THE UNIVERSITY OF MANITOBA

THE AMINO ACID SEQUENCE OF *ESCHERICHIA COLI* CITRATE SYNTHASE

BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TO MY PARENTS

Sh. Atam Parkash & Smt. Parkash Wati

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ABSTRACT

The amino acid sequence of Escherichia coli citrate synthase is presented. To elucidate this sequence, S-carboxymethylated protein was subjected to enzymatic and chemical fragmentation and the individual peptides were isolated by electrophoretic and column chromatographic techniques. High pressure liquid chromatography proved superb for peptide purification. Automated sequencing performed on these peptides established the amino acid sequence of 416 amino acids, of the total 426 in the enzyme subunit. Certain features of the sequence were confirmed from the amino terminal sequences of the fragments generated previously by partial proteolysis with subtilisin (Bell, A.W., Bhayana, V. & Duckworth, H.W. (1983) Biochemistry 22, 3400) and from the gene sequence (Ner, S.S., Bhayana, V., Bell, A.W., Giles, I.G., Duckworth, H.W., & Bloxham, D.P. (1983) Biochemistry 22, 5243).

A sequence homology of 27% is observed between the allosteric E. coli enzyme and non-allosteric pig heart citrate synthase when the two are aligned by inserting gaps at appropriate positions. Substantial homology is observed along the region believed to be involved in the active site. Evidence is presented indicating that at least some features of the folding patterns are similar in the two enzymes. Regions of considerable differences are noted between the two enzymes which could explain why the E. coli enzyme is hexameric and allosterically inhibited by NADH, while the pig heart enzyme is dimeric and insensitive to that molecule.

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ABBREVIATIONS

AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
α-KG	α-ketoglutarate
Acetyl-CoA	acetyl coenzyme A
A. anitratum	*Acinetobacter anitratum*
approx.	approximately
A	absorbance
CoA	coenzyme A
cm, mm, nm	centimeter, millimeter, nanometer
C	centigrade
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
DNAase	deoxyribonuclease
DT	Difference total
DEAE	diethylaminoethyl-
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetate
FDNB	fluorodinitrobenzene
g	grams
Gdn-HCl	guanidine hydrochloride
HPLC	high pressure liquid chromatography
HFBA	heptafluorobutyric acid
hr	hour
IAA	iodoacetic acid
LB	Luria broth
M, mM, nM, μM	molar, millimolar, nanomolar, micromolar

MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced form)
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OAA	oxaloacetic acid
PDS	pyridine disulfide
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	polyacrylamide gel electrophoresis
PITC	phenylisothiocyanate
PMSF	phenylmethanesulfonyl fluoride
PTH	phenylthiohydantoin
PCA	pyrrolidone carboxylic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	tricarboxylic acid cycle
Tris	trihydroxymethylaminomethane
TFA	trifluoroacetic acid
TPCK-trypsin	trypsin pretreated with L-1-(tosylamido)--2-phenyl ethyl chloromethyl ketone
UV	ultraviolet
U	units

INTRODUCTION

Citrate synthase catalyses the condensation reaction :

Acetyl-CoA + Oxaloacetate ⇋ Citrate + CoA.

The reaction effects the entry of carbon into the citric acid cycle, and thus citrate synthase is an important potential site for the control of this metabolic pathway. In some organisms the enzyme is indeed regulated by various metabolic effectors, which have control over the energetic and biosynthetic roles of the cycle. Furthermore, the patterns of regulation of citrate synthase from diverse organisms are correlated with its molecular structure and the taxonomy of the organisms (Weitzman & Danson, 1976; Weitzman, 1981). Eukaryotes and Gram-positive bacteria have "small" enzymes, of molecular weight 90-100,000 g/mole, which have been shown to be dimeric (Wu and Yang, 1970; Singh et al., 1970; Wiegand et al., 1979). These enzymes are isosterically inhibited by ATP but are insensitive to NADH and α-ketoglutarate (Harford and Weitzman, 1975; Weitzman and Danson, 1976; Weitzman,1981). On the other hand, the "large" citrate synthases, of molecular weight 240-280,000 g/mole are probably hexameric (Tong and Duckworth, 1975) and these are found exclusively in Gram-negative bacteria (Weitzman & Jones, 1968). These citrate synthases are allosterically inhibited by NADH; the inhibition is relieved by AMP in the aerobic members of this group, whereas the enzyme from facultative anaerobes is subjected to additional inhibition by α-ketoglutarate (Wright et al., 1967; Weitzman & Dunmore, 1969 a)

The "small" citrate synthases have been well characterized from a

number of eukaryotic sources (Weitzman and Danson, 1976; Bloxham et al., 1980, 1981) and of these the three dimensional structure of pig heart citrate synthase is also known (Wiegand et al., 1979; Remington et al., 1982)

It is possible that NADH-sensitive and NADH-insensitive citrate synthases possess similar types of subunits, whose molecular weights and tertiary structures are basically the same; the differences in their kinetics and regulatory properties would then be due to differences in their quaternary structures. The studies on mutant E. coli citrate synthase are in support of this notion (Danson et al., 1979). In these studies citrate synthase-deficient mutants were produced and revertants were selected which had regained citrate synthase activity. The enzyme isolated from one of these revertants was less sensitive to NADH and α-KG, than the original. In addition, several other features of the citrate synthase from mutant E. coli were similar to those of pig heart enzyme. Thus, the molecular weight of the enzyme was 100,000 g/mole, characteristic of "small" citrate synthases. This conversion of the "large" to the "small" form, supports the above hypothesis that extent of polymerization, rather than substantial differences in the subunits, determines the functional characteristics of a citrate synthase.

Furthermore, in favor of the above hypothesis partial proteolysis studies upon citrate synthases from pig heart (Bloxham et al., 1980), and from E. coli (Bell et al., 1983) suggested that at least some features of the three dimensional folding of the subunit are the same

in both enzymes.

To strengthen the above hypothesis, in this thesis I set out to determine the amino acid sequence of citrate synthase from Escherichia coli. The methodologies of sequencing were conventional and have been described earlier in several reviews (Konigsberg, 1977; Niall, 1977; Perham, 1978; & Walsh, 1981). The two major sets of peptides from carboxymethylated citrate synthase were obtained by specific cleavages at arginine and methionine, using trypsin after citraconylation of the protein and cyanogen bromide respectively. Other cleavage reagents such as o-iodosobenzoic acid and hydroxylamine were also used to obtain peptides in the region where the residues had been assigned tentatively or not at all from the previous two sets of data. The peptides were isolated by techniques such as gel filtration, ion exchange chromatography (on Dowex 50-X2, Dowex 1-X2 and DEAE cellulose), high voltage paper electrophoresis and high pressure liquid chromatography (HPLC) on a reverse phase C-8 column. Of all these techniques of peptide purification, HPLC proved extremely fast and powerful. In fact, on several occasions this technique alone was employed for isolating pure peptides. The use of volatile solvents in the HPLC system eliminated the need to desalt peptide solutions; therefore these samples, dissolved in appropriate volumes of solvent, could be subjected directly to automatic sequencing. The automatic Edman degradation of peptides was performed on a Beckman 890C Sequencer using a 0.1 M Quadrol as a coupling buffer.

The results of the sequence of 416, of the total 426 residues are

presented in this thesis. The complete sequence is then inferred with the help of the base sequence of the E. coli citrate synthase gene (Ner et al., 1983) and it is aligned with that of pig heart enzyme to identify the homology and differences between the two. The secondary structure is also predicted from the primary structure by the method of Chou and Fasman (1978), and Garnier (1978).

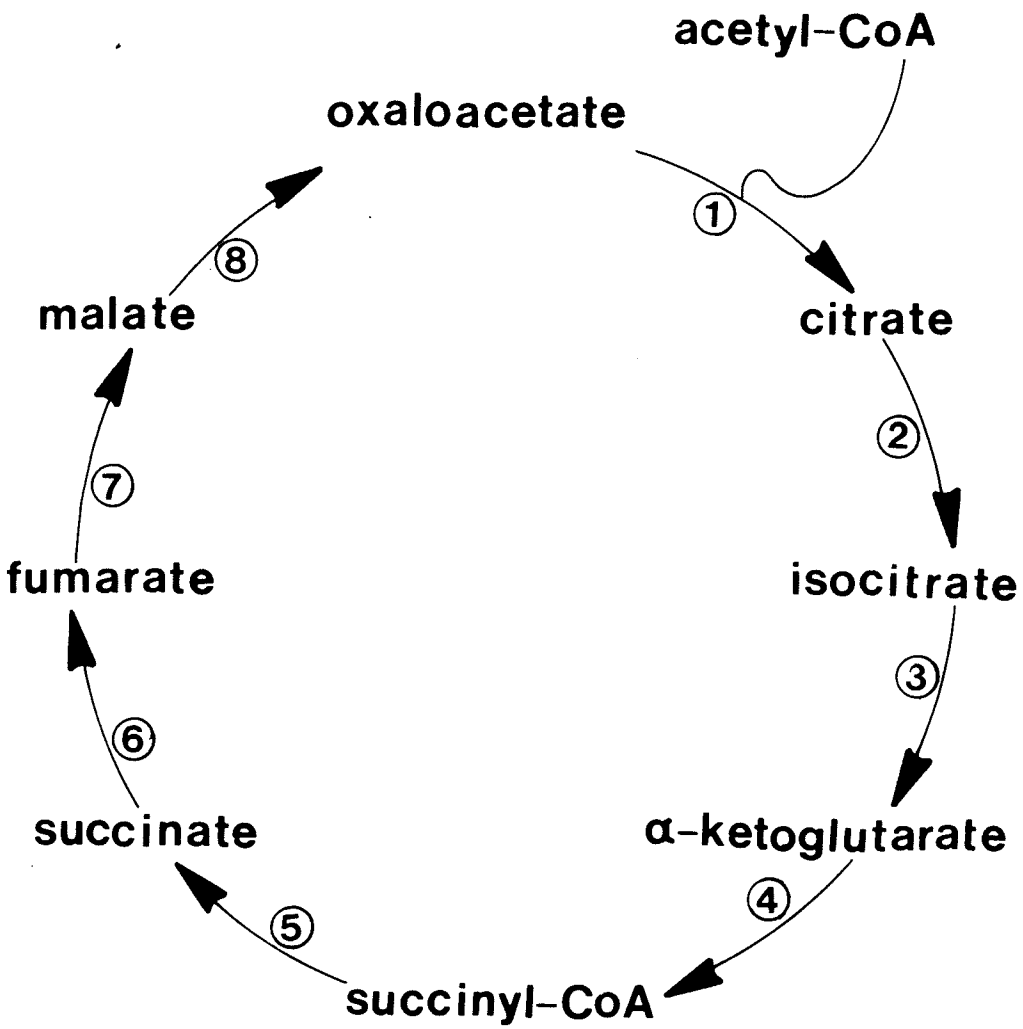
HISTORICAL REVIEW

The Tricarboxylic Acid Cycle :

By 1950 the work of Krebs (1943) had demonstrated that citrate is formed from oxaloacetic acid and pyruvate. This discovery allowed the already established oxidation reaction sequence from citrate to oxaloacetate to be arranged into a cyclic pathway, which was linked with the glycolytic pathway of carbohydrate metabolism. With the discovery of acetyl coenzyme A, it became clear that the purpose of the TCA cycle was to produce energy by oxidation of acetate obtained from pyruvate in the form of acetyl-CoA. The TCA cycle was soon accepted as the terminal pathway for the oxidation of foodstuff in all respiring animal tissues. The scheme of the cycle originally proposed still holds good, with very few modifications (Figure 1). In summary, this cycle involves the degradation of acetyl-CoA derived from the oxidation of carbohydrates, fatty acids and amino acids. The net production of this cycle is two molecules of CO_2 and four pairs of hydrogen atoms. These hydrogen atoms result in the reduction of three molecules of NAD^+ and one FAD, which in turn are fed into the respiratory chain containing a series of electron carriers. ATP is generated as the electrons are transferred from these carriers to oxygen.

After the discovery of the TCA cycle in animal tissues, it was proposed that the same cycle may also be responsible for terminal respiration in microorganisms. The major problem in proving this was that the procedures employed with animal tissues to demonstrate the occurrence of the TCA cycle were unsuccessful in microorganisms. The difficulty was due

Figure 1. The tricarboxylic acid cycle (general scheme). The enzymes are: 1. citrate synthase 2. aconitase 3. isocitrate dehydrogenase 4. α-ketoglutarate dehydrogenase 5. succinyl-CoA synthase 6. succinate dehydrogenase 7. fumarase 8. malate dehydrogenase.



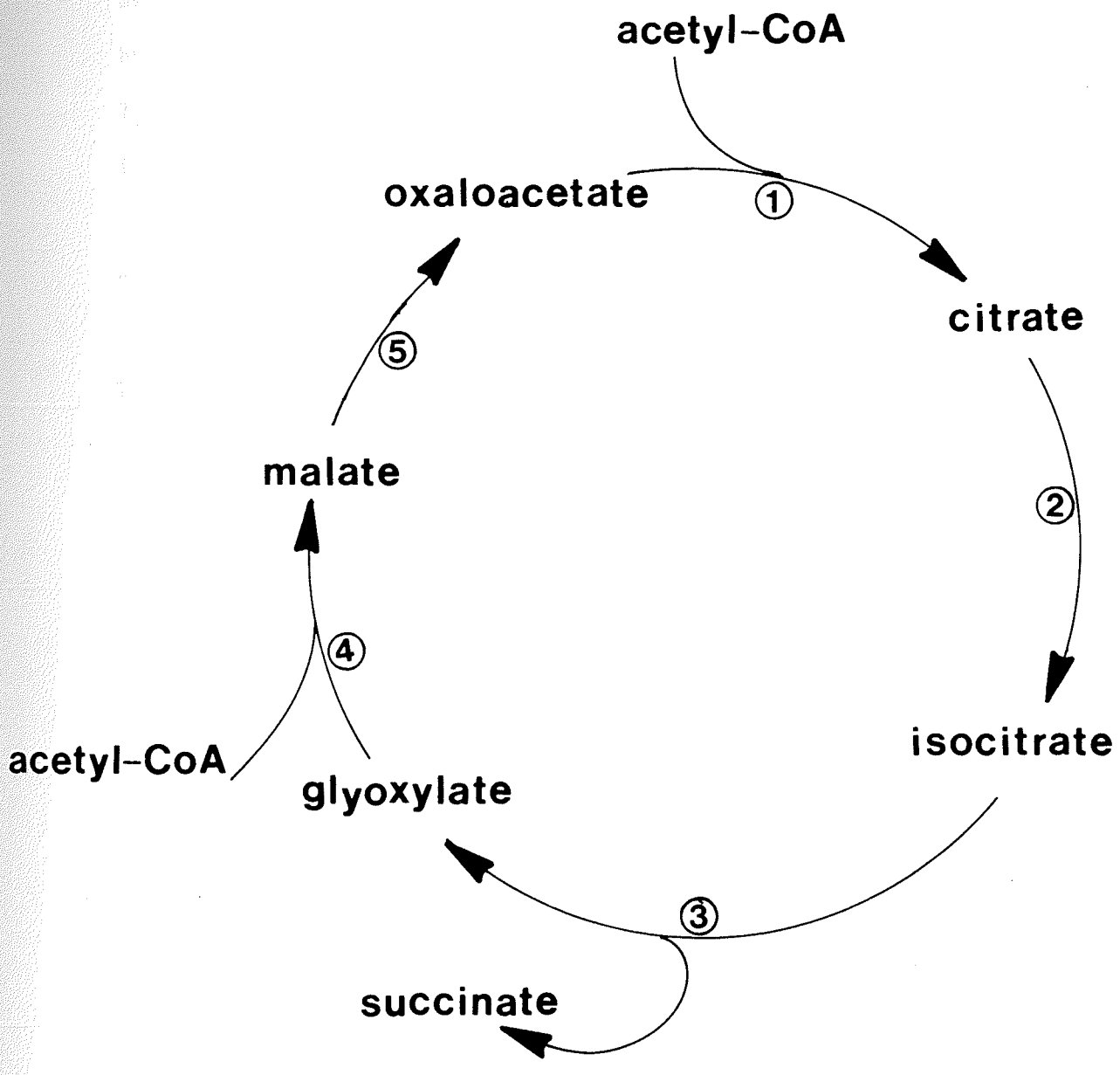
THE TRICARBOXYLIC ACID CYCLE

to permeability barriers towards metabolites and inhibitors, which microorganisms possess to a more marked extent than do animal tissues. The problems encountered and the ways in which the cycle was finally established as the major pathway of terminal respiration in microorganisms have been reviewed (Kornberg, 1959).

In addition to its role as an energy yielding catabolic pathway, Krebs et al. (1952) further suggested that the TCA cycle has a biosynthetic role in the cell, providing precursors for various cell components. The metabolic processes responsible for conversion of two-carbon compounds such as acetate and ethanol to cell constituents were not known. Thunberg and later Knoop (cited from Kornberg, 1959) had postulated a direct condensation of two acetates to form a succinate, but the true solution to the problem was not solved until the discovery of the enzymes isocitrate lyase and malate synthase. Kornberg and his coworkers later formulated the concept of the glyoxylate cycle, which would allow cells to grow on acetate as a sole carbon source and supply intermediates to the TCA cycle.

A simplified picture of the glyoxylate cycle is given in Figure 2. The cycle bypasses the CO_2 evolving steps of the TCA cycle and it accounts for the net synthesis of succinic acid (C4) which is used for biosynthetic purposes as a precursor in gluconeogenesis, the biosynthesis of new sugars by reversal of glycolysis. In fact organisms may possess both the tricarboxylic acid and glyoxylate cycles, where the former provides energy needs and the latter fulfills biosynthetic requirements.

Figure 2. The glyoxylate cycle. The enzymes are: 1. citrate synthase 2. aconitase 3. isocitrate lyase 4. malate synthase 5. malate dehydrogenase.



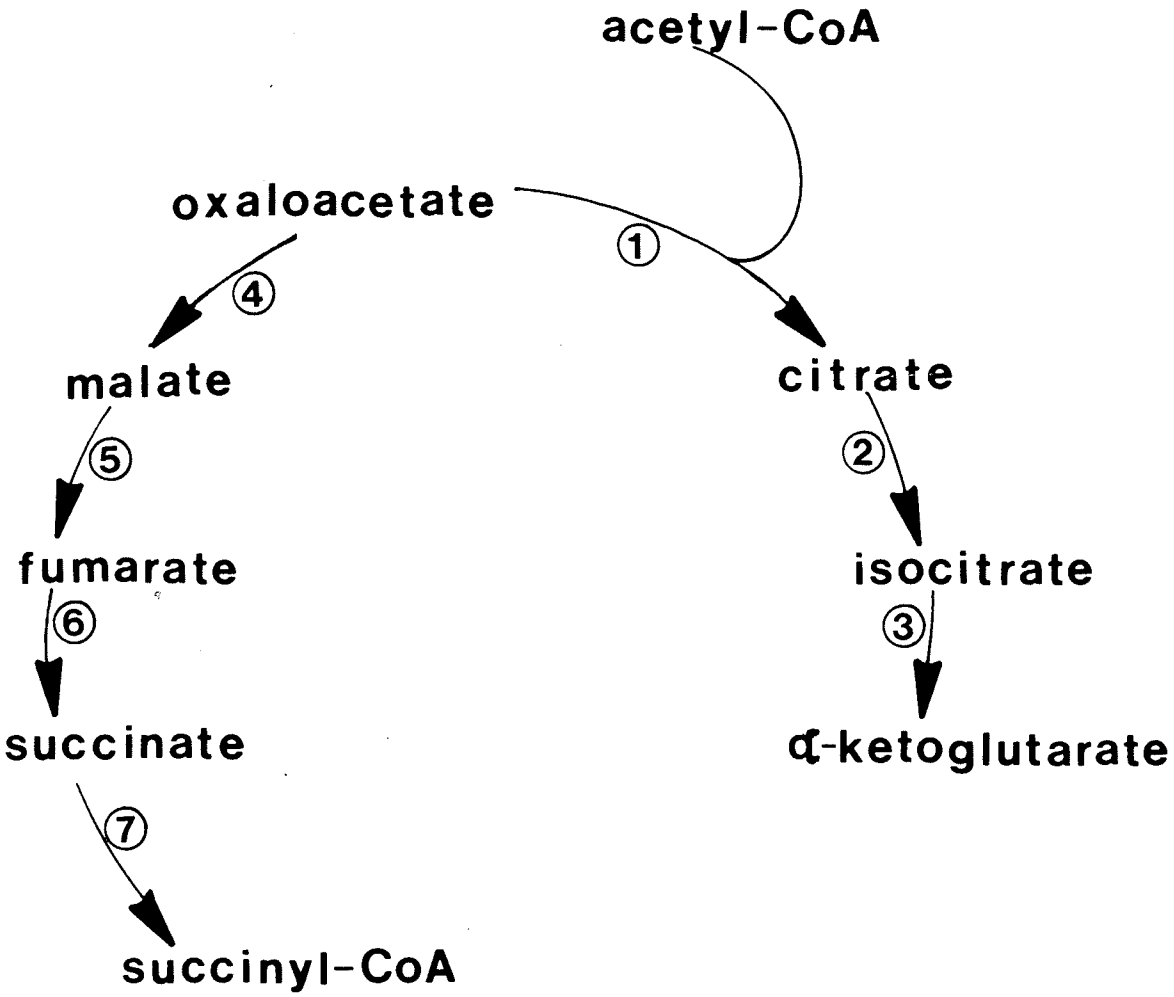
THE GLYOXYLATE CYCLE

It is evident that the TCA cycle enzymes do form a true cycle under aerobic conditions, but under anaerobic conditions they provide a branched non-cyclic pathway (Figure 3) to meet biosynthetic demands. In fact this split pathway also operates under aerobic conditions in E. coli, when glucose is the substrate and glycolysis is the energy yielding pathway (Amarasingham and Davis, 1965). In this non-cyclic pathway one arm is called the oxidative arm,where the sequence of oxidation reactions is started by citrate synthase to yield α-KG as the end product. The other arm, called the reductive arm, leads to the formation of succinyl-CoA; this arm does not depend on the activity of citrate synthase. Succinyl-CoA serves as the precursor for biosynthesis of porphyrins and some amino acids. Production of α-KG is important since it is enzymatically converted to glutamate and this reaction is one of the primary pathways for the formation of α- amino groups directly from ammonia.

Amarasingham and Davis (1965) reported that the connecting enzyme for the two arms (non cyclic TCA), α-ketoglutarate dehydrogenase, is not synthesized under anaerobic conditions. The same enzyme was also missing in cells growing exponentially on glucose under aerobic conditions.

In summary, in all the three pathways mentioned above, the TCA cycle, the glyoxylate cycle and the non-cyclic TCA pathway, citrate synthase occupies an important position. It catalyzes the condensation reaction between acetyl-CoA and oxaloacetate and thus ensures the entry of carbon into the pathways. Control of its activity has been postulated to be important for various metabolic processes, and indeed there is evidence

Figure 3. A non-cyclic tricarboxylic acid pathway in Escherichia coli (Amargsingham and Davis, 1965). The enzyme are: 1. citrate synthase 2. aconitase 3. isocitrate dehydrogenase 4. malate dehydrogenase 5. fumarase 6. fumarate reductase 7. succinyl-CoA synthase.



A NON-CYCLIC TCA PATHWAY

that citrate synthase catalyzes the rate limiting step in these pathways. Due to the fact that citrate synthase holds such a key position, a number of laboratories are involved in investigating its molecular, catalytic and regulatory properties. Four major reviews have appeared in the literature. Srere (1972) reviewed citrate synthase with respect to its physical and chemical properties, kinetics and regulatory features. He discussed apparent variations among citrate synthases from different sources and attempted to identify common characteristics. Spector (1972) discussed molecular properties, and the stereospecificity and mechanism of the condensation reaction catalyzed by citrate synthase. Weitzman & Danson (1976) in their review stressed mainly the comparative properties of the enzymes obtained from diverse organisms, while in a further review article, Weitzman (1981) discussed correlations between the bacterial taxonomic divisions and the regulatory and molecular properties of citrate synthase.

Citrate Synthase :

Stern and Ochoa (1951) were the first to show the synthesis of citrate from acetate, ATP and OAA, in the presence of a soluble enzyme preparation from pigeon liver. The newly-discovered Coenzyme A, and magnesium or manganese ions, were required as cofactors in the system. These investigators showed that the above reaction was catalyzed probably because the extract also contained an enzyme which converts acetate into " active acetate", acetyl CoA.

Lynen and Reichert (1951) succeeded in isolating acetyl-CoA from baker's yeast incubated aerobically in the presence of alcohol or acetic acid. Ochoa's group used a sample of acetyl-CoA from Lynen and demonstrated that acetyl-CoA readily reacts with OAA to produce citrate in the presence of condensing enzyme (the original name given to citrate synthase).

Stern, Shapiro, Stadtman and Ochoa (1951) showed first time that the condensing enzyme catalyzes a reversible reaction. The equilibrium was in favor of citrate formation. Ochoa et al. (1951) later reported that the equilibrium constant:

$$K = \frac{(\text{citrate})\ (\text{CoA})}{(\text{acetyl-CoA})\ (\text{OAA})} = 5 \times 10^5$$

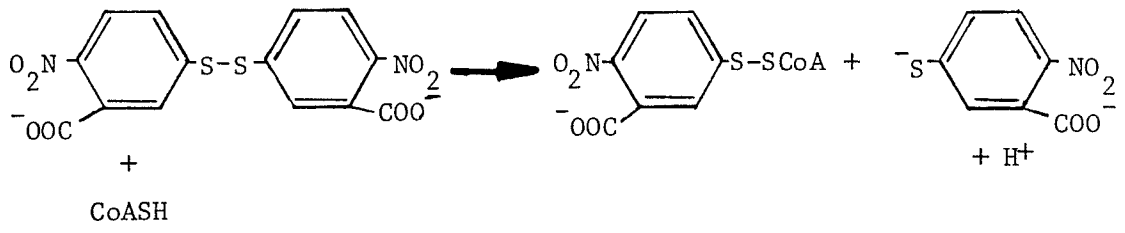
is strongly in favor of citrate.

Ochoa et al. (1951) crystallized the condensing enzyme from pig heart and also reported the presence of this enzyme in liver, kidney, brain, skeletal muscle, heart muscle, tumor, various bacteria, and yeast. The enzyme has now been found in almost all living cells and has been purified to homogeneity from some of these (Srere, 1972; Weitzman & Danson, 1976; Weitzman, 1981). The enzyme from Escherichia coli has been purified and the method of purification is described by several investigators (Faloona & Srere, 1969; Wright & Sanwal, 1971; Tong & Duckworth, 1975; and Duckworth & Bell, 1982). The apparent absence of citrate synthase has also been reported in Lactobacillus plantarum and Streptococcus faecalis (Weitzman & Jones, 1968) and Gemella haemolysans (Weitzman & Jones, 1975).

(i) Assay Procedures

In the early studies, two ultra-violet spectrophotometric methods were employed to assay citrate synthase. One was based on coupling with malate dehydrogenase in which the appearance of NADH was measured at 340 nm (Ochoa, 1955). The other method was based on following the decrease in absorbance at 233 nm consequent to the consumption of acetyl-CoA (Srere & Kosicki, 1961). Neither of these assays is well suited to crude extracts, as these may contain high concentrations of UV absorbing material and some preparaions may exhibit NADH oxidase activity.

Srere, Brazil and Gonen (1963) developed an assay procedure based on the reaction of CoASH with Ellman's reagent, 5,5'-dithio bis (2-nitrobenzoic acid) or DTNB :



DTNB reacts rapidly, nonenzymatically and stoichiometrically with the free thiol group of CoA-SH to form a mixed disulfide and to liberate the yellow thionitrobenzoate anion which absorbs strongly at 412 nm (E_m=13,600). The formation of CoA-SH in the course of the citrate synthase reaction may be measured continuously. This assay procedure is convenient and sensitive; it is now the most widely used method.

One potential difficulty with DTNB is that it can attack thiol groups on the enzyme so as to cause inactivation or desensitization towards effectors. To overcome this, another method was devised based on the polarographic signal, produced at the dropping mercury electrode by CoA-SH (Weitzman & Danson 1976; Weitzman 1981).

Regulatory Control of Citrate Synthase

(i) Inhibition by ATP:

Hathaway and Atkinson (1965) first showed an inhibitory effect of adenine nucleotides on citrate synthase isolated from Saccharomyces cerevisiae. The inhibition was competitive with AcCoA, while there was no change in the affinity for OAA in the presence of 5 mM ATP. The order of effectiveness for the inhibition was reported to be ATP >ADP >AMP. Similar effects were found for the enzyme isolated from pig heart. The discovery of ATP inhibition was of great interest because ATP is produced as a source of energy by the TCA cycle in higher organisms, and its ability to inhibit citrate synthase, the first enzyme in the cycle, would mean that the TCA cycle is under tight feedback control. When ATP is degraded to ADP or AMP, the inhibition of citrate synthase would be diminished as the latter nucleotides are less effective inhibitors of the enzyme.

The finding of ATP inhibition of citrate synthase from yeast led several investigators to look for a similar type of inhibition of the enzyme from various organisms including animals, plants, fungi and

bacteria (Weitzman & Danson, 1976). The studies revealed that only citrate synthases from eukaryotes and Gram-positive bacteria are inhibited by ATP.

Kosicki and Lee (1966) studied the effect of divalent metal ions on the reaction catalyzed by pig heart citrate synthase. They reported that the inhibition of enzyme by ATP was reduced at physiological concentrations of the divalent cations. It was suggested that the effect was due to the chelation of metal ions by the polyphosphate moiety of ATP. Consistent, with this idea , and with the order of inhibitory effectiveness of the nucleotides as stated above, Srere et al.(1973) suggested that electrostatic interactions may make an important contribution to enzyme binding of the nucleotide. The more negative charge resulting from the increased phosphate content in the adenine nucleotides produces stronger inhibition.

It was reported that nicotinamide nucleotides act as weak inhibitors for those citrate synthases which are sensitive to adenine nucleotides and the order of effectiveness was found to be NADPH > NADH > $NADP^+$ > NAD^+ (Kosicki & Lee, 1966; Lee and Kosicki, 1967; Srere et al., 1973; Danson et al., 1979). This finding was in accordance with the above observations since the additional phosphate groups in the reduced and oxidized forms of $NADP^+$, compared with those of NAD^+, cause greater inhibition.

The inhibition by ATP, of those citrate synthases which are sensitive to it, was found to be competitive with respect to acetyl-CoA (Hathaway and Atkinson, 1965). Again, in the case of weaker inhibitors, the nicotinamide nucleotides, the inhibitory effect was competitive with respect to

acetyl-CoA (Kosicki & Lee, 1966; Lee & Kosicki, 1967; Harford & Weitzman, 1975). From these observations it was suggested that the inhibition arises from the structural similarity between nucleotides and the substrate acetyl-CoA. The idea of a general non-specific affinity of the active site for adenine and nicotinamide adenine nucleotide was advanced, according to which the inhibition of enzyme activity by adenine-based nucleotides is isosteric (Weitzman & Danson, 1976).

Harford and Weitzman (1975) conducted multiple-inhibition studies on citrate synthase from various sources. They found that ATP acts in all cases as an isosteric inhibitor at the AcCoA site whereas NADH, an allosteric inhibitor for Gram-negative bacterial enzyme, acts isosterically with eukaryotic and Gram-positive bacterial citrate synthases.

Further evidence for such isosteric inhibition was obtained from the comparison of the apparent affinities of various citrate synthases for both ATP and acetyl-CoA. In general, it was found that when the affinity is high for acetyl-CoA (low Km) there is substantial inhibition by ATP. On the other hand when the affinity is low for acetyl-CoA (high Km) there is decreased sensitivity to ATP inhibition (Weitzman, 1981). It was concluded from these observations that ATP and related nucleotides act as inhibitors of citrate synthase (ATP sensitive) by binding to that portion of the active site of the enzyme which is occupied by acetyl-CoA.

Remington et al. (1982) studied the binding of CoA in the pig heart citrate synthase by X-ray crystallographic analysis. They found that the binding of CoA is characterized by a number of hydrophobic interactions;

the phosphate groups interact with three arginines (46, 164 and 324); and the adenine ring is recognized by the main chain which folds edgewise around the ring and forms the "adenine recognition loop". This loop may be the place where the competition takes place between acetyl-CoA and ATP or the competition may occur at the phosphate binding sites or at both.

When citrate synthase from Escherichia coli was tested for sensitivity to ATP, the enzyme was found to be weakly inhibited under the assay conditions used (Weitzman, 1966a). Weitzman clearly demonstrated, however, that NADH is an effective inhibitor for this enzyme (see below). Jangaard et al.(1968) also observed an ATP effect on E. coli citrate synthase but found that the response was very weak as compared to that of the mammalian enzyme. At pH 6.0, ATP inhibited the E. coli enzyme whereas at pH 7.6 no effect was observed. At pH 6.4, where ATP inhibits, the affinity for acetyl-CoA was found to be high (Km 50 μM). This observation is consistent with that of Srere (1972) who reported that in the presence of KCl, the Km for acetyl-CoA is lowered for E. coli citrate synthase and the response to ATP is enhanced. These results are in favor of the suggestion that ATP acts isosterically with acetyl-CoA.

Harford and Weitzman (1975) performed multiple inhibition studies also on citrate synthase from Pseudomonas aeruginosa, a Gram-negative bacterium in the same class as E. coli . They reported that ATP acts as an isosteric inhibitor at the acetyl-CoA site, whereas NADH acts as a specific allosteric inhibitor. After desensitization by treatment of the enzyme with DTNB, only a weak isosteric inhibition by NADH was observed.

Talgoy & Duckworth (1979) presented strong evidence indicating that a number of derivatives of adenylic acid bind to the NADH binding sites of E. coli citrate synthase. This means that the enzyme has two types of adenylate binding sites, allosteric sites and active sites (see below for details)

(ii) Inhibition by NADH

It was Weitzman (1966 a) who first showed that ATP had little effect on the inhibition of citrate synthase from the Gram-negative bacteria Escherichia coli , Azotobacter vinelandii and Pseudomonas ovalis. However, NADH was found to be an effective inhibitor for citrate synthase from the above sources, whereas 0.1 mM NADH caused complete inhibition of activity of citrate synthase from E. coli, only 75% inhibition occurred with as high as 20 mM ATP. The NADH inhibition was reported to be competitive with respect to acetyl-CoA. No inhibition of citrate synthase activity was observed by NAD^+, $NADP^+$ or NADPH. It was postulated from these results that NADH acts as a feedback control of energy production in bacteria, in contrast to the role of ATP in mammals and yeast.

Examination of another Gram-negative microorganism, Acinetobacter lwoffi, showed that citrate synthase again was sensitive to NADH, like that of E. coli, but differed in that the inhibition was completely overcome by low concentrations of AMP (Weitzman & Jones, 1968). This variation in the regulatory behaviour prompted a survey of the citrate synthases from a range of bacterial genera by Weitzman & Jones (1968), who found that the

organisms can be divided into two groups according to the susceptibility of their citrate synthases to inhibition by NADH. The enzymes from all Gram-negative organisms showed inhibition by NADH, whereas no such inhibition was observed in any Gram-positive bacteria. Moreover, the Gram-negative bacteria could be subdivided into two groups on the basis of response of NADH inhibition to AMP. In the citrate synthases from strict aerobes, NADH inhibition was relieved by AMP but with facultative anaerobes, AMP had no effect. Weitzman & Jones (1968) proposed a rationale for this further subdivision in terms of energy control mechanisms. The strict aerobes are absolutely dependent on the citric acid cycle for their energy supply, therefore in response to energy requirements there is need for an effector (AMP) to turn on the first enzyme of the cycle, citrate synthase, when energy supply is low (high AMP/ATP ratio). The facultative anaerobes, on the other hand, can also generate energy by fermentation and it is not so important for an effector (AMP) to regulate citrate synthase in such a situation.

Weitzman & Dunmore (1969 a) compared molecular weights of citrate synthase from various sources. The NADH sensitive enzymes (from Gram-negative bacteria) all belonged to a "large" group and those insensitive to NADH were placed in a "small" group (from eukaryotes and Gram-positive bacteria).

Weitzman (1966 b) presented evidence for the allosteric nature of NADH inhibition of bacterial citrate synthase by showing that alkaline pH or elevated salt concentration brought about desensitization of the

enzyme to the inhibitor.

Wright & Sanwal (1971) studied the binding of NADH to E. coli citrate synthase. They found that the binding of NADH to the enzyme was very weak, though it was considerably enhanced in the presence of acetyl-CoA. The binding of NADH was negatively cooperative in the absence of acetyl-CoA, but positively cooperative in its presence. An unexpected finding was that even in the presence of acetyl-CoA, the binding of NADH was too weak to account for the inhibition observed in kinetic experiments. The results presented in this paper are in conflict with later studies, probably because the enzyme used by Wright & Sanwal contained large amounts of ammonium sulfate. As already noted high concentrations of salts desensitize E. coli citrate synthase to NADH.

Duckworth & Tong (1976) have studied the binding of NADH to E. coli citrate synthase using a fluorescence enhancement technique. Contrary to the results of Wright & Sanwal (1971), they found a strong binding of NADH with a K_D of 1.6 μM; the binding was weakened by the presence of acetyl-CoA. The authors also reported that the dissociation constant for the NADH citrate synthase complex increases towards alkaline pH as if the binding depends on the protonation of a group with a pK value of 7.05. In addition to its effect on the K_D, an increase in pH from 6.2 to 8.7 reduced the total number of NADH binding sites from 0.65 to about 0.25 per subunit. This observation was explained from the fact that enzyme polymerizes in this pH range (Tong & Duckworth, 1975) and it could be that different polymeric forms have different affinities for NADH. These

authors also found that NAD^+ and $NADP^+$ are weak competitive inhibitors, whereas 5'-AMP and 3'-AMP are strong inhibitors of NADH binding.

Talgoy and Duckworth (1979) studied several analogues of NADH, including 2',5' and 3',5'-ADP, 5'-AMP, 3'-AMP and NADPH. They presented evidence that a number of derivatives of adenylic acid bind to the allosteric NADH binding site of E. coli citrate synthase. This evidence consisted of the facts that the adenylates inhibit NADH binding in a competitive manner and that they protect the enzyme from DTNB reaction in the same way as NADH does (see sulfhydryl groups for details). This finding that adenylates bind to the allosteric site of E. coli citrate synthase suggests a close similarity between this enzyme and the citrate synthases of strictly aerobic Gram-negative bacteria, whose NADH inhibition is reversed by 5'-AMP (Weitzman & Jones, 1968; Morse & Duckworth, 1980).

The results described above can be explained by the existence of a T $\leftrightarrow$ R state equilibrium of the enzyme in terms of the Monod, Wyman and Changeux model (1965). According to this model, two conformational states of the enzyme, active (R) and inactive (T), are in equilibrium. The R state has a high affinity for substrates and activators, whereas the T state has a high affinity for inhibitors. The position of the resting enzyme in T $\leftrightarrow$ R equilibrium determines the nature of saturation curve, hyperbolic or sigmoid.

It has been shown for E. coli citrate synthase that in the absence of KCl, the enzyme displays sigmoid saturation with acetyl-CoA indicating that the resting state of the enzyme is in the T state (Faloona & Srere,

1969; Wright & Sanwal, 1971). The effect of KCl, in inducing a conformational change, is indicated by change of acetyl-CoA saturation to hyperbolic in the presence of KCl (Faloona & Srere, 1969). Thus the effect of KCl most likely is to convert the T state to an active R state. That this is the case was also indicated by the fact that in the presence of 0.2 M KCl, the enzyme is desensitized to NADH (Weitzman, 1966 b).

Duckworth & Tong (1976) and Talgoy & Duckworth (1979), from the binding studies of NADH and other adenylates, suggested two types of adenylate binding sites, an active site and an allosteric site. Acetyl-CoA and KCl were shown to inhibit the binding of NADH; this finding was explained by suggesting that acetyl-CoA causes a conformational change in the enzyme and destroys the allosteric sites. The effect of KCl is to promote the same change so as to abolish NADH binding and stimulate acetyl-CoA binding and enzyme activity simultaneously. All these binding studies are consistent with the kinetic observations as described above.

Other adenylates inhibit NADH binding competitively by binding at the NADH site, but they are activators, in contrast to NADH which is an inhibitor (Talgoy & Duckworth, 1979; Duckworth & Tong, 1976). It is suggested that adenylates binding at the allosteric site would shift the equilibrium to the R state thus making the binding of substrate easier; conversely, when the active site is occupied, NADH cannot bind to the allosteric site whereas the simpler adenylates can. The binding sites have a high affinity for NADH only in the T state. This characteristic binding of NADH was attributed to a critical part on the allosteric

site, responsible for binding the dihydronicotinamide part of NADH,which is present in the T state but disappears as a result of the T to R transition (Talgoy & Duckworth, 1979). However, the finding that both NADH and adenylates protect the SH group which is reactive towards DTNB suggests that they occupy a common area close to the allosteric site.

The finding of Talgoy and Duckworth (1979) that acetyl-CoA inhibits the DTNB reaction with E. coli citrate synthase, seems to be consistent with the previous conclusion that acetyl-CoA induces conformational changes at the allosteric site. On the other hand there is the possibility that acetyl-CoA protects the SH group by binding at the allosteric site as other adenylates do. If this is the case then acetyl-CoA may be inhibiting the NADH binding for steric reasons, and may bind to both kinds of sites on a subunit, which could also explain the sigmoid nature of the acetyl-CoA saturation curves.

A direct proof of the conclusion from kinetic studies that ligand binding induces a conformational change, as stated above, has been obtained by Rowe and Weitzman (1969) for citrate synthase from Acinetobacter calcoaceticus, a Gram-negative strict aerobe. They examined the enzyme in the electron microscope by negative-contrast staining with uranyl acetate or ammonium molybdate. The mean diameter was reported to change in the presence of NADH, whereas no such change was observed with 0.2 M KCl. They also observed that the swelling of the enzyme in the presence of NADH, was reversed when AMP was added; AMP reverses NADH inhibition of this enzyme. These structural changes were consistent with the changes

in activity induced by effectors; NADH converts the enzyme to an inactive state and AMP reverts it to active state. No such study has been done on the E. coli enzyme.

(iii) Inhibition by α-ketoglutarate:

Wright, Maeba and Sanwal (1967) first reported the inhibition of E. coli citrate synthase by α-ketoglutarate, and it was suggested that this inhibition was allosteric since desensitization was brought about by KCl or high pH. Weitzman and Dunmore (1969b) investigated this type of inhibition in citrate synthases from diverse organisms and found that the inhibition was restricted to Gram-negative facultative anaerobic bacteria, such as E. coli. There was no inhibition of the enzyme from aerobic Gram-negative, Gram-positive or eukaryotic organisms. The rationale for this type of inhibition of an anaerobic citrate synthase was explained again in terms of metabolism. During anaerobic growth energy is generated by fermentation rather than the TCA cycle. Under such conditions, the enzyme α-ketoglutarate dehydrogenase is absent and the citric acid cycle becomes a branched chain non-cyclic pathway which permits the production of α-ketoglutarate and succinyl-CoA (Amarasingham et al., 1965). Citrate synthase thus plays a biosynthetic role in a reaction sequence leading to α-ketoglutarate and the effect of the latter on citrate synthase activity is an example of feedback inhibition.

Duckworth & Tong (1976) showed that α-KG has no effect on NADH binding at pH 7.8 at a concentration of 0.99 mM, although it has pronounced

inhibitory effect on enzyme activity at this level. Since the molecule had no effect on NADH binding in the presence of KCl or acetyl-CoA, the authors suggested that it does not stabilize the T state as NADH does.

α-ketoglutarate is a homologue of OAA and it is reasonable to expect that it would show affinity for the OAA binding site. Talgoy & Duckworth (1979) reported that α-KG inhibits OAA binding to the enzyme-CoA complex. At the same level, the molecule (α-KG) does not inhibit binding of OAA to the enzyme if KCl is present. It was suggested from these results that α-KG can bind to the OAA sites, and these sites become particularly much more selective for OAA in the presence of KCl. Since KCl promotes the formation of the R state and NADH stabilizes the T state, it appears that α-KG is not involved in allosteric mechanisms; its inhibitory effect is isosteric in nature, with α-KG behaving as a structural analogue of OAA.

Functional Residues of E. coli Citrate Synthase

(i) Sulfhydryl groups:

The sulfhydryl groups of E. coli citrate synthase have been widely investigated with regard to the functional characteristics of the enzyme. Weitzman (1966 b) was the first to investigate the sensitivity of the enzyme to modification of SH groups. He found that the activity of the enzyme was abolished when incubated for 5 min with 0.1 mM DTNB at pH 8.0. However, in the presence of 0.2 M KCl, no inactivation was observed. Moreover,

the addition of KCl to the DTNB-inactivated enzyme resulted in more than 50% reactivation.

Faloona and Srere (1969) presented different results from Weitzman (1966 b) with respect to the reaction of DTNB with E. coli citrate synthase, for they claimed that DTNB reacts with the enzyme with no loss of enzyme activity. Later Weitzman & Danson (1976) explained this discrepancy by noting that Faloona & Srere (1969) used high ionic strength buffers in the assay mixture (0.1 M Tris-HCl and 0.1 M KCl), thus preventing any DTNB inactivation.

Wright and Sanwal (1971) investigated the reactivity of SH groups with DTNB and 4,4'-PDS. They reported that reaction of 0.1 mM DTNB over a period of 6 hrs resulted in a loss of 90% enzyme activity. They found that the number of titratable SH groups was not altered in the presence of acetyl-CoA, NADH or α-KG. This finding was in contrast to the results reported by other investigators (see below). With another sulfhydryl reagent, 4,4'-PDS (1.0 mM), Wright & Sanwal showed that enzyme activity dropped to zero in 20 min.

Danson and Weitzman (1973) concluded from the results of photo-oxidation of E. coli citrate synthase that cysteinyl residues are involved in catalysis and in the inhibition of activity by NADH. They verified the results by further modification studies with DTNB. Danson and Weitzman (1977) suggested from the kinetic studies that DTNB modification leads to a total loss of NADH inhibition whereas there is only partial

loss of enzyme activity.

Further evidence that SH groups are associated with the regulatory site was given by Talgoy and Duckworth (1979). They clearly demonstrated that the reaction between the enzyme and DTNB is markedly inhibited in the presence of NADH, indicating that the reactive SH group is closely associated with the NADH binding site on the enzyme. Other adenylic acid derivatives also protected the SH group from DTNB reaction in the same way as NADH does (Talgoy and Duckworth, 1979).

One possibility was that the DTNB modification of the enzyme was causing conformational changes so as to prevent NADH binding indirectly. This possibility was investigated by Talgoy *et al.* (1979) who found that acetyl-CoA and KCl saturate the DTNB modified enzyme in a cooperative way. This observation suggests that modification leaves the enzyme still in the T state and does not cause a substantial conformational change (Talgoy *et al.* 1979).

Citrate synthase from *E. coli* reacts with 4,4'-PDS resulting in rapid but complete inactivation whereas DTNB causes complete loss of NADH inhibition and only partial loss of activity (Wright & Sanwal, 1971; Danson & Weitzman, 1973, 1977; Talgoy & Duckworth, 1979). The fact that the two reagents (DTNB and PDS) are similar in structure and mode of action led Talgoy *et al.* (1979) to investigate the reason for their different functional effects upon citrate synthase. They observed that when

citrate synthase is modified with DTNB to the extent of 1 equivalent of TNB released per subunit, it loses its ability to bind NADH (as measured by fluorescence enhancement). Modification of citrate synthase to the extent of about 1 equivalent per subunit with 4,4'-PDS also results in loss of NADH binding. Conversely, saturating levels of NADH and some other adenylic acid derivatives inhibited the reaction of both reagents with enzyme. These results suggest that DTNB and 4,4'-PDS probably react with the same SH group. That this is the case was further shown by direct evidence (Talgoy et al., 1979). The modification of the enzyme with one equivalent of DTNB or 4,4'-PDS resulted in a substantial decrease in reacitvity towards the other reagent.

Although there is considerable evidence to support the notion that DTNB and 4,4'-PDS react at the same SH group, there is evidence against it. DTNB modification completely desensitizes the enzyme to NADH but does not cause inactivation, whereas 4,4'-PDS inactivates to 99.9%. Prior modification of the enzyme with DTNB affords no protection against later inactivation by 4,4'-PDS (Talgoy et al., 1979). These observations suggest that these two reagents are not reacting at the same SH group. In conclusion, it seems that there is interaction of some kind between DTNB and 4,4'-PDS modifications, but the exact relationship is not known.

(ii) Other residues:

Modification of E. coli citrate synthase by photo-oxidation and by treatment with specific chemical reagents was carried out by Danson and

Weitzman (1973), to gain information on the amino acid residues involved in enzymatic activity, and in the inhibition of activity by NADH and α-ketoglutarate.

Photo-oxidation of E. coli citrate synthase at pH 8.0 with Methylene Blue (cationic dye) resulted in a decay of enzyme activity and loss of NADH inhibition, indicating the presence of photo-oxidizable residues at the active and allosteric site. The pH profiles for the rates of photo-oxidation induced inactivation indicated that more than one class of histidinyl residues is involved in catalysis. Furthermore, the presence of histidines at the active site was indicated by using a photosensitive dye, Rose Bengal (anionic dye). This dye has been shown to cause preferential photo-oxidation of histidine. The rate of loss of citrate synthase activity was found to be greater with Rose Bengal than with Methylene Blue (Danson and Weitzman, 1973).

Consistent with the inference from photo-oxidation studies, it was found that chemical modification of E. coli citrate synthase with diethyl pyrocarbonate, a specific reagent for histidine, caused 80% inactivation of the enzyme (Danson and Weitzman, 1973). It was concluded from these results that histidine residues are involved in catalytic activity. They also presented evidence for the participation of histidine at the α-ketoglutarate binding site.

Chemical modification of E. coli citrate synthase with 2-hydroxy-5-nitrobenzyl bromide, a tryptophan specific reagent, at pH 4.6, indicated the presence of a tryptophan residue at the active site

of E. coli citrate synthase (Danson and Weitzman, 1973).

The presence of amino groups (from lysyl or N-terminal α-amino group) have been indicated in the active or allosteric site of E. coli citrate synthase, by reaction with pyridoxal 5-phosphate (Bell, 1983). From the chemical modification of citrate synthase with two arginine specific reagents, butanedione and 1,2-cyclohexanedione, Bell (1983) suggested the involvement of arginine in the catalytic and the allosteric properties of the enzyme.

Remington et al.(1982) have defined a number of residues in the active site of pig heart citrate synthase from the X-ray crystallographic studies of this enzyme. Of these residues, histidines and arginines are the key amino acids, which are involved in catalysis. A comparison of the pig heart and E. coli citrate synthase sequences, indicates that these residues are conserved in the active site of the enzyme (see Discussion section). This observation is clearly in agreement with the chemical modification studies.

Molecular Weight of E. coli Citrate Synthase:

From the kinetic and regulatory properties, there emerged a clear picture that Gram-negative bacterial citrate synthases are functionally more complex than those from Gram-positive bacteria and eukaryotes (Weitzman, 1981). Moreover, many of the regulatory properties operate via allosteric mechanisms which are associated with subunit-subunit interactions. The possibility that the functional complexity might be correlated with the

structural complexity was first explored by Weitzman and Dunmore (1969a). The molecular weights of a variety of citrate synthases were estimated by gel filtration of partially purified preparations on Sephadex G-200 using catalase (molecular weight approx. 250,000) and lactate dehydrogenase (molecular weight approx. 140,000) as marker proteins. All the citrate synthases examined fell into two groups- "large" molecules that eluted before catalase and had a molecular weight higher than 250,000 g/mole, and "small" molecules that eluted after lactate dehydrogenase and had a molecular weight around 100,000 g/mole. Moreover, as shown in Table 1, large citrate synthases were found only in Gram-negative bacteria, whereas the small type belonged to Gram-positive bacteria. Eukaryotic citrate synthases fell into the "small" group (Weitzman & Danson, 1976). The E. coli enzyme was "large" type. Faloona and Srere (1969) determined a molecular weight of 280,000 g/mole for E. coli citrate synthase, from the elution volume on a precalibrated Bio-Gel column.

Wright and Sanwal (1971) reported from their zonal sedimentation velocity experiments that E. coli citrate synthase exists as an equilibrium mixture of monomers, tetramers and octamers. They rep ted that the tetrameric form had the enzymatic activity and that the association-dissociation phenomenon is pH dependent. A subunit molecular weight of 60-65,000 g/mole was determined by SDS-PAGE, which is too large according to later investigations (Tong & Duckworth, 1975; Ner et al., 1983; Bhayana & Duckworth, 1984). From the tryptic peptide map, it was postulated

TABLE: 1 Regulatory patterns and molecular sizes among bacterial citrate synthases.[a]

Large citrate synthases	
NADH inhibition	
AMP reactivation	No AMP reactivation
Acinetobacter anitratus	*Aeromonas formicans*
Acinetobacter calcoaceticus	*Escherichia coli*
Azotobacter vinelandii	*Klebsiella aerogenes*
Pseudomonas denitrificans	*Salmonella anatum*
Pseudomonas aeruginosa	*Serratia marcescens*
Rhodopseudomonas sphaeroides	

Small citrate synthases[b]	
No NADH inhibition	
Arthrobacter globiformis	*Kurthis zopfii*
Bacillus stearothermophilus	*Mycobacterium rhodochrous*
Bacillus subtilis	*Streptomyces somaliensis*
Brevibacterium linens	
Haemophilus vaginalis	

[a]Taken from Weitzman (1981).

[b]All eukaryotic citrate synthases fall into this group.

that all protomers of E. coli citrate synthase are equivalent.

Srere (1972) reported from sedimentation equilibrium studies that E. coli citrate synthase is a tetramer of molecular weight 210,000 g/mole. Danson and Weitzman (1973) reported a value of 230,000 g/mole and confirmed the finding of Wright and Sanwal (1971) that at alkaline pH the enzyme is dissociated into monomers.

Tong and Duckworth (1975) performed a detailed study on the molecular weight of E. coli citrate synthase and these authors were first to report a correct value for subunit molecular weight (47,000 g/mole). From the SDS-polyacrylamide gel electrophoresis they found 46,000 ± 2000 g/mole as the subunit molecular weight. When the enzyme was first cross-linked with dimethyl suberimidate and then submitted to SDS-polyacrylamide gel electrophoresis, six bands were observed, each a multiple of 47,000 g/mole. By high speed sedimentation equilibrium experiments in 6 M Gdn-HCl at pH 7.0, an average molecular weight of 43,000 was obtained. In accordance with the previous investigations, pH and salt concentration were reported to affect the aggregation state of the enzyme under non-denaturating conditions. At pH 7.0 and pH 7.8, in the absence of salt, species ranging from monomers to decamers were present; at pH 9.0, only dimers were seen. At pH 7.8 in the presence of 0.05 M KCl, dimers and hexamers were the only species present but increasing the KCl concentration to 0.1 M converted all of the enzyme to hexamer.

Danson, Harford and Weitzman (1979) found that the molecular weight of their mutant E. coli citrate synthase was close to 100,000 g/mole. Since

the enzyme was believed to be dimeric, the molecular weight of a subunit would be approximately 50,000 g/mole, a number which is close to the one reported by Tong and Duckworth (1975).

Phillips et al. (1980) performed two dimensional gel electrophoresis by the O'Farrell technique in order to identify various proteins from E. coli. The protein identified as citrate synthase had an apparent molecular weight of 46,000 g/mole.

Robinson et al.(1983) presented results in complete agreement with those of Tong and Duckworth (1975) for E. coli citrate synthase. The molecular weight of 269,000 was determined by zonal filtration studies, and the enzyme was shown to be a hexamer of the equivalent polypeptide chain by sedimentation velocity analysis.

Bloxham et al.(1983) reported a subunit molecular weight of 47-49,000 g/mole by SDS-polyacrylamide gel electrophoresis. By using the same technique, Rubin et al.(1983) reported a value of 42-44,000 g/mole. The true value is 47,930 g/mole as determined from the complete amino acid sequence (Bhayana and Duckworth, 1984).

In summary, the subunit molecular weight of E. coli citrate synthase was reported as 52,500 (Srere, 1972); 55,000 (Danson and Weitzman, 1973); 60,000 (Wright and Sanwal, 1971) and 47,000 (Tong and Duckworth, 1975). Later on, values for subunit molecular weight were reported which were in close agreement with that of Tong and Duckworth (1975). As stated above, the true subunit molecular weight, 47,930 g/mole has now been obtained from the amino acid sequence (Bhayana and Duckworth, 1984; Ner et al. 1983).

The native molecular weight has been reported to be greater than 250,000 (Weitzman & Dunmore, 1969a), 280,000 (Faloona & Srere, 1969); 210,000 (Srere,1972); and 245,000 (Wright and Sanwal, 1971). Tong and Duckworth (1975) suggested a hexameric structure with a molecular weight of 280,000 g/mole. Robinson et al.(1983) provided further evidence for the hexameric structure of the enzyme by glutaraldehyde cross-linking, in agreement with the results of Tong and Duckworth (1975) who performed cross-linking with dimethyl suberimidate.

Preliminary crystallographic data for E. coli citrate synthase were interpreted to indicate a tetrameric structure for the enzyme with a molecular weight of 188,000 g/mole (Rubin et al.1983). This molecular size is inconsistent with all the other evidence. The unit cell, a 220 Å cube, was estimated to contain about 100 subunits; this indicates a low degree of symmetry and it seems quite possible that the tetrameric symmetry proposed is only one possible interpretation of the data.

Structural Studies of Citrate Synthase

(i) Pig heart citrate synthase:

The only citrate synthase for which the X-ray crystallographic structure and complete amino acid sequence have been determined, is that isolated from pig heart. This enzyme is one of the most widely studied examples of citrate synthase (Srere, 1972; Weitzman & Danson, 1976; Weitzman, 1981).

In 1970, Wu and Yang presented their preliminary observations on the structural and physicochemical properties of pig heart citrate synthase. They reported that the enzyme has a molecular weight of 100,000 g/mole and is made up of two identical subunits. A subunit molecular weight of 49,000 g/mole was obtained by the sedimentation equilibrium method. Singh et al.(1970) reported value of 96,000 and 46,000 g/mole for native enzyme and monomer respectively, thus confirming the dimeric structure. Tryptic digestion and peptide mapping also indicated that the subunits are structurally identical. (Singh et al. 1970).

Wiegand et al. (1979) presented a structural analysis of a tetragonal crytalline form of the enzyme at 0.35 nm resolution. At this resolution chain tracing was possible from which a model was constructed. According to the model, chain folding featured much α-helix structure while β-sheet was absent. The authors accounted for 355 residues and claimed that 80 residues from the amino terminal were disordered in the crystal. A two-domain structure for each subunit was proposed. The authors also showed that the enzyme is susceptible to attack by trypsin at low concentrations.

Bloxham et al.(1980) studied the effect of limited proteolysis by subtilisin, chymotrypsin and trypsin in the presence of palmitoyl-CoA. At low concentrations, all the three proteolytic enzymes cleaved intact subunit into a large fragment (35-35.8 K) and a small

fragment (9.0-12.0 K). The small fragment was further cleaved into smaller pieces by both chymotrypsin and trypsin but was relatively stable in the presence of subtilisin. The cleavage products of subtilisin were separated by gel filtration under denaturating conditions and their amino terminal sequences were determined. The large fragment was found to be from the amino-terminal of the intact protein, and the small one from the carboxyl end.

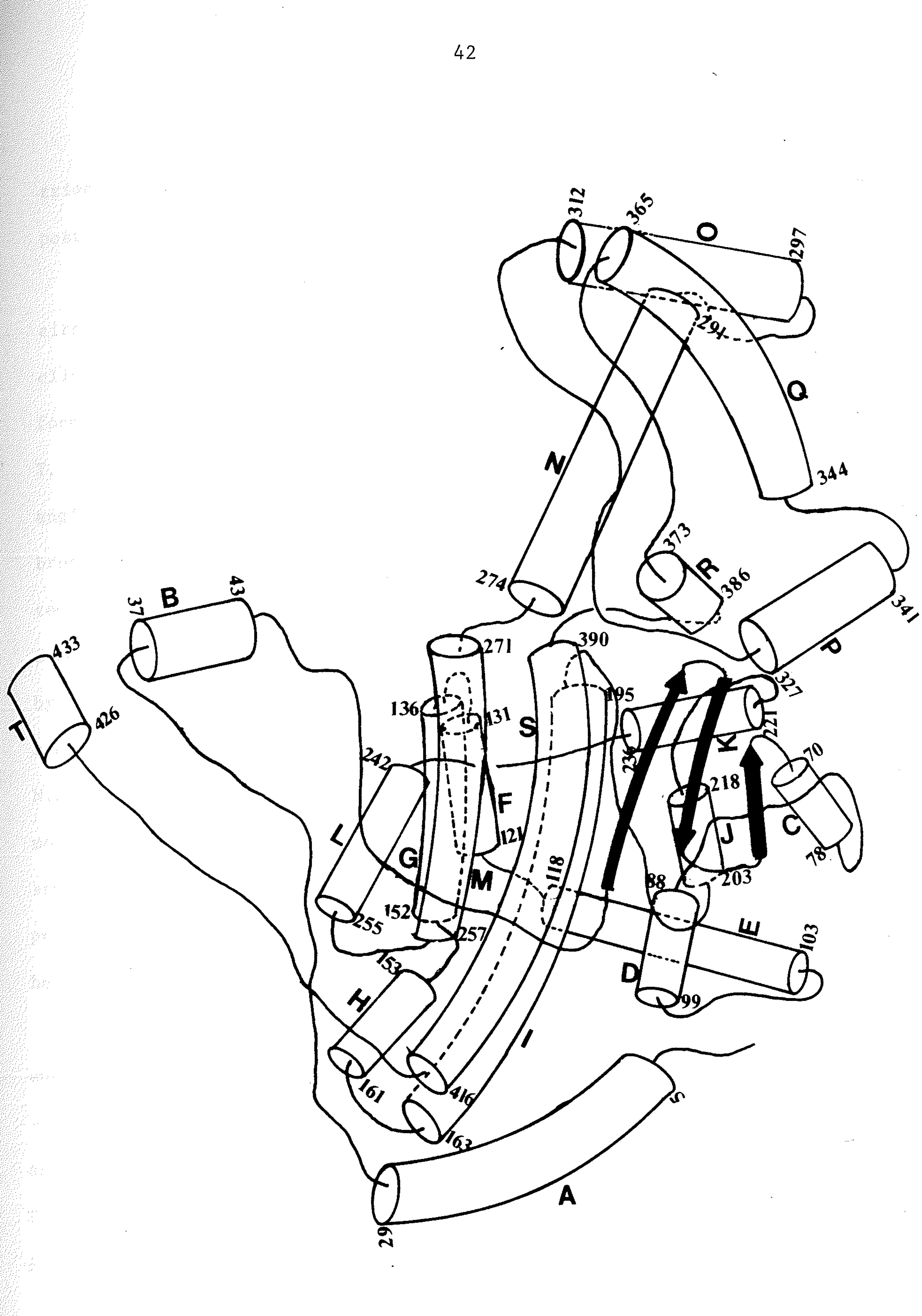
When the effects of various ligands on proteolytic cleavage were studied, palmitoyl-CoA proved to be required absolutely for the cleavage (Bloxham et al.1980). Other ligands, acetyl-CoA, CoA-SH, ATP and NADH, did not protect against proteolysis, whereas OAA, citrate and ammonium sulfate all completely protected the enzyme. From these observations Bloxham et al. (1980) proposed that citrate synthase consists of two functional domains; one responsible for acetyl-CoA binding and the other for binding oxaloacetate or citrate. These two domains cooperate with each other for catalysis. The site of proteolysis was suggested to be in the fringe region located between the domains.

Bloxham et al. (1981, 1982) reported the complete sequence of the 437 amino acid residues of pig heart citrate synthase. To obtain the sequence the methionine peptides were generated by cleavage with cyanogen bromide and were sequenced as completely as possible. These peptides were then ordered by isolation and analysis of peptides generated by tryptic cleavage (of citraconylated protein), hydroxylamine cleavage, limited proteolysis and mild acid cleavage. The complete sequence is shown in Fig.31 . One of the special features of the sequence is the presence of

Figure 4. The structure of the monomer of the pig heart citrate synthase. The schematic diagram is drawn according to Remington et al. (1982). The starting and ending residues of the helical and β-sheet segments are as follows:

A	5-29	K	221-236
B	37-43	L	242-255
C	70-78	M	257-271
D	88-99	N	274-291
E	103-118	O	297-312
F	121-131	P	327-341
G	136-152	Q	344-365
H	153-161	R	373-386
I	163-195	S	390-416
J	203-218	T	426-433

β-sheet 56-69


312
365
O
297
291
Q
N
344
373
R
386
274
341
P
B
37
43
433
T
426
271
390
136
131
195
S
327
K
221
236
70
242
F
218
C
L
121
J
78
G
M
118
88
203
152
255
257
E
103
153
D
99
H
I
161
416
163
5
A
29

trimethyllysine at residue 368, suggesting that the enzyme has undergone post-translational modification.

Remington et al.(1982) presented the crystal structure of pig heart citrate synthase in two forms, a tetragonal form at 2.7 Å and a monoclinic form at 1.7 Å resolution. As shown in Figure 4, the tetragonal form of the citrate synthase subunit contains 20 α-helices, A through T. These helices are either straight or smoothly curved over a large angle. The curvature is attributed to the presence of proline, a helical breaker, in the center of the helices showing it. The helices are connected by extended regions, consisting of residues in random coil or rarely β-sheet. A small section of three strands of antiparallel β-sheet is formed by residues 56 to 69 on the surface of the subunit. The subunit can be seen (Fig. 4) as consisting of two domains, a small domain with helices N,O,P,Q,R (residues 275 to 380) and large domain with the rest of the molecule. A total of 315 or 72% of the residues are found in helical structure. This value for helical content is much higher than the previously reported value of 40% by Wu and Yang (1970), who determined helical content by optical properties.

Remington et al.(1982) studied the subunit-subunit contact regions and reported that the two subunits are tightly associated by eight α -helical structures FF', GG', MM', and LL'; the axes of these helices are perpendicular to the two-fold axis of the dimer. Two helices B and T (see Fig. 4) which are connected to the large domain by the extended β -sheet-like chain, are deeply buried in grooves on the surface of

each two-fold related monomer. The authors calculated that 28% of the monomer becomes inaccessible to solvent upon dimerization; the buried residues are mostly hydrophobic. Hydrophilic residues are also involved in hydrogen bonding and salt bridge formation between the two subunits.

The residues involved in the substrate binding were also identified and a probable active site was located. Citrate in the monoclinic form (closed form) is very tightly bound at the active site, which is deeply buried in the enzyme. In this binding three arginine and three histidine residues are involved, Arg-329, Arg-401, Arg-421'(from the other subunit), His-238, His-274 and His-320. In addition to these, citrate is also held by several hydrogen bonds and Van der Waals forces (Asp-375). Three residues, His-320, His-274 and Asp-375, were suggested to be involved in catalysis (Remington et al. 1982).

The citrate binding in the tetragonal (Open) form of the molecule was quite different; citrate was much more loosely held as compared to that in closed form. His-320 and Arg-329 are in close contact with citrate in the closed form but these residues were found to be too distant in the open form of the crystal (Remington et al. 1982).

Binding of CoA to the tetragonal form was not detected, whereas in the closed form (monoclinic) binding of this ligand was quite obvious and was characterized by hydrophobic interactions and hydrogen bonding (Remington et al. 1982). The three phosphates of the CoA molecule form salt bridges with arginine residues: the 3'-phosphate with Arg 164' and the 5'-phosphate with Arg-324 and Arg-46. The adenine ring is recognized

by the peptide backbone of residues 314 to 320, forming an "adenine recognition loop". The pantothenate arm also interacts with this loop. The investigators observed that the electron density around the pantothenate arm is connected to Asp-375, raising the possibility of aspartyl-CoA formation during catalysis.

The availability of the amino acid sequence for E. coli citrate synthase allowed the comparison of its sequence with that of pig heart (see discussion). A detailed alignment of the two sequences is shown in Figure 31 , where it is quite obvious that the regions of apparently significant homology are scattered throughout the sequence, however most of the residues believed to be involved in the active site are the same in two enzymes (Bhayana and Duckworth, 1984; Ner et al., 1983; Bell et al., 1983). In this thesis, I have attempted to correlate the structure and functional properties of E. coli citrate synthase with that of pig heart enzyme (see discussion).

(ii) Escherichia coli citrate synthase:

(a) Protein sequence:

In preliminary studies of the primary structure of E. coli citrate synthase, Wright and Sanwal (1971) obtained a tryptic peptide map of S-carboxymethylated enzyme. From the number of peptides detected on a two dimensional "fingerprint", they concluded that the subunits of the enzyme are identical.

Singh(1980) attempted to isolate and sequence the peptides generated from cyanogen bromide cleavage of S-carboxymethylated citrate synthase. He reported the isolation of six peptides by using methods such as gel filtration, ion-exchange chromatography (DE-52) and countercurrent distribution. Of the six peptides isolated, five were blocked at the amino terminus and one, reported to be about ninety residues long contained threonine at the amino terminus, although no such peptide was found in later studies (Bhayana & Duckworth, 1984; Ner et al.1983). Aggregation of peptides was reported to be one of the major problems in purification.

Duckworth and Bell (1982) reported an amino terminal sequence of 20 residues, as determined by automated Edman degradation on intact E. coli citrate synthase. The sequence is in complete agreement with the results presented in this thesis and by Bhayana and Duckworth (1984).

Bell, Bhayana and Duckworth (1983) have studied the limited subtilisin digestion of E. coli citrate synthase. It was shown that the intact subunit, 47K, was converted to 32K and 13.5K fragments. At later times the smaller fragment of 13.5K was further cleaved to a 7.5K fragment. Automated Edman degradation of the 32K piece gave the same N-terminal sequence as intact subunit. The 13.5K fragment gave a sequence of 19 residues, the major portion of which was confirmed by isolating and sequencing two arginine peptides. An N-terminal sequence of 18 residues was obtained from the 7.5K fragment. The sequences of these proteolytic fragments were compared with the sequence of pig heart

citrate synthase and some of the residues involved in the active site of citrate synthase were found to be conserved. The authors suggested that some features of the folding patterns are similar in the two enzymes. The hypothesis was advanced that the allosteric properties of the E. coli citrate synthase are due to different subunit-subunit interactions rather than to a different kind of subunit from the non-allosteric pig heart enzyme. The determination of the complete amino acid sequence of E. coli citrate synthase was the extension of this work, so as to study the above hypothesis more closely.

While the amino acid sequence work was in progress in our laboratory, investigators in two other laboratories were involved in determining the base sequence of gltA, the structural gene for E. coli citrate synthase (see below). Some DNA base sequence was also obtained by Dr. Duckworth while working in T. Rabbitts' laboratory at the Laboratory of Molecular Biology, Cambridge.

(b) gltA gene sequence:

Ashworth, Kornberg and Nothman (1965) were the first to isolate mutants of E. coli which were devoid of citrate synthase activity (gltA mutants). These mutants were unable to grow on glucose minimal medium unless provided with a nutritional supplement of L-glutamate or L-proline. This finding indicates that in the presence of glucose, growth of the mutants can be supported by the energy from glycolysis, if the biosynthetic functions of TCA cycle, particularly L-glutamate

biosynthesis, are replaced by supplementation.

The citrate synthase structural gene (gltA) has been located at 16.2 min in the E. coli linkage map (Ashworth et al. 1965; Bachmann and Low, 1980).

Guest (1981) screened the colony bank of Clarke and Carbon (1976, cited from Guest,1981) containing Col E1-E. coli hybrid plasmids representing the entire E. coli genome. Three plasmids were found to contain the gltA gene; pLC26-17 (26.0 Kb), pLC27-18 (16.35 Kb) and pLC31-28 (26.0 Kb). The citrate synthase activity of strains containing these hybrid plasmids was amplified by a factor of 3 to 10.

Spencer and Guest (1982) reported the subcloning of a 3.1 Kb fragment, generated by a HindIII and EcoRI double digest of the Col E1-gltA$^+$ plasmids (pLC26-17, pLC27-18 and pLC31-28) into a bacteriophage lambda vector. The resulting phages λG-112 and λG-113 complemented the lesion of gltA mutant, showing that the 3.1 Kb fragment contains the functional gltA gene. It was shown that this HindIII-EcoRI contained also the promotor for gltA gene. The transcription of this gene was in the direction of HindIII → EcoRI, and counterclockwise relative to E. coli chromosome map.

Duckworth and Bell (1982) digested Guest's plasmid pLC26-17 with the restriction enzymes HindIII and SalI, and cloned the resulting 2.9 Kb fragment into the plasmid pBR322; this subclone was designated pHSgltA. They prepared a HB101/pHSgltA strain by transforming competent E. coli strain HB101 with pHSgltA, and selecting for ampicillin resistance.

This plasmid containing strain was used for large scale production of citrate synthase which accounted for 8% of the soluble protein. The enzyme was reported to be identical to the chromosomal enzyme by several criteria such as amino acid analysis, N-terminal sequence analysis, apparent subunit molecular weight and response to NADH and KCl. Plasmid-specified enzyme was also used for part of the amino acid sequencing presented in this thesis.

Bloxham et al. (1983) and Robinson et al.(1983) have also reported the purification of citrate synthase from cultures harbouring the gltA gene in cloned form. In both cases, the gltA gene was derived from Guest's plasmids pLC26-17 or pLC26-18. Their findings were similar to those reported earlier by Duckworth and Bell (1982).

The first report on the nucleotide sequence of a portion of the gltA gene encoding citrate synthase was from Hull et al.(1983) who published a sequence of 752 basepairs containing the gltA promoter and the first 96 codons of the structural gene. Of the 96 residues inferred from this work, the first 90 were in agreement with the results presented in this thesis and by Ner et al. (1983; see below) but the last remaining six were different. When the base sequence results of Hull et al. (1983) are compared with those of Ner et al.(1983) it can be seen that the former group introduced reading frame errors by omitting adenines at their positions 735, 744 and 745. The sequence which is obtained by inserting these adenines, is the same as that of Ner et al.(1983).

Bloxham et al.(1983a)also reported the DNA base sequence of the

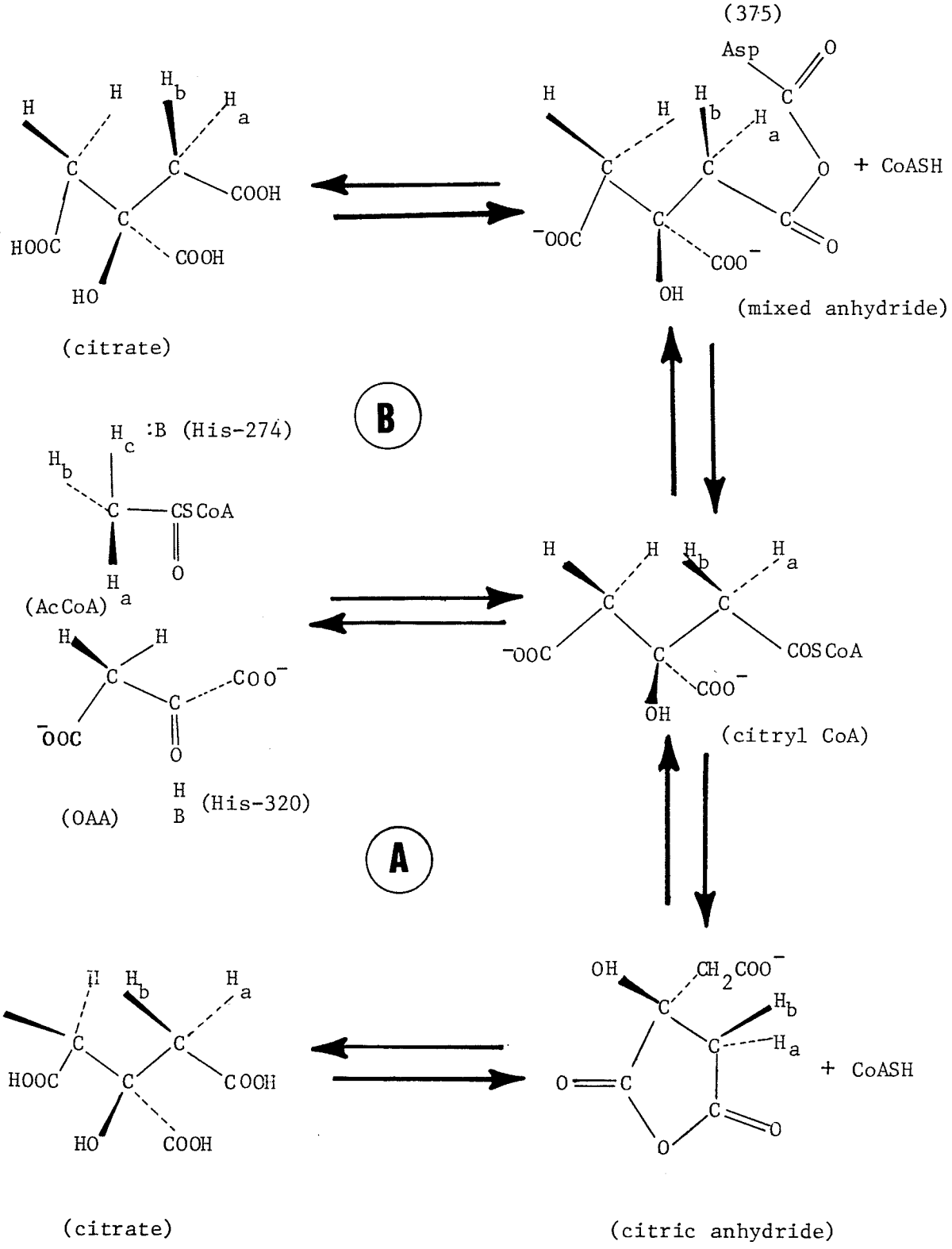
promoter region and codons for the first 50 amino acid residues of citrate synthase from the gltA gene. Their results were found to be completely consistent with the data of Hull et al.(1983) and later Ner et al.(1983).

The sequence of 3265 bases from the gltA gene, encoding the entire E. coli citrate synthase (427 amino acids), was reported in a joint publication between our group and Bloxham's group (Ner et al. 1983). Of the 427 amino acids predicted from the DNA, the sequence of 377 residues could be confirmed at that time by protein sequence work. In a separate publication (Bhayana and Duckworth, 1984), we have since presented detailed evidence for the amino acid sequence of 415 residues. The residues which have been determined show agreement with the predicted sequence except that the N-terminal Met is missing from the isolated protein and Asp is present at position 10 rather than Asn as predicted from the gene sequence. The homology of the sequence with that of pig heart citrate synthase has also been discussed (Bhayana and Duckworth, 1984), the details of which are given in the "Discussion" section of this thesis.

A Note On The Mechanism of Citrate Synthase Reaction:

Citrate synthase catalyzes the condensation of acetyl-CoA with OAA to form citrate and CoA. The mechanism of this reaction has been studied by several investigators and the details have been reviewed by Spector (1972) and Srere (1972) and the latter has presented the most probable model of Buckel and Eggerer (1969) in terms of the sequence of events occuring in this reaction. According to the proposed mechanism the oxaloacetate binds to the enzyme with the re side facing the enzyme and si side exposed. This binding results in a conformational change in the enzyme so that acetyl-CoA can be held tightly (Srere, 1972). It is proposed that there must be an adenine binding site in the active site since many adenine compounds such as ATP, ADP, NADPH and NADH, compete with acetyl-CoA for its site on pig heart citrate synthase. As shown in Scheme A (Figure 5), the reaction takes place with the abstraction of a proton from the methyl group of acetyl-CoA; thus there must be a base in its vicinity to accept this proton. The acetyl moiety of the acetyl-CoA is then added to the si side of the oxaloacetate with inversion of configuration at the methyl carbon. As a result condensation takes place with the formation of an enzyme bound citryl-CoA. The fate of this intermediate citryl-CoA is hydrolysis to citrate and CoA. It is proposed that this hydrolysis step is not direct, but that it occurs through the formation of another intermediate, citryl anhydride. This anhydride is formed as a result of intramolecular nucleophilic attack by the carboxylate anion (Buckel and Eggerer, 1969). The final step would be hydrolysis of citryl anhydride to form citrate.

Figure 5. Mechanism of citrate synthase catalyzed reaction. (A) mechanism as presented by Srere (1972). (B) mechanism as suggested by Remington et al. (1982) from the X-ray crstallographic studies. B: represents the base group on the enzyme with lone pairs of electron. His-274, His-320, Asp-375 have been indicated to be involved in catalysis (Remington et al., 1982).


(375)
Asp
+ CoASH
(citrate)
(mixed anhydride)
B
:B (His-274)
(AcCoA)
(OAA)
(His-320)
(citryl CoA)
A
+ CoASH
(citrate)
(citric anhydride)

The Buckel and Eggerer model as described by Srere (1972) is represented in Figure 5. From the proposed mechanism it is evident that citrate synthase is involved in three types of activities. First, there must be enolase activity which requires the presence of a base near the methyl group of acetyl CoA so as to abstract a proton and convert the acetyl group into its enol form. Second is a ligase activity which ensures the activation of the keto group of OAA and its nucleophilic attack at the carbonyl carbon so as to form citryl-CoA, a thioester intermediate. Third, there is a hydrolase activity, which is involved in the hydrolysis of the thioester to form citrate and CoA, probably through an intermediate citryl anhydride.

Remington et al.(1982) described the functional properties of pig heart citrate synthase from the structural analysis on the two crystal forms studied by them. The tetragonal or open form of the enzyme is inactive since it does not bind CoA at all and binds citrate only weakly. Because the open form crystal cracks upon OAA binding it appears that OAA induces conformational change. This change in conformation was postulated to be from the open to the closed form found in monoclinic crystals, where CoA and citrate binding sites exist. The existence of open and closed forms also explain the previous observation that the binding of OAA increases the binding constant of acetyl-CoA by immobilization of the acyl portion of CoA (Johansson & Pettersson, 1977, Weidman et al. 1973)

From the model building studies Remington et al. (1982) located

the active site of the enzyme and proposed a mechanism of reaction which is similar to the one described by Srere (1972) except that no intramolecular anhydride formation is suggested by Remington.
In the model building studies it was assumed that OAA would bind similarly to citrate with its re side facing the enzyme; the si side would be facing a narrow pocket near to His-274 and the incoming methyl group from the immobilized acetyl group. The environment in the vicinity of C-3 of OAA would be extremely polar with contributions from residues His-320, His-238, Arg-329 and Arg-401. Since His-320 would be close to the carbonyl group of oxaloacetate and His-274 close to the methyl group of acetyl-CoA, it is possible that these two residues are involved in proton donation and abstraction respectively. As a result condensation would take place with the inversion of configuration of the methyl group of acetyl-CoA (Remington et al.1982).

The idea that citryl-CoA is an intermediate in the reaction was also supported by Remington et al.(1982), and they proposed that the closed form is involved in catalyzing the hydrolysis of this intermediate. Asp-375 would attack the carbonyl carbon of citryl-CoA to form a mixed anhydride, and CoA would be released. Since there was evidence for formation of asparty1(375)-CoA in the crystals, transesterification may then take place, releasing citrate. The sequence of events is shown in Figure 5, where the important activities of citrate synthase enolase, ligase and hydrolase are represented.

MATERIALS & METHODS

Materials

Iodoacetic acid, protamine sulfate, Trizma base, sodium dodecyl sulfate, Coomassie Brilliant Blue R250, Polybrene, Carboxypeptidase A and B, DNAse, TPCK-trypsin and trypsin inhibitor were purchased from Sigma. Ammonium sulfate (ultra pure) and Gdn-HCl (ultra pure) were from Schwarz/Mann. Iodo(2-^{14}C)acetic acid and ACS scintillation cocktail were from Amersham/Searle. Citraconic anhydride, p-cresol, Ellman's reagent (DTNB) were from Aldrich. DE 52(DEAE Cellulose) was from Whatman. Dowex 50-X2 and Dowex 1-X2, acrylamide and N, N'-Methylene-bis-acrylamide were from Bio-Rad. N,N,N',N'-Tetramethylenediamine (TEMED) was from Eastman. Cyanogen bromide was from J.T. Baker Chemicals Co. Polyamide sheets were from Cheng Chin Trading Co. Taiwan. Reagents and solvents for the 890C Sequencer, PTH amino acid standard kit, amino acid standard, ninhydrin, trifluoroacetic acid and o-iodosobenzoic acid were from Pierce. Butyl chloride was also purchased from Beckman. Acetonitrile, propanol-2, methanol and water used for HPLC, were purchased from Fisher and were HPLC grade. Glass distilled HPLC grade propanol-1 was from Burdick and Jackson. All other chemicals were of the highest purity commercially available.

METHODS

Purification of citrate synthase:

(i) *Escherichia coli* citrate synthase:

Escherichia coli K12 strain CGSC 259 was grown in a 200 L fermenter containing minimal acetate medium at the Biochemistry Department, University of Alberta. The cells were delivered in the frozen state in packages of approximately 800 g each.

The method of Tong and Duckworth (1975) was used to purify citrate synthase from the above source. To start with 200 g of cells were thawed and suspended in 600 mL of 20 mM Tris-HCl buffer pH 7.8, containing 1 mM EDTA (hereafter called standard Tris buffer). The cells were broken by one passage through a 40 mL Aminco French Pressure Cell, applying a cell pressure of 18,000 psi. The broken cells were treated with a pinch of DNAse to reduce viscosity and the solution was stirred for 15 min at 4°C. The resulting cell suspension was centrifuged for 1 h at 48,000 X g_{max} in Sorvall RC 2-B ultracentrifuge using the SS-34 rotor head (20,000 rpm). The pellet was discarded and to the supernatant was added slowly 0.42 mL of 2% aqueous protamine sulfate solution per gram of cells wet weight. The solution was stirred for 30 min after protamine sulfate addition and the precipitate was removed by centrifuging as described above. The supernatant was fractionated with ammonium sulfate to obtain protein precipitating between 55% and 70% saturation. Centrifugation was at 5000 X g_{max} for 30 min using Sorvall RC 2-B centrifuge GSA rotor head (6000 rpm).

The 55-70% protein fraction was dissolved in a minimum volume of standard Tris buffer containing 50 mM KCl and the mixture was dialyzed overnight in the cold against three changes of 4 L of the same buffer.

The dialyzed solution was applied to a DEAE-cellulose (Whatman DE-52) column (2.5 cm X 23 cm) previously equilibrated with standard Tris buffer containing 50 mM KCl. The column was washed overnight with this buffer to remove unbound proteins. The elution of bound proteins was achieved by running a linear gradient of 50 mM to 300 mM KCl, each in 600 mL standard Tris buffer. Fractions of 20 mL were collected and analyzed for protein content and enzyme activity. Those fractions with specific activity of 15 units/mg or more were pooled and then concentrated to about 3 mL by ultrafiltration. The concentrated solution was applied to a Sephadex G-200 column (2 cm X 90 cm) equilibrated with standard Tris buffer containing 50 mM KCl at 4 °C. Fractions having a specific activity of citrate synthase of 30 units/mg or more were pooled and concentrated by ultrafiltration. Purity of the protein was tested by running SDS/polyacrylamide gel electrophoresis. The progress of purification is shown in Table 2.

The large scale production of citrate synthase was achieved from pHS glt A transformed E. coli HB 101, by the method of Duckworth and Bell (1982). The strain harbouring the citrate synthase plasmid, HB 101/pHS glt A, was prepared in our laboratory by transforming competent HB 101 cells with purified plasmid pHS glt A and selecting

TABLE: 2

Purification of citrate synthase from Escherichia coli (strain # CGSC 259).

Fraction	Volume mL.	Protein mg/mL	Units/mL[a]	Total Units	Specific Activity Units/mg	Recovery %
Crude Extract	600	ND[c]	33.8	20,300	-	(100)
Protamine sulfate supernatant	660	ND	24.8	16,300	-	80
Dialyzed 55-70% ammonium sulfate pellet	110	ND	115	12,600	-	62
DEAE-cellulose	126	7.03[b]	110	13,900	15.7	68
Sephadex G-200	4.72	36.3[b]	1930	9,100	53.1	45

[a]Citrate synthase activity measured in the standard assay (see Methods)

[b]Protein determined spectrophotometrically using extinction coefficient of 1.0 mg/mL at 280 nm for a 1 cm light path.

[c]Not determined.

TABLE: 3

Purification of citrate synthase from pHS gltA transformed Escherichia coli.

Fraction	Volume mL.	Protein mg/mL	Units/mL[a]	Total Units	Specific Activity Units/mg	Recovery %
Crude Extract	395	39.5[b]	394	156,000	9.98	(100)
Protamine sulfate supernatant	425	40.0[b]	368	156,000	9.20	100
Dialyzed 55-70% ammonium sulfate pellet	160	29.0[b]	630	101,000	21.7	65
DEAE-cellulose	140	9.90[c]	584	81,800	59.0	52
Sephadex G-200	5.29[d]	124	11,000	58,200	88.7	37

[a]Citrate synthase activity measured in the standard assay (see Methods)

[b]Protein determined according to the method of Lowry et al. (1951)

[c]Protein determined spectrophotometrically using an extinction coefficient of 1.0 mg/mL at 280 nm for a 1 cm light path.

[d]Value represents the weight of the enzyme solution in gm.

for ampicillin resistance. The transformed strain was grown in 15 L of LB liquid medium containing 100 mg/L ampicillin, at 37°C for 24 h under forced aeration. A second batch of 1.5 g ampicillin was added after 10 h. Cells were harvested using continuous flow centrifugation and were suspended in 3 volumes of standard Tris buffer containing 50 mM KCl and 3 mM phenylmethanesulphonyl fluoride (PMSF). The rest of the purification procedure was the same as described for the chromosomal E. coli citrate synthase with the difference that fractions with higher specific activity (35 units/mg at the DEAE cellulose step and 60 units/mg at the Sephadex G-200 step) were pooled so as to achieve maximum purity. The purification scheme is shown in Table 3.

(ii) A. anitratum citrate synthase

A wild type strain of Acinetobacter anitratum was obtained from the Department of Microbiology, University of Mantitoba. It is numbered 371 in their collection. The strain was maintained in medium containing 40% glycerol at -20°C and it was sent to the Biochemistry Department, University of Alberta where large scale cultures were grown in minimal acetate medium. The cells were shipped to us in the frozen state packed in dry ice. Citrate synthase was purified from this source by using the method of Morse and Duckworth (1980). The method employed was similiar to that used for E. coli citrate synthase with the difference that the precipitates at 55% and at 70% ammonium sulfate saturation were spun down at 48,000 X g_{max} for 1 h as compared to a 30 min spin at 5000 X g_{max} for E. coli enzyme This change was necessary since lower speeds failed to sediment all

TABLE: 4

Purification of citrate synthase from Acinetobacter anitratum.

Fraction	Volume mL	Protein mg/mL	Units/[a] mL	Total Units	Specific Activity Units/mg	Recovery %
Crude Extract	240	23.0[b]	30.9	7410	1.34	(100)
Protamine sulfate Supernatant	280	16.4[b]	25.7	7210	1.56	97
Dialyzed 55-70% ammonium sulfate pellet	120	22.6[c]	41.9	5030	1.85	68
DEAE-cellulose	110	1.08[c]	29.4	3230	27.2	44
Sephadex G-200	46	0.90[c]	55.7	2560	62.5	35

[a]Citrate synthase activity measured in the standard assay. Unlike E. coli citrate synthase no KCl was added to the assay mixture.

[b]Protein determined according to the method of Lowry et al. (1951)

[c]Protein determined spectrophotomerically using an extinction coefficient of 1.0 mg/mL at 280 nm for a 1 cm light path.

precipitates. The progress of the purification is summarized in Table 4.

(iii) Pseudomonas aeruginosa citrate synthase :

P. aeruginosa cells were also grown at the Biochemistry Department, University of Alberta (University of Manitoba, Microbiology Dept. strain # 74, ATCC 7700) and delivered to us in the frozen state. For the preparation of citrate synthase, 145 g (wet weight) of cells were thawed and suspended in 300 mL of standard Tris buffer containing 2.5 mM PMSF. The suspension was stirred continuously on ice for 2 h. The cells were then lysed by passing the suspension through a French press at a pressure of 18,000 psi. A pinch of DNAse and more PMSF to a final concentration of 2.5 mM were added. The debris was removed by centrifugation at 48000 X g_{max}. The supernatant was treated with ammonium sulfate and the precipitates appearing between 50% to 70% ammonium sulfate saturation were collected. The precipitates were dissolved in a minimum volume of standard Tris buffer and dialyzed overnight against three changes of 4 L of standard Tris buffer containing 50 mM KCl. The dialyzed protein sample was applied to a column (3 cm X 30 cm) of DEAE cellulose previously equilibrated with standard Tris buffer containing 50 mM KCl. The column was washed with a further 200 mL of this buffer and a linear gradient of 0.05 - 0.30 M KCl was applied, total volume 1200 mL. Fractions containing a specific activity of 1.9 units/mg or more were pooled and dialyzed at 4°C for 24 h against three

TABLE: 5

Purification of citrate synthase from Pseudomonas aeruginosa.

Fraction	Volume mL	Protein mg/mL	Units/mL[a]	Total Units	Specific Activity Units/mg	Recovery %
Crude Extract	126	ND[c]	10.2	1290	-	(100)
Dialyzed 50-70% ammonium sulfate pellet	51	ND	12.2	622	-	48
DEAE-cellulose	60	0.47[b]	7.37	442	15.7	34
Hydroxylamine apatite	26	0.15[b]	11.9	310	22.6	24
Sephadex G-200	2	0.98	30.5	61	31.1	4

[a]Citrate synthase activity measured in the standard assay (see Methods)

[b]Protein determined spectrophotometrically using an extinction coefficient of 1.0 mg/mL at 280 nm for 1 cm light path.

[c]Not determined.

changes of 4 L of 10 mM potassium phosphate buffer pH 7.0 containing 0.1 mM EDTA and 0.2 M KCl (hereafter called standard phosphate buffer). The product was applied to a column (1.8 cm X 5.0 cm) of hydroxylapatite (Higa et al.,1977) equilibrated with standard phosphate buffer. A linear gradient of potassium phosphate 10 mM to 150 mM was applied, total 400 mL,and 4 mL fractions were collected. Those having a specific activity of 13 units/mg protein or more were pooled and concentrated to about 2 mL by ultrafiltration. The concentrated protein solution was applied to a Sephadex G-200 (2.8 cm X 80 cm) column equilibrated previously with standard Tris buffer containing 50 mM KCl. The protein was eluted with the same buffer and 5 mL fractions were collected. Those containing enzyme at a specific activity of 20 units/mg or more were pooled and tested for purity by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Table 5 and Figure 26).

Enzyme Assay

One unit of citrate synthase is defined as the amount which is necessary to catalyze the reaction:

acetyl-CoA + oxaloacetate ⇋ citrate + CoA

at the rate of 1 μmole product formed per minute under the standard assay conditions. The standard assay mixture contains 0.1 mM acetyl-CoA, 0.1 mM oxaloacetate, 0.025 mM DTNB and 20 mM Tris-HCl pH 7.8 containing 1 mM EDTA in a volume of 1 mL. A sufficient amount of enzyme was added such that the steady state was maintained for at least

10 min at room room temperature. The formation of CoA was monitored at 412 nm by reaction with DTNB, according to Srere et al.,(1963). The specific activity is defined as the number of μmoles of product formed per minute per mg of enzyme.

Chemical Modifications of E. coli Citrate Synthase:

(i) Carboxymethylation

Citrate synthase at a concentration of 30 mg/mL was reduced and carboxymethylated under nitrogen in 4 times volume of 0.4 M Tris-HCl, pH 8.2, containing 6 M Gdn-HCl and 0.01 M dithiothreitol (DTT) for up to 4 h at 60°C in a water bath. The solution was then cooled to room temperature and iodo (2-^{14}C) acetic acid was added equivalent to the total thiols present in the solution (protein + dithiothreitol). The mixture was kept in the dark for 1 h before the reaction was terminated by addition of a pinch of DTT. The solution was dialyzed against three changes of 4 L of deionized water for 36-48 h at 4 C. The dialysis resulted in precipitation of the carboxymethylated protein. The solution from the dialysis tube including precipitated protein was collected and was lyophilized.

(ii) Citraconylation

Freeze dried (^{14}C)-carboxymethylated citrate synthase was subjected to citraconylation by the method of Atassi and Habeeb(1972).

The protein (171 mg) was dissolved in 40 mL of 6 M Gdn-HCl containing 0.2 M N-ethylmorpholine acetate, pH 8.5. Citraconic anhydride, a 100 fold molar excess over the calculated free amino groups present, was added in 100 µL aliquots to the rapidly stirred protein solution. The pH was maintained between 8 and 9 by dropwise addition of 6N NaOH. After the last addition of citraconic anhydride, the reaction was allowed to proceed for another 2 h with constant stirring, adjusting the pH to 8.5 when needed. At the completion of the reaction, Gdn-HCl and reaction products were removed by dialysis against two changes of 4 L each of 0.05 M N-ethylmorpholine.

Whenever necessary, the removal of citraconyl groups was effected by dissolving the modified protein or peptide in 10% formic acid, allowing the solution to stand overnight at room temperature, and then lyophilizing.

The degree of citraconylation was determined by FDNB reaction. About 1 mg of citraconylated, carboxymethylated citrate synthase was dissolved in 1 mL of 0.05 M N-ethylmorpholine acetate buffer, pH 8.5, and to it was added 150 µL of pure liquid FDNB with vigorous shaking. The dinitrophenylation reaction was carried out at room temperature for 14 min. The reaction was stopped by dropwise addition of 6 M HCl to lower the pH to 2.0. Unreacted FDNB was extracted from the mixture with benzene. The resulting sample was dried *in vacuo*, hydrolyzed with 6 M HCl for 24 h at 110°C in sealed tube and then subjected to amino acid analysis. A control sample was also run under

the same conditions except that it was not treated with FDNB.

FDNB would only react with those lysine residues which were not blocked by citraconylation. The number of DNP-lysines produced as a result of this reaction was determined by taking the difference between the recoveries of lysines in the control sample and in the test sample. This was used to calculate the degree of citraconylation.

Enzymatic Cleavages

(i) Trypsin cleavage:

For cleavage, citraconylated and carboxymethylated citrate synthase was dissolved at a concentration of 10 mg/mL in 0.1 M NH_4HCO_3 buffer and then hydrolyzed at 37°C with a 1:100 ratio by weight of TPCK-trypsin. The reaction mixture was constantly stirred and a second, identical amount of trypsin was added after 3 h to ensure complete hydrolysis. The digestion was terminated after 5-12 h by adding 1.0 mg of soybean trypsin inhibitor per mg of trypsin used. Trypsin caused cleavage at the carboxyl sides of arginine residues, and no cleavage of lysine bonds was observed in the present study.

At times it was necessary to cleave arginine peptides further into short pieces with trypsin. To carry out this digestion peptides were first decitraconylated as described above and then were treated with trypsin (1% by weight) in 0.2 M N-ethylmorpholine acetate buffer, pH 8.0, for 5 h at 37°C. The digest was then either lyophilized or applied

directly to the HPLC coulumn.

(ii) Endoproteinase "Lys-C" Cleavage:

This commercial preparation of Lysobacter enzymogenes endoproteinase "Lys-C" (Boehringer Manheim) is specific for cleavage at the carboxyl terminal lysine residues and no cleavage at the arginine bonds was observed in the present study. The method of Chung et al.(1982) was used for digestion of (^{14}C)-carboxymethylated citrate synthase and peptide CB-1 (see Nomenclature). The sample was dissolved at a concentration of 2mg/mL in 20 mM Tris-HCl buffer pH 7.8, containing 1 mM EDTA, and the hydrolysis was carried out at 37°C for 6 h using 2.5 mg of the enzyme (5% by weight). The digest was lyophilized and the resulting peptides were then separated on the C-8 reverse phase HPLC column.

Chemical Cleavages

(i) Cyanogen bromide cleavage:

The cyanogen bromide cleavage was carried out by the method of Steers et al. (1965). S-carboxymethylated citrate synthase was dissolved in 70% formic acid at a concentration of 15 mg/mL. To this solution, crystalline cyanogen bromide, 18 mg/mL , was added and the reaction was carried out at room temperature for 24 h in the dark. The reaction was stopped by diluting the mixture with five volumes of water. Excess reagent was removed by lyophilization. A portion of the dried mixture

was dissolved in 6 M Gdn-HCl and then loaded on a C-8 reverse phase HPLC column equilibrated with 0.1% TFA. The adsorbed peptides were eluted by a linear gradient of 2-propanol.

(ii) Hydroxylamine cleavage:

The method of Bornstein (1970) was used for the cleavage at the single Asn-Gly linkage in the protein. To carry out the reaction, 2 M hydroxylamine solution was prepared fresh by dissolving 3.48 gm of hydroxylamine in 13 mL of 6 M Gdn-HCl in an ice bath and then adding 2.5 mL of 12.5 N NaOH. Five mL of freshly prepared 1 M K_2CO_3 was then added to this solution, the pH was adjusted to 10.5 and the final volume was brought upto 20 mL with 6 M Gdn-HCl. To 5 mL of freshly prepared 2 M hydroxylamine solution was added 22 mg of S-carboxymethylated citrate synthase, which dissolved. The reaction was allowed to proceed for 7 h at 45°C, and then was stopped by adding enough HCl to lower the pH to 6.0. This mixture was loaded on a Sephadex G-75 column (2.5 cm X 120 cm) equilibrated with 0.1 M NH_4HCO_3 in 6 M Gdn-HCl. Fractions were collected and were monitored for their peptide content by taking absorbance at 280 nm. Suitably pooled fractions were dialyzed against water and examined by SDS/polyacrylamide gel electrophoresis, amino acid analysis and sequence analysis.

(iii) Ortho - Iodosobenzoic acid cleavage:

The cleavage, which is specific for bonds C-terminal to tryptophan,

was carried out by the method of Mahoney et al. (1979). Eighty mg of o- iodosobenzoic acid was dissolved in 4 mL of 80% acetic acid-4 M Gdn-HCl. To it was added 86 µL of a solution of para-cresol in ethanol (100 mg/mL) and the mixture was kept for 4 h at room temperature to remove cresol oxidizing activity. Then 40 mg of freeze dried S-carboxymethylated citrate synthase was dissolved in this mixture and the reaction was maintained at room temperature for 135 h in the dark. The reaction was then stopped by adding 100 µL of β-merceptoethanol. The mixture was concentrated to 2.5 mL in vacuo and then loaded directly on a column of Sephadex G-50 (Superfine, 2.0 cm X 100 cm) previously equilibrated with 9% formic acid. Furthermore, 5 mL fractions were collected and were examined for their peptide content by reading the absorbance at 280 nm.

Chromatographic Methods for Peptide Purification

(i) Gel filtration:

The tryptic digest of citraconylated, carboxymethylated citrate synthase was initially fractionated on a Sephadex G-50 (2.5 cm X 160 cm) column equilibrated with 0.1 M NH_4HCO_3 buffer. The column was run at room temperature at a flow rate of 30 mL/h. Collected fractions were analyzed for their peptide content at OD_{280} and a 10 µL aliquot was taken from each fraction for radioactive counting. Appropriate pools were made and then lyophilized. In some cases, short peptides were desalted on Sephadex G-25 (1.5 cm X 120 cm) or Biogel P2 (0.5 cm X 90 cm) column in

0.05 M NH_4HCO_3 buffer. The eluted peptides were detected by absorbance at 230 nm.

(ii) Ion exchange chromatography:

Ion exchange chromatographic methods were used only for the purification of arginine (Tc) peptides and no other types of peptides were separated by this procedure. Sephadex G-50 peaks containing larger peptides were chromatographed on DEAE cellulose (Whatman DE52) and peaks with smaller peptides were fractionated on Dowex 50-X2 and Dowex 1-X2.

For chromatography on DEAE-cellulose, the peptide mixture was dissolved in 0.03 M NH_4HCO_3 buffer, and applied to a column (1.5 cm X 30 cm) previously equilibrated with the same buffer. Peptides were eluted with a linear gradient of 0.03 to 0.50 M NH_4HCO_3 buffer, total volume 600 mL. Fractions of 5 mL were collected and peptides were located by absorbance at 230 nm. A small portion of each fraction was also taken for radioactive counting. Pooled fractions were immediately lyophilized since hydrolysis of peptide was observed if they were kept in NH_4HCO_3 for a long time.

For chromatography on Dowex 50-X2, the method of Schroeder (1972) was used. The peptides were decitraconylated, dissolved in 2 mL of 0.20 M pyridine-acetic acid buffer, pH 3.1, and applied to a column of Dowex 50-X2 (0.6 cm X 60 cm) equilibrated with the same buffer. A gradient vessel like that designed by Bock and Ling (1954) was employed for developing the column. The linear gradient was obtained by using 100 mL

of 0.02 M pyridine-acetic acid buffer, pH 3.1, and 200 mL of 2 M pyridine-acetic acid buffer, pH 5.0. After running 0.60 of the above gradient, 2 M pyridine-acetic acid buffer, pH 5.0, was replaced by an exactly equal volume of 8.5 M pyridine-acetic acid buffer, pH 8.5; and the elution was completed with this gradient. The column was run at 40°C with a flow rate of 10 mL/h and 2 mL fractions were collected. Peptides were detected by absorbance at 570 nm with ninhydrin after alkaline hydrolysis (see below)

For chromatography on Dowex 1-X2, the dried peptides, obtained from the Dowex 50-X2 column, were dissolved in 2 mL of starting buffer, pH 9.4 (prepared by mixing 60 mL of N-ethylmorpholine, 80 mL of α-picoline, 40 mL of pyridine, acetic acid sufficient to attain pH 9.4 and water to bring the total volume to 4 L) and applied to a Dowex 1-X2 column (0.60 cm X 60 cm) previously equilibrated with the same buffer. The gradient was produced by using an automatic device, as designed by Schroeder (1972). In this device, the mixing chamber initially containing starting buffer, was constantly stirred and the following solutions were delivered to the column through the mixing chamber.

1. 100 mL of starting buffer, pH 9.4
2. 50 mL of pH 8.4 buffer (same quantities as for starting buffer except that the quantity of acetic acid was increased to attain pH 8.4)
3. 25 mL of pH 6.5 buffer (same quantities as above except acetic acid was increased to attain pH 6.5)

4. 25 mL of 0.5 N acetic acid.

5. 25 mL of 2.0 N acetic acid.

The column was maintained at 40°C and fractions of 2 mL were collected at a flow rate of 10 mL/min. Collected fractions were analyzed by the ninhydrin reaction after alkaline hydrolysis.

Ninhydrin reaction was performed according to the method of Hirs et al.(1956) to analyze fractions obtained from Dowex 50-X2 or Dowex 1-X2 column. An aliquot of 0.1 mL was taken from each fraction into a Pyrex test tube and was completely dried by placing them in hot water bath. To each tube 1.0 mL of 2.5 M NaOH was added with thorough mixing, and the mixture was heated at 90°C for 2.5 h in an open water bath. The alkaline hydrolysis was stopped by adding 1 mL of 30% acetic acid, reducing the pH to 5.0. The tubes were vigourously shaken and then 2 mL of freshly prepared ninhydrin solution (2 gm ninhydrin, 75 mL of methyl cellosolve, 25 mL of 4 N sodium acetate buffer pH 5.5 and 10 mg of ascorbic acid) was added to each tube with mixing. The tubes were incubated for 20 min in a boiling water bath and then cooled immediately in ice water. Absorbance readings were taken at 570 nm after diluting each tube with 5 mL of 50% aqueous ethanol.

(iii) High pressure liquid chromatography

Most of the peptide fractions obtained after fractionation by gel filtration and ion exchange chromatography, were still not pure enough for sequencing. These peptides were purified completely, however, in a single step by reverse phase high pressure liquid chromatography on a Perkin

Elmer Series 4 Liquid Chromatograph, equipped with a microprocessor controlled delivery system, dual beam variable wavelength LC 85 spectrophotometer and a Sigma 15 console chart recorder.

Peptides were decitraconylated, dissolved in 6 M Gdn-HCl and injected through a 100 µL loop on to Perkin Elmer C-8 column (4 mm X 250 mm) equilibrated with 0.1% TFA. A linear gradient of 0-50% propanol-2 or propanol-1 or acetonitrile was generated to elute the peptides. The organic phase also contained 0.1% TFA for stabilization of the base line in the chromatogram. The column was maintained at a flow rate of 0.8 mL/min and the rate of change of gradient was 1% per minute. Back pressure on the column varied from 12 MPa in the beginning to 23 MPa at the end of the gradient. The peptides eluting from the column were detected at 210 nm and were collected in quantities sufficient for amino acid analysis and amino acid sequencing.

For peptide mapping, the same procedure was used as just described except that the rate of change of gradient was slowed to 0.5% per minute. Different peaks appearing on the chromatogram were manually collected for composition and sequence analysis. In practice, 4-6 identical runs were required to obtain enough material for all these analyses.

Electrophoretic Methods

(i) High voltage paper electrophoresis:

High voltage paper electrophoresis was performed at pH 2.1

(2% formic acid and 0.5% acetic acid) or pH 6.5 (10% pyridine and 0.5% acetic acid) on Whatman No. 3 MM chromatography paper, both to determine the purity of short peptide fractions and to isolate them in quantities sufficient for composition and sequence analysis. Electrophoresis was carried out in a Savant Flat Plate electrophoresis apparatus for 45 to 60 min at 3000 V (52 V/cm). The electropherogram was dried and peptides were visualized by dipping the paper in a ninhydrin-cadmium solution made up from 85 mL of solution A (1% ninhydrin in acetone) and 15 mL of solution B (1 gm cadmium acetate, 50 mL acetic acid and 10 mL water). The paper was allowed to dry at room temperature for 2-3 h and then heated for a few minutes in an oven at 100°C (Perham, 1978).

(ii) SDS-polyacrylamide gel electrophoresis:

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed either in tube gels or in vertical slab gels.

For tube gel electrophoresis, the method of Weber et al.(1972) was used. The denatured protein samples were loaded gently on top of 7.5% acrylamide gels and the electrophoresis was performed at a constant current of 8 mA/gel in Shandon disc elecrophoresis unit. The electrode compartment of the tank was filled with 0.2 M phosphate buffer, pH 7.2, containing 0.2% SDS. Electrophoresis was carried out until the tracking dye had travelled 90% of the way through the gels. The gels were stained overnight with Coomassie Brilliant Blue, and destaining was effected with a solution of 10% acetic acid - 10% isopropanol in water until bands

were visible with a clear background.

For slab gel electrophoresis, a discontinuous buffer system was used as described by Laemmli (1979). The resolving gel was prepared with 10% monomer in 0.75 M Tris/SDS buffer, pH 8.8 and the stacking gel contained 3% monomer in 0.25 M Tris/SDS buffer, pH 6.8. Proteins were denatured prior to application, by the method of Fairbanks et al. (1971). This involved incubation at 100°C for 10 min in sample buffer. Samples were loaded on top of the stacking gel and electrophoresis was performed in an LKB 2001 Vertical Electrophoresis Unit at 20 mA/plate for 10-12 h. The temperature was maintained at 15°C.

Slab gels were stained and destained according to the method of Fairbanks et al. (1971). Gels were first stained overnight with stain A (0.03% Coomassie Brilliant Blue R250, 25% isopropanol and 10% acetic acid), followed by staining for 6 h in stain B (0.005% Coomassie Brilliant Blue R250, 10% isopropanol, 10% acetic acid) and finally by staining for 12 h in stain C (0.003% Coomassie Brilliant Blue R250, 10% acetic acid). Destaining was carried out in 10% acetic acid until the background was clear.

Sequence Methodologies

(i) Automated Edman degradation:

Automated Edman degradations (Edman and Begg 1967) were carried out in a Beckman 890C Sequencer. A 0.1 M Quadrol buffer program (Beckman

Catalogue Number 030176) was used routinely for degradation of peptides and proteins (Brauer et al. 1975). Polybrene (4 mg) was added to the cup to help retain peptide (Tarr et al. 1978, Klapper et al.1978, Hunkapiller et al.1978) and one complete cycle was run to remove any contaminating material. Peptide or protein samples were introduced into the cup as solutions in water or 10% acetic acid. Samples were dried in the cup using a drying subroutine program (Beckman Catalogue Number 02772).

The automatic sequencing is started by introducing a solution of 5% phenylisothiocyanate (PITC) in heptane, sufficient to rise to the undercut of the cup. Heptane is then removed by low vacuum and 0.1 M Quadrol buffer (N,N,N',N', tetrabis(2-hydroxypropyl)-ethylenediamine) is introduced at high cup speed. The coupling reaction is carried out over a 21 min period at 55°C. Vacuum is used to remove excess water and propanol from the buffer. The protein/peptide sample is then precipitated with benzene. Subsequently, benzene is removed and the film is washed with a combination of benzene and ethyl acetate, resulting in the removal of nonvolatile PITC and Quadrol and the breakdown products of PITC. Anhydrous heptafluorobutyric acid is next added to cleave the N-terminal amino acid as its 2-anilino-5-thiazolinone derivative; and finally butyl chloride is used to extract the cleaved product from the cup to the fraction collector. The butyl chloride contained 0.1% dithiothreitol (DTT) to protect the unstable serine and threonine derivatives from oxidative decomposition.

The butyl chloride extracts were removed from the fraction collector and divided into two parts. One part, about three fourths of the extract was converted to the free amino acid (Mendez and Lai, 1975), and the remainder was converted to the isomeric 3-phenyl-2-thiohydantoins (PTH) amino acids. In later studies, the whole butyl chloride extract was converted to the PTH form of the amino acid.

For conversion to free amino acids the butyl chloride extract was transferred to a hydrolysis tube and butyl chloride was removed in vacuo. The dry thiazolinone derivatives were hydrolyzed with 6 N HCl containing 0.1% stannous chloride in vacuo at 150°C for 4 h. The HCl was removed in vacuo over solid NaOH and the free amino acids analyzed with a Technicon NC-2P amino acid analyzer.

For conversion to the PTH amino acids, the butyl chloride extracts were taken to dryness under a stream of nitrogen at 60°C ; 0.2 mL of 1 N HCl was added ; the tubes were flushed with nitrogen and stoppered. Conversion was carried out by heating at 80°C for 10 min. The HCl was then removed in vacuo over solid NaOH.

The conversions were carried out in general in batches of 6-10 samples to minimize the decrease in yield which is caused by prolonged storage of the unstable thiazolinone derivatives. However, recoveries of PTH amino acids were normalized to PTH-norleucine, added to each tube of the sequencer fraction collector as an internal standard. Repetitive yields from cycle to cycle were calculated by the relative yields of a specific amino acid at two different cycles. The average repetitive yield

was calculated by least squares linear regression plot.

(ii) Identification of PTH amino acids:

In the early phase of this work, PTH amino acids were identified on a Perkin-Elmer gas chromatograph equipped with an SP 400 column (Pisano et al. 1972). The column was run at 190°C for 5 min, followed by a linear increase of 5°C/min to 245°C; held for 20 min at this temperature and then increased to 270°C at the rate of 5°C/min. PTH's of Asn/Asp and Gln/Glu were identified following derivatization with N,O-bis(trimethylsilyl) acetamide or BSA.

A more convenient and faster method of identification of PTH amino acids for most of this study was by HPLC on a C-18 reverse phase column according to the method of Skiados (1981). Dried PTH amino acid samples were dissolved in 100 µL methanol and injected on a Perkin-Elmer HS-3/C-18 column through a 6 µL injector loop. Elution was achieved with a step gradient between acetonitrile and 0.01 M sodium acetate buffer, pH 4.5, as shown in Figure 6. The column was maintained at 45°C with constant flow of the solvents at 1.5 mL/min and effluent was monitored at 250 nm.

The standard conversion procedure followed by HPLC analysis gave unequivocal identification of all the PTH amino acids except the unresolved pair Ile/Lys. However, when a Ile/Lys ambiguity was encountered the sample was identified by running a modified program

Figure 6 Separation of PTH amino acids (standard B from Pierce Chemicals) by HPLC on a C-18 reverse phase column. PTH-His and PTH-Arg elute in this programme at about 7.32 min and 9.60 min, respectively, as characteristic broad peaks. PTH-CM-cysteine elute at about 0.96 min, barely resolved from PTH-Asp; its identity was confirmed by radioactivity. The details of the prgramme are given below:

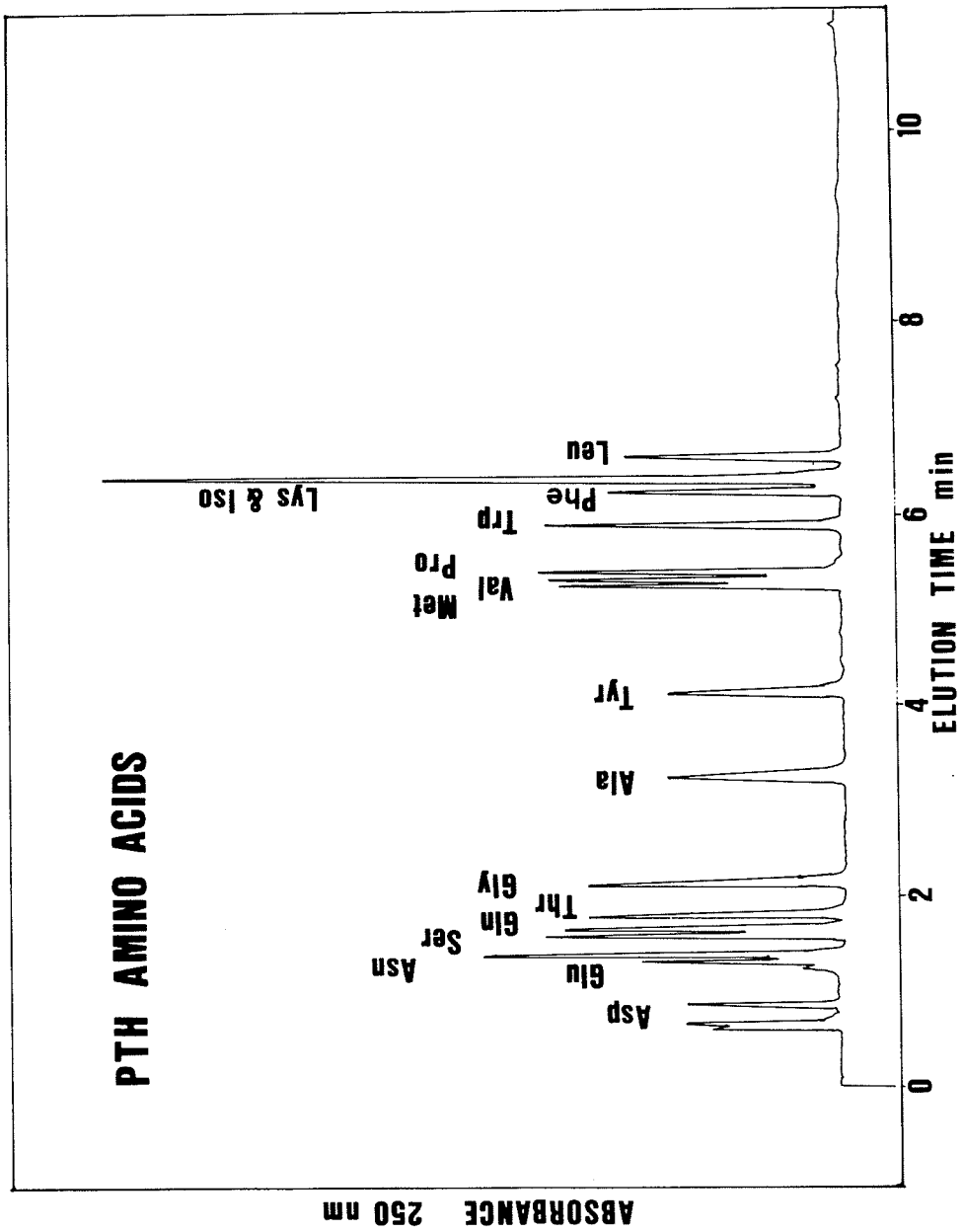
SECTOR	TIME	A%	B%	CURVE	
EQUIL.	8.0	74.5	25.5	0	
1	1.0	74.5	25.5	0	
2	3.0	55.0	45.0	0	FLOW RATE 1.5 mL/min
3	4.0	35.0	65.0	0	
4	2.0	0.0	100.0	0	

The following program was used to resolve Ile/Lys pair (elution profile not shown)

EQUIL.	6.0	74.5	25.5	0	
1	1.0	74.5	25.5	0	
2	2.0	55.0	45.0	0	FLOW RATE 1.0 mL/min
3	5.0	35.0	65.0	0	
4	2.0	0.0	100.0	0	

A = 0.01 M sodium acetate buffer, pH 4.5; B = Acetonitrile

CONDITIONS	
Column Packing	HS-3/C-18
Injector Loop	6 µL
Detection	250 nm
Temperature	45°C


PTH AMINO ACIDS
Asp
Glu
Asn
Ser
Gln
Thr
Gly
Ala
Tyr
Met
Val
Pro
Trp
Phe
Lys & Iso
Leu
0
2
4
6
8
10
ELUTION TIME min
ABSORBANCE 250 nm

as shown in caption of Figure 6.

End Group Analyses

(i) Amino terminus

For a few peptides, the dansyl method of Gray (1972) was used in combination with thin layer chromatography of the derivatives as recommended by Woods and Wang (1967). To perform the analysis approximately 5 nmoles of the peptide was taken into a hydrolysis tube, dried *in vacuo*, dissolved in 15 µL of 0.2 M NH_4HCO_3 solution and dried again *in vacuo*. The peptide was then dissolved in 15 µL deionized water and the pH adjusted to 8.5 if necessary. Then 15 µL of dansyl chloride solution (5 mg/mL in acetone) was added and the tube was covered with Parafilm. The dansylation reaction was carried out by incubation at room temperature for 2 h.

Dansylated peptide was hydrolyzed in 100 µL of 6N HCl in an evacuated and sealed tube at 105°C for 12 h. The hydrolyzate was dried, and the dansyl amino acid was extracted with 20 µL water-saturated ethyl acetate. The residue was identified by two dimensional thin layer chromatography on a Cheng-Chin Polyamide layer sheets as described by Woods and Wang (1967). The first dimension involved the solvent system water/90% formic acid (200:3 v/v) and the second dimension involved first the system benzene/acetic acid (9:1 v/v) and then after drying, the solvent system ethyl acetate/methanol/acetic acid (20:1:1 v/v). Dansylated N-terminal

amino acids were visualized under UV light and identified by comparison of their positions with those of standard dansyl amino acids, run under similar conditions.

(ii) Carboxy terminus:

The sequence of carboxy terminus of citrate synthase from E. coli was explored by carboxypeptidases A and B digestion as described by Ambler (1972). The general procedure was to dissolve approximately 100 nmoles of (^{14}C)-carboxymethylated citrate synthase in 1 mL of 0.2 M N-ethylmorpholine acetate buffer, pH 8.5. To the protein solution, 50 µL each of carboxypeptidases A and B (concentration 1.0 mg/mL) were added and the reaction was carried out at 37°C. At appropriate times 0.25 mL aliquots were removed from the mixture and mixed with 0.60 mL of 20% acetic acid to stop the digestion. The precipitated protein was removed by centrifugation and the supernatant was subjected to quantitative amino acid analysis.

Other Methods

(i) Amino Acid Analysis:

Amino acid compositions of proteins/peptides were determined after acid hydrolysis, by automated ion exchange chromatography on a Technicon NC-2P analyzer. Peptide samples were routinely hydrolyzed in vacuo for 20-24 h at 110°C in 6 N HCl (constant boiling). The hydrolyzates were dried over

solid NaOH *in vacuo*. Amino acid composition data were used to calculate peptide concentrations prior to loading into the sequencer cup.

For proteins, acid hydrolysis was usually carried out for 24, 48 and 72 h at 110°C in 6 N HCl. Values for threonine and serine were calculated by first order extrapolation to zero time. For valine and isoleucine, the 72 h values were taken. Tryptophans were determined by the method of Edelhoch (1967) and cysteine plus cystine were estimated together as cysteic acid after performic acid oxidation by the method of Hirs (1967).

(ii) Deblocking reaction:

L-pyroglutamate amino peptidase was used in an attempt to remove pyrrolidone carboxylic acid (PCA) from the N-terminus of peptide TC-22, by the procedure of Podell *et al.*(1978). Freeze dried peptide was dissolved in deblocking buffer, which consists of 0.1 M sodium phosphate buffer, pH 8.0, containing 5 mM DTT, 10 mM EDTA and 5% (v/v) glycerol. The reaction was started by adding 25 µL of 1 mg/ml freshly prepared enzyme solution and was then flushed with nitrogen. The reaction mixture was incubated for 10 h at 4°C under an atmosphere of nitrogen. After 10 h an additional 25 µg of the enzyme was added and the solution was flushed again with nitrogen. The solution was now incubated at room temperature for 14 h with occasional mixing. At the end of the incubation, the peptide was loaded on a Biogel P2 column (0.5 cm X 90 cm) in 0.05 M NH_4HCO_3 buffer, mainly to remove reaction products. The collected peptide was subjected to composition and sequence analysis.

Peptide Nomenclature

Peptides isolated from tryptic digests of citraconylated, (^{14}C)-carboxymethylated citrate synthase are designated as TC followed by an Arabic numeral indicating their relative locations in the polypeptide chain. For example, TC-1 is the peptide comprising residues from the NH_2-terminus through the first arginine in the sequence that is cleaved by trypsin. Tryptic peptides produced by tryptic digestion of decitraconylated TC fragments (e.g. TC-2) are symbolized by TC2-T followed by Arabic numerals specifying their sequential alignment in the parent fragment. This system follows the convention (cleavage 1)(position in sequence)-(cleavage 2)(position in parent fragment) etc. The cyanogen bromide cleavage products are designated as CN peptides and follow the same convention as TC peptides.

RESULTS

Strategies for Sequence Determination:

In order to elucidate the primary structure of citrate synthase from Escherichia coli, tryptic peptides of (^{14}C)-carboxymethylated, citraconylated citrate synthase were isolated and their amino acid sequences were determined as far as possible. Ordering of these TC peptides (see nomenclature in Methods) was achieved by isolation and sequence analysis of peptides generated from CNBr cleavage, hydroxylamine cleavage and o-iodosobenzoic acid cleavage. Certain features of the sequence were also confirmed by the amino terminal sequences of fragments generated previously by partial proteolysis with subtilisin (Bell et al., 1983).

The summary of the strategy used for sequence determination is given in Figure 7 , and the complete sequence of E. coli citrate synthase is given in Figure 8 . Of the 426 amino acids indicated, the sequence of 416 was determined by protein sequencing in this work, whereas the remaining 10 were placed by DNA base sequence of gltA gene encoding citrate synthase (Ner et al., 1983).

Although the strategies used for the elucidation of amino acid sequence are conventional, a novel feature of this work is the use of reverse phase high pressure liquid chromatography to isolate fairly pure peptides from a complex mixture in a single step. The HPLC method not only proved to be powerful tool for separating large denatured peptides but also the peptides obtained were free of salt and were best for automated sequencing. The details of the sequence determination are given in the following sections.

Figure 7 Summary of the strategy used in the sequence determination. The top row locates the arginine (R,●) and methionine (M,▲) residues, and the other rows show peptides expected from the indicated cleavage methods; shaded portions were those actually sequenced by automated Edman degradation. In the last row, the shading at the C-terminus shows the information obtained by carboxypeptidase digestion.

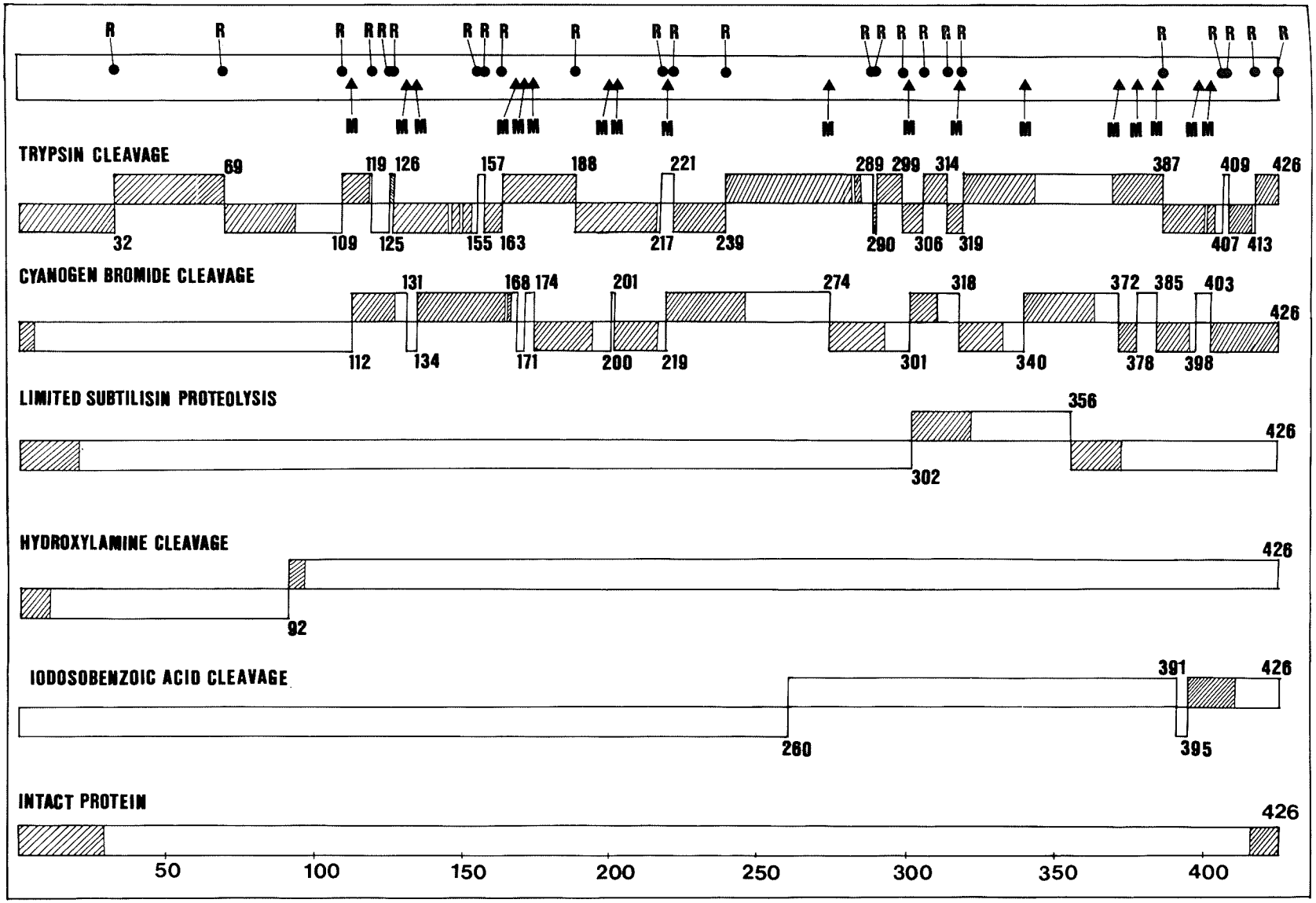

TRYPSIN CLEAVAGE
CYANOGEN BROMIDE CLEAVAGE
LIMITED SUBTILISIN PROTEOLYSIS
HYDROXYLAMINE CLEAVAGE
IODOSOBENZOIC ACID CLEAVAGE
INTACT PROTEIN
50
100
150
200
250
300
350
400

Figure 8 Complete amino acid sequence of Escherichia coli citrate synthase.


20
40
ADTKAKLTLDGDTAVELDVLKGTLGQDVIDIRTLGSKGVF
60
80
TFDPGFTSTASCESKITFIDGDEGILLHRGFPIDQLATDS
100
120
NYLEVCYILLNGEKPTQEQYDEFKTTVTRHTMIHEQITRL
140
160
FHAFRRDSHPMAVMCGITGALAAFYHDSLDVNNPRHREIA
180
200
AFRLLSKMPTMAAMCYKYSIGQPFVYPRNDLSYAGNFLNM
220
240
MFSTPCEPYEVNPILERAMDRILILHADHEQNASTSTVRT
260
280
AGSSGANPFACIAAGIASLWGPAHGGANEAALKMLEEISS
300
320
VKHIPEFFRRAKDKNDSFRLMGFGHRVYKNYDPRATVMRE
340
360
TCHEVLKELGTKDDLLEVAMELENIALNDPYFIEKKLYPN
380
400
VDFYSGIILKAMGIPSSMFTVIFAMARTVGWIAHWSEMHS
420
426
DGMKIARPRQLYTGYEKRDFKSDIKR

Arginine Peptides:

The amino acid composition of citrate synthase from E. coli indicates the presence of 24 arginines in a subunit (Duckworth and Bell, 1982). Since the carboxy terminus of the enzyme is arginine (see below), 24 TC peptides would be expected at most. It was later determined that the cleavage did not occur at Arg-407 (an Arg-Pro sequence) and that there are two Arg-Arg sequences, thus reducing the number of peptides to 21. Of these 21 peptides, 15 were isolated by a combination of gel filtration, ion exchange chromatography, paper electrophoresis and reverse phase HPLC methods, and four more were isolated by the one step purification method on the C8 reverse phase HPLC column; two were not isolated.

Figure 9 summarizes the purification scheme for the TC peptides. To start with, 3.1 μmoles of (^{14}C)-carboxymethylated citrate synthase were citraconylated and then digested with trypsin as described in the Method section. Since gel filtration provides size fractionation with minimum loss of material, this digest was initially resolved on a Sephadex G-50 column (2.5 cm X 160 cm) in 0.1 M NH_4HCO_3 buffer. Fractions of 10 mL were collected and analyzed by absorbance at 280 nm. An aliquot of 10 μL was taken from each tube to monitor the distribution of radioactivity. As may be seen in the elution profile depicted in Figure 10, the digest was resolved into six major pools, which are labelled by Roman numerals in the order in which they eluted from the column. Each pool was lyophilized, except for pool III which was used directly for the next purification step. The lyophilization step

Figure :9 Scheme for purification of arginine peptides from Escherichia coli citrate cynthase. RP: reverse phase high pressure liquid chromatography; DE: DEAE-cellulose chromatography; CE: cation exchange chromatography on Dowex 50-X2; AE: anion exchange chromatography on Dowex 1-X2; PE: high voltage paper electrophoresis; P2 : Biogel P2 column chromatography. The arabic numerals represent TC peptides.

PURIFICATION SCHEME FOR ARGININE PEPTIDES FROM *E. coli* CITRATE SYNTHASE.

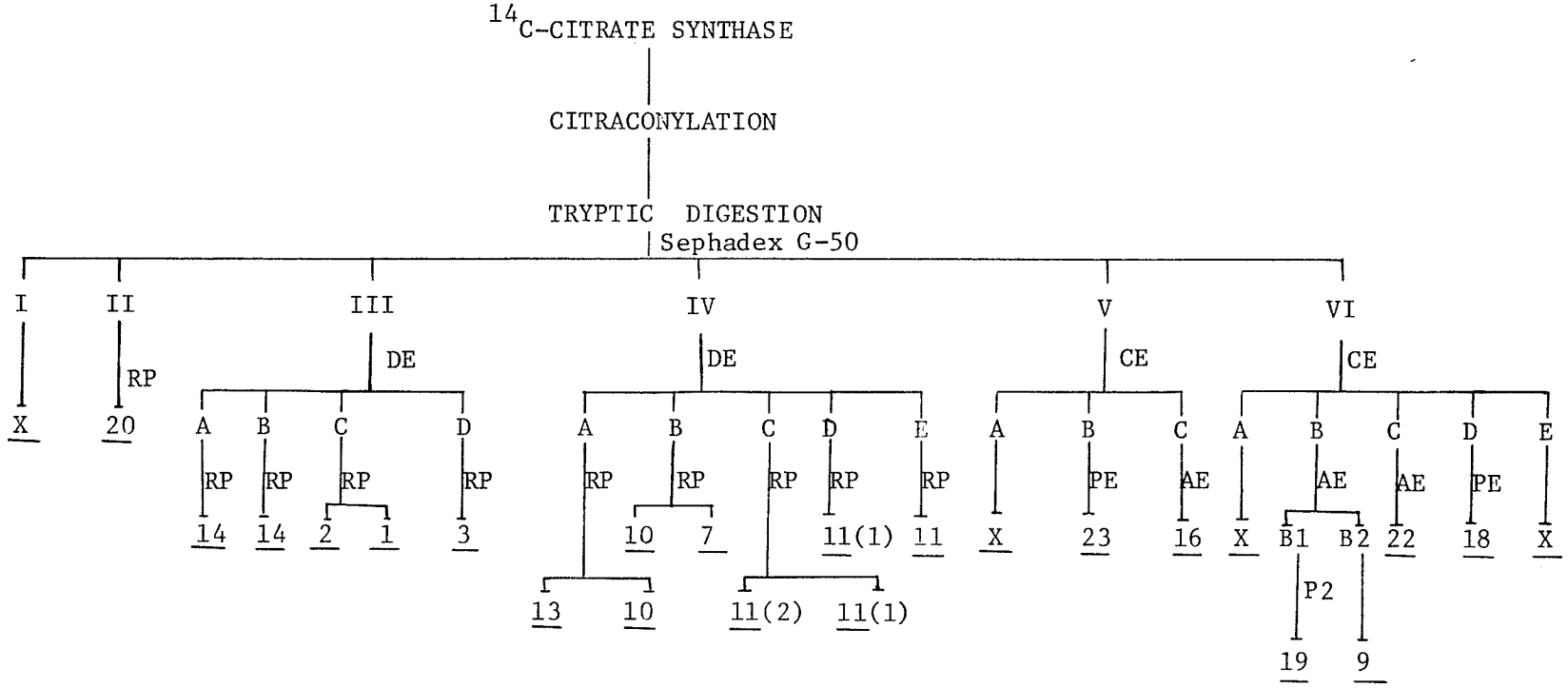
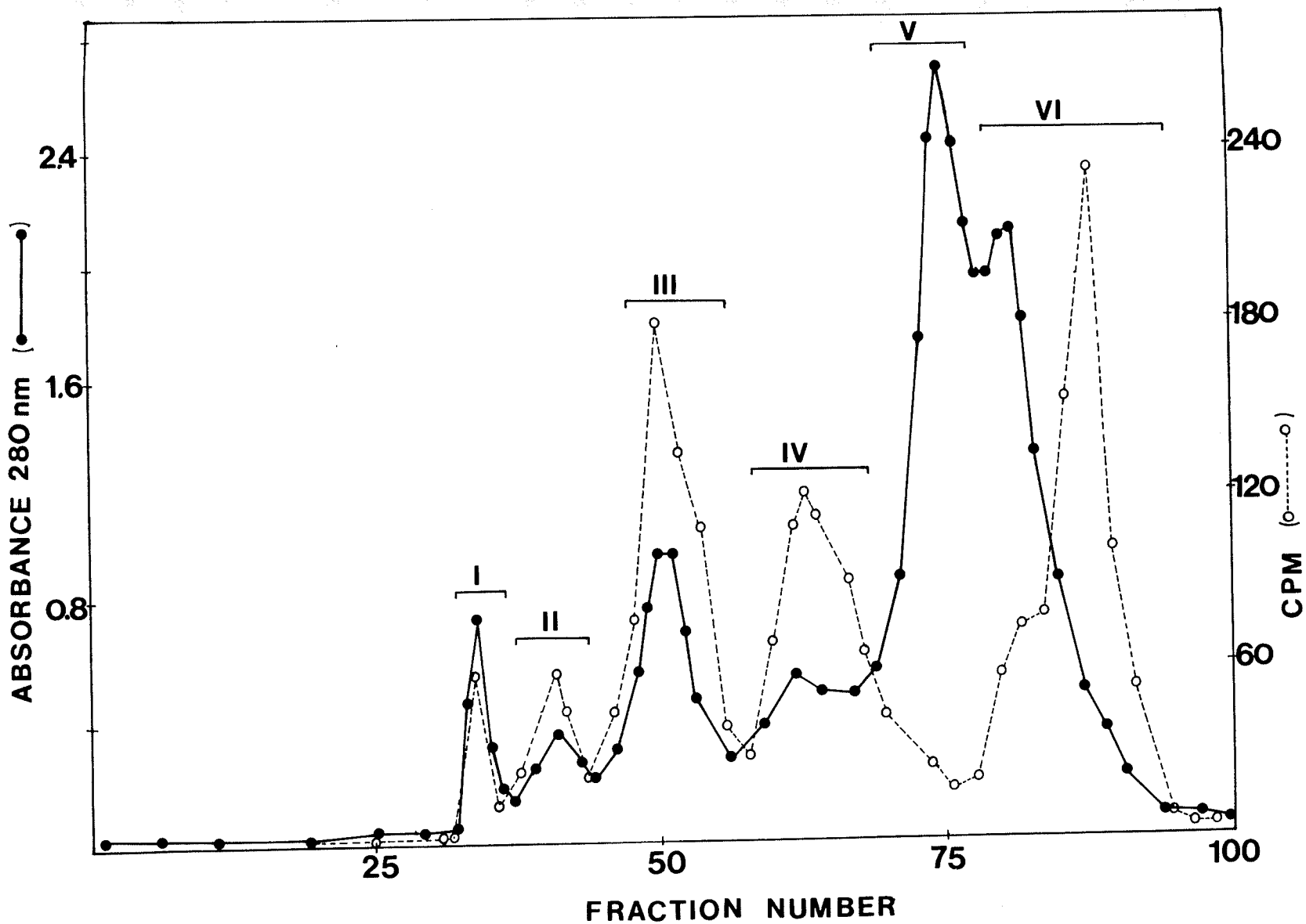


Figure 10 Gel filtration of the peptides produced by tryptic digestion of S-(^{14}C)-carboxymethylated citraconylated citrate synthase (3.1 μmol). Separation was on a Sephadex G-50 column (2.5 x 160 cm) in 0.1 M NH_4HCO_3. Fractions of 10 mL were collected and monitored for A_{280} (—) and radioactivity in 20 μL aliquots (--). The solid bars indicate the six pools made.


V
VI
III
IV
I
II
2.4
1.6
0.8
240
180
120
60
25
50
75
100
ABSORBANCE 280 nm
CPM
FRACTION NUMBER

was necessary because peptides stored in this salt solution for prolonged time have been reported to be hydrolyzed (Weng et al., 1978). This type of hydrolysis was also observed in this study, when the peptide TC-3 was stored in NH_4HCO_3 buffer. Henceforth, peptides in this buffer were immediately lyophilized.

In order to fractionate these pools from Sephadex G-50 further, techniques such as ion exchange chromatography (Dowex 50-X2, Dowex 1-X2 and DEAE cellulose), paper electrophoresis and gel filtration were employed. As a result, six short TC peptides from pools V and VI were isolated, but none of the large peptides was obtained in a pure state. The final purification of these large, denatured peptides was achieved by reverse phase HPLC. The detailed handling of each pool with regard to its purification, composition and sequencing is described in the following section.

(i) Pool 1

The mixture from this pool was initially chromatographed on a DEAE cellulose column (1.5 cm X 30 cm) equilibrated with 0.03 M NH_4HCO_3 buffer. The bound material was eluted in a gradient of 0.50 M to 1.0 M NH_4HCO_3. Fractions were monitored for peptide content by absorbance at 230 nm and by measuring radioactivity. The material appeared in a single peak at about 0.60 M NH_4HCO_3. After decitraconylation the purity of the collected fractions was checked by dansylation

and composition analysis; both indicated that it was still a mixture of peptides. Another evidence for its impurity was obtained from automated degradation; each cycle contained more than one amino acid, proving that it needed further purification.

An attempt to fractionate this mixture by reverse phase HPLC was unsuccessful since the material was not eluted from the C-8 column by any of the acetonitrile, propanol-2 or propanol-1 systems (see Methods). Because the material was from the first peak on the Sephadex G-50 column and because it was not resolved by ion exchange and HPLC techniques, it was concluded that the pool contained partially cleaved, aggregated large fragments. No further attempts were made to resolve this mixture.

(ii) Pool II

After decitraconylation, this pool was fractionated by reverse phase HPLC using the propanol-1 system. Several small peaks appeared early in the elution profile, but the bulk of the peptide emerged in a single peak at about 30% propanol-1. All peaks suspected to contain peptide material were collected and then analyzed for composition. The small peaks contained insignificant amounts of peptide material and were not studied further. The major peak proved to contain peptide TC-20, which was 68 residues long, the largest TC peptide.

Automated Edman degradation of TC-20 provided sequence for 25 residues (320 to 344). Phenylthiohydantoins in each cycle were identified only by the HPLC method; CM-cysteine at cycle 3 was also confirmed by measuring radioactivity. The sequence of 17 residues from the C-terminal of TC-20

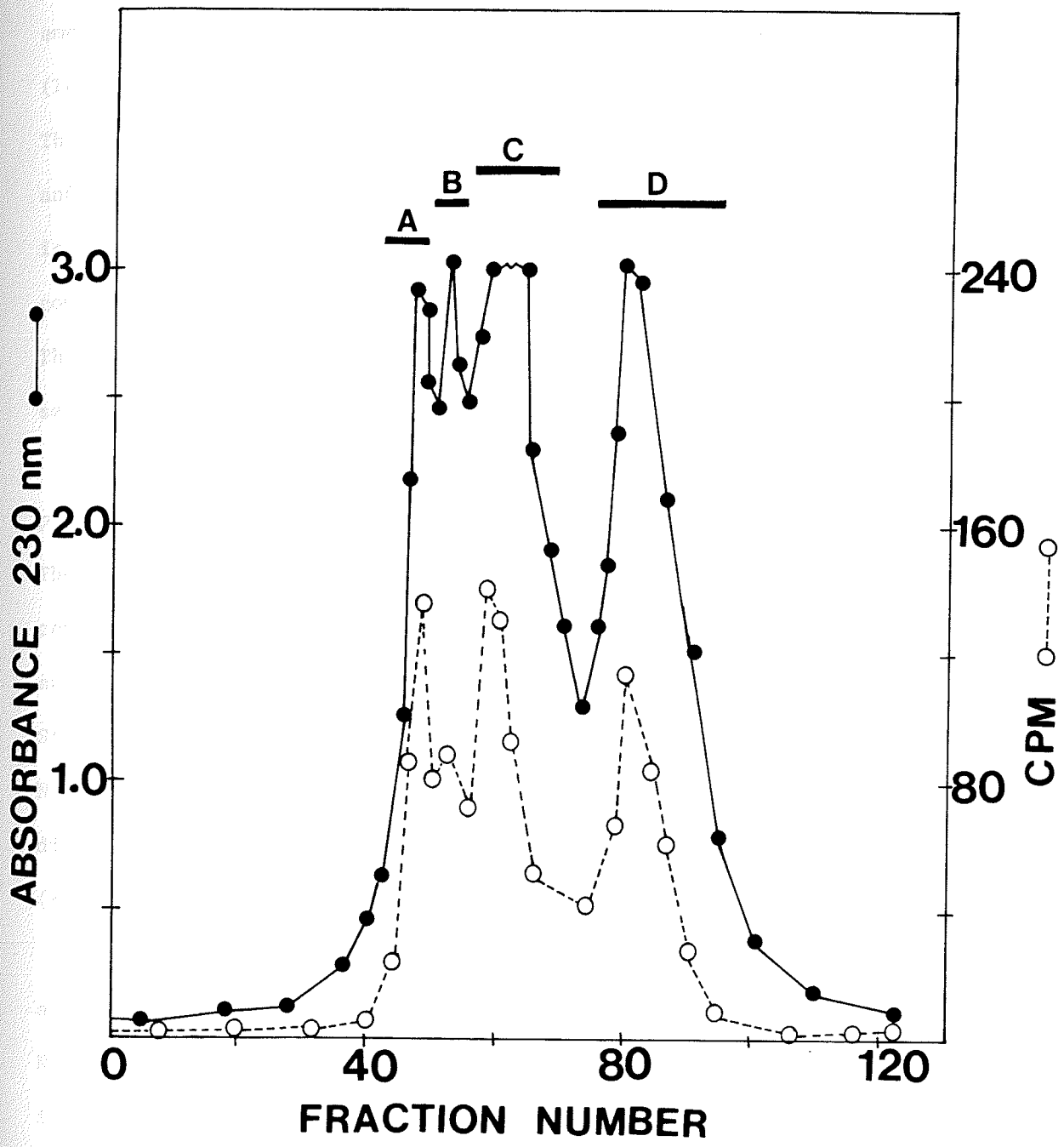
was obtained by isolating and sequencing a tryptic peptide (see later for details).

(iii) Pool III

Initial fractionation of this pool was by chromatography on a DEAE cellulose column equilibrated with 0.03 M NH_4HCO_3 buffer, and eluted with a linear gradient of 0.03 to 0.50 NH_4HCO_3. The mixture was resolved into four fractions as shown in Figure 11 . Pooled fractions are indicated by solid bars. None of the fractions proved to be pure by amino acid composition and dansylation. Five cycles of automated Edman degradation on fraction III C and III D showed more than one amino acid in each cycle. Therefore, further fractionation was required to obtain pure peptides.

Fraction III A was decitraconylated and chromatographed by reverse phase HPLC method using the propanol-2 system. Figure 12A represents the elution profile, where the first two peaks are due to Gdn-HCl and citraconic acid respectively; these were present in all profiles, and will not be discussed further. The third peak, later identified as TC-14 eluted at 45% concentration of propanol-2. The fourth peak at about 65% propanol-2 was also collected, but only the third peak (TC-14) contained significant amount of peptide material. Automated Edman degradation on TC-14 provided continuous sequence of 43 residues (240 to 282); His-283 was not identified and Ile-284 and Pro-285 were only tentatively assigned since the degradation was out of phase by one complete cycle by this point and also the background levels of these were high (see Fig. 30).

Figure: 11 Chromatography of pool III (form Sephadex G-50) on a column (1.5 cm X 30 cm) of DEAE-cellulose (DE52) that was equilibrated with 0.03 M NH_4HCO_3 buffer. The elution was achieved with a linear gradient between 0.03 M and 0.50 M NH_4HCO_3, total volume 600 mL. Fractions of 5 mL were collected at a flow rate of 20 mL per hour and monitored by absorbance at 230 nm (——). Aliquots of 20 µL from every second fraction were counted for radioactivity (-------). Solid bars indicate pooled fractions.

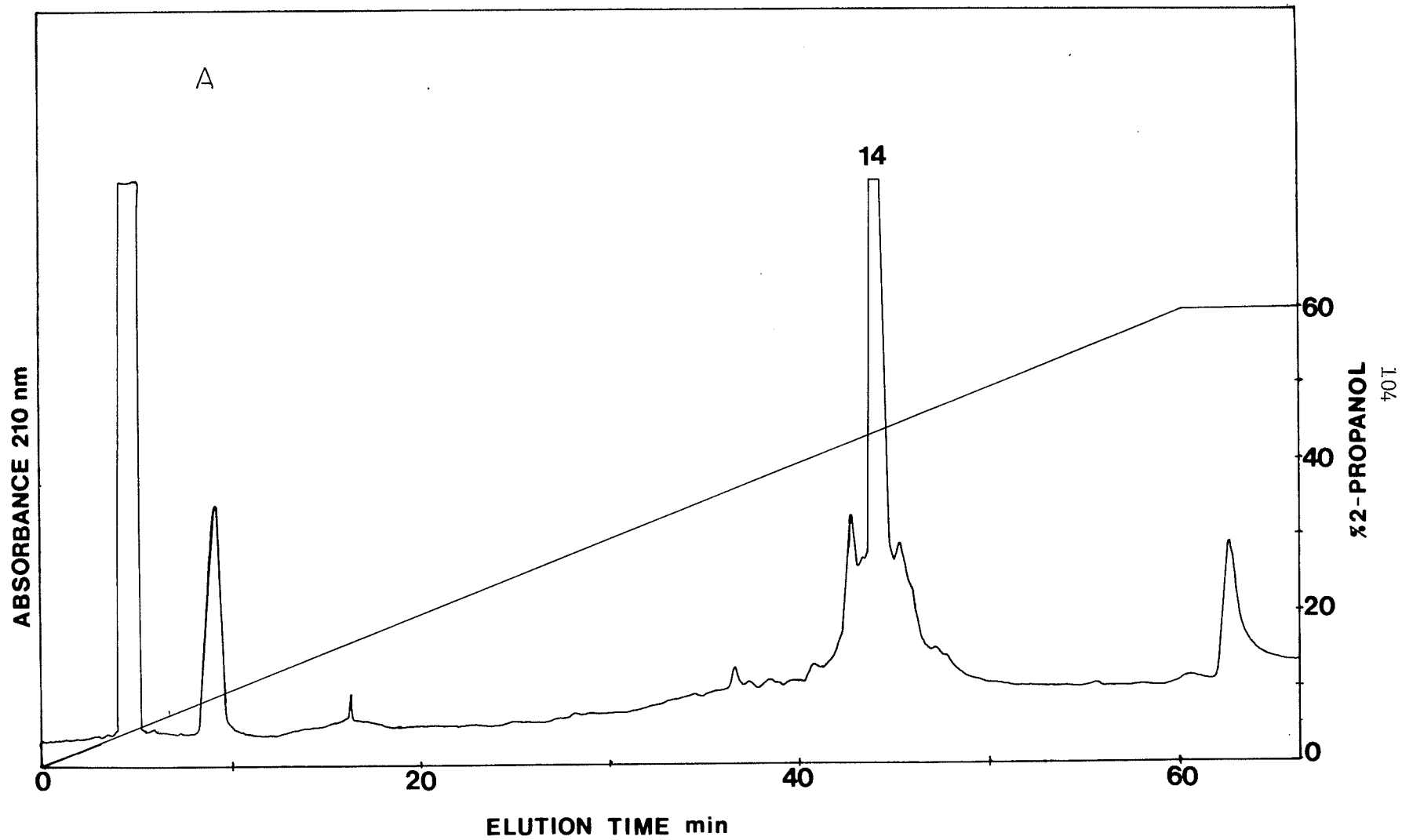

A
B
C
D
3.0
2.0
1.0
ABSORBANCE 230 nm
240
160
80
CPM
0
40
80
120
FRACTION NUMBER

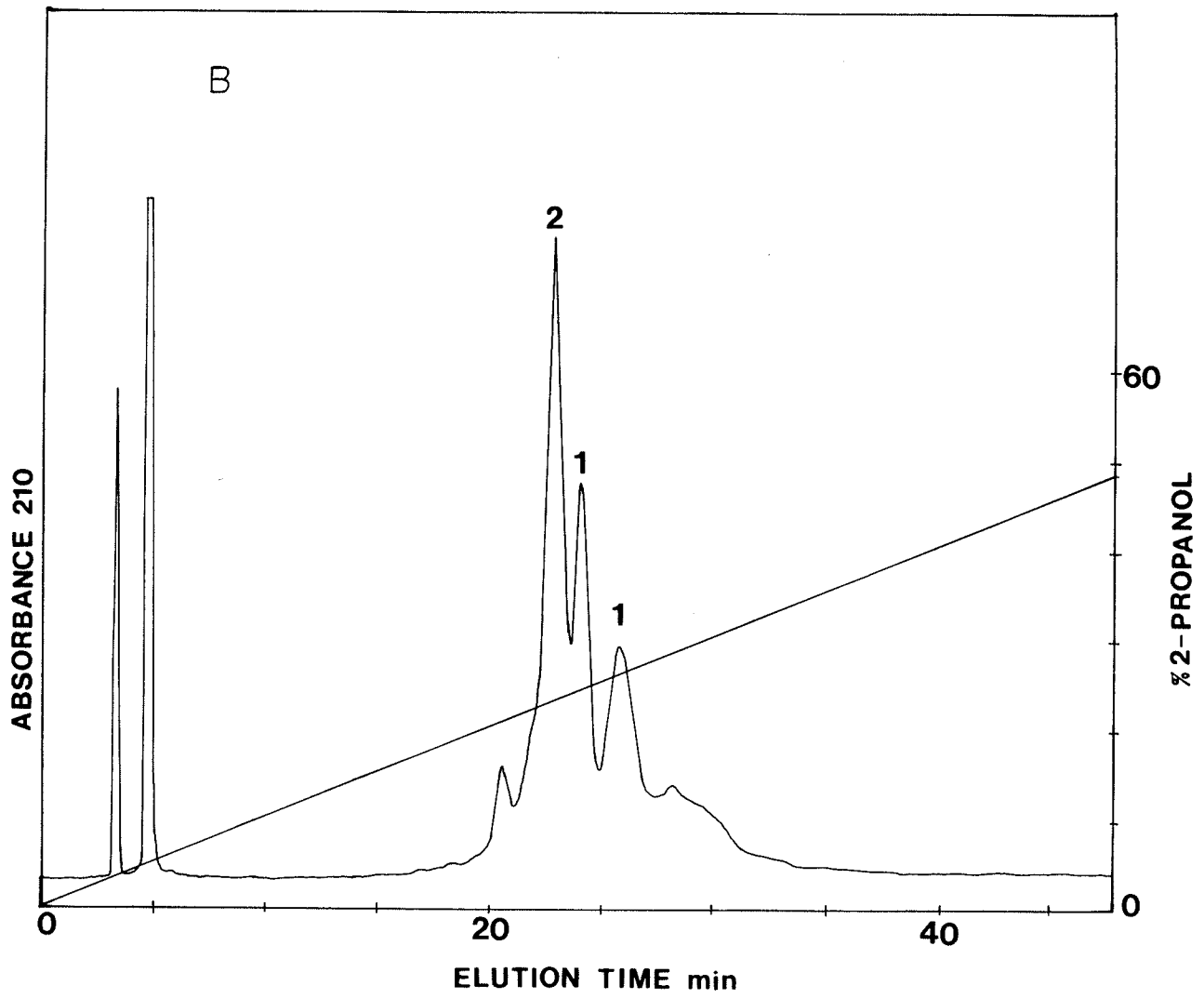
The fractionation of decitraconylated III B by reverse phase HPLC was achieved by using the propanol-2 system. The bulk of peptide material emerged at 45% propanol-2. By composition analysis and by sequencing (14 residues) the peptide proved to be the same as sequenced above (TC-14). This means that TC-14 was distributed in two peaks on DEAE cellulose (III A and III B). Although the reason for this type of behaviour of this peptide is not exactly known, it can however be suggested that deamidation or conformational differences might have caused the splitting into two peaks. This type of splitting was found to be very common on reverse phase HPLC separation profiles.

Fraction III C, after decitraconylation, was resolved by reverse phase HPLC using the propanol-2 system into three subfractions (Figure 12B). The first fraction contained peptide TC-2, and the other two had similar composition to each other, and proved to be the peptide TC-1. The automated sequencing of TC-2 gave a sequence of 29 residues (33 to 61). Similarly, the sequence of 29 residues of TC-1 showed that it was the N-terminal sequence of intact subunit. Both peptides TC-1 and TC-2 were digested with trypsin and their carboxy terminal sequence was determined (see below).

The fourth peak, fraction III-D was well resolved from other peaks as shown in Figure 11. After decitraconylation, this peak showed a single N-terminal amino acid residue glycine by dansylation procedure. Composition analysis also gave numbers which were close to integral, again indicating high purity. But later this fraction was found to be hydrolyzed because

Figure:12 High pressure liquid chromatography of fraction III A (A) and III C (B) from Figure 11 , on a Perkin Elmer C8 reverse phase column (4mm X 200 mm) using the propanol-2 solvent system (see Methods). The peptide mixture was decitraconylated, dissolved in 1.0 mL of 6 M Gdn-HCl and 100 µL was applied to the column. The rate of increase of the gradient was 60% propanol-2 per hour and the flow rate was 0.8 mL/min. The profiles show A_{210} (full scale: 5.12 A and 2.56 A for (A) and (B) respectively) The numbers above the peaks refer to the TC peptides subsequently identified in those peaks.


A
14
ABSORBANCE 210 nm
%2-PROPANOL
0
20
40
60
ELUTION TIME min


B
2
1
1
60
0
ABSORBANCE 210
% 2-PROPANOL
0
20
40
ELUTION TIME min

it was stored in NH_4HCO_3 buffer for a long time before being used for sequencing. That the peptide had been hydrolyzed, was also indicated by automated Edman degradation, where several amino acids were seen in each cycle. An attempt was made to purify an intact peptide from the above mixture by rechromatography on DEAE cellulose; four peaks were obtained, but their composition analysis still suggested mixtures. One of these was further fractionated into three peaks by reverse phase HPLC using the propanol-2 system; the major one of these gave a sequence of five residues from the amino terminus of TC-3, and the other two were still a mixture. Since it became difficult to obtain new information from these complex mixtures, none of these were further analyzed.

Peptide TC-3 was later obtained by a single step reverse phase HPLC purification of TC peptides (peptide map) and the sequence of its 24 residues (70 to 93) was achieved. This sequence contained an Asn-Gly linkage, a site for the hydroxylamine cleavage.

(iv) Pool IV

This pool was initially chromatographed on a DEAE-cellulose column in the NH_4HCO_3 buffer system. Figure 13 shows the elution profile; five fractions were pooled as indicated. Since the resolution was very poor each fraction was rechromatographed on DEAE cellulose. Single peaks were obtained but composition analysis indicated that they were still not pure. The same conclusion was reached by dansylation of these fractions. Therefore, reverse phase HPLC was employed for their final purification after

decitraconylation as usual.

The peak IV A was resolved by HPLC into four peaks as shown in Figure 14A. Of these four peaks, the first two had the same composition and the sequence of its 17 residues was determined; arginine at position 18 was placed only from composition analysis. This sequence is that of TC-13(222-239). The last two peaks in Figure 14A proved to be the peptide TC-10(164-188); the sequence of its 25 residues was determined including the C-terminal arginine, by automated Edman degradation.

The peak IV B was resolved by HPLC into two peaks, as shown in Figure 14B, which are TC-10 and TC-7. The sequence of peptide TC-10 had already been determined. Peptide TC-7(125-155) yielded a continuous sequence of 19 residues; His-146 was not identified and Asp-147, Ser-148, Leu-149, Val-151, Asn-152 and Asn-153 were placed tentatively because the sequencing data were of rather inferior quality. However, these residues in the C-terminal portion of TC-7 were established later by sequencing a cyanogen bromide peptide CN-4.

The peak IV C was separated into three peaks by reverse phase HPLC, using the propanol-2 system (Figure 14C). The first peak TC-11(2) was sequenced for 14 residues and arginine at position 15 was placed by composition analysis. The second peak TC-11(1) yielded a sequence of 11 residues. In later studies, peptide TC-11 (189-217) was isolated and its composition was the sum of those of TC-11(1) and TC-11(2). The amino acid sequence of its 21 residues proved that TC-11(1) and TC-11(2) originated from it by a hydrolysis that had taken place at the carboxyl

Figure: 13 Chromatography of pool IV (from Sephadex G-50) on a column (1.5 cm X 30 cm) of DEAE-Cellulose (DE52) that was equilibrated with 0.03 M NH_4HCO_3. The elution was achieved with a linear gradient between 0.03 M and 0.50 M NH_4HCO_3, total volume 600 mL. Fractions of 5 mL were collected at a flow rate of 30 mL per hour and monitored by absorbance at 230 nm (——). Aliquots of 20 µL, from each alternate fraction were counted for radioactivity (-----). Solid bars indicate pooled fractions.

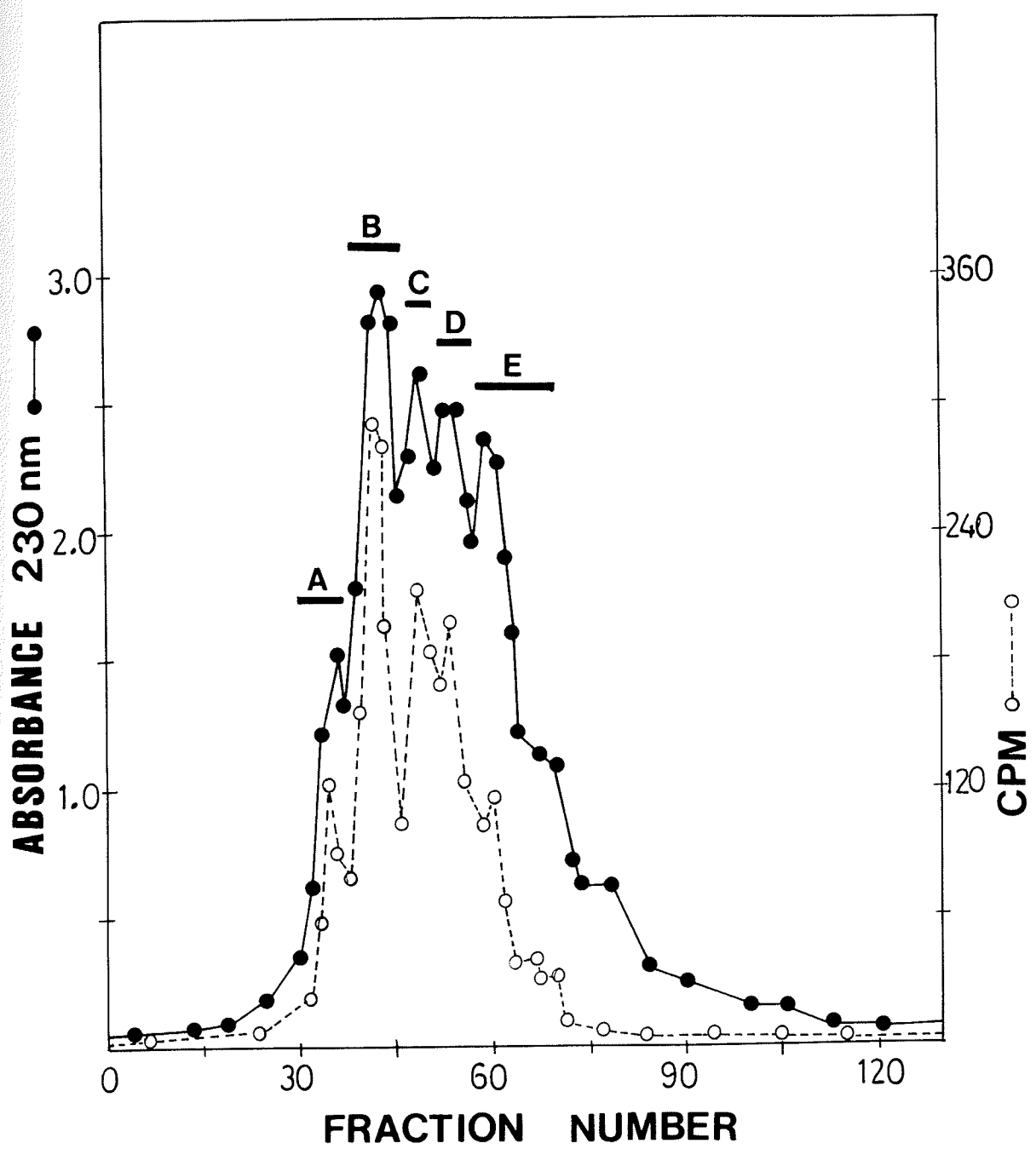
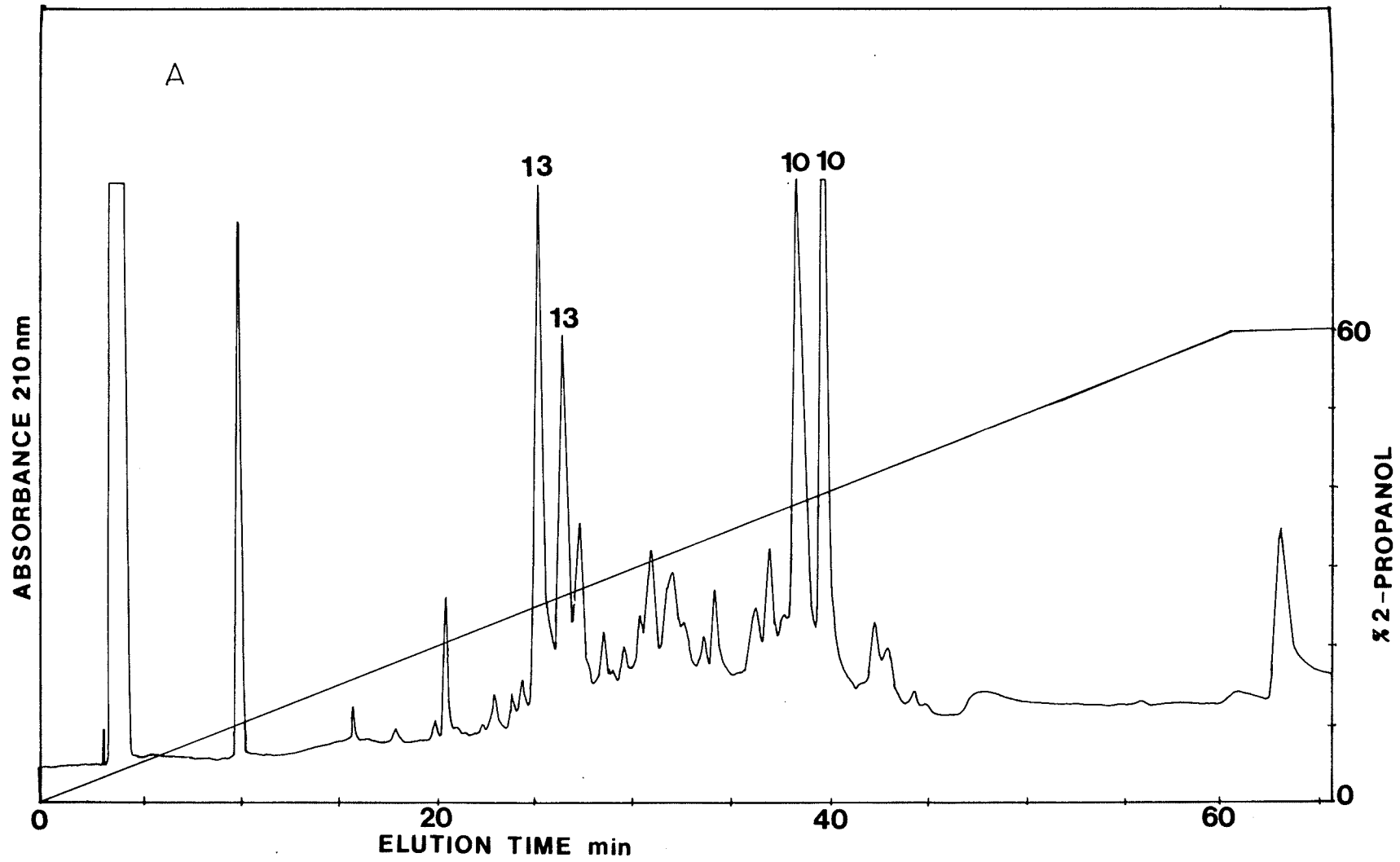
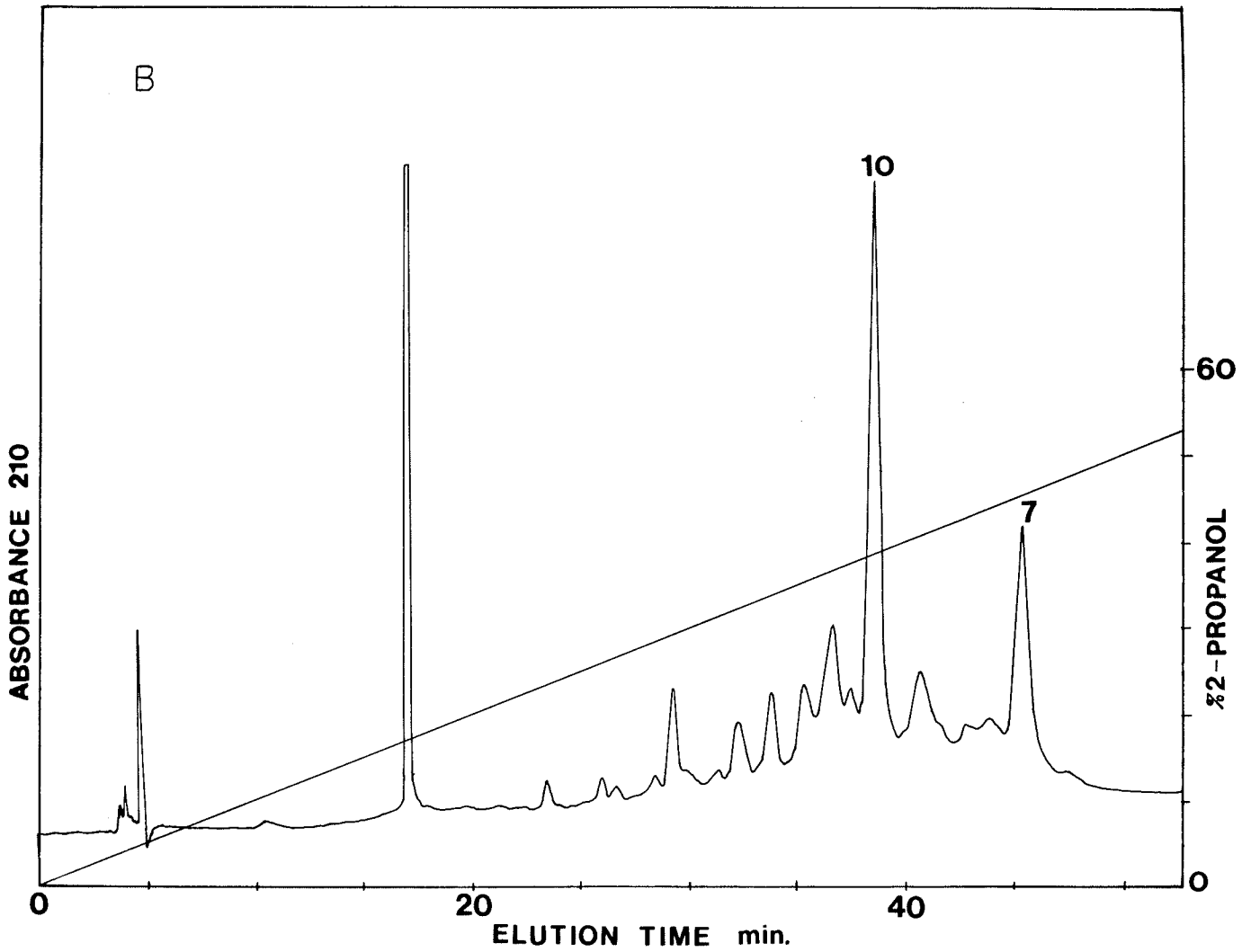
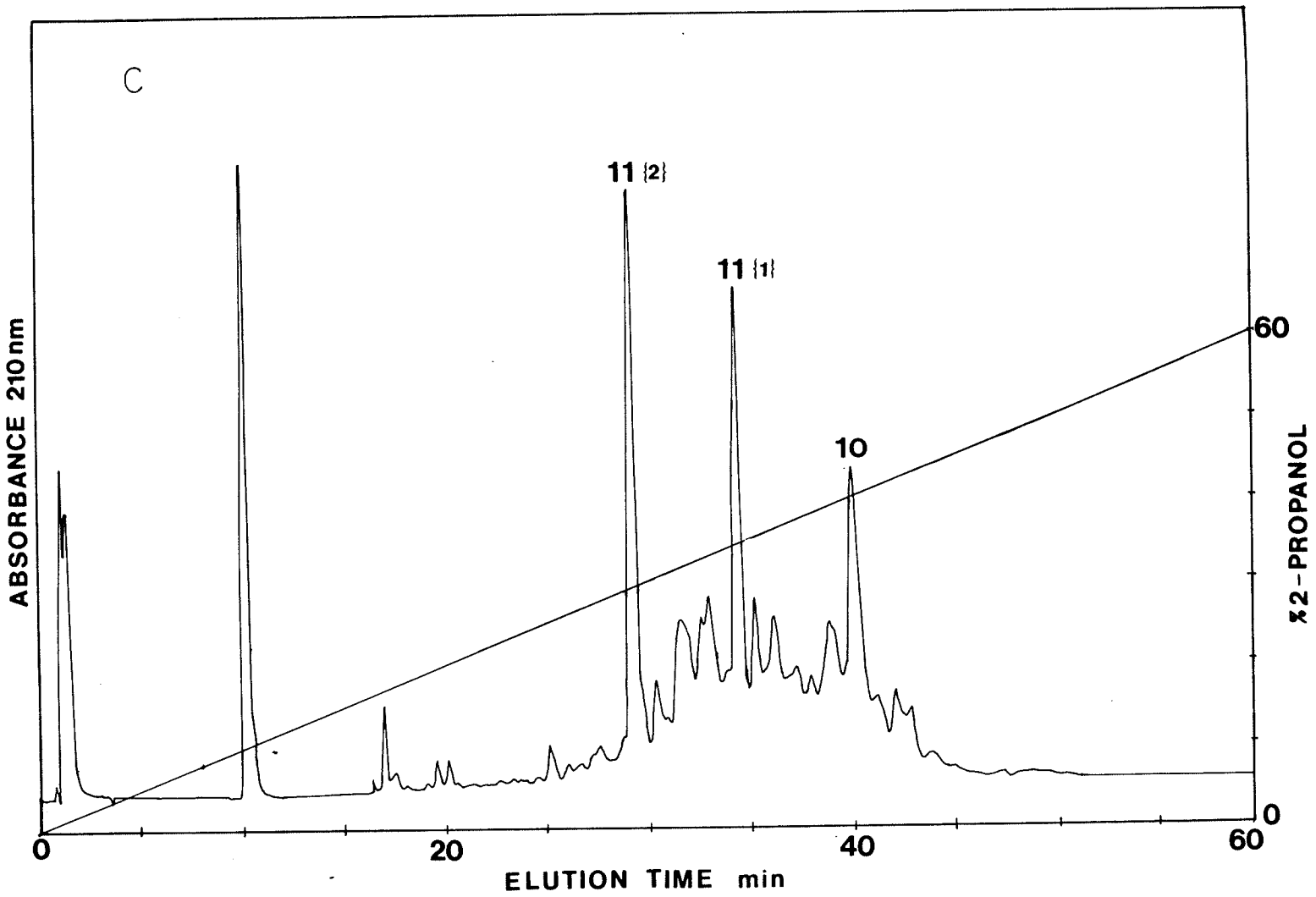
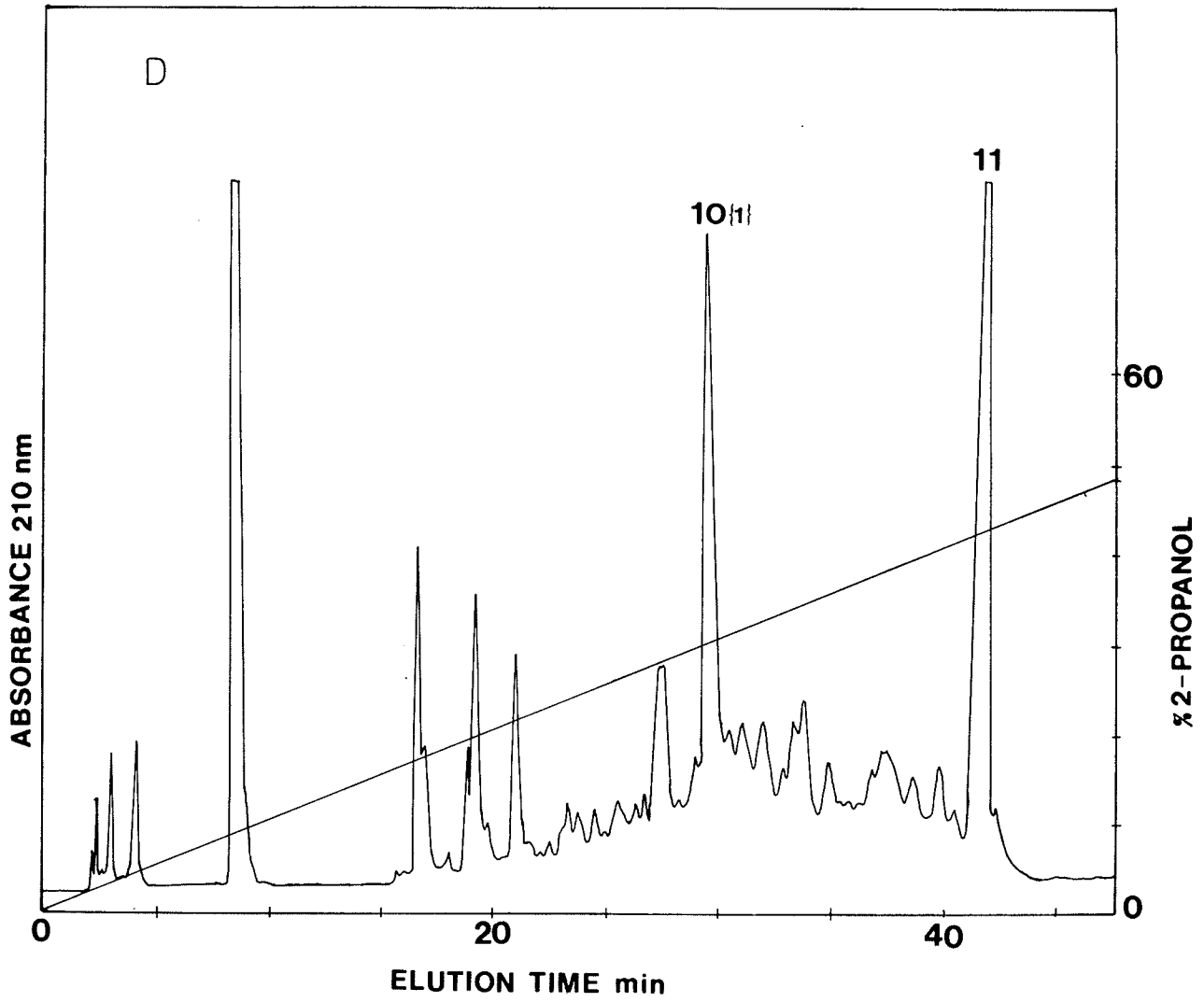

B
C
D
E
A
3.0
2.0
1.0
360
240
120
ABSORBANCE 230 nm
CPM
0
30
60
90
120
FRACTION NUMBER

Figure:14 Reverse phase HPLC of the fractions IV A, IV B, IV C and IV E [(A), (B), (C) and (D) respectively]. The peptide mixture was decitraconylated, dissolved in 1.0 mL of 6 M Gdn-HCl and 200 µL was applied to a Perkin Elmer reverse phase C8 column (4mm X 250mm) equilibrated with 0.01% TFA. Peptides were eluted using 2-propanol system (see Methods). The rate of increase of the gradient was 60% aqueous 2-propanol per hour and the flow rate was 0.8 mL/min. The profiles show A_{210} (full scale= 5.12 A). The numbers above the peaks reresents TC peptides subsequently identified in those peaks.


A
13
13
10 10
ABSORBANCE 210 nm
60
0
% 2-PROPANOL
0
20
40
60
ELUTION TIME min


B
10
7
60
0
ABSORBANCE 210
%2-PROPANOL
0
20
40
ELUTION TIME min.


C
11 {2}
11 {1}
10
ABSORBANCE 210nm
%2-PROPANOL
60
0
0
20
40
60
ELUTION TIME min


D
11
10(1)
60
0
ABSORBANCE 210 nm
% 2-PROPANOL
0
20
40
ELUTION TIME min

side of Phe-202. The third peak in Figure 14 C was a further amount of TC-10.

Fraction IV D, when chromatographed by HPLC appeared as a single major peak (not shown). From composition it was identified as more of peptide TC-11(1). Since the complete sequence of TC-11 had already been established, no sequence analysis was done to this fraction.

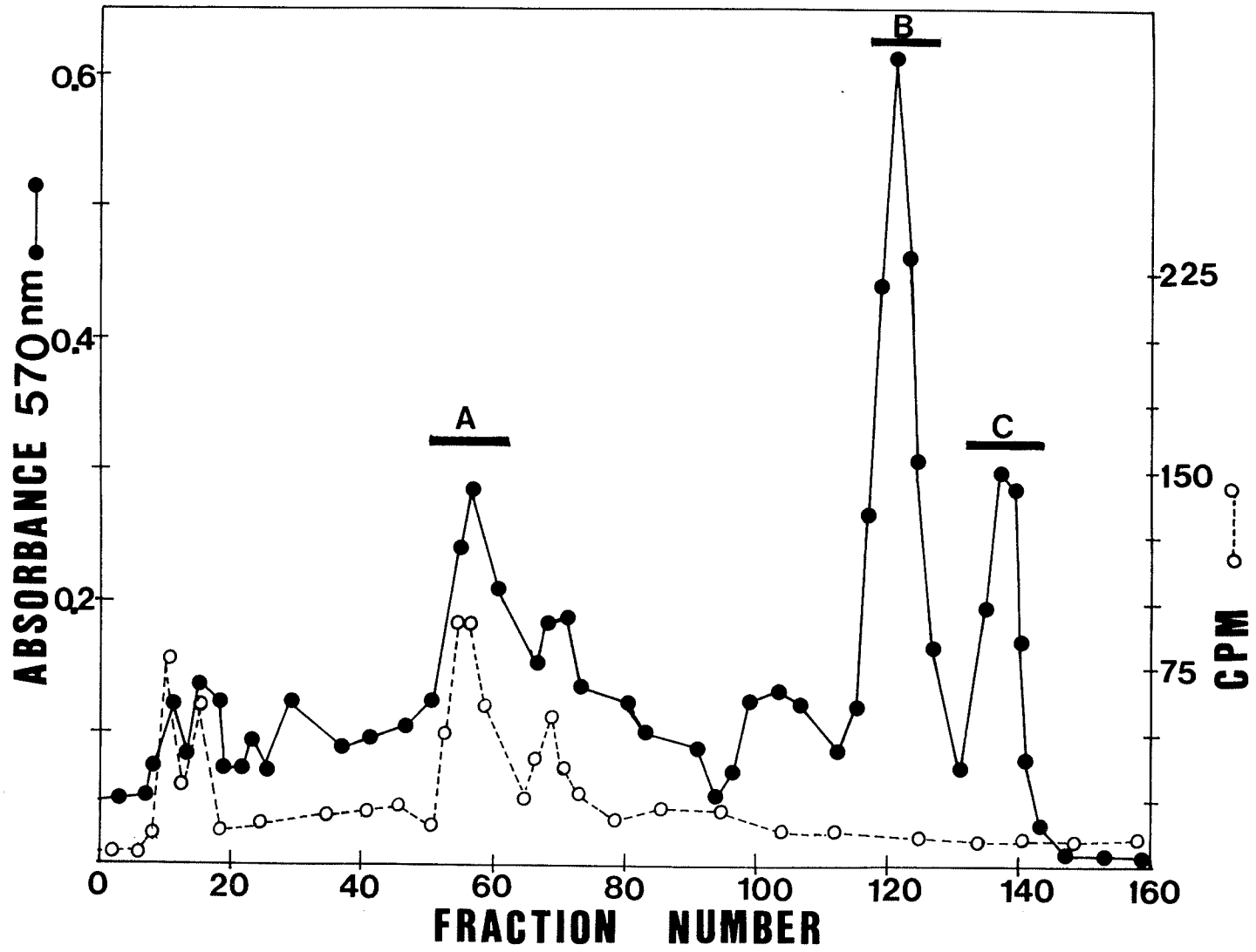
The peak IV E was resolved into two peaks by reverse phase HPLC in propanol-2 (Figure 14D). The first designated as TC-10(1) had composition corresponding to residues 164-178 and five cycles of automated Edman degradation confirmed that it was from the N-terminus of TC-10. These observations indicate that TC-10(1) was generated by hydrolysis of TC-10 at the carboxyl side of Tyr-178. It is interesting to note here that peptides TC-10 and TC-11 were hydrolyzed by a similar chymotryptic-like activity which occurred between an aromatic amino acid and serine.

The second peak in Figure 14D, proved to be the peptide TC-11. The sequence analysis of this peptide has already been discussed above.

(v) Pool V

After decitraconylation, pool V was chromatographed on a cation exchange column, Dowex 50-X2 as described in Methods. Figure 15 shows the elution profile, where the mixture is resolved into seven prominent peaks. Of these only three, designated as V-A, V-B and V-C, contained significant material as determined by amino acid analysis. Therefore, only these three fractions were further analyzed.

Figure : 15 Chromatography of pool V from Sephadex G-50 (Figure 10) on a Dowex 50-X2 column (0.6 cm X 60 cm) at 40°C. The mixture was decitraconylated, dissolved in 2 mL of 0.20 M pyridine acetic acid buffer, pH 3.1 and applied to the column equilibrated with the same buffer. Peptides were eluted according to the method of Schroeder (1972). Fractions of 1.2 mL were collected at a flow rate of 10 mL per hour, and assayed by ninhydrin after alkaline hydrolysis of 100 µL samples (———). Aliquots of 20 µL were counted for radioactivity. Solid bars represent the pools containing significant amount of material as determined by subsequent analysis.


B
A
C
0.6
0.4
0.2
ABSORBANCE 570nm
225
150
75
CPM
0
20
40
60
80
100
120
140
160
FRACTION NUMBER

The peak V A was rechromatographed on an anion exchange column, Dowex 1-X2. The elution profile showed no peaks, and the material was not analyzed further.

The peak V B was fractionated by high voltage paper electrophoresis at pH 6.5. As a result, three spots were observed and peptide TC-23 (419-426), corresponding to the middle darkest spot was eluted from the paper using 10% formic acid. The peptide was analyzed for composition and the sequence of its 8 residues was determined. Phenylthiohydantoins were identified by back hydrolysis in 6 M HCl containing 0.1% $SnCl_2$, and also by gas chromatography.

The peak designated V C was rechromatographed on Dowex 1-X2 and the material emerged in a single large peak containing peptide TC-16 (291-299). Seven of the nine amino acids were placed in sequence by automated Edman degradation. The remaining two residues, Phe-298 and Arg-299, were assigned from the composition data.

(vi) Pool VI

This pool was from the last peak of Sephadex G-50 column and should contain short peptides. Thus the mixture was decitraconylated and initially fractionated on a Dowex 50-X2 column. The elution profile is shown in Figure 16, where the portion pooled for each peak is represented by a solid bar. From the amino acid analysis it was determined that the first peak which was highly radioactive did not contain any peptide. The other five fractions (VI A to VI E) contained significant amounts of peptide

Figure: 16 Chromatography of pool VI from Sephadex G-50 (Figure 10) on a Dowex 50-X2 column (0.6 cm X 60 cm) at 40°C. The mixture was decitraconylated, dissolved in 1.5 mL of 0.2 M pyridine acetic acid buffer pH 3.1 and applied to the column previously equilibrated with the same buffer. Peptides were eluted according to the method of Schroeder (1972). Fractions of 1.0 mL were collected at a flow rate of 10 mL per hour, and assayed by ninhydrin after alkaline hydrolysis of 100 µL samples (——). Aliquots of 20 µL were counted for radioactivity. Solid bars represent the pools containing significant amount of material as determined by subsequent analyses.

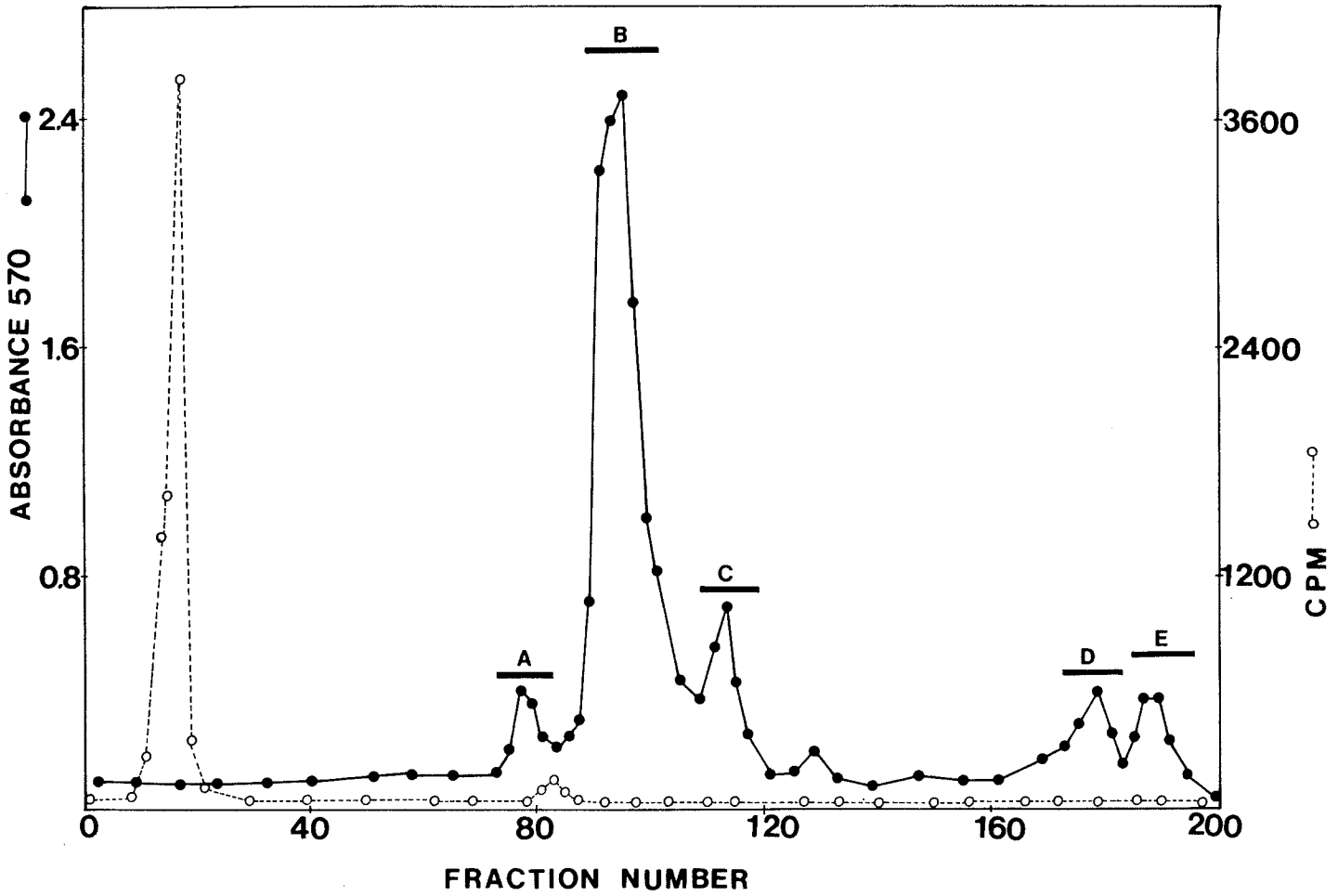

B
A
C
D
E
2.4
1.6
0.8
ABSORBANCE 570
3600
2400
1200
CPM
0
40
80
120
160
200
FRACTION NUMBER

Figure:17 Chromatography of fraction VI B (Figure 16) on a column (0.6 cm X 60 cm) of Dowex 1-X2 at 40°C. The mixture was dissolved in 2 mL of pH 9.4 buffer (prepared by mixing 60 mL of N-ethylmorpholine, 8 mL of α-picoline, 40 mL of pyridine, acetic acid to attain pH 9.4 and water to bring the toatal volume to 4 L) and applied to the column previously equilibrated with the same buffer. Peptides were eluted as described by Schroeder (1972). Fractions of 2 ml were collected at a flow rate of 10 mL per hour, and assayed by ninhydrin analysis after alkaline hydrolysis of 100 µL samples. Solid bars represent the pooled fractions.

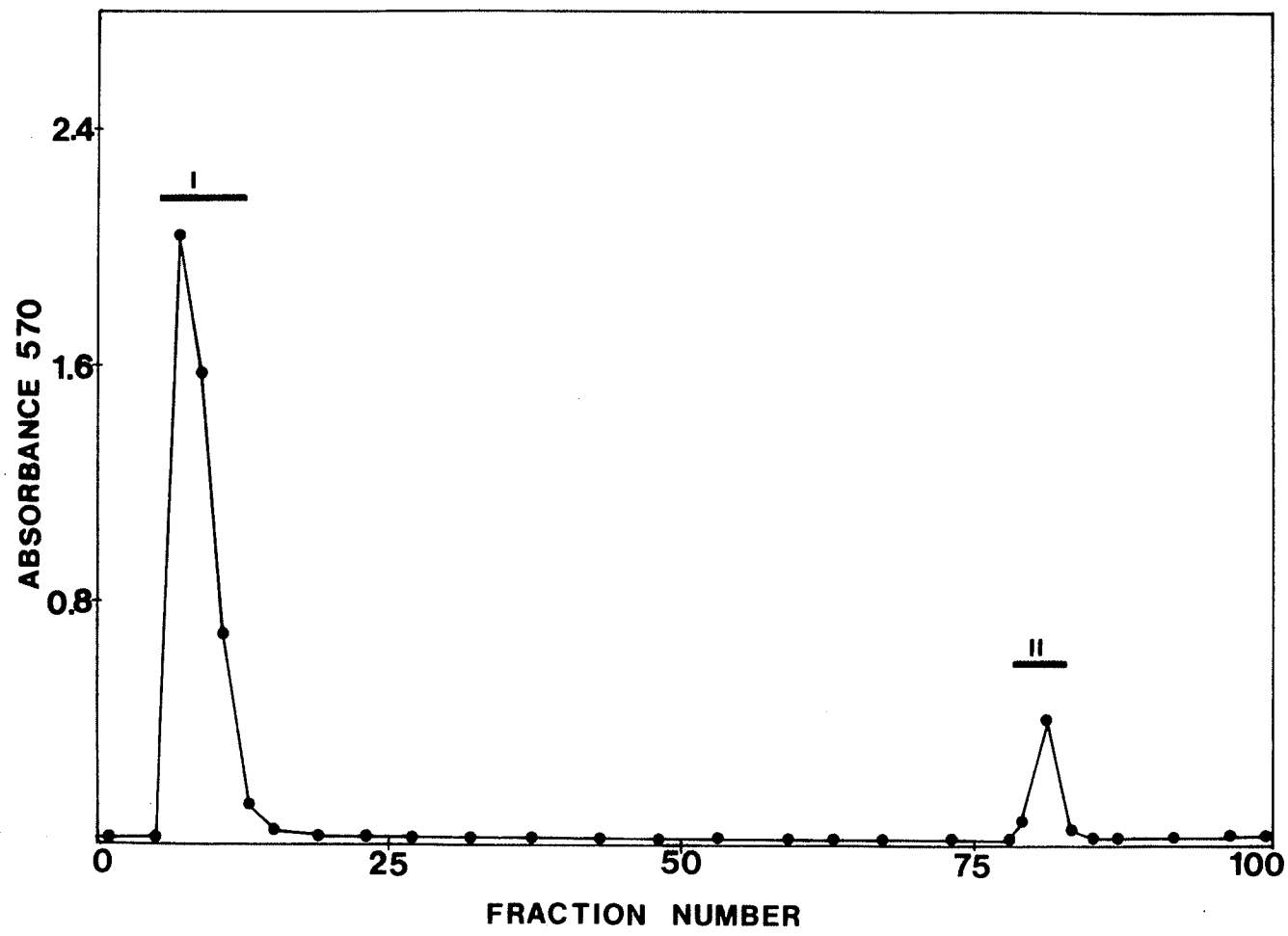
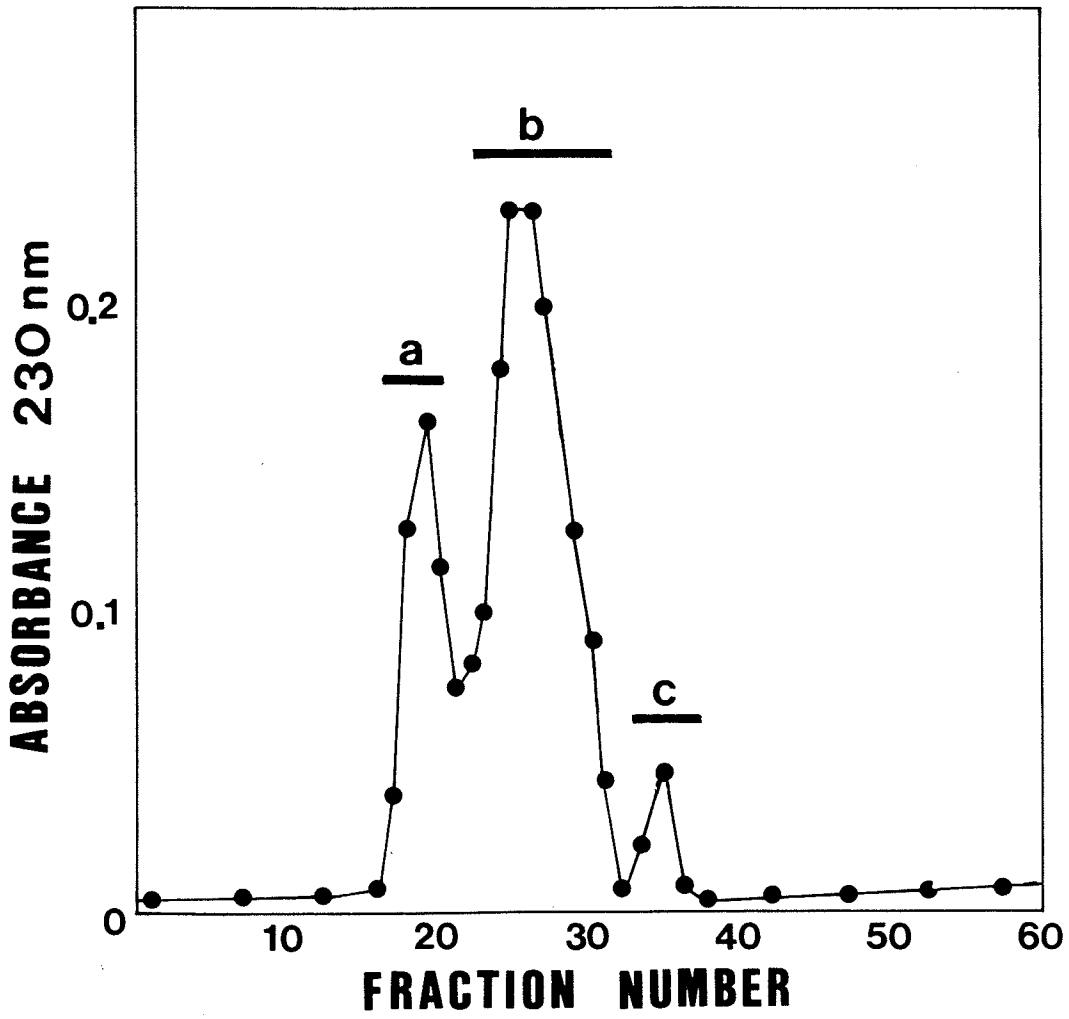

2.4
1.6
0.8
ABSORBANCE 570
I
II
0
25
50
75
100
FRACTION NUMBER

Figure: 18 Chromatography of fraction VI BI (from Figures 16 & 17) on a Biogel P2 column (0.5 cm X 90 cm) in 0.1 M NH_4HCO_3. Fractions of 2 mL were collected at a flow rate of 15 ml/hr and monitored by absorbance at 230 nm. Solid bars indicate pooled fractions. Subsequent analysis indicate that the peak 'a' contained peptide TC-19 (315-319), and peaks 'b' and 'c' contained free arginine.


b
a
c
ABSORBANCE 230nm
0.2
0.1
0
10
20
30
40
50
60
FRACTION NUMBER

material and all of these were further analyzed. However, material from two fractions, VI A and VI E was exhausted in attempts to fractionate them by high voltage paper electrophoresis at pH 6.5.

Fraction VI B, a large peak, was rechromatographed on a Dowex 1-X2 column and the elution profile shown in Figure 17 was obtained. Two peaks designated as VI BI and VI BII were pooled. Fraction VI BI was rechromatographed on Biogel P2 column and a further three peaks, containing peptides TC-19, TC-6 and TC-15 were obtained (Figure 18). The sequence of TC-19(315-319) was determined through its carboxy terminal arginine. TC-6 and TC-15 turned out to contain free arginine. Although such a high absorbance at 230 nm in the Biogel column is unexpected for a single amino acid it is possible that some other light absorbing material may be coeluting with free arginine. Fraction VI BII contained peptide TC-9 (158-163) and its complete amino acid sequence was determined by automated Edman degradation.

Fraction VI C was rechromatographed on a Dowex 1-X2 column, and only one large peak containing peptide TC-22 was obtained (not shown). The amino acid composition indicated that the peptide is pure and contains 9 residues (1 Thr, 2 Glu, 1 Leu, 2 Tyr, 1 Lys, 1 Gly and 1 Arg). However, every attempt to determine its sequence by automated Edman degradation was unsuccessful. Furthermore, attempts to determine its amino terminus by dansylation reaction were also futile. These results indicated that the peptide has a blocked amino terminus. One possibility, that the amino terminus is blocked due to the cyclization of glutamine to pyroglutamic acid was examined by treating the peptide with deblocking enzyme,

L-pyroglutamate amino peptidase. Still no sequence was obtained when the products of the enzymatic reaction were run in the sequencer, indicating that the amino terminus was still blocked. No further analysis was done, since most of the peptide was already consumed.

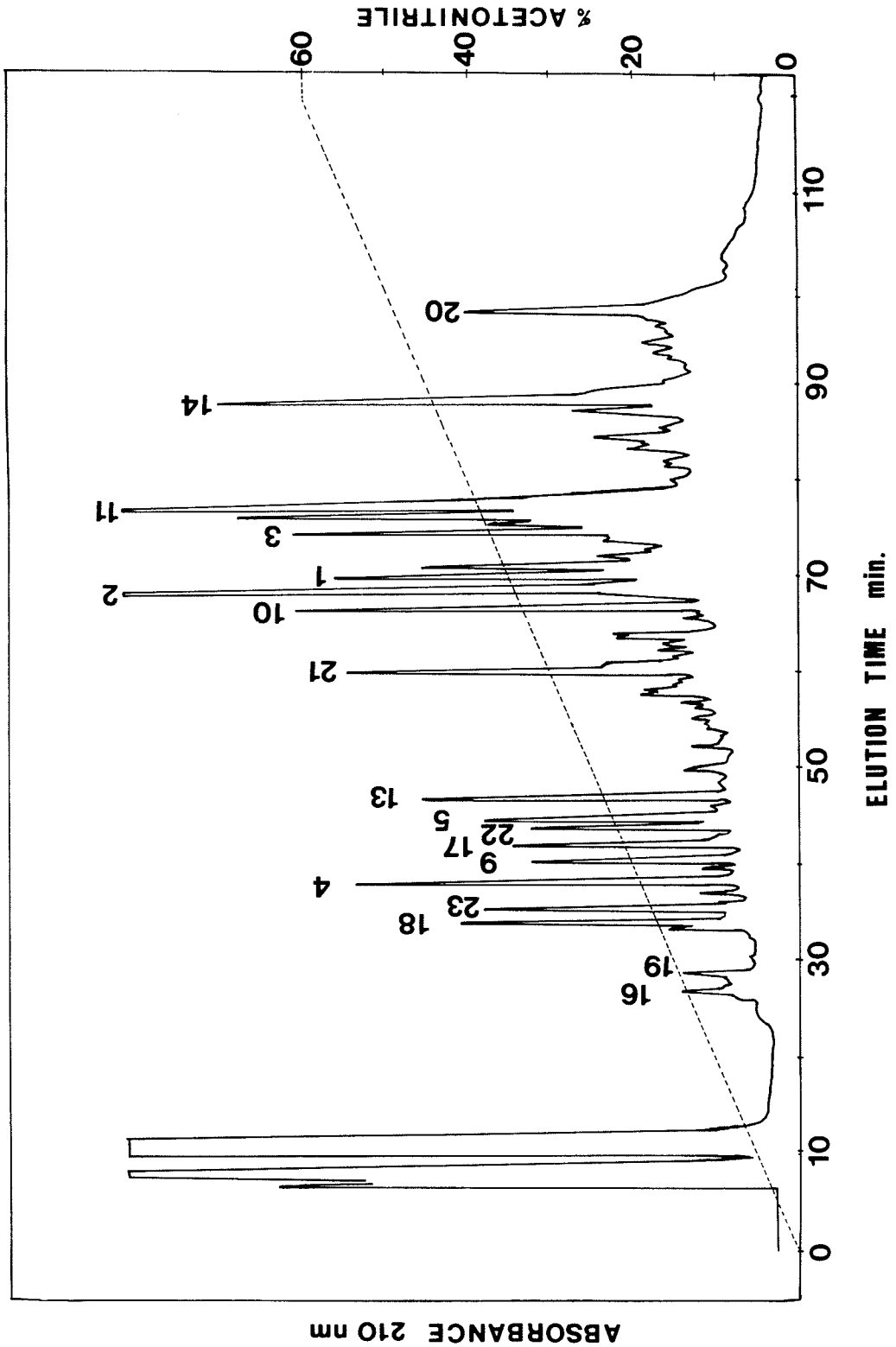
In a later experiment, the peptide TC-22 was isolated from a fresh batch of arginine peptides fractionated by a single step reverse phase HPLC method. The peptide was sequenced successfully by automated Edman degradation and Gln was identified as its amino terminal residue. The degradation went through its carboxy terminus arginine; obviously the peptide was not blocked in this case. It seems that the lengthy procedures used to isolate TC-22 in the previous case, caused cyclization of Gln to pyroglutamic acid. Failure to remove this residue by deblocking enzyme means that either that the enzyme used was inactive or that the conditions used for the reaction were not adequate.

Fraction VI D from Figure 16 was purified by high voltage paper electrophoresis at pH 6.5. A single peptide TC-18 (307-314) was isolated in a pure state. The sequence of its eight residues was also determined.

(vii) One step purification of arginine peptides

In the previous section the use of reverse phase HPLC was confined to the final purification following more conventional gel filtration and ion exchange chromatography. However, it has been demonstrated that the resolving power of reverse phase HPLC is sufficiently high that this technique alone can be used to isolate pure peptides (Walsh

Figure 19 Reverse phase HPLC of the tryptic digest of S-(^{14}C)-carboxymethylated citraconylated citrate synthase. The digest (268 nmoles) was decitraconylated, dissolved in 1.0 mL of 6 M Gdn-HCl and 200 µL was applied to a Perkin Elmer reverse phase C8 column (4 x 250 mm) equilibrated with 0.1% TFA. Peptides were eluted with a linear gradient between equlibration solvent and 60% aqueous acetonitrile containing 0.1% TFA, over a period of 120 min, flow rate 1.0 mL/min. The profile shows A_{210} (full scale= 2.56 A). The numbers above the peaks refer to the TC peptides subsequently identified in those peaks.


% ACETONITRILE
0
20
40
60
ELUTION TIME min.
0
10
30
50
70
90
110
ABSORBANCE 210 nm
16
19
18
23
4
9
17
22
5
13
21
10
2
1
3
11
14
20

et al., 1981). In view of the fact that this technique has proved to be an extremely powerful tool for peptide purification, it was used to isolate missing arginine peptides.

To start with, the tryptic digest of the (^{14}C)-carboxymethylated citraconylated citrate synthase (268 nmoles) was decitraconylated and the mixture was resolved on C-8 reverse phase column, by elution with a linear gradient using the acetonitrile or propanol-1 system (see Methods for details). The rate of change of gradient was kept at 0.5% per minute; the flow rate was 1.0 mL/min in the case of the acetonitrile system but 0.8 mL/min for the propanol-1 system. The elution profile is depicted in Figure 19, where each peak is labelled by the peptides it was found to contain. Recoveries of TC peptides from these columns were used to calculate the total yields of TC peptides.

Four arginine peptides, those designated TC-4, TC-5, TC-17 and TC-21, were identified from this batch although they had not been located in the fractions from the previous batch. In addition, a few other peptides obtained by this procedure were used for sequencing since the material from the first, larger batch had been exhausted. As described previously peptide TC-22, which had become blocked in the previous case, was isolated here in an unblocked state.

Of the four TC peptides isolated only by the one step purification, the sequence of one (TC-5) had already been established by a cyanogen bromide CN-2 peptide (see below), therefore it was not sequenced again.

The peptide TC-4 actually gave two residues in each cycle of automated Edman degradation. One was identified as the known sequence of TC-22 and the other one proved to be the peptide TC-4 (110-119). The sequence of its 9 residues was determined.

The peptide TC-17 (300-306) was sequenced for 7 residues through its carboxyl terminal arginine.

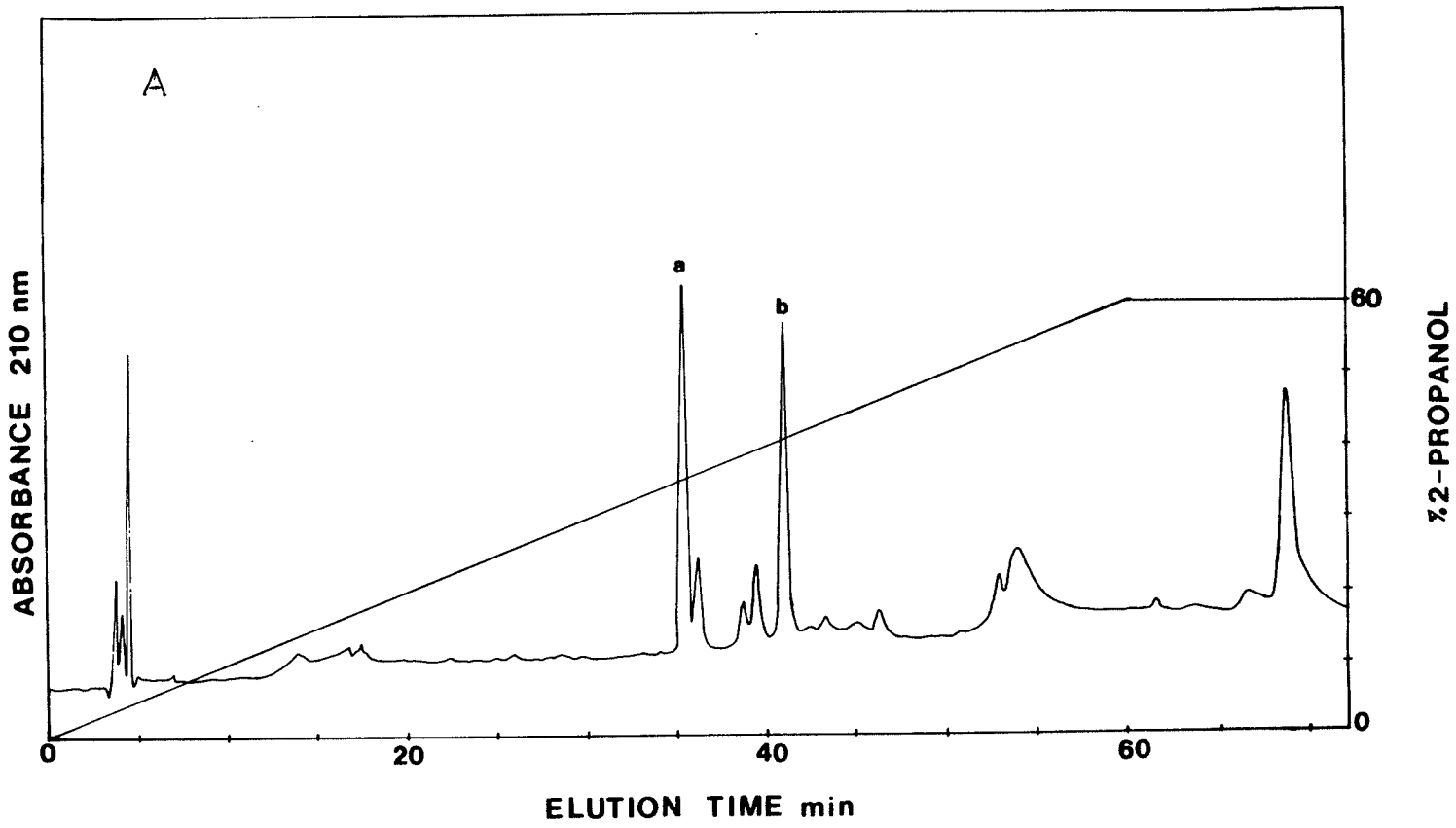
The last peptide isolated in the TC series was TC-21 (388-409). The sequence of its 15 residues was determined. A feature of this sequence was the presence of 2 tryptophans, of the total 3 in the subunit of citrate synthase, thus providing a site for the o-iodoso-benzoic acid cleavage. Another feature of the peptide TC-21 was that it contained two arginines, a fact with means that one arginine is associated with a trypsin resistant bond. This resistant bond was later identified as the Arg-Pro bond at residues 407 and 408.

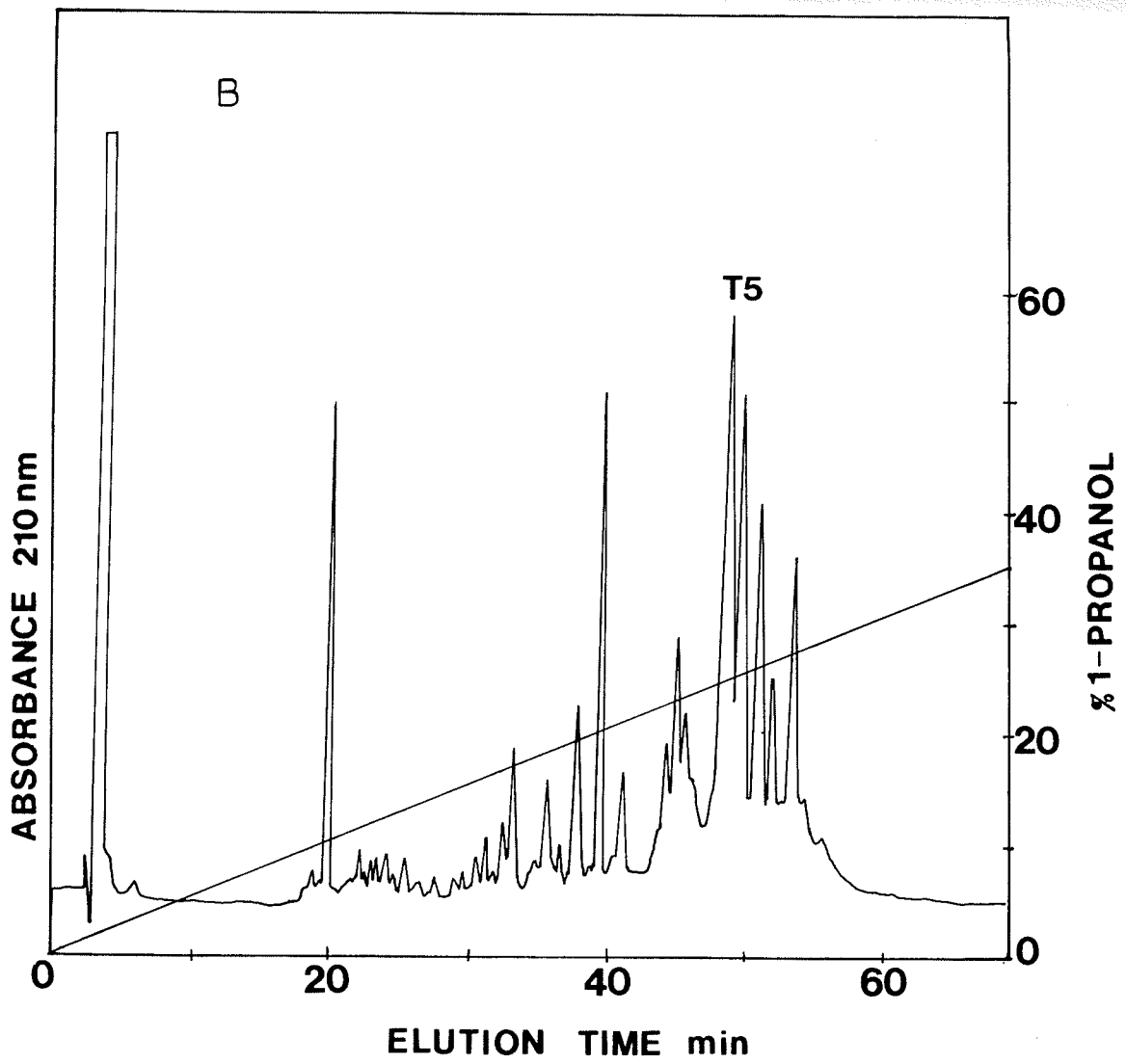
(viii) Subdigestion of arginine peptides:

Three arginine peptides (TC-1, TC-2 and TC-20) were decitraconylated and digested with trypsin in order to determine their complete amino acid sequences. TC-1 digest, expected to contain peptides TC1-T1, TC1-T2, TC1-T3 and TC1-T4, was resolved into two peaks by reverse phase HPLC using the propanol-2 system (Figure 20A). The first peak had the composition of peptide TC1-T3 and the second peak turned out to be the peptide TC1-T4. The sequence of the second peak was Gly-Thr-Leu-Gly-Gln-Asp-Val-Ile-Asp-Val-Ile-Asp-Ile-Arg. This was obviously the C-

Figure:20 A:Reverse phase HPLC of the tryptic digest of TC-1 (82 nmol). The digest was dissolved in 400 µL of 0.1% TFA and 100 µL was applied to a Perkin Elmer reverse phase C8 column (4 mm X 250 mm) equilibrated with 0.1% TFA. Peptides were eluted using 2-propanol system (see Methods). The rate of increase of the gradient was 60% aqueous 2-propanol per hour and the flow rate was 0.8 mL/min. The profile show A_{210} (full scale = 0.64 A). The peaks 'a' and 'b' were found to contain peptide TC1-T3 and TC1-T4 respectively.

B:Reverse phase HPLC of the tryptic digest of TC-20 (54 nmol). The digest was dissolved in 250 µL of 6 M Gdn-HCl and 100 µL was applied to a Perkin Elmer reverse phase C8 column (4 mm X 250 mm) equilibrated with 0.1% TFA. Peptides were eluted using 1-propanol system. The rate of increase of the gradient was 30% aqueous 1-propanol per hour and the flow rate was 0.8 mL/min. The profile shows A_{210} (full scale = 1.28 A). Subsequent analyses indicated that the peak T5 contained the C-terminal peptide of TC-20.


A
a
b
ABSORBANCE 210 nm
%2-PROPANOL
60
0
0
20
40
60
ELUTION TIME min


B
T5
ABSORBANCE 210 nm
% 1-PROPANOL
0
20
40
60
ELUTION TIME min

terminal peptide in TC-1. The other two peptides TC1-T1 and TC1-T2 being very short were not isolated.

In the case of TC-2, the tryptic digest containing the three peptides TC2-T1, TC2-T2 and TC2-T3, was subjected to sequence analysis without prior separation on HPLC. For the first four cycles, the three expected residues appeared in each cycle, but hereafter the peptide TC2-T1 sequence disappeared. Of the other two residues appearing in each cycle one was from the known sequence of TC2-T2 and the other one by difference yielded the sequence of TC2-T3 through its carboxy terminal arginine.

The tryptic digest of TC-20 was resolved into five peaks by reverse phase HPLC using the propanol-1 system as shown in Figure 20B Of these, the third peak, designated TC20-T5, proved to be the C-terminal peptide of TC-20 and provided the sequence of 17 residues, 371 to 387.

(ix) Summary

Of the 23 peptides expected from the complete sequence, 19 are listed in Table 6 , where the compositions, recoveries and sequencing of these peptides are given. It is obvious from the Table that the composition data are in good agreement with the sequencing data in most of the cases. The recovery of each peptide was calculated from the one step HPLC purification, except for TC-7 for which the overall yield through the large scale purification is given. From the remaining four TC peptides not listed two were free arginine and two (TC-12 and TC-8) were not isolated.

Table 6 Compositions and Sequencing of Peptides from Tryptic Digestion of Citraconylated Citrate Synthase.

Peptide & Residues Covered	TC-1[a] (1-32)	TC-2 (33-69)	TC-3 (70-109)	TC-4 (110-119)	TC-5 (120-125)	TC-7 (127-155)	TC-9 (158-163)	TC-10 (164-188)	TC-11 (189-217)	TC-13 (222-239)	TC-14 (240-289)	TC-16 (291-299)	TC-17 (300-306)	TC-18 (307-314)	TC-19 (315-319)	TC-20 (320-387)	TC-21 (388-409)	TC-22 (410-419)	TC-23 (420-426)
Asp	6.1(6)	3.4(3)	4.9(5)			5.1(5)		1.0(0)	4.9(5)	2.0(2)	2.2(2)	3.1(3)		2.1(2)		7.0(7)	1.3(1)		1.8(2)
Thr	4.4(4)	5.1(5)	4.5(5)	2.0(2)		0.9(1)		1.5(1)	1.4(1)	2.0(2)	0.9(1)				1.3(1)	3.0(3)	1.0(1)	0.7(1)	
Ser		3.6(4)	1.3(1)			1.6(2)		1.9(2)	1.8(2)	1.9(2)	4.2(5)	0.9(1)				3.4(3)	2.0(2)		0.8(1)
Glu	2.3(2)	2.4(2)	6.6(7)	2.4(2)		0.4(0)	1.3(1)	1.5(1)	3.2(3)	2.0(2)	4.0(4)					6.4(7)	1.3(1)	1.7(2)	0.3(0)
Pro		1.3(1)	1.8(2)			2.1(2)		2.8(3)	2.6(3)		3.3(3)			0.9(1)		3.8(3)	1.2(1)		
Gly	3.3(3)	4.8(5)	2.6(2)			1.8(2)		1.5(1)	1.7(1)	0.3(0)	6.0(6)		2.0(2)			3.2(3)	2.0(2)	1.0(1)	
Ala	3.3(3)	1.6(1)	2.1(1)	0.7(0)	1.0(1)	3.8(4)	2.0(2)	2.2(2)	1.9(1)	2.3(2)	9.5(10)	1.4(1)			1.4(1)	5.2(5)	1.9(2)		
Val	2.7(3)	1.7(1)	1.7(2)			1.8(2)		1.1(1)	1.3(1)	1.1(1)	2.1(1)			0.9(1)	1.1(1)	3.8(4)	1.1(1)		
Met		0.4(0)		0.6(1)		1.8(2)		2.2(3)	1.5(2)		1.3(1)		1.0(1)		0.9(1)	4.0(4)	1.7(2)		
Iso	1.6(2)	2.9(3)	1.8(2)	1.7(2)		1.5(1)	1.1(1)	1.3(1)	1.5(1)	2.1(2)	4.2(4)		0.4(0)			5.5(6)	1.9(2)		1.0(1)
Leu	4.9(5)	3.0(3)	4.5(4)		1.0(1)	2.4(2)		2.1(2)	2.8(3)	2.1(2)	3.3(3)		1.1(1)			7.0(8)		1.1(1)	
Tyr			2.1(3)			0.8(1)		2.5(3)	1.7(2)					1.8(2)		3.1(3)		1.6(2)	
Phe		3.9(4)	1.6(2)		1.9(2)	0.9(1)	0.8(1)	1.2(1)	2.0(2)		2.3(3)	0.8(1)	1.1(1)			3.0(4)			1.1(1)
His	0.2(0)	0.8(1)	2.4(2)	1.6(2)	1.1(1)	1.7(2)				1.7(2)	1.8(2)		1.0(1)			1.3(1)	1.6(2)		
Lys	3.2(3)	2.0(2)	2.3(2)					1.8(2)		0.3(0)	1.8(2)	1.9(2)		0.9(1)		4.1(5)	1.1(1)	1.1(1)	1.7(2)
Arg	1.0(1)	0.9(1)	1.0(1)	1.0(1)	1.0(1)	0.9(1)	0.8(1)	1.0(1)	1.0(1)	1.0(1)	1.0(1)	1.3(1)	1.2(1)	1.0(1)	1.0(1)	1.2(1)	1.5(1)	1.0(1)	1.0(1)
Cys		1.0(1)	+[b](1)			+ (1)		+ (1)	+ (1)		+ (1)					+ (1)			
Yield[c], %	68	85	54	90	36	17[c]	86	56	60	75	63	39	73	90	59	45	86	58	99
Number of Residues Sequenced	32	37	24	9	not sequenced	25	6	25	28	18	45	9	7	8	5	42[d]	14	9	8
Unsequenced Residues	-	-	94-109	119	120-125	146,150 154,155	-	-	217	-	283, 286-289	-	-	-	-	345-370	400, 403-409	-	-

[a] Numbers of residues found by amino acid analysis. In brackets are numbers expected from the sequence (Figure 8).
[b] Present but not quantitated.
[c] Calculated from the one-step HPLC purification, except for TC-7, for which the overall yield through the large-scale purification is given (see the text)
[d] Residues 371-387 were obtained by sequencing of the C-terminal tryptic peptide derived from TC-20. See the text.

Cyanogen Bromide (CN) Peptides:

In view of the dramatic success achieved in the purification of TC peptides by reverse phase HPLC in a single step, it was decided to use a similar technique for the purification of methionine peptides. The cleavage product obtained as a result of CNBr treatment of 0.44 μmoles of (^{14}C)-carboxymethylated citrate synthase, was loaded directly on C-8 reverse phase HPLC column. The peptides were eluted as already described for TC peptides. The elution profile is shown in Figure 21A, where each peak is labelled by arabic numerals which identify the peptide(s) found in it. In the following section the peaks are described in the order in which they emerged from the column.

CN-15

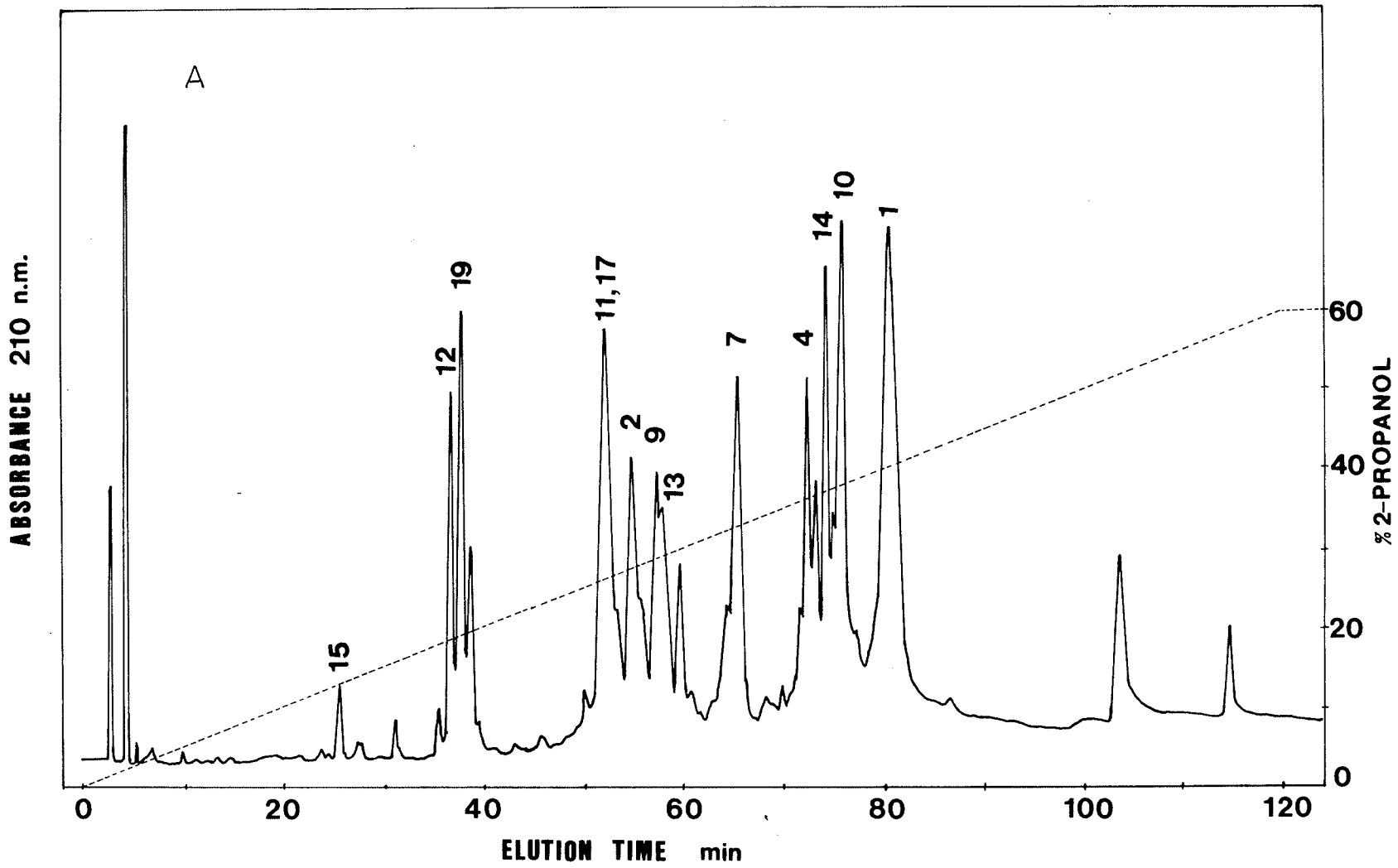
This peptide appeared first in the elution profile, and supposedly contained minimum numbers of hydrophobic residues. The sequence of this short peptide confirmed the residues 373 through 377 which had already been established within the TC-20 sequence.

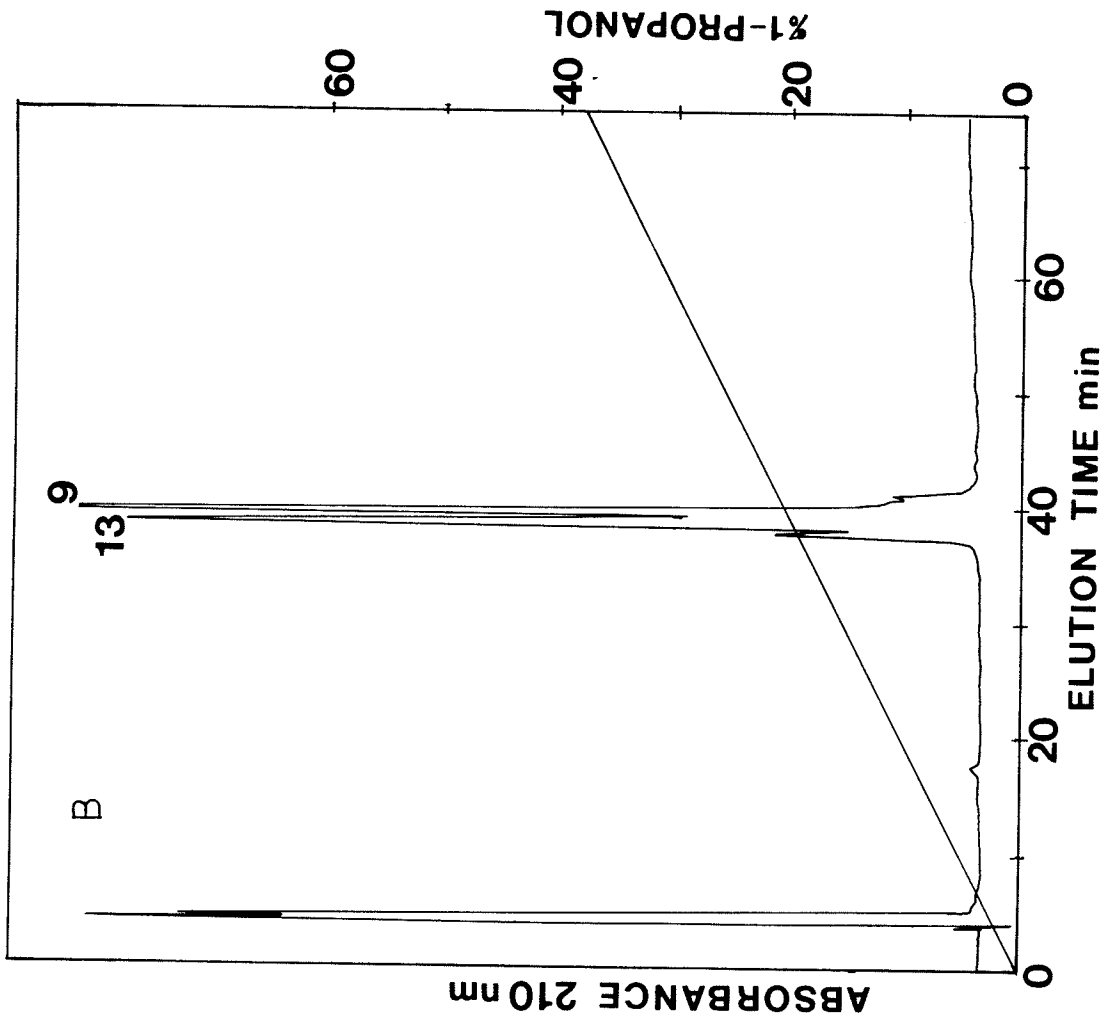
CN-12

Ten cycles of automated Edman degradation on this peak provided the sequence of residues 302 to 311. A contaminating sequence also appeared, which was present only in the first five cycles; it proved to be the C-terminus of CN-12 and accounted for residues 313 to 317. This observation indicated that the peptide CN-12 was hydrolyzed at Asp-Pro an acid sensitive bond. The combined results of the sequences yielded overlaps between arginine peptides TC-17, TC-18 and TC-19. In addition these overlaps were

Figure 21A Reverse phase HPLC of the CNBr cleavage products from S-(^{14}C)-carboxymethylated citrate synthase. The mixture (440 nmoles) was dissolved in 1.0 mL of 6 M Gdn-HCl and 200 µL was applied to a Perkin Elmer C8 column (4 x 250 mm) equilibrated with 0.1% TFA. Peptides were eluted with a linear gradient between equilibration solvent and 60% aqueous propanol-2 containing 0.1% TFA, over a period of 120 min, flow rate 0.8 mL/min. The profile shows A_{210} (full scale= 2.56). The number above the peaks refer to the CN peptides subsequently identified in these peaks

B Reverse phase HPLC of the CN-9 and CN-13 mixture under the same conditions as stated above except that the propanol-1 gradient system was used instead of propanol-2.


A
ABSORBANCE 210 n.m.
%2-PROPANOL
ELUTION TIME min
0
20
40
60
80
100
120
0
20
40
60
15
12
19
11,17
2
9
13
7
4
14
10
1


%1-PROPANOL
60
40
20
0
ABSORBANCE 210 nm
0
20
40
60
ELUTION TIME min
B
9
13

confirmed by the sequence of a subtilisin generated fragment (Bell et al. 1983).

CN-19

The fraction turned out to be the peptide from the carboxy terminus of citrate synthase. The sequence of its 22 residues, established the order of residues 404 to 425 and provided overlaps between TC-21, TC-22 and TC-23. A single trypsin resistant Arg-Pro bond was also identified from this sequence.

CN-11 and CN-17

This peak contained two unresolved peptides, CN-11 and CN-17. An attempt to resolve these by rechromatography on reverse phase HPLC column using the propanol-1 system, was unsuccessful. Therefore, these peptides were sequenced together; rapid wash out of CN-17 after the removal of arginine at cycle 2 led to a de facto pure sequence for CN-11 after a few cycles. Sequence of 19 residues (275-293) of CN-11 yielded overlaps between peptides TC-14, TC-15 and TC-16; and the CN-17 sequence provided a minimum overlap of two residues between TC-20 and TC-21.

CN-2

This peak contained the single peptide CN-2. Automated Edman degradation allowed the placement of 16 residues from positions 113 to 128. Ordering of peptides TC-4, TC-5, TC-6 and TC-7 was established from this sequence. It should be noted that the above sequence of CN-2 contains the sequence of TC-5 in itself. Therefore, the arginine peptide TC-5, although isolated in pure state late in the study, was not subjected to sequencing.

CN-9 and CN-13

These two poorly resolved peaks were collected as a single fraction in the propanol-2 system. In order to fractionate these, the mixture was rechromatographed on the same reverse phase HPLC column, but this time using the propanol-1 system. The mixture was resolved into two peaks as shown in Figure 21B. The first peak proved to be CN-13 and sequence determination of its 15 residues provided an overlap of one arginine amino acid between TC-19 and TC-20. However, this overlap is better defined from the sequence of one subtilisin fragment (Bell et al., 1983).

The second peak contained a single peptide CN-9. Fifteen cycles of automated Edman degradation provided the sequence of residues 202 to 216. Although these results do not provide any overlap, the sequence confirms the residues at the end of TC-11. In order to extend the sequence further, Arg-217 was tentatively placed from the composition analysis of TC-11, and Ala-218 and Met-219 were tentatively assigned their respective positions from the composition of CN-9. Since peptide TC-12 was not isolated the overlap in this region was inferred from the DNA sequence (Ner et al.1983).

CN-7

This peak, containing a single peptide CN-7 provided the sequence of residues 175 through 194, and therefore established the order of TC-10 and TC-11.

CN-4

This peak contained peptide CN-4 and the sequence of its 34 amino acids

established residues 135 to 167. These results unequivocally proved the placement of peptides TC-7, TC-8, TC-9 and TC-10.

CN-14

Automated Edman degradation on this peak showed that it contained peptide CN-14, commencing at residue 341. The sequence of 26 residues was determined and the results were used to extend the peptide TC-20 sequence through residue 366. The sequence was further extended to residue 373 by the sequence information available from the subtilisin minor fragment (Bell et al., 1983). This in turn provided overlap sequence to the tryptic peptide derived from the C-terminus of TC-20, thus establishing the sequence through residue 387.

CN-10

This peak contained a peptide, which was 55 residues long. When subjected to sequencing, residues 220 through 246 were placed. This sequence provided the C-terminal part of TC-12 and also the overlap between TC-13 and TC-14.

CN-1

This peak contained the amino terminal peptide, CN-1. Since this was a large peptide (112 residues) and since its amino terminal sequence was available from sequenator runs on peptide TC-1 and intact protein, CN-1 was sequenced for only six cycles to confirm its identity. The sequence of 94 residues was built up from the peptides TC-1, TC-2 and TC-3, which were placed in order by the DNA base sequence (Ner et al., 1983). In order to establish the sequence of the carboxy-terminal part, attempts were made to cleave

CN-1 at the Asn-Gly sequence, residues 91 and 92, with hydroxylamine. Although this reaction was successful when it was used on the intact protein, no cleavage of CN-1 was detected by reverse phase HPLC of the reaction mixture, in two attempts. Perhaps the acidic conditions used in the original cyanogen bromide cleavage, or some aspect of the subsequent peptide purification, had led to complete deamidation of Asn.

In another attempt to explore the C-terminal of CN-1, the peptide was digested with Lysobacter enzymogenes endoproteinase "Lys C", and the products were fractionated by reverse phase HPLC. Peptides having the compositions for the larger expected products, including residues 7-21, 22-37, 38-55 and 56-104 were readily identified, thus giving further evidence for the order of TC-1, TC-2 and TC-3. Since residues 56-104 were found in a single peptide, the enzyme did not apparently cleave the Lys-Pro bond at residues 94 and 95. Failure to isolate smaller Lys peptides covering the region 105-112, and failure to extend CN-1 sequence beyond residue-100, means that there is no proof from my work for a portion of sequence form 101 to 108.

Summary of CN peptides:

Of the 19 peptides expected from the sequence in Figure 8, 13 are listed in Table 7 , where compositions, recoveries and sequencing of these peptides are given. From the missing six, one is free homoserine and three are tripeptides which were not isolated. The remaining two

Table 7 : Compositions and Sequencing of Peptides from CNBr Cleavage of Citrate Synthase.

Peptide and Residues Covered	CN-1 (1-112)	CN-2 (113-131)	CN-4 (135-168)	CN-7 (175-200)	CN-9 (202-219)	CN-10 (220-274)	CN-12 (302-318)	CN-13 (319-340)	CN-14 (341-372)	CN-15 (373-378)	CN-19 (404-426)	CN-11(275-301) CN-17^{+}(386-398)
Aspartic Acid	12.3(14)	1.7(1)	3.4(4)	4.2(4)	1.3(1)	6.6(5)	2.1(2)	2.4(2)	6.0(5)		2.3(2)	3.9(3+1)[c]
Threonine	16.7(15)	1.6(1)	1.5(1)	0.5(0)	1.3(1)	3.0(3)	1.1(1)	2.0(2)	0.6(0)		1.3(1)	1.5(0+1)
Serine	5.2(5)	1.0(1)	2.0(2)	2.1(2)	0.9(1)	4.1(5)	0.3(0)		1.5(1)	1.9(2)	0.9(1)	3.3(3+1)
Glutamic Acid[b]	11.3(12)	3.0(3)	1.9(2)	1.7(2)	4.1(4)	4.8(4)	0.6(1)	4.8(5)	4.1(4)	1.1(1)	2.4(2)	5.3(4+2)
Proline	3.7(3)	0.5(1)	0.7(1)	2.5(2)	2.8(3)	2.5(2)	0.6(1)		1.7(2)	0.8(1)	0.5(1)	2.1(1+0)
Glycine	9.3(10)	0.7(0)	2.4(2)	2.3(2)	0.4(0)	5.9(6)	1.8(2)	1.2(1)	2.1(1)	1.3(1)	1.2(1)	1.2(0+1)
Alanine	7.7(5)	1.2(1)	5.0(5)	2.0(1)	1.2(1)	11.4(12)	0.9(1)	1.2(1)	3.6(2)		1.1(1)	3.0(1+2)
Valine	4.9(6)	1.1(0)	1.4(1)	1.2(1)	1.2(1)	1.6(1)	1.6(2)	1.9(2)	1.3(1)		0.5(0)	2.8(1+1)
Isoleucine	5.9(7)	1.4(2)	2.5(2)	1.1(1)	0.8(1)	4.0(4)			3.5(4)	0.8(1)	1.8(2)	3.2(2+1)
Leucine	10.8(12)	1.1(1)	3.1(4)	2.0(2)	1.2(1)	4.3(4)		3.5(4)	4.1(4)		0.8(1)	2.0(2+0)
Tyrosine	2.4(3)	0.2(0)	1.0(1)	2.7(4)	0.7(1)	1.0(0)	1.3(2)		2.7(3)		1.9(2)	0.2(0+0)
Phenylalanine	4.8(6)	1.3(2)	1.7(2)	1.6(2)	1.0(1)	1.7(1)	0.5(1)		2.0(2)		0.9(1)	1.8(3+0)
Histidine	2.3(2)	1.3(3)	1.1(2)			2.1(3)	0.4(1)	+ (1)			0.3(0)	1.6(1+1)
Lysine	5.7(7)	0.7(0)	1.2(1)	1.0(1)	0.4(0)	2.1(1)	1.2(1)	1.4(2)	2.6(3)	0.5(0)	3.0(4)	2.7(3+0)
Arginine	3.3(3)	2.6(3)	2.5(3)	1.1(1)	0.8(1)	2.0(2)	2.1(2)	0.9(1)	0.6(0)	0.6(0)	3.8(4)	3.7(3+1)
CM-Cysteine	+ (2)		+ (1)	0.7(1)	0.7(1)	0.7(1)	0.1(0)	0.7(1)	0.5(0)	0.5(0)		0.2(0+0)
Yield	75	75	70	107	36	48	63	27	27	35	72	89
Number of Residues Sequenced	6	15	32	20	15	27	10	15	25	5	22	18(CN-11) 11(CN-17)
Unsequenced Residues	7-112	128-131	166 168	195-200	217-219	247-274	312-318	334-340	365, 367-372	378	426	292,294-301 397-398

[a] Arrangement is as for Table 6

[b] Includes homoserine.

[c] As explained in the text, these two peptides were not resolved, and were sequenced together. In the bracket, the composition of CN-11 is given first, followed by CN-17.

peptides are of five (residues 399-403) and seven (residues 379-385) amino acids; they could not be located in the fractions from the HPLC run, but a six residue peptide, CN-15, was isolated in good yield (Table 7) although it is not clearly more hydrophobic than the two missing peptides. Other methods evidently would have been needed to ensure dependable isolation of all short peptides.

The sequence of these thirteen CN peptides established the overlap for 18 arginines. The overlaps at two places, Arg-217 and Arg-299, were difficult to obtain since these residues were in the C-terminal portions of CN peptides. The other three overlaps at Arg-32, Arg-69 and Arg-109 were present in CN-1 and the evidence for their presence is only given by composition analysis in the present work; however, as mentioned earlier the gene sequence information was used to establish these overlaps. The lengths of CNBr peptides sequenced and the ordering of TC peptides established by them, is represented in Figure 30.

Hydroxylamine Cleavage:

(^{14}C)- carboxymethylated citrate synthase (77.8 nmoles) was reacted with hydroxylamine by the method of Bornstein (1970). The cleavage products were then run in the sequenator, and two sequences were identified; the expected N-terminal sequence of the protein and one commencing with glycine at position 92, which suddenly disappeared after cycle 4. The yields of Ala and Gly in cycle 1 were 83.2 and 25.9 nmoles respectively, indicating a cleavage yield of about 33%.

In another attempt 468 nmoles of the hydroxylamine cleavage products from citrate synthase were fractionated on a Sephadex G-75 column (2.5 cm X 125 cm) in 0.1 M NH_4HCO_3 buffer containing 6 M Gdn-HCl. As shown in the elution profile, Figure 22A, the mixture was resolved into three peaks. The first large peak was pooled in six fractions (I to VI); the second in four fractions (VII to X) and the third in one fraction (XI). All these fractions were dialyzed against water and then analyzed for composition. The single fraction from the third peak (XI) did not contain any amino acids and therefore it was not analyzed further.

Five fractions from the first large peak were also analyzed by SDS-polyacrylamide gel electrophoresis (Figure 22B). Fraction I had one major band, perhaps the uncleaved citrate synthase. Fractions II and III mostly contained a fragment of apparent molecular weight

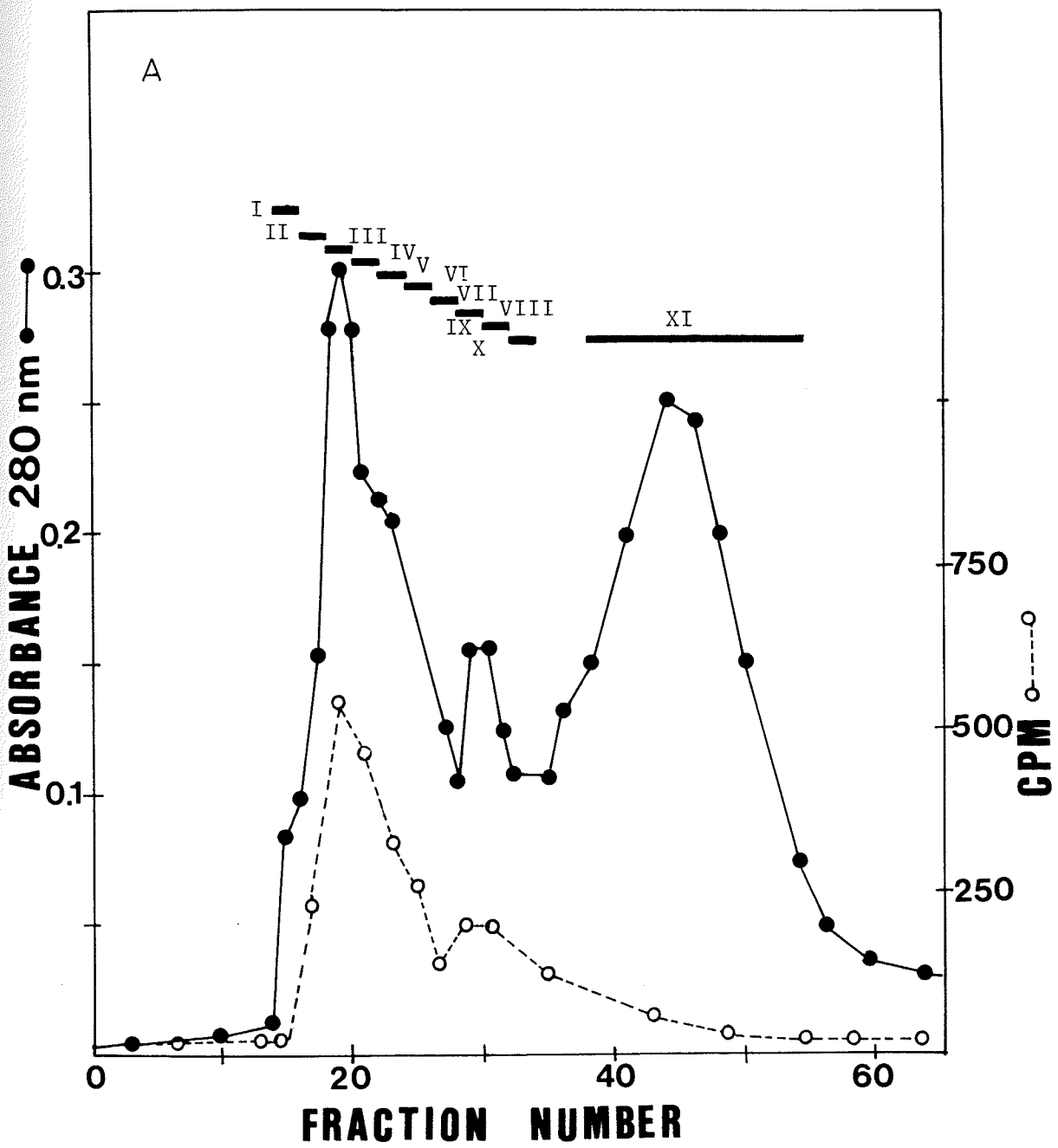
36,000 (36K), which was not present in the subsequent fractions. Fractions IV and V had low molecular weight cleavage products (14K and 10K). Other minor faint bands were also observed in later fractions (IV and V), perhaps arising from non-specific cleavage of citrate synthase. These results by themselves are not explained by the presence of single Asn-Gly bond as indicated from the sequence run on the previous batch. However when these fractions were subjected to N-terminal sequencing, most showed the amino terminal sequence of the intact E. coli citrate synthase; fractions II and III also contained a sequence from Gly-92 onwards and provided information up to Tyr-100.

The fractions from the smaller peak contained fragment 1-91, as identified from the composition analysis (not given). The N-terminal sequence of the intact protein was also observed over five cycles, when either of fractions V or VII were subjected to sequencing. These results indicate that the 14K or 10K band may be the fragment 1-91, which was present in the trailing portion of the large peak as a contamination from the short middle peak.

It should be noted that no evidence was found for cleavage between residues 10 and 11 of the protein, which are Asp-Gly according to this work but were predicted to be Asn-Gly by the gltA gene sequence of Ner et al. (1983).

Figure: 22 (A) Chromatography of the hydroxylamine cleavage products from S-carboxymethylated citrate synthase (468 nmol) on a Sephadex G-75 column (2.5 cm X 125 cm) in 0.1 M NH_4HCO_3 containing 6 M Gdn-HCl. The fractions of 10 mL were collected at a flow rate of 15 mL per hour, and were assayed by absorbance at 280 nm and by counting 50 µL samples for radioactivity. Solid bars represent the pooled fractions.

(B) SDS-polyacrylamide gel electrophoresis of pool I to V (Figure 22A). About 15% of each pool was dialyzed overnight against 4 L of water, freeze dried, and then prepared the samples for electrophoresis. The discontinuous buffer system of Laemmli (1970) was used. The resolving gel concentration was 10% and stacking gel 3.5% acrylamide. The Coomassie Blue stained protein/peptide band are shown in the photograph.


A
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
ABSORBANCE 280 nm
0.3
0.2
0.1
CPM
750
500
250
0
20
40
60
FRACTION NUMBER

NOTICE/AVIS

PAGE(S) 150 IS/ARE EST/SONT black & white photo

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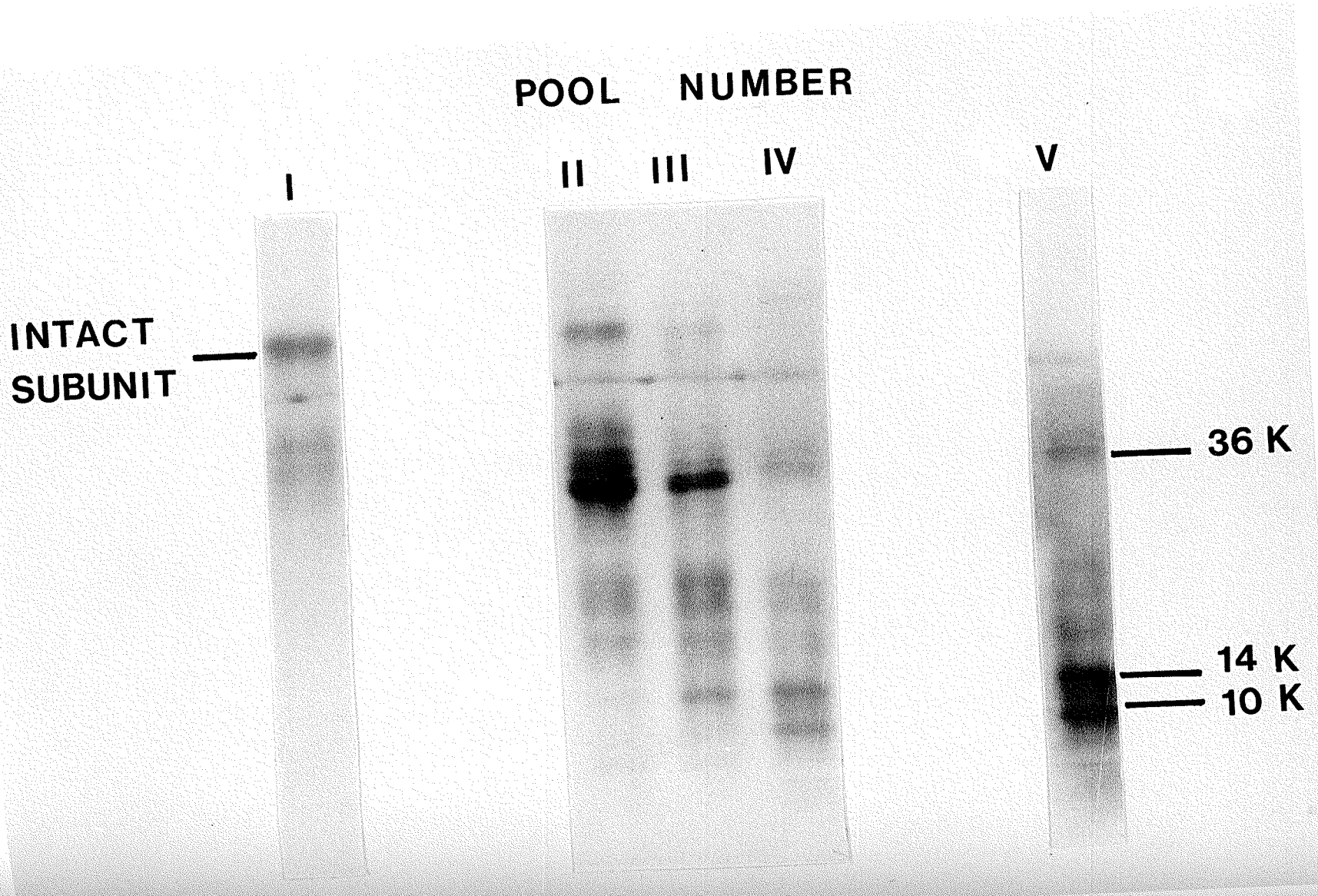
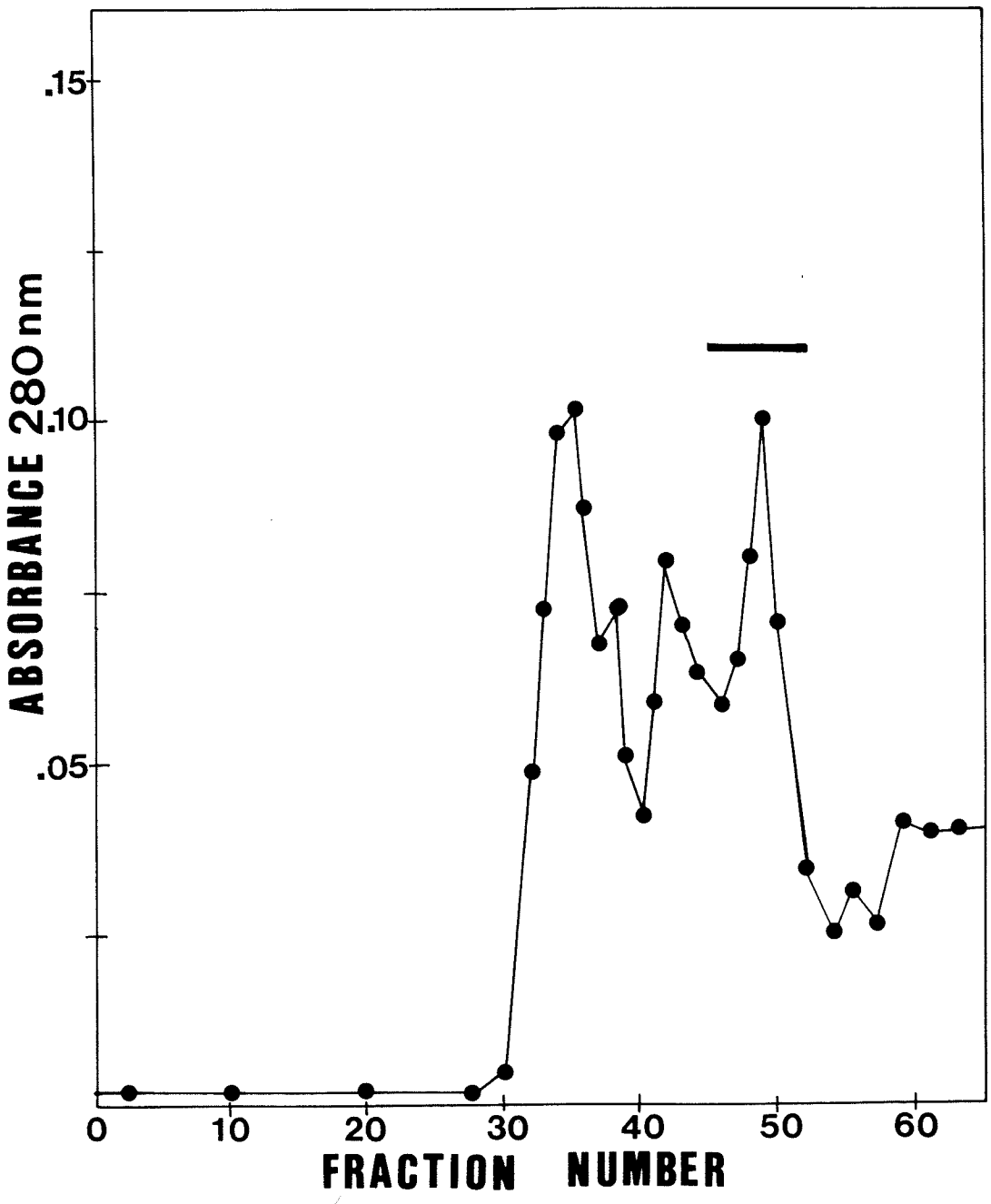
VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.


POOL NUMBER
I
II
III
IV
V
INTACT
SUBUNIT
36 K
14 K
10 K

Oxidative Cleavage at Tryptophan:

Since the sequence of TC-21 did not overlap with that of CN-19 and since the short peptide CN-18 was not isolated, further evidence was needed to establish the sequence between residues 398 and 403. The only fragment likely to yield the above sequence information was that arising from the cleavage at tryptophan (395). Accordingly, (^{14}C)-carboxymethylated citrate synthase (freeze dried, 40 mg) was reacted with o-iodosobenzoic acid and the cleavage products were fractionated on a Sephadex G-50 column (2.0 cm X 100 cm) in 9% formic acid. The column was run at room temperature and the fractions were analyzed by reading absorbance at 280 nm. The mixture was resolved into three major peaks as shown in Figure 23. Of these, the last one most likely containing the short peptides was rechromatographed by reverse phase HPLC, using the acetonitrile system (not shown). One of the two major peaks obtained from this fractionation was sequenced, and proved to be the peptide required, commencing with Ser-396. Sixteen cycles of automated Edman degradation were run, giving the expected sequence and confirming residues 396 to 411. An unusual PTH amino acid, found in cycle 3 and 8 of this degradation was identified as PTH-methionine sulfone by running standards. This conversion of methionine sulfone was expected from the oxidative conditions used to perform the cleavage.

Figure: 23 Fractionation of the Ortho-iodosobenzoic acid cleavage product of S-carboxymethylated citrate synthase (40 mg) on a Sephadex G-50 column (2.0 cm X 100 cm) in 9% formic acid. Collected 7 mL of fractions at a flow rate of 15 mL/min. Each fraction was analyzed for absorbance 280 nm. Solid bar represent the peak which was further fractionated by reverse phase HPLC on a C8 column.


.15
.10
.05
ABSORBANCE 280 nm
0
10
20
30
40
50
60
FRACTION NUMBER

End Group Analysis :

(i) Amino terminal sequence

The amino terminal sequence of E. coli citrate synthase was determined by automated Edman degradation on the intact protein. Sequencing was performed on approximately 1 mg of protein in the presence of 4 mg Polybrene. Phenylthiohydantoins were identified solely by reverse phase HPLC. Analysis of 30 cycles provided the sequence of 28 residues. The sequence was in complete agreement with the published amino terminal 20 residues (Duckworth and Bell, 1982) and it was confirmed by the complete amino acid sequence of peptide TC-1 (32 residues).

(ii) Carboxy terminal sequence

A sample of (^{14}C)-carboxymethylated citrate synthase, 106 nmoles, was digested with 1:100 (w/w) of both carboxypeptidase A and B. A time course study of the release of free amino acids is presented in Table 8. As shown, Arg (1.0 mole), Lys (0.7 mole) and Ile (0.2 mole) were rapidly released at 10 minutes, showing that ---Ile-Lys-ArgCOOH is the carboxy terminal sequence. The rate of appearance of other amino acids at 30 min, 60 min and 240 min gave a sequence (Table 8) which is in agreement with the carboxy terminal sequence obtained from fragments TC-23 and CB-19.

TABLE: 8

Amino acid released by Carboxypeptidase A plus B from 106 nmol S-carboxymethylated Citrate Synthase.[a]

Amino Acid	Number of nmol after Digestion Times			
	10 min	30 min	60 min	240 min
Arginine	117	122	164	240
Lysine	70	130	193	253
Isoleucine	17	57	70	81
Aspartic Acid	-	50	95	144
Serine	-	28	95	131
Phenylalanine	-	20	58	75

Sequence Deduced -(Lys, Arg, Asp, Phe, Lys)[b]Ser-Asp-Ile-Lys-Arg COOH

[a]The enzymatic reaction of carboxypeptidases A and B was stopped by adding 20% acetic acid and released amino acids were quantitated on amino acid analyzer.

[b]From the quantitative analysis the sequence of these residues is not deduced.

Sequence Analysis of Peptides : Examples of Results

Automated Edman degradation of peptides was performed in the Beckman 890C Sequencer, using 0.1 M Quadrol as the coupling buffer. A polyquaternary amine, Polybrene (4 mg) was always used to retain peptide in the Sequencer cup. Peptide samples were added in volumes 400 µL of 10% acetic acid or distilled water, and then dried along the side of the cup in a thin film by using a sample application subroutine program (see Methods). In most cases double coupling was performed at the first cycle.

In the initial stages of this work, the butyl chloride extract collected at the end of each cycle was analyzed in the amino acid analyzer after back hydrolysis in 6 M HCl containing 0.1% $SnCl_2$. The conditions of this hydrolysis have been shown to cause conversion of amino acid derivatives such that serine and CM-cysteine are converted completely to alanine, threonine completely to α-amino butyric acid and isoleucine partially to alloisoleucine. Tryptophan is converted to glycine and alanine; aspargine and glutamine are converted to aspartic acid and glutamic acid respectively as in all acid hydrolysis (Mendez and Lai, 1975).

To illustrate the sensitivity of the Sequencer, a representative example of peptide TC-23 sequence analysis is shown in Table 9 . In this case, the residues in each cycle were analyzed quantitatively by back hydrolysis and the relative recovery of any amino acids from cycle to cycle was calculated by normalizing to the internal standard

TABLE : 9

Automated sequence analysis of peptide TC-23.[a]

Cycle Number	Residues Identified [b]	GLC analysis	%Yield in nmoles
1.	Asx	Asp	114
2.	Phe	Phe	74
3.	Lys	-	93
4.	Ala [c]	Ser	63
5.	Asx	Asp	77
6.	Ile [c]	Ile	28
7.	Lys	-	47
8.	Arg	-	19

[a] Peptide TC-23 is sequenced using 0.1 M Quadrol program (Beckman Cat. #030176) in 890 C Beckman Sequencer. The yield of each residue is corrected according to the recovery of Nle, an internal standard.

[b] Residues are identified by amino acid analyzer after back hydrolysis in 6 M HCl containing 0.1% $SnCl_2$.

[c] Under the hydrolysis conditions, serine is completely converted to alanine and isoleucine partially to alloisoleucine. The yield for isoleucine represents the sum of isoleucine and alloisoleucine.

Figure: 24 Sequence determination of peptide TC-23. The recoveries of an individual amino acid over the total number of cycles run is shown. The results of the sequencing are also given in Table 9. Alanine in cycle 4 was identified as serine by gas chromatography. Isoleucine in cycle 6 represents the sum of isoleucine and allo-isoleucine. Cycle 1 and 5 were identified as Aspartic acid by gas chromatography (see Methods). The inferred sequence is : Asp-Phe-Lys-Ser-Asp-Ile-Lys-Arg.

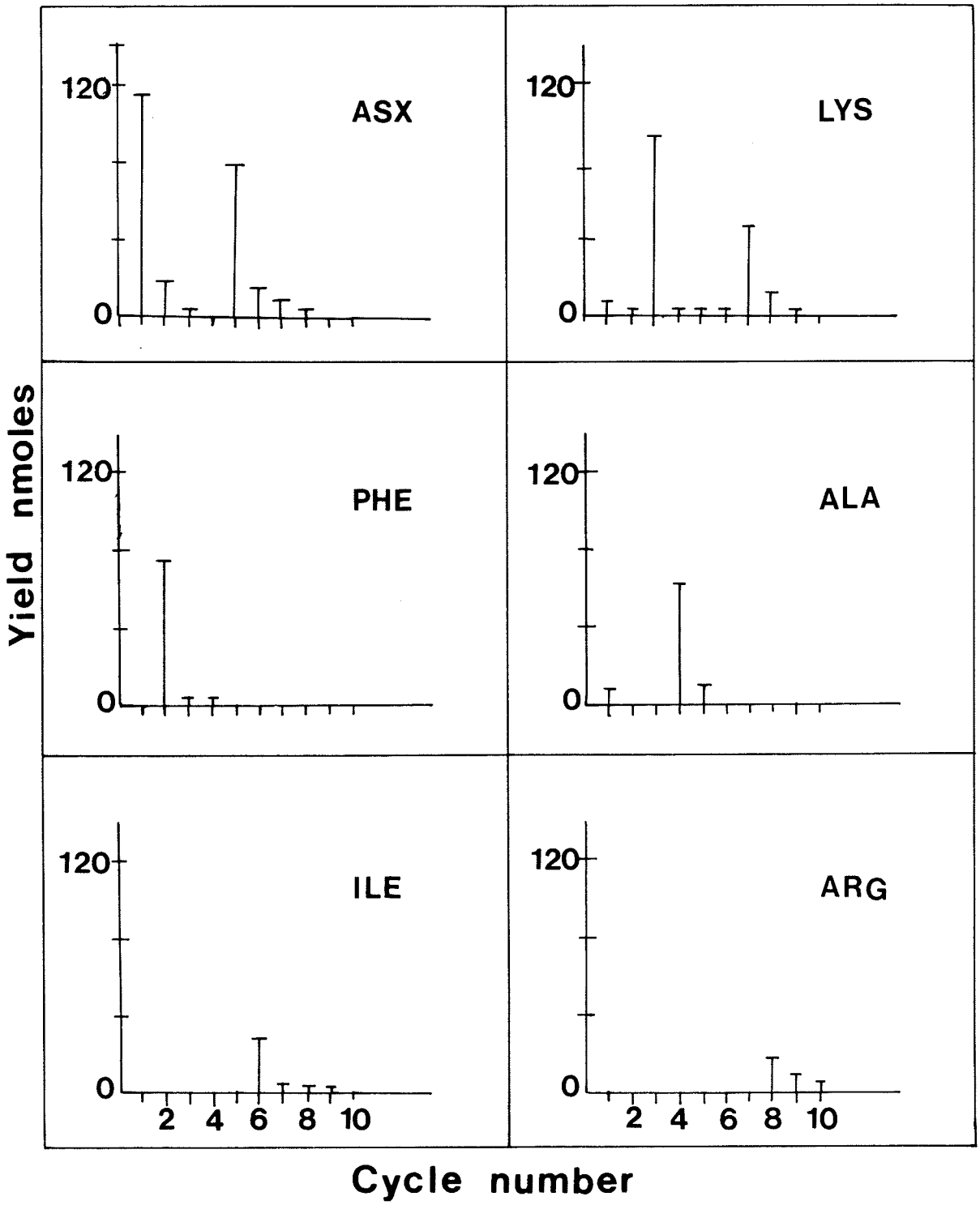
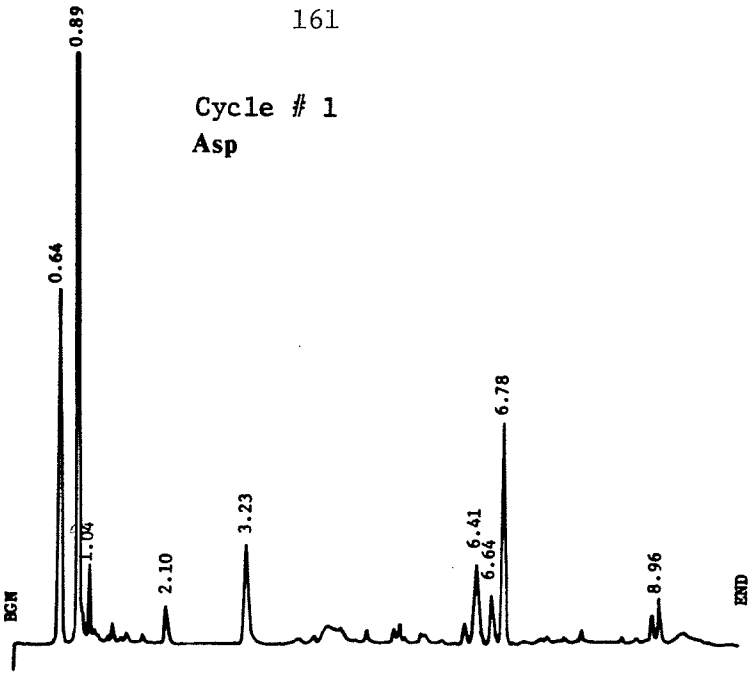
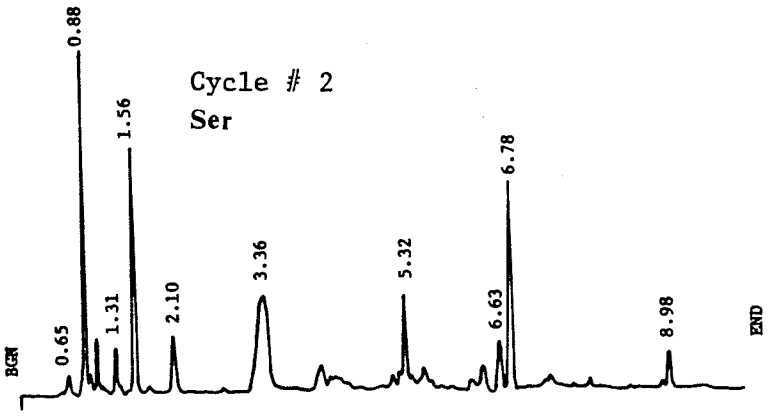

ASX
LYS
PHE
ALA
ILE
ARG
120
0
2 4 6 8 10
Yield nmoles
Cycle number

Figure:25 Sequence determination of peptide TC-7. PTH amino acids were identified by reverse phase HPLC on a C18 column according to the method of Skiados (1981). Dried samples of PTH amino acid were dissolved in 100 µL methanol and injected 6 µL through an injector loop. In most cases, PTH's of Asn and Gln were converted partially to their acid forms, the yields of serine and threonine were low due to their unstability. Twenty nmoles of PTH-norleucine were used as the internal standard. The standard (Standard B from Pierce) represents 0.2 µ moles/mL of each amino acid. The profiles show A_{250} (full scale 0.16 A).

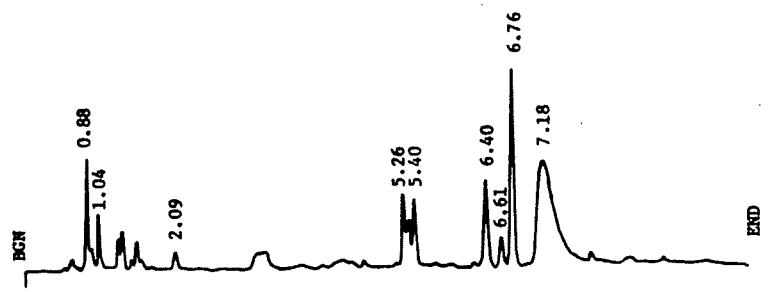
Cycle # 1
Asp



Cycle # 2
Ser

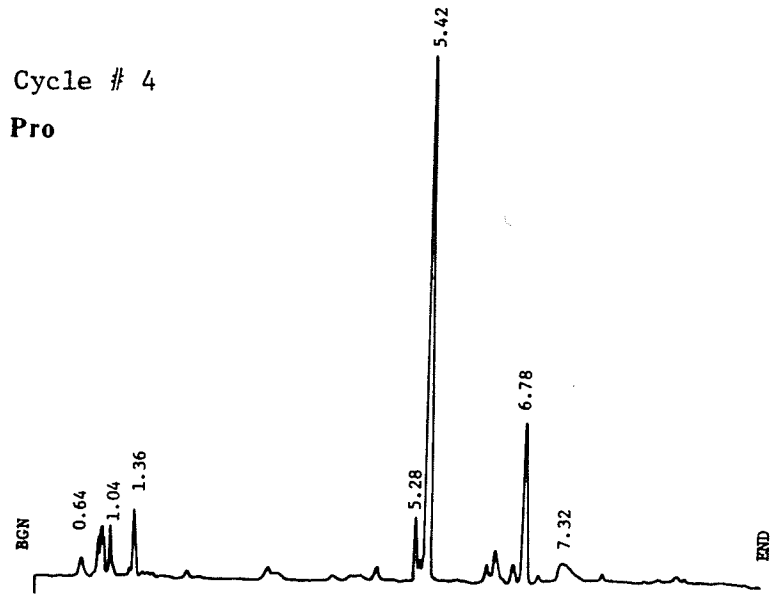


Cycle # 3
His



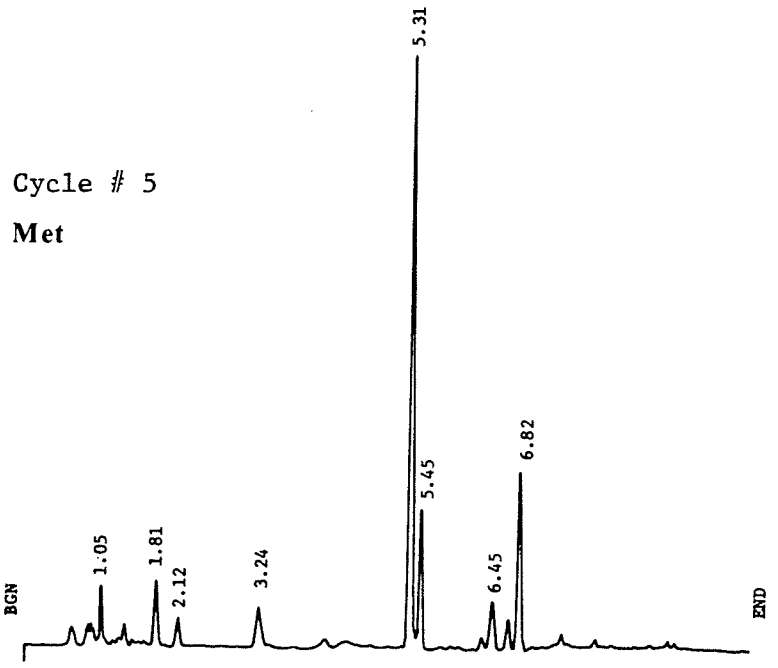
Cycle # 4

Pro



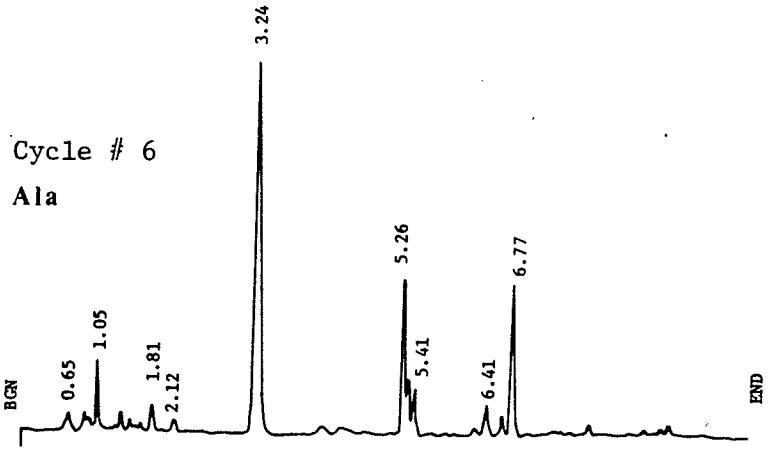
Cycle # 5

Met



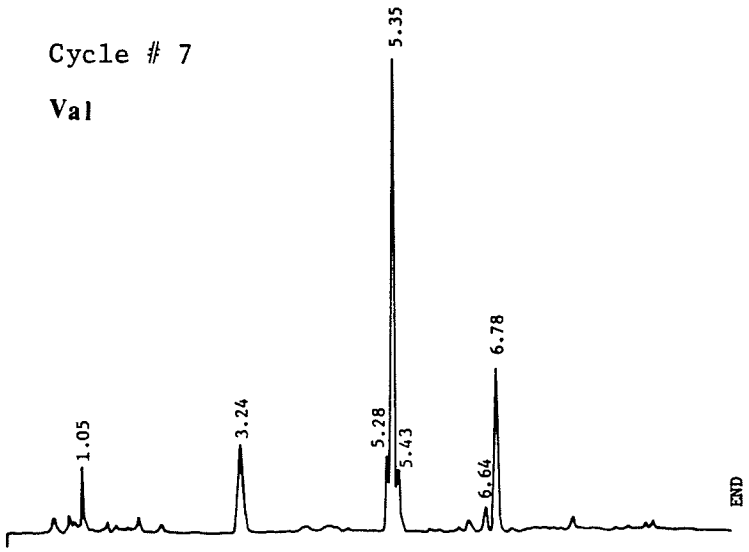
Cycle # 6

Ala



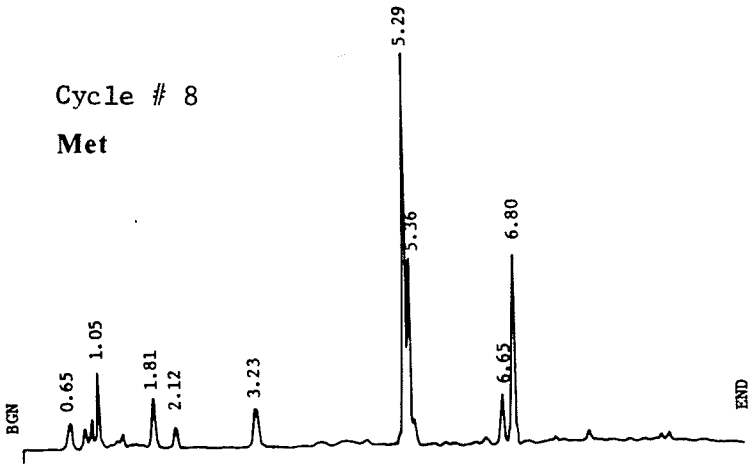
Cycle # 7

Val



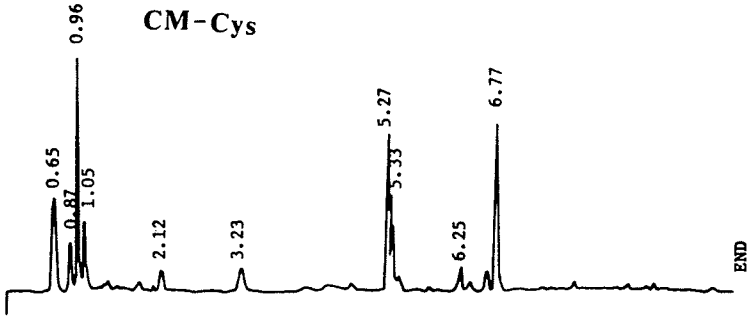
Cycle # 8

Met

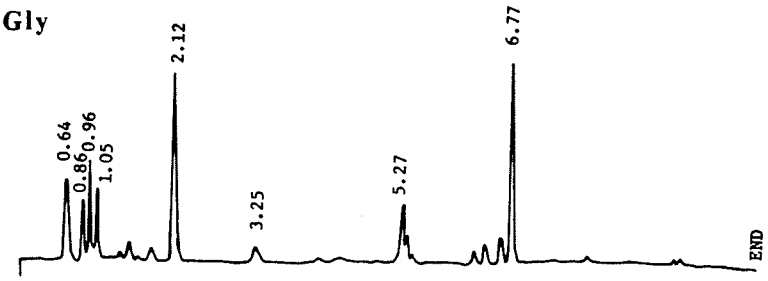


Cycle # 9

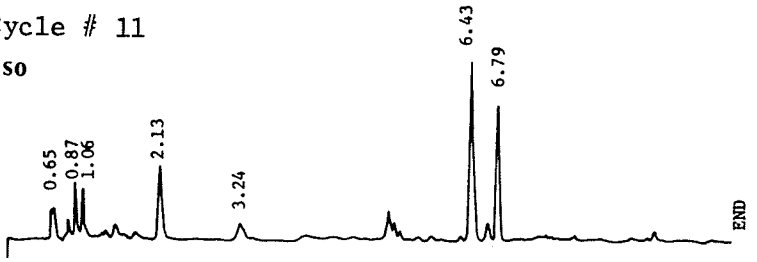
CM-Cys



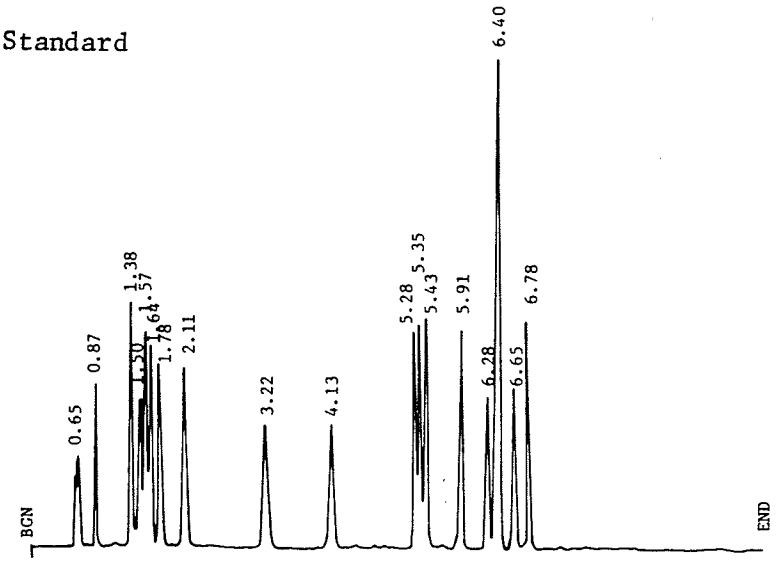
Cycle # 10
Gly



Cycle # 11
Iso



Standard



PTH-norleucine. Figure 24 shows the results of sequencing with respect to recoveries of an individual amino acid as a function of cycles run. Aspartate at cycle 1 and cycle 5, and serine at cycle 4 were identified by gas chromatography (see Methods for details). Initial coupling was calculated to be 55.6% and the repetitive yield was 90.7% when calculated for aspartate at cycle 1 and 5.

Another method of identification of phenylthiohydantoins was by reverse phase HPLC using C-18 column. This method was employed for most of the sequencing data presented in this work. In this case, the identifications at individual cycles were said to be proven, if a single phenylthiohydantoin appearing in a given cycle clearly rose above the background of the previous cycle and dropped in the next cycle. Sometimes, the accumulated carryover during the Edman degradation led to high background values of the other amino acids and made identification difficult. Under such circumstances, the collection of the data was discontinued.

A representative example of the sequence data assigned by only HPLC is shown for peptide TC-7 in Figure 25. These observations were regarded as semiquantitative in nature, therefore yields of PTH's are not documented.

Purification and Characterization of Citrate Synthases

(i) Escherichia coli citrate synthase :

The purification scheme for E. coli citrate synthase (strain # CGSC 259) is shown in Table 2 . It is observed that the purified preparation has a specific activity of 53.1 U/mg which is consistent with the values reported earlier from our laboratory, 30-50 U/mg (Tong & Duckworth, 1975; Duckworth & Bell, 1982). Specific activities as high as 135-160 U/mg have been reported from other laboratories (Faloona & Srere, 1969, Wright & Sanwal, 1971), but these high values have been attributed to the different assay conditions and differences in the buffers in which the enzyme was stored (Tong & Duckworth, 1975). Above all, it has been observed that long time incubation of the enzyme in buffer containing 1 M KCl results in 2-3 fold increase in specific activity, irrespective of whether the enzyme is stored in the cold or at room temperature (Tong and Duckworth, 1975).

The purified preparation of citrate synthase from pHS gltA transformed E. coli HB101 cells, has a specific activity of 88.8 U/mg (Table 3) which is considerably higher than that of the chromosomal enzyme preparation. The reason for the higher specific activity of the plasmid preparation is probably the relative ease of carrying large amounts of enzyme through various purification steps with little loss of activity, rather than intrinsic differences between the enzymes (Duckworth & Bell, 1982). Evidence has been presented indicating that the chromosomal and

plasmid enzyme preparations contain the same citrate synthase (Duckworth & Bell, 1982).

The purity of the enzyme for both plasmid and chromosomal preparations of citrate synthase was determined by SDS-polyacrylamide gel electrophoresis. As may be seen in Figure 26, the chromosomal preparation shows one major protein band and a few low molecular weight protein bands which in total do not seem to exceed 10% of the major band. The plasmid-borne enzyme migrates as a single major protein band and contaminating bands are more or less absent from this preparation. The purified preparation was subjected to amino acid analysis after HCl hydrolysis for 24, 48 and 72 h , and also to N-terminal sequence determination. With 1 mg of citrate synthase (in the presence of 4 mg Polybrene, using the 0.1 M Quadrol Sequenator program) a single amino terminal sequence of 28 residues was obtained. This also indicated that the enzyme contains a single type of subunit. The enzyme preparation was 95% inhibited at pH 7.8 by a saturating level of NADH and the concentration of NADH needed to achieve half maximal inhibition was 1.7 μM. KCl (0.1 M) activated the enzyme activity 40 fold in the standard assay mixture. These kinetic results are characteristic of E. coli citrate synthase and are useful for identification (Faloona & Srere, 1969; Tong & Duckworth, 1975; Morse & Duckworth, 1980; Duckworth & Bell, 1982). Both chromosomal and plasmid preparations of the enzyme were used for the elucidation of the complete amino acid sequence.

Figure: 26 SDS-polyacrylamide gel electrophoresis of various citrate synthases. The electrophoresis is performed according to the method of Laemmli (1970) on a 10% polyacrylamide slab gel: samples were prepared in the presence of 1% SDS plus 1% 2-merceptoethanol. Photograph shows Coomassie Blue stained gel of 20 µg samples of each enzyme except P. aeruginosa citrate synthase for which 2 µg was used. The apparent molecular weight is found to be 47,000 g/mol from standard plot as recommended by Weber and Osborn (1967) using marker proteins: Bovine serum albumin 67,000 g/mol; Catalase 60,000 g/mol; Ovalbumin 43,000 g/mol; Glyceraldehyde -3-phosphate dehydrogenase 36,000 g/mol; Lysozyme 14,400 g/mol.

NOTICE/AVIS

PAGE(S) 169 IS/ARE EST/SONT black & white photo

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VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.

Citrate synthases

Pigeon breast muscle

E.coli chromosomal

A anitratum

E.coli plasmid

Pig heart

P. aeruginosa

(ii) Acinetobacter anitratum citrate synthase:

Citrate synthase purified from A. anitratum had a specific activity of 62.5 U/mg as shown in Table 4. The purification factor, 46.7 fold and 35% recovery of the enzyme, are comparable with the values obtained by Morse and Duckworth (1980).

Johnson and Hanson (1974) described a procedure for obtaining a highly purified preparation of A. anitratum citrate synthase, but their enzyme preparation was completely insensitive to allosteric inhibitor NADH. In contrast, studies of Weitzman and Danson (1976) showed that the enzyme is inhibited by NADH and reactivated by AMP. They claimed that the enzyme preparation of Johnson and Hanson was desensitized to NADH inhibition, probably because of harsh treatments during purification. The procedure of Morse and Duckworth, which I used, provided citrate synthase which showed both NADH inhibition and AMP reactivation.

SDS-polyacrylamide gel electrophoresis (Figure 26) revealed that the major band in the preparation had the same mobility as E. coli citrate synthase, indicating that the subunits are equivalent in size with a molecular weight of about 47,000 g/mole. A minor protein band (molecular weight approx. 80,000) could be clearly seen in the same track which was perhaps a dimer of the main species, not completely denatured under the conditions used. Amino acid comosition is given in Table 10.

(iii) Effect of pH on NADH inhibition of A. anitratum citrate synthase:

Morse and Duckworth (1980) have demonstrated that 89% of the activity

TABLE 10

Amino acid composition of A. anitratum citrate synthase.

Amino Acid	Average or Extrapolated Values (nmol)	Moles per 47,000 g	Nearest integer
Asx D & N	376.4	42.8	43
Thr T	217.0[a]	24.7	25
Ser S	167.0[a]	19.0	19
Glx E & Q	353.1	40.2	40
Pro P	204.7	23.3	23
Gly G	277.4	31.4	31
Ala A	385.7	43.9	44
Val V	244.3	27.8	28
Met M	89.0	10.1	10
Ile I	223.2[b]	25.4	25
Leu L	317.8	36.1	36
Tyr Y	148.8[c]	16.9	17
Phe F	154.5	17.6	18
His H	105.5	12.0	12
Lys K	210.6	23.9	24
Arg R	178.0	20.3	20
Trp W	47.2[c]	5.4	5
1/2-Cys C	25.8[d]	4.9	5
Total			425

[a]First-order extrapolation to zero hydrolysis time. [b]Values for 72 h hydrolysis taken. [c]Method of Edelhoch (1967). [d]As cysteic acid after performic acid oxidation.

of A. anitratum citrate synthase is inhibited at a saturating level of NADH, at pH 7.8. The saturation curve is sigmoidal in shape for the allosteric inhibitor NADH, and hyperbolic for the substrate acetyl-CoA. The results from steady state kinetics and inhibition studies have been interpreted in terms of the Monod et al.(1965) model, assuming that the resting enzyme is in R or active state (Morse and Duckworth, 1980). On the other hand, many of the kinetic properties of E. coli citrate synthase can be explained by assuming that the resting enzyme is in T or inactive state. For E. coli citrate synthase NADH inhibition is hyperbolic and has been shown to be pH dependent; the inhibition is strongest at pH values below 7.5 and falls off rapidly as the pH is raised to 9.0 (Weitzman, 1966 b). From NADH binding studies, Duckworth & Tong (1975) observed that the dissociation constant (Kd) for the NADH-enzyme complex increases as the pH is raised from 6.2 to 8.7, and the number of NADH sites decreases. It has been suggested that Kd is dependent on the protonation of a group with pKa value of about 7.05, whereas variation in number of NADH binding sites may reflect changes in the quaternary structure of the enzyme (Duckworth and Tong, 1975).

I have studied the effect of pH on the NADH inhibition of A. anitratum citrate synthase. NADH saturation curves were obtained at pH 6.6, 7.2, 7.8 and 8.4, using the standard assay mixture. The concentration of NADH needed to achieve half maximal inhibition (K_{NADH}) and the Hill number (n) were calculated from the kinetic Hill's plot. As shown in Figure 27, the saturation curve for NADH is hyperbolic at pH 6.6 and becomes sigmoid

TABLE : 11

Effect of pH on NADH inhibition of Acinetobacter anitratum citrate synthase.[a]

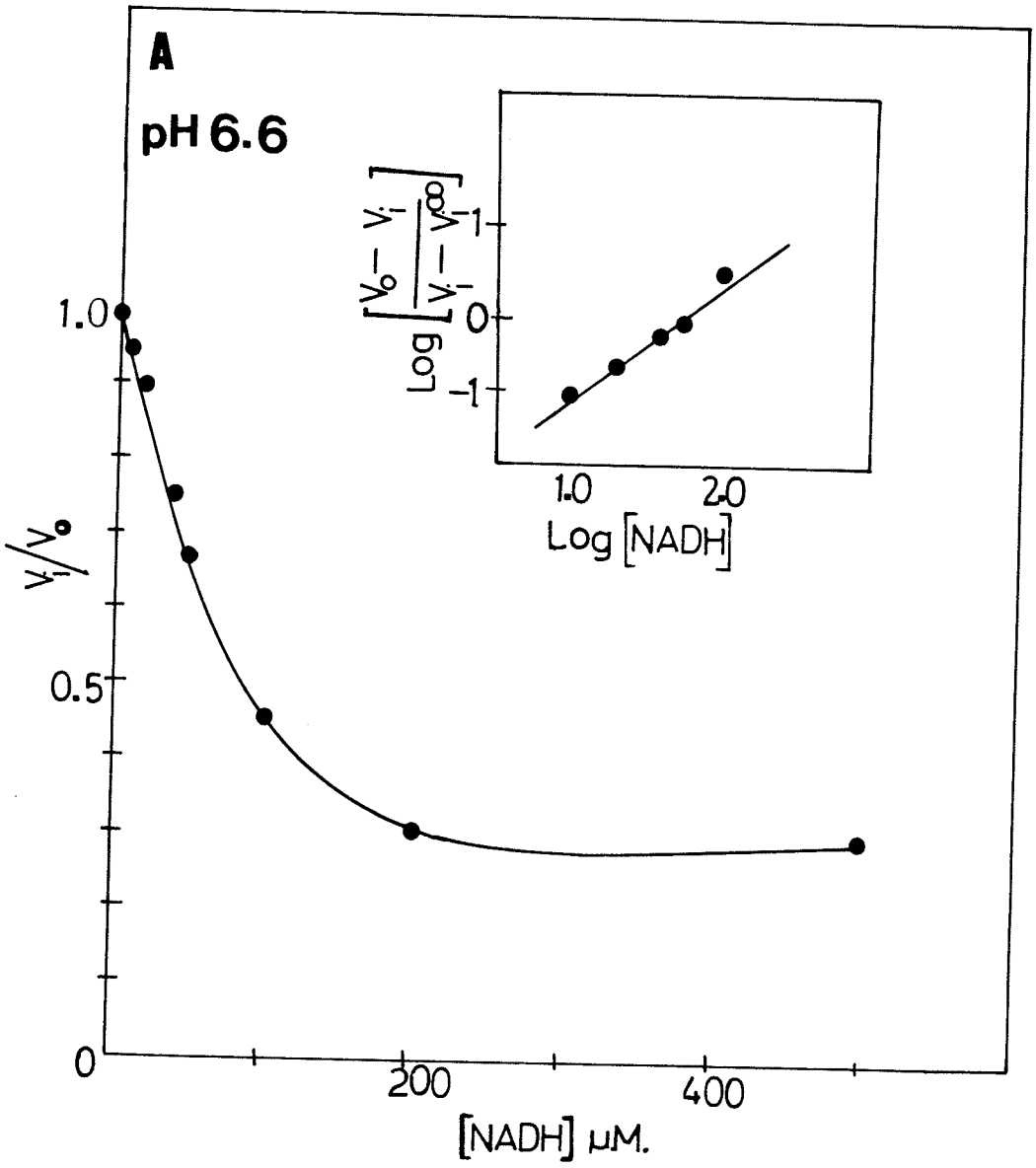
pH	K_{NADH}[b]	Hill number "n"
6.6	50.2	1.65
7.2	80.4	2.47
7.8	109.0	2.68
8.4	526.0	3.48
9.0	NI[c]	-

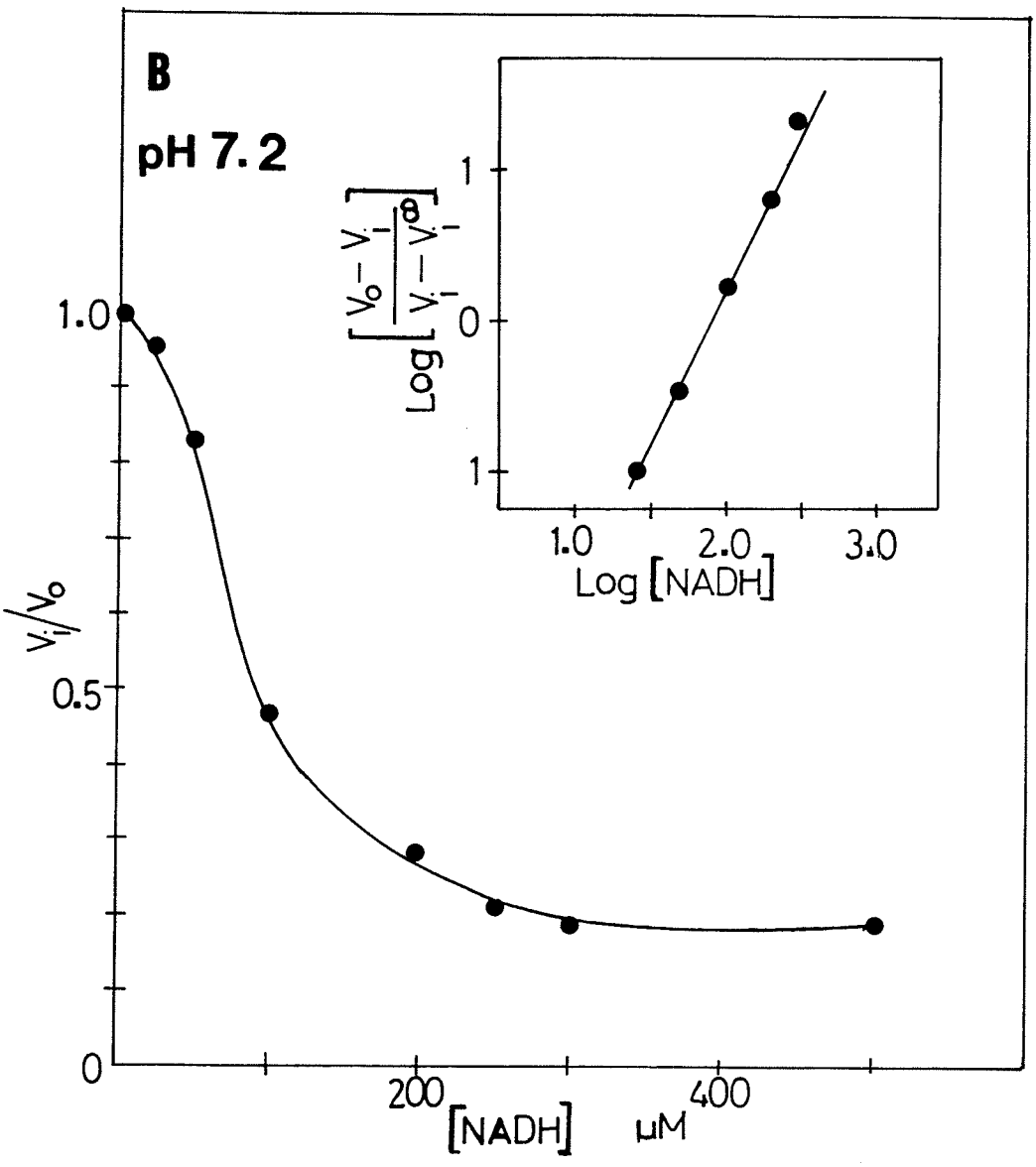
[a] From Figure

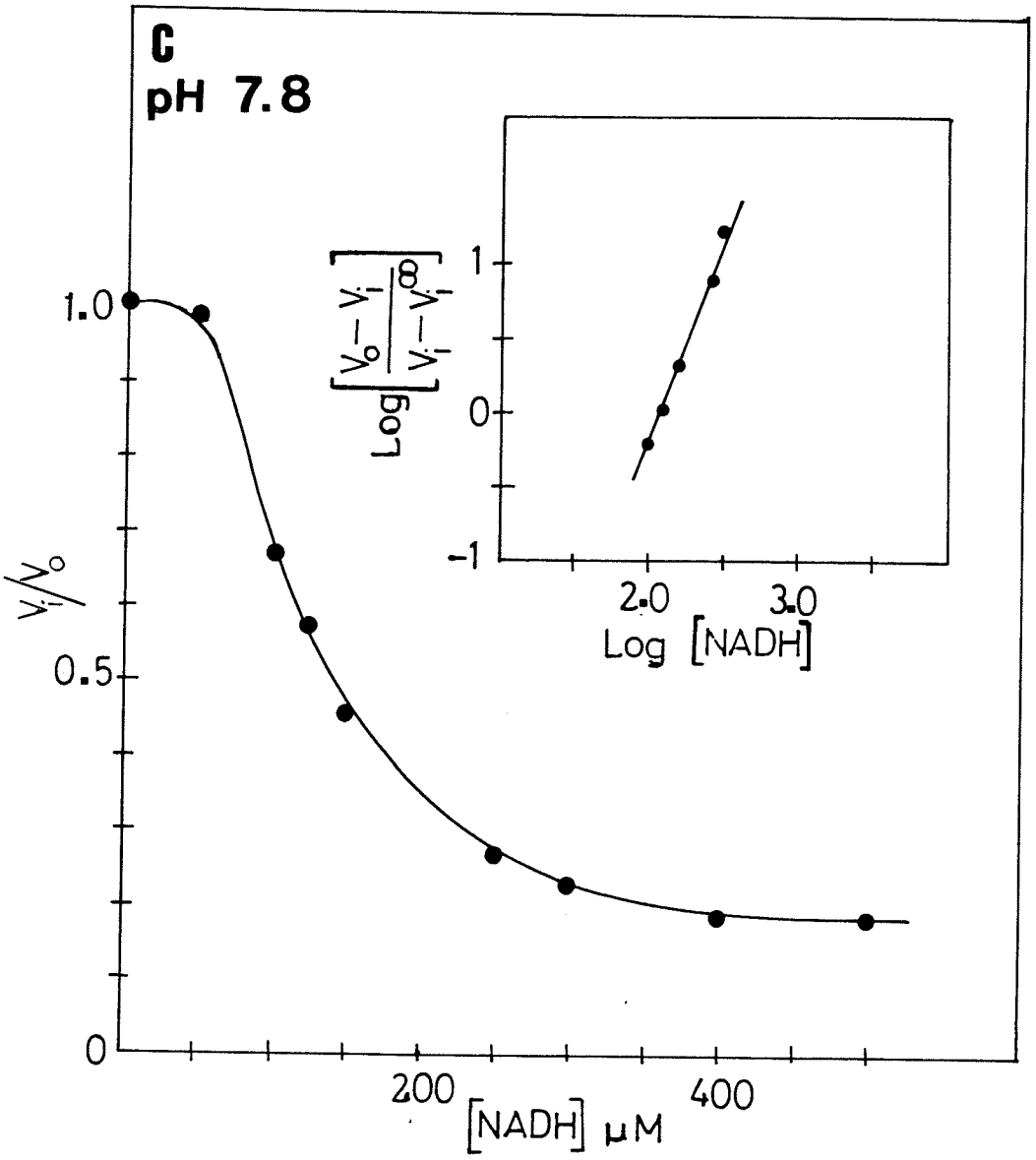
[b] K_{NADH} values represents the concentration of NADH needed to achieve half-maximal inhibition A. anitratum enzyme. Calculations are according to Morse and Duckworth (1980).

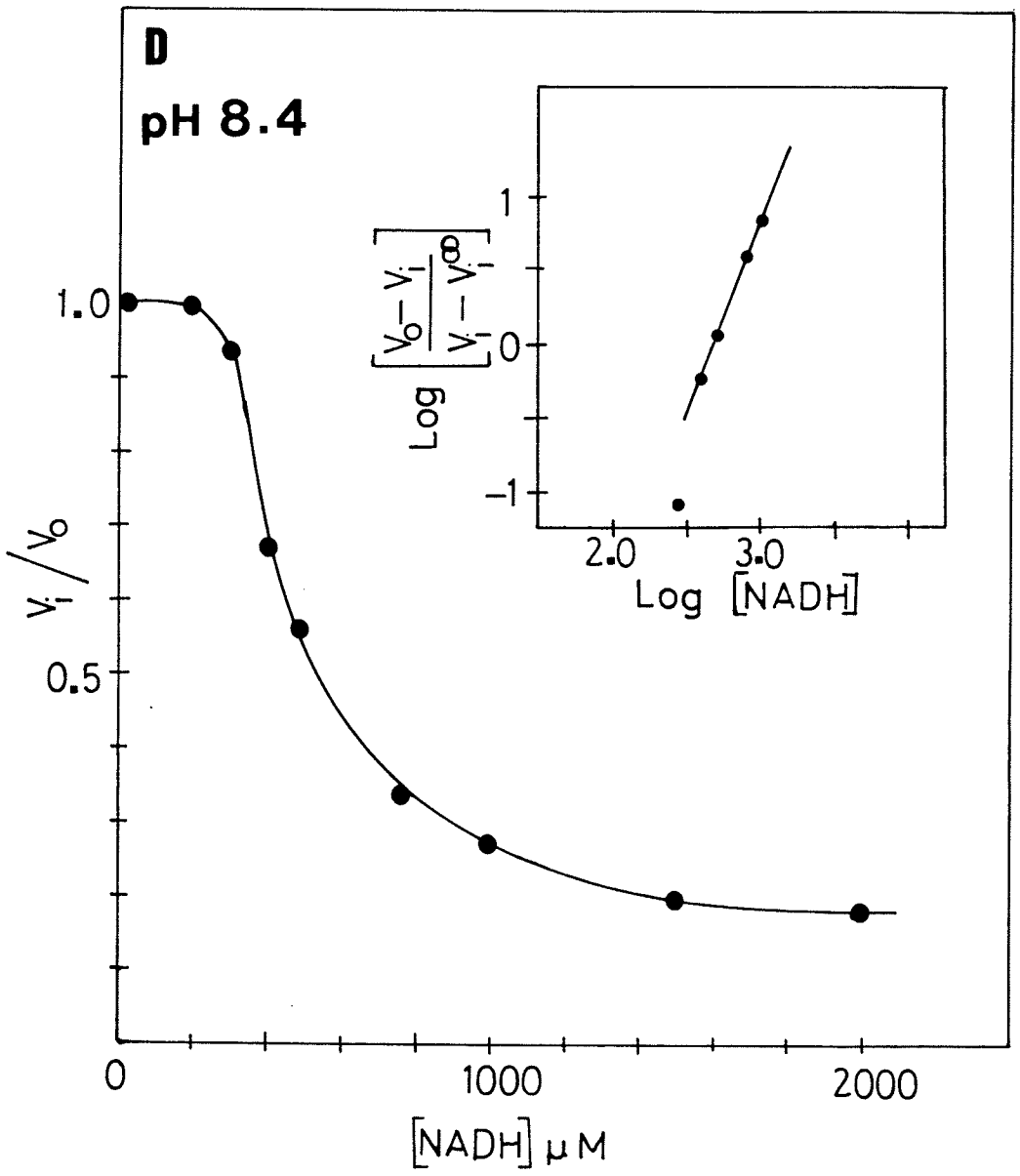
[c] No inhibition by NADH at pH 9.0.

Figure: 27 Effects of pH on NADH inhibition of Acinetobacter anitratum citrate synthase. The concentrations of acetyl-CoA, OAA and DTNB were same as those of standard assay mixture; and NADH concentrations were varied. The exact concentration of NADH was determined spectrophotometrically by A_{340} using E_m = 6220 $M^{-1}cm^{-1}$. All the solutions except DTNB were made fresh just before the analyses. Enzyme assays were performed in either 0.20 M Tris-HCl buffer (pH 7.2, 7.8, 8.4 and 9.0) or sodium phosphate (pH 6.6). In all cases, citrate synthase was added at the end to start the reaction. Vi represent the activity of the enzyme in the presence of NADH (allosteric inhibitor); Vo is the activity in the absence of inhibitor and Vi^{∞} is the activity in the presence of infinite concentration of inhibitor. Inserts show the Hill plots.


A
pH 6.6
1.0
0.5
0
V_i/V_o
200
400
[NADH] µM.
$\log\left[\frac{V_o - V_i}{V_i - V_\infty}\right]$
1
0
−1
1.0
2.0
Log [NADH]


B
pH 7.2
1.0
0.5
0
V_i/V_o
200
400
[NADH] μM
$\log\left[\frac{V_o - V_i}{V_i - V_\infty}\right]$
1
0
1
1.0
2.0
3.0
Log [NADH]


C
pH 7.8
1.0
0.5
0
V_i/V_o
200
400
[NADH] μM
$\text{Log}\left[\frac{V_o - V_i}{V_i - V_i^\infty}\right]$
1
0
-1
2.0
3.0
Log [NADH]


D
pH 8.4
1.0
0.5
V_i / V_o
0
1000
2000
[NADH] μM
$\log \left[\frac{V_o - V_i}{V_i - V_\infty}\right]$
1
0
−1
2.0
3.0
Log [NADH]

at higher pH's. The K_{NADH} value increases with the increase in pH. No NADH inhibition is observed at pH 9.0. There is a steady increase in the Hill number from 1.65 to 3.48 as the pH is raised from 6.6 to 8.4, indicating that pH has an effect on cooperative interactions (Table 11).

Although no detailed model can be made from the above information, the results can be explained qualitatively in terms of a shift in $R \leftrightarrow T$ equilibrium of the kind proposed by Monod et al. (1965). Assuming that NADH has a preferential affinity for the T state of the enzyme, it may be proposed that the T state predominates at pH 6.6. As the pH is increased, the enzyme conformation changes so as to convert the enzyme to the R state; this would explain the sigmoid saturation curves obtained at higher pH values with the allosteric inhibitor NADH.

The protonation of an acid dissociable group in the enzyme may also play a role in affecting the K_{NADH} values for this enzyme, similar to the one observed for E. coli citrate synthase, but it is difficult to separate the possible pH effects on K_{NADH} and the allosteric equilibrium.

(iv) Pseudomonas aeruginosa citrate synthase:

P. aeruginosa citrate synthase has been purified to a specific activity of 31.1 U/mg (Table 5). The recovery of enzyme preparation is very low, as only 1 mg of purified enzyme has been obtained from 145 gms of wet cells (the yield is only 4% as compared to 45% for E. coli enzyme). The explanation for low recovery may be the instability of the enzyme under the purification conditions used in the present study. In addition to low

TABLE : 12

Amino acid composition of Pseudomonas aeruginosa citrate synthase

Amino Acid		Average or Extrapolated Values (nmol)	Moles per 47,000 g	Nearest Integer
Asx	D & N	61.72	38.26	38
Thr	T	32.42[a]	20.10	20
Ser	S	32.48[a]	20.01	20
Glx	E & Q	66.46	41.20	41
Pro	P	30.39	18.84	19
Gly	G	54.46	33.76	34
Ala	A	78.77	48.83	49
Val	V	53.56	32.20	32
Met	M	22.97	14.24	14
Ile	I	41.02[b]	25.43	25
Leu	L	55.39	34.24	34
Tyr	Y	16.86	10.45	11
Phe	F	24.09	14.93	15
His	H	20.77	12.88	13
Lys	K	42.37	26.27	26
Arg	R	30.67	19.01	19
Trp	W	ND[c]	-	- (3)
1/2-Cys	C	ND[c]	-	- (7)
Total				420

[a]First-order extrapolation to zero hydrolysis time. [b]Value from 72 h hydrolysis time. [c]It is assumed that the subunit contains three tryptophan and seven half-cystine residues.

recovery, the purified enzyme was desensitized to NADH inhibition, although it showed two fold activation with 0.1 M KCl in the standard assay mixture. Evidently, a better purification procedure is required to isolate a higher quantity of the enzyme, which also retains NADH sensitivity.

SDS-polyacrylamide gel electrophoresis of the above enzyme preparation is shown in Figure 26. The enzyme migrates as a single major protein band with an apparent molecular weight of 47,000 g/mole. The material used for electrophoresis is 10 times less for P. aeruginosa citrate synthase, than for the samples in the other tracks of the gel. This is why even the major band is very light in case of P. aeruginosa enzyme, and it is not possible to observe contaminating bands. The results of the amino acid composition are presented in Table 12.

Comparison of Molecular Properties of Citrate Synthase

The NADH-sensitive group of citrate synthases are divided into two groups on the basis of response to AMP. In one such group the inhibition by NADH is relieved by AMP, whereas in the other, AMP produces no effect; the former group includes the enzyme from Gram-negative aerobic organisms and the latter group enzymes are from Gram-negative facultative bacteria (Danson and Weitzman, 1975). These two types of citrate synthases from Gram-negative organisms show differences in their kinetic behaviour. The enzyme from facultative anaerobes exhibits hyperbolic dependence of NADH, whereas either sigmoidal or hyperbolic saturation curves are obtained for different citrate synthases of aerobic bacteria; the latter group also shows sigmoidal dependence of reactivation by AMP (Weitzman, 1967;

Kleber & Tauchert, 1974; Higa et al.,1978; Morse & Duckworth, 1980). With the enzyme from facultative anaerobe (E. coli) inhibition by NADH is competitive with respect to acetyl-CoA, while both competitive and non-competitive inhibition have been observed with various citrate synthases from aerobic bacteria (Morse and Duckworth, 1980). The latter group shows hyperbolic saturation with the substrate acetyl-CoA whereas the former group shows sigmoidal saturation (Faloona & Srere, 1969; Wright and Sanwal, 1971; Johnson & Hanson, 1974; Morse & Duckworth, 1980). It is thus obvious that different citrate synthases display characteristics of both 'R' and 'T' state according to the allosteric model of Monod et al. (1965). Thus, the differences among these enzymes in the allosteric kinetics may originate mainly because of differences in the positions of their R ⇋ T equilibria.

Although the kinetic properties are quite different in the two groups of Gram-negative bacterial enzymes, there are clear similarities in their molecular features. Indeed, molecular similarities have been pointed out between NADH-sensitive ("large" type) and NADH-insensitive ("small" type) citrate synthases (Weitzman & Danson, 1975; Morse & Duckworth, 1980; Ner et al., 1983; Bhayana & Duckworth, 1984).

Figure 26 shows the SDS-polyacrylamide gel electrophoresis of citrate synthases from bacterial and eukaryotic sources. Evidently, all citrate synthases in Figure 26 have subunit molecular weight close to 47,000 g/mole. This close similarity in subunit size may be accompanied by more extensive molecular similarities as stated above, perhaps

extending to significant amino acid sequence homology. I have investigated this issue by comparing some molecular features of citrate synthases from the different organisms.

(i) Amino acid composition:

The amino acid compositions of three bacterial and three eukaryotic citrate synthases are given in Table 13. One way to compare amino acid compositions of equal or near equal lengths of proteins, is by determining the "difference total" (DT) values as described by Cornish-Bowden (1979). By using an empirical relationship, the DT values can be used to predict the sequence homology between two proteins. Morse & Duckworth (1980) predicted a 92% sequence homology between E. coli and A. anitratum citrate synthases, using for E. coli enzyme the composition data of Tong & Duckworth (1975). A sequence homology of 55.6% can be now predicted, using the composition values from Table 13. From the composition of P. aeruginosa another Gram-negative strict aerobe, a sequence homology of 16% with E. coli and 75% with A. anitratum enzyme, can be predicted. A higher degree of sequence homology (more than 50%) is predicted among eukaryotic citrate synthases.

The compositions of E. coli and pig heart citrate synthases are exact, since their complete amino acid sequences are known. The comparison of their compositions by Cornish-Bowden method predicted 0% homology, whereas 27% identities have actually been located by alignment, although a few deletions and insertions are introduced (Bhayana & Duckworth, 1984). It is evident from the above composition calculations that bacterial

TABLE 13

AMINO ACID COMPOSITION OF VARIOUS CITRATE SYNTHASES

AMINO ACID	*ESCHERICHIA COLI*	*ACINETOBACTER ANITRATUM*[a]	*PSEUDOMONAS AERUGINOSA*[a]	PIG HEART[b]	CHICKEN HEART[c]	PIGEON BREAST MUSCLE[c]
Asp	44	43	38	39	39	35
Thr	28	25	20	23	21	20
Ser	25	19	20	29	31	24
Glu	34	40	41	41	45	34
Pro	19	23	19	22	23	24
Gly	28	31	34	33	40	35
Ala	37	44	49	33	38	33
Val	19	28	32	28	28	32
Met	18	10	14	15	13	16
Iso	28	25	25	19	16	20
Leu	35	36	34	53	53	59
Tyr	16	17	11	19	17	17
Phe	23	18	15	12	12	14
His	15	12	13	14	13	13
Lys	23	24	26	25	24	25
Arg	24	20	19	19	20	20
Trp	3	5	ND(3)	9	11	8
Cys	7	5	ND(7)	4	5	14
Total	426	425	420	437	449	443

[a]From Table 10 & Table 12. [b]From Bloxham et al., (1982) [c]From Bell(1983).

citrate synthases are more closely related to one another than to the enzyme from eukaryotes, but the accuracy of this method is questionable.

(ii) N-terminal sequences:

The N-terminal sequences of three bacterial and three eukaryotic citrate synthases are aligned in such a way so as to obtain maximum homology (Figure 28). It is observed that there is considerable homology among Gram-negative citrate synthases, assuming that E. coli and P. aeruginosa enzyme start at the same place whereas A. anitratum enzyme has an extension of three residues at the N-terminus. Similarly, eukaryotic citrate synthases have a high degree of sequence homology among their N-terminal sequences. Of the 16 N-terminal amino acids determined for A. anitratum 5 are identical in eukaryotic citrate synthases. On the other hand, only 2 residues from E. coli and P. aeruginosa citrate synthases are the same in eukaryotic enzymes. This observation reveals an intermediate position for A. anitratum enzyme, between eukaryotic and the other Gram-negative bacterial citrate synthases.

(iii) Immunological cross reactivity:

The Ouchterlony double diffusion method was used to test the cross reactivity of citrate synthases from P. aeruginosa, pig heart and pigeon breast muscle with anti-E. coli citrate synthase. As shown in Figure 29, P. aeruginosa citrate synthase forms a precipitin line and a spur is clearly visible on the E. coli citrate synthase side. This

Figure: 28 N-terminal sequences of citrate synthases from prokarotic and eukaryotic sources. Single letter codes for amino acids are used. Gaps have been introduced to increase sequence homology.
The amino-terminal sequences of citrate synthases from E. coli, P. aeruginosa and A. anitratum were determined by automated Edman degradation in Beckman 890C Sequencer using 0.1 M Quadrol program (# 030176).

[a] from Bloxham et al. (1983).

[b] from Beeckmans et al. (1983).

[c] from Bell (1983).

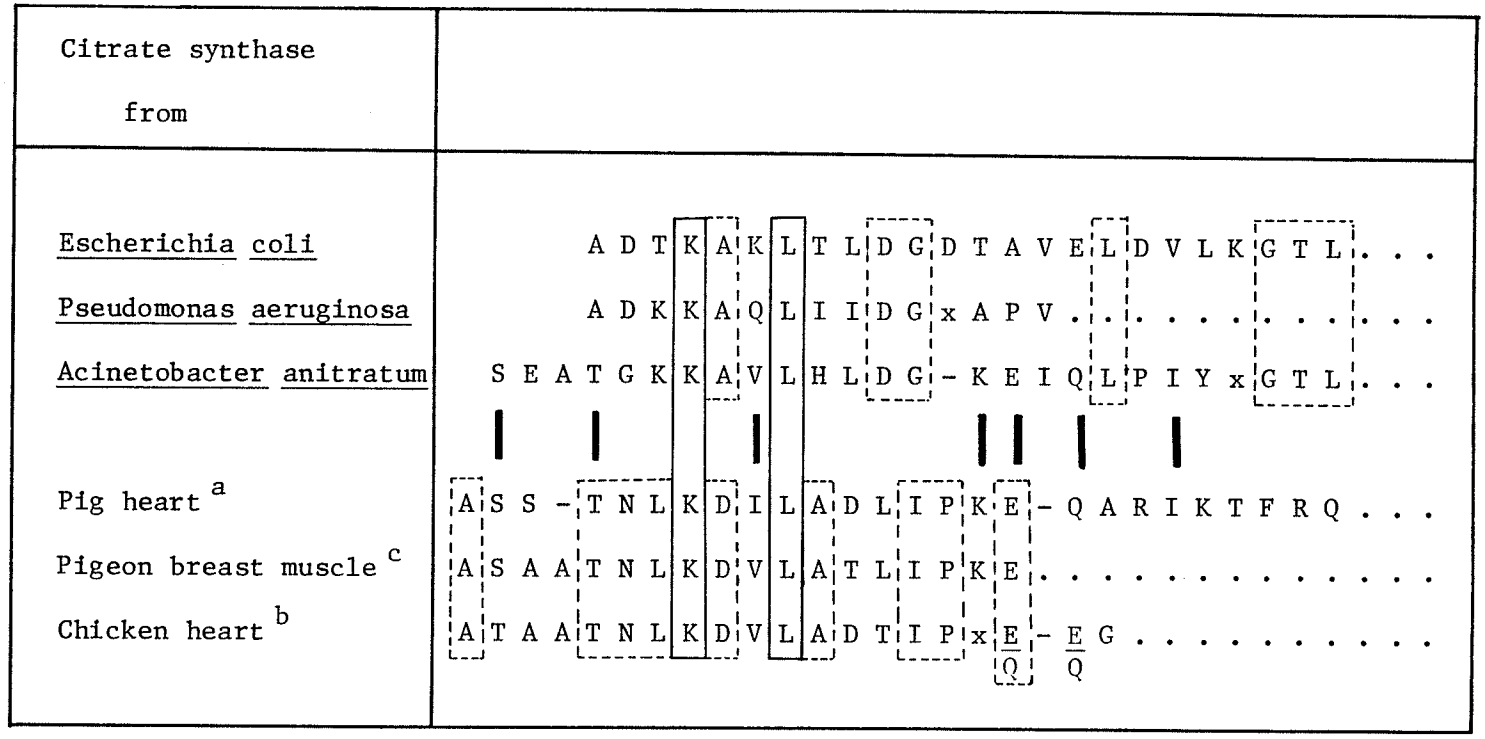

Citrate synthase from
Escherichia coli
Pseudomonas aeruginosa
Acinetobacter anitratum
Pig heart a
Pigeon breast muscle c
Chicken heart b
A D T K A K L T L D G D T A V E L D V L K G T L . . .
A D K K A Q L I I D G x A P V . . . . . .
S E A T G K K A V L H L D G - K E I Q L P I Y x G T L . . .
A S S - T N L K D I L A D L I P K E - Q A R I K T F R Q . . .
A S A A T N L K D V L A T L I P K E . . . . . .
A T A A T N L K D V L A D T I P x E/Q - E/Q G . . . . . .

Figure:29 The Ouchterlony double diffusion analysis to determine immunological cross-reactivity between citrate synthases. 'Ab' well contained 8 µL of undiluted anti-*E. coli* citrate synthase antiserum, and other wells contained 8 µL of one of the following antigen:

(1) Plasmid-borne *E. coli* citrate synthase (75 µg/mL).

(2) Chromosomal *E. coli* citrate synthase (75 µg/mL).

(3) Pig heart citrate synthase (400 µg/mL)

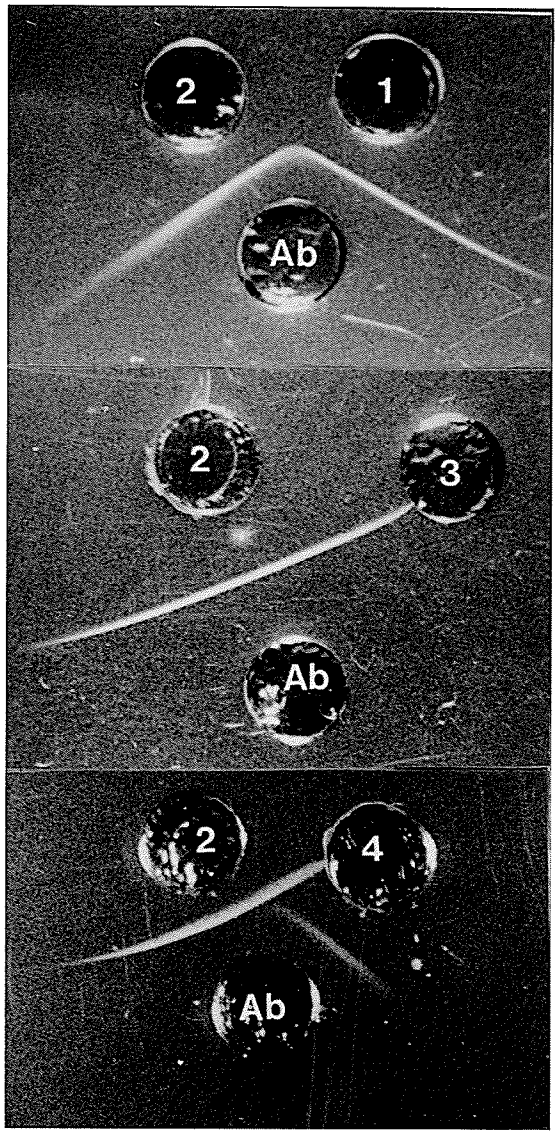
(4) *Pseudomonas aeruginosa* citrate synthase (500 µg/mL)

NOTICE/AVIS

PAGE(S) 189 IS/~~ARE~~ EST/~~SONT~~ black + white photo

PLEASE WRITE TO THE AUTHOR FOR INFORMATION, OR CONSULT THE ARCHIVAL COPY HELD IN THE DEPARTMENT OF ARCHIVES AND SPECIAL COLLECTIONS, ELIZABETH DAFOE LIBRARY, UNIVERSITY OF MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.

VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.


2
1
Ab
2
3
Ab
2
4
Ab

test reveals partial antigenic identity between E. coli and P. aeruginosa citrate synthases. The same type of pattern has previously been observed for A. anitratum enzyme when it was tested for cross reactivity with anti- E. coli citrate synthase (Morse and Duckworth, 1980). No precipitin bands were observed when anti- E. coli was tested with either of pig heart or pigeon breast muscle citrate synthases.

It may be concluded from the above observation that some of the antigenic determinants on the E. coli enzyme are similar to those of A. anitratum and P. aeruginosa citrate synthases. Thus it seems that the outer surface of citrate synthase molecule is structurally conserved, at least in part, in these three Gram-negative bacterial sources.

DISCUSSION

The first successful attempt to determine the complete primary structure of a polypeptide was made by Sanger on the hormone insulin, as a result of which he received the Nobel Prize in 1957. Since then an increasing number of protein sequences have appeared in the literature. Walsh et al. (1981) made a statistical analysis of the proteins which had been sequenced between the years 1950 and 1978. They showed that the number of sequenced proteins has dramatically increased due to automated methodologies. However, 95% of the proteins for which the complete sequence is available, are below the subunit molecular weight of 40,000 g/mole and very few proteins with higher molecular weight have been sequenced. One of the reasons for this drop in the number of sequenced proteins in the high molecular weight range is the requirement of more sophisticated strategies than those needed for smaller proteins.

The present project is involved with sequencing of E. coli citrate synthase with subunit molecular weight of 47,930 g/mole. Several problems were encountered during the course of sequencing, of which the major ones are discussed below.

(i) Cleavage reactions:

Tryptic cleavage of the citraconylated citrate synthase proceeded exactly as expected. All of the peptides isolated from this tryptic digest contained single arginines except TC-22 which had two arginines as it contained a tryptic resistant Arg-Pro bond. In addition,

chymotryptic-like activity caused further cleavage of two peptides TC-10 and TC-11 (see Results). This type of cleavage has been noticed before in tryptic digests and is described as due to the contamination by chymotrypsin (Rovery 1967; Smyth, 1967). It has been reported that this chymotryptic-like activity is usually diminished but not completely removed by treatment with chymotrypsin inhibitors such as L-(1-tosylamide -2-phenyl) ethyl chloromethyl ketone (TPCK). Keil-Dlouha et al.(1971) have suggested that surviving chymotryptic-like activity in commercially available TPCK treated trypsin (used in the present study) is due to the presence of pseudo (Ψ)-trypsin, which is produced by autolysis of α-trypsin during prolonged incubation.

Cleavage with CNBr reagent was selective and trouble free. A mixture of homoserine and homoserine lactone is produced from the original methionine residue (Gross, 1967); this heterogeneity may be the reason for doublet peaks for a single peptide as commonly observed on HPLC. No attempt was made in this study to avoid this complexity.

Hydroxylamine cleavage, which should have proceeded smoothly because of the presence of only a single susceptible Asn-Gly linkage, actually produced a rather complex mixture. This complexity must have been because of non-specific cleavages which have been found to occur at Asn-Leu and Asn-Ala (Bornstein 1970; Steinman et al. 1974). To reduce this complexity, the reaction was performed on CN-1 peptide (112 residues), but disappointingly the cleavage did not occur, although other peptides generated by CNBr reaction have been shown to be cleaved with

hydroxylamine (Bornstein, 1970).

The o-iodosobenzoic acid reaction also produced a complex mixture as was expected because of the presence of 3 tryptophans. Methionine was converted to methionine sulfone and tyrosine was completely destroyed in the peptide in spite of taking precautionary measures to prevent oxidation (Mahoney & Hermodson, 1979; Mahoney et al.1981).

(ii) Peptide isolation:

In this section, the initial problem was the tendency of many peptides to associate with other peptides and become insoluble in volatile solvents. This tendency to form aggregates, especially by larger peptides, is not surprising in view of the number and distribution of hydrophobic residues in many parts of the protein. This problem was experienced at the very first purification step, the gel filtration, when most of the decitraconylated arginine peptides eluted in two broad peaks in 40% formic acid (not shown). Further attempts to fractionate these aggregated mixtures by gel filtration, ion exchange chromatography or paper electrophoresis were futile. The use of denaturing solvents such as urea and Gdn-HCl was almost always avoided (except that peptides were dissolved in 6M Gdn-HCl before loading on a reverse phase HPLC column) because of difficulties in desalting peptides later by gel filtration. Therefore, to escape from the problem of aggregation, the peptides were left in the citraconylated state. A citraconylated group at the ε-NH_2 group of lysine results in a change in charge from +1 to -1 at neutral

pH and electrostatic repulsion between the ω-carboxyl of the citraconyl group and carboxyl groups from the polypeptide exerts a solubilizing effect. One limitation of this procedure is that buffers of alkaline pH are required throughout the chromatographic fractionation procedure because the citraconyl group is removed in the acidic pH range. Ammonium bicarbonate buffer was routinely used for fractionation of citraconylated peptides by gel filtration and ion exchange chromatography. This buffer system has the advantage that it does not absorb UV light, so that the peptides are easily detected by taking absorbance readings at 230 nm. However, care had to be taken to lyophilize the peptides immediately because they tend to be hydrolyzed if stored for a long time in this buffer solution.

The second major problem was in purifying each component of the peptide mixture to homogeneity. Although, initial fractionation of the arginine peptides on Sephadex G-50 provided appropriate pools on the basis of molecular size, ion exchange chromatographic procedures were unable to purify any of the larger peptides completely. In the case of smaller arginine (TC) peptides little success was achieved by using polystyrene ion exchangers (Dowex 50-X2 and Dowex 1-X2). The procedure for analyzing fractions collected from these ion exchange columns by ninhydrin analysis after alkaline hydrolysis, was also cumbersome. Yet another difficulty with the Dowex columns was the low recovery of peptides - in the range of 30% to 60%. A few of the smaller peptides (TC-4, TC-5, TC-8, TC-12 and TC-17) were actually completely lost on

these columns.

High voltage paper electrophoresis of the mixture of smaller peptides was not as easy as would be expected from the results observed in other systems (Perham, 1978). Also, the recovery of the peptides from the paper was low- approximately 20%.

High pressure liquid chromatography proved superb for purifying various peptide mixtures. As can be seen in the Results section, the final purification of most of the peptides was obtained by using this technique. The problem encountered with this procedure were much less as compared to other methods described above. The following minor troublesome aspects were experienced with HPLC.

(i) The first peak in the elution profile was due to Gdn-HCl (when samples were dissolved in 6 M Gdn-HCl) and must have contained polar peptides which did not bind to the column. These short polar peptides went undetected due to the difficulties in separating them by gel filtration. As a result of this problem, a few smaller peptides, TC-8, TC-12, CN-3, CN-5, CN-16 and CN-18 were never identified by HPLC procedures (Figure 19 and 21).

(ii) The larger peptides were recovered in lower yield than the smaller peptides. In the propanol-1 gradient system the yield was better for larger peptides but on the other hand resolution of smaller peptides was sometimes poor in this system. This was not the case all the time, as peaks which were poorly resolved in the propanol-2 system were nicely separated in the propanol-1 system, for example CN-9 and CN-13.

(iii) Well separated pairs of peaks, of almost equal size, which exhibited the same sequence, were commonly seen in HPLC elution profile. Although the reason for this type of behaviour has not been explained it is possible that HPLC may easily distinguish the alterations caused by oxidation or deamidation of an amino acid in the parent peptide.
(iv) It is difficult to know if all of the peptides are eluted from the column unless the exact number of peptides in the mixture is known.
(v) In order to protect the column from plugging, very clean samples were required. Also, HPLC grade solvents were needed, in order to obtain a stable baseline at high sensitivity detection.

(iii) Automated sequencing:

Several problems were encountered during amino acid sequencing of peptides/proteins in the Beckman 890 C spinning cup Sequencer. Most of these were routine problems and have been discussed in several review articles (Niall, 1977; Waterfield & Bridgen, 1975; Walsh et al. 1981). One problem which was quite annoying, was the high carry over of amino acids from one step into subsequent steps (Figure 25). In the first few cycles of degradation the carry over was low but it increased constantly as the degradation proceeded. Moreover, in a few instances the degradation went out of phase by one complete cycle towards the end of the peptide sequence. This carry over problem is common with spinning cup Sequencers, and it cannot be removed, although it can be reduced. It is believed

that incomplete coupling and/or cleavage, and technical problems with the vacuum system in the Sequencer, are the root causes of high carry over (Waterfield & Bridgen, 1975).

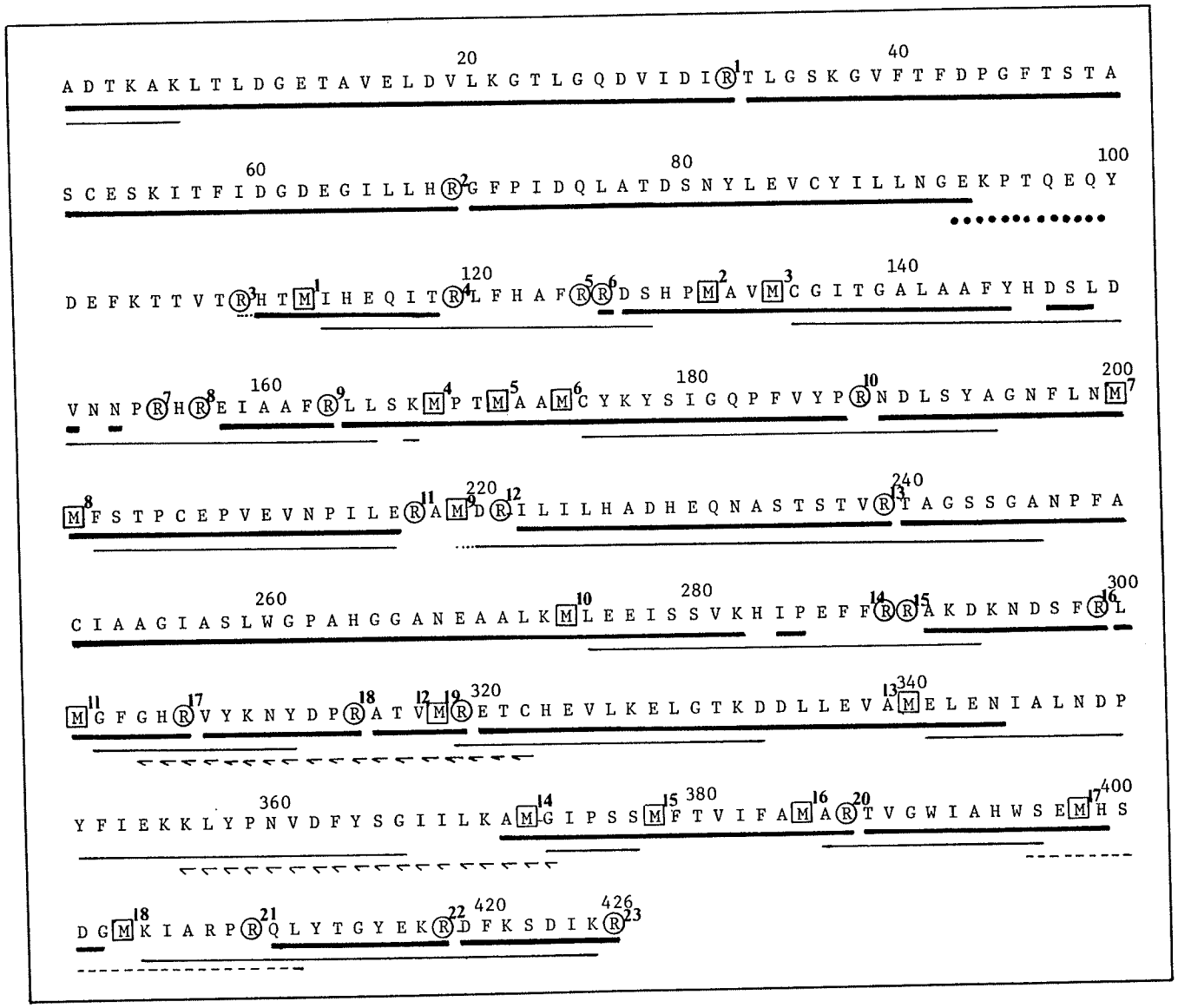
Proof of Escherichia Coli Citrate Synthase Amino Acid Sequence :

The complete amino acid sequence of E. coli citrate synthase is shown in Figure 30. The sequence is deduced by the isolation and sequenci of 19 arginine (TC) peptides which are aligned by overlapping peptides generated from CNBr, hydroxylamine and o-iodosobenzoic acid cleavage. At certain places in the sequence the overlaps are obtained from the sequence of fragments generated by subtilisin partial proteolysis (Bell et al. 1983) and from the base sequence of gltA , the gene for citrate synthase (Ner et al., 1983). Most of the overlaps from protein chemistry are established by two or more residues. For the sake of convenience the proof of the sequence can be summarized into four sections:

Residues 1-109

The amino terminal sequence of the intact protein (Duckworth & Bell, 1982) allowed the initiation codon in the gene to be recognized, and two laboratories have reported the same DNA sequence up to the codon for leu-90 (Ner et al. 1983; Hull et al. 1983). Peptides TC-1, -2 and -3 are placed in order by reference to the DNA sequence. A small piece (94-100) is placed by sequencing a fragment generated by hydroxylamine cleavage. I have made a few attempts to extend this sequence beyond

Figure:30 Proof of Escherichia coli citrate synthase amino acid sequence. The numerals after arginine and methionine residues refer to the order of TC and CN peptides respectively, in the polypeptide chain starting from the N-terminal end. The length of the peptide sequenced is indicated; ▬▬ TC peptides, ——— CN peptides ------- o-iodosobenzoic acid cleaved peptide, ⌐⌐⌐⌐ subtilisin fragments, •••• hydroxylamine fragment.


A D T K A K L T L D G E T A V E L D V L K G T L G Q D V I D I R T L G S K G V F T F D P G F T S T A
S C E S K I T F I D G D E G I L L H R G F P I D Q L A T D S N Y L E V C Y I L L N G E K P T Q E Q Y
D E F K T T V T R H T M I H E Q I T R L F H A F R R D S H P M A V M C G I T G A L A A F Y H D S L D
V N N P R H R E I A A F R L L S K M P T M A A M C Y K Y S I G Q P F V Y P R N D L S Y A G N F L N M
M F S T P C E P V E V N P I L E R A M D R I L I L H A D H E Q N A S T S T V R T A G S S G A N P F A
C I A A G I A S L W G P A H G G A N E A A L K M L E E I S S V K H I P E F F R R A K D K N D S F R L
M G F G H R V Y K N Y D P R A T V M R E T C H E V L K E L G T K D D L L E V A M E L E N I A L N D P
Y F I E K K L Y P N V D F Y S G I I L K A M G I P S S M F T V I F A M A R T V G W I A H W S E M H S
D G M K I A R P R Q L Y T G Y E K R D F K S D I K R

residue 100. In the first attempt, the intact protein was cleaved with hydroxylamine and the mixture was sequenced. The information obtained from this sequence was good only up to residue 100. In the second attempt the peptide CN-1 was treated with hydroxylamine. No cleavage was detected by HPLC in two separate attempts. In the third attempt, peptide CN-1 was cleaved at lysine by the enzyme endoproteinase Lys-C and the products were isolated. From the composition (not given) overlapping peptides 22-37 and 56-104 were identified indicating that the Lys(94)-Pro(95) bond was not cleaved probably because it is resistant to hydrolysis by this enzyme as it is to trypsin. The short peptide (105-109) was not isolated; this means the results presented in this thesis do not confirm the sequence of residues 101-108 as derived from the DNA, although compositions of TC-3 and CN-1 are consistent with this sequence.

It is obvious from the above discussion, that the sequence of residues 101 to 108 would be difficult to obtain unless peptide TC-3 was sequenced through its C-terminus. One way to obtain the sequence of residues 105 to 109 would be to cleave TC-3 with trypsin and sequence the whole digest. Only two sequences, one starting at position 70 and another at position 105, would appear. The sequence of residues 101-104 would not be obtained, however, except by a long degradation.

Yet another way to obtain the missing sequence (101-108) would be by effecting further cleavage of the peptide CN-1 at cysteines 52 and 86, and then isolating peptide 87-112. This cleavage could be achieved by modification of the cysteine to S-(2 amino ethyl) cysteine, which mimics

lysine and should be a target for the enzyme endoproteinase "Lys-C". If the lysine residues in CN-1 were previously blocked, the cleavage would occur only at Cys-52 and Cys-86.

Residues 110-219

Continuous sequence information all the way through this region was obtained as far as Glu-216. Since a short peptide TC-12, which comprises residues 218-221 in the full sequence, was not isolated; there is no evidence from protein chemistry that peptide CN-10 follows CN-9 directly. For what it is worth, the compositions of TC-11 and CN-9 are in complete agreement with the sequence 217-219, inferred from the DNA. Isolation of the chymotryptic peptide 210-249 would seem to be a good way to elucidate this missing sequence.

Residues 220-299

The sequence of these residues may be assembled from the relevant peptides with no ambiguities. Since the sequence of peptide CN-11 was not obtained beyond Lys-294, there is no proof from this work that TC-17 follows directly upon TC-16. The isolation and characterization of a endoproteinase "Lys-C" peptide (295-309) would evidently prove this sequence. In an attempt to isolate this peptide, the endoproteinase "Lys-C" digest of citrate synthase was fractionated in a single step by reverse phase HPLC. Unfortunately, the desired peptide (295-309) could not be identified by composition analysis of the collected fractions. Apparently, other purification procedures would be needed to isolate this peptide.

Residues 300-426

This stretch is mostly proved by relevant arginine and methionine peptides. To prove the stretch between 364 and 387, it is necessary to use the sequence of the C-terminal tryptic peptide from TC-20, and the N-terminal sequence of the subtilisin partial proteolysis fragment, commencing at Lys-356 (Bell et al. 1983). Proof of the sequence of residues 396-404 is provided by the peptide isolated after oxidative cleavage at Trp-395. The carboxy-terminus sequence is provided by kinetic studies using carboxypeptidase A and B.

Composition and Subunit Molecular Weight of Escherichia coli Citrate Synthase.

The overall composition of the protein, as derived from the complete sequence, agrees quite well with the amino acid analysis as shown in Table 14. The values observed for most of the amino acid residues are within the accuracy limit of the analysis method; however, a few exceptions require further comments.

Serine is an unstable amino acid under the acid hydrolysis conditions and therefore its correct value is calculated by first order extrapolation to zero hydrolysis time. Surprisingly, even after correction the value for serine residues was low (22) from composition analysis as compared to sequence(25).

The sulfur containing amino acids (methionine and cysteine) are susceptible to oxidation during the course of acid hydrolysis in 6 M HCl.

TABLE 14

AMINO ACID COMPOSITION OF *E. COLI* CITRATE SYNTHASE.[a]

Amino acid	Composition
Ala	35.2 (37)
Arg	24.0 (24)
Asn	44.3 (16)
Asp	(28)
Cys	7.40 (7)
Gln	31.6 (8)
Glu	(26)
Gly	28.9 (28)
His	15.5 (15)
Ile	27.1 (28)
Leu	36.1 (35)
Lys	23.0 (23)
Met	15.9 (18)
Phe	21.1 (23)
Pro	19.6 (19)
Ser	21.9 (25)
Thr	27.5 (28)
Trp	3.20 (3)
Tyr	16.1 (16)
Val	20.1 (19)

[a]Composition is from Duckworth & Bell (1982); values in parentheses are from amino acid sequence. Total number of residues are 426 and the subunit molecular weight is 47,930 g/mol.

Frequently, methionines are protected by using oxygen scavengers such as merceptoethanol or thiodiglycol in the acid (6 M HCl). Since no such special protection was provided for methionine in the analysis performed by Duckworth and Bell (1982), low values of methionine might be expected from their results. Cysteine and cystine must be converted to a more stable derivative, cysteic acid, prior to hydrolysis. As shown in Table 14, the values obtained for cysteine (1/2 cystine) are completely consistent with sequence results.

Since tryptophan is labile during the standard hydrolysis with HCl, its quantitation is usually performed by spectrophotometric measurements by the method of Edelhoch (1967). Evidently, the values determined by this procedure are correct as determined from the complete sequence (Table 14).

A subunit molecular weight of 47,930 g/mole is calculated from the complete sequence of E. coli citrate synthase. This value compares very well with 47,000 g/mole obtained from SDS-polyacrylamide gel electrophoresis (Figure 26 , and Tong & Duckworth, 1975).

Similarities and Differences Between Allosteric and Non-allosteric Citrate Synthase.

As explained in the Introduction, there are two major groups of citrate synthases, the "large" and the "small". The "large" citrate synthases are allosteric enzymes and are hexameric in structure (Tong and Duckworth, 1975; Morse and Duckworth, 1980). On the other hand the "small"

citrate synthases are non-allosteric and consist of two identical subunits (Weitzman, 1981). It is possible that the polymeric state of the enzyme accounts for the allosteric properties of the "large" citrate synthases, a statement which is consistent with the allosteric model of Monod et al. (1965).

In favor of the above hypothesis evidence has been presented to show that "small" citrate synthases can be derived from the "large" enzyme. Higa et al.(1978) showed the presence of both the "small" (CS-I) and the "large" (CS-II) citrate synthase in a marine Pseudomonas. CS-II was the native form of the enzyme that exhibited characteristics of an allosteric enzyme such as inhibition by NADH, insensitivity to ATP, and reversal of NADH inhibition by AMP. However, CS-I had characteristics of non-allosteric citrate synthase such that it was inhibited by ATP and was insensitive to NADH. The authors also demonstrated the dissociation of CS-II into CS-I in the presence of 2.0 mM phosphate and the reassociation of CS-I to the higher molecular weight form upon removal of phosphate. This finding that the two forms of the enzymes are interconvertible led Higa et al. (1978) to suggest that allosteric properties are closely related to the state of aggregation of the enzyme.

More evidence in favor of the above hypothesis was presented by Danson et al. (1979). They described the isolation and characterization of a mutant of E. coli which produces a varient form of citrate synthase that resembles the "small" non-allosteric enzyme. It was demonstrated by gene mapping studies that the mutation leading to small citrate synthase

is located in the same gene as that producing the "large" enzyme in the original wild type organism. It was suggested from their findings that regulatory properties of the "large" citrate synthase are crucially dependent on its quaternary structure.

The best studied example of non-allosteric citrate synthase is that from pig heart. The enzyme has two identical subunits of 48,969 g/mole; its complete amino acid sequence (437 residues) and three dimensional structure are known (Bloxham et al. 1981, 1982; Remington et al. 1982). E. coli citrate synthase, the most widely studied example from the allosteric group consists of a single type of subunit of molecular weight 47,930 g/mole (Ner et al.1983; Tong and Duckworth, 1975).

The hypothesis has been advanced that the E. coli and pig heart enzymes consist of similar kinds of subunits, whose tertiary structures are basically the same (Morse and Duckworth, 1980; Bell et al. 1983). The existence of 'T' or inactive state in E. coli enzyme would then be caused by additional subunit interactions present in this enzyme as compared to that in pig heart enzyme. In favor of this notion evidence has been presented from partial proteolysis studies that the major subtilisin cleavage site is homologous in the two enzymes (Bell et al. 1983; Bloxham et al.1980). The complete amino acid sequence of E. coli citrate synthase (Bhayana and Duckworth, 1984; Ner et al.1983) as presented in this thesis, now allows me to investigate further the similarities and differences with the pig heart enzyme.

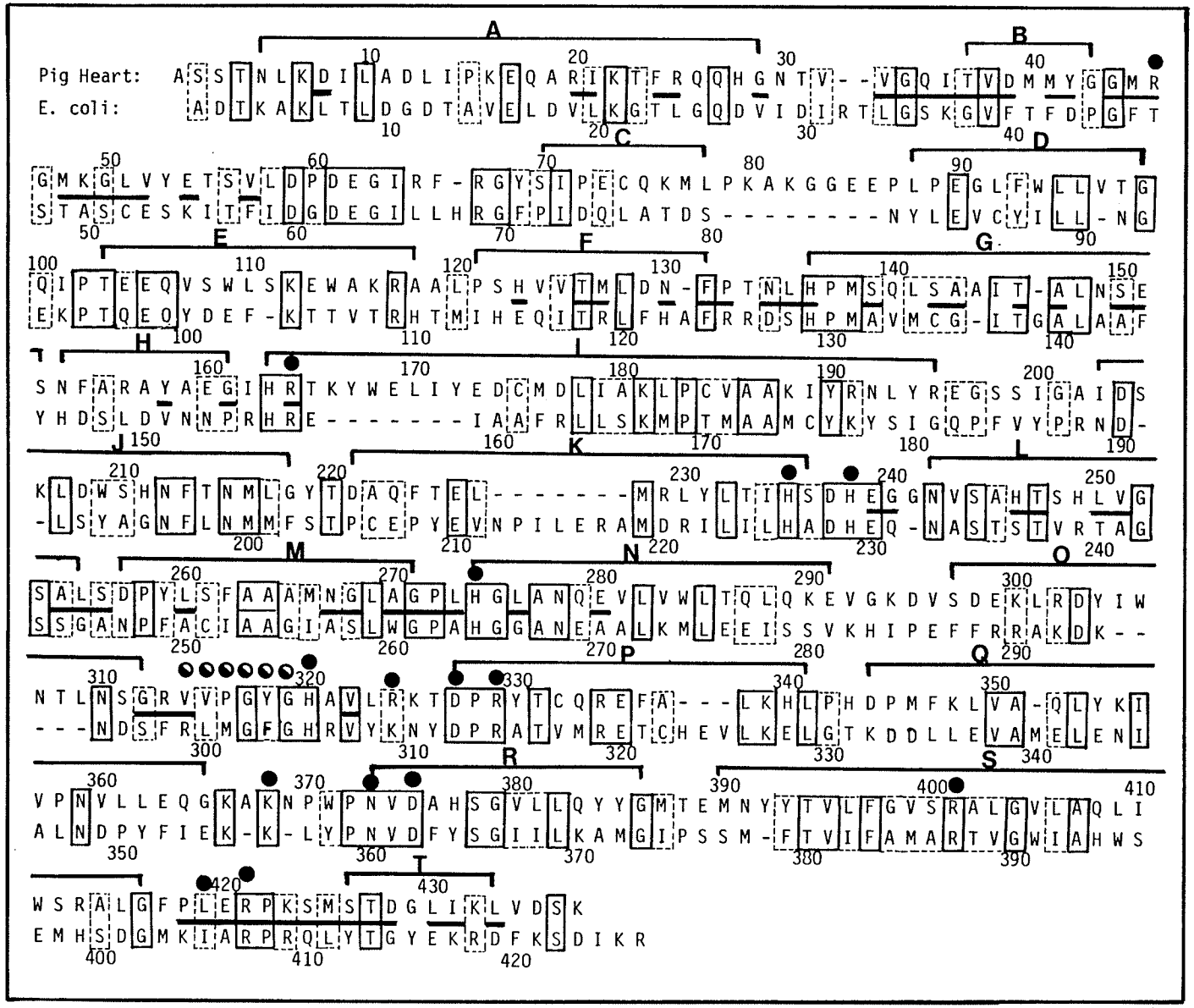
(i) Sequence Homology:

The compositions of E. coli and pig heart citrate synthase are basically the same. Although large differences are apparent for individual amino acids, if similar residues are taken in groups the sums are equivalent. Thus Asp, Asn, Glu and Gln sum to 78 and 80; Ala Gly and Pro 80 and 84; Cys, Ser and Thr 60 and 56; Tyr, Phe and Trp 42 and 40; Lys, Arg and His 62 and 58; Ile, leu, Val and Met, 115 and 110; in E. coli and pig heart respectively. In spite of these similarities in composition, no sequence homology (0%) is predicted from the Cornish-Bowden procedure (1978). Significant homology is observed, however, by alignment of the two sequences. As shown in the homology diagram (Fig.31) 16 insertions and 30 deletions have been introduced in the pig heart sequence to maximize the homology. Of the total 437 amino acids in pig heart sequence 391 have counterparts in E. coli enzyme; 117 of these are identical and 85 are conservatively replaced. A conservative replacement is identified when the replacement is by another amino acid in the same group; the groups are: Ser, Thr, Pro, Ala, Cys and Gly; Asn, Asp, Glu and Gln; His, Arg and Lys; Met, Ile, Leu and Val; Phe, Tyr and Trp.

It is evident from Figure 31 that homologous residues are distributed throughout the sequence and are not completely localized. However, it may not be denied that homology is less in the amino terminal region (1-58) than in the rest of the sequence.

The crystal structure of pig heart citrate synthase (Remington et al. 1982) predicts that Asp-375, His-274 and His-320 are the residues most

Figure: 31 Alignment of amino acid sequence of pig heart (top) and Escherichia coli (bottom) citrate synthases. Gaps have been introduced in both sequences to maximize homologies, and are shown by dashes with in the sequence. Boxes with solid line are drawn around identities and boxes with broken lines represent conservative replacements. Black dots (●) have been placed above residues involved in catalysis or substrate binding, according to Remington et al.(1982). Residues marked (◒) in the pig heart sequence, according to the same authors, provide peptide backbone atoms but not side chains to the CoA binding site. Solid lines over the pig heart sequence represent helical regions. The residues predicted to be involved in subunit-subunit contact region in pig heart enzyme, are shown by solid lines (▬▬) between the two sequences.


Pig Heart:
E. coli:
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T

probably involved in catalysis. His-274 is in a position to abstract a proton from the acetyl-CoA, His-320 could donate a proton to the carbonyl group of oxaloacetate, and Asp-375 could be involved in releasing citrate from citryl-CoA either by transesterification or by forming a mixed anhydride. Clearly, equivalent residues are present in E. coli sequence and are found as Asp-362, His-264 and His-305. The strong homology exhibited in residues involved in catalysis, reflects the point that both enzymes catalyze the same reaction and probably have similar tertiary structures in the active site region.

From the crystal structure of pig heart citrate synthase the residues involved in citrate and CoA binding have been identified (Remington et al., 1982); these residues are indicated in Figure 31. Interestingly, there is strong homology for these residues in E. coli enzyme. Of the 21 residues indicated 14 are identical in E. coli and 4 are conservatively replaced. Residues 314 to 320 in pig heart sequence are wrapped around the adenine ring of CoA and form hydrogen bonds with the peptidyl backbone rather than amino acid side chains (Remington et al., 1982). A reasonable homology can be seen along this region in the E. coli enzyme, indicating that the same basic structure is conserved.

Arginine-46 is responsible for making an ionic interaction with 5'-diphosphate of CoA in heart citrate synthase (Remington et al., 1982), and there seems to be no equivalent homologous residue in the sequence of the E. coli enzyme. It has been suggested that the loss of this ionic interaction in E. coli enzyme explains the fact that this enzyme has

lower affinity for acetyl-CoA than pig heart enzyme does (Ner et al., 1983). This seems to be a reasonable interpretation from the homology diagram in Figure 31, but another possibility is that Arg-46 may correspond to E. coli Lys-37 if a further gap is introduced into the E. coli sequence corresponding to the residues 25 to 32 of pig heart. It should be noted that the alignment shown in Figure 31 has been made so as to obtain maximum homology; single residue deletions or insertions have been used at several places, which may be questionable.

Another point of interest is to locate the conserved areas in the tertiary structure of heart citrate synthase. It is reasonable to believe that homologous areas would have similar functions in the two enzymes, and that nonconserved areas are associated with functional differences between the two enzymes. Accordingly, the allosteric NADH-binding site and the additional subunit contacts in E. coli citrate synthase are anticipated to be in the areas of minimum homology. In Figure 31, the areas with more than 60% homologous residues (including conservative replacements) include pig heart helices G, M, P and R; slightly less conservative (between 50% to 60% homology) are helices I, J, K, L, N and S. Of these, helices P, R and N are parts of the active site and G, M and L are involved in dimeric contacts, whereas S and I form the core structure of dimeric particle. This striking homology in the active site and subunit contact surface and the core areas, suggests that the tertiary structures of subunits of the prokaryotic and eukaryotic citrate synthase are very similar. This conclusion, that the basic dimeric structure

of pig heart citrate synthase is conserved in the E. coli enzyme is not surprising since dimers of the latter enzyme have been observed under native conditions and also under mildly denaturating conditions (Tong and Duckworth, 1975)

Although the preceding discussion stresses the areas of similarities there are also regions with critical differences between E. coli and pig heart enzyme. One of the areas of minimum homology is the C helix, where only 25% of the residues are homologous. Other regions of minimum homology include helices E, F, Q and T with 35%, while D helix exhibits 42% homology. Any of these regions may participate in dimer-dimer contacts (assuming that the dimer is still the basic unit in E. coli enzyme) or in forming the allosteric site which is present in E. coli but absent in pig heart enzyme.

Remington et al. (1982) have presented the list of residues which are involved in the subunit contacts in the pig heart dimer. Residues which participate in hydrogen bonds and salt bridges between subunits have also been identified. The extent of homology at these residues with the corresponding E. coli positions is also shown in Figure 31. Of the 84 residues indicated only 18 are identical and 15 are conservatively replaced. The homologous contact region residues are concentrated in G helix and ST loop (6/8, i.e 6 out of 8 for both), whereas in helices F (2/5), L (2/7), M (3/7), B (2/7) and T (2/6), most contact residues are obviously not conserved. This observation brings out the point that although the basic structure seems to be the same in the dimeric contact region (as

observed from strong general homology in this area), there are striking differences in the actual intersubunit interactions made within the dimers of the two enzymes.

(ii) Antigenic determinants

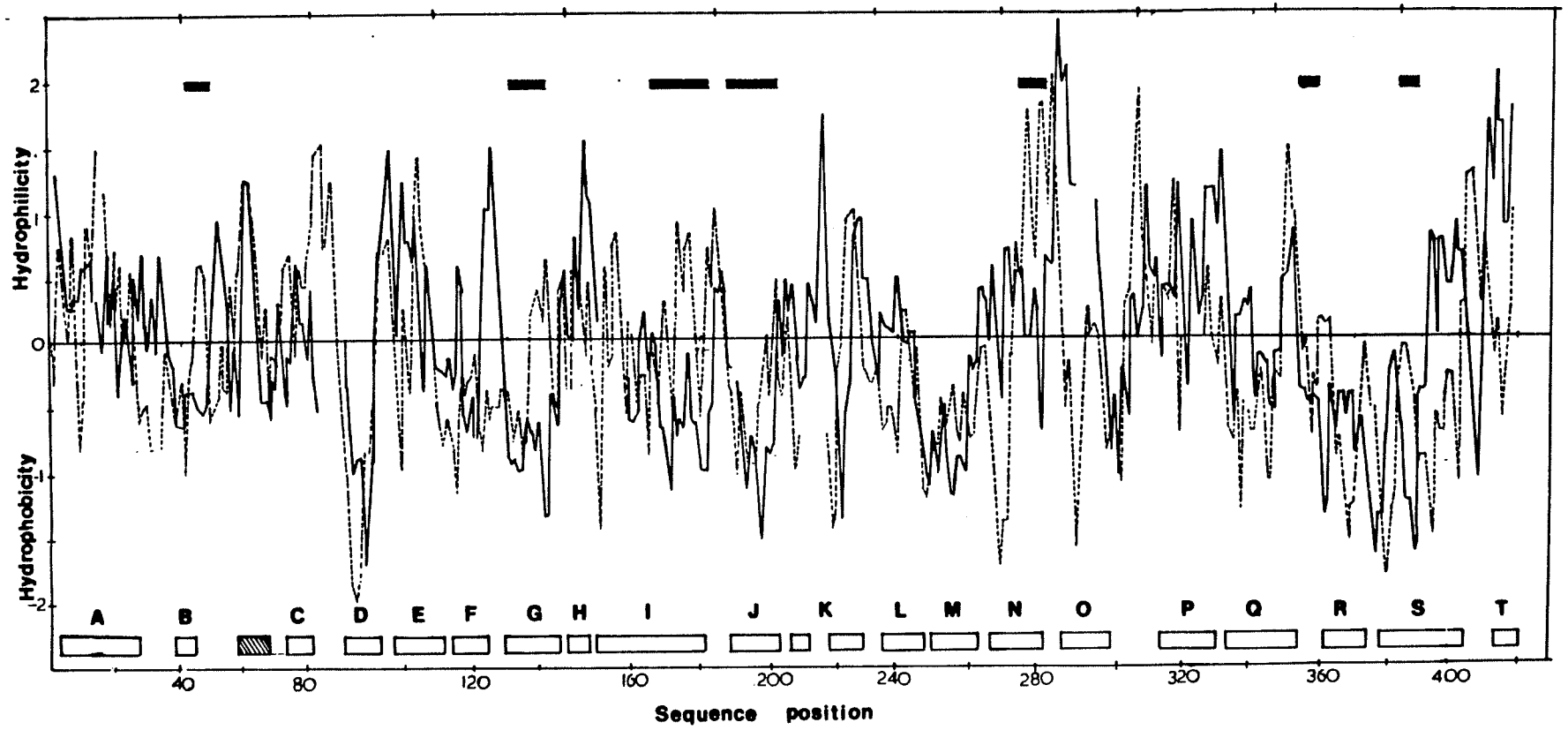
Bloxham et al. (1980) have performed some immunological studies to locate the main antigenic determinant site on pig heart citrate synthase. They reported that the 36 K fragment generated from the partial proteolysis of citrate synthase with trypsin was unreactive towards antisera raised against native enzyme, as studied by Ouchterlony double diffusion analysis and quantitative immunoprecipitation. On the other hand the mixture of 37 K and 10 K fragments generated by subtilisin partial proteolysis was as reactive towards antisera as towards native citrate synthase. It is concluded from this study that the major antigenic site is located on the 10 K fragment or on the portion present in the 37 K but not in the 36 K fragment.

A method is now available to predict the location of antigenic determinants from the complete amino acid sequence of a protein (Hopp and Woods, 1981). This method is based on locating the point of the greatest hydrophilicity in a protein, which is predicted to be the antigenic determinant. To accomplish this, a hydrophilic value (a numerical value) is assigned to each amino acid, then these values are averaged in groups of six residues down the length of polypeptide chain. This prediction method is reported to be 100% correct for predicting a single important antigenic determinant (Hopp and Woods, 1981). The second and third most

Figure: 32 Hydrophilicity/Hydrophobicity profiles for Escherichia coli (solid line) and pig heart (broken line) citrate synthases. The following numerical values representing hydrophilicity were suggested by Hopp and Woods (1981).

Amino acid	Hydrophilicity value	Amino acid	Hydrophilicity value
Arg	3.0	Ala	-0.5
Asp	3.0	His	-0.5
Glu	3.0	Cys	-1.0
Lys	3.0	Met	-1.3
Ser	0.3	Val	-1.5
Asn	0.2	Ile	-1.8
Gln	0.2	Leu	-1.8
Gly	0.0	Tyr	-2.3
Pro	-0.0	Phe	-2.5
Thr	-0.4	Trp	-3.4

The above values are assigned to the amino acids in the sequence and then these are repetitively averaged in a group of six. The averaged values are plotted versus position along the amino acid sequence. The profiles for E. coli and pig heart enzyme are superimposed on each other according to the homology diagram (Figure 31). ☐ represent the helical and ▨ shows the β-sheet region in pig heart sequence. ▬ depicts the area of higher hydrophobicity in E. coli than pig heart enzyme.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Sequence position

hydrophilic points did not prove reliable for predicting other antigenic determinants on the protein.

The hexapeptide profile for pig heart citrate synthase is shown in Figure 32 , where the point of highest hydrophilicity is at position 298. This position represents the average value for the hexapeptide Asp^{298}-Glu-Lys-Leu-Arg-Asp^{303}, which thus is predicted to constitute the antigenic site. Interestingly, this hexapeptide is located in the 'O' helix which makes the outermost boundary of the small domain (see Fig. 10 of Remington et al., 1982) and that exposed sequence is the place one would expect an antigenic site to be. This location is also consistent with the results of Bloxham et al.(1981) as the 37 K fragment (amino terminal 321 residues, Bloxham et al., 1982) which contains the proposed antigenic site was indeed reactive towards antisera. The site was probably destroyed by trypsin in the 36 K fragment, explaining the loss of immunological reactivity in this fragment. The hexapeptide has two potential sites for tryptic cleavage.

When similar predictions of an antigenic site are performed on E. coli citrate synthase (Figure 32), the site of maximum hydrophilicity is located at position 289, which means that the hexapeptide ^{289}Arg-Arg-Ala-Lys-Asp-Lys^{294} is predicted to be an antigenic site. Interestingly, this sequence is homologous with the proposed antigenic site in pig heart citrate synthase as can be seen in the homology diagram (Fig. 31).

Antibodies to E. coli citrate synthase have been found to be completely unreactive towards pig heart enzyme (Figure 29). This situation is

consistent with the fact that there is no actual sequence homology between the two enzymes at their predicted antigenic sites.

(iii) Secondary structure predictions:

In this section, the secondary structure of E. coli citrate synthase is predicted from the sequence by two methods, those of Garnier et al., (1978) and Chou and Fasman (1978).

The method of Garnier has been chosen because it is completely computerized and is based on a set of unambiguous rules. Four state assessments are made in which each residue is assigned one conformational state; the four possibilities are α -helix, β-sheet, random coil and turn. Kabsch and Sander (1983) reported that 56% of residue states are predicted correctly by this method using a three state definition (α,β, turn/random coil). The secondary structure of E. coli citrate synthase predicted by using Garnier's method is shown in Fig. 33A. The residues are aligned with the pig heart sequence according to the sequence homology diagram (Fig. 31) and the predicted structure of the E. coli enzyme is compared with the known structure of the pig heart enzyme as determined by X-ray crystallographic studies (Remington et al. 1982). These predictions indicate large differences between the two enzymes especially for helices B, C, D, H, J, L, R and T which are not found in the E. coli prediction, while helices E, M and S are rather poorly predicted for E. coli by the Garnier method. The correspondence between the two enzymes is not very convincing in spite of the fact that the sequences have been

aligned in such a way as to obtain maximum homology. These observations prompted me to investigate the efficiency of this method in predicting the known structure of pig heart citrate synthase. Surprisingly, only 42% of the residues are correctly predicted and the degree of success for the helical portion is even less, only 37% by the method of Garnier et al. (1978) as shown in Figure 33B. Thus, if the known structure is predicted rather poorly, no more success would be anticipated for an unknown structure.

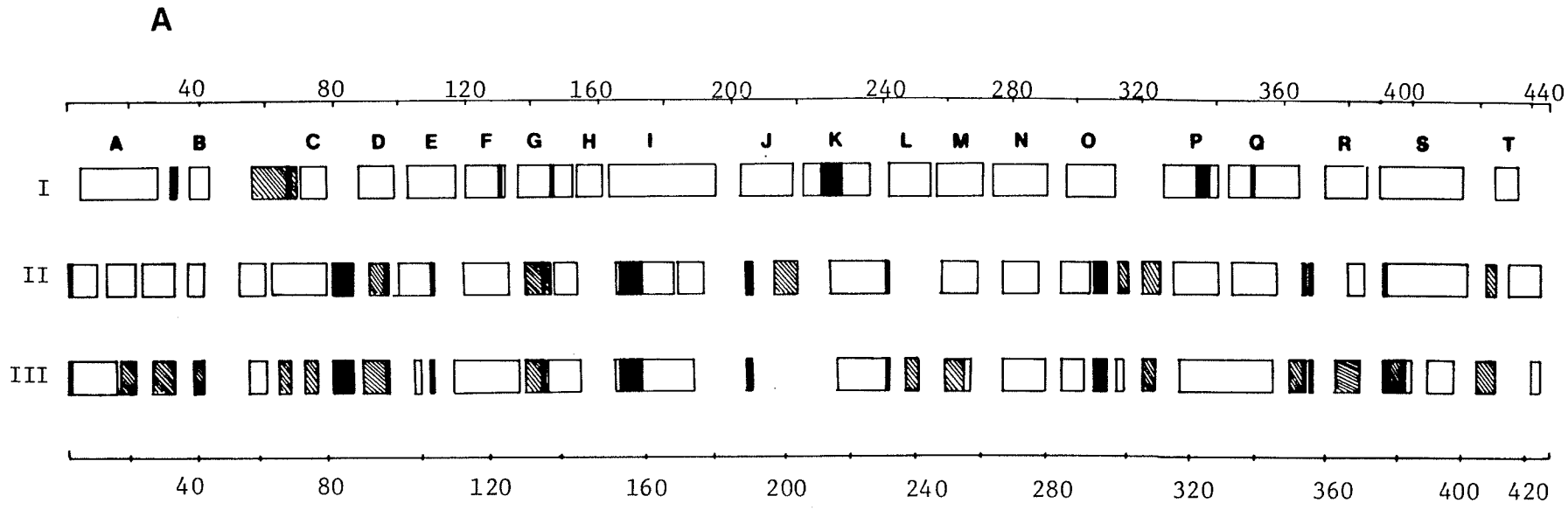
The Chou and Fasman (1978) method for predicting the secondary structure of a protein utilizes the conformational parameters P_α and P_β. These parameters represent helix (P_α) and βsheet (P_β) potentials of amino acids; their values have been determined by statistical analysis of 29 proteins with known conformations. The method utilizes empirical rules for predicting the helical, sheet, turn and coil regions in proteins. In an early report, an accuracy of 77% was reported for the three state conformations, helix, β-sheet and coil (Chou and Fasman, 1974). I used this method first to predict the secondary structure of pig heart enzyme. When the results were compared with the known X-ray structure, 68.4% of the residues in helical regions are correctly predicted. Most of the helices found by X-ray studies of pig heart enzyme were located in this prediction, except helix F which was not predicted and helices L, M and O which were poorly predicted.

The secondary structure of E. coli citrate synthase was also predicted by Chou and Fasman rules (Chou and Fasman,1978). It is quite obvious from

Figure 33 A. The prediction of secondary structure of Escherichia coli citrate synthase by Chou and Fasman (1978) and Garnier et al. (1978) as shown in row II and III respectively. In row I, the secondary structure as determined from from crystallographic studies is shown(Remington et al., 1982). The lay out of the residues is according to homology diagram (Figure 31); the bottom scale represents position of E. coli residues, while the scale on top shows the position of pig heart enzyme residues.

B. The secondary structure of pig heart citrate synthase. Row I shows the structure according to crystallographic studies (Remington et al., 1982) and II & III depict the structure as predicted by Chou and Fasman (1978) and Garnier et al.(1978). The scale is according to pig heart amino acid sequence (Bloxham et al., 1981).

▬ Gaps in the sequence (Figure 31), ▭ Helix, ▨ β-sheet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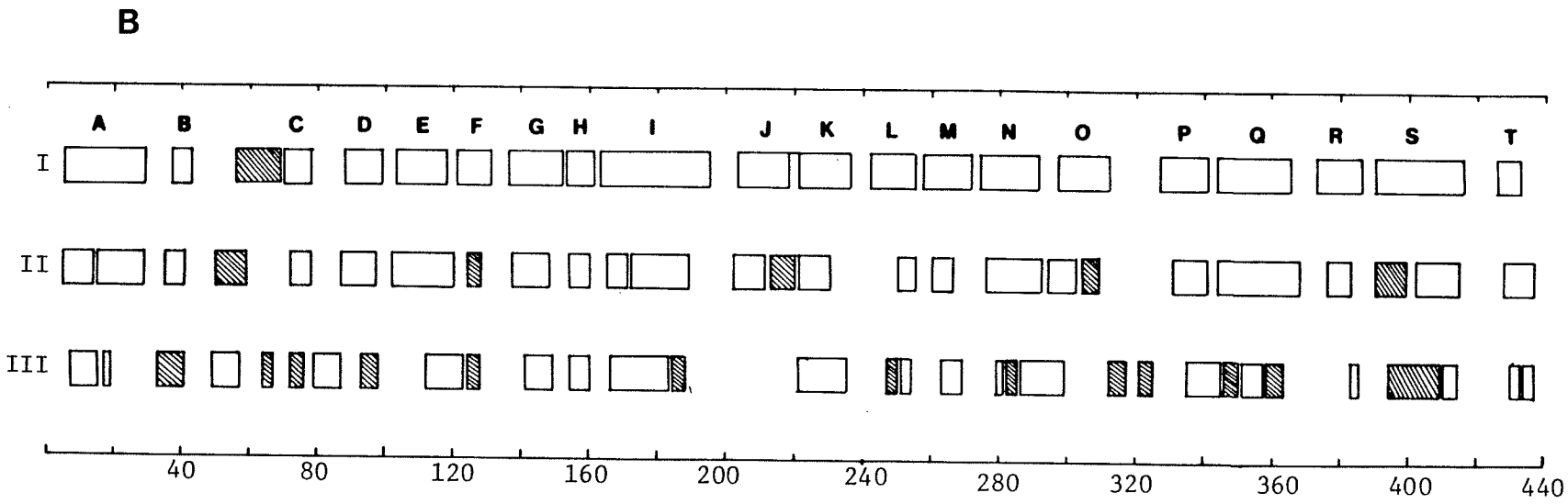
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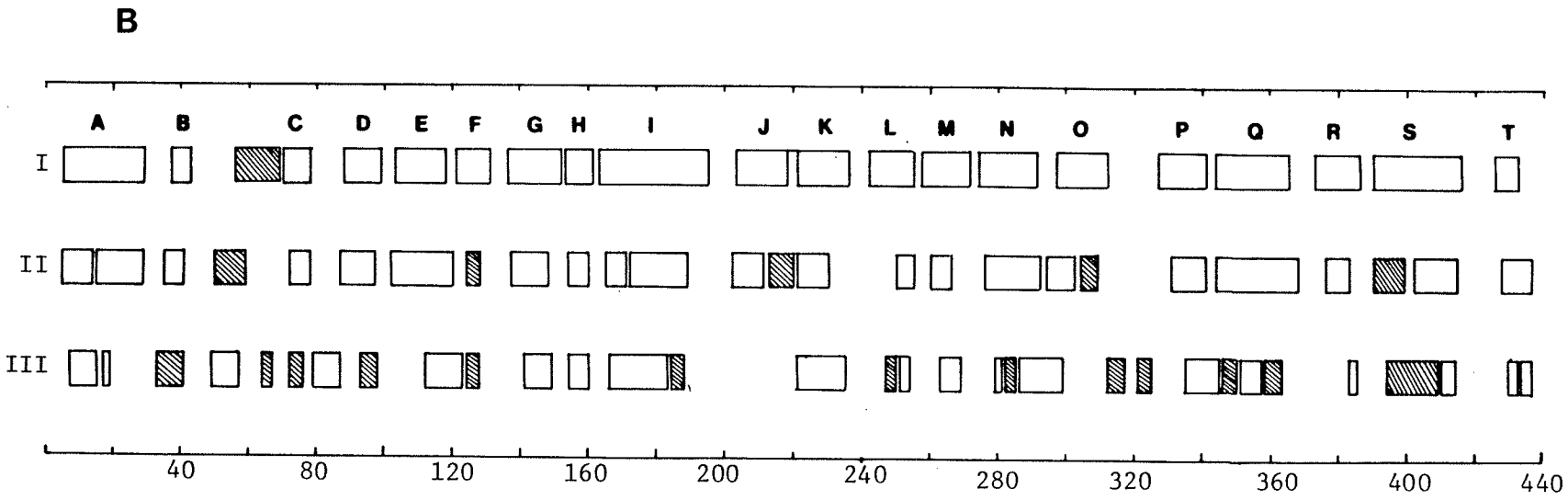
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Figure 33A that there are striking similarities between the predicted structure of this enzyme and the X-ray strucure of pig heart enzyme. The results predict that helix A is split into three small helices in E. coli citrate synthase; alternatively, it is possible that A is a single bent helix as in pig heart enzyme. The predicted helices I and S in E. coli have strong internal helical breakers, proline and glycine respectively, and thus they may be bent and resemble the corresponding helices in pig heart. Both of these helices are important features of the pig heart enzyme structure since they form the core structure of the dimer (Remington et al. 1982). In addition, helices B, E, F, K, M, N, O, P, Q and T are predicted to be almost identical in the two enzymes.

Apart from large similarities, a few apparent differences appear in the predictions. Thus, the β-sheet in pig heart enzyme is replaced by a helical structure in E. coli enzyme; the C helix is longer; the G and R helices are poorly defined and the D, H, J and L helices are not predicted at all.

In a recent analysis, the Chou and Fasman method has been completely computerized so as to avoid ambiguities arising because of bias in hand analysis (Nishikawa, 1983). An overall success rate of 58% has been found by the computer based method, which is comparable to that of other methods.

Location of The Extra Subunit Contact Surface in E. coli Citrate Synthase:

E. coli citrate synthase is an hexameric enzyme (Tong and Duckworth, 1975) as compared to the pig heart enzyme which is dimeric. It has already been suggested above from the sequence homology and secondary structure prediction studies that the dimeric structure is basically the same in the two enzymes, although the residues involved in subunit-subunit interactions are different. In addition to the subunit contact surfaces within dimers, the E. coli enzyme must have a second contact surface, presumably between dimers, to form the polymeric structure. One would expect these new contact surfaces to be hydrophobic. Therefore, I have attempted to locate areas of increased hydrophobicity in the E. coli citrate synthase sequence in comparison to heart enzyme. Hydrophilicity/hydrophobicity profiles for pig heart and E. coli citrate synthases are superimposed according to the sequence homology diagram, to identify the areas of increased hydrophobicity. The observations are discussed assuming that the tertiary structures of the two enzymes are similar and also making the rather large assumption that the sequence alignment shown in Figure 31, is valid.

As shown in Figure 32, the areas of higher hydrophobicity in the E. coli sequence include helices B, C, G (C-terminal portion), I (C-terminal portion), J, and N (C-terminal portion). Of these, B, I, G and N are involved in making the subunit contact surface within the dimer and thus are expected to be hydrophobic. Therefore, the C and J helices have the potential of being involved in dimer-dimer contact surfaces. The D helix, which is hydrophobic in both enzymes, appears to be slightly

buried (see Figure 10 of Remington et al., 1982). Since residues corresponding to the CD loop are missing in the E. coli enzyme sequence, it is possible that the D helix is closer to the C helix in this enzyme and a little above it, and that it becomes more accessible for making a new contact surface in this enzyme. It is quite probable that the areas surrounding the C, J, and D helices are different in E. coli, suggesting that these are likely to be involved in different functions in the allosteric citrate synthase.

Possible Location of Allosteric Site:

Chemical modifications of E. coli citrate synthase have been performed to determine the residues involved in allosteric and catalytic activity (Danson & Weitzman, 1973; Talgoy et al., 1979; Wright & Sanwal, 1971). There is general agreement over the finding that modification with the sulfhydryl reagent DTNB, to the extent of one TNB released, causes desensitization of the enzyme to NADH without substantial loss of activity. However, there are conflicting reports about the extent of activity loss upon DTNB modification (Talgoy, 1979). The DTNB modified enzyme spontaneously releases TNB by reaction with another protein sulfhydryl group to form an intrasubunit disulfide (Talgoy et al., 1979).

Modification of E. coli citrate synthase with another sulfhydryl reagent, PDS, is associated with complete loss of activity and NADH binding (Wright & Sanwal, 1971; Talgoy et al.,1979). The reagent reacts

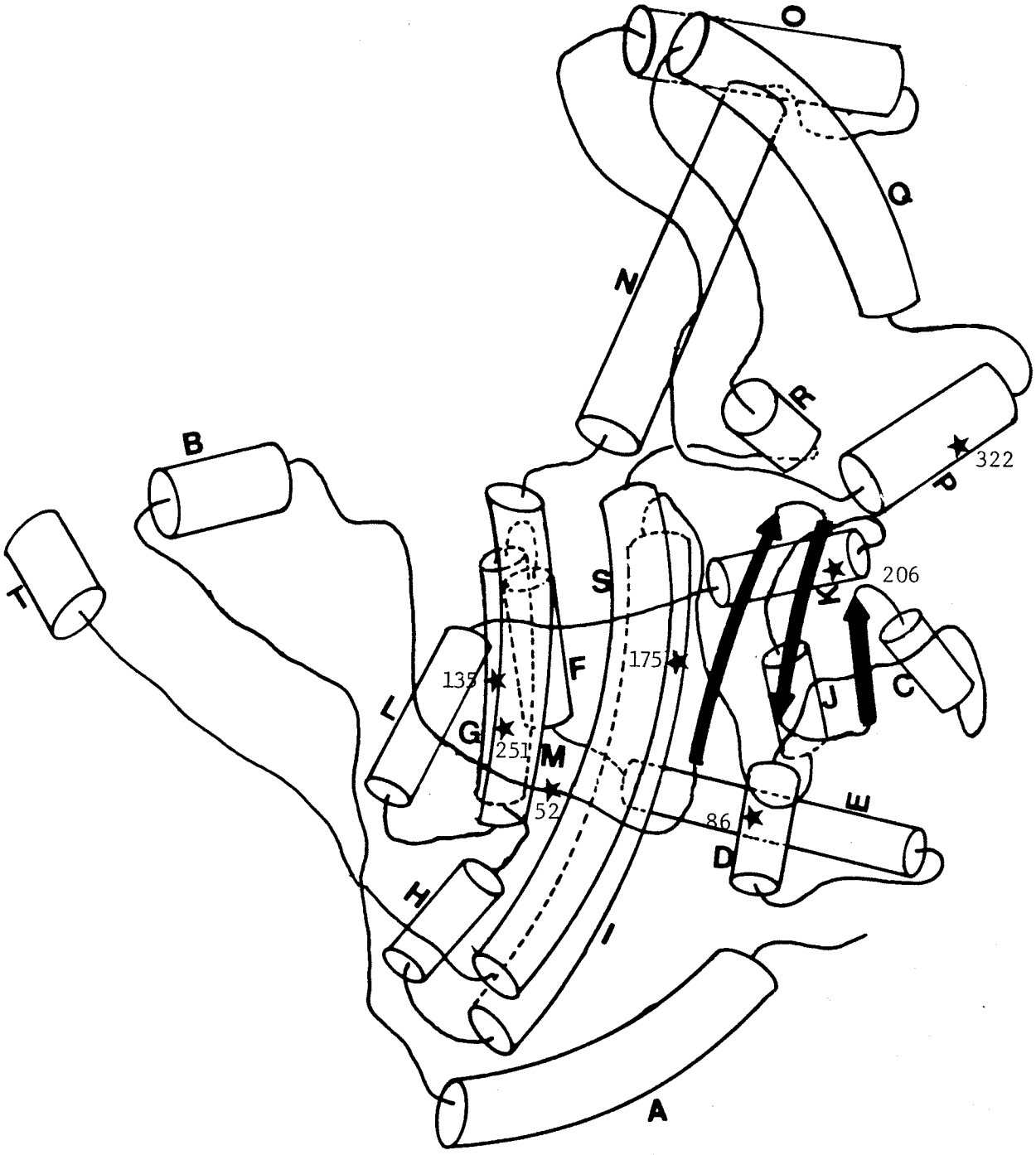
with two sulfhydryl groups per subunit at different rates. The more reactive SH is associated with inactivation and with spontaneous release of PT from the modified protein, again to form an intrasubunit disulfide (Talgoy et al., 1979). The effect of modifying the second cysteine with PDS could not be determined since the modification of the first cysteine caused complete inactivation and almost 89% loss of NADH binding sites (Talgoy et al., 1979).

Some evidence has been presented indicating that DTNB and PDS react with different cysteines while other evidence suggests that both reagents react with the same cysteine (Talgoy et al., 1979). The true relationship between DTNB and PDS reactive cysteines is thus uncertain.

There are two possible ways by which DTNB could inhibit NADH binding. One is that the DTNB modification may physically prevent NADH binding and the second possibility is that the modification may result in a conformational shift in the enzyme so that NADH cannot bind. Talgoy et al. (1979)have favored the first explanation for two reasons: (i) NADH (allosteric inhibitor) and a number of adenylic acid derivatives (slight activators), which are believed to bind at the allosteric site, have all been shown to prevent the DTNB reaction (Talgoy & Duckworth, 1979); (ii) both acetyl-CoA and KCl saturate the DTNB modified enzyme in a cooperative way as they do the native enzyme, indicating that the modification leaves the enzyme still in the T state and does not cause a conformational change.

From the preceding discussion, it is reasonable to believe that at least one cysteine is located in or near the allosteric site. Therefore,

Figure: 34 Positions of cysteine residues of E. coli citrate synthase in the tertiary structure of pig heart enzyme. The approximate position of the cysteine α-carbon (★) is indicated with reference to homology diagram (Figure 31) The residues are represented with E. coli citrate synthase numbers.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to guess the possible location of allosteric site it becomes necessary first to predict the locations of cysteine residues in the tertiary structure of the subunit. Figure 34 shows the positions of cysteines in the E. coli enzyme subunit, assuming that its folding resembles that of pig heart subunit. Pairs of cysteines which appear to have potential of forming disulfides are Cys-135 and Cys-251; Cys-206 and Cys-322; Cys-52 and Cys-251 or Cys-135; and Cys-135 and Cys-175. The pairs 135 & 251 and 52 & 251 or 135, are in a region predicted to be involved in dimeric subunit contacts, while 135 & 175 are in the core area of the contacts within the dimers. It seems unlikely that any of these are accessible to DTNB or PDS. Cysteine-206 and Cysteine-322 are in helices K and P respectively, and both of these are exposed on the surface of the subunit. The P helix also contributes residues to the active site; Arg-329 and Asp-327 (pig heart sequence) in this helix are invloved in citrate binding. It is possible that Cys-206 and Cys-322 are located in or near the allosteric site. This possibility is further strengthened by the finding that there are substantial differences between the allosteric and non-allosteric subunit along this region. According to the sequence alignement (Figure 31) the presumed K helix and P helix in E. coli subunit show extensions of 7 and 3 residues respectively. The insertion of these residues into the K and P helices in the E. coli citrate synthase subunit should produce significant structural differences from pig heart enzyme, perhaps creating an allosteric site.

The second major difference between E. coli and pig heart enzyme is

along the β-sheet region (corresponding to the residues 56 to 69 of pig heart). β-sheet was not predicted at all for E. coli in this region by the Chou and Fasman method, while it was poorly predicted by the Garnier's method (Figure33A). The third difference in E. coli enzyme subunit, again from the sequence alignment, is that the CD loop is apparently missing, a feature which may bring about tremendous changes in the tertiary structure along this region.

The above differnces between the E. coli and pig heart citrate synthase subunit are all clustered in one part of the subunit (Fig. 34) close to the K and P helices. A part of this region has already been suggested to be involved in forming dimer-dimer contacts, since it is more hydrophobic than the corresponding pig heart region, but this added hydrophobicity might also be needed for NADH binding.

Although the above findings identify a region which is structurally different in E. coli and which has the potential of being involved in allosteric site, it should be noted that these conclusions are based on rather large assumptions. The true picture should become clearer upon isolation and characterization of peptides containing DTNB or PDS reactive cysteines. The X-ray crystallographic studies, of course, should reveal the exact location of allosteric site.

The bimanes, another group of sulfhydryl reagents, react irreversibly with cysteines and the reaction products are fluorescent (Kosower et al., 1979). These reagents have been shown to affect the allosteric and catalytical properties of E. coli citrate synthase (Bell, 1983).

Furthermore, it has been found that the modification with monobromobimanes to the extent of close to one sulfhydryl group per subunit results in complete loss of sensitivity to NADH, but partial loss in activity, an effect which is similar to DTNB reaction (Duckworth, unpublished). The reactivity of this modified enzyme was reduced by about one equivalent towards DTNB and about 0.7 equivalent towards 4-4' PDS. Experiments are in progress in our laboratory to isolate and identify peptides containing bimane reactive cysteine(s).

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