studied. The ratios of amino acids released at various positions were quantitated and shown to be 1:1:1 indicating a similar ratio of the three subunits present in the complex. Singh et al. (1976) have suggested that two of the six ACP's probably lack the prosthetic group since all assays of prosthetic group components have indicated approximately four prosthetic groups per mol of the enzyme.

Subunit stoichiometry of S. faecalis citrate lyase determined by gel scans of coomassie blue stained SDS-polyacrylamide gels have also indicated molar ratios of α -, β - and γ -subunits of 1:1:0.7 like the K. aerogenes enzyme (Hiremath, 1977). Pantothenate assays in both S. faecalis (Hiremath, 1977) and S. diacetylactis (Singh and Srere, 1975) yield 4 mol pantothenate in the holoenzyme molecule. Chemical analysis of the pantothenate components also showed four prosthetic groups per mol holoenzyme of S. faecalis (Hiremath, 1977).

Preliminary electron microscopic studies on the enzymes from R. gelatinosa (Beuscher et al., 1974) and K. aerogenes (Dimroth and Eggerer, 1975a) have been reported to indicate a hexameric structure.

Amino acid composition of subunits:

Singh et al. (1976) determined the amino acid compositions of the α -subunit (M_r 54 000) and β -subunit (M_r 32 000) of the enzyme from K.aerogenes separated by gel-filtration through agarose beads after dissociation in 6 M guanidine.HCl. They also reported the partial amino acid sequence for the ten residues from the amino terminus of each of the three subunits. All the three subunits had methionine at the NH_2 -terminus. The sequence of the β -subunit indicated microheterogeneity, equal amounts of proline and arginine being detected at position 4. Hiremath (1977) reported the amino acid compositions of the α -subunit (M_r 54 000) and β -subunit (M_r 37 000) from S.faecalis and showed marked differences in the amino acid contents of both subunits as compared to those of the corresponding subunits of the K.aerogenes enzyme.

Amino acid analysis of acid hydrolysates of the separated ACP (r -) subunit showed the presence of one cysteine and one cysteamine residue per mol of r -subunit (ACP) of the K.aerogenes enzyme (Dimroth et al., 1973). The cysteine and cysteamine were found qualitatively to be acylated in the ACP subunit. This was established by treatment of the ACP subunit with cold iodoacetate, followed by deacylation with hydroxylamine and treatment now with iodo($2-^{14}C$)acetate. The ^{14}C -labelled ACP was then acid hydrolysed and the carboxymethylated products carrying the ^{14}C -label were separated and their identity established as S-carboxymethyl cysteine and S-carboxymethyl cysteamine. Of the two sulfhydryls only the SH-group of cysteamine residue was found

to be biologically active (Dimroth and Eggerer, 1975a). This was shown by specific alkylation of the prosthetic cysteamine group and not of the cysteine in the HS-enzyme by affinity labelling with low concentration (0.1 mM) of iodoacetate in presence of excess dithioerythritol (10-20 mM). The cysteamine-S-carboxymethyl deacetyl citrate lyase could not be reactivated even by treatment with acetic anhydride, indirectly indicating that the prosthetic group cysteamine and not the cysteine residue carried the essential acetyl group.

The analysis of the complete amino acid composition of the ACP subunit determined for the citrate lyase enzyme of K.aerogenes indicated excess of acidic amino acid residues and one residue each of cysteamine and β -alanine (Dimroth et al., 1975). Beyreuther et al. (1978) have reported the complete amino acid sequence of the ACP subunit by automated sequential Edman degradations. The ACP is composed of 78 residues and lysine and histidine are lacking. Circular dichroism studies indicated that the ACP has a high degree of α -helical structure. Hiremath (1977) has reported significant differences in the amino acid compositions of the ACPs of K.aerogenes and S.faecalis enzymes, a marked difference being the presence of 4 histidine residues in the ACP subunit of S.faecalis enzyme while that from the K.aerogenes enzyme has none. However, the ACP from this source also was shown to contain a single cysteine as an S-acylated residue.

The ACPs of citrate lyase from K.aerogenes and fatty acid synthetase from E.coli have similar function and molecular weight (Dimroth et al., 1973; Vagelos, 1973).

However, they differ in their amino acid compositions and in the nature of their prosthetic groups. The fatty acid synthetase ACP prosthetic group contains only 4'-phosphopantetheine while the lyase ACP contains besides phosphopantetheine, adenine, phosphate and sugar residues. It was observed that an acyl carrier protein from crude extracts of K.aerogenes could replace the ACP of E.coli fatty acid synthetase in the malonyl-CoA/CO₂ exchange reaction. However, the isolated deacetylated ACP of citrate lyase from the same organism could not substitute for the ACP of fatty acid synthetase (Dimroth et al., 1973). This indicated the presence of two different ACPs in K.aerogenes, one being specific for citrate lyase and the other for the fatty acid synthetase. The two ACPs of fatty acid synthetase in E.coli and K.aerogenes were interchangeable and were probably similar but the ACP for citrate lyase was distinct. This was also established with tryptic digests of carboxymethylated ACP's of citrate lyase (K.aerogenes) and fatty acid synthetase (E.coli). The two were very different and their tryptic digests had no common peptides as shown by fingerprint analysis (Dimroth, 1975).

CITRATE LYASE PROSTHETIC GROUP

The first evidence for citrate lyase being a pantothenate-containing enzyme was presented by Srere et al. (1972). Pantothenate was determined microbiologically in alkaline hydrolysates of citrate lyase from K.aerogenes which had been treated with alkaline phosphatase (EC 3.1.3.1). Since no

pantothenate could be assayed without alkaline phosphatase treatment and from an analogy with the fatty acid synthetase system, it was assumed that the enzyme carried a covalently bound 4'-phosphopantetheine and that this moiety carried the essential acetyl group in thioester linkage. The association of the 4'-phosphopantetheine with the 10 000 dalton subunit in K.aerogenes was established by Dimroth et al. (1973), thus characterising this as the acyl carrier protein (ACP).

The prosthetic group of citrate lyase was shown in later work to be more complex than the 4'-phosphopantetheine of a fatty acid synthetase and was found to contain adenine phosphate, sugar, cysteamine, β -alanine and pantoic acid (Dimroth, 1975; 1976).

From analysis of products of chemical and enzymic degradation, Robinson et al. (1976) proposed the structure 3'(or 2') $\rightarrow$ 1''-(5''-phosphoribosyl) dephospho-CoA for the prosthetic group. From NMR and mass spectrometry studies the prosthetic group was assigned the structure (1'' $\rightarrow$ 2')-(5''-phosphoribosyl) dephospho-CoA (Fig. 2) (Oppenheimer et al., 1979).

The prosthetic group of K.aerogenes was shown to be linked to serine-14 of the ACP (γ -subunit) from a characterisation of the products obtained after β -elimination of the prosthetic group in 0.1 N NaOH at 65°C followed by NaB^3H_4 reduction of the dehydroalanine in the apo-ACP to (^3H)-alanine (Beyreuther et al., 1978).

Robinson et al. (1976) observed that the prosthetic group obtained after pronase digestion of citrate lyase from

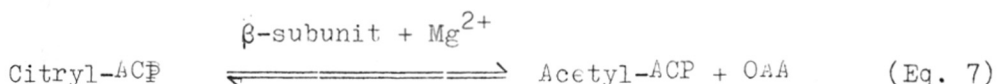
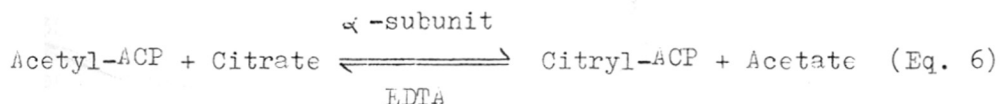
K.aerogenes could act as substrate for pure pig heart citrate si-synthase (EC 4.1.3.7); pure rat liver ATP-citrate lyase (EC 4.1.3.8) and for acetate: HS-citrate lyase ligase (AMP) from K.aerogenes. The citrate lyase prosthetic group is the first compound which has been shown to substitute for CoA in the ATP-citrate lyase and si-citrate synthase catalysed reactions. Similarly acetyl-CoA substitutes for its prosthetic group in reactions catalysed by HS-citrate lyase (Buckel et al., 1973) or its separated α -subunit (Dimroth et al., 1977b). This would establish a close similarity in the mechanism of action of the three groups of enzymes that catalyse the cleavage or formation of citrate by a lyase reaction.

Dimroth and Loyal (1977) have shown that the prosthetic group of citramalate lyase from Clostridium tetanomorphum is similar to that of citrate lyase from K.aerogenes. Citramalate lyase has the same type of structural organisation and a similar mechanism of action as citrate lyase (Buckel and Bobi, 1976).

CITRATE LYASE- A MULTISUBUNIT COMPLEX

The active citrate lyase was reconstituted from its isolated α - β - and γ -subunits by Dimroth and Eggerer (1975b). It was shown that citrate cleavage could occur only in the presence of all three subunits and the reconstituted enzyme was identical to the native enzyme complex in its sedimentation behaviour and specific activity. The 54 000 daltons α -subunit catalysed the formation of (1,5-¹⁴C) citryl-ACP and acetate from the 10 000 daltons acetyl-ACP (γ -)subunit in presence of (1,5-¹⁴C) citrate.

This established the acyl transferase function of the α -subunit and the transferase reaction was shown to require no metal ion for activity. The catalytically active form of the α -subunit was found to be a dimer of the 54 000 dalton polypeptide chain (Dimroth and Eggerer, 1975b). The (1,5- ^{14}C)-citryl-ACP when incubated with the 32 000 dalton β -subunit in presence of Mg^{2+} resulted in formation of (4- ^{14}C) OAA with the liberation of the acetylated ACP, demonstrating the lyase function of the β -subunit in citryl-ACP cleavage. These findings confirmed the earlier postulate of Buckel *et al.* (1973) and of Singh *et al.* (1974) that the citrate lyase from K.aerogenes is a multienzyme complex composed of an acyl carrier protein and two enzymes, an acyl transferase and a lyase which together catalyse the reactions below (Eq. 6 and 7) to give an overall dissimilation of citrate.



Citramalate lyase from C.tetanomorphum resembles the citrate lyase complex of K.aerogenes in its molecular weight, subunit composition, mechanism of action and also in the functions of its individual subunits (Buckel and Bobi, 1976). The close relationship between the two enzymes from the two distinct microorganisms was confirmed by cross-reactions between the isolated subunits of the two enzyme complexes,

resulting in the formation of a hybrid enzyme complex with acetyl transferase (α -subunit, M_r 54 000) and the lyase (β -subunit, M_r 32 000) subunits contributed by the citrate lyase of K.aerogenes and the ACP from the citramalate lyase of C.tetanomorphum (Dimroth et al., 1977a). Other hybrid combinations of heterologous subunits did not show catalytic activity.

Mechanism of action:

The isolated transferase (α -)subunit (M_r 54 000), the largest subunit from the citrate lyase of K.aerogenes, has been shown also to be an acetyl-CoA: citrate-CoA transferase (Dimroth et al., 1977b). The transferase subunit also catalyses an exchange of acetyl residues between acetyl-ACP or acetyl-CoA and acetate as a citrate independent reaction. The isolated subunit, like the HS-enzyme, also catalyses the formation of (3S)-citryl-CoA from acetyl-CoA and citrate. Evidence has been presented for the generation of anhydride intermediates on the transferase subunit of citrate lyase; the anhydrides being citric-acetic anhydride in the acetyl exchange reaction with citrate, and acetic anhydride in the absence of citrate. However, unlike other CoA-transferases like succinyl-CoA: 3-oxoacid CoA-transferase, the transferase-catalysed CoA transfer from acetyl-CoA to citrate in citrate lyase does not apparently involve an enzyme-CoA intermediate (Dimroth et al., 1977b).

PRESENT INVESTIGATION

It will be evident from the foregoing survey of literature that considerable attention has been focussed on citrate lyases in recent years, mainly after the discovery of its multienzyme character and its built-in ACP with a covalently attached coenzyme A-like prosthetic group.

We have in our present studies attempted to look at the mechanism of action of the enzyme complex from K.aerogenes. We have also examined in detail the nature of the half-cystine residues in the complex as well as in the separated subunits and in particular the behaviour of the S-acylated cysteine residue known to be present in the ACP in addition to the essential acetyl residue of the prosthetic group. The nature and content of these groups in the complex and subunits are described for the first time and direct evidence is presented for the non-involvement of the acylated cysteine in the reaction mechanism.

The spatial location of the 3 non-identical subunits of the enzyme complex have been studied for the first time using bifunctional cross-linking reagents. Besides throwing new light on the structural organization of the complex in solution, evidence is presented for the first time for the conformational changes brought about in the complex by the presence of the metal cofactor, Mg^{2+} .

The complex has been immobilized for the first time by covalent binding to CNBr-activated Sepharose as carrier. The immobilized complex behaves like the native molecule in its reaction-inactivation as well as enzymic reactivation by

acetate:HS-citrate lyase ligase (AMP) in presence of ATP and acetate. The attachment of the complex to the carrier has been shown to be multipoint involving several lysine residues/mol enzyme. An immobilized multicomponent system in which both citrate lyase and malate dehydrogenase are covalently attached simultaneously to the Sepharose support is described for the first time and its usefulness for the repeated determination of citrate has been established.

The present work is described in the following four parts:

- Part I: Studies on Mechanism of action and OAA inactivation of citrate lyase.
- II: Studies on the content and nature of the -SH groups in citrate lyase from K.aerogenes.
- III: Subunit contacts in citrate lyase complex.
- IV: Studies on immobilization of citrate lyase.

The references for the introduction as well as component parts are collectively presented in the final Section.

The significant findings are summarised in the following Section.

GENERAL SUMMARY

GENERAL SUMMARY

1. Citrate lyase (EC 4.1.3.6) from K.aerogenes has been obtained pure by a modification of the earlier methods and the enzyme obtained with a uniformly higher specific activity of 90 U/mg. Studies on mechanism of action of the enzyme using tritiated NaBH_4 ruled out the formation of a covalent enzyme-CoA type intermediate as well as the formation of any schiff-base during the course of the reaction.

Citroyl formic acid, a common contaminant in commercial ^{al}oxoacetate has been shown to have no inhibitory effect on the enzyme.

2. A procedure for indirect determination of S-acylated sulfhydryl groups in proteins has been standardized.

The enzyme from K.aerogenes has 48 half-cystine residues/mol and no disulfide linkages. The native enzyme has a total of 42 -SH residues/mol of which only 3 are reactive towards DNB under non-denaturing conditions. The complex has 10 S-acylated residues of which 4 are reactive and deacylated during reaction-inactivation. Iodo[2- ^{14}C]acetate labelling studies showed the reactive S-acylated groups to be on the cysteamine residues of the prosthetic group. This is the first direct evidence that only the S-acetylated cysteamine residue is biologically active.

The cysteine content of the α -subunit has been shown to be 5 CySH/mol and of the β -subunit, 2 CySH/mol. In the ACP subunit 6 S-acylated cysteine residues have been shown to be present. The presence of S-acylated cysteine residues had been qualitatively indicated in earlier reports.

3. Inter-subunit cross-linking studies carried out with bifunctional reagents gave different cross-linked species with dimethyl adipimidate (DMA), dimethyl suberimidate (DMS) and the cleavable reactive ester dithiobis (succinimidyl propionate) (DSP). The cross-linked α_2 dimer has been obtained with DMA. In addition DMA gave a $\alpha_2\beta$ species and DMS gave an $\alpha_2\gamma_2$ species. DSP treatment in presence of Mg^{2+} gave a 178 000 dalton species corresponding to the dimer of cross-linked α - and β -subunits and this was confirmed after subsequent cleavage. An allosteric effect brought about by the metal cofactor Mg^{2+} has been indicated in DSP cross-linking experiments carried out with and without the metal. In the absence of Mg^{2+} the $\alpha\beta_2$ aggregate has been obtained in markedly lower yield together with a $\alpha\beta_2\gamma_2$ species. Reaction-inactivated citrate lyase gave only the $\alpha\beta_2$ species like the native enzyme in presence of Mg^{2+} .

4. The citrate lyase complex from K.aerogenes has been immobilized for the first time by covalent attachment to cyanogen bromide-activated agarose. The attachment of the enzyme to the carrier is through an average of 23 lysyl residues/mol enzyme. The immobilized enzyme, like the native complex, undergoes reaction-inactivation and is reactivated enzymically by acetate: HS-citrate lyase ligase (AMP) in presence of acetate and ATP.

An immobilized multienzyme system containing both citrate lyase complex and malate dehydrogenase covalently attached to cyanogen bromide-activated Sepharose has been prepared and its use for citrate assay standardized.

STUDIES ON CITRATE LYASE (EC 4.1.3.6)

from Klebsiella aerogenes

I: MECHANISM OF ACTION AND OAA INACTIVATION

STUDIES ON MECHANISM OF ACTION AND OAA INACTIVATION OF CITRATE LYASE

Summary

A method is described for preparation of citrate lyase of specific activity 90 U/mg which is higher than that reported earlier.

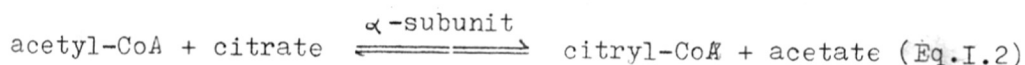
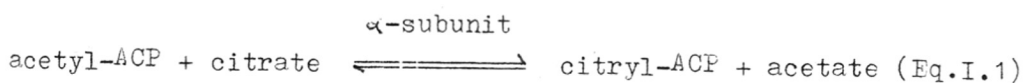
Treatment of the enzyme complex in presence of citrate with tritiated NaBH_4 did not result in the specific covalent ^3H -labelling of any of the subunits. This extends the earlier evidence against a covalent enzyme-CoA-acyl intermediate, obtained with the isolated transferase subunit, to the complex with its built-in acetyl-ACP substrate. The failure to obtain covalent tritium labelling by treatment with NaB^3H_4 during the course of the reaction would also rule out the possibility of a schiff-base complex formation between the enzyme and the product, OAA.

Citroyl formic acid which has been reported to occur as a contaminant in commercial OAA showed no inhibitory effect on the enzyme.

Introduction

Citrate lyases have been obtained homogeneous from K.aerogenes (SivaRaman, 1961), S.diacetilactis (Singh and Srere, 1975; Kummel et al., 1975), S.faecalis (Hiremath et al., 1976) and R.gelatinosa (Beuscher et al., 1974; Giffhorn and Gottschalk, 1978) and are all multienzyme complexes with marked similarity in their structural organisation. They are

all of about the same molecular weight (M_r 550 000) and are all composed of three non-identical subunits of about 55 000, 35 000 and 10 000 daltons. The smallest subunit has a covalently bound CoA-like prosthetic group carrying an essential acetyl function in thioester linkage. In the enzyme from K.aerogenes, which has been studied the most, the largest subunit (M_r 54 000) functions as an acetyl-ACP; citrate-ACP transferase (Eq. I.1) (Dimroth and Egger, 1975b) as well as a acetyl-CoA; citrate-CoA transferase (Eq.I.2) (Dimroth et al., 1977b).



The reaction catalysed by the transferase subunit thus involves the conversion of citrate to an energy rich citryl thioester at the cost of the original thioester substrate, acetyl-ACP. The transferase subunit thus functions as a CoA-transferase. The mechanism of action of the classical CoA transferases has been elucidated mainly through studies on succinyl-CoA: 3-keto acid-CoA transferase (Jencks, 1973). The enzyme reaction in this case has been shown to proceed through two half reactions. An enzyme-CoA intermediate is formed first from the succinyl-CoA or acetoacetyl-CoA and the enzyme through a specific reactive glutamate residue on the enzyme molecule. The intermediate has been isolated by gel-filtration and shown to react with succinate to give succinyl-CoA. Sodium borohydride reduction of the complex or the enzyme in presence

of the acyl-CoA substrate inactivates the enzyme and with NaB^3H_4 the radioactive label has been shown to be incorporated into the enzyme through the reduction of the γ -COOH group on the reactive glutamate residue. Preliminary studies reported by Dimroth et al. (1977b) on isolated transferase subunit of citrate lyase indicate that this subunit differs from the classical CoA-transferase in not being inactivated by sodium borohydride in presence of acetyl-CoA.

In the present study these investigations have been extended to the citrate lyase complex with its built in acetyl-ACP. Tritium labelled borohydride treatment was used as a more sensitive probe since the failure to observe inactivation in the earlier report could have been due to an extremely unstable enzyme-CoA intermediate, particularly at the alkaline pH of 8.0 used.

A modified procedure for the isolation of the enzyme from K.aerogenes in uniformly high purity and specific activity is also described. The possibility of citroyl formic acid (CFA), which has been recently reported to be an impurity in commercially available oxaloacetate (OAA) samples (Johnson et al., 1978), being responsible for the OAA-inactivation of the enzyme reported earlier (Dagley and Dawes, 1955; Singh and Srere, 1971) has also been studied.

Materials

Streptomycin sulfate was obtained from Hindustan Antibiotics Ltd., Poona. Acrylamide and N-N'-methylene-bisacrylamide were of electrophoresis grade and were obtained

from Eastman Organic Chemicals, USA.

Adenosine 5'-triphosphate (disodium salt) from Equine muscle, Malate dehydrogenase from pig heart (specific activity $945 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$), NADH from yeast (disodium salt- $3\text{H}_2\text{O}$ grade III), crystalline bovine serum albumin and tris were obtained from Sigma Chemical Co., USA.

Sodium borohydride was from BDH, England and Tritium labelled NaB^3H_4 (293 mCi/mmol) was from the Radiochemical Centre, Amersham. Naphthalene was a product of Koch-Light Laboratories Ltd., England and 2,5-Diphenyloxazole (FPO) of Beckman Instruments Inc., USA. Sodium dodecylsulphate was obtained from Hico Products Pvt. Ltd., Bombay. Oxaloacetic acid was from Fluka, AG., Switzerland.

All other chemicals were of the highest purity available.

Organism:

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

Buffers:

All the buffers were prepared in glass distilled water by mixing and diluting 1 M stock solutions of K_2HPO_4 and KH_2PO_4 in given proportions (Green and Hughes, 1955). All the buffers, excepting the 0.03 M potassium phosphate buffer pH 7.0 used in the extraction of intracellular enzymes from the

sonicated cells, contained MgSO_4 (1.6 mM). The tris-HCl buffer was prepared from Trizma base.

Alumina C_g 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 and was used repeatedly after washing. The exchanger was initially washed with water and then equilibrated with buffer. About 10 g of anion exchanger was washed repeatedly with three lots of 100 ml 0.5 M potassium phosphate buffer pH 7.5 containing 1.6 mM MgSO_4 and then washed with water. This was followed by washing with three lots of 100 ml 0.01 M potassium phosphate buffer pH 7.5 containing 1.6 mM MgSO_4 . The anion exchanger was packed into columns of 1.5 cm diameter to a height of 30 cm. The packing of the column was done under pressure and the column was also run under pressure at flow rates of 18-20 ml/h.

Sepharose CL-6B:

The gel was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The sodium azide used as a preservative was removed by washing the gel repeatedly with glass distilled water. The gel was then washed thoroughly with 0.05 M potassium phosphate buffer, pH 7.5, containing MgSO_4 (1.6 mM). The Sepharose CL-6B was packed into a column (110 x 2.5 cm) under gravitational pressure. The column flow rates were 14-16 ml/h.

Ammonium sulfate fractionation:

In the early steps of the purification procedure, powdered ammonium sulfate was used and the quantities of solid ammonium sulfate required for the fractionation were read off a nomogram where the concentrations are expressed in terms of saturation at 0-3°C. After the DEAE-cellulose chromatography step, a neutralised, cold saturated solution of ammonium sulfate (pH 7.0) was used for precipitating protein.

Experimental Procedures

Purification of citrate lyase:

Protein determination: Protein was determined at all stages of purification by the method of Lowry et al. (1951) standardised against pure citrate lyase from K.aerogenes. Streptomycin sulfate containing samples were dialysed for 24 h to remove the antibiotic prior to protein determination, and the colour was read either at 500 nm or 750 nm. Protein in the purified preparation of the enzyme was also estimated by the method of Singh et al. (1976) using the absorbance index $E_{1\text{ cm}}^{1\%}$ of 6.2 at 278 nm.

Enzyme assay: The enzyme activity was assayed by two different methods. The method routinely used in the early stages of purification was that described by Mahadik and SivaRaman (1968). The enzyme was assayed at 28°C using 3.0 ml of the test system containing sodium citrate 100 mM; MgSO_4 , 10 mM; Tris-HCl buffer, 50 mM; pH 8.0. Reaction was initiated by adding 5-10 μg of the enzyme. The reaction was stopped by adding 3.0 ml of 10% (w/v) trichloroacetic acid.

The keto acids formed were estimated by the Friedmann and Haugen method (1943). Initial rates were determined from the values obtained at 15 sec.

Purified enzyme preparations were assayed by a slight modification of the coupled assay procedure described by Singh and Srere (1971). The citrate cleavage reaction was coupled with malate dehydrogenase in presence of NADH and the change in optical density at 340 nm was recorded. In 1.0 ml the test system contained 100 μmol sodium citrate; 10 μmol MgSO_4 ; 50 μmol Tris-HCl buffer, pH 8.0; excess of malate dehydrogenase (approx. 15 μg) and 0.1 - 0.2 μmol NADH. 0.1-0.5 μg citrate lyase in 0.01 ml was added to start the reaction in the test system contained in a 1 ml cuvette of 10 mm light path. The temperature was maintained at 28°C. A decrease of 6.22 in the optical density corresponds to 1 μmol of keto acid formed.

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

Optical density: All optical density measurements were made on a Beckman DU or Model 26 spectrophotometer and cuvettes of 10 mm light path were used. For enzyme assay by the malate dehydrogenase coupled assay procedure the Beckman model 26 spectrophotometer equipped with strip-chart recorder was used.

pH measurement: A Philip's precision pH meter Model PR 9405 with a combination glass electrode was used for all pH measurements.

Ultracentrifugation: The homogeneity of all enzyme preparations was checked by ultracentrifugation in an analytical

Spinco Model E ultracentrifuge having phase plate schlieren optics and a rotor temperature indicator and control device for maintaining constant temperature during the run. All the runs were carried out at 2 to 5°C and 59 780 rpm.

Polyacrylamide gel-electrophoresis: Disc gel electrophoresis was carried out according to Davis (1964) using 5% polyacrylamide gels. Polymerisation was done using 0.07% ammonium persulphate. The buffer system of Davis was used with the separating gel containing Tris-HCl buffer, pH 8.3. The sample and stacking gels were omitted. Unreacted ammonium/persulphate was removed in a preliminary electrophoresis of two hours. Then 50-100 µg of the sample protein, made dense with 30% sucrose, was loaded on top of the gels in a volume of about 200 µl and the Tris-glycine, pH 8.3, electrode buffer was carefully layered on top. Bromophenol blue was incorporated as the tracking dye. Electrophoresis was carried out at 4°C and 4 mA/tube till the tracking dye reached the bottom of the gel (3-4 h). After completion of the run the protein bands were visualised by staining the gels either with 0.25% Coomassie Brilliant Blue prepared in water containing 45.4% methanol and 9.2% glacial acetic acid or with 1% Amido Black 10B in 7% acetic acid. Destaining was done by diffusion in a solution containing 7.5% glacial acetic acid and 5% methanol in water.

Ultrafiltration: Concentration of the enzyme solution obtained in the final step of purification was done in Amicon ultrafiltration cells of 10 ml capacity using a PM-10 membrane.

Growth and Extraction of Enzymes from *K.aerogenes* cells

Growth of *Klebsiella aerogenes* cells: *K.aerogenes*

NCTC 418 was grown without aeration at 30°C for 16 h in 10 L flasks filled with the medium of Dagley and Dawes (1953c) and containing in each flask trisodium citrate. 2H₂O, 90 g; KH₂PO₄, 20 g; (NH₄)₂SO₄, 10 g; MgSO₄·7H₂O, 4 g; adjusted to pH 7.0 with 40.0 ml of 2 N NaOH and made up to volume with distilled water. MgSO₄ and (NH₄)₂SO₄ were dissolved in 1 L of water and autoclaved separately to avoid precipitation of magnesium phosphate. This was mixed with the rest of the medium just before inoculation. An inoculum of 10% final volume containing cells adapted to the citrate growth medium was used. After 18 h the cells were harvested in a Sharples supercentrifuge at 12 000 g and the yield of cells was 18-20 g wet cells/10 L of the medium. The wet cells were stored at -20°C and were active for more than a week.

Extraction of cells: Batches of 110 to 120 g (wet weight) cells were processed for purification of the enzyme.

The enzyme was extracted at 0 - 4°C. The frozen cells were thawed overnight and then suspended in 0.03 M potassium phosphate buffer, pH 7.0, (4 ml/g of cell paste) and sonicated in Biosonik III (Brönwill Scientific Co., USA) sonicator twice at 20 Kc (300 W) for 3 min. The sonicate was centrifuged at 50 000 g for 60 min at 4°C to remove the cell debris and the clear supernatant was adjusted to a protein concentration of 0.5% with 0.03 M potassium phosphate buffer, pH 7.5

Purification of the Enzyme

Purification of the enzyme was carried out continuously at 0-4°C without storage at any step of the purification procedure. All the buffers contained 1.6 mM MgSO_4 to stabilise the enzyme.

ATP and acetate treatment:

The clear sonicate extract adjusted to 0.5% protein concentration was treated with 0.1 M ATP and 1 M acetate to give final concentrations of 0.5 mM and 1 mM, respectively and kept stirred for 15 min to reactivate any HS-citrate lyase present.

Streptomycin sulphate treatment:

The above extract was kept stirring and streptomycin sulfate was added slowly to a final concentration of 1.4%. The nucleic acids which precipitated out were removed after 1 h by centrifugation at 50 000 g for 45 min.

Adsorption on Alumina C_1 gel:

Different gel preparations showed variations in their capacity to adsorb the enzyme and were therefore standardised in preliminary experiments. The conditions for a representative batch are as described below:

First gel treatment: The supernatant obtained after removal of nucleic acids was treated with the alumina C_1 gel under constant stirring till about 5% of the enzyme activity was adsorbed (10 ml/600 ml streptomycin sulfate treated supernatant). The gel was separated by centrifugation at 3 000 g for 20 min and discarded.

Second gel treatment: The supernatant obtained after the first gel treatment contained most of the activity and was treated under agitation with slow addition of the alumina C_Y gel (200 ml/600 ml of the streptomycin sulfate treated supernatant), About 90% of the activity was adsorbed on the gel. The suspension was stirred for 15 min and then allowed to stand for 15 min. The gel was separated by centrifugation at 3 000 g for 20 min and the supernatant with only slight enzymic activity was discarded.

Elution of the enzyme from alumina C_Y gel:

The gel was homogenised with 0.05 M potassium phosphate buffer, pH 7.0, (200 ml/600 ml streptomycin sulfate treated extract) in a glass tube with a close fitting ground-glass pestle and after 15 min the gel was again separated by centrifugation at 3 000 g for 20 min. The gel was discarded and the clear supernatant was treated further.

Ammonium sulfate fractionation:

To the supernatant obtained in the above step, powdered ammonium sulfate was added to 0.2 saturation (20 g/200 ml) under constant stirring. After complete addition, the precipitate was removed by centrifugation at 50 000 g for 30 min and discarded. The supernatant was further treated with powdered ammonium sulfate to 0.5 saturation (38 g/200 ml) and kept stirring for 15 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 dialysed against 500 ml of the same buffer for 16 h.

DEAE-cellulose chromatography:

The dialysed solution (6-7 ml) was centrifuged at 10 000 g for 10 min to remove any turbidity and the clear solution was then loaded on a DEAE-cellulose column (30 x 1.5' cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. Ion-exchange chromatography was carried out using continuous gradient elution with potassium phosphate buffer, pH 7.5, of increasing buffer strength from 0.01 M to 0.3 M. This concentration gradient was obtained by using 200 ml of 0.01 M buffer solution in the mixing chamber attached to the column and addition of 0.5 M buffer to this solution at the same flow rate as the column. Fractions of 3.0 ml were collected on an automatic fraction collector and enzyme activity was detected in the fraction by sampling a drop from each tube. The enzyme eluted out between about 90-120 ml. Fractions having high activity were pooled (about 25 ml) and precipitated by the slow addition of an equal volume of neutralised, cold saturated ammonium sulfate solution. The precipitate was collected by centrifugation at 50 000 g for 30 min and then dissolved in about 2.0 ml of 0.05 M potassium phosphate buffer, pH 7.5.

Gel filtration:

The enzyme solution from the above step was loaded on a Sepharose CL-6B column (110 x 2.5 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. The enzyme was eluted with buffer of the same composition. Fractions of 4.0 ml were collected on an automatic fraction collector. The fractions were tested for enzyme activity by sampling a drop

from each fraction. The enzyme eluted between 180-200 ml. Fractions with high enzyme activity were pooled (about 20 ml) and were concentrated by ultrafiltration through a PM-10 membrane.

Studies on mechanism of action:

Sodium borohydride reduction: The reduction of citrate lyase with sodium borohydride was carried out essentially by the procedure of Horecker et al. (1961).

Citrate lyase enzyme (8-10 mg), was treated at 30°C in 1.0 ml final volume of a solution containing 9 μ moles sodium citrate, 1.6 μ moles MgSO_4 and Tris buffer, pH 8.0, 50 μ moles. The reaction mixture was cooled to 0-4°C and made upto 4.0 ml with 0.05 M potassium phosphate buffer, pH 6.5, and adjusted to maintain pH 6.5. Reduction was carried out at 0°C by the addition first of a solution of 5 mg tritium labelled sodium borohydride containing 10 mCi of NaB^3H_4 in 0.001 M NaOH. The additions were made in lots of 0.01 ml and were followed by the addition of a solution of 30 mg non-radioactive sodium borohydride in 1.0 ml of 0.001 N NaOH in aliquots of 0.01 ml over a period of 30 min at pH 6.3 - 6.5 to reduce non-specific labelling. The pH of the mixture was maintained constant by the addition of 0.06 ml aliquots of 1 N acetic acid. On completing the addition of sodium borohydride, the reaction mixture was kept at 0°C for 30 min and then treated with an equal volume of saturated ammonium sulfate, pH 7.0, to precipitate the protein. Protein was collected by centrifugation and dissolved in 2.0 ml of 0.05 M potassium phosphate buffer, pH 7.5, containing 1.6 mM Mg^{2+} and reprecipitated 5 times with ammonium sulfate as before.

The final precipitate was dialysed 24 h against 3 changes of the 0.05 M phosphate buffer, pH 7.5, exhaustively. An aliquot of the final solution was taken for determination of radioactivity in a Beckman Liquid Scintillation Model LS 100C.

SDS-gel electrophoresis: Non-specific tritium incorporation into the protein occurs on NaB^3H_4 reduction (Soloman and Jencks, 1969). To detect the presence of any covalently linked ^3H in the reduced protein, SDS-gel electrophoresis was carried out under denaturing conditions according to the procedure of Weber and Osborn (1969). ^3H -labelled protein (700 μg , 16 000 cpm) was treated with SDS to a final concentration of 1% and heated at 100°C for 3 min. The treated protein was loaded on 7.5% polyacrylamide gel of 1.5 cm diameter. A control without NaB^3H_4 treatment was run simultaneously. After the electrophoresis, gels were stained with Amido Black and destained with 7.5% acetic acid. The gel with NaB^3H_4 treated protein was cut into slices of 0.5 cm thickness and taken into small loosely stoppered glass tubes. Each gel piece was broken up and treated with 0.5 ml of 30% H_2O_2 at 50°C for 12 h to solubilise the gel. Each sample was then taken to dryness over P_2O_5 , dissolved in 1 ml BSS-3 Beckman Bio-solv solubiliser and taken in 10 ml scintillation fluid. The scintillation cocktail contained PPO (5 g), Naphthalene (100 g) and 1,4-Dioxane (to make 1 L). The ^3H counts were registered in a Beckman LS 100 C liquid scintillation counter.

Preparation of Citroyl formic acid: Citroyl formic acid (CFA) was prepared from oxaloacetic acid according to

the procedure described by Wiley and Kim (1973). An aqueous solution of oxaloacetic acid adjusted to pH 7.5 with NaOH was kept at 25-30°C for 48 h and then the calcium salt of CFA was precipitated by the addition of 60% CaCl_2 solution. The precipitate was collected and dried over P_2O_5 in a vacuum desiccator. The calcium salt of CFA (2 g) was taken in 30 ml dry ether along with 0.6 g of anhydrous MgSO_4 and treated with dropwise addition of 0.6 ml concentrated H_2SO_4 in 20 ml dry ether, under constant stirring. The solution was filtered and the clear liquid evaporated to give a residue of CFA.

Inhibition by OAA: In studies on OAA inhibition, the enzyme was incubated at 30°C with OAA (2.2 mM) in presence of Mg^{2+} (1.6 mM) for 1 min. MDH and excess of NADH were added first, followed by citrate after complete reduction of the excess OAA. Reaction was then monitored at 340 nm.

CFA at the final concentrations in the assay system was found to have no effect on malate dehydrogenase activity.

Results

Purification of citrate lyase:

The summary of a typical purification procedure of citrate lyase from K.aerogenes is presented in Table I.1. The purification procedure was carried out without storage at any stage although the undialysed ammonium sulfate fraction is stable at -20°C for 2 to 3 days.

The enzyme preparations were homogeneous as checked by analytical ultracentrifugation (Fig. I.1), analytical polyacrylamide gel electrophoresis (Fig. I.2) and SDS

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	440	5.0	4400	2.0	-
Cell-free extract + acetate + ATP	443	5.0	5538	2.5	100
Streptomycin sulfate treatment followed by centrifugation to remove precipitated nucleic acids.	430	3.8	4902	3.0	89
0.05 M buffer eluate of second alumina C _γ gel	200	2.66	3192	6.0	58
Ammonium sulfate fraction (0.2 - 0.5 Sat.) dissolved in 0.05 M buffer, pH 7.5	4.7	40.0	3008	16.0	54
DEAE-cellulose fraction pooled and fractionated with saturated (NH ₄) ₂ SO ₄ and the precipitate dissolved in 0.05 M buffer, pH 7.5	1.25	42.6	2813	52.8	51
Sephacrose CL-6B fractions after concentration over PM-10 membrane by ultrafiltration	3.0	4.8	1296	90.0	23

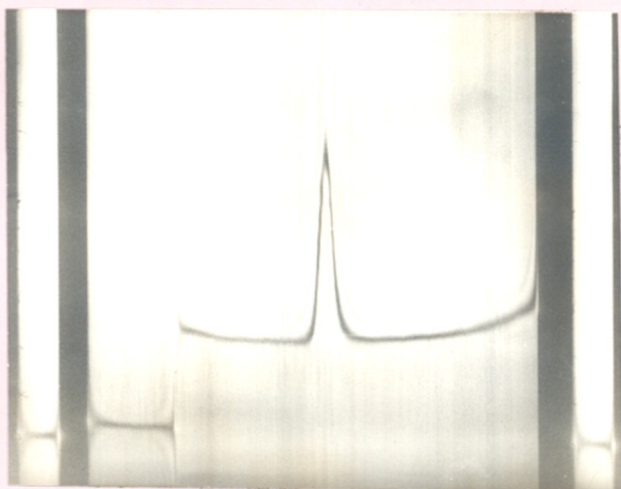
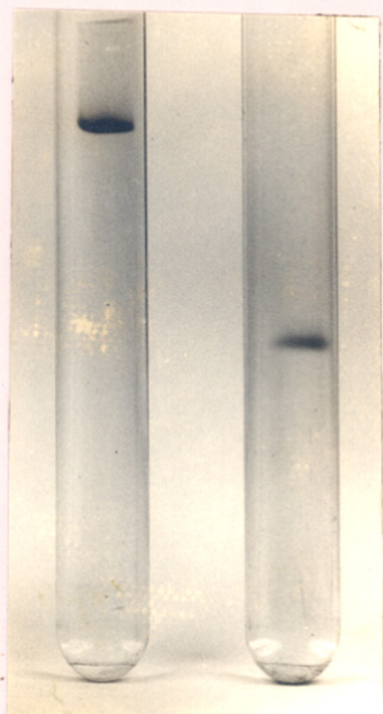


FIG. I. 1: SEDIMENTATION PROFILE OF NATIVE CITRATE LYASE
FROM K.aerogenes

Protein concentration	- 4.5 mg/ml
Buffer	- 0.05 M potassium phosphate buffer, pH 7.5 with 1.6 mM MgSO_4
Speed	- 59 780 rpm
Temperature	- 4.5°C
Phase plate	- 60°
Time	- 32 min



+

(a)

(b)

FIG.I.2: POLYACRYLAMIDE GEL ELECTROPHORESIS OF
NATIVE CITRATE LYASE FROM K.aerogenes

5% gel; Tris-glycine buffer, pH 8.3;
4 mA/tube; 4°C; Protein loaded 50 µg.
(a) 3 h (b) 6 h.

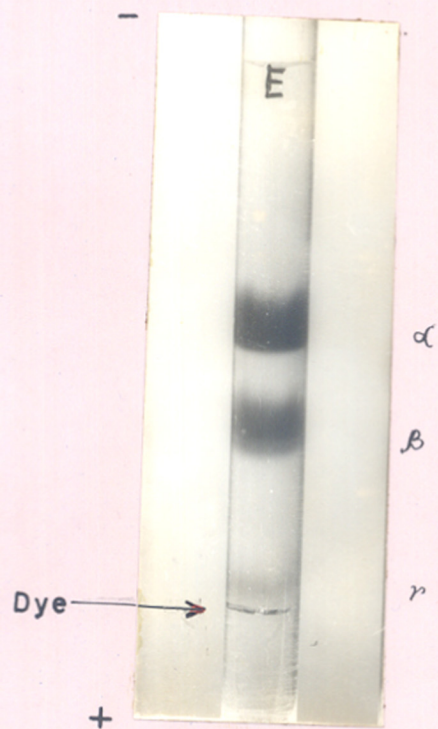


FIG.I.3: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS
OF CITRATE LYASE FROM K.aerogenes

7.5% gels; sodium phosphate buffer, pH 7.3,
containing 0.1% SDS; 8 mA/tube; 28°C; 4 h.
Protein loaded 100 μ g.

polyacrylamide gel electrophoresis showed the presence of the α -subunit (M_r 54 000), β -subunit (M_r 32 000) and γ -subunit (M_r 10 000 to 11 000) (Fig. I.3).

Mechanism of action:

The results obtained on tritium labelled sodium borohydride treatment of the enzyme in presence of citrate are presented diagrammatically in Fig. I.4. Electrophoresis of the treated enzyme in SDS-polyacrylamide gel electrophoresis showed the characteristic three non-identical subunits. None of the subunit bands after staining and destaining nor the unstained region of the gel showed any radioactivity after solubilising the gel. Experiments in which the reaction mixture was initially incubated for 30 min before treatment with tritiated NaBH_4 also resulted in subunits which showed no radioactivity.

OAA inactivation of citrate lyase:

Results of experiments on the effect of OAA and CFA on citrate lyase activity are summarised in Table I.2.

TABLE I: EFFECT OF OAA AND CPA ON ACTIVITY OF CITRATE LYASE

20 μ l of a solution containing citrate lyase (0.1 to 1.5 μ g/ml), test compound (OAA or CPA) (2.2 mM), Mg^{2+} (1.6 mM), potassium phosphate buffer, pH 7.6 (50 mM) was incubated at 30°C for 10 min. Controls were run in which the test compound was omitted. At the end of the incubation period, the reaction was stopped by the addition of 10% trichloroacetic acid (TCA) and the mixture was centrifuged at 10,000 g for 5 min. The supernatant was removed and the pellet was washed with 50% TCA. The complete conversion of OAA to citrate was confirmed by the activity assay by addition of citrate lyase to the supernatant and the Experimental Procedures.

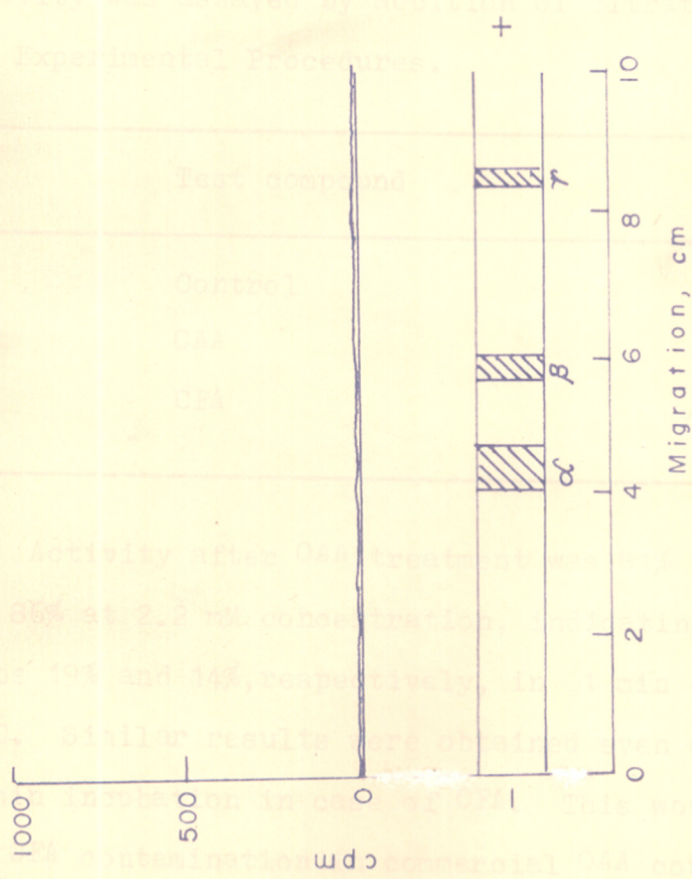


FIG. 1.4: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CITRATE LYASE TREATED WITH $NaB^{3}H_{4}$ IN PRESENCE OF SUBSTRATE. Diagrammatic representation of gel indicating relative positions of α , β and γ - subunits. Gel fractions were uniformly without ^{3}H -label and counts were in range of background after staining and destaining.

TABLE 1.2: EFFECT OF OAA AND CFA ON ACTIVITY OF CITRATE LYASE

20 μ l of a solution containing citrate lyase (0.1 to 0.5 μ g); test compound (OAA or CFA) (2.2 mM); Mg^{2+} (1.6 mM); potassium phosphate buffer, pH 7.5 (50 mM) was incubated 1 min at 30°C. Controls were run in which the test compounds alone were omitted. At the end of the incubation period, malate dehydrogenase and NADH were added to remove any OAA. Following the complete conversion of OAA to malate, citrate lyase activity was assayed by addition of citrate as described in the Experimental Procedures.

Test compound	% activity
Control	100
OAA	81
CFA	86

Activity after OAA treatment was 81% and that with CFA was 86% at 2.2 mM concentration, indicating inactivation to be 19% and 14%, respectively, in 1 min of incubation at 30°C. Similar results were obtained even after a period of 15 min incubation in case of CFA. This would indicate that any CFA contamination in commercial OAA could not have been responsible for the inactivation reported in the earlier studies.

Discussion

Purification of citrate lyase:

The present purification procedure was a slight modification of the methods described earlier (SivaRaman, 1961; Mahadik and SivaRaman, 1968). The treatment with ATP and acetate was included to reactivate any HS-citrate lyase present in cell extracts of K.aerogenes. The increase in specific activity of the enzyme in the cell sonicates from 2.0 to 2.5 would confirm the presence of some inactive deacetylated enzyme at this stage. The final gel filtration step was carried out through Sepharose CL-6B in place of Sephadex G-200 as the latter gel beads did not uniformly separate higher molecular weight components from the enzyme fraction. The use of ultrafiltration in place of ammonium sulfate precipitation uniformly gave preparations homogeneous in the ultracentrifuge and by gel electrophoretic techniques. The specific activity of 90 U/mg dry weight of pure enzyme obtained by this procedure is also higher than that reported earlier. Singh et al. (1976) have reported enzyme preparations with specific activities of 73 U/mg dry protein and Dimroth and Eggerer (1975a) report a value of 50 U/mg dry protein. A modification of their earlier procedure for purification has been reported by Dimroth et al. (1977b) which involves fractionation with polymin HS after elution from the second alumina C γ gel treatment, followed by extraction from the polymin HS precipitate and gel-filtration through Sephadex G-200. This procedure is reported to yield enzyme with specific activity of only 55 U/mg protein.

Recovery of activity of over 20% is comparable to yields reported by others (SivaRaman, 1961; Mahadik and SivaRaman, 1968; Dimroth et al., 1977b).

Mechanism of action:

The report by Dimroth et al. (1977b) on the mechanism of action of the isolated transferase subunit (α -subunit) of citrate lyase from K.aerogenes appeared during the course of our studies on this aspect. In this report the authors have separated the active transferase subunit and investigated this using CoA as substrate. Their attempts to isolate an enzyme-CoA intermediate were unsuccessful. Treatment of the subunit in presence of acetyl-CoA with KBH_4 at pH 8.0 did not result in the inactivation of the transferase activity. This was taken as evidence against the formation of an enzyme-CoA intermediate under the conditions tested. In this respect the transferase subunit was found to differ from the classical CoA transferases represented by succinyl CoA: 3-keto acid transferase (Jencks, 1973). However, the formation of anhydride intermediates were indicated from (a) affinity labelling of HS-citrate lyase with acetic anhydride or iodoacetate in presence of excess DTT (b) shielding effect of citrate against iodoacetate-inactivation of HS-citrate lyase (c) kinetic studies on acetic anhydride activation of HS- citramalate lyase which closely resembles the citrate lyase complex.

In the present study, the enzyme complex was used in place of the isolated transferase subunit. It must be

mentioned here that NaBH_4 -treatment of native citrate lyase complex in the absence of citrate has no effect on its cleavage activity (Srere and Singh, 1974). On an analogy with the classical CoA transferases, the possible intermediate in the case of the citrate lyase complex would be at the first step of the sequence of reactions involving the transferase subunit and the ACP substrate. Such an intermediate on reduction with tritiated NaBH_4 would result in radioactive labelling of the transferase subunit with the $^3\text{H-C}$ linkage expected from the reduction of an $\text{E}-\overset{\text{O}}{\underset{\text{||}}{\text{C}}}-\text{S-ACP}$ type intermediate where E represents the transferase subunit (Hersh and Jencks, 1967; Solomon and Jencks, 1969; Sramek and Frerman, 1975; Tung and Wood, 1975). The reaction mixture containing citrate and Mg^{2+} at pH 8.0 was treated with citrate lyase and the pH adjusted to 6.5 before reduction with NaB^3H_4 since the enzyme-CoA intermediate obtained from pig heart succinyl-CoA: 3-keto acid CoA transferase has been shown to be labile to base and stable in dilute acids (Hersh and Jencks, 1967).

Citrate lyase after NaB^3H_4 treatment when separated by SDS-polyacrylamide gel electrophoresis carried no radioactive label. The counts on the protein loaded on the gel were evidently due to non-specific labelling (Solomon and Jencks, 1969) which is removed during the process of electrophoresis followed by staining and destaining. The present results would extend the earlier finding of Dimroth et al. (1977b) that no covalent intermediate formation takes place between the transferase subunit of citrate lyase and the substrate, which in the present case is acetyl-ACP.

OAA inactivation:

Reaction inactivation of citrate lyase has been shown to be distinct from that caused by OAA (Singh and Srere, 1971). Both the enol and keto forms of OAA have been reported to inhibit the enzyme. However, even at extremely high concentrations compared to that produced in the course of the reaction, the isomers have been shown to cause only partial inactivation. Reaction inactivation as well as OAA inhibition results in the loss of the essential acetyl groups of citrate lyase to yield the HS-enzyme (Buckel et al., 1971a). In the former case the inactivation is presumed to be due to the unstable nature of the citryl-ACP while the mechanism by which OAA deacetylates the enzyme is not known. The present observation that tritiated NaBH_4 treatment, both during the course of the reaction and at the end of 30 min does not result in covalent tritium labelling of any of the enzyme subunits would indicate that OAA does not form a ketimine intermediate with enzyme since borohydride reduction of such a schiff-base complex would be favoured under the acidic conditions used (Schellenberg, 1963; Morse and Horecker, 1968). In experiments carried out with 6- ^{14}C -citrate, Singh and Srere (1971) have reported that some of the OAA formed was "tightly" bound with the protein, although the amount formed was stoichiometrically insignificant. Our studies rule out a schiff-base type complex. The earlier observed binding could have been as an OAA-Mg-protein complex.

The failure of CFA to inhibit the enzyme would also rule out the possibility of this compound, which is reported to be a common contaminant of commercial OAA preparations, being implicated in the OAA-inhibition reported earlier.

STUDIES ON CITRATE LYASE (EC 4.1.3.6)

from Klebsiella aerogenes

II: CONTENT AND NATURE OF THE -SH GROUPS

STUDIES ON THE CONTENT AND NATURE OF THE -SH GROUPS IN CITRATE
LYASE FROM K.aerogenes

Summary

A rapid procedure for the indirect determination of S-acylated sulphhydryl groups in proteins has been standardized. The procedure involves spectrophotometric titration with DTNB under denaturing conditions both in the presence of the deacylating agent, hydroxylamine, and in its absence.

The citrate lyase complex from K.aerogenes has been shown to contain 48 half-cystine residues/mol, none of which are involved in -S-S-linkages. The native enzyme has a total of 42 -SH residues/mol as estimated by DTNB titration and only 3 of the sulphhydryl groups of the complex are reactive towards DTNB under native conditions. The enzyme complex has 10 S-acylated residues of which only 4 are reactive towards DTNB after deacylation. The 4 reactive S-acylated sulphhydryl residues are also deacylated during the course of reaction-inactivation. The reactive S-acylated groups have been shown to be on the cysteamine residues of the prosthetic group by reaction-inactivation and iodo [2-¹⁴C]acetate labelling. The reaction-inactivated enzyme initially labelled with cold iodoacetate followed by deacylation with hydroxylamine and subsequent labelling with iodo [¹⁴C]acetate had all the radioactive label on the cysteine residue. This is the first direct evidence for the involvement of the cysteamine residue of the prosthetic group, and not the S-acetylated cysteine residue, as the functional acyl carrier.

The distribution of the cysteine residues in the complex has been shown to be 5 CySH/mol α -subunit and 2 CySH/mol β -subunit. The presence of a single cysteine residue in the γ -subunit has been established in earlier work (Dimroth et al., 1975) and qualitatively shown to exist as an S-acylated residue. The present study indicates that 6 S-acylated cysteine residues are present in the complex, evidently contributed by the six ACP subunits.

Introduction

Citrate lyase (EC 4.1.3.6) carries an essential acetyl group in thioester linkage with the (phosphoribosyl)-dephospho-CoA prosthetic group (Buckel et al., 1971a; Singh et al., 1977). In the course of substrate turnover, the acetyl groups are exchanged first with citrate to yield the citryl-enzyme and this intermediate is subsequently cleaved with liberation of OAA and regeneration of acetyl-enzyme (Singh et al., 1974; Dimroth and Eggerer, 1975b). During the course of the reaction it catalyses, the enzyme undergoes deacylation, presumably through hydrolysis of the unstable citryl-enzyme (Singh and Srere, 1971; Dimroth and Eggerer, 1975b) to give the HS- enzyme which is inactive towards citrate as substrate.

The 10 000 dalton subunit of the citrate lyase complex from K.aerogenes which functions as the ACP (Dimroth et al., 1973) has been shown qualitatively to carry an acylated cysteine residue in addition to the acetyl-S-cysteamine on the prosthetic group (Dimroth et al., 1973). Affinity

labelling with ^{14}C -iodoacetate (0.1 mM) of the cysteamine residue alone was achieved by treating the HS- citrate lyase with the alkylating agent in presence of excess of DTE (10 - 20 mM) and this resulted in complete loss of the ability to be reactivated by acetylation, both chemically and enzymically (Dimroth and Eggner, 1975a). The involvement of the cysteamine residue alone as the functional acyl carrier was postulated on the basis of these results.

Despite the importance of -SH residues in the mechanism of action of citrate lyase, little is known about the nature of the half-cystine residues in the enzyme molecule. Results are presented here on the cysteine and S-acylated cysteine residues in the holoenzyme as well as component subunits of citrate lyase from K.aerogenes. The reactivities of these residues and the effect of reaction-inactivation on the S-acylated groups are also reported.

Materials

All chemicals used were of the highest purity available. Sodium dodecyl sulfate (SDS) was a preparation of Hico Products, Pvt. Ltd., Bombay. Analytical grade hydroxylamine hydrochloride ($\text{NH}_2\text{OH}.\text{HCl}$) was obtained from BDH Ltd. and a neutralised hydroxylamine solution, pH 7.0, was prepared by mixing 14% NaOH and $\text{NH}_2\text{OH}.\text{HCl}$ (Stadtman, 1957). Fresh solutions were prepared just before use.

5-5'-Dithiobis (2-nitrobenzoate) (DTNB) was from Calbiochem, USA; L-cysteine hydrochloride. H_2O was from Fluka, AG., Switzerland and reduced glutathione, Trizma base

and acetyl-CoA were obtained from Sigma Chemical Co., USA. Dithiothreitol (DTT) and cysteamine were also from Sigma Chemical Co., USA.

Glass-distilled, constant boiling HCl used for protein hydrolysis was obtained from Beckman Inc., USA.

Iodide $2\text{-}^{14}\text{C}$ acetic acid, specific activity 57 mCi/mmol, was obtained from the Radiochemical Centre, Amersham. The radioactive iodoacetate was diluted before use with cold iodoacetate to a specific activity of 20 mCi/mmol.

Cellogel strips were obtained from Reeve Angel Scientific Ltd., London.

The S-carboxymethyl derivatives of cysteine and cysteamine were prepared as described below:

S-carboxymethyl cysteine derivative: The derivative was prepared as described by Dickens (1933). A solution of cysteine hydrochloride was neutralised with 0.1 M $\text{Ba}(\text{OH})_2$ solution and then treated under nitrogen atmosphere with a solution of iodoacetic acid also previously neutralised with $\text{Ba}(\text{OH})_2$. The reaction mixture was maintained neutral with $\text{Ba}(\text{OH})_2$ addition and then left overnight after addition of 15 ml alcohol. A further 30 ml of alcohol was then added and the resulting precipitate of barium salts separated by centrifugation. The precipitate was dissolved in 3 ml water and sulphuric acid was added to precipitate out barium. The filtered solution was concentrated in vacuum to give white crystals of S-carboxymethyl derivative of cysteine.

S-carboxymethyl cysteamine: This derivative was prepared according to De Marco *et al.* (1964). An aqueous solution

of cysteamine hydrochloride was treated with iodoacetic acid and neutralised with a 10 N KOH solution under nitrogen atmosphere. Nitrogen was bubbled through the solution for 30 min till the reaction was complete. This reaction mixture was loaded on a Dowex 50 x 8 column (2.5 x 5 cm) and the column washed with water. The S-carboxymethyl derivative was then eluted using 0.5 N NH_4OH . Fractions of the eluate were spotted, dried and tested with ninhydrin. The ninhydrin positive fractions were pooled and concentrated on a boiling water bath under reduced pressure. From the concentrate the S-carboxymethyl derivative was crystallised out by addition of alcohol followed by cooling to 0-4°C.

Citrate lyase: The enzyme from K.aerogenes was purified and assayed as described in Part I of the thesis. The enzyme preparation was homogeneous as checked by both analytical ultracentrifugation and polyacrylamide gel electrophoresis. The enzyme had a specific activity of 90 U/mg.

Experimental Procedures

Protein estimation:

Protein estimations were carried out as described earlier (Part I) either by the method of Lowry et al. (1951) or by the method of Singh et al. (1976) using an absorbance index of $E_{1\text{ cm}}^{1\%} = 6.2$ at 278 nm. For SDS-containing samples the values were obtained from a calibration curve obtained with crystalline bovine serum albumin in solutions containing SDS at a concentration equal to that in the test samples.

Estimation of -SH groups:

The analysis of sulfhydryl groups in the citrate lyase complex was carried out using DTNB. Molecular weight of 575 000 (Mahadik and SivaRaman, 1968) was assumed for calculation of $[SH]/[Enzyme]$. The titrations were carried out both under non-denaturing as well as denaturing conditions. DTNB titration was also performed in the presence of DTT and arsenite. The native enzyme complex possessed acylated -SH groups and these were determined by difference between the values obtained from DTNB titrations under denaturing conditions with prior incubation with hydroxylamine and without treatment with hydroxylamine.

DTNB titration: The titration of the enzyme with DTNB was performed essentially according to the procedure of Ellman (1959).

Estimation of accessible -SH groups in native enzyme: The native enzyme (0.5 - 1.0 mg) was taken in 0.25 M Tris buffer, pH 8.0, in a 1.0 ml capacity silica cuvette of 10 mm light path and the titration was started by the addition of 0.01 ml of 0.01 M DTNB in 0.25 M Tris buffer, pH 8.0. The change in absorbance at 412 nm was read against a reagent blank and recorded on a Beckman model 26 double beam spectrophotometer equipped with a strip chart recorder. Sulfhydryl content was calculated from the molar absorbance $\epsilon_{412 \text{ nm}}$ value of $13600 \text{ M}^{-1} \text{ cm}^{-1}$ for the free thiol ion of the reagent.

The -SH groups exposed after incubation with DTT (3 mM) were also estimated in the enzyme. DTNB titration was carried out in presence of arsenite (5 mM in 40 mM EDTA) according to the procedure of Habeeb (1972). The DTT treated enzyme was completely inactive.

Estimation of -SH groups in denatured enzyme: The total free sulfhydryl content of the enzyme was determined by carrying out the titration in the presence of 0.9% SDS.

Estimation of total -SH and acylated -SH groups: The usual method for the estimation of total free -SH and acylated -SH groups in proteins involves unmasking of any acylated -SH groups by treatment with hydroxylamine followed by removal of excess reagent by gel-filtration through Sephadex G-25. The determination of the -SH groups by this procedure when applied to citrate lyase was unsatisfactory and highly erratic values were obtained, probably due to the reactivity of the exposed -SH groups and their spontaneous oxidation during the course of this elaborate procedure. A procedure which has been described for the estimation of thioesters of CoA (Stegink and Coon, 1968) was therefore standardized for the estimation of both masked and free sulfhydryl groups in proteins. The procedure involves DTNB titration in presence of low concentrations of hydroxylamine.

Standardization of the estimation of -SH groups in presence of hydroxylamine: It was observed that hydroxylamine reacted with DTNB at pH 8.0 to produce free thiol ions as evident from the increase in absorbance at 412 nm. However this reaction was slow and in 30 min the increase in optical density

for a final hydroxylamine concentration of 0.1 M was only 0.06. This was corrected for by including both hydroxylamine and DTNB in the blank at identical levels to the test system.

Equal volumes of standard solutions containing mercaptans or S-acylated mercaptans and varying concentrations of neutralised hydroxylamine (1 M, 2 M and 4 M) were incubated for 30 min and an aliquot (0.1 ml) of the incubation mixture was taken in silica cuvettes of 1.0 ml capacity and 10 mm light path. To this 0.5 M Tris buffer, pH 8.0 containing 1.8% SDS (0.49 ml) was added. The volume was made up to 0.99 ml with water and reaction started by addition of 0.01 ml of 0.01 M DTNB solution in 0.25 M Tris-buffer, pH 8.0. The reference cuvettes contained the solutions in which only the solution containing the test substance was omitted and replaced by an equal volume of water. Absorbance changes at 412 nm were recorded for 30 min using an automatic recorder with the Beckman model 26 spectrophotometer. This procedure was checked with cysteine, reduced glutathione, acetyl-CoA and citrate lyase. Solutions of acetyl-CoA were standardized by enzymic assay with pig heart citrate synthase and OAA in presence of DTNB as described by Tubbs and Garland (1969).

In these reactions the color was seen to fade slowly probably due to slow oxidation of thiol ions. This was corrected for by extrapolating the titration curves to zero time. The presence of 2.5 mM EDTA or nitrogen atmosphere did not affect the titration curves.

Estimation of acylated -SH groups of proteins: The test solution in 0.05 M potassium phosphate buffer, pH 7.4,

containing 1.6 mM Mg^{2+} was incubated with equal volume of neutralised 2 M hydroxylamine for 30 min at 30°C. An aliquot of 0.1 ml corresponding to 0.2 - 0.5 mg protein was taken in the cuvette for DTNB titration as described above.

The number of acylated -SH groups in the enzyme were calculated by difference between the -SH groups obtained by DTNB titration in the presence and absence of hydroxylamine.

Deacylation of enzyme sulphydryls during reaction-inactivation:

The native enzyme (1%, 1.5 mg) was incubated with equal volume of 2 M sodium citrate containing 1.6 mM Mg^{2+} , pH 8.0, and incubated for 15 min at 30°C. That the enzyme was completely inactivated at the end of the period of incubation was established by the coupled enzyme assay with malate dehydrogenase. The citrate-treated, inactive enzyme was used for -SH estimation by DTNB titration in presence of 0.9% SDS. The number of acylated -SH groups unmasked during reaction-inactivation were obtained by difference between the -SH values obtained with the inactivated and the native enzyme.

Characterization of S-acylated residues in reaction-inactivated citrate lyase using Iodo[2- ^{14}C]acetate:

The pure native enzyme (1mg/0.1ml) was reaction-inactivated as described above by incubation with an equal volume of 2 M sodium citrate, pH 8.0, containing 1.6 mM Mg^{2+} for 15 min at 30°C. To the inactive enzyme 2 mg of iodoacetic acid in 0.2 ml water was added and the pH was maintained between 7.5 to 8 by the addition of 20 mg of potassium bicarbonate. The mixture was incubated at 30°C for 60 min in the dark under an atmosphere of nitrogen and then dialysed against 0.01 M

potassium phosphate buffer, pH 7.0, at 0 to 4°C. The dialysed solution was saturated with nitrogen and then reacted with 0.1 ml of 2 M hydroxylamine solution, pH 7.0, containing 10 mM DTT. The reaction mixture was incubated at 30°C for 60 min in the dark and left for dialysis against 0.01 M potassium phosphate buffer, pH 7.4 at 0 to 4°C. The dialysed solution was taken to 1.0 ml in a lyophiliser and the solution incubated for 60 min in the dark at 30°C under nitrogen with 20 μ Ci of iodo[14 C]acetate (1 μ mol) in 0.1 ml water. The labelled enzyme was loaded on a Sephadex G-25 column (40 x 1.8 cm) equilibrated with 0.1% NH_4HCO_3 . The protein fractions were pooled and lyophilised.

An aliquot of the S-carboxymethylated enzyme (21 000 cpm) and 2.2 μ mol of S-carboxymethyl cysteamine were taken in a glass tube and hydrolysed by the procedure of Moore and Stein (1963) for 24 h using 6 N HCl. The acid was removed on a rotary evaporator at 40°C. The sample was dissolved in water, taken to dryness, and redissolved in water and dried again. About 2.0 μ mol of S-carboxymethyl cysteine was added as carrier to the sample and dissolved in 0.2 ml water. The labelled derivative was separated on Cellogel strips using a solvent system of pyridine, acetic acid and water (1:10:89) (Dimroth *et al.*, 1973). Electrophoresis was carried out at 2 to 3 mA for 1 h. A mixture of the reference compounds, S-carboxymethyl cysteine and S-carboxymethyl cysteamine (2 μ mol each) was electrophoretically separated under identical conditions on a separate Cellogel strip and their positions after migration visualised by spraying with 0.1% ninhydrin

in acetone. The Cellogel strip with the hydrolysed enzyme was cut into pieces at the end of the run and radioactivity located by suspending in a scintillation solution (PPO 5 g, Naphthalene 100 g and Dioxane to make 1 L) and counting on a Beckman LS 1000 liquid scintillation counter.

Separation of citrate lyase subunits:

The separation of subunits of citrate lyase reported in the literature make use of urea (Carpenter et al., 1975; Dimroth and Eggerer, 1975) or guanidine.HCl (Singh et al., 1976) to dissociate the protein, followed by either gel filtration or resolution on DEAE-cellulose. Since in the present study the separated subunits were required only for determination of their half-cystine contents, the simpler procedure of dissociating with 2% SDS in presence of 2-ME followed by gel-filtration through Sephadex was standardised to give total separation of the component subunits.

A solution of the pure holoenzyme (8 mg/0.5 ml 0.05 M potassium phosphate buffer, pH 7.5) was treated with an equal volume of a solution containing 4% SDS and 2% 2-ME and heated 3 min in a boiling water bath to dissociate the complex. The solution was cooled and filtered through a 90 x 1 cm Sephadex G-100 column which had been previously washed and equilibrated with 0.05 M sodium phosphate buffer, pH 7.3, containing 0.1% SDS. The protein was eluted with 0.05 M sodium phosphate buffer, pH 7.3 containing 0.1% SDS and fractions (3 ml) were tested for eluted protein spectrophotometrically by measuring absorbance at 280 nm. repeat

Determination of total half-cystine residues by amino acid analysis:

Total half-cystine residues in the enzyme complex and in the separated α - and β -subunits were determined as cysteic acid residues by performic acid oxidation followed by hydrolysis with 6 N HCl. Determinations with the γ -subunit were not carried out as this subunit was obtained in very low yield.

The holoenzyme (about 0.3 mg), α -subunit (0.5 mg) and β -subunit (0.2 mg) were separately taken in glass tubes (18 x 150 mm), lyophilised and oxidised with performic acid according to the procedure described by Hirs (1967). The performic acid reagent was prepared by mixing 1.0 ml of 30% H_2O_2 and 9.0 ml of 88% formic acid and allowing the mixture to stand for one hour at room temperature, followed by cooling to 0°C in an ice bath just before use. The cold reagent (2.0 ml) was then added to the protein sample and left at 0°C for 4 h. To each sample 48% HBr (0.3 ml) was added separately with swirling at 0°C to destroy excess of the performic acid reagent and the samples taken to dryness in a rotary evaporator at 40°C.

The oxidised samples were separately hydrolysed at 110°C for 24 h by the procedure of Moore and Stein (1963) using 3.0 ml of 6 N HCl. Excess acid was removed from the hydrolysates in a rotary evaporator and each sample then dissolved in 2.0 ml water and again taken to dryness. The evaporation after addition of water was repeated once. The residue was finally dissolved in citrate buffer, pH 2.2, and an aliquot used for

amino acid analysis on a Beckman Model 120C amino acid analyser. The Beckman calibration mixture used for standardisation was treated identically.

Results

Estimation of -SH groups by DTNB titration:

The titration of the holoenzyme with DTNB was carried out both in the absence and in the presence of 0.9% SDS as denaturant and the results are summarised in Table II.1 and the titration curves are represented in Fig. II.1 (A and B).

TABLE II.1: DETERMINATION OF SULFHYDRYL GROUPS OF CITRATE LYASE (K.aerogenes) WITH DTNB

Citrate lyase $M_r = 575\ 000$; Temp. 28°C ; Wavelength 412 nm .

i) Determination of -SH groups in absence of denaturant:

Citrate lyase (about 1 mg) in 1.0 ml of 0.25 M Tris-HCl buffer, pH 8.0, titrated with 0.01 ml of 0.01 M DTNB in 0.25 M Tris buffer, pH 8.0.

ii) Determination of -SH groups in presence of 0.9% SDS:

Citrate lyase (about 0.3 mg) in 1.0 ml of 0.25 M Tris buffer, pH 8.0, containing 0.9% SDS treated with 0.01 ml of 0.01 M DTNB.

System	[SH] / [Enzyme]
i) Citrate lyase in 0.25 M Tris buffer, pH 8.0 + DTNB	3.0 3.7 3.0
Average	3.2
ii) Citrate lyase in 0.25 M Tris buffer, pH 8.0, containing 0.9% SDS + DTNB	42.3 42.6 41.4 41.8
Average	42.0

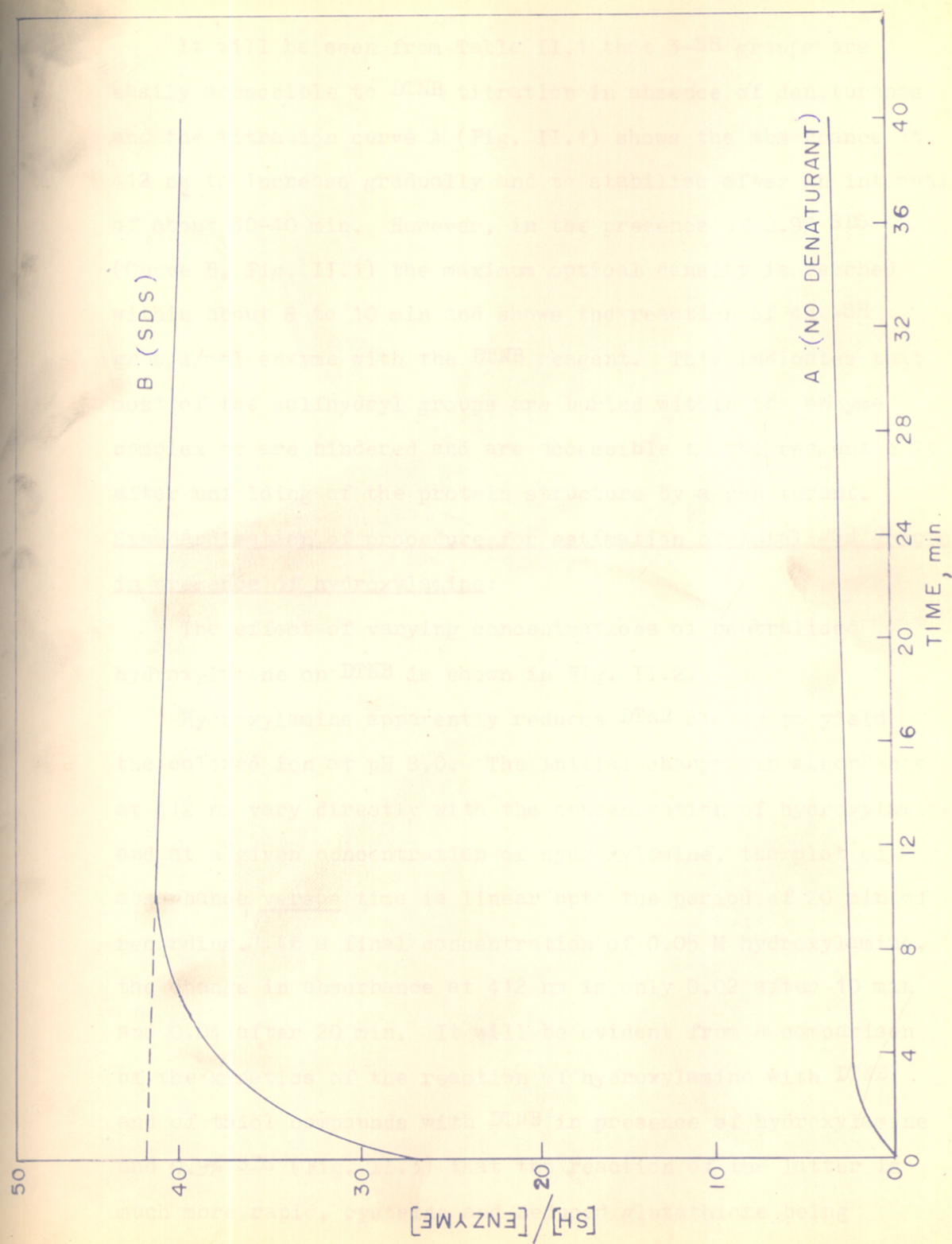


FIG. II. 1: REACTION OF -SH GROUPS OF CITRATE LYASE (*K. aerogenes*) WITH DTNB

It will be seen from Table II.1 that 3-SH groups are easily accessible to DTNB titration in absence of denaturants and the titration curve A (Fig. II.1) shows the absorbance at 412 nm to increase gradually and to stabilise after an interval of about 30-40 min. However, in the presence of 0.9% SDS (Curve B, Fig. II.1) the maximum optical density is reached within about 8 to 10 min and shows the reaction of 42 -SH groups/mol enzyme with the DTNB reagent. This indicates that most of the sulphhydryl groups are buried within the enzyme complex or are hindered and are accessible to the reagent only after unfolding of the protein structure by a denaturant.

Standardisation of procedure for estimation of total -SH groups in presence of hydroxylamine:

The effect of varying concentrations of neutralised hydroxylamine on DTNB is shown in Fig. II.2.

Hydroxylamine apparently reduces DTNB slowly to yield the colored ion at pH 8.0. The initial changes in absorbance at 412 nm vary directly with the concentration of hydroxylamine and at a given concentration of hydroxylamine, the plot of absorbance versus time is linear upto the period of 20 min of recording. At a final concentration of 0.05 M hydroxylamine, the change in absorbance at 412 nm is only 0.02 after 10 min and 0.04 after 20 min. It will be evident from a comparison of the kinetics of the reaction of hydroxylamine with DTNB and of thiol compounds with DTNB in presence of hydroxylamine and 0.9% SDS (Fig. II.3) that the reaction of the latter is much more rapid, cysteine and reduced glutathione being completely reacted within 10 s and acetyl-CoA and citrate

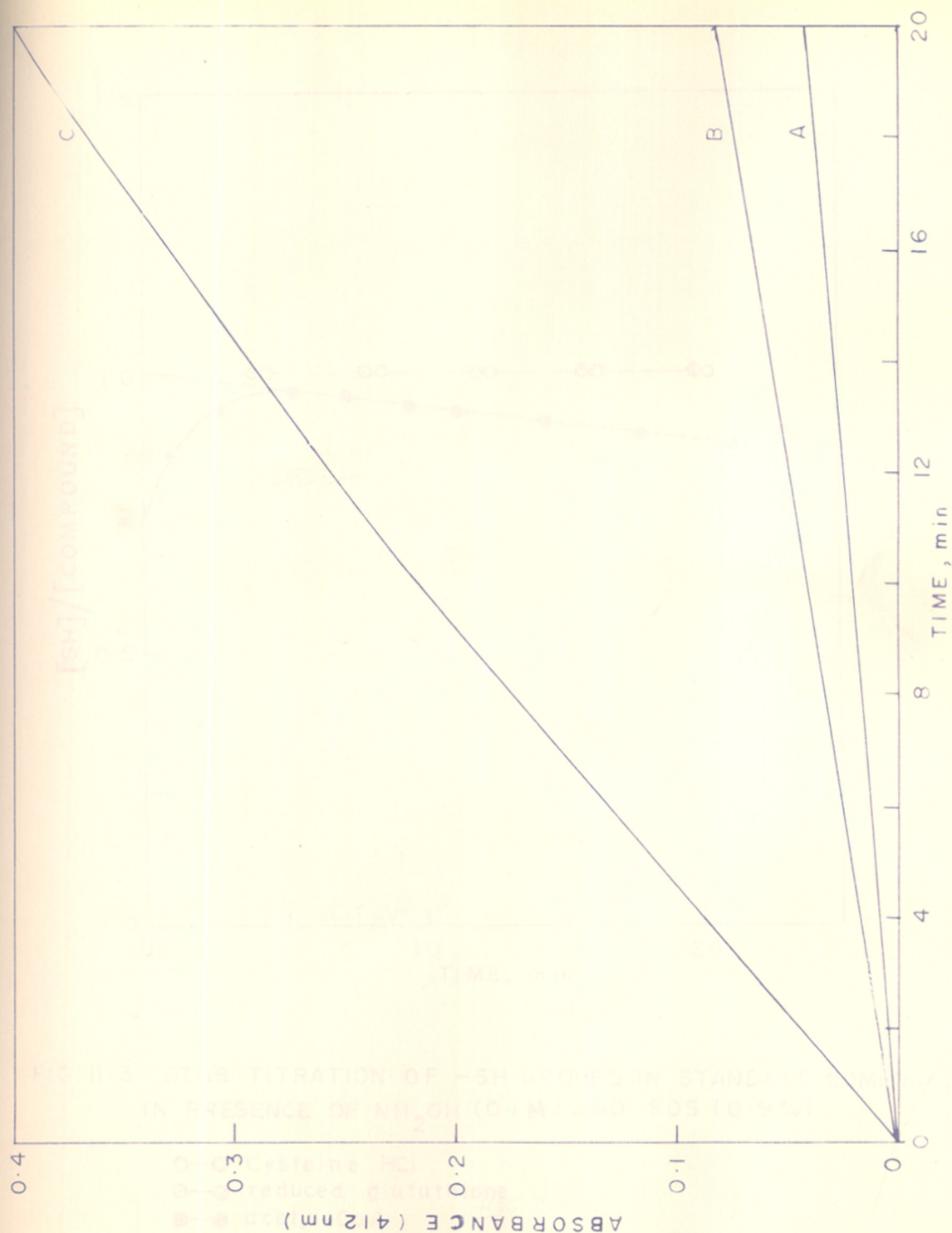


FIG. II. 2 : EFFECT OF HYDROXYLAMINE ON DTNB (0.25 M Tris-HCl buffer, pH 8.0, with 0.9% SDS) Final concentrations of NH_2OH in cuvette : A-0.05 M, B-0.10 M, C-0.50 M

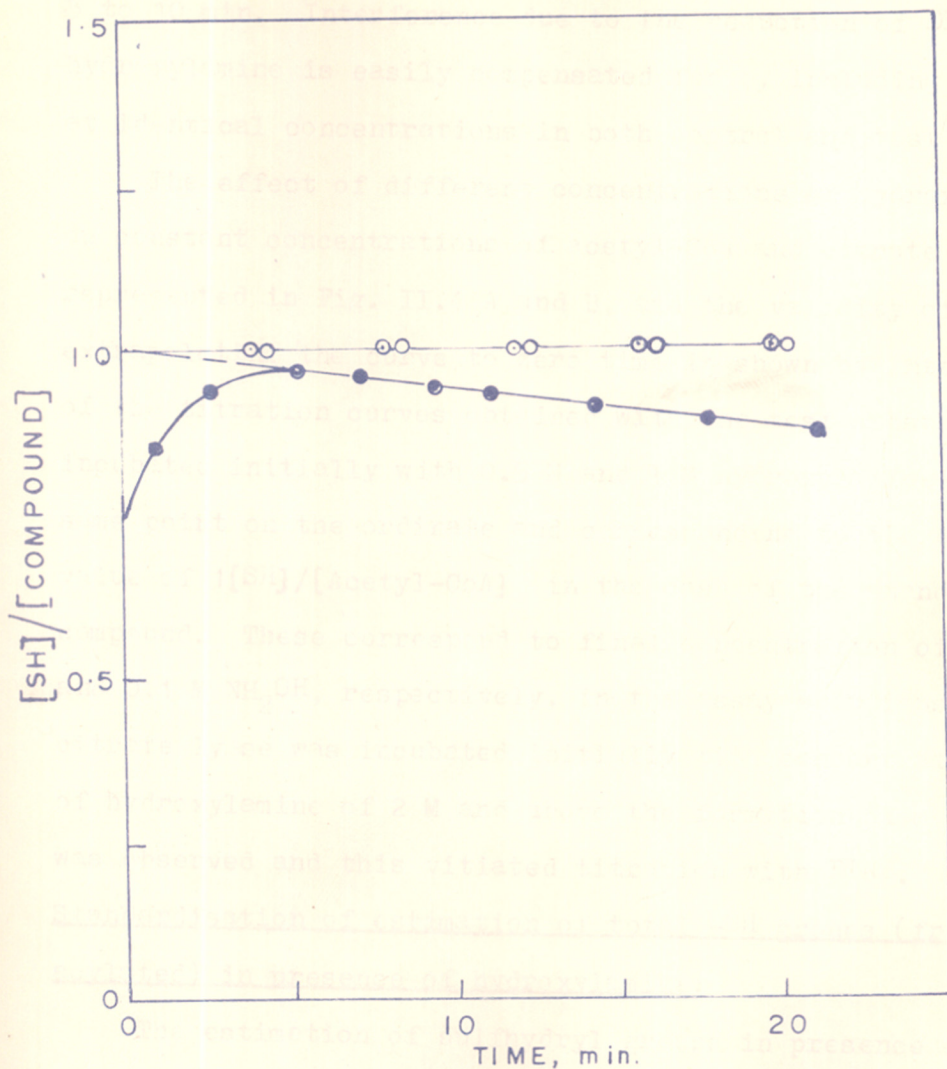


FIG. II-3 : DTNB TITRATION OF $-SH$ GROUPS IN STANDARD COMPOUNDS IN PRESENCE OF NH_2OH (0.1M) AND SDS (0.9%)

- Cysteine HCl
- ⊙—⊙ reduced glutathione
- acetyl-CoA

lyase (Fig. II.4 A and B) showing maximum absorbance within 5 to 10 min. Interference due to the reduction of DTNB by hydroxylamine is easily compensated for by including hydroxylamine at identical concentrations in both control and test sample.

The effect of different concentrations of hydroxylamine on constant concentrations of acetyl-CoA and citrate lyase is represented in Fig. II.4 A and B, and the validity of extrapolating the curve to zero time is shown by intersection of the titration curves obtained with the test substances incubated initially with 0.5 M and 1 M hydroxylamine at the same point on the ordinate and corresponding to the expected value of $1[\text{SH}]/[\text{Acetyl-CoA}]$ in the case of the standard compound. These correspond to final concentration of 0.05 M and 0.1 M NH_2OH , respectively, in the assay solutions. When citrate lyase was incubated initially with concentrations of hydroxylamine of 2 M and above the formation of a precipitate was observed and this vitiated titration with DTNB.

Standardisation of estimation of total -SH groups (free and acylated) in presence of hydroxylamine:

The estimation of sulphhydryl groups in presence of hydroxylamine was tested with standard L-cysteine, reduced glutathione and acetyl-CoA. The spectrophotometric titration curves are reproduced in Fig. II. 3. It is evident that hydroxylamine does not interfere with the sulphhydryl group estimation of cysteine and glutathione as the studies give the expected value of $1[\text{SH}]/\text{mol}$. Reaction of the sulphhydryl groups with DTNB is almost instantaneous and complete within 10 s of addition of DTNB. The color is stable and no decrease

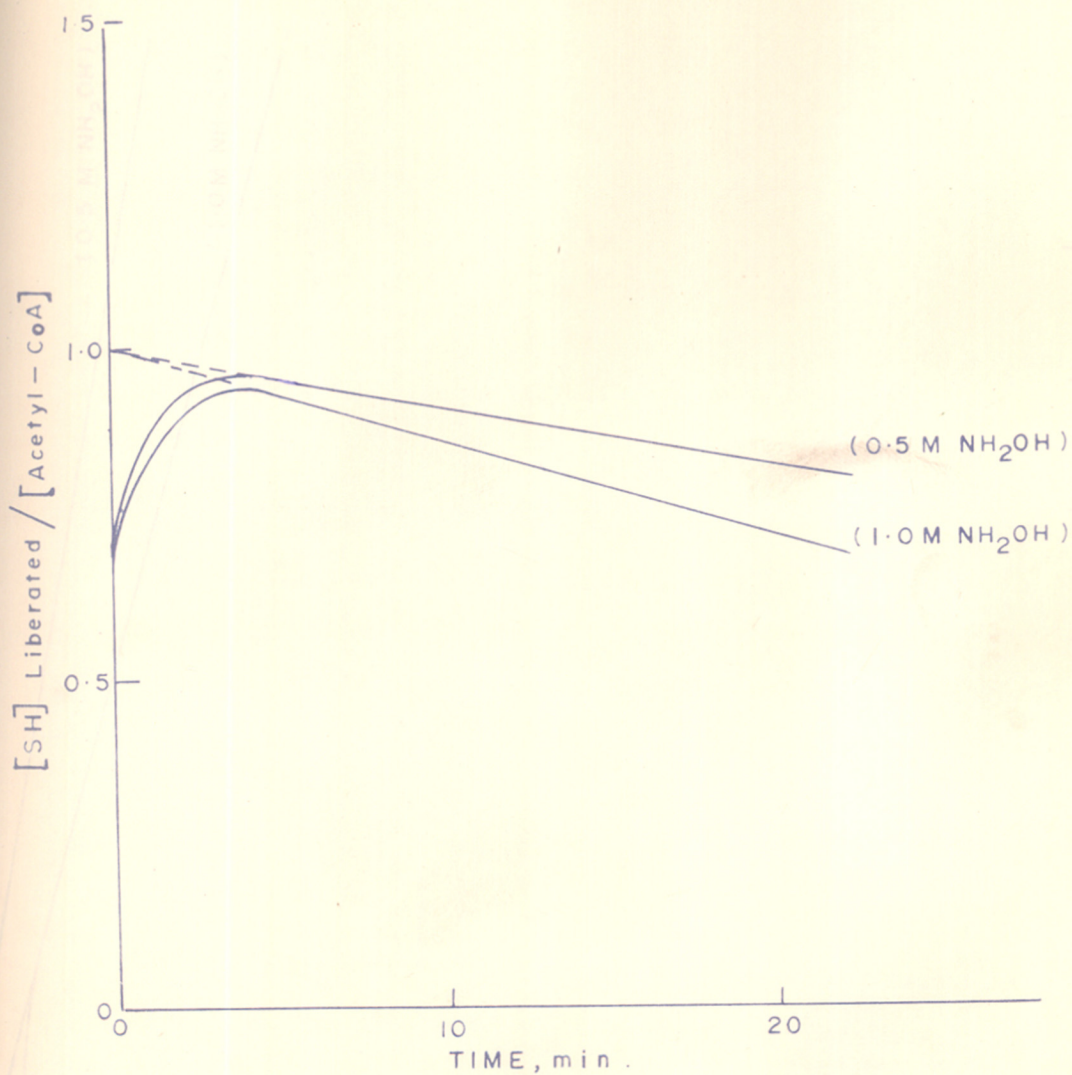


FIG. II.4 A : EFFECT OF VARYING CONCENTRATIONS OF NH_2OH IN INCUBATION MIXTURE ON ESTIMATION OF -SH RELEASED FROM ACETYL-CoA BY DTNB TITRATION IN PRESENCE OF 0.9% SDS

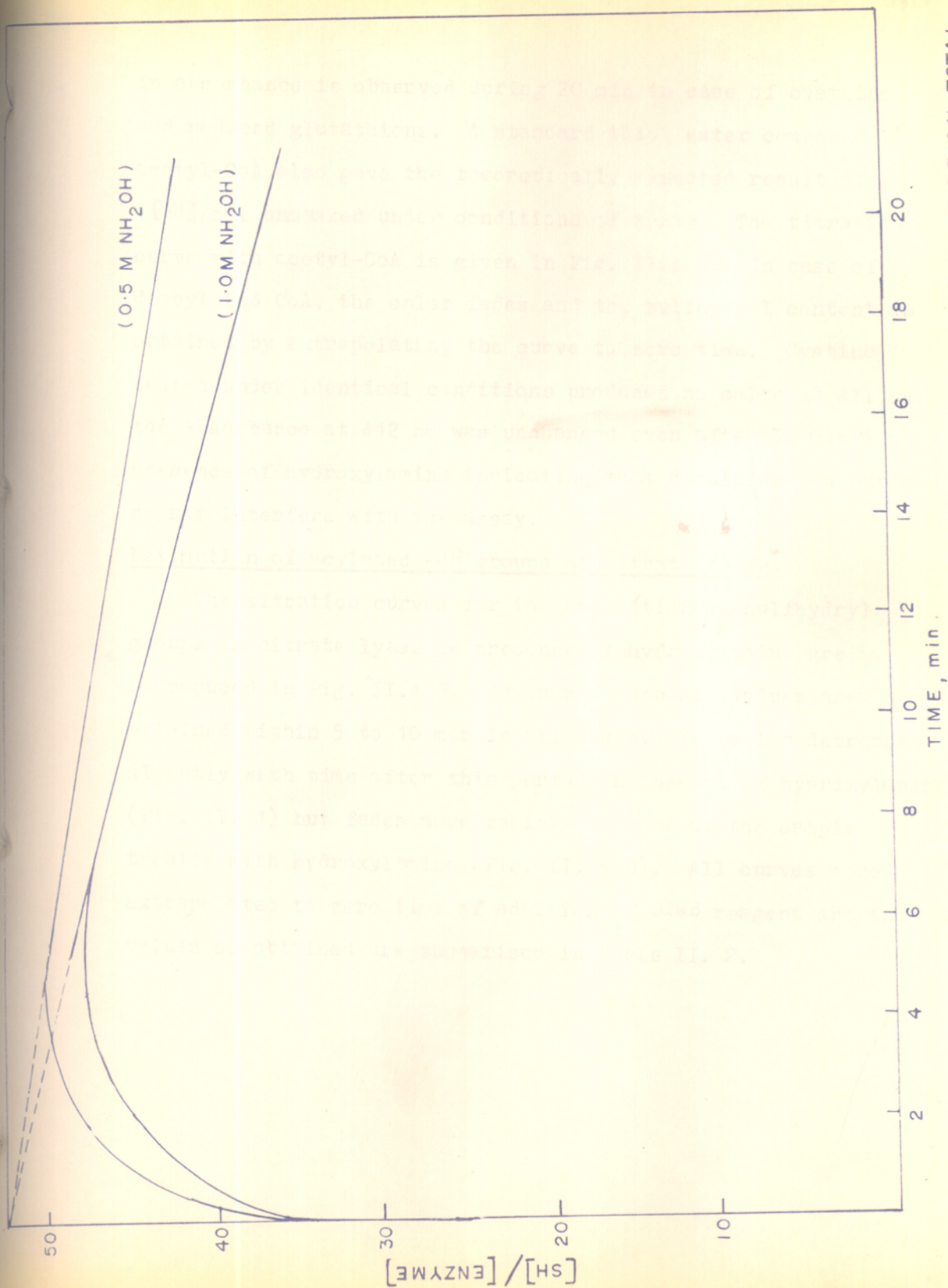


FIG. II.4B: EFFECT OF VARYING CONCENTRATIONS OF NH_2OH IN INCUBATION MIXTURE ON TOTAL -SH ESTIMATION IN CITRATE LYASE BY DTNB TITRATION IN PRESENCE OF 0.9% SDS.

in absorbance is observed during 20 min in case of cysteine and reduced glutathione. A standard thiol ester compound like acetyl-CoA also gave the theoretically expected result of $1[\text{SH}]/\text{mol}$ unmasked under conditions of assay. The titration curve with acetyl-CoA is given in Fig. II.4 A. In case of deacylated CoA, the color fades and the sulfhydryl content is obtained by extrapolating the curve to zero time. Cystine tested under identical conditions produced no color at all and the absorbance at 412 nm was unchanged even after 30 min in presence of hydroxylamine indicating that disulfide compounds do not interfere with the assay.

Estimation of acylated -SH groups of citrate lyase:

The titration curves for the estimation of sulfhydryl groups in citrate lyase in presence of hydroxylamine are reproduced in Fig. II.4 B. Maximum absorbance values are obtained within 5 to 10 min in all cases. The color decreases slightly with time after this period in absence of hydroxylamine (Fig. II. 1) but fades more rapidly in case of the sample treated with hydroxylamine (Fig. II. 4 B). All curves were extrapolated to zero time of addition of DTNB reagent and the values so obtained are summarised in Table II. 2.

TABLE II.2: ESTIMATION OF TOTAL -SH GROUPS (FREE + ACYLATED) OF CITRATE LYASE

Citrate lyase (0.5 - 1.0 mg in 0.1 ml) incubated with equal volume of 2 M NH_2OH , pH 7.0, at 25°C for 30 min. An aliquot of 0.1 ml of incubation mixture (0.25 - 0.5 mg protein) taken in 1 ml capacity silica cuvette of 10 mm light path. The volume was adjusted to 0.5 ml with water and 0.5 ml of 0.5 M Tris buffer, pH 8.0, containing 1.8% SDS was added. The solution was then mixed and treated with 0.01 ml of 0.01 M DTNB.

Citrate lyase $M_r = 575\ 000$.

System	[SH] / [Enzyme]
Citrate lyase - 0.1 M NH_2OH - 0.9% SDS	52.0
	52.8
- 0.25 M Tris buffer, pH 8.0 + DTNB	51.5

Average	52

Estimation of total acylated -SH groups:

The acylated -SH groups in the holoenzyme are obtained by difference between the -SH groups/mol enzyme reacting with DTNB in presence of 0.9% SDS after prior deacylation with 1 M NH_2OH , pH 7.0, and the values obtained by reaction with DTNB in presence of 0.9% SDS without hydroxylamine treatment. The data are summarized in Table II.3.

TABLE II.3: ESTIMATION OF S-ACYLATED GROUPS IN CITRATE LYASE

	[SH]/[Enzyme]		[Acyl-S-]/[Enzyme]
	In presence of NH_2OH	In absence of NH_2OH	
	(A)	(B)	(A - B)
	52.0	42.3	9.7
	52.8	42.6	10.2
	51.5	41.4	10.1
Average	52	42	10

It will be seen that a total of 52[SH]/mol are estimated after treatment of the enzyme with NH_2OH followed by SDS. In the absence of the deacylating reagent, 42[SH]/mol are estimated indicating that 10 of the total of 52 are present as S-acylated residues in the complex.

Determination of -SH groups in reaction-inactivated citrate lyase:

Table II.4 summarises data on -SH groups in citrate lyase rendered inactive in the course of the reaction it catalyses.

TABLE II.4: DETERMINATION OF SULPHYDRYL GROUPS IN REACTION-
INACTIVATED CITRATE LYASE

All determinations were carried out in presence of 0.9% SDS.

Active Enzyme	Reaction- inactivated enzyme	NH ₂ OH - treated enzyme	-SH groups exposed on reaction- inactivation	Reaction- inactivated enzyme
[SH]/ [Enz]	[SH]/ [Inact.Enz]	[SH + Acyl-S]/ [Enz]	[SH]/[Enz]	[Acyl-S]/ [Enz]
(A)	(B)	(C)	(B - A)	(C - B)
42.3	45.3	51.6	3.0	6.3
42.6	46.7	52.8	4.1	6.1
41.4	44.9	52.0	3.5	7.1
42.1	46.5	52.1	4.4	5.6
-----	-----	-----	-----	-----
Average 42	46	52	4	6

It will be seen from Table II.4 that reaction-inactivation of citrate lyase results in the deacylation of about 4 -SH residues/mol enzyme and the inactive enzyme still retains 6 S-acylated residues/mol enzyme.

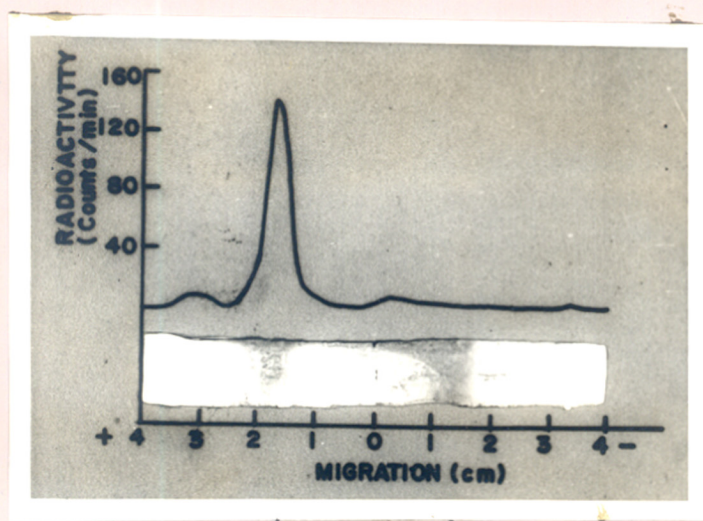
Characterization of S-acylated residues in citrate lyase:

As shown in Table II.4 citrate lyase inactivated by treatment with citrate in presence of Mg²⁺ is partially deacylated while retaining some S-acylated residues. The nature of the S-acylated residues resistant to deacylation during reaction-inactivation was characterised as described under Experimental Procedures by S-carboxymethylation of the inactive enzyme first with cold iodoacetate followed by

complete deacylation with hydroxylamine and labelling of the newly unmasked -SH groups with iodo[2-¹⁴C]acetate. The characterization of the labelled S-carboxymethylated residues obtained after acid hydrolysis and electrophoresis of the hydrolysate is shown in Fig. II.5. Under the conditions of electrophoresis standard S-carboxymethyl cysteine migrates approximately 1.8 cm towards the anode and S-carboxymethyl cysteamine migrates approximately 1.0 cm towards the cathode. The distribution of radioactivity after electrophoresis of the acid hydrolysates of iodo[2-¹⁴C]acetate labelled protein clearly indicates that the activity is associated almost exclusively with the S-carboxymethyl derivative of cysteine while none of the radioactive label is associated with the S-carboxymethylated cysteamine. These results show that the cysteamine residue alone is deacylated during reaction-inactivation while the cysteine remains S-acylated.

Determination of exposed S-acylated groups:

The titration of sulfhydryl groups exposed in presence of DTT was carried out using DTNB in the presence of arsenite. The results are tabulated in Table II.5.



S-Carboxymethyl cysteine

S- carboxymethyl cysteamine

FIG.II.5: CHARACTERIZATION OF S-ACYLATED RESIDUES IN
REACTION-INACTIVATED CITRATE LYASE

Inactivated enzyme treated with cold iodoacetate followed by deacylation with NH_2OH and subsequent treatment with iodo[2- ^{14}C] acetate. The acid hydrolysate of the enzyme so treated was separated by electrophoresis.

Distribution of ^{14}C -label is shown graphically above electrophoretogram of standard S-carboxymethyl derivatives of cysteine and cysteamine visualised with ninhydrin.

Electrophoresis on Cellogel strips; pyridine-acetic acid-water (1:10:89); 1 h, 2 mA/strip.

TABLE II.5: DETERMINATION OF -SH GROUPS BY DTNB TITRATION
IN PRESENCE OF DTT AND ARSENITE

Citrate lyase (1 to 2 mg in 0.2 ml) incubated in presence of 0.1 ml of 0.05 M Tris buffer, pH 9.0 with 0.1 ml of 3 mM DTT. for 30 min at 28°C. 0.2 ml of 0.1 M Tris buffer, pH 8.1 and 1.5 ml sodium arsenite (5 mM in 40 mM EDTA) were then added followed by water to make volume upto 3.0 ml. Final solution was treated with 0.1 ml of 3 mM DTNB in 0.05 M acetate buffer, pH 5.0.

Citrate lyase M_r = 575 000.

System	[SH] / [Enzyme]
Citrate lyase - Tris buffer - DTT	6.0
and arsenite + DTNB	6.3
Average	6

Table II.5 indicates that a total of 6 -SH groups/mol enzyme are available for reaction with DTNB in the presence of a mercaptan like DTT.

DTNB titration of the enzyme in presence of hydroxylamine but absence of denaturants gave a value of 6.8 [SH] /mol enzyme which is in close agreement with the value obtained by titration with DTNB in presence of DTT and arsenite (Table II.5).

Isolation of subunits of citrate lyase:

The elution profile of the subunits separated by filtration through Sephadex G-100 column in presence of 0.1% SDS is shown

in Fig. II.6. The three subunits are separated on filtration. The α -subunit (M_r 54 000) emerges between 27 to 33 ml, the β -subunit (M_r 32 000) between 39 to 48 ml and the γ -subunit (M_r 10 000) between 90 to 102 ml.

All isolated subunits moved as single bands in SDS-polyacrylamide gel electrophoresis, indicating their homogeneity. Aliquots of the fractions containing α - and β -subunits were pooled separately and lyophilised before performic acid oxidation and estimation of half-cystine residues as cysteic acid.

Determination of total half-cystine residues:

The half-cystine residues in the holoenzyme and the separated subunits were determined as cysteic acid by amino acid analysis and the results obtained are presented in Table II.6.

TABLE II.6: DETERMINATION OF TOTAL HALF-CYSTINE RESIDUES IN CITRATE LYASE (K.aerogenes) AND ITS SUBUNITS

Sample	Protein in mg	M_r	No. of cysteic acid residues/mol
Citrate lyase holoenzyme	0.2 - 0.4	575 000	49.0
			47.0
			average 48.0
α -subunit	0.5	54 000	5.2
β -subunit	0.2	32 000	2.3
γ -subunit*	-	10 000	1.0

* Value obtained by Dimroth (1975).

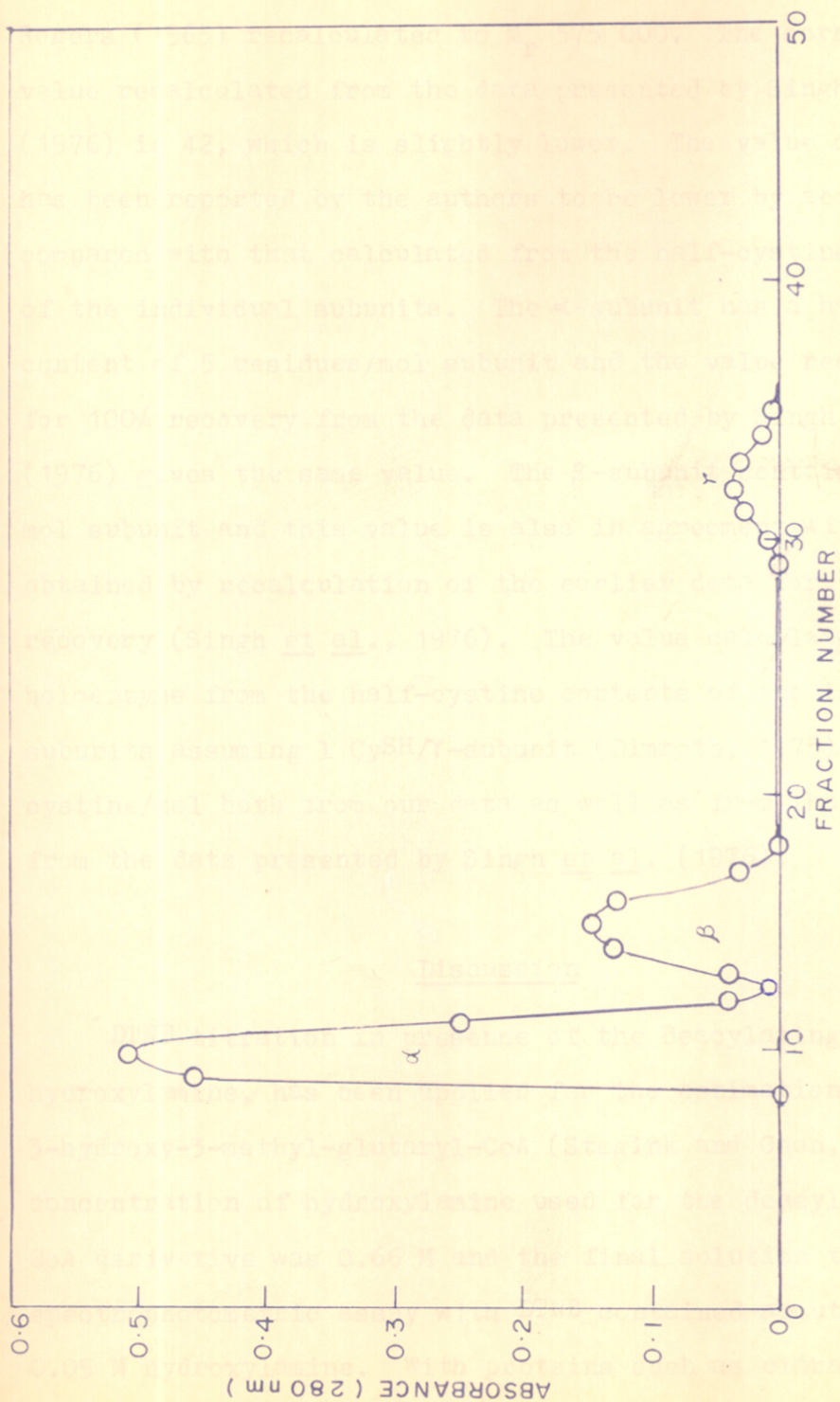


FIG.II 6: SEPARATION OF CITRATE LYASE ON SEPHADEX G-100 COLUMN (90 x 1 cm) IN PRESENCE OF 0.1% SDS INTO COMPONENT SUBUNITS (α , β , γ)

The half cystine content of 48 residues/mol holoenzyme is in close agreement with the value of 47 reported by Bowen and Rogers (1965) recalculated to M_r 575 000. The corresponding value recalculated from the data presented by Singh et al. (1976) is 42, which is slightly lower. The value of 42 residues has been reported by the authors to be lower by about 14% compared with that calculated from the half-cystine contents of the individual subunits. The α -subunit has a half-cystine content of 5 residues/mol subunit and the value recalculated for 100% recovery from the data presented by Singh et al. (1976) gives the same value. The β -subunit contains 2 residues/mol subunit and this value is also in agreement with that obtained by recalculation of the earlier data for 100% recovery (Singh et al., 1976). The value calculated for the holoenzyme from the half-cystine contents of the individual subunits assuming 1 CySH/ α -subunit (Dimroth, 1975) is 48 half-cystine/mol both from our data as well as from that calculated from the data presented by Singh et al. (1976).

Discussion

DTNB titration in presence of the deacylating agent, hydroxylamine, has been applied for the estimation of 3-hydroxy-3-methyl-glutaryl-CoA (Stegink and Coon, 1968). The concentration of hydroxylamine used for the deacylation of the CoA derivative was 0.66 M and the final solution taken for the spectrophotometric assay with DTNB contained about 0.04 - 0.05 M hydroxylamine. With proteins such as citrate lyase (Buckel et al., 1971a), citramalate lyase (EC 4.1.3.22)

(Buckel and Bobi, 1976) and the acylated acyl carrier protein of fatty acid synthetases (Prescott and Vagelos, 1972) which contain acylated sulphhydryl groups, the concentration of neutral hydroxylamine solution required for quantitative deacylation in a period of about 30 min is usually 1 M to 2 M. We observed, particularly at higher concentrations of hydroxylamine, that the direct spectrophotometric titrations with DTNB are vitiated through an apparent reduction of DTNB by the hydroxylamine.

A procedure has been standardised by us in which the hydroxylamine treated protein is diluted to a final concentration of 0.1 M or less of the reagent before spectrophotometric titration. The addition of an equivalent amount of NH_2OH to the reagent blank compensated for the DTNB- NH_2OH reaction. The validity of extrapolating titration curves to zero time of DTNB addition was established by varying the final NH_2OH concentration upto 0.1 M and showing that the absorbance versus time plots intercept at the same point on the ordinate. That the values obtained by this procedure are valid is established with sulphhydryl determination in cysteine and reduced glutathione where the expected values were obtained even in presence of NH_2OH . The precision of the new procedure was proven with an authentic acetyl-CoA sample. The method gives a rapid procedure for the assay of S-acylated sulphhydryl groups in proteins from the amount of -SH groups released after hydroxylaminolysis. In our hands the more tedious procedure of preliminary deacylation with NH_2OH followed by removal of excess reagent by dialysis or gel-filtration prior to

determination of the -SH groups has proved impracticable. Values obtained were erratic and low, probably due to the high reactivity of the unmasked -SH groups and consequent reoxidation during the separation procedure. The present procedure of estimating the -SH groups liberated in the presence of hydroxylamine without any separation of the deacylating reagent on the other hand is rapid and yields uniformly reproducible results.

The results of the various assays of -SH groups under different conditions of treatment are summarised in Table II.7.

TABLE II.7: -SH GROUPS OF CITRATE LYASE ACCESSIBLE TO DTNB
UNDER VARYING CONDITIONS

Citrate lyase (M_r 575 000), 48 half-cystine/mol.

Conditions	[SH] / [Enzyme complex]
Native enzyme	3
Native enzyme + 1 M NH_2OH	7
Native enzyme + DTT	6
Enzyme + 0.9% SDS	42
Enzyme + 1 M NH_2OH + 0.9% SDS	52
Reaction-inactivated enzyme + 0.9% SDS	46

Although under denaturing conditions 42 -SH groups are available for DTNB reaction, only about 3 of these apparently are exposed in the native complex (Table II.7). The large number of sulfhydryls not estimated by this reagent under non-denaturing conditions may indicate that these are either

buried within the complex or are unavailable for a bulky reagent like DTNB through steric hindrances. Since the 3 non-identical subunits are known to be present in 6 copies each in the complex, it would appear that only a single -SH group on an individual subunit is available for reaction and of these only half react with DTNB. The reactive -SH would have to be in the α - or β -subunit since the ACP is known to have only a single cysteine as an S-acylated derivative. The presence of such a masked -SH has been indicated qualitatively by Dimroth et al. (1973) and quantitatively shown to be 1 acyl-S / γ -subunit in the present work.

The total number of -SH groups estimated under non-denaturing conditions after deacylation by treatment either with DTT or hydroxylamine are about 6 - 7 indicating that 3 - 4 exposed S-acylated groups are present in the native complex. Both DTT and hydroxylamine treatment resulted in the total inactivation of the enzyme. The -SH groups exposed on treatment with these reagents are evidently contributed by the essential S-acetylated cysteamine residues of the (phosphoribosyl) dephospho-CoA prosthetic group. One would indeed expect the prosthetic group to be in the exposed hydrophilic region of the complex. The S-acylated cysteine residues which are known to be present in the ACP subunit are deacylated with NH_2OH under non-denaturing conditions as shown in iodoacetate labelling experiments but possibly not being exposed to the outside environment, do not react with DTNB.

The number of sulfhydryl groups estimated after deacetylation with hydroxylamine under denaturing conditions

(0.9% SDS) is 52. Since the denatured complex in the absence of the deacylating reagent shows 42 -SH groups/mol enzyme, the total number of S-acylated -SH residues are 10/mol enzyme. Of these 3 - 4 would be contributed by the S-acetyl cysteamine residue of the prosthetic group. Although citrate lyase has been shown to contain six ACP subunits (Singh et al., 1976) only 3 - 4 of these apparently carry the prosthetic group since a variety of procedures used for the assay of prosthetic group components have resulted in the identification of only 3 or 4 instead of the total possible of six. This would indicate the presence of about 6 S-acylated cysteine residues in the enzyme complex. These are evidently contributed by the single cysteine residues present in each of the six 10 000 dalton ACP subunits which have been qualitatively shown earlier to be present as S-acylated residues (Dimroth et al., 1973).

On the complete reaction-inactivation of the enzyme by incubating with citrate in presence of Mg^{2+} , a total of 46 -SH groups/mol enzyme complex were estimated after denaturing with 0.9% SDS. This would suggest that all the 3 - 4 cysteamine residues are deacylated under the process of inactivation while the 6 S-acylated cysteine residues in the ACP subunit remain intact. This was confirmed by treatment of the reaction-inactivated complex with unlabelled iodoacetate followed by deacylation with NH_2OH and then treatment with iodo[2- ^{14}C]acetate. On subsequent total acid hydrolysis of the labelled protein and separation of the products, the radioactive ^{14}C -label was detected exclusively

in the cysteine and not the cysteamine, indicating that the former existed as the S-acylated residue in the reaction-inactivated enzyme. The S-acylated cysteamine residues in the native protein on the other hand, are completely deacetylated during reaction-inactivation. This is the first report of the stability of the S-acylated cysteine to deacylation under conditions of reaction-inactivation. This would also exclude unequivocally the possibility of the S-acylated cysteine residues functioning as acyl carriers in the reaction mechanism of citrate lyase.

A value of 48 total half-cystine residues/mol in the citrate lyase complex was obtained by performic acid oxidation. Determination of the total -SH groups in the complex by spectrophotometric titration with DTNB under denaturing conditions and after deacylation with NH_2OH gave a value of 52 -SH groups/mol enzyme. Since about 4 of these -SH groups are contributed by the cysteamine residue of the prosthetic group all half-cystine residues in the complex are present evidently as cysteine residues in the enzyme. Citrate lyase from K.aerogenes evidently has no cystine residues.

The nature of the half-cystine residues in the complex has not been reported hitherto. In the present study the separated subunits of the citrate lyase complex in K.aerogenes have been shown to have 5 CySH/mol α -subunit (M_r 54 000) and 2 CySH/mol β -subunit (M_r 32 000). Earlier studies by others have established the presence of 1 CySH/mol ACP (M_r 10 000) (Dimroth et al., 1973). This has been shown in our work to be completely as S-acylated residue in the native protein.

The complex has no cystine residue.

Beuscher et al. (1974) have reported data on the -SH groups of citrate lyase from R.gelatinosa. The DTE treated enzyme has been reported to give 21 -SH/mol with DTNB while under denaturing conditions the values reported are 54 with 1% SDS and 36 with 8 M urea. With urea containing systems, low and erratic values have also been observed by us, particularly when ascorbate is omitted from the samples (Kaplan and Flavin, 1966). This might explain a value of 18 -SH reported by Singh et al. (1976) for the enzyme from K.aerogenes in presence of 4 M urea.

Marked differences apparently exist in the nature and content of the half-cystine residues present in the citrate lyase complex from different sources. The nature of the half-cystine residues in the complex from S.faecalis (Hiremath, 1977) are summarised in Table II.8 and compared with the results obtained in the present studies on the K.aerogenes enzyme.

TABLE II.8: COMPARISON OF -SH GROUPS IN SEPARATED SUBUNITS
OF THE ENZYMES FROM *K.aerogenes* AND *S.faecalis*

K.aerogenes citrate lyase (M_r 575 000) total half-cystine/mol, 48.

Subunits	Cystine	Cysteine	S-acylated cysteine	Total [$\frac{1}{2}$ cystine] /[Enzyme]
1. <u><i>K.aerogenes</i></u>				
α - (M_r 54 000)	0	5	0	
β - (M_r 32 000)	0	2	0	
γ - (M_r 10 000)	0	0	1	48
2. * <u><i>S.faecalis</i></u>				
α - (M_r 54 000)	0	1	0	
β - (M_r 37 000)	2	0	0	
γ - (M_r 14 000)	0	0	1	36

* Results taken from Hiremath (1977).

It will be apparent from the table that although the two enzyme complexes have several common features such as subunit structure and stoichiometry, prosthetic group content and composition, and approximate molecular size; significant differences in the amino acid composition of their subunits exist.

STUDIES ON CITRATE LYASE (EC 4.1.3.6)

from Klebsiella aerogenes

III: SUBUNIT CONTACTS

SUBUNIT CONTACTS IN CITRATE LYASE COMPLEX

Summary

Citrate lyase complex from Klebsiella aerogenes is composed of 3 non-identical subunits: acyl transferase α (M_r 54 000), the lyase β (M_r 32 000) and ACP γ (M_r 10 000) subunits. Interactions between subunits of this complex have been explored by means of diimido esters and a cleavable cross-linking reagent, dithiobis(succinimidyl propionate) (DSP).

Inter-subunit cross-linking experiments with dimethyl adipimidate (DMA), dimethyl suberimidate (DMS) and DSP showed cross-linking of the subunits to occur. DMA gave a cross-linked species of 107 000 daltons probably corresponding to α_2 dimer and another of 135 000 corresponding probably to $\alpha_2\beta$. DMS gave a 126 000 daltons aggregate corresponding probably to an $\alpha_2\gamma_2$ cross-linked species. The DSP cross-linked species obtained with the reagent in presence of Mg^{2+} had an average molecular weight of 178 000 as estimated by SDS-gel electrophoresis. This molecular weight corresponds to a dimer of cross-linked α - and β -subunits and this has been confirmed after cleavage of the cross-link. On cross-linking with DSP in the absence of metal an extra band was obtained on SDS-gel electrophoresis and had a molecular weight of 200 000, probably being $\alpha_2\beta\gamma_2$. Reaction-inactivated enzyme on treatment with DSP in presence Mg^{2+} showed the same banding pattern as the cross-linked native enzyme in presence of metal.

The difference in cross-linked species obtained with

DSP in presence and in absence of Mg^{2+} are indicative of metal dependent conformational modulations.

Introduction

The citrate lyase multienzyme complex comprises of 3 different types of polypeptide chains (α -, β -, γ -) (Singh et al., 1974). The enzyme from K.aerogenes which has been studied the most, has an acyl carrier protein (γ -subunit, M_r 10 000) (Dimroth et al., 1973), which carries an essential acetyl group in thioester linkage with (phosphoribosyl) dephospho-CoA (Robinson et al., 1976; Dimroth, 1976). This acetyl group exchanges with the substrate in presence of the α -subunit (M_r 54 000) which functions as an acyl transferase (Dimroth and Eggerer, 1975b). The β -subunit (M_r 32 000) catalyses the cleavage of the citryl-intermediate to OAA regenerating the acetyl enzyme (Dimroth and Eggerer, 1975b). The enzyme complex has the hexameric structure $(\alpha\beta\gamma)_6$ and the component subunits can be taken apart after disrupting the structural organisation of the complex with denaturants like urea (Dimroth and Eggerer, 1975b). The separated subunits can be reassembled in vitro in the absence of denaturants to form the original quaternary structure (Dimroth and Eggerer, 1975b). Detailed description of the spatial arrangement of subunits in the complex is not known although it has been suggested from the appearance of citrate lyases from R.gelatinosa (Beuscher et al., 1974) and K.aerogenes (Dimroth and Eggerer, 1975a) in electron micrographs that the subunits could be arranged in a hexameric structure.

The precise structural organization and contact domains between subunits can be obtained only by X-ray crystallography. However, useful information can be obtained by the much simpler method of cross-linking of the polypeptide chains with bifunctional reagents. Thus bifunctional imido esters have been used extensively to study the subunit spatial arrangement of oligomeric proteins e.g. aldolase, (Davies and Stark, 1970), catalase, fumarase and pyruvate kinase (Hucho et al., 1975) and multicomponent proteins like RNA polymerase (Coggins et al., 1977) and ATPase (Bragg and Hou, 1975; 1976; 1977). Bisimido esters used in several of these studies covalently cross-link lysine ϵ -NH₂ groups of proteins without altering the charge of the protein and the products are characterised by SDS-polyacrylamide gel electrophoresis. In studies of complex systems the use of cleavable bifunctional reagents such as the bis imido ester, dimethyl-3-3'-dithiobispropionimidate (Wang and Richards, 1974) and the reactive ester, dithiobis (succinimidyl propionate) (DSP) (Lomant and Fairbanks, 1976), permit unambiguous identification of the protein chains contributing to a cross-linked species. After an initial electrophoretic separation of the cross-linked species, the disulfide bonds can be readily cleaved to regenerate the monomers. The regenerated monomers are characterised by a second dimension gel electrophoresis.

We report for the first time studies on subunit contacts of citrate lyase from chemical cross-linking studies using the bis imido esters, DMA and DMS, and the cleavable bifunctional Lomant's reagent, DSP. Circumstantial evidence

is presented regarding the contact domains in the enzyme, both in presence and absence of Mg^{2+} . Evidence is presented from these chemical cross-linking studies for the divalent metal ion dependent conformational modulation of the complex.

Materials

The chemicals used were of the highest purity available commercially. Sodium dodecyl sulfate was obtained from Hico Products, Pvt. Ltd., Bombay. Acrylamide and N-N'-methylene bisacrylamide were from Eastman Organic Chemicals, Rochester, USA. DMA and DMS were products of Polysciences, Warrington and Dithiobis (succinimidyl propionate) was a gift from Prof. Peter Marfey, Department of Biological Sciences, State University of New York, Albany. Dithiothreitol was obtained from Fluka, AG., Switzerland.

Citrate lyase enzyme from K.aerogenes was prepared as described in Part I and had a specific activity of 90 U/mg. Protein and enzyme assays were carried out as described earlier (Part I).

Experimental Procedures

Cross-linking of citrate lyase with diimidoesters:

Reactions were carried out essentially as described by Davies and Stark (1970). Citrate lyase was dialysed at 0-4°C against 0.2 M triethanolamine buffer, pH 8.0, containing 1.6 mM Mg^{2+} . Samples of the dialysed enzyme (0.4 - 0.5 mg in 0.1 ml buffer) were separately treated with an equal volume of a freshly prepared solution of the diimido ester (DMA or DMS;

8 mg/ml in triethanolamine buffer, pH 8.0, containing 1.6 mM Mg^{2+}) and the pH of the reaction mixture adjusted to 8.0 with NaOH. The reaction mixture was incubated at 25°C for 3 h and the reaction stopped by the addition of 10% solution of SDS to a final SDS concentration of 1%. The sample was heated at 100°C for 3 min before subjecting it to SDS-polyacrylamide gel electrophoresis.

Cross-linking of enzyme subunits with DSP:

Citrate lyase enzyme in 0.05 M potassium phosphate buffer, pH 7.5, obtained from the Sepharose CL 6B column used in the final step of purification was concentrated by ultrafiltration through PM-10 membrane and then subjected to cross-linking essentially as described by Lomant and Fairbanks (1976).

In studies on the enzyme in presence of metal, citrate lyase (1.0 mg) in 0.25 ml of 0.05 M potassium phosphate buffer, pH 7.5, containing 1.6 mM Mg^{2+} was treated with an equal volume of a solution of DSP (final concentration $3 \times 10^{-4} M$). The DSP solution was prepared fresh by dissolving 6 mg of the reagent in 0.1 ml of DMF and diluting to 25 ml with 0.005 M potassium phosphate buffer, pH 7.6, containing 1.6 mM Mg^{2+} . The reaction mixture was incubated at 25°C for different time intervals and the reaction stopped by addition of a 10% SDS solution to give a final concentration of 1% SDS, followed by heating at 100°C for 3 min.

In experiments involving cross-linking of the enzyme in absence of metal, the enzyme was initially dialysed against 0.05 M potassium phosphate buffer, pH 7.5, containing 2 mM EDTA. The metal-free enzyme was treated with the DSP reagent

prepared in buffer containing no metal.

To study whether the arrangement of subunits in the citrate lyase complex is altered on reaction - inactivation, cross-linking with DSP was carried out as described earlier after prior treatment of the enzyme (1.0 mg in 0.1 ml) with an equal volume of 2 M sodium citrate, pH 8.0, containing 1.6 mM Mg^{2+} and incubating at 30°C for 15 min when the enzyme was completely inactivated.

Detection of cross-linked subunits:

Products of cross-linking reactions were analysed by SDS-polyacrylamide gel electrophoresis and characterised by molecular weight determination. SDS gel electrophoresis was carried out in 7% polyacrylamide gels according to the procedure of Weber and Osborn (1969). The gels were stained after the runs for 2 h with a 0.25% Coomassie brilliant blue solution and destained using a methanol: acetic acid: water mixture (Weber and Osborn, 1975).

Two-Dimensional SDS-gel electrophoresis:

Identification of the cross-linked species obtained by cross-linking experiments with the cleavable reactive ester, DSP, was attempted by two-dimensional SDS polyacrylamide gel electrophoresis. Citrate lyase treated with DSP as described earlier was denatured using 1% SDS at 100°C for 3 min and then loaded on a 7% slab gel (8 x 12 cm) of 2 mm thickness. Electrophoresis was carried out at 32 mA/slab for 6 h. At the end of the run a slice of the slab gel was cut out and stained with coomassie blue to locate the protein bands. A second slice of the slab gel was cut out and layered on top of a

10% slab gel using 2% agarose in 0.01 M sodium phosphate buffer, pH 7.3, containing 10% 2-mercaptoethanol. Electrophoresis was carried out in the second dimension at 32 mA/slab for 6 h using bath buffer containing 2% mercaptoethanol. At the end of the run the second dimension slab gel was stained with 0.25% coomassie blue for 6 h and destained with methanol-acetic acid-water mixture.

Molecular weight of cross-linked species:

Molecular weight of the cross-linked protein was obtained from a plot of log molecular weight versus relative mobility of the protein. The standard curve was obtained from electrophoretic runs of marker protein, cytochrome C, ovalbumin and bovine serum albumin as well as the α -, β - and γ -subunits of native citrate lyase (Fig. III.1).

Results

Citrate lyase not treated with cross-linking agents when denatured in SDS disaggregates into 3 non-identical subunits, the α -subunit (M_r 54 000), β -subunit (M_r 32 000) and γ -subunit (M_r 10 000). The SDS-polyacrylamide gel electrophoresis pattern obtained with the untreated enzyme in absence of mercaptans is shown in Fig. III.2(i). As seen in Fig. III.2(i) no intersubunit disulfide bonds are present and separation is complete even in the absence of mercaptans in the dissociating system.

The types of cross-linking between subunits varies with the bifunctional reagent used (Fig. III.2). Treatment of the complex with DMA (Fig. III.2(ii)) causes the formation

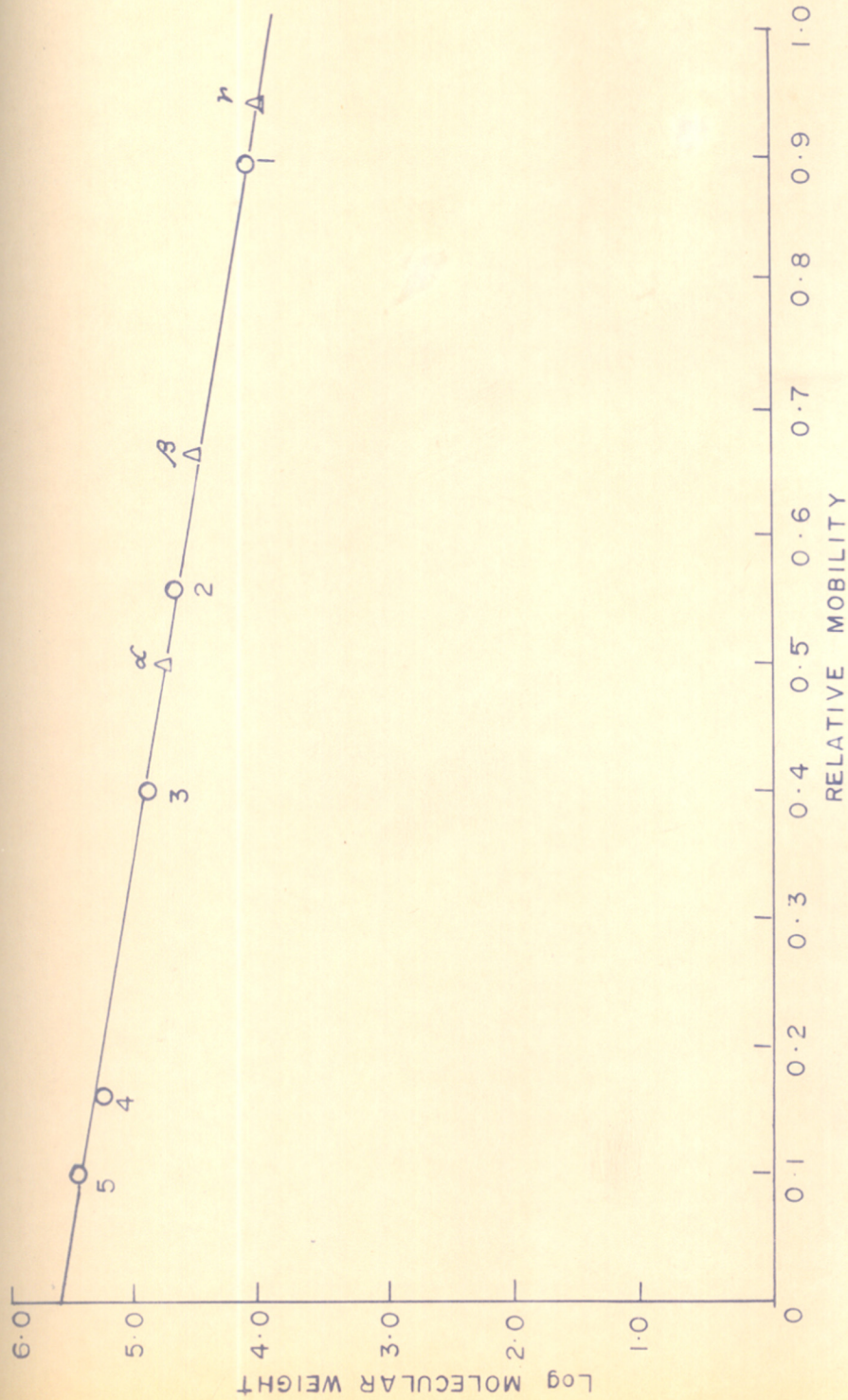


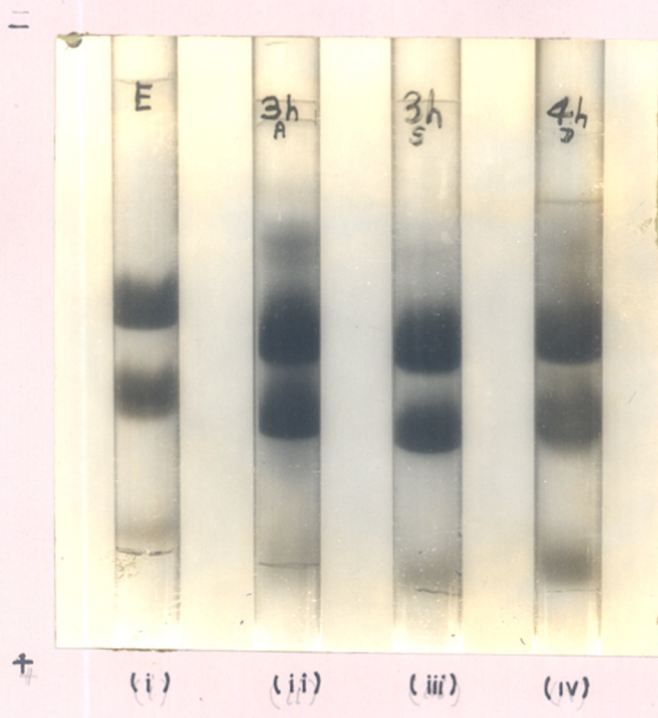
FIG. III. 1 : MOLECULAR WEIGHT DETERMINATION BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS
Calibration curve for 7% gel.

○ - Mol. wts. of marker proteins : 1. cytochrome C (11700); 2. ovalbumin (43000);

3. bovine serum albumin, monomer (68000); 4. bovine serum albumin, dimer (136000),

5. bovine serum albumin, tetramer. (272 000)

Δ - α - (54000), β - (32000) and γ - (10000) chains of citrate lyase from K. aerogenes



TREATMENT

NIL

DMA

DMS

DSP

FIG. III.2: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF CITRATE
LYASE FOLLOWING CROSS-LINKING WITH BIFUNCTIONAL
REAGENTS

- (i) Control with untreated enzyme
- (ii) Enzyme cross-linked with DMA (3 h)
- (iii) Enzyme cross-linked with DMS (3 h)
- (iv) Enzyme cross-linked with DSP (4 h)

7% gel; Sodium phosphate buffer, pH 7.3,
containing 0.1% SDS; 8 mA/tube; 28°C; 4 h.
Protein loaded, about 200 μ g.

of two bands, one of M_r 107 000 and the other of M_r 135 000, in addition to the separated subunit chains. The M_r 107 000 species corresponds probably to the dimer α_2 and the M_r 135 000 band might probably be $\alpha_2\beta$. With DMS (Fig. III.2(iii)) only a single band of M_r 126 000 was observed in addition to the individual subunits. This probably represents linked $\alpha_2\gamma_2$ chains.

In the case of the enzyme treated with the cleavable bifunctional reagent, DSP, (Fig. III.2(iv)) a single sharp band is observed in addition to the separated individual subunits when cross-linking is carried out in the presence of Mg^{2+} . The calculated M_r of 178 000 of this band would correspond to an $\alpha_2\beta_2$ species. The $\alpha_2\beta_2$ structure was confirmed by separation of the cross-linked species by electrophoresis in presence of SDS followed by cleavage of the cross-linked species with 2-ME and electrophoresis in the second dimension in presence of the reducing agent (Fig. III.3). In the second dimension the monomeric α - and β -subunits are generated from the band of M_r 178 000. This confirms that the $\alpha_2\beta_2$ aggregate is formed on cross-linking with DSP.

The rapid reaction with DSP is seen from Fig. III.4. Even at the earliest sampling at 30 min the reaction is apparently complete.

An interesting observation is the effect of Mg^{2+} on the nature of the aggregate obtained by cross-linking with DSP. In the absence of metal and with EDTA, conditions under which the enzyme is inactive, cross-linking with DSP results in the formation of two aggregates which separate as very faint bands

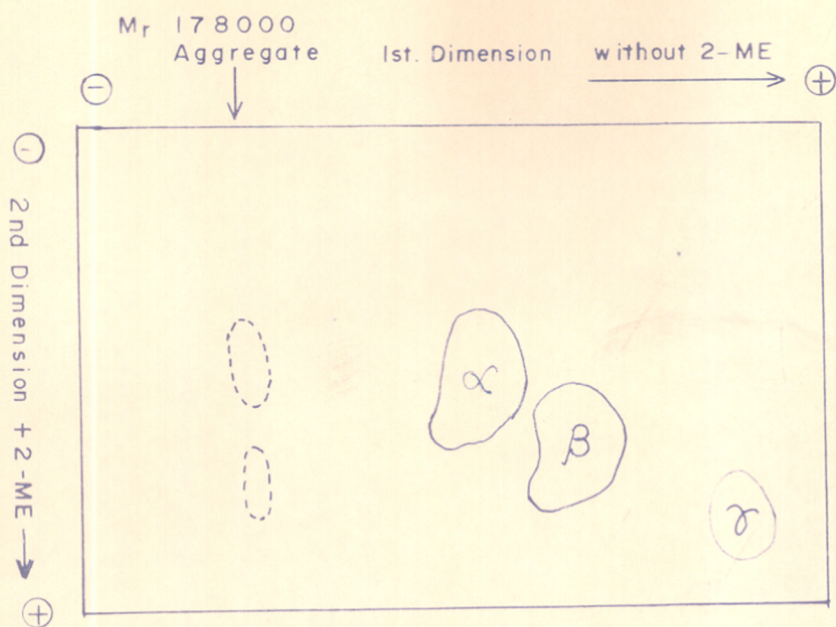
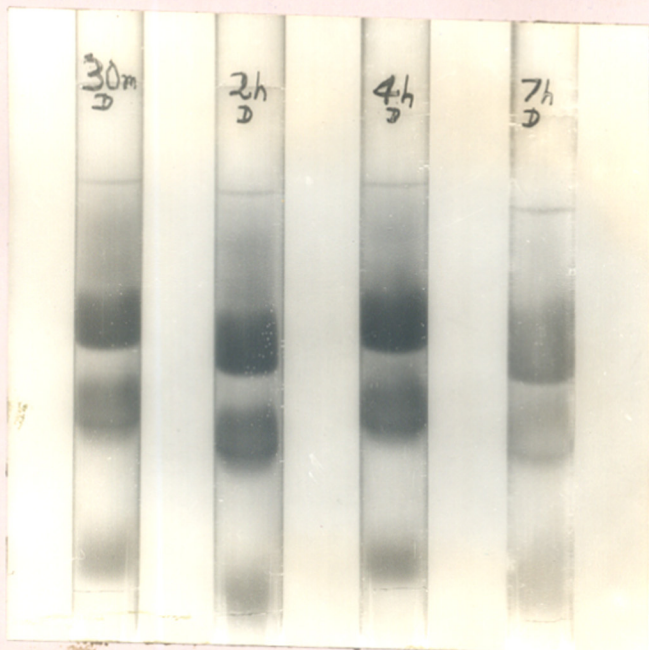


FIG. III. 3 : DIAGRAMMATIC REPRESENTATION OF TWO-DIMENSIONAL SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF DSP-TREATED CITRATE LYASE.

PROTEIN : 200 μ g ; 32 mA / slab ; 6 to 8 h each run.

-



+

FIG.III.4: SDS-GEL ELECTROPHORESIS OF CITRATE LYASE CROSS-
LINKED WITH DSP FOR VARYING TIME INTERVALS IN
PRESENCE OF Mg^{2+}

Citrate lyase (about 1 mg) treated with DSP reagent. Samples removed at different time intervals as indicated in channel and subjected to SDS-gel electrophoresis. Protein loaded, about 200 μ g; 8 mA/tube; 4 h.

in SDS-gels, a faint band corresponding to the 178 000 daltons $\alpha_2\beta_2$ species and in addition an even fainter band of 200 000 daltons (Fig. III.5(iii)). The yield of this new band was too low to permit characterisation after cleavage of the cross-links. Though visible to the eye, photographs show no distinct contrast and the positions of the bands are indicated by arrows in Fig. III.5(iii). The M_r 200 000 band could probably be an $\alpha_2\beta_2\gamma_2$ species which is not seen when cross-linking is done in presence of the metal cofactor, Mg^{2+} .

Reaction-inactivated enzyme in which the enzyme is known to lose its essential acetyl function to give the HS-enzyme, however, behaves like the native enzyme in presence of Mg^{2+} in cross-linking reactions with DSP (Fig. III.5).

Discussion

A model of citrate lyase from R.gelatinosa was proposed by Beuscher et al. (1974) from the molecular profiles of the complex observed in electron micrographs. The two most distinctive projections have the appearance of ring-shaped doughnut-like structures and a sandwich pattern consisting of two parallel rows of density. The number of subunits in the doughnut structure was not evident directly from the profiles but analysis using the rotation technique of Markham et al. (1963) indicated grossly an arrangement of 3 large (ellipse of axis $58 \text{ \AA}$ and $49 \text{ \AA}$) and 3 smaller (spherical with diameter of $43 \text{ \AA}$) subunits in alternating sequence in the ring structure. Assuming that the rings were face-on views of the citrate lyase molecule and the

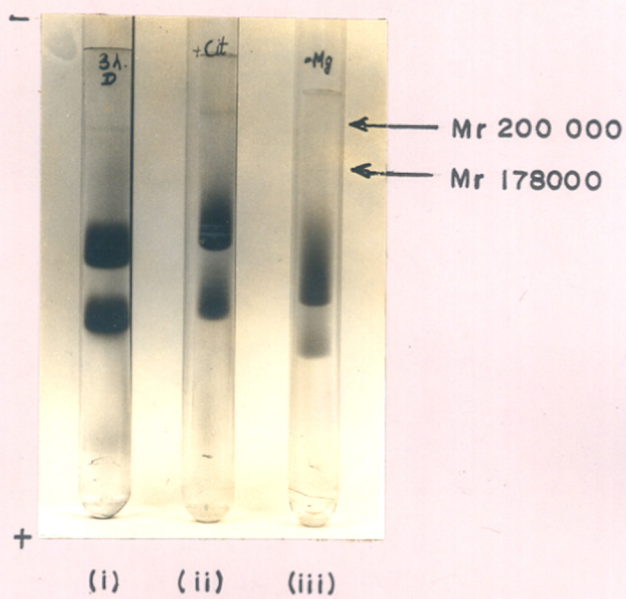


FIG.III.5: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS OF NATIVE,
REACTION-INACTIVATED AND METAL-FREE CITRATE LYASE
FOLLOWING CROSS-LINKING WITH DSP

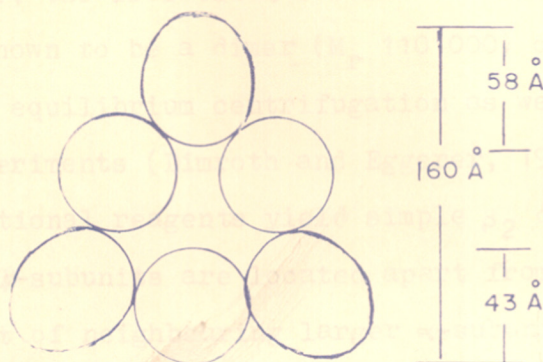
- (i) Native enzyme cross-linked in presence of Mg^{2+} (1.6 mM)
- (ii) Enzyme inactivated during cleavage reaction by treatment with sodium citrate (1 M) in presence of Mg^{2+} (1.6 mM) followed by cross-linking with DSP.
- (iii) Enzyme previously dialysed against 0.05 M potassium phosphate buffer, pH 7.5, containing 2 mM EDTA and the metal-free enzyme cross-linked with DSP

Cross-linking reaction at 25°C for 3 h followed by SDS-gel electrophoresis; 8 mA/tube; 4 h.
Protein loaded, about 200 μ g.

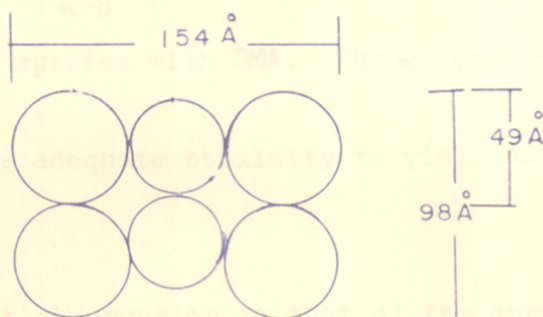
sandwich structure a side-on view, it was suggested that the major subunits could be arranged in a hexameric structure with alternating dimers of the large and the smaller subunits at the corners of a hexagon in which the two rings are stacked one above the other. A representation of the model is reproduced in Fig. III.6. At the time Beuscher et al. (1974) proposed the model, the enzyme from R.gelatinosa was thought to consist of six copies each of only two types of subunits and the model took no account of the ACP subunit component of about 11 000 daltons now known to be present in this enzyme also (Giffhorn and Gottschalk, 1978). The profile of the citrate lyase complex in K.aerogenes has also been reported to resemble the R.gelatinosa enzyme in its gross structural features (Dimroth and Eggerer, 1975a). Although the spatial location of the ACP (γ -subunit) has not been indicated in the electron microscopy studies, the reaction mechanism of citrate lyase complex requires an oscillation of the acylated prosthetic group of the subunit between the transferase (α -subunit) and the lyase (β -subunit) components of the complex (Dimroth and Eggerer, 1975b). The extended length of the 4'-phosphopantetheine component is about 20 Å (Srere and Singh, 1974) and could readily permit interactions of the prosthetic group arm with the larger subunit.

The present chemical cross-linking studies carried out with solutions of the enzyme from K.aerogenes support the proposed model based on evidence obtained from electron microscopy of dried enzyme preparations. They also permit further inferences, although circumstantial, on the topological

FACE-ON VIEW



SIDE-ON VIEW

FIG. III.6: MODEL OF CITRATE LYASE FROM *R. gelatinosa*

(Reproduced from Beuscher et al., 1974)

proximity of the subunits in the citrate lyase complex from K.aerogenes. The cross-linked product of reaction of M_r 107 000 obtained with DMA would support the earlier model where α_2 dimers are located symmetrically at the corners of the hexagon structure. Further, the isolated α -subunit that is enzymically active, has been shown to be a dimer (M_r 110 000) of α -chains from sedimentation equilibrium centrifugation as well as from gel-filtration experiments (Dimroth and Eggerer, 1975b). That none of the bifunctional reagents yield simple β_2 dimers might indicate that the β -subunits are located apart from each other, probably on account of neighbouring larger α -subunits as shown in the speculative model (Fig. III.7). Neighbouring α - and β -subunits on the other hand are probably located spatially close to one another since α - β species is obtained with DSP cross-

$$\begin{array}{c} \alpha-\beta \\ | \\ \alpha-\beta \end{array}$$

linking and a α - β species with DMA. The α - and γ -subunits are

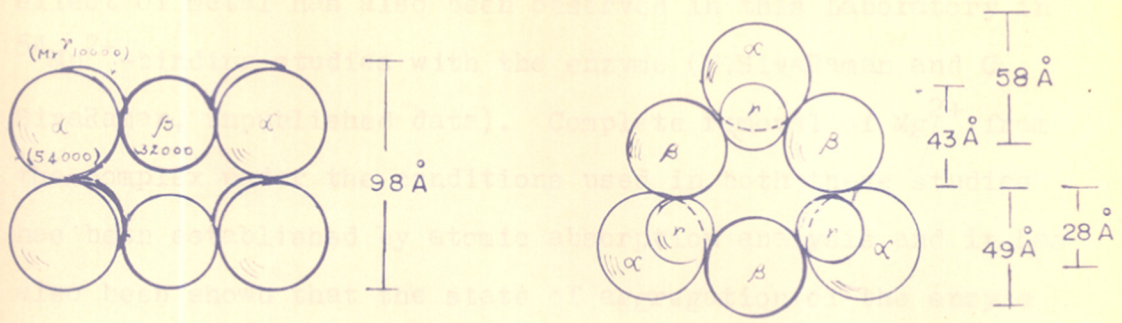
$$\begin{array}{c} \alpha \\ | \\ \alpha \end{array}$$

also apparently in adequate proximity to yield an α - γ

$$\begin{array}{c} \alpha-\gamma \\ | \\ \alpha-\gamma \end{array}$$

species with DMS.

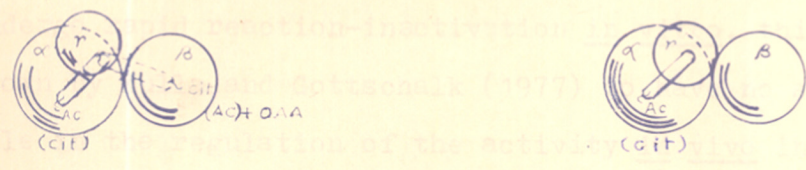
A significant observation is that of the apparent effect of Mg^{2+} on the conformation of the enzyme complex. In the presence of the metal which is required for substrate cleavage by this enzyme, cross-linking with DSP gives a sharp and distinct single $\alpha_2\beta_2$ cross-linked species the identity of which has been established after cleavage of this species. However, in the absence of metal and presence of EDTA, DSP treatment results in a markedly lower yield of the $\alpha_2\beta_2$ aggregate and an even lower amount of a second cross-linked



SIDE-ON VIEW

FACE-ON VIEW

(i)



WITH Mg^{2+}

WITHOUT Mg^{2+}

(ii)

FIG. III. 7 SPECULATIVE MODEL OF CITRATE LYASE (K. aerogenes)

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

species, probably $\alpha_2\beta_2\gamma_2$. This is suggestive of conformational modulation of the complex in presence of the metal. Such an effect of metal has also been observed in this Laboratory in $^{54}\text{Mn}^{2+}$ -binding studies with the enzyme (H.SivaRaman and C. SivaRaman, unpublished data). Complete removal of Mg^{2+} from the complex under the conditions used in both these studies has been established by atomic absorption analysis and it has also been shown that the state of aggregation of the enzyme is unaltered. Mn^{2+} -binding has been shown to be cooperative. Evidence is obtained in the present study, by an entirely different approach, which would support such a divalent metal-dependent conformational change in the complex. A speculative representation of the Mg^{2+} -effect is depicted in the models shown in Fig. III.7(i and ii).

These are the first reports on the allosteric behaviour of the citrate lyase complex. Such behaviour constitutes an important mechanism for the regulation of enzyme activity at the molecular level. While citrate lyase has been shown to undergo rapid reaction-inactivation in vitro, this has been shown by Kulla and Gottschalk (1977) to have no significant role in the regulation of the activity in vivo in K.aerogenes. Again, K.aerogenes unlike R.gelatinosa has no detectable deacetylase activity and regulation through enzymic deacetylation is not available in this organism. Experimental evidence has been presented for the energy dependent inactivation of citrate lyase in K.aerogenes (Kulla and Gottschalk, 1977). Several key enzymes of metabolic pathways, such as nitrate reductases of K.aerogenes, P.mirabilis and E.coli (de Groot

and Stouthamer, 1969; 1970a,b,c; Oltmann et al., 1976; Showe and De Moss, 1968; Van't Riet et al., 1968) and aspartate transcarbamylase of B.subtilis (Waindle and Switzer, 1973) have also been shown to be inactivated in vivo only in the presence of an energy source. In these, although the mechanism is not clearly understood, regulation has been assumed to be through the energy dependent generation or utilization of metabolite(s) which function as modulators. The allosteric behaviour of citrate lyase in K.aerogenes might also be the mechanism for its regulation in vivo. The earlier speculation of Blair et al. (1967) that a possible mechanism for regulation of citrate lyase activity in the cell may be through the ability of ATP to chelate metal ions could indeed be so through modulations in the conformation of citrate lyase when Mg^{2+} is abstracted from the complex.

No difference in the structural organisation of the native and the reaction-inactivated enzyme was detected in DSP cross-linking studies. The SDS-gel profiles of the native enzyme treated with DSP in presence of Mg^{2+} and of reaction-inactivated complex (Fig. III.5(ii)) are similar, both showing a distinct $\alpha\beta_2$ species as the only aggregate. Reaction-inactivation as shown earlier (Part II of this thesis) results in deacylation with exposure of reactive sulphydryls of the prosthetic group. DSP is known to participate in mercaptan-disulfide interchange with reactive sulphydryl groups in addition to amine acylation. However, even if such a reaction did occur, no subsequent cross-linking by acylation through the reactive ester group takes place

since this should have resulted in a cross-linked species containing the γ -subunit.

Different cross-linked species have been obtained with the two bis imidates, DMA° (extended span 8.6 Å; Hitchcock, 1975) and DMS° (extended span 11.2 Å; Hitchcock, 1975) and the bifunctional reactive ester DSP° (extended span 12 Å; Bragg and Hou, 1976). Such variations have also been observed by others (Hucho and Janda, 1974) and the differences have been explained on the basis of varying reactivities of the reagents and different accessibilities as well as reactivities of the functional groups of the protein. Yields of the cross-linked species are invariably low for various reasons as discussed by Bickle *et al.* (1972). The low yield has been interpreted on the basis of a competition between different reaction pathways undergone by the bifunctional reagent. The reagent can hydrolyse at both ends without reacting or can lead to a single-hit with one end reaction and the hydrolysis of the other end, or the double-hit with either inter-or intra- molecular cross-linking. These alternate pathways of reaction result in low yields of the inter-molecular cross-linked species.

Conclusions on distances between the different components of a complex from the extended chain lengths of the reagents are not warranted. Conclusions on subunit contacts and relative locations based on chemical cross-linking studies have inherent limitations since cross-linking requires both adequate proximity as well as availability of appropriate reactive groups at such domains. However, this technique is amongst the few available for the study of subunit locations in oligomeric proteins in solution.

STUDIES ON CITRATE LYASE (EC 4.1.3.6)

from Klebsiella aerogenes

IV: IMMOBILIZATION OF CITRATE LYASE

STUDIES ON IMMOBILIZATION OF CITRATE LYASE

Summary

Citrate lyase (EC 4.1.3.6) from K.aerogenes has been immobilized by covalent attachment to cyanogen bromide-activated agarose. The immobilized enzyme shows about 15% of the original activity on the basis of protein bound. The immobilized enzyme, like the native complex, undergoes reaction-inactivation and is reactivated by the enzyme, acetate: HS-citrate lyase ligase (AMP) in presence of acetate and ATP. The immobilized enzyme has been shown to be attached to the carrier through an average of 23 lysyl residues/mol citrate lyase complex.

An immobilized multienzyme system containing both citrate lyase and malate dehydrogenase has been prepared by covalent attachment to cyanogen bromide-activated agarose and the use of this system for citrate assay has been standardised.

Introduction

Citrate lyase (EC 4.1.3.6) has been shown to be a multienzyme complex composed of three non-identical subunits; an acyl carrier protein and two subunits with enzymic activities, one catalysing a transferase and the other a lyase reaction as shown in Equation IV.1 and IV.2, respectively (Dimroth and Eggerer, 1975b).





Several immobilized multienzyme systems such as combinations of β -galactosidase + hexokinase + glucose-6-phosphate dehydrogenase (Mattiasson and Mosbach, 1971) and malate dehydrogenase + citrate synthetase + lactate dehydrogenase (Srere et al., 1973), have been reported in the literature in which the product of one reaction is the substrate for another, in a sequence of reactions. These systems differ from a built-in complex such as citrate lyase wherein the continuous turnover of substrate involves oscillation of the acylated prosthetic group between the enzymically active subunits within the complex. In a system such as citrate lyase where conformational changes are likely to interfere with the prosthetic group oscillation, it was of interest to determine whether the covalent attachment of the complex to a carrier would result in loss of enzyme activities.

The present work reports for the first time the immobilization of citrate lyase from K.aerogenes and the properties of the enzyme covalently attached to CNBr-activated Sepharose.

It was also of interest to determine whether immobilized citrate lyase could be applied to citrate assay. Assay systems using citrate lyase coupled with either malate dehydrogenase (EC 1.1.1.37) (Ward and Srere, 1965) or a combination of malate dehydrogenase and lactate dehydrogenase

(EC 1.1.1.27), the latter to account for any pyruvate formed non-enzymically from oxaloacetate (Moellering and Gruber, 1966), have been described for the rapid and specific assay of citrate. Enzymes used hitherto in the coupled assay of citrate have been soluble, native preparations. The advantages of applying immobilized enzymes repetitively are obvious. Immobilized systems also lend themselves to automatic methods of analysis. However, the reuse of any citrate lyase preparation poses problems since the enzyme undergoes reaction-inactivation (Singh and Srere, 1971; Buckel et al., 1971). During the course of the reaction, the enzyme undergoes deacetylation to yield an inactive deacetyl (HS-) enzyme (Singh and Srere, 1971; Srere et al., 1972). The inactive HS-citrate lyase can however be reactivated either chemically (Buckel et al., 1971a; Srere et al., 1972) or enzymically by an acetate: HS- citrate lyase ligase (AMP) in presence of acetyl adenylate or of acetate and ATP (Schmellenkamp and Eggerer, 1974).

The preparation of an immobilized multicomponent system containing both the citrate lyase complex and malate dehydrogenase is also described. The multicomponent system can be applied repetitively for citrate assay with enzymic reactivation of the lyase activity after each use.

Materials

Reagents were generally of analytical grade or of the highest purity available commercially. Citric acid. $1\text{H}_2\text{O}$, Analar Grade, was obtained from BDH, England. Oxaloacetic acid, analytical grade, and cyanogen bromide were obtained

from Fluka AG., Switzerland. NADH (Disodium salt. $3H_2O$, grade III from yeast) was obtained from Sigma Chemical Co., USA. Malate dehydrogenase (from pig heart, specific activity $945 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) was also obtained from Sigma Chemical Co., USA.

Citrate lyase from K.aerogenes was prepared as described previously (Part I). Homogeneity of the preparation was established by analytical ultracentrifugation and by polyacrylamide gel electrophoresis. The enzyme preparations had a specific activity of 90 U/mg.

Experimental Procedures

Protein determination:

Protein assays were carried out as described in Part I of the thesis.

Preparation of Immobilized citrate lyase:

Pure citrate lyase complex was covalently coupled to cyanogen bromide activated Sepharose 4B. The activation of the support was carried out by a modification of the procedure described by Porath et al. (1967) using 1 M Na_2CO_3 for controlling the pH during activation as described by March et al. (1974).

One volume of an aqueous slurry of washed Sepharose 4B beads (1:1 w/v) was treated with 2 M Na_2CO_3 (1.5 vol). An aqueous solution of cyanogen bromide (50 mg/ml; 0.5 vol) was added to the beads and agitated gently for 10 min at 18 to 20°C. The beads were then cooled to 0°C with addition of

crushed ice. The activated beads were washed on a glass filter, first with ice-cold 0.1 M NaHCO_3 containing 0.17 M NaCl (20 to 25 vol) and then with ice-cold 0.1 M potassium phosphate buffer, pH 7.5, containing 1.6 mM MgSO_4 and 0.17 M NaCl (20 to 25 vol). The filtration and washings were completed within a period of 10-15 min, care being taken to keep the contents of the funnel cool with addition of crushed ice.

Coupling of citrate lyase to cyanogen bromide-activated Sepharose beads was carried out at 0 to 4°C. Citrate lyase solution (containing 2 to 10 mg protein/ml) was previously dialysed extensively against 0.05 M potassium phosphate buffer, pH 7.5, containing 1.6 mM MgSO_4 and added to a suspension (1:1 w/v) of cyanogen bromide-activated Sepharose beads in the same buffer and the mixture gently agitated for 16 to 18 h. The beads were then filtered and washed thoroughly with cold coupling buffer. The washed beads were separately tested for protein and citrate lyase activity. The filtrate and washings were also analysed for protein content.

Estimation of bound protein:

The protein bound to the support was determined by the procedure described by Lowry et al. (1951) for insoluble proteins. Samples were preliminarily treated with 1 N NaOH and heated for 10 min at 100°C. The standardization with crystalline bovine serum albumin was also carried out under similar conditions.

Assay of immobilized citrate lyase activity:

The enzyme immobilized on the beads was preliminarily activated with acetate: HS- citrate lyase ligase (AMP) as described later and washed thoroughly with buffer before assay for activity.

The activity of citrate lyase immobilized on Sepharose beads was determined as follows: An aliquot corresponding to 50-100 μg bound protein was tested for activity by adding to the citrate test medium, described under assay of soluble enzyme (Part I) and incubating at 28°C with gentle agitation followed by determination of the keto acids formed.

Estimation of the number of lysyl groups involved in attachment of enzyme to CNBr-activated Sepharose support:

The estimation was carried out by a modification of the method described by Koelsch et al. (1975). Samples of Sepharose-bound enzyme were washed thoroughly with water and taken to dryness in vacuum at 30°C over P_2O_5 . Acid hydrolysis of the dry beads was carried out with 6 N HCl in tubes in which the acid treated samples were frozen at -72°C, evacuated twice, warmed on a water bath and again frozen and evacuated before sealing. Hydrolysis was carried out at 110°C for 24 h. The acid was removed on a rotary evaporator and the residue dissolved in 0.02 N HCl (2.0 ml). The sample was applied to a Dowex 50 x 8 column (3.5 x 10 cm) and eluted with 25 ml of 4 N HCl. The eluate was taken to dryness on a rotary evaporator. The residue was dissolved in 5.0 ml water and again taken to dryness and the process repeated once. The final residue was dissolved in citrate buffer, pH 2.2 and

an aliquot used for amino acid analysis by standard procedures on a Beckman Model 120C automatic amino acid analyser. A calibration mixture of amino acids was treated similarly and amino acid contents were calculated by integration of the areas under the peaks. The protein content was calculated from the estimated amounts of the neutral amino acids: alanine, glycine and isoleucine in the hydrolysate of the immobilized enzyme and the separately determined amino acid composition of the pure soluble enzyme.

Effect of metals on activity of immobilized citrate lyase:

The enzyme immobilized on Sepharose beads was activated using acetate: HS- citrate lyase ligase (AMP) as described later and then checked for activity. The active beads were treated with 0.05 M Tris-HCl, pH 8.0 buffer containing 2 mM EDTA for 10 min and then washed repeatedly with the same buffer. This was followed by washing twice with the buffer containing no EDTA. Equal aliquots of the beads were treated separately as follows: One aliquot was treated directly with 3 ml of the citrate test medium containing no divalent metal and assayed for citrate lyase activity as described earlier. Three other aliquots were separately incubated preliminarily at 28°C for 15 min with 1.0 ml solutions of 0.05 M Tris-HCl buffer, pH 8.0 containing 10 mM MgSO_4 , 10 mM ZnSO_4 or 10 mM MnCl_2 and to each was then added 2.0 ml of the citrate test medium containing 10 mM concentrations of the respective divalent metals, and assayed as described already for citrate lyase activity.

Preparation of Sepharose-bound multienzyme system containing citrate lyase complex and malate dehydrogenase:

Cyanogen bromide-activated Sepharose 4B beads were prepared as described above and were coupled with a combination of citrate lyase and malate dehydrogenase, both of which were previously dialysed separately against 0.05 M potassium phosphate buffer, pH 7.5, containing 1.6 mM MgSO_4 . The coupling was carried out by the addition of malate dehydrogenase followed by citrate lyase after a period of about 5 min. The coupling reaction was allowed to proceed with gentle agitation for 16-18 h at 0-4°C. The beads were then washed as described for immobilized citrate lyase.

Assay of malate dehydrogenase activity:

Malate dehydrogenase activity in solution was assayed spectrophotometrically (Ochoa, 1955). Malate dehydrogenase activity on the Sepharose beads was checked quantitatively by the same procedure using a suspension of beads gently agitated in the test medium. Aliquots were removed at intervals for determination of A_{340} nm values.

Preparation of acetate: HS- citrate lyase ligase (AMP):

Cell-free extracts of K.aerogenes containing the ligase activity and a partially purified ligase, free from citrate lyase activity, was prepared as described below:

Klebsiella aerogenes cells were grown as described in Part I. Batches of 10 g cell paste were suspended in 30 ml 0.03 M potassium phosphate buffer, pH 7.0, cooled in an ice bath and the cells disrupted by sonic oscillation for 3 min at 20 Kc (300 W) in a Biosonik III sonic oscillator.

Clear extracts were obtained on removal of cell debris by centrifugation at 50 000 g for 60 min at 4°C. The extracts containing about 0.5% protein were used fresh or stored for periods upto a week at -20°C.

Acetylating enzyme activity tested in the various steps of citrate lyase purification indicated that the ligase activity separates from citrate lyase during the second Alumina C_{γ} treatment. Ligase activity free from citrate lyase was obtained by the following procedure: Cell-free extracts prepared as described above were treated with streptomycin sulfate for nucleic acid removal. The precipitated nucleic acids were separated by centrifugation and the clear supernatant was treated with Alumina C_{γ} gel (0.35 vol gel, dry weight 33 mg/ml) and centrifuged. The supernatant was free from citrate lyase activity but contained acetate: HS-citrate lyase ligase (AMP) activity. The partially purified ligase was used fresh and was not stable to storage at -20°C.

Preparation of HS- citrate lyase and estimation of the ligase activity was carried out according to the procedure described by Schmellenkamp and Eggerer (1974). Ligase activity is defined in terms of citrate lyase units formed per minute. Crude extracts of K.aerogenes had about 3 U/mg and the Alumina C_{γ} treatment resulted in about 2-3 fold purification. Determination of citrate with immobilized citrate lyase - malate dehydrogenase:

Solutions:

Buffer solution (A): 0.05 M potassium phosphate, pH 7.5, with 1.6 mM $MgSO_4$.

NADH solution (B): An aqueous solution containing 1.5 - 3.0 μ mol NADH/ml was prepared fresh every week and stored at 0°C.

Standard citrate: A stock solution of 0.02 M citric acid was made and stored frozen. The stock solution (5.0 ml) was neutralised with sodium bicarbonate and diluted with water to give a 1 mM solution. This solution was prepared fresh daily. The standard solutions were checked by coupled assay with native citrate lyase and malate dehydrogenase (Ward and Srere, 1965).

Citrate assay:

A small packed bed (2 x 1 cm, corresponding to about 1.5 g wet beads) of the immobilized multienzyme system containing both citrate lyase and malate dehydrogenase, was taken over a sintered glass disc of a jacketed column maintained at 30°C. The column was provided with a short, narrow bore outlet controlled by a stopcock immediately below the sintered disc. The beads were preliminarily activated in the column with the acetate: HS- citrate lyase ligase (AMP) in presence of ATP and acetate at final concentrations of 1.5 mM and 0.75 mM; respectively. Sepharose beads treated with the activating system were washed free of protein with buffer solution (A) (10 vol). NADH solution (B) (0.5 ml) was added to the packed bed followed by the addition of solution containing citrate (0.1 - 1.0 ml). The Sepharose bed was stirred with a thin glass rod for 5 min and then allowed to settle, drained and washed with buffer (A) and the eluate (10 ml) was collected in a stoppered graduated cylinder. A control was run without added citrate. The $A_{340 \text{ nm}}$ values of the eluates were read

on a Beckman Model 26 spectrophotometer in cells of 10 mm light path and the difference between the absorbance of the control and the citrate samples calculated.

Results

Immobilization of citrate lyase:

The effect of enzyme concentration on efficiency of immobilization was studied. The data are summarized in Table IV.1.

TABLE IV.1: PREPARATION OF IMMOBILIZED CITRATE LYASE

Sepharose 4B was activated with 50 mg CNBr/g wet beads for 10 min. The activated beads were treated with the indicated amounts of citrate lyase for 16-18 h at 0-4°C. Activity of the immobilized enzyme was determined for citrate cleavage and is compared with the corresponding activity of the native enzyme on the basis of protein bound.

Citrate lyase mg/g Sepharose	Protein bound (%)	Activity (%)
8.0	75	
2.5	86	
2.0	97	
1.5	100	15

The amount of protein bound was calculated from the differences between the total protein taken and that recovered in the filtrate and washings. The calculated values were in agreement with direct determinations of bound protein carried

out on a few samples. It is apparent that almost quantitative binding of protein to cyanogen bromide-activated Sepharose is obtained at enzyme levels of about 2 mg/g support under the conditions used. No leakage of protein into solution occurred when the beads with bound enzyme were treated with 1 M NaCl, 0.05 M potassium phosphate buffer, pH 6.0 or 0.05 M sodium acetate buffer, pH 4.5, respectively, indicating covalent attachment of the enzyme complex to the support.

The immobilized enzyme showed only about 15% of the original activity in presence of 10 mM MgSO_4 in the test system, calculated on the basis of protein bound. The immobilized enzyme, when treated with excess of citrate, was inactivated during the course of the enzyme reaction. Such a preparation when washed with 0.05 M potassium phosphate buffer, pH 7.5, containing 1.6 mM MgSO_4 and assayed was completely inactive. Reaction-inactivated preparations could, however, be completely reactivated by either crude K.aerogenes extracts containing acetate: HS-citrate lyase ligase (AMP) or the partially purified ligase free from citrate lyase activity (2:1 v/w in both cases). Estimations of citrate lyase activity in washings from beads treated with the crude bacterial extract indicated that no activity was adsorbed on the support. The reactivation of the inactivated enzyme by the partially purified ligase, free from citrate lyase activity, confirmed that the inactivated enzyme is enzymically reactivated in presence of acetate and ATP as in case of the soluble enzyme (Schmellenkamp and Eggerer, 1974). Repeated treatment with citrate solutions followed by enzymic reactivation resulted

in no measurable loss of activity.

Effect of metals:

In addition to Mg^{2+} , divalent metals such as Mn^{2+} and Zn^{2+} have been shown to activate the native enzyme (Dagley and Dawes, 1955). Zn^{2+} has also been shown to result in only a weak reaction-inactivation in case of the soluble enzyme (Singh and Srere, 1971; Gruber and Moellering, 1966). The immobilized enzyme preparation which was rendered metal-free by treatment with EDTA (2 mM) and then washed free of the chelating agent was tested for activity separately in presence of Mg^{2+} , Zn^{2+} and Mn^{2+} as described in Experimental Procedures. The beads with bound enzyme showed equal activity in presence of all three metal ions, but were completely inactive in the absence of metal.

Effect of denaturants:

The Sepharose beads with covalently attached enzyme were treated with 1% sodium dodecyl sulfate and heated to 100°C for 5 min to dissociate the complex. The beads lost their structure and went into solution. This solution was used for SDS-polyacrylamide gel electrophoresis to detect the released subunits. It was observed that all three subunits (α -, β - and γ -) were released but visually only a fraction of the bound protein appeared to be released on treatment with the denaturant, judging from the intensities of the stained subunits.

Estimation of the number of lysine residues involved in covalent attachment to support:

Amino acid analysis data for alanine, glycine, isoleucine

and lysine of the native enzyme are summarized in Table IV.2.

TABLE IV.2: AMINO ACID ANALYSIS OF NATIVE CITRATE LYASE

Citrate lyase, M_r 575 000

Amino acid	Residues/mol
Alanine	528
Glycine	413
Isoleucine	309
Lysine	203

The lysine content of the immobilized enzyme was computed from the total protein values calculated separately from the alanine, glycine and isoleucine peaks in the elution profile of the acid hydrolysate of the immobilized enzyme and are shown in Table IV.3.

TABLE IV.3: LYSINE CONTENT OF IMMOBILIZED CITRATE LYASE

Citrate lyase, M_r 575 000

Control amino acid used as reference for calculation	Lys residues/mol
Alanine	172
Glycine	186
Isoleucine	183

Average	180

Covalent attachment of protein to CNBr-activated carriers is mainly through coupling with ϵ -NH₂ group of Lysine and the α -NH₂ group of N-terminal amino acid (Porath and Axen, 1976). Amino acid residues so linked are destroyed on acid hydrolysis. Thus while native citrate lyase molecule has 203 Lysine residues the immobilized enzyme molecule on acid hydrolysis yields only about 180 Lysine residues.

This would indicate that the enzyme is linked by multipoint attachment through approximately 23 lysine residues/mol enzyme complex to the CNBr-activated carrier. Citrate lyase contains only methionine residues at the amino terminus of all the three subunits (Singh *et al.*, 1976) and the control amino acids used as reference cannot participate in the covalent linking of the complex to the support.

Immobilized multienzyme system containing citrate lyase and malate dehydrogenase:

Table IV.4 summarizes the binding data for combinations of citrate lyase and malate dehydrogenase.

TABLE IV.4: PREPARATION OF IMMOBILIZED MULTICOMPONENT SYSTEM CONTAINING CITRATE LYASE AND MALATE DEHYDROGENASE

CNBr-activated Sepharose 4B was washed and treated with indicated amounts of citrate lyase and malate dehydrogenase for 16-18 h at 0-4 °C.

Citrate lyase mg/g Sepharose	Malate dehydrogenase mg/g Sepharose	Total protein bound %
1.0 - 1.4	0.430 - 0.625	92-100

Under the conditions used for coupling, both enzymes were almost quantitatively bound. The immobilized citrate lyase component was inactivated completely in presence of excess citrate during the course of the reaction as in the case of the single enzyme. However, the malate dehydrogenase activity was apparently unaffected. The inactive citrate lyase component could be reactivated by the ligase present both in the crude extracts and in the partially purified preparations. The conditions of reactivation were as described for the single component system. Reactivation under these conditions had no effect on the malate dehydrogenase activity.

Quantitative estimation of citrate by coupled assay with immobilized citrate lyase and malate dehydrogenase:

Assay of citrate was carried out in the range 0 to 1 μmol . The standard curve with citrate is represented in Fig. IV.1. It will be evident from Fig. IV.1 that there is a linear relationship between the $A_{340 \text{ nm}}$ values and the amount of citrate (0.0 - 1.0 μmol) taken. It will also be evident from the standard curve that a stoichiometric assay of citrate is obtained since 1 μmol of citrate yields a $A_{340 \text{ nm}}$ value of 0.625 in the final volume of 10 ml, the theoretical value being 0.622 calculated from the molar extinction coefficient of NADH ($\epsilon_{340 \text{ nm}} = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$) (Horecker and Kornberg, 1948).

The column was used and reactivated repetitively over more than 35 cycles, through a period of ten days, without any apparent loss in activity of the immobilized enzymes.

Recovery experiments carried out with rabbit serum using

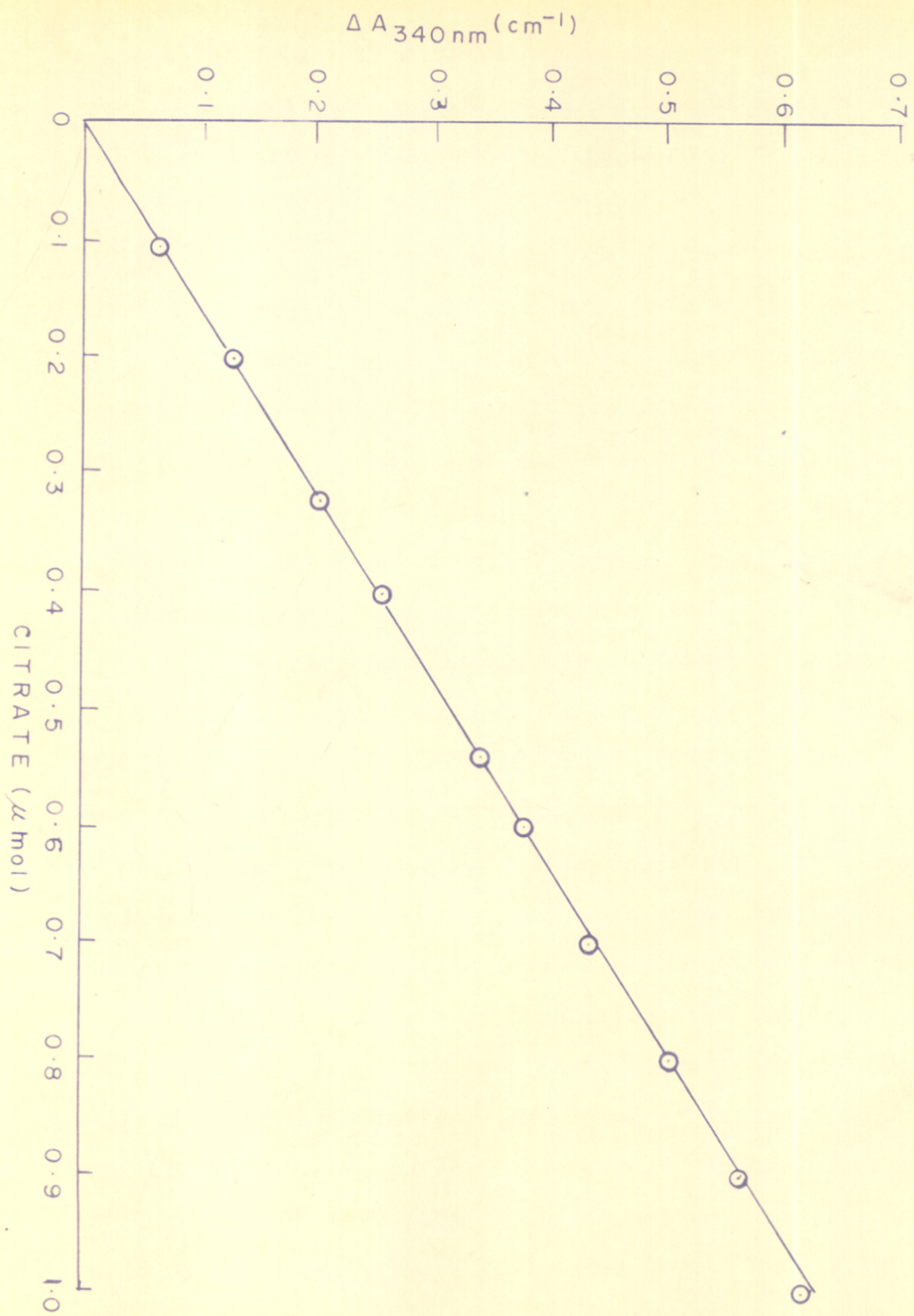


FIG. IV. 1: STANDARD CURVE OF CITRATE BY COLUMN ASSAY USING
CITRATE LYASE-MALATE DEHYDROGENASE-SEPHAROSE 4B

1 1.4 mg citrate lyase and 0.4 - 0.6 mg malate dehydrogenase per g support. Treated with NADH (0.5 ml; 0.75 - 1.5 μ mol) and eluted with 0.05M phosphate buffer, pH 7.4; 10.0 ml total volume collected.

added citrate gave a recovery of 100%. The sample of rabbit serum used gave a citrate concentration of 0.168 mg/ml.

Discussion

The immobilization of citrate lyase complex is described for the first time. The enzyme from K.aerogenes is a multienzyme complex with equimolar amounts of three non-identical subunits: an acetyl-ACP component of about 10 000 daltons (Buckel et al., 1971a; Dimroth et al., 1973), a 54 000 dalton subunit which functions as a transferase in the formation of citryl-ACP with the liberation of acetate (Dimroth and Eggerer, 1975b) and a 32 000 dalton subunit with lyase activity which cleaves the citryl-ACP releasing oxaloacetic acid and reforming the acetylated-ACP (Dimroth and Eggerer, 1975b). Such a mechanism of action would require the oscillation of the acetyl function on the acyl carrier protein component between the transferase and the lyase subunits during the course of the cleavage reaction (Srere and Singh, 1974; Dimroth and Eggerer, 1975b). These functional aspects are evidently not entirely abolished on immobilization. The enzyme immobilized with cyanogen bromide-activated Sepharose retains about 15% of the original activity as compared to the free enzyme. Steric hindrances imposed by binding to a matrix as well as diffusional restrictions may probably be responsible for the observed low activity. The immobilized enzyme complex behaves like the native enzyme in undergoing reaction-inactivation. The inactivated

immobilized enzyme is also reactivated enzymically with acetate: HS- citrate lyase ligase (AMP) in presence of acetate and ATP.

Amino acid analysis of the immobilized enzyme carried out to study the involvement of ϵ -NH₂ groups of lysyl residues in the covalent attachment of the protein to the polysaccharide support showed the participation of about 23 lysyl residues/mol complex, in the immobilization process. Such multiple point anchoring of protein has been reported recently for leucine aminopeptidase covalently bound to CNBr-activated Sepharose 6B through approximately 22 lysine residues/mol (Lasch and Koelsch, 1977). Although CNBr-activated polysaccharides have been used extensively for enzyme immobilization, relatively little is known about the nature and extent of the covalent linkage between the carrier and the coupled protein molecule. The reaction presumably is through an isocyanate derivative of the carrier as an unstable intermediate which can yield in the coupling step atleast three products: imidocarbonate, substituted carbamate and isourea derivatives (Porath and Axen, 1976). Even less is known about the number of linkages that attach the protein molecule to the support. The few isolated studies on the number of linkages would indicate that under the usual conditions of coupling to CNBr-activated support, attachment is through several points on the oligomeric enzyme molecule.

Whether all the subunits were attached was checked by dissociating the immobilized citrate lyase with 1% SDS. SDS-polyacrylamide gel electrophoresis indicated the presence

of all three subunits. This might indicate random attachment of the complex to the support, assuming that the bonds involved in covalent attachment are not susceptible to cleavage during SDS-treatment.

The usefulness of an immobilized multienzyme system containing citrate lyase and malate dehydrogenase for column assays of citrate has been demonstrated. This system has been repetitively used and reactivated enzymically in the column for over 35 cycles without any apparent loss in activity during a period of about 10 days. However, the use of such a system would require prior removal of any oxaloacetate present in biological samples. This could be achieved by preliminary heating of the sample at acid pH to decarboxylate oxaloacetate quantitatively (Stern, 1957) to pyruvate which does not interfere in the assay.

REFERENCES

REFERENCES

- Antranikian, G., Giffhorn, F. and Gottschalk, G. (1978)
 FEBS Letters, 88, 67-70.
- Barker, H.A. (1971) Arch. Microbiol. 59, 4-12.
- Beuscher, N., Mayer, F. and Gottschalk, G. (1974) Arch.
 Microbiol. 100, 307-328.
- Beuscher, N. and Gottschalk, G. (1972) Z.Naturforsch, 27b,
 967-973.
- Beyreuther, K., Böhmer, H. and Dimroth, P. (1978) Eur. J.
 Biochem. 87, 101-110.
- Bickle, T.A., Hershey, J.W.B. and Treut, R.R. (1972) Proc.
 Natl. Acad. Sci., USA, 69, 1327-1331.
- Blair, McD. J., Datta, S.P. and Tate, S.S. (1967) Eur. J.
 Biochem. 1, 26-28.
- Bowen, T.J. and Mortimer, M.G. (1971) Eur. J. Biochem. 23,
 262-266.
- Bowen, T.J. and Rogers, L.J. (1963a) Biochim. Biophys. Acta,
67, 633-640.
- Bowen, T.J. and Rogers, L.J. (1963b) Biochim. Biophys. Acta,
77, 685-687.
- Bowen, T.J. and Rogers, L.J. (1965) Nature 205, 1316-1317.
- Bowen, T.J. and SiveRaman, C. (1960) Biochem. J. 75, 9P.
- Bowien, S. and Gottschalk, G. (1977) Eur. J. Biochem. 80,
 305-311.
- Bragg, P.D. and Hou, C. (1975) Arch. Biochem. Biophys. 167,
 311-321.
- Bragg, P.D. and Hou, C. (1976) Biochem. Biophys. Res. Comm.
72, 1042-1048.

- Bragg, P.D. and Hou, C. (1977) Can. J. Biochem. 55, 1121-1124.
- Brewer, C.R. and Werkman, C.H. (1939) Enzymologia, 6, 273-281.
- Buckel, W. and Bobi, A. (1976) Eur. J. Biochem. 64, 252-262.
- Buckel, W., Buschmeier, V. and Eggerer, H. (1971a) Hoppe-Seylers. Z. Physiol. Chem. 352, 1195-1205.
- Buckel, W., Lenz, H., Wunderwald, R., Buschmeier, V., Eggerer, H. and Gottschalk, G. (1971b) Eur. J. Biochem. 24, 201-206.
- Buckel, W., Ziegert, K. and Eggerer, H. (1973) Eur. J. Biochem. 37, 295-304.
- Burton, K. (1955) Biochem. J. 59, 44-46.
- Cahn, R.S., Ingold, C. and Prelog, V. (1966) Angew. Chem. Int. Ed. 5, 385-415.
- Carpenter, D.E., Singh, M., Richards, E.G., and Srere, P.A. (1975) J. Biol. Chem. 250, 3254-3260.
- Coggins, J.R., Lumsden, J. and Malcolm, A.D.B. (1977) Biochemistry 16, 1111-1116.
- Collins, E.B. and Harvey, R.J. (1968) J. Dairy Sci. 45, 32-35.
- Dagley, S. (1954) J. Gen. Microbiol. 11, 218-227.
- Dagley, S. (1969) Meth. Enzymol. 8, 160-163.
- Dagley, S. and Dawes, E.A. (1953a) Nature 172, 345-346.
- Dagley, S. and Dawes, E.A. (1953b) J. Bact. 66, 259-265.
- Dagley, S. and Dawes, E.A. (1953c) Enzymologia 16, 226-230.
- Dagley, S. and Dawes, E.A. (1955) Biochim. Biophys. Acta, 17, 177-184.
- Davis, B.D. (1956) in "Enzymes - Units of Biological Structure and Function", Ed. O.H. Gaebler, pp 509-522, Academic Press, Inc. New York.
- Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-427.

- Davies, G.E. and Stark, G.R. (1970) Proc. Natl. Acad. Sci. 66, 651-656.
- Defner, M. and Franke, W. (1939) Ann. 541, 85-117.
- de Groot, G.N. and Stouthamer, A.H. (1969) Arch. Mikrobiol. 66, 220-233.
- de Groot, G.N. and Stouthamer, A.H. (1970a) Biochim. Biophys. Acta, 208, 414-427.
- de Groot, G.N. and Stouthamer, A.H. (1970b) Arch. Mikrobiol. 74, 326-339.
- de Groot, G.N. and Stouthamer, A.H. (1970c) Arch. Mikrobiol. 74, 340-349.
- De Marco, C., Riva, F. and Dupre, S. (1964) Anal. Biochem. 8, 269-271.
- Dimroth, P. (1975) FEBS Letters, 51, 100-104.
- Dimroth, P. (1976) Eur. J. Biochem. 64, 269-281.
- Dimroth, P., Buckel, W., Loyal, R. and Eggerer, H. (1977a) Eur. J. Biochem. 80, 469-477.
- Dimroth, P., Dittmar, W., Walther, G. and Eggerer, H. (1973) Eur. J. Biochem. 37, 305-315.
- Dimroth, P. and Eggerer, H. (1975a) Eur. J. Biochem. 53, 227-235.
- Dimroth, P. and Eggerer, H. (1975b) Proc. Natl. Acad. Sci., USA, 72, 3458-3462.
- Dimroth, P. and Loyal, R. (1977) FEBS Letters, 76, 280-283.
- Dimroth, P., Loyal, R. and Eggerer, H. (1977b) Eur. J. Biochem. 80, 479-488.
- Dimroth, P., Mayer, K. and Eggerer, H. (1975) Eur. J. Biochem. 51, 267-273.

- Dickens, F. (1933) Biochemical J. 27, 1141-1151.
- Eagon, R.G. and Asbell, M.A. (1966) Biochem. Biophys. Res. Comm. 24, 67-73.
- Eagon, R.G. and Wilkerson, L.S. (1972) Biochem. Biophys. Res. Comm. 46, 1944-1950.
- Eggerer, H., Buckel, W., Lenz, H., Wunderwald, P., Gottschalk, G., Cornforth, H.W., Donninger, C., Mallaby, R. and Redmond, J.W. (1970) Nature 226, 517-519.
- Eisenthal, R., Tate, S.S. and Datta, S.P. (1966) Biochim. Biophys. Acta, 128, 155-164.
- Fillman, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.
- Englard, S. (1959) J. Biol. Chem. 234, 1004-1006.
- Englard, S. and Siegel, L. (1969) Meth. Enzymol. 13, 99-106.
- Evans, M.C.W. and Buchanan, B.B. (1965) Proc. Natl. Acad. Sci. 53, 1402-1425.
- Evans, M.C.W. and Buchanan, B.B. and Arnon, D.I. (1966) Proc. Natl. Acad. Sci. 55, 928-934.
- Friedmann, J.E. and Haugen, G.I. (1943) J. Biol. Chem. 147, 415-442.
- Giffhorn, F., Beuscher, N. and Gottschalk, G. (1972) Biochem. Biophys. Res. Comm. 49, 467-472.
- Giffhorn, F. and Gottschalk, G. (1975a) J. Bact. 124, 1046-1051.
- Giffhorn, F. and Gottschalk, G. (1975b) J. Bact. 124, 1052-1061.
- Giffhorn, F. and Gottschalk, G. (1978) FEBS Letters, 96, 175-178.
- Gillespie, D.C. and Gunsalus, I.C. (1953) Bact. Proc. p. 80.
- Glusker, J.P. and Srere, P.A. (1973) Bioorganic Chem. 2, 301-310.

- Gottschalk, G. and Barker, H.A. (1966) *Biochem.* 5, 1125-1133.
- Green, A.A. and Hughes, W.L. (1955) *Meth. Enzymol.* 1, 67-90.
- Gruber, W. and Moellering, H. (1966) *Biochem. Z.* 346, 85-88.
- Grunberg-Manago, M. and Gunsalus, I.C. (1953) *Bact. Proc.*
p. 73-74.
- Gunsalus, I.C. (1958) in *Proc. 4th Int. Congr. Biochem. Vienna*,
Vol. 13, pp 226-250, Pergamon Press Ltd., London.
- Guynn, R.W., Gelberg, H.J. and Veech, R.L. (1973) *J. Biol.*
Chem. 248, 6957-6965.
- Habeeb, A.F.S.A. (1972) *Meth. Enzymol.* 25, 457-464.
- Hanson, K.R. and Rose, I.A. (1963) *Proc. Natl. Acad. Sci.* 50,
981-988.
- Harvey, R.J. and Collins, E.B. (1961) *J. Bact.* 82, 954-959.
- Harvey, R.J. and Collins, E.B. (1962) *J. Bact.* 83, 1005-1009.
- Harvey, R.J. and Collins, E.B. (1963) *J. Biol. Chem.* 238,
2648-2653.
- Hersh, L.B. and Jencks, W.P. (1967) *J. Biol. Chem.* 242,
3481-3486.
- Hiremath, S.T. (1977) Ph.D. Thesis, University of Poona.
- Hiremath, S.T., Paranjpe, S. and SivaRaman, C. (1976)
Biochem. Biophys. Res. Comm. 72, 1122-1128.
- Hirs, C.H.W. (1967) *Meth. Enzymol.* 11, 59-62.
- Hirschmann, H. and Hanson, K.R. (1971) *Eur. J. Biochem.* 22,
301-309.
- Hitchcock, S.E. (1975) *Biochem.* 14, 5162-5167.
- Horecker, B.L. and Kornberg, A. (1948) *J. Biol. Chem.* 175,
385-389.

- Horecker, B.L., Pontremoli, S., Ricci, C. and Cheng, T.
(1961) *Proc. Natl. Acad. Sci.* 47, 1949-1955.
- Hucho, F. and Janda, M. (1974) *Biochem. Biophys. Res. Comm.*
57, 1080-1088.
- Hucho, F., Müllner, H. and Sund, H. (1975) *Eur. J. Biochem.*
59, 79-87.
- Imai, K. (1978) *J. Gen. Appl. Microbiol.* 24, 279-285.
- Imai, K., Iijima, T. and Hasegawa, T. (1973) *J. Bact.* 114,
961-965.
- Jencks, W.P. (1973) in "The Enzymes" Vol. 9, 3rd edn. Ed.
Boyer, P.D. pp 483-496, Academic Press, New York.
- Johnson, C.L., Cha, Y. and Stern, J.R. (1975) *J. Bact.* 121,
682-687.
- Johnson, R.W., Grossman, A. and Boctor, A. (1978) *Anal.*
Biochem. 86, 679-691.
- Kaplan, M.M. and Flavin, M. (1966) *J. Biol. Chem.* 241,
5781-5789.
- Kempler, G.M. and McKay, L.L. (1979) *Appl. Environ. Microbiol.*
37, 316-323.
- Klinman, J.P. and Rose, I.A. (1971) *Biochemistry* 10, 2267-2271.
- Koelsch, R., Lasch, J., Marquardt, I. and Hanson, H. (1975)
Anal. Biochem. 66, 556-567.
- Kulla, H. and Gottschalk, G. (1977) *J. Bact.* 132, 764-770.
- Kümmel, A., Behrens, G. and Gottschalk, G. (1975) *Arch.*
Microbiol. 102, 111-116.
- Lasch, J. and Koelsch, R. (1977) *Eur. J. Biochem.* 82, 181-186.
- Lenz, H., Buckel, W., Wunderwald, P., Biedermann, G.,
Buschmeier, V., Eggerer, H., Cornforth, J.W., Redmond, J.W.

- and Mallaby, R. (1971) *Eur. J. Biochem.* 24, 207-215.
- Lomant, A.J. and Fairbanks, G. (1976) *J. Mol. Biol.* 104, 243-261.
- Lowenstein, J.M. (1969) in "Citric Acid Cycle", Marcel-Dekker, New York.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
- Mahadik, S.P. and SivaRaman, C. (1968) *Biochem. Biophys. Res. Comm.* 32, 167-172.
- March, S.C., Parikh, I. and Cuatrecasas, P. (1974) *Anal. Biochem.* 60, 149-152.
- Markham, R., Frey, S. and Hills, G.J. (1963) *Virology*, 20, 88-102.
- Mattiasson, B. and Mosbach, K. (1971) *Biochim. Biophys. Acta*, 235, 253-257.
- Moellering, H. and Gruber, W. (1966) *Anal. Biochem.* 17, 369-376.
- Moore, S. and Stein, W.H. (1963) *Meth. Enzymol.* 6, 819-831.
- Morse, D.E. and Horecker, B.L. (1968) *Adv. in Enzymol.* 31, 125-181.
- O'Brien, R.W. (1975a) *J. Bact.* 122, 468-473.
- O'Brien, R.W. (1975b) *J. Bact.* 124, 1084-1088.
- O'Brien, R.W., Frost, G.M. and Stern, J.R. (1969) *J. Bact.* 99, 395-400.
- O'Brien, R.W. and Geisler, J. (1974) *J. Bact.* 119, 661-665.
- O'Brien, R.W. and Stern, J.R. (1969) *J. Bact.* 98, 388-393.
- Ogston, A.G. (1948) *Nature* 162, 963.
- Oltmann, L.F., Reijnders, W.N.M. and Stouthamer, A.H.

- (1976) Arch. Microbiol. 111, 37-43.
- Oppenheimer, N.J., Singh, M., Sweeley, C.C., Sung, S.-J.
and Srere, P.A. (1979) J. Biol. Chem. 254, 1000-1002.
- Porath, J., Axen, R. and Ernback, S. (1967) Nature (London) 215, 1491-1492.
- Porath, J., Axen, R. (1976) Meth. Enzymol. 44, 19-45.
- Prescott, D.J. and Vagelos, P.R. (1972) Adv. in Enzymol. 36, 269-311.
- Retey, J., Luthy, J. and Arigoni, D. (1970) Nature 226, 519-521.
- Robinson, J.B. Jr., Singh, M. and Srere, P.A. (1976) Proc. Natl. Acad. Sci., USA. 73, 1872-1876.
- Sachan, D.S. and Stern, J.R. (1971a) Biochem. Biophys. Res. Comm. 45, 402-408.
- Sachan, D.S. and Stern, J.R. (1971b) Fed. Proc. 30, 1115.
- Sata, G., Asagi, M., Oka, C., Ishiguro, N. and Terakado, M. (1978) Microbiol. Immunol. 22, 357-360.
- Schaab, C., Giffhorn, F., Schoberth, S., Pfenning, N. and Gottschalk, G. (1972) Z. Naturforsch. 27b, 962-967.
- Schellenberg, K.A. (1963) J. Org. Chem. 28, 3259-3261.
- Schmellenkamp, H. and Eggerer, H. (1974) Proc. Natl. Acad. Sci. USA, 71, 1987-1991.
- Showe, M.K. and De Moss, J.A. (1968) J. Bact. 95, 1305-1313.
- Singh, M., Carpenter, D.E. and Srere, P.A. (1974) Biochem. Biophys. Res. Comm. 59, 1211-1218.
- Singh, M., Robinson, J.B. Jr. and Srere, P.A. (1977) J. Biol. Chem. 252, 6061-6068.
- Singh, M. and Srere, P.A. (1971) J. Biol. Chem. 246, 3847-3850.

- Singh, M. and Srere, P.A. (1975) J. Biol. Chem. 250, 5818-5825.
- Singh, M., Srere, P.A., Klapper, D.G. and Capra, J.D. (1976)
J. Biol. Chem. 251, 2911-2915.
- SivaRaman, C. (1961) Biochim. Biophys. Acta, 52, 212-213.
- Smith, R.A., Stamer, J.R. and Gunsalus, I.C. (1956) Biochim.
Biophys. Acta, 19, 567-568.
- Solomon, F. and Jencks, W.P. (1969) J. Biol. Chem. 244,
1079-1081.
- Spector, L.B. (1972) in "The Enzymes" Vol. 7, 3rd edn. Ed.
Boyer, P.D. pp. 357-389, Academic Press, New York.
- Sramek, S.J. and Frerman, F.E. (1975) Arch. Biochem. Biophys.
171, 27-35.
- Srere, P.A. (1965) Nature 205, 766-770.
- Srere, P.A. (1968) in Biochemical Society Symposia 27,
"Metabolic Roles of Citrate", pp 11-22 Ed. Goodwin, T.W.,
Academic Press, Inc. New York.
- Srere, P.A. (1972) in "Current topics in Cellular Regulations",
5, 229-284.
- Srere, P.A. (1974) Life Sciences 15, 1695-1710.
- Srere, P.A. (1975) Adv. in Enzymol. 43, 57-101.
- Srere, P.A. and Bhaduri, A. (1964) J. Biol. Chem. 239, 714-718.
- Srere, P.A., Bottger, B. Brooks, G.C. (1972) Proc. Natl. Acad.
Sci. USA. 69, 1201-1202.
- Srere, P.A., Mattiasson, B. and Mosbach, K. (1973) Proc. Natl.
Acad. Sci. USA. 70, 2534-2538.
- Srere, P.A. and Singh, M. (1974) in Lipman Symposium, "Energy,
Biosynthesis and Regulation in Molecular Biology" pp.
575-588, Walter de Gruyter and Co., Berlin.

- Stadtman, E.R. (1957) Meth. Enzymol. 3, 228-231.
- Stegink, L.D. and Coon, M.J. (1968) J. Biol. Chem. 243, 5272-5279.
- Stern, J.R. (1957) Meth. Enzymol. 3, 425-434.
- Stern, J.R. (1967) Biochem. 6, 3545-3551.
- Stern, J.R. and Bambers, G. (1966) Biochem. 5, 1113-1118.
- Stern, J.R. and Sachan, D.S. (1970) Fed. Proc. 29, 932.
- Sullivan, A.C., Singh, M., Srere, P.A. and Glusker, J.P. (1977) J. Biol. Chem. 252, 7583-7590.
- Switzer, R.L. (1977) Ann. Rev. of Microbiol. 31, 135-157.
- Tate, S.S. and Datta, S.P. (1964) Biochem. J. 91, 18c-19c.
- Tate, S.S. and Datta, S.P. (1965) Biochem. J. 94, 470-477.
- Tubbs, P.K. and Garland, P.B. (1969) Meth. Enzymol. 13, 535-551.
- Tung, K.K. and Wood, W.A. (1975) J. Bact. 124, 1462-1474.
- Vagelos, P.R. (1973) in "The Enzymes" Vol. 8, Part A, pp 155-199 3rd edn. Ed. Boyer, P.D. Academic Press, New York.
- van'T Riet, J., Stouthamer, A.H. and Planta, J.R. (1968) J. Bact. 96, 1455-1464.
- Villarreal-Moguel, E.I. and Ruiz-Herrera, J. (1969) J. Bact. 98, 552-558.
- Windle, L.M. and Switzer, R.L. (1973) J. Bact. 114, 517-527.
- Walther, R., Hippe, H. and Gottschalk, G. (1977) Appl. Environ. Microbiol. 33, 955-962.
- Wang, K. and Richards, F.M. (1974) J. Biol. Chem. 249, 8005-8018.
- Ward, R.L. and Srere, P.A. (1965) Biochim. Biophys. Acta, 99, 270-274.

- Watson, J.A., Fang, M. and Lowenstein, J.M. (1969) Arch. Biochem. Biophys. 135, 209-217.
- Weber, K. and Osborn, M. (1969) J. Biol. Chem. 246, 4406-4416.
- Weber, K. and Osborn, M. (1975) in "The Proteins" Vol. 1, 3rd edn. Ed. Neurath, H. and Hill, R.L., pp. 179-223, Academic Press, New York.
- Weckesser, J., Drews, G. and Tauschel, H.D. (1969) Arch. Microbiol. 65, 346-358.
- Wheat, R.W. and Ajl, S.J. (1955a) J. Biol. Chem. 217, 897-907.
- Wheat, R.W. and Ajl, S.J. (1955b) J. Biol. Chem. 217, 909-920.
- Wiley, R.H. and Kim, K. (1973) J. Org. Chem. 38, 3582-3585.
- Wilkerson, L.S. and Eagon, R.G. (1972) Arch. Biochem. Biophys. 149, 209-221.
- Wilkerson, L.S. and Eagon, R.G. (1974) J. Bact. 120, 121-124.
- Willecke, K. and Pardee, A.B. (1971) J. Biol. Chem. 246, 1032-1040.
- Wunderwald, P., Buckel, W., Lenz, H., Buschmeier, V., Eggerer, H., Gottschalk, G., Cornforth, J.W., Redmond, J.W. and Mallaby, R.W. (1971) Eur. J. Biochem. 24, 216-221.