THE UNIVERSITY OF MANITOBA

ESCHERICHIA COLI CITRATE SYNTHASE: CYANOGEN BROMIDE PEPTIDES AND EFFECT OF pH ON ALLOSTERIC KINETICS

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 dearest friend, Sukhdev.

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ABSTRACT

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E.coli citrate synthase has been subjected to chemical cleavage with cyanogen bromide and some of the resulting peptides have been isolated and analysed. The steady state kinetics of this enzyme have also been studied.

There are seventeen peptides which should be generated by cyanogen bromide cleavage of E.coli citrate synthase and of these, six have been isolated. Purification of these peptides involved gel filtration, ion exchange chromatography and counter current distribution. Citraconylation of peptide mixtures was necessary to avoid aggregation and solvation problems. Of the six peptides isolated, five appeared to contain blocked amino terminals. One of the six peptides was ninety residues long, contained threonine as amino terminal and did not contain tryptophan.

Steady state kinetic analysis of E.coli citrate synthase in the absence of KCl has led to the conclusion that the allosteric equilibrium displayed by this enzyme does not change with pH. The K_M values for acetyl-CoA decreased with a decrease in pH. Hill values revealed that higher concentrations of OAA do not effect the cooperativity of the acetyl-CoA saturation curves. At pH 6.56, no cooperativity was observed. The trend in K_{cat} values observed was similar to that observed by Bell (1978) in the presence of KCl. These observations indicate that the enzyme might follow similar reaction mechanisms in the presence and absence of KCl.

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LIST OF ABBREVIATIONS

AcCoA, acetyl-CoA	acetyl Co-enzyme A
AMP	adenosine monophosphate
BIS	N,N'-methylene-bis-acrylamide
C	centigrade
cm, mm, nm	centimeter, millimeter, nanometer
DEAE-cellulose	diethylaminoethyl cellulose
DTNB	5-5' dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetate
g, mg, µg	gram, milligram, microgram
HFBA	heptafluorobutyric acid
hr	hour
IAA	iodoacetic acid
log	logarithm
M, mM, µM	molar, millimolar, micromolar
min	minutes
M. Wt.	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced
OAA	oxaloacetate
SDS	sodium dodecyl sulfate
Tris	trihydroxymethylaminomethane
UV	ultraviolet

INTRODUCTION

INTRODUCTION

Primary structure or amino acid sequence determines the higher order structure of proteins. Knowledge of primary structure is essential for the definitive interpretation of data from experiments determining secondary and tertiary structure.

The first successful attempt at determining the complete primary structure of a protein was made by F. Sanger and his associates (1955) working on the peptide hormone insulin. This landmark in the field of protein chemistry resulted in the award to Sanger of the 1957 Nobel Prize for chemistry. The techniques and concepts developed by Sanger were further developed by the work of Hirs, Moore and Stein (1960) who determined the complete amino acid sequence of ribonuclease. These studies led to many other successful attempts at amino acid sequencing, as a result of which the primary structures of a large number of proteins have been reported in the literature. These reports have given insight at the molecular level to processes of concern to biochemists, biologists and geneticists.

Sequence determination studies are still carried out by classical multistep methods which involve determination of the molecular weight and amino acid composition as the first step. The proteins are then degraded to known numbers of peptide chains using specific chemical or enzymic cleavage, and these peptides are then isolated and purified. The complete sequence is determined by overlapping the sequences of these peptides.

The evolutionary relationships among species can be established by comparing sequences of proteins with identical biological functions. Using this approach evolutionary trees can be constructed such as the one reported by Schwartz and Dayhoff (1978) which uses cytochrome c sequences to

establish evolutionary distances between species.

The secondary structure of a protein can be predicted from its primary structure using approaches such as that proposed by Chou and Fasman (1974). This approach uses known probabilities of finding amino acid sequences in different secondary structures with varying degree of success and has been tested on several proteins using methods such as ORD and X-ray crystallography (Chou and Fasman, 1978). Although X-ray crystallography is still the definitive method of determining higher order structure of proteins, secondary structural predictions are useful in the recognition of structural domains in homologous sequences.

The classical methods of protein sequencing have been used more recently in conjunction with chemical modifications in attempts to gain insight into particular regions of proteins. The reactivity of given amino acids in the native state with chemical modification reagents such as structural analogues of substrates or specific inhibitory reagents has provided information about the position of those amino acids in the protein (Dayhoff, et al., 1972). Frequently the amino acid sequence at and around the active site of distantly related proteins is conserved during evolution.

Citrate Synthase

Citrate synthase (E.C.4.1.3.7) is a key enzyme in the tricarboxylic acid cycle. Metabolites are oxidized by this central metabolic pathway to NADH and $FADH_2$ which are converted to ATP through coupling with oxidative phosphorylation. This cycle also provides metabolites for biosynthesis. The regulatory effects of citrate synthase have been widely studied and reported in the literature (Weitzman and Danson, 1976; and Srere, 1972). This enzyme

might be involved in controlling the rate of entry of carbon into this cycle, at least in some organisms.

Citrate synthase catalyzes the condensation of acetyl Co-enzyme A and oxaloacetic acid to form citrate:

$$AcCoA + OAA^{2-} + H_2O \quad === \quad Citrate^{3-} + CoA + H^+$$

This reaction is unique in the tricarboxylic acid cycle because it is the only reaction involving the condensation of two carbon compounds in this cycle. It is significant that although the reaction is a chemical event requiring an input of energy, the overall reaction is energy-yielding ($\Delta G^{\circ\prime}$ = -9 kcals; Johnson, 1960).

Faloona and Srere (1969) reported that the mechanism of E.coli citrate synthase is sequential with either random or ordered addition of substrates. As CoA binding studies on E.coli citrate synthase carried out in the absence of OAA by Wright and Sanwal (1971) and Duckworth and Tong (1976) suggest that AcCoA is the preferred first substrate in an ordered mechanism. Johansson and Petterson (1974a) have shown by gel equilibrium diffusion studies that the catalysis of pig heart citrate synthase proceeds by a mechanism which does not require a fixed order of addition of substrates. However, these authors (Johansson and Petterson, 1974b) have also reported other kinds of experiments which suggest that the mechanism is ordered with OAA as the first substrate to bind.

Studies on citrate synthase have been carried out on extracts from various aerobic, facultative anaerobic bacteria (Weitzman and Jones, 1968), strictly anaerobic bacteria (Gottschalk, 1968), higher plants (Sarkissian, 1970) and fungi (Flavell and Fincham, 1968; Kobr et al., 1969). Citrate synthase has been purified from many of these sources and molecular weights have been determined. The molecular weight information led Weitzman and

Danson (1976) to divide citrate synthases into two molecular types: "large" and "small". Mammalian citrate synthases are "small" dimers with molecular weights around 100,000 while bacterial citrate synthases are "large" multimers with molecular weights in the range of 220,000-300,000. All citrate synthase subunits are seen to have similar molecular weights (Weitzman and Danson, 1976; Tong and Duckworth, 1975; Srere, 1972; Higa et al., 1978; and Köller and Kindl, 1977).

Weitzman and Jones (1968) reported three kinds of citrate synthases on the basis of their regulatory properties: (a) NADH inhibited, (b) NADH inhibited, but NADH inhibition reversed by AMP, (c) ATP inhibited. Citrate synthases from the Gram-negative facultative anaerobic bacteria belong to class (a); from the strict anaerobes belong to class (b); from Gram-positive and eukaryotic organisms belong to class (c); with some exceptions as outlined by Weitzman and Danson (1976).

NADH inhibition of citrate synthase from E.coli, a Gram-negative bacterium, was indicated to be allosteric, pH dependent and abolished in the presence of 0.2M KCl (Weitzman, 1966). Wright and Sanwal (1971) and Faloona and Srere (1969) obtained sigmoidal substrate saturation curves for AcCoA in the absence of KCl, which is regarded as an allosteric activator of E.coli citrate synthase (Duckworth and Tong, 1976). It has also been shown that NADH binding to this enzyme is a function of pH. The binding is tight at low pH and weakens progressively as the pH is increased (Duckworth and Tong, 1976). This observation can be explained by assuming that the NADH binding is dependent upon the protonation of an acid dissociable group of pKa of 7.08. Using stopped flow fluorescence studies, Sadar et al. (1977) have confirmed that the dissociation of NADH-enzyme complex is pH-dependent. They have also shown that KCl increases the rate of dissociation

of the NADH-enzyme complex, but does not affect the rate of complex formation.

In a previous study from this laboratory, Bell (1977) studied the effect of pH on steady state kinetics of E.coli citrate synthase in the presence of KCl. This author observed the cooperativity in AcCoA saturation curves at pH 6.6, even in the presence of KCl, an increase in K_{cat} values with increasing pH, and a pH optimum of 8.0.

One of the studies reported in this thesis is an extension of Bell's work to the steady state kinetics in the absence of KCl.

Introduction to the Sequencing Work

As previously mentioned, citrate synthases from different sources have the same subunit size and similar subunit structure (for references see above). It is very likely that the difference in their functional properties is the result of their subunit interactions. Although no sequence information has yet been published on any citrate synthase, there are some indications that the enzymes from different sources have only a limited number of amino acid differences. Such differences must be the reason for their different catalytic and regulatory properties.

There have been several studies to date that have inferred that a limited number of amino acid substitutions in citrate synthase will result in very different regulatory and catalytic properties. Peptide mapping experiments on E.coli and pig heart citrate synthase (Singh et al. 1970, Wright and Sanwal, 1971) have led to the conclusion that subunits of these enzymes have the same or very similar amino acid sequence. One estimate of the probable amount of homology between E.coli and pig heart citrate synthase suggests that 30% of the residues in these two molecules are

identical (Morse and Duckworth, 1980). As would be expected, these enzymes differ in both their multimeric forms and their allosteric properties. A comparison between Acinetobacter anitratum and E.coli citrate synthases by Morse and Duckworth (1980), however, has shown that while these bacterial enzymes are quite different in their functional properties, there is a high degree of homology. E.coli citrate synthase is more sensitive to NADH inhibition and DTNB reaction than the A. anitratum enzyme. NADH inhibition of A. anitratum citrate synthase is reversible by AMP, but that of E.coli citrate synthase is not. Despite these differences in their functional behavior, these enzymes share antigenic determinants, have good similarities in their amino acid compositions and, as judged by the statistical method of Cornish-Bowden (1978), appeared to have 90% sequence homology. While these studies indicate that substitutions of amino acids at key positions in a protein result in significant evolutionary and functional consequences, direct comparison of the complete amino acid sequences is necessary to identify more closely the nature and consequences of these substitutions.

The use of sequencing in chemical modification studies could be a valuable technique in showing the relationship of amino acids with a known topology. It is also possible to locate and compare specific modified peptides from different sources. Danson and Weitzman (1973) have used photo oxidation, diethylpyrocarbonate modification and sulfhydryl reagents to locate histidine and cysteine residues in E.coli citrate synthase. These authors concluded that the histidine residues are involved in catalytic activity and α-keto-glutarate inhibition and that cysteine residues are involved in NADH inhibition. Talgoy et al. (1979) have extended the work on cysteine modification and have proposed the presence of one cysteine

residue close to the allosteric site of E.coli citrate synthase, while at least one other cysteine residue may be located in or close to the active site. The relationship between these cysteine residues is not fully understood, but the amino acid sequence of E.coli citrate synthase will lend insight into this problem.

As stated earlier, the secondary and tertiary structure is determined by the primary structure of proteins, and so the amino acid sequence of E.coli citrate synthase will help in determining the secondary structure and the folding patterns of this enzyme. This will also help future X-ray crystallographic studies to determine the location of the amino acid side chains.

The sequence of no coenzyme A-utilizing enzyme has yet been reported. The attempt to determine amino acid sequence of E.coli citrate synthase is a step in this direction.

In the work described in this thesis, chemical cleavage with cyanogen bromide of E.coli citrate synthase was performed to generate peptides. These peptides were subjected to various methods of peptide isolation in attempts to purify them. N-terminal and amino acid analysis of the peptide fractions were also performed. Although little success was achieved in isolating and purifying the seventeen expected cyanogen bromide peptides due to the difficulties faced in solvation and aggregation of the peptides, the results presented here will be helpful in future attempts to determine the complete sequence of this enzyme.

MATERIALS AND METHODS

MATERIALS

Protamine sulfate, trizma (Tris), iodoacetic acid, SDS, DNAse were obtained from Sigma; oxaloacetate was from Boehringer; acetyl-CoA was from P-L Biochemicals; Ellman's reagent (DTNB) was from Aldrich; ammonium sulfate enzyme grade and GuHCl were from Schwarz/Mann; ^{14}C-IAA and ACS scintillation cocktail were from Amersham/Searle; DEAE-cellulose, preswollen microgranule (DE 52) was from Whatman; Ninhydrin was from Pierce, Sephadex and SP-Sephadex were from Pharmacia Fine Chemicals; cyanogen bromide was from J. T. Baker Chemical Co.; Polyamide Sheets were from Cheng Chin Trading Co. Ltd., Taiwan; reagents for the sequencer were supplied by Beckman. All other chemicals used were reagent grade.

Organism

Citrate synthase was purified from E.coli K12 3000 which was grown by the following procedure.

Media

Stock LB Medium is a solution containing

Bacto tryptone	1.0%
Yeast extract	0.5%
NaCl	1.0%
1M NaOH	1.0 ml/liter
Water to volume	

Stock LB Medium is autoclaved at 121 °C for 20 minutes before use.

Minimal Medium A is a solution containing

K_2HPO_4	10.5 gm/liter
KH_2PO_4	4.5 gm/liter
$(NH_4)_2SO_4$	1.5 gm/liter
$MgSO_4 \cdot 7H_2O$	0.1 gm/liter
Water to volume	

The minimal acetate medium A also contains 1% sodium acetate and 0.001% thiamine in addition to the above components.

Growth of Organism

Growth was initiated by inoculating 2x10 ml LB medium with a loop full of E.coli K12 3000 strain. These two tubes were grown overnight and were used to inoculate 2x250 ml minimal acetate medium A and, after growth overnight, these were combined to inoculate 1x15 liter minimal acetate medium A in a carboy. This carboy was grown overnight with good aeration and then used to inoculate 4x15 liter minimal medium A in carboys.

After growth overnight, the cells were harvested using a Sharples continuous flow centrifuge.

At each stage the culture was allowed to grow overnight at 37 °C with sufficient agitation to ensure good aeration. A typical yield is about 210 grams of wet E.coli cells from the final four carboys.

At each stage of growth, the purity of culture was checked. Samples were plated on LB plates using sterile techniques and were also streaked on λ, T_6 and T_7 phage plates which are specific for E.coli. Visual inspection of the LB and phage plates indicated that our preparations were essentially pure.

For later experiments, cells grown in a 200ℓ fermenter at the Department of Biochemistry, University of Alberta, were used.

Enzyme Purification

The method of enzyme purification as described by Weitzman (1966) with the modifications as described by Tong and Duckworth (1975) was used.

The harvested cells were suspended in 5 times the wet weight of cells, of 20 mM Tris-Cl buffer containing 1 mM EDTA, pH 7.8 and were stirred for a few hr at 4 °C. The cells were then broken by one passage through a 40 ml Aminco French Pressure cell at a cell pressure of 18,000 pounds per square inch while keeping the cells and extract in ice. The broken cell suspension was treated with a small amount of DNAse and stirred for 15 min at 4 °C.

The DNAse treated cell suspension was centrifuged for one hr at 20,000 rpm in a Sorvall RC2-B ultracentrifuge using an SS-34 head. The supernatant was collected and to this 0.42 ml of 2% protamine sulfate was added, for every gram of cells wet weight. This addition was carried out over a

period of 20 min with constant stirring. The solution was centrifuged again as described above and the supernatant was saved.

To the supernatant sufficient solid ammonium sulfate was slowly added over a period of 15 min to achieve 55% saturation and the mixture was allowed to stir for an additional 15 min at 4 °C. The solution was then centrifuged at 6,000 rpm in the RC2-B, in a GSA head for 30 min. The pellets were discarded. The supernatant was then made 70% saturated in ammonium sulfate by slowly adding a sufficient amount of solid ammonium sulfate over a period of 15 min. After stirring for an additional 15 min the solution was centrifuged for 30 min at 6,000 rpm (GSA head) and the pellets were saved. The pellets were dissolved in a minimum amount of standard buffer containing 50 mM KCl and the solution was layered on the top of a Sephadex G-25 (coarse) column (6x32 cm). The column was eluted with standard buffer containing 50 mM KCl. Fractions of about 20 ml size were collected and their OD_{280} and conductivity were measured. Fractions with conductivity lower than 10 mho were pooled together and the entire pool was applied to DEAE-Cellulose column (2.5x23 cm) which had been previously equilibrated with standard buffer containing 50 mM KCl. The column was washed overnight with standard buffer containing 50 mM KCl and then a linear gradient of KCl from 50 mM to 300 mM in standard buffer, total volume 2 liters, was applied to elute the column. Fractions of about 20 ml were collected and those with citrate synthase activity of 2 U/ml or more were pooled and concentrated to less than 3 ml by ultrafiltration. This concentrated solution was applied to a Sephadex G-200 column (2.8x80 cm) and eluted with standard buffer containing 50 mM KCl at 4 °C. Fractions

with specific activity above 30 U/mg were pooled and concentrated as above. The concentrated solution contained 270-280 mg of protein at a specific activity 70 U/mg and SDS gel electrophoresis results revealed that it was pure.

Standard Buffer

The standard buffer used throughout the enzyme purification is 20 mM Tris-Cl, 1.0 mM EDTA pH 7.8.

Enzyme Assay

One unit of enzyme is defined as the amount of enzyme which is necessary to catalyse the reaction:

acetyl-CoA + oxaloacetate = citrate + CoA

at the rate of 1 μmole of product formed per min under standard assay conditions. The enzyme was assayed by the method of Srere et al. (1963) involving the measurement with DTNB of the appearance of the free sulfhydryl group of co-enzyme A. The assay mixture contains 0.1 mM AcCoA, 0.1 mM OAA, 100 mM KCl, 1 mM EDTA and 0.05 mM DTNB and 20 mM Tris-Cl pH 7.8, in a final volume of 1 ml. A sufficient amount of enzyme is added such that the steady state is maintained for at least 10 min at room temperature. These conditions define the standard assay.

Steady State Kinetic Studies

For the pH studies KCl was omitted; amount of acetyl-CoA and oxaloacetate were varied as stated, and the buffer was either 20 mM Tris-HCl (pH 7.72 and 8.37) or 15 mM sodium phosphate (pH 6.56) containing 1 mM EDTA. The same amounts of enzyme were used to determine the effect of pH on the steady state kinetics using the assay mixtures described above.

Enzyme concentration was determined by the standard assay at pH 7.8.

Enzyme was added to the cuvette only after substrates and reagents showed no change in absorbance at 412 or 232 nm. As alkaline hydrolysis of DTNB presents difficulty at higher pH's (Bell, 1978), enzyme activity at pH 8.37 was determined by measuring the disappearance of acetyl-CoA at 232 nm.

Reduction and Carboxymethylation

Reduction of any disulfide bonds was carried out in a solution containing 6M guanidine hydrogen chloride, 0.4M Tris-base and 0.1M DTT. The pH of the solution was adjusted with concentrated HCl to 8.2 and the solution was incubated for 4 hr at 60 °C in a water bath and then cooled to room temperature. Carboxymethylation of cysteinyl residues was done by adding to the reduced protein solution an amount of IAA-^{14}C equimolar to sulfhydryl groups present in solution. Total sulfhydryl groups represented were the sum of sulfhydryl groups present in DTT and in the protein. Total sulfhydryl groups were determined using formula [1].

$$\text{Total moles of -SH} = \left(2^{*} \times [\text{DTT}] \times \frac{\text{ml of denaturing solution}}{10^{3}}\right) + \left(7^{\dagger} \times [\text{protein}]^{\oplus} \times \frac{\text{ml}}{10^{3}} \times \frac{1}{\ddagger 47 \times 10^{3}}\right) \quad \ldots\ldots\ldots\ldots [1]$$

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*There are two sulfhydryl groups per DTT molecule.

†There are seven cysteine residues per mole of protein.

‡Molecular weight of E.coli citrate synthase according to Tong and Duckworth (1975).

⊕Protein concentration was determined from absorbance at 278 nm, using $\varepsilon = 9.76$.

The reaction was allowed to proceed in the dark at room temperature for 1 hr and then terminated by adding a pinch of solid DTT. The solution was dialysed against 4 liters of deionized water for 20-24 hr at 4 °C. The carboxymethylated protein which precipitated was collected and lyophilized.

Cyanogen Bromide Cleavage

The cyanogen bromide reaction was carried out as described by Steers, Craven and Anfinsen (1965). Lyophilized, reduced and carboxymethylated protein was dissolved in 70% formic acid solution to give a final concentration of about 20 mg/ml. A 50-fold molar excess over methionine of crystalline cyanogen bromide was added and the reaction was allowed to proceed at room temperature. After 20 hr, the reaction was stopped by diluting the solution with five volumes of deionized water. Excess reagent was removed by lyophilization and amino acid analysis was used to determine the extent of the reaction.

Radioactive Counting Procedure

A 10-200 µl aliquot of the sample was mixed in a clean scintillation vial with 5 ml of the ACS scintillation cocktail, placed in a Packard Tri-Carb Liquid Scintillation Counter, Model 3003 and allowed to cool. The counting was carried out so as to maximize counts in the [^{14}C] channel.

Amino Acid Analysis

Of the total peptide solution, 5% was analyzed to determine amino acid composition.

The peptides were hydrolyzed in 1 ml of constant boiling 6N HCl in evacuated and sealed tubes at 105 °C for 20-24 hr. The hydrolyzate was dried under vacuum over NaOH pellets, dissolved in 1 ml of deionized water and applied to the column of the Technicon NC-2P Automatic Amino Acid Analyzer.

Gel Filtration

Gel filtration chromatography was used to fractionate the peptide mixture at room temperature on columns of Sephadex of appropriate porosity using appropriate eluants. Fractions of suitable size were collected and analysis was performed by monitoring absorbances at 280 and 230 nm, radioactivity and ninhydrin positive material.

Ion Exchange Chromatography

Ion exchange chromatography was performed at room temperature using Whatman DE-52 resins. Columns were eluted with a NH_4HCO_3 gradient of increasing ionic strength and the fractions were analysed by monitoring absorbances at 280 and 230 nm and radioactivity.

Ninhydrin Analysis

A method described by Hirs et al. (1956) utilizing ninhydrin analysis with alkaline hydrolysis was routinely used to analyse effluent fractions obtained from gel filtration and counter current distribution. Sample aliquots of 0.3 ml were mixed with 1.0 ml of 2.5M NaOH in Pyrex test tubes which had been cleaned by boiling 3 hr in 2% Alcojet solution. Duplicate blanks were prepared using eluant buffer rather than effluent samples in the procedure. The tubes were then placed, unstoppered, in an open water bath at 90 °C for 2.5 hr. To each of the cooled tubes, 1.0 ml of 30% acetic acid was added in order to bring the final solution to pH 5.0. After the tubes were shaken vigorously, ninhydrin analysis was performed by adding 1.0 ml of ninhydrin reagent to each tube and mixing vigorously. Ninhydrin reagent contained 20.0 g ninhydrin, 100 mg ascorbic acid, 250 ml 4N sodium acetate buffer and 750 ml 50% Methyl Cellosolve prepared shortly before use. The tubes were then incubated for 20 min in a vigorously boiling covered water bath and then diluted with 5.0 ml of 50:50 ethanol-water diluant.

After dilution the tubes were kept away from direct light and cooled to 30 °C in front of an electric fan. The tubes were read on a Gilford Recording Spectrophotometer, Model 2400-2 at 570 nm with the instrument set so that the blank read zero.

Urea Gels

Urea gels were used to determine the purity of the isolated peptides. The procedure of Laboy, Cox and Flaks (1964) was followed and gels containing 10% acrylamide, 0.15% BIS and 8M urea were prepared and loaded with about 100 µg of the peptide samples previously mixed with 5 µl of tracking dye, pyronine G, a drop of glycerol and 5 µl of mercaptoethanol. Electrophoresis was performed in a Shandon Disc Electrophoretic Apparatus cooled with circulating tap water for 90-110 min at a constant current of 3mA/tube. Running buffer was β-alanine-acetate buffer pH 4.5 and migration was toward the cathode.

The gels were removed from tubes, stained in Coomassie blue for 2 hr and destained by diffusion according to Weber *et al.* (1972).

SDS Gel Electrophoresis

The method described by Weber *et al.* (1972) was followed using 7.5% gel concentration. Standards used to draw a calibration curve for M. Wt. determination included *E.coli* citrate synthase, ovalbumin, cytochrome C, lysozyme, hemoglobin (sheep), glyceraldehyde 3 phosphate dehydrogenase and catalase.

All the standards and sample (CNBr I) were treated with 1% SDS and 1% mercaptoethanol in boiling water for 2 min and left overnight at room temperature. Gels of 7.5% acrylamide concentration were poured and left overnight at room temperature. The samples were mixed with 5 µl Bromo-

phenol Blue (tracking dye), 5 µl of mercaptoethanol, a drop of glycerol and layered gently on top of gels. Electrophoresis was performed in a Shandon Disc Electrophoresis Apparatus by passing a constant current of 8mA/gel until the tracking dye reached the bottom of the gels.

The running buffer was 0.1M phosphate buffer, pH 7.2, and the migration was toward the anode. The gels were removed from the gel tubes and stained with Coomassie blue overnight at room temperature. Gels were destained by diffusion in a solution containing methanol:glacial acetic acid:deionized water (50:75:875 V/V).

Counter Current Distribution

To resolve the mixture of cyanogen bromide peptides the method of counter current distribution was used as described by Craig (1960). It was performed on an Automatic Quickfit Steady State Distribution Machine equipped with a train of 100 glass cells. It has a standard lower phase volume of 25 ml and a variable volume of top phase (25 ml in this case), which could be added after each transfer. The solvent system used was 2-butanol:0.5M acetic acid:10% trichloroacetic acid (400:500:10 V/V). Trichloroacetic acid was used to increase the solubility of the peptides in the organic phase. The solvent system was mixed on a floor shaker for 15 min and left at room temperature overnight in order to equilibrate the two phases. Before loading the sample the train was loaded with lower phase.

The sample to be subjected to counter current distribution was dried and dissolved by stirring in the 1:1 mixture of two phases. After dissolving it was left overnight to ensure equilibration of two phases. The undissolved material at the interface was discarded, sample in the lower phase loaded

into the emptied first two cells of the train and then topped with 25 ml of upper phase. The sample in the upper phase was loaded into the next two cells of the train. Upper phase was added to the 10 cells ahead of the sample to ensure equilibration of two phases.

The machine was run for 95 transfers using 30 sec agitation time and 15 min settling time. After 95 transfers the samples from the machine were removed by a syringe fitted with a long Teflon tube and were analysed by ninhydrin analysis and absorbances at 280 nm.

High Voltage Paper Electrophoresis

High voltage paper electrophoresis in combination with paper chromatography in the second dimension was used to separate certain peptide mixtures. Electrophoresis was performed in a Savant Flat Plate Electrophoresis Apparatus for 45 min at 40 V/cm on Whatman No. 3 paper using 10% formic acid buffer pH 1.9. After electrophoresis the paper was dried and descending chromatography was carried out for 10 hr using the solvent system n-butanol/pyridine/acetic acid/water (90:60:18:72 V/V). The chromatogram was dried, sprayed with ninhydrin-collidine solution (absolute ethanol 600 ml, glacial acetic acid 200 ml, collidine 80 ml and ninydrin 1 gm) and developed at 80 °C to locate the peptides.

Citraconylation

The citraconylation procedure described by Atassi and Habeeb (1972) was followed. Freeze-dried peptide mixture was dissolved in 200 ml of 0.2M $NaHCO_3$ solution and the pH was adjusted to 8.2 with 10M NaOH solution. Aliquots of 100 µl citric anhydride were added at 30 min intervals while the solution was stirred with a magnetic stirrer. A total of 800 µl citraconic anhydride was added at room temperature and reaction was allowed to proceed

for another 2 hr by constant stirring at pH 8.2. During this entire time, the pH was monitored by a Radiometer pH Meter 26 and a pH of 8.2 was maintained by the addition of 10M NaOH. The solution was then centrifuged using a bench centrifuge and the supernatant was applied to a Sephadex G-15 (fine) column (4.5x23 cm) and eluted with 0.2M NH_4HCO_3 to get rid of excess reagent.

Amino Terminal Analysis

The dansyl method of Gray (1974) was used in combination with thin layer chromatography of the derivatives as reported by Woods and Wang (1967). To perform the analysis a 5% aliquot of the total peptide solution was delivered into a hydrolysis tube with the help of a fine tipped micropipette, dried in vacuo, dissolved in 15 µl of 0.2M NH_4HCO_3 solution and dried again in vacuo. The peptide was then dissolved in 15 µl of deionized water in order to adjust the pH to 8.5 - 9.0 if necessary. After checking the pH, 15 µl of dansyl chloride solution was added and the tube was covered with Parafilm. The dansylation reaction was carried out by incubation at 37 °C for 1 hr.

Dansylated peptide was then hydrolysed in 100 µl of 6N HCl in an evacuated and sealed tube at 105 °C for 6-18 hr. The hydrolysate was dried, and the N-terminal amino acid was extracted with 20 µl 50% pyridine or 20 µl of water saturated ethyl acetate. The N-terminal amino acid was identified as its dansyl derivative by two dimensional thin layer chromatography on Cheng Chin Polyamide layer sheets as described by Woods and Wang (1965). The first dimension involved the solvent system water/90% formic acid (200:3 V/V) and the second dimension involved first the solvent 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

identified by comparison of their positions on the two dimensional thin layer chromatograms with those of standard dansyl amino acids.

Automated Edman Degradation

Automated Edman degradations were performed on a Beckman Sequencer Model 890C using one of the following programmes: DMBA peptide (Cat. No. 120171) or Quadrol (Cat. No. 072172) as supplied by Beckman. The initial step of the programme was HFBA treatment which denatures the sample. DTT (15 mg/liter) or ethylmercaptan (0.1% V/V) was added to the butyl chloride in order to protect threonine and serine residues. When less than 2 mg of protein was added to the cup, 2 mg of Polybrene were also added to obtain a better sample film. Before loading the protein sample was dialysed overnight againt 0.1M NH_4HCO_3. Peptides were decitraconylated by incubating at 40 °C for 4 hr in 400 µl of 5% acetic acid and were loaded in this solution. Peptide or protein samples of 100-200 nmoles were loaded in a volume of 0.4 - 0.6 ml and were dried using the "sample application sub-routine" (Beckman Cat. No. 02772). Sample concentrations were determined by amino acid analysis of 20 µl aliquots. For an internal standard, a known amount of PTH-norleucine (about 50 nmoles) was added to each fraction collected.

The PTH-derivatives were identified by a combination of gas chromatography and amino acid analysis as described below:

1) Half of the BuCl extracts were transferred to hydrolysis tubes and were taken to dryness. The residue was hydrolysed in constant boiling 5.7N HCl containing 0.1% $SnCl_2$ at 150 °C for 4 hr in vacuo. The hydrolyzate was then dried under vacuum and redissolved in a known amount of deionized water for amino acid analysis. Most of the amino acids were recovered in

good yield although threonine is recovered as α-aminobutyric acid; serine and cysteine as alanine, tryptophan as glycine and alanine, glutamine as glutamate and asparagine as aspartate.

2) The other half of the BuCl extracts was taken to dryness at 50 °C under a N_2 stream and the residue was heated in 200 µl of 1M HCl containing 0.1% ethylmercaptan at 80 °C for 10 min to convert 5-anilinothiazolinones to PTH's. PTH-amino acids were extracted twice with 0.70 ml of ethyl acetate, and the extracts were taken to dryness. The residues, dissolved in 30 µl ethyl acetate, were analyzed on a GLC equipped with an SP 400 column. The column was run 10 min at 200 °C followed by a linear increase in temperature to 250 °C, at 8°/min.

Initial coupling and repetitive yields were calculated from amino acids such as ala, leu, val and gly which are known to be recovered quantitatively by amino acid analysis after back hydrolysis. The initial coupling obtained for E.coli citrate synthase was 46% although 81% coupling has since been obtained (Duckworth, personal communication) on a fresh preparation of this enzyme.

RESULTS

RESULTS

N-terminal amino acid sequence determination of E.coli citrate synthase was performed with several objectives in mind. First, the purity of the protein preparation can be confirmed. Second, the number of different kinds of subunits in a multimeric protein can be determined. Third, the N-terminal amino acid sequence is necessary in identifying the N-terminal peptide generated by chemical cleavage.

N-Terminal Sequence

The N-terminal amino acid sequence of E.coli citrate synthase has been determined up to 15 residues and is reported in Table I. In a previous report (Tong and Duckworth, 1975), dansylation of E.coli citrate synthase indicated that the N-terminal amino acid is methionine. Edman degradation of this protein indicates that the N-terminal amino acid is alanine. This discrepancy may be explained by the difficulties inherent in identifying the dansyl amino acids, when working with a large protein. The results obtained here indicate that there is a unique 15 amino acid sequence at the N-terminal of E.coli citrate synthase which is consistent with the previous reports (Weight and Sanwal, 1971 and Tong and Duckworth, 1975) that E.coli citrate synthase subunits are all of the same size. This sequence does not contain methionine and thus the minimum length of the N-terminal cyanogen bromide peptide would have to be 16 residues.

Cyanogen Bromide Cleavage

Disulfide bonds of 5.85 μmoles purified citrate synthase from E.coli K12 3000 strain were reduced, and cysteinyl residues were then carboxymethylated with [^{14}C]-IAA, as described in Methods. Reduced and carboxymethylated protein was then lyophilized after dialysis against 4 ℓ of deionized water overnight

TABLE I

Amino Terminal Sequence Analysis of E.coli Citrate Synthase*

Cycle No.	GLC	AAA	n mole above background	Other Major Amino Acids (AAA)
1	ala	ala	59.4	
2	asp	asx	44.8	
3	thr	aba†	26.9	
4	-	lys	22.5	
5	ala	ala	21.5	
6	-	lys	9.3	
7	leu	leu	16.4	
8	thr	aba†	11.5	
9	leu	leu	10.4	
10	-	asx	10.4	
11	-	gly	17.0	
12	-	asx	14.0	
13	thr	(thr)	1.8	
14	ala	(ala)	15.0	glu (15.5); val (10.3)
15	val	-		

*143 nmoles of E.coli citrate synthase was subjected to automated Edman degradation on a Beckman Sequencer Model 890C

†α-aminobutyric acid, derived from threonine

at 4 °C.

Cyanogen bromide cleavage of the above material was performed as described in Methods. The cleaved product was lyophilized and dissolved in a minimum amount of 20% acetic acid. The cleavage was almost 100% as judged by amino acid analysis which showed an absence of any detectable methionyl residue. A new homoserine-lactone peak between the His and Lys peaks also appeared in the hydrolyzate of the cyanogen bromide reaction material. Quantitation of this peak was difficult due to partial conversion of homoserine lactone to homoserine. Homoserine coelutes with glutamate during amino acid analysis. This problem was kept to a minimum by dissolving the dried hydrolyzates immediately before analysis.

Gel Filtration of Cyanogen Bromide Fractions

Cleaved protein solution was divided into two equal fractions and chromatographed on a Sephadex G-75 column (2.5x160 cm) with 20% acetic acid as the eluant. Fractions of 10 ml each were collected and analyzed for radioactivity and ninhydrin positive material. The elution profile obtained was as shown in Figure 1. Fractions were pooled as shown by solid bars and lyophilized. The four pools obtained were designated as CNBr I, CNBr II, CNBr III and CNBr IV.

These pools were further purified by using gel filtration, ion exchange chromatography, counter current distribution or high voltage paper electrophoresis and paper chromatography. The general scheme of purification is described in Figure 2.

Urea gel electrophoresis of CNBr I, CNBr II, CNBR III and CNBR IV was performed as described in Methods. The CNBR I fraction was considered pure because it showed one major doublet in these gels. Cyanogen bromide peptides migrate as doublets during urea gel electrophoresis because

FIGURE 1: Elution of peptide fractions from the cyanogen bromide cleavage reaction from a Sephadex G-75 column (2.5x160 cm) in 20% acetic acid at 20 ml/hr. Fractions of 10 ml were collected and analyzed by ninhydrin analysis of 0.3 ml aliquots and by radioactivity of 50 µl aliquots. Fractions were pooled as shown by solid bars and pools were called CNBr I, CNBr II, CNBr III and CNBr IV in the order of their elution.

(Note: Some data points were omitted for the sake of clarity.)

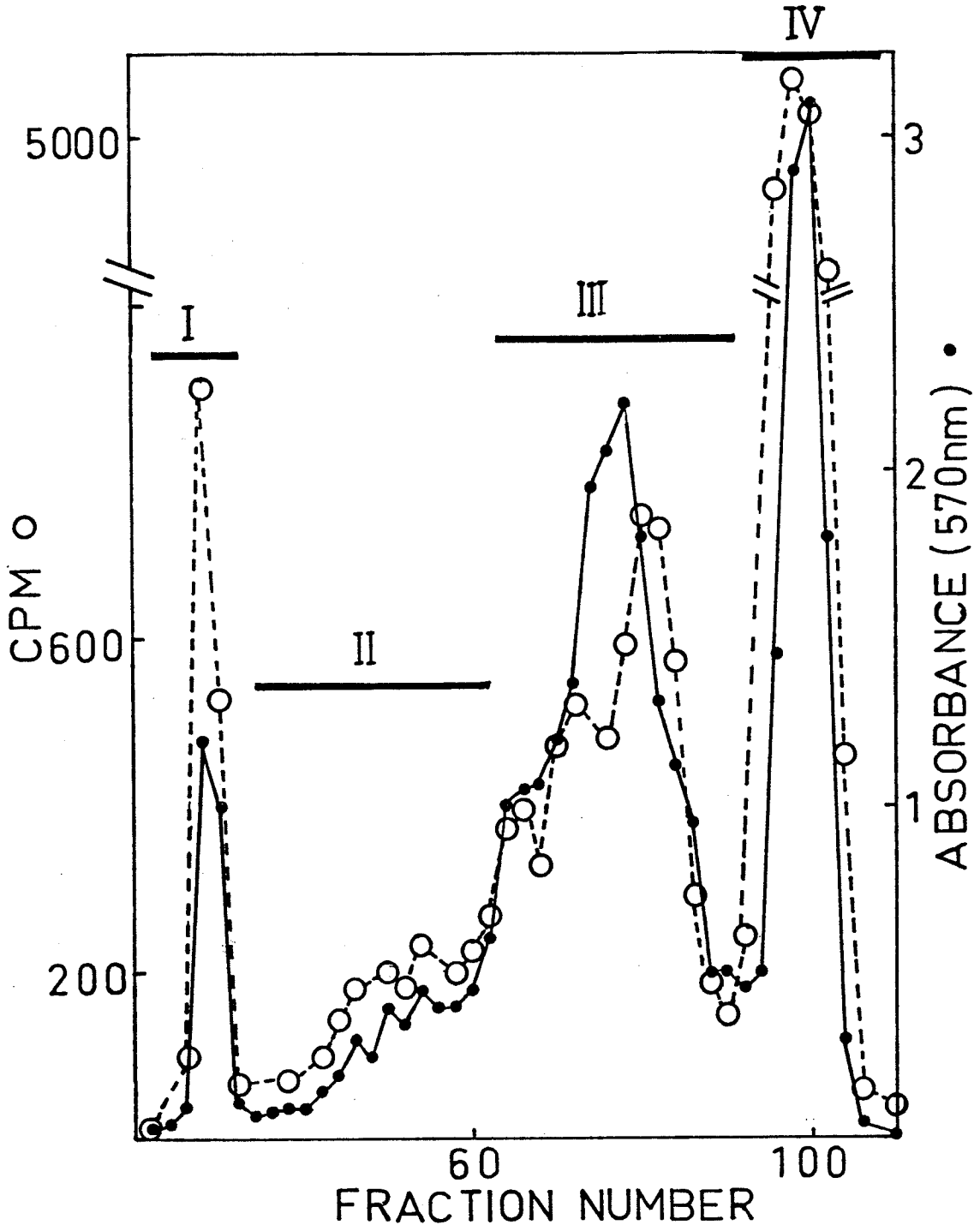
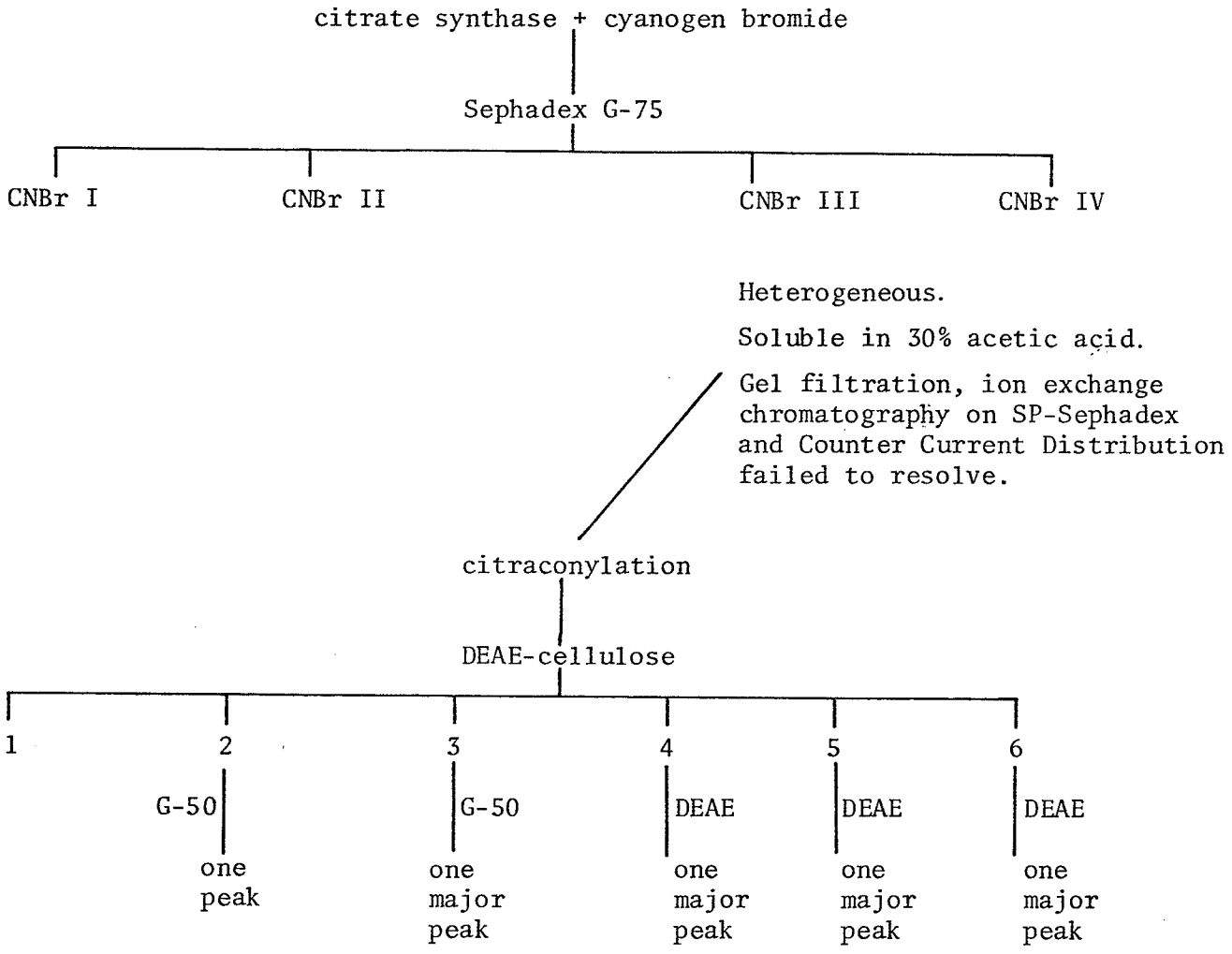

IV
5000
3
I
III
CPM o
600
II
2
ABSORBANCE (570nm) •
1
200
60
100
FRACTION NUMBER

FIGURE 2: Purification Scheme of Cyanogen Bromide Peptides.


citrate synthase + cyanogen bromide
Sephadex G-75
CNBr I
CNBr II
CNBr III
CNBr IV
Heterogeneous.
Soluble in 30% acetic acid.
Gel filtration, ion exchange chromatography on SP-Sephadex and Counter Current Distribution failed to resolve.
citraconylation
DEAE-cellulose
1
2
3
4
5
6
G-50
one peak
G-50
one major peak
DEAE
one major peak
DEAE
one major peak
DEAE
one major peak

homoserine lactone formed during the cleavage exists in equilibrium with homoserine and these two forms differ in charge and thus in electrophoretic mobility. CNBr II and CNBr III appeared to have 7-10 peptides each as determined by these gels. CNBr IV appeared to contain 4-5 peptides on the basis of urea gel electrophoresis results.

CNBr I

The early elution of fraction CNBr I on Sephadex G-75 suggested that this material was either high molecular weight peptide or uncleaved protein. To distinguish between these possibilities, SDS gel electrophoresis was performed as described in Methods. CNBr I gave one major band and a minor band visible only at higher sample loads. The major band corresponded to a molecular weight of 9000 Daltons as compared to native citrate synthase which has subunit molecular weight of 47000 Daltons. This indicates that CNBr I was an essentially pure high molecular weight cleavage product of citrate synthase.

Amino Acid Analysis

Amino acid analysis of CNBr I was performed as described in Methods. A 3% sample of it was hydrolysed in 1 ml of 6N HCl in an evacuated and sealed tube at 105 °C for 20 hr. The hydrolysate was dried *in vacuo* over NaOH pellets and dissolved in 1 ml of deionized water to load on the automatic amino acid analyzer. The results obtained were as shown in Table II.

This peptide did not contain tryptophan as determined by its UV spectrum which gave an absorption maximum at 274 nm.

N-Terminal Amino Acid

An aliquot of 10 µl of CNBr I was dansylated as described in Methods and after acid hydrolysis extracted with 50% pyridine. This was then

spotted on Cheng Chin Polyamide Layer Sheets. Standard dansyl amino acids were also spotted for the sake of comparison. CNBr I had a single fluorescent spot on these sheets which, on comparison with standards, corresponded to Threonine.

CNBr III

After obtaining this fraction from Sephadex G-75 column, it was analyzed by urea gel electrophoresis. This fraction was heterogeneous as was shown by the appearance of 7-10 doublets on these gels. As this fraction contained many of the expected peptides and these peptides were soluble in 30% acetic acid, most of the work reported here dealt with purification and analysis of peptides in this fraction.

Gel Filtration of CNBr III

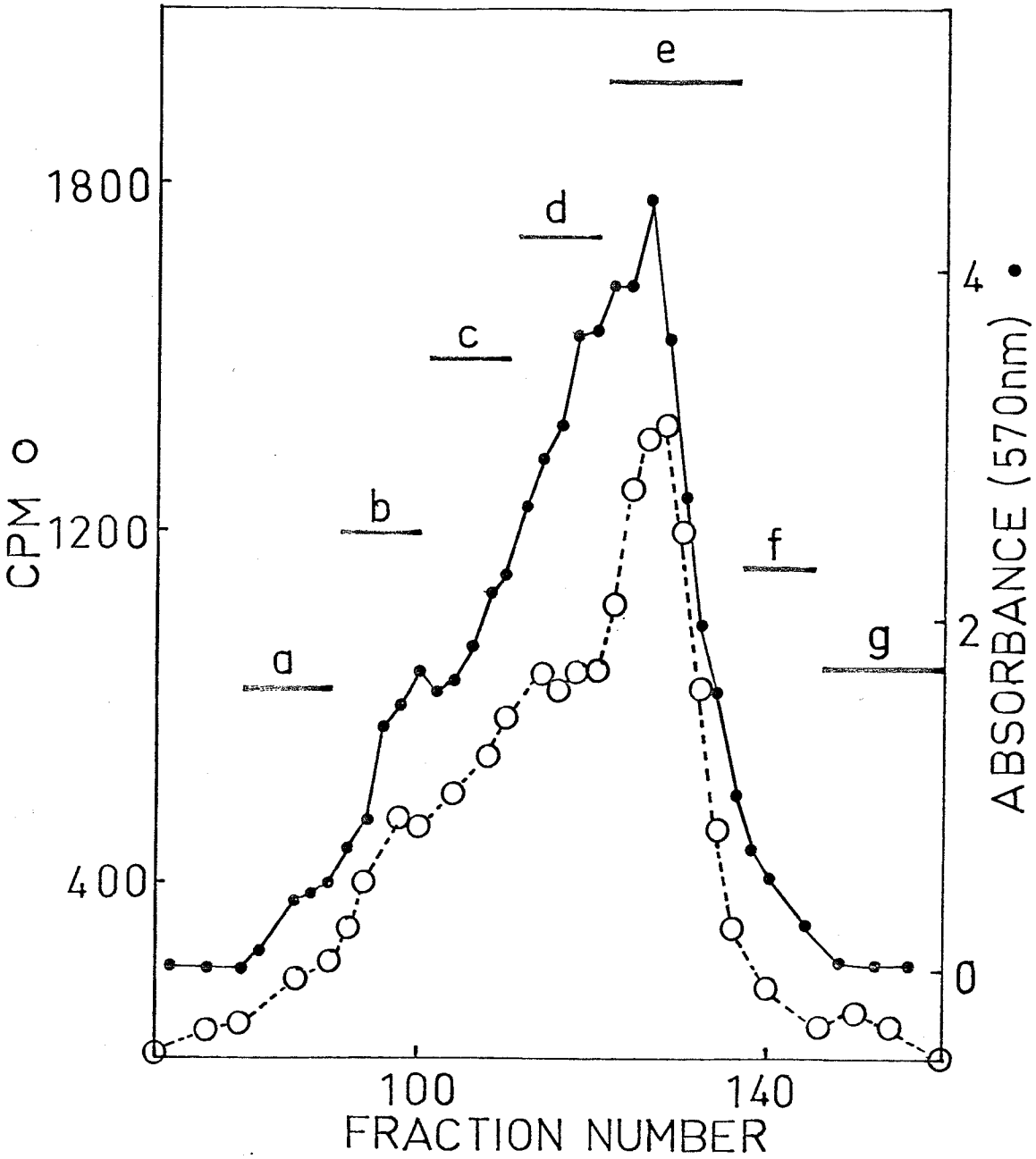
CNBr III was further purified by gel filtration on Sephadex G-50. The concentrated fraction CNBr III was applied to a Sephadex G-50 (medium) column (2.5x100 cm) and eluted with 30% acetic acid at a flow rate of 30 ml/hr. Fractions of 5 ml were collected and monitored for radioactivity and ninhydrin positive material. The elution profile obtained is shown in Figure 3 and fractions were pooled as shown by solid bars. These pools were concentrated by rotary evaporation and rechromatographed on Sephadex G-50 as above. Urea gel electrophoresis of the resulting pools indicated no further purification of peptides and so all subfractions obtained from Sephadex G-50 chromatography were remixed.

Counter Current Distribution

Fraction CNBr III was then subjected to counter current distribution as described in Methods. The solvent system used was 2-butanol:0.5M acetic acid:10% trichloroacetic acid (40:50:1 V/V). The machine was loaded with

FIGURE 3: Elution profile of CNBr III on a column (2.5x100 cm) of Sephadex G-100 in 30% acetic acid at 30 ml/hr in 5 ml fractions. Ninhydrin analysis was performed on 0.3 ml aliquots and 50 µl aliquots were taken for radioactivity from every second fraction. Fractions were pooled as shown by solid bars and were called CNBr IIIa-g in the order of their elution.

(Note: Some data points were omitted for the sake of clarity.)


a
b
c
d
e
f
g
1800
1200
400
CPM o
4
2
0
ABSORBANCE (570nm) •
100
140
FRACTION NUMBER

lower phase and 25 ml of upper phase was introduced after each transfer. CNBr III was dried using rotary evaporation, dissolved by stirring in 100 ml of a 1:1 mixture of upper and lower phases and left overnight at room temperature to ensure equilibration. The emulsion at the interface was discarded, sample containing lower phase loaded into the first two emptied cells of the train and then each topped with 25 ml of upper phase (without sample). The sample containing upper phase was loaded into the next two cells of the train. Fresh upper phase was added to the next 10 cells of the train to ensure equilibration of both phases.

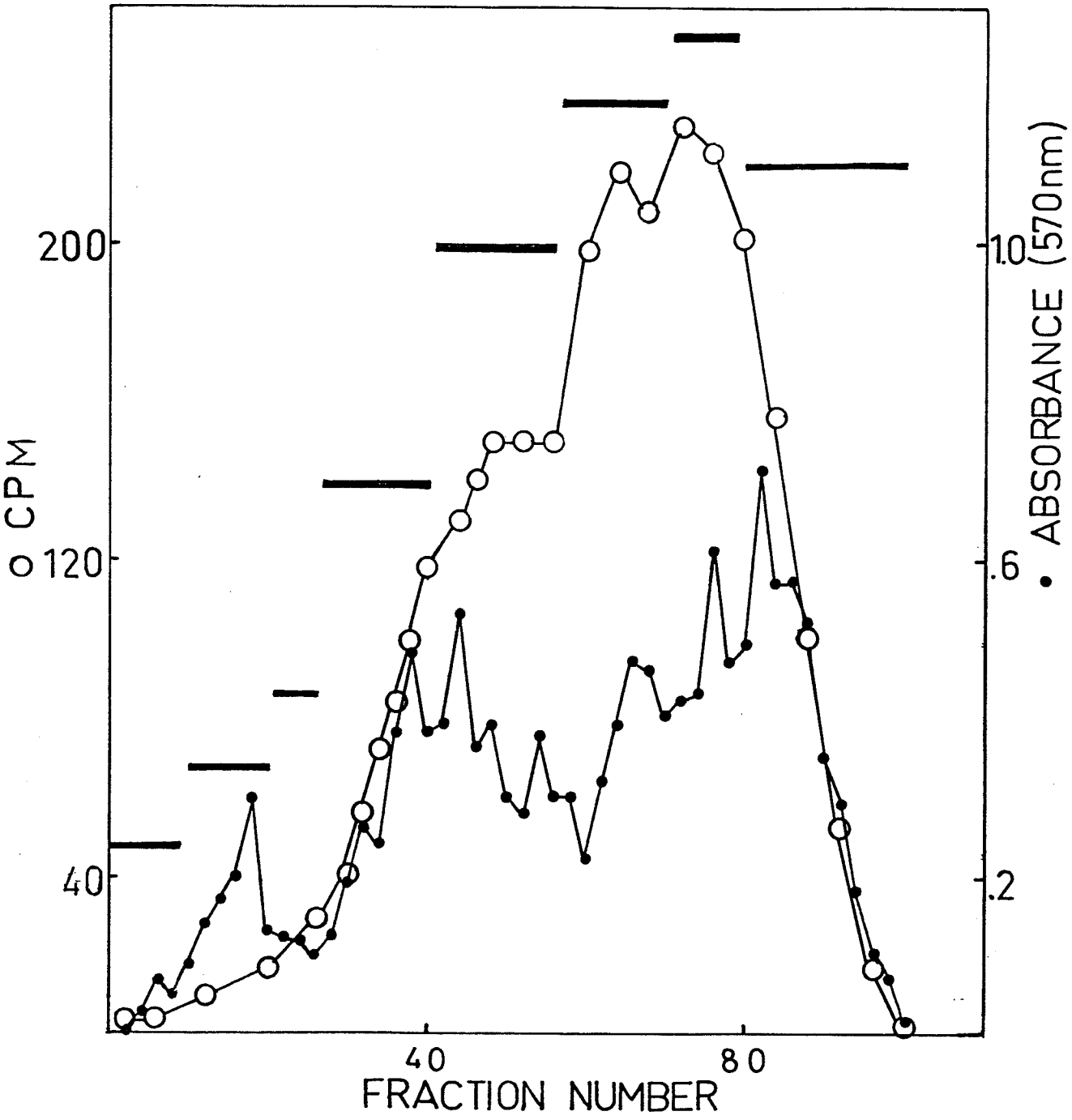
The machine was set to do 95 transfers using 30 seconds of agitation time and 15 min of settling time. At the end of the run the fractions were removed by aspiration using a syringe fitted with a long Teflon tube and analyzed by ninhydrin analysis and radioactive counts. The profile obtained is shown in Figure 4. Fractions were pooled as shown by solid bars, concentrated using an Evapo-mix and passed through Sephadex G-25 (medium) column to remove TCA. After a second concentration of samples as above, they were analyzed by urea gel electrophoresis which did not show any further purification of peptides. All fractions obtained from counter current distribution were remixed, dried and redissolved in 30% acetic acid.

Ion Exchange Chromatography on SP-Sephadex

CNBr III was then subjected to ion exchange chromatography using a strong cation exchanger SP-Sephadex (C-25). The sample was applied to the SP-Sephadex column (1.6x4.0 cm) pre-equilibrated with 0.1M ammonium acetate in 30% acetic acid and washed overnight with the same buffer. Radioactivity was determined in sample aliquots before loading. The column was eluted with a linear gradient of 0.1M to 0.5M ammonium acetate in 30%

FIGURE 4: Countercurrent distribution of peptides from fraction CNBr III (from Figure 1). Bars refer to fractions pooled on the basis of ninhydrin analysis and radioactive counts. (Background counts were subtracted before plotting.)

(Note: Some data points were omitted for the sake of clarity.)


200
o 120
40
CPM
1.0
.6
.2
• ABSORBANCE (570nm)
40
80
FRACTION NUMBER

acetic acid. Fractions of 5 ml were collected and analyzed for absorbances at 280 nm, radioactivity and ninhydrin positive material. All of the CNBr III preparation was recovered in the 0.1M ammonium acetate/30% acetic acid wash. This would seem to indicate that the basic groups of CNBr III peptides are not accessible to exchange on the SP-Sephadex resin. This may be partly due to blockage of amino terminals of these peptides (see Discussion).

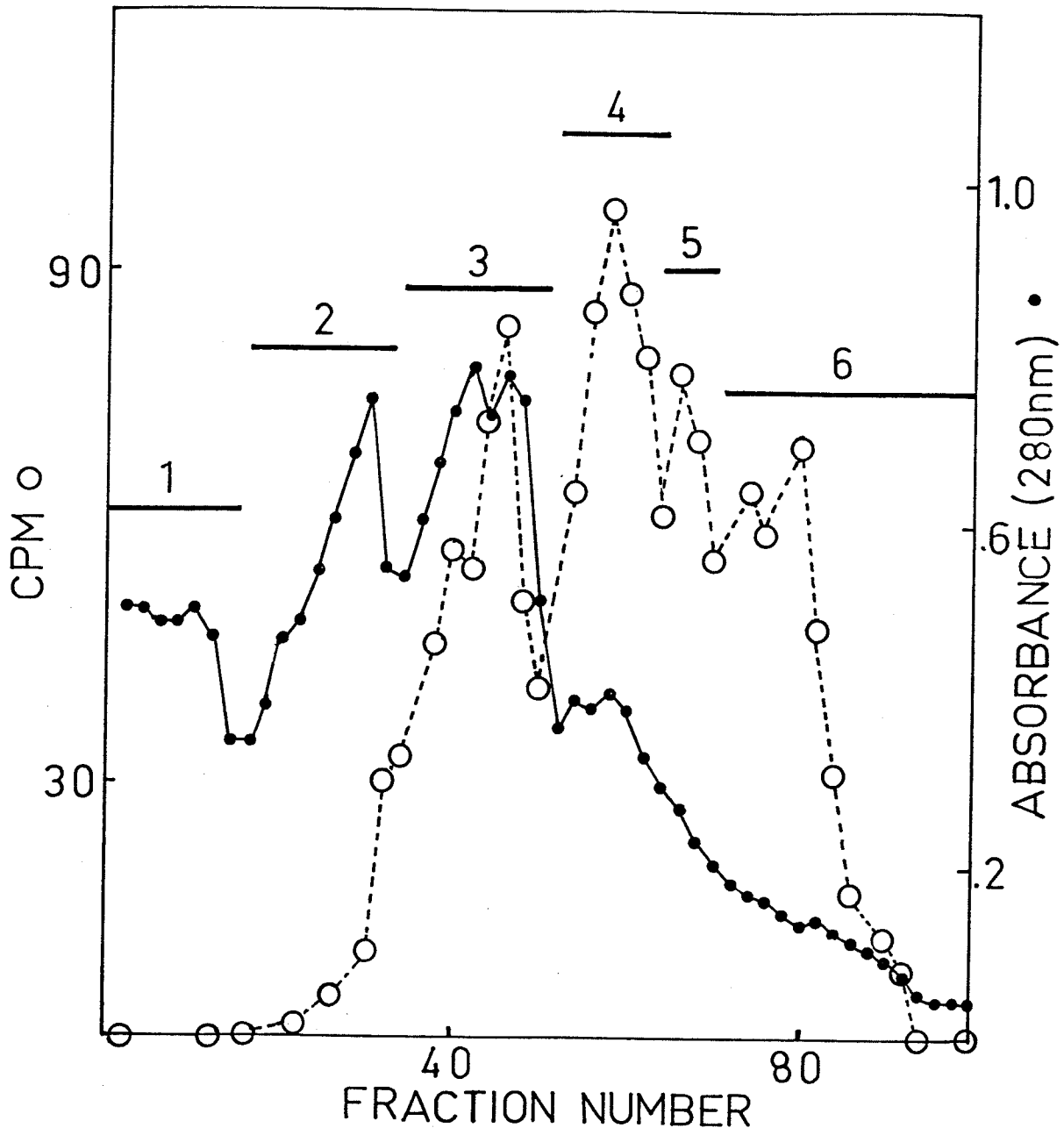
The difficulties faced in dissolving this fraction in different buffers, and the failure of gel filtration, counter current distribution and cation exchange chromatography on SP-Sephadex to resolve this complex mixture of peptides, are probably due to aggregation of peptides as seen, for example, by Cossar-Gheerbrant et al. (1978) with aspartokinase 1-homoserine dehydrogenase 1 from E.coli K12.

Citraconylation and Ion Exchange Chromatography

Material eluted in the initial wash of SP-Sephadex column was dried and redissolved in 0.2M $NaHCO_3$ for citraconylation as described in Methods to solve the aggregation problem. Even after citraconylation a clear solution was not obtained. The undissolved material was separated by centrifugation using a bench centrifuge. The pellet was saved, the supernatant was passed through a Sephadex G-15 (fine) column (4.5x35 cm) and eluted with 0.04M NH_4HCO_3 in order to remove the unreacted reagent and unwanted reaction products. Fractions with high absorbances at 280 nm were pooled and applied to an ion exchange column (2.5x35 cm) of DE52, pre-equilibrated with 0.04MNH_4HCO_3. The column was eluted with a .04M-0.5M NH_4HCO_3 linear gradient at a flow rate of 30 ml/hr. Fractions of 4 ml were collected and radioactivity and absorbances at 280 nm were determined. The elution profile obtained is shown in Figure 5.

FIGURE 5: Chromatography of citraconylated peptides of fraction CNBr III (from Figure 1) on a column (2.5x35 cm) of DEAE-cellulose in .04M NH_4HCO_3. Peptides were eluted by a linear gradient of 0.04 to 0.5M NH_4HCO_3 (600 ml total volume) at a flow rate of 30 ml/hr. Fractions (4 ml) were analyzed by absorbance at 280 nm and by radioactivity of 50 µl aliquots. Fractions were pooled as shown by solid bars and called CNBr III1-6 in the order of their elution. Finally the column was eluted with 0.5M NH_4OH.

(Note: Some data points were omitted for the sake of clarity.)


4
1.0
90
3
5
2
6
1
CPM o
.6
ABSORBANCE (280nm) •
30
.2
40
80
FRACTION NUMBER

Fractions were pooled as indicated by solid bars and lyophilized. Each fraction was further purified as described below:

CNBr III1

CNBr III1 was pooled with material eluted with equilibration buffer.

CNBr III2

CNBr III2 was applied to a Sephadex G-50 (fine) column (2.5x100 cm) and 4 ml fractions were eluted with 0.05M NH_4HCO_3 at a flow rate of 30 ml/hr. Fractions were analyzed by monitoring radioactivity and absorbances at 280 nm and 230 nm. As this fraction gave a single peak (see Figure 6), it was considered to be a pure peptide, the amino acid analysis of which is given in Table II.

CNBr III3

CNBr III3 was applied to DE-52 column (2.5x35 cm) and eluted with 500 ml of a linear 0.04M to .5M NH_4HCO_3 gradient at a flow rate of 25 ml/hr. Analysis of the 4 ml fractions revealed a non-alignment of radioactivity and absorbance peaks which indicates no further purification by this method.

Fractions under both peaks were pooled, concentrated and chromatographed on a Sephadex G-50 (fine) column (2.5x100 cm) as above. Two peaks were obtained from this column as shown in Figure 7. They were pooled separately and lyophilized. Amino acid analysis of these fractions revealed that the large peak (CNBr III3b) does not contain any detectable amino acids.

CNBr III4, 5 and 6

These fractions were further purified on a DE-52 column (2.5x38 cm). CNBr III4 was eluted from the column with a 600 ml 0.04M to 0.5M NH_4HCO_3 linear gradient (Figure 8). CNBr III5 was rechromatographed on a DE-52

TABLE II

Amino Acid Composition of Cyanogen Bromide (CNBr) Peptides of E.coli Citrate Synthase

Amino Acid	CnBr I†	CNBr III 2*	CNBr III 3a*	CNBr III 4b*	CNBr III 5c*	CNBr III 6*
Asp	11.6(11)	3.5(3)	5.5(5)	3.8(4)	7.4(7)	9.4(9)
Thr	11.0(11)	1.5(1)	2.2(2)	1.4(1)	2.8(3)	2.7(3)
Ser	5.4(5)	1.8(2)	3.2(3)	2.2(2)	3.1(3)	1.8(2)
Glu	12.8(11-13)	2.7(3)	6.3(5-6)	4.3(4)	8.2(8)	5.4(5)
Pro	5.1(5)	1.9(2)	3.3(3)	1.6(2)	1.7(2)	2.0(2)
Gly	8.5(8)	3.2(3)	4.2(4)	2.2(2)	4.7(5)	2.4(3)
Ala	7.8(8)	5.1(5)	6.6(6)	2.8(3)	4.1(4)	2.4(2)
Cys	-	-	-	-	-	-
Val	6.0(6)	1.6(2)	3.5(3)	2.4(2)	4.9(5)	1.6(2)
Met	-	-	-	-	-	-
Ile	6.9(7)	1.5(2)	3.1(3)	1.9(2)	2.6(3)	1.8(2)
Leu	9.7(10)	2.6(3)	4.8(5)	3.0(3)	6.2(6)	3.7(4)
Tyr	3.3(3)	1.0(1)	2.4(2)	1.4(1)	2.3(2)	2.3(2)
Phe	5.6(6)	1.8(2)	2.4(2)	1.5(1)	1.6(2)	1.7(2)
His	3.2(3)	1.0(1)	1.3(1)	1.1(1)	1.0(1)	0.7(1)
Homoserine Lactone	1.0(1)	1.0(1)	1.0(1)	1.0(1)	-	-
Lys	6.0(6)	1.7(2)	3.5(3)	2.5(2)	4.0(4)	2.7(3)
Arg	3.8(4)	2.1(2)	3.7(4)	2.4(2)	3.0(3)	1
nmoles of pure peptides	1028	172	526	1680	307	280
% yield'	(17.57%)	(2.94%)	(8.99%)	(28.72%)	(5.25%)	(4.79%)

†3% of total peptide fraction was taken for amino acid analysis.

*5% of total peptide fraction was taken for amino acid analysis.

For a given peptide numbers are normalized to homoserine lactone (CNBr I, CNBr III2, CNBr III3a and CNBr III4b), histidine (CNBr III5c) and arginine (CNBr III6).

'This is based on 5850 nmoles of starting protein.

FIGURE 6: Chromatography of CNBr III2 on a column (2x100 cm) of Sephadex G-50 (fine) in 0.04M NH_4HCO_3 at 30 ml/hr. Fractions of 4 ml were collected and monitored for absorbances at 230 and 280 nm and radioactivity. Fractions 54-61 were pooled and amino acid analysis performed.

(Note: Some data points were omitted for the sake of clarity.)

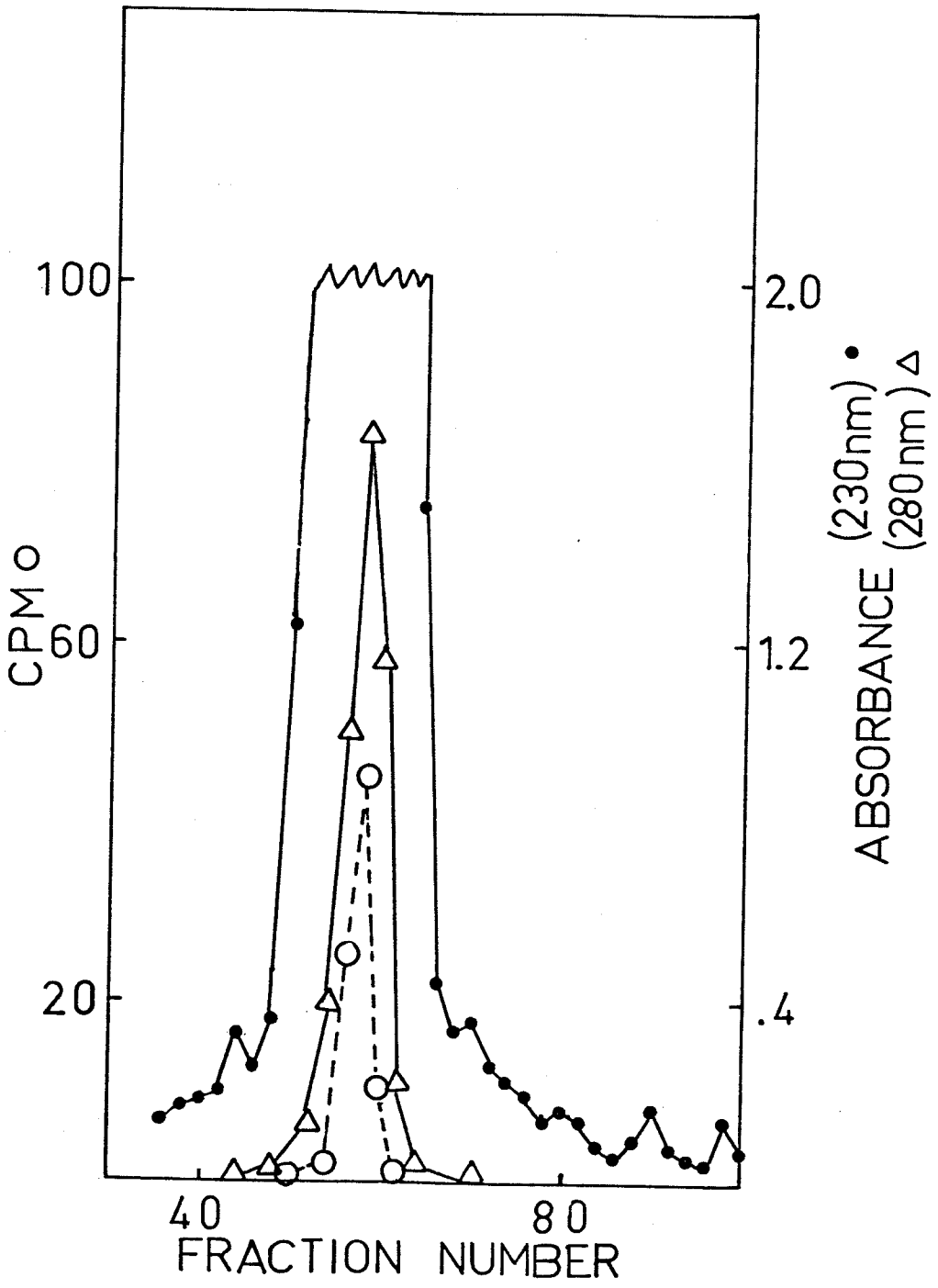

100
60
20
CPM o
2.0
1.2
.4
ABSORBANCE (230nm) •
(280 nm) △
40
80
FRACTION NUMBER

FIGURE 7: Resolution of CNBr III3 on a column (2x100 cm) of Sephadex G-50 (fine) in 0.04M NH_4HCO_3. Conditions were the same as in Figure 6. Fractions were pooled as shown by solid bars and called CNBr III3a and CNBr III3b in the order of their elution. Amino acid analysis revealed CNBr III3b does not contain any amino acids.

(Note: Some data points were omitted for the sake of clarity.)

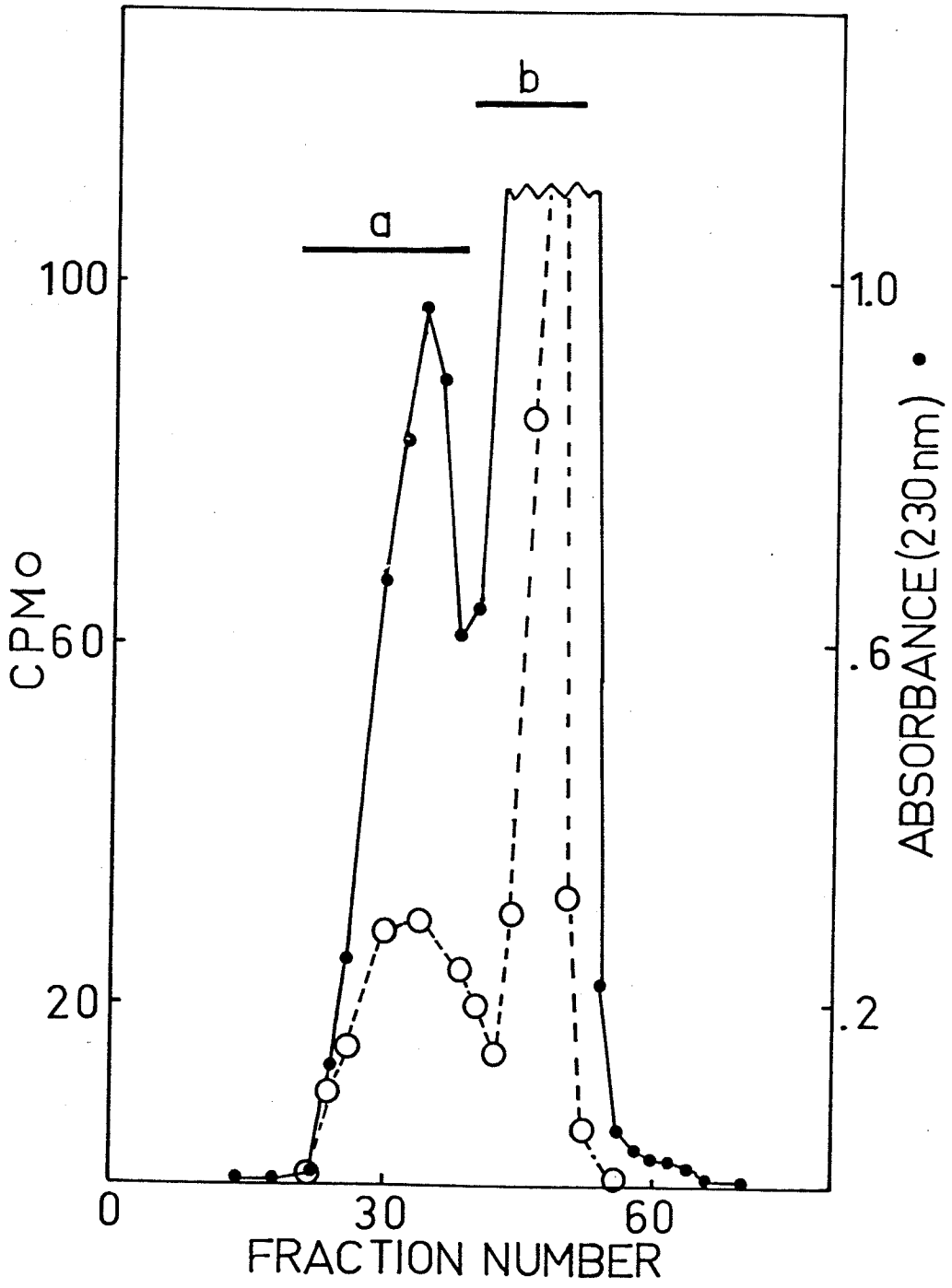
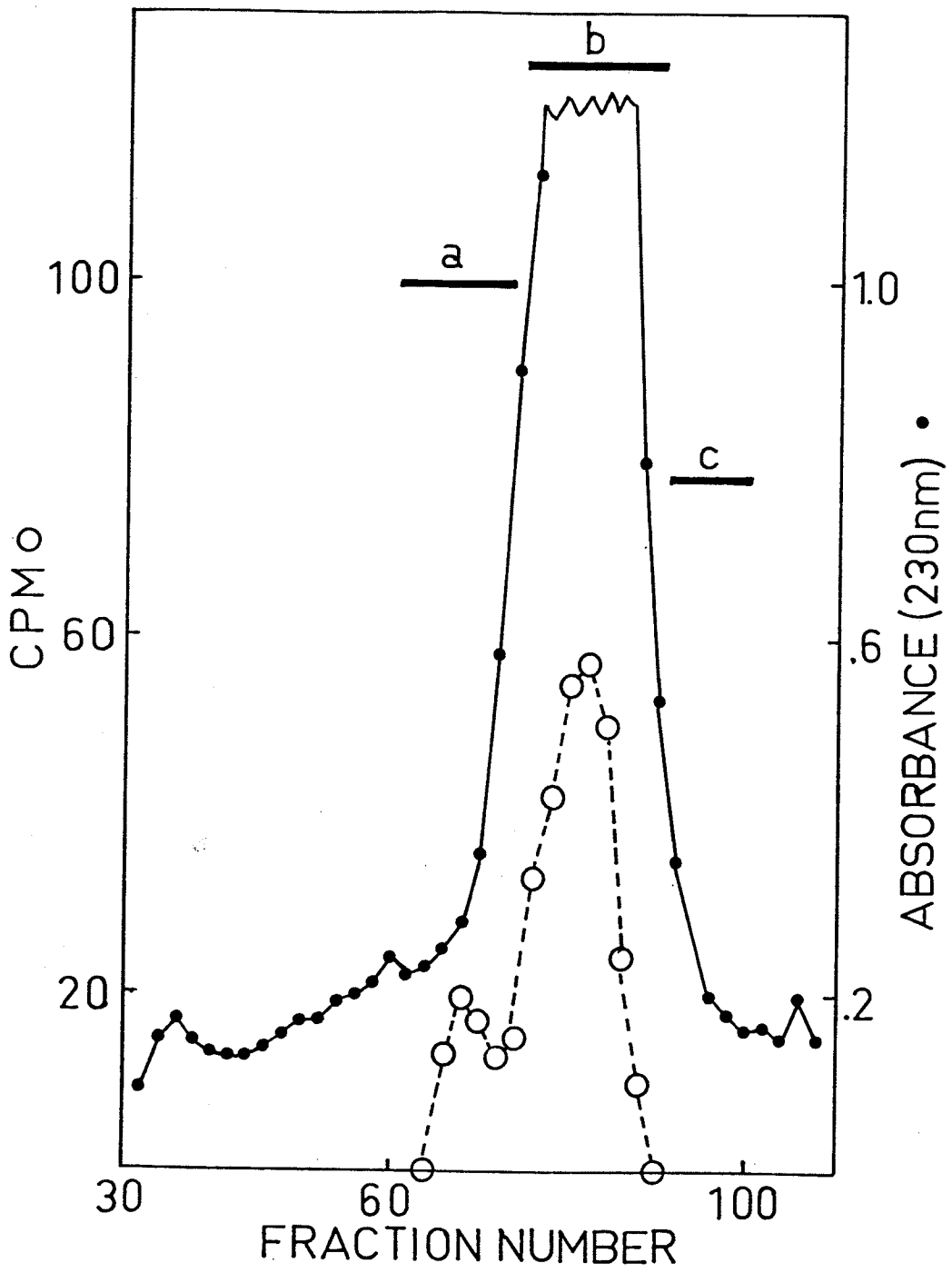

b
a
100
60
20
CPMo
1.0
.6
.2
ABSORBANCE (230 nm) •
0
30
60
FRACTION NUMBER

FIGURE 8: Chromatography of CNBr III4 on a column (2.5x35 cm) of DEAE-cellulose. Conditions are the same as in Figure 5. Fractions were pooled as shown by solid bars and called CNBr III4a-c. Amino acid analysis revealed that only CNBr III4b contains detectable amounts of amino acids.

(Note: Some data points were omitted for the sake of clarity.)


b
a
c
100
60
20
CPMo
1.0
.6
.2
ABSORBANCE (230nm) •
30
60
100
FRACTION NUMBER

column under identical conditions (Figure 9). CNBr III6 was eluted from DE-52 column using a 600 ml 0.04M to 1.0M NH_4HCO_3 gradient (Figure 10). Analysis of fractions was performed as for CNBr III (Figure 5). Major peaks obtained by ion exchange chromatography of these fractions were pooled as indicated in the elution profiles, concentrated and analyzed for amino acid composition as shown in Table II. CNBr III4b and CNBr III5c were subjected to automated Edman degradation as previously described. Low yield during coupling reactions indicated that the amino terminal residues of these peptides were blocked.

FIGURE 9: Chromatography of CNBr III5 on a column (2.5x30 cm) of DEAE-cellulose. Conditions are the same as in Figure 5. Fractions were pooled as shown by solid bars and called CNBr III5a-e. Amino acid analysis after concentrating them revealed that only fraction CNBr III5c contains detectable amounts of amino acids.

(Note: Some data points were omitted for the sake of clarity.)

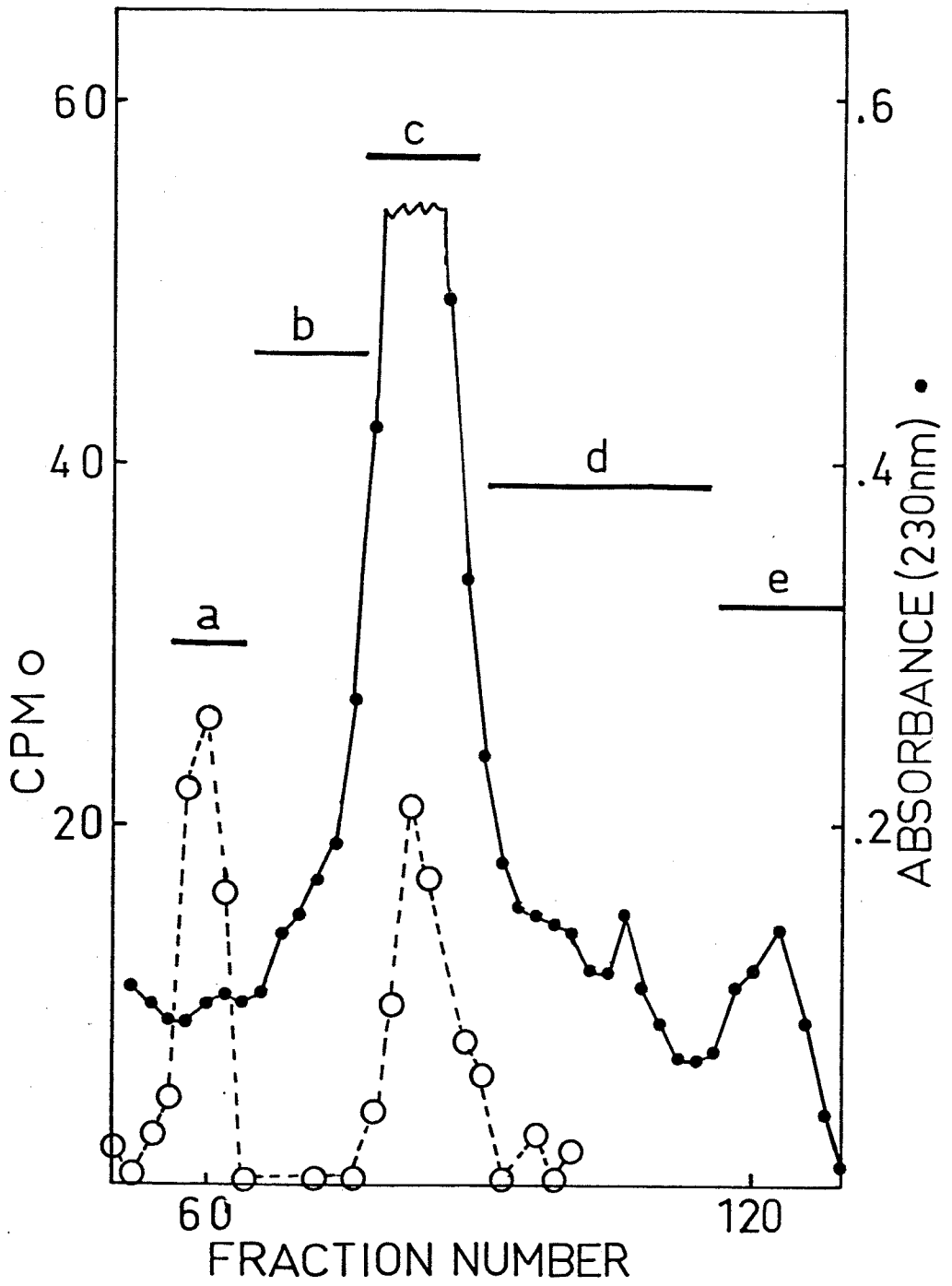
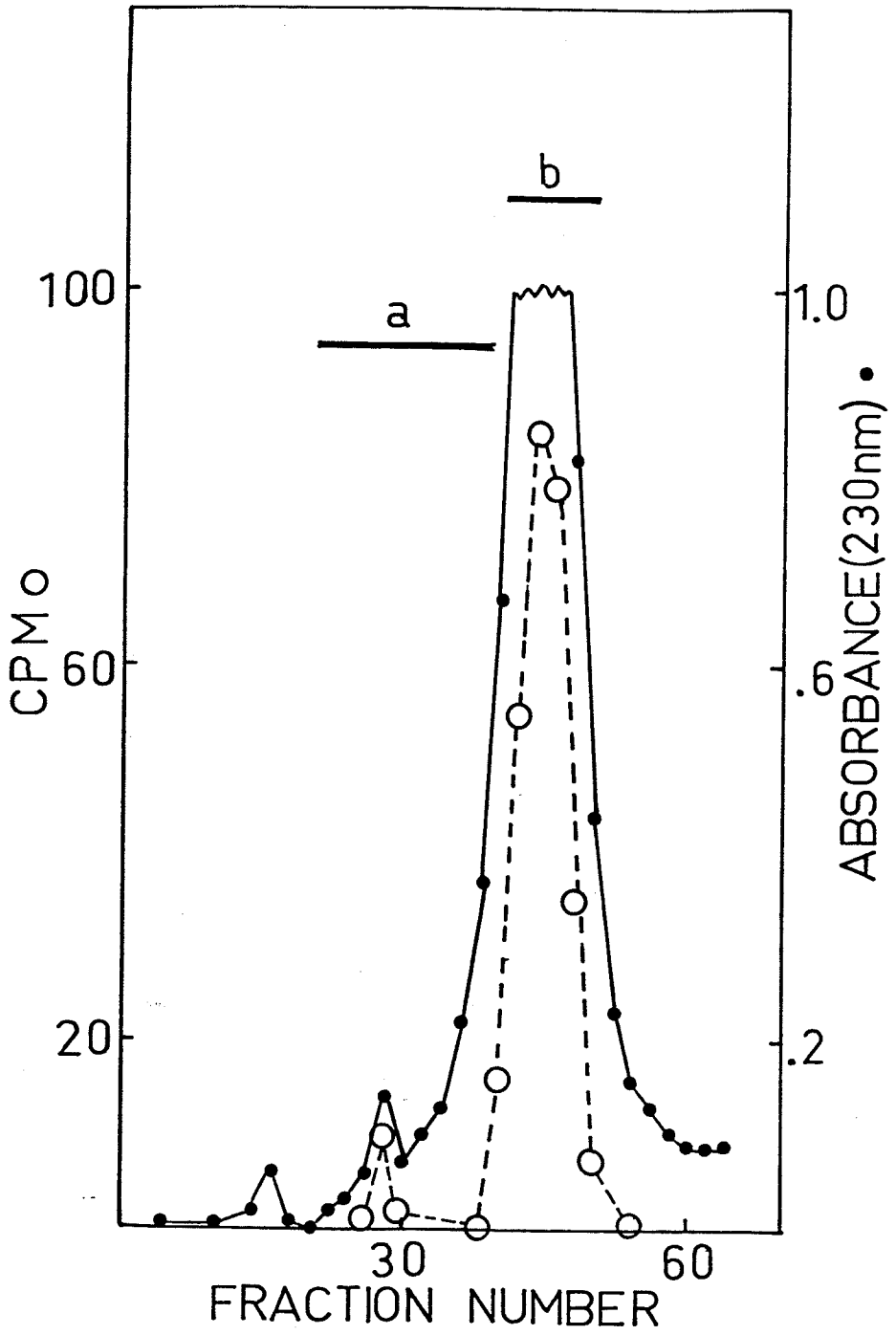

60
40
20
CPM o
.6
.4
.2
ABSORBANCE (230nm) •
a
b
c
d
e
60
120
FRACTION NUMBER

FIGURE 10: Chromatography of CNBr III6 on a column (2.5x35 cm) of DEAE-cellulose in .04M NH_4HCO_3. Column was eluted with a linear gradient of 0.04M to 1.0M NH_4HCO_3 (600 ml total volume) at a flow rate of 25 ml/hr. Fractions of 4 ml each were collected and analyzed for A_{230} and radioactivity. Fractions were pooled as shown by solid bars and called CNBr III6a and CNBr III6b. Only CNBr III6b contains amino acids as revealed by amino acid analysis.

(Note: Some data points were omitted for the sake of clarity.)


b
a
100
60
20
CPM o
1.0
.6
.2
ABSORBANCE(230nm) •
30
60
FRACTION NUMBER

Steady State Kinetics

Potassium ions induce several changes in the properties of E.coli citrate synthase which include an increased stability of enzyme activity, an increase in substrate affinities, a decrease in the effectiveness of several inhibitors, an increased resistance to heat and urea denaturation and a shift in UV spectra (Faloona and Srere, 1969). These changes apparently result from changes in the enzyme conformation.

Weitzman (1967), Faloona and Srere (1969) and Wright (1970) obtained a roughly bell-shaped pH curve for E.coli citrate synthase, with a maximum at pH 8.0. Steady state kinetics of E.coli citrate synthase have been studied as a function of pH in the presence of 100 mM KCl (Bell, 1978). No cooperativity was expected in the acetyl-CoA saturation curves in this medium. However, a slight positive cooperativity was not affected by increasing the level of KCl, although it appeared to induce substrate inhibition at substrate levels greater than 4 K_m.

In the absence of KCl, Faloona and Srere (1969) and Wright and Sanwal (1971) observed non-linear kinetics, a pH optimum of 8.0 and a several-fold increase in the apparent K_m for AcCoA, but they did not observe any change in maximum velocity. In the study reported here, steady state kinetics of E.coli citrate synthase were studied as a function of pH in the absence of KCl. This is essentially a continuation and extension of Bell's (1978) work.

Substrate saturation curves were obtained for E.coli citrate synthase at pH's 6.56, 7.72 and 8.34. The Lineweaver-Burk plots obtained from these data are shown in Figures 11-13. In all cases, OAA saturation curves obtained were non-cooperative while acetyl-CoA saturation curves showed positive cooperativity. The sigmoidity of acetyl-CoA saturation curves

FIGURE 11a: Lineweaver-Burk plots. Double reciprocal plots of velocity versus acetyl-CoA concentration at several fixed levels of OAA, pH 6.56. OAA concentrations: 0.01, 0.02, 0.04, 0.10, 0.20 and 0.40 mM from top to bottom. In all cases the reaction mixture contains 1 mM EDTA, no KCl and either buffer 0.015M phosphate (pH 6.56) or 0.02M Tris-Cl (pH 7.72 and 8.37). The formation of CoA is monitored at 232 nm by the loss of acetyl-CoA according to Srere et al. (1963). Where straight lines are drawn the parameters were obtained by hyperbolic fitting of the individual curves by the method of Wilkinson (1961).

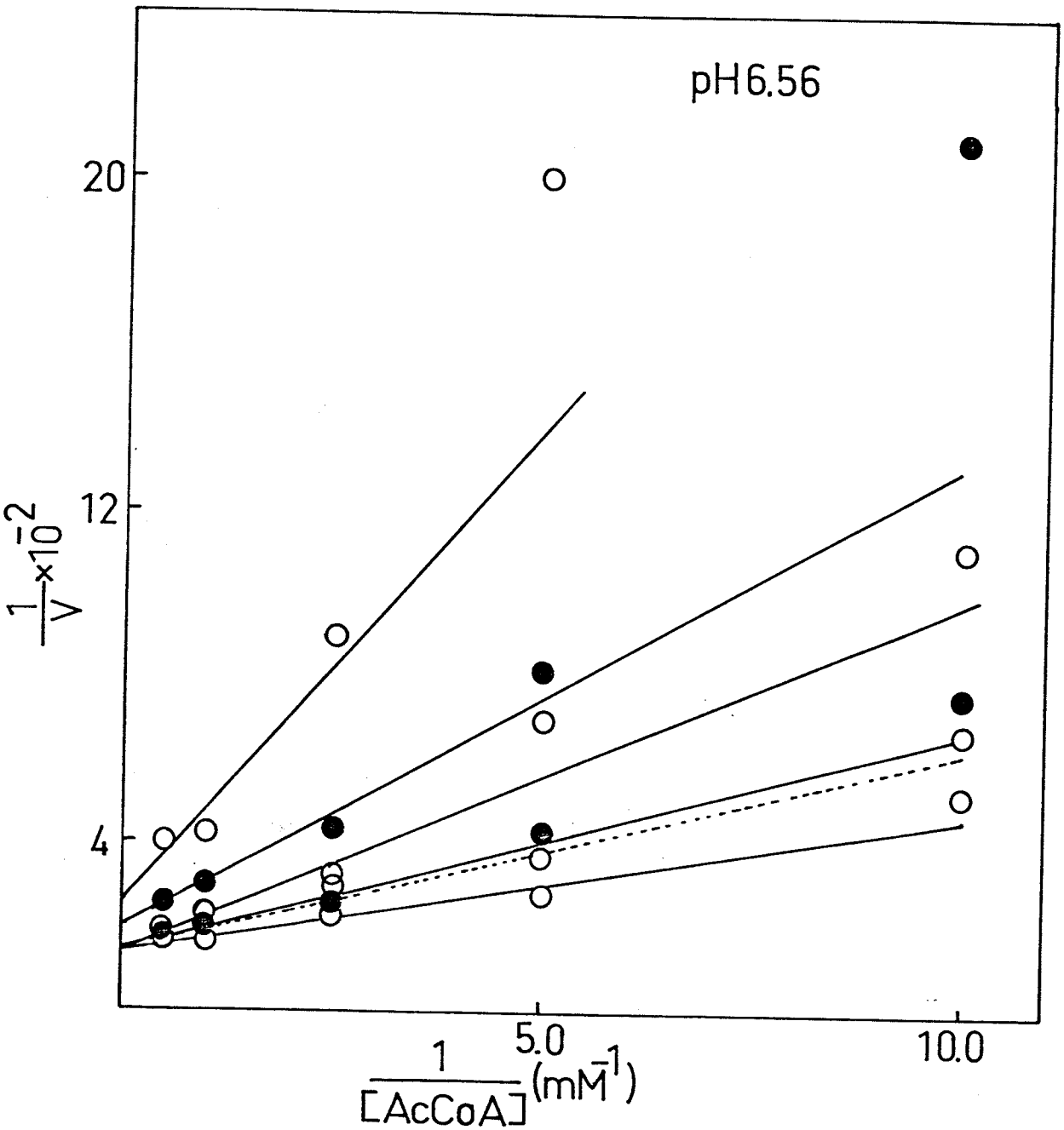

pH6.56
20
12
4
$\frac{1}{V} \times 10^{-2}$
5.0
10.0
$\frac{1}{[AcCoA]}$ (mM^{-1})

FIGURE 11b: Lineweaver-Burk plots (see caption to Figure 11a). Double reciprocal plots of velocity against OAA concentration at several fixed levels of acetyl-CoA, pH 6.56. Acetyl-CoA concentrations: 0.1, 0.2, 0.4, 1.0 and 2.0 mM from top to bottom.

Inset: Intercepts ($I/\bar{V}$) and slopes ($K/\bar{V}$) of double reciprocal plots versus reciprocal of acetyl-CoA concentration.

(Note: Some data points were omitted for the sake of clarity.)

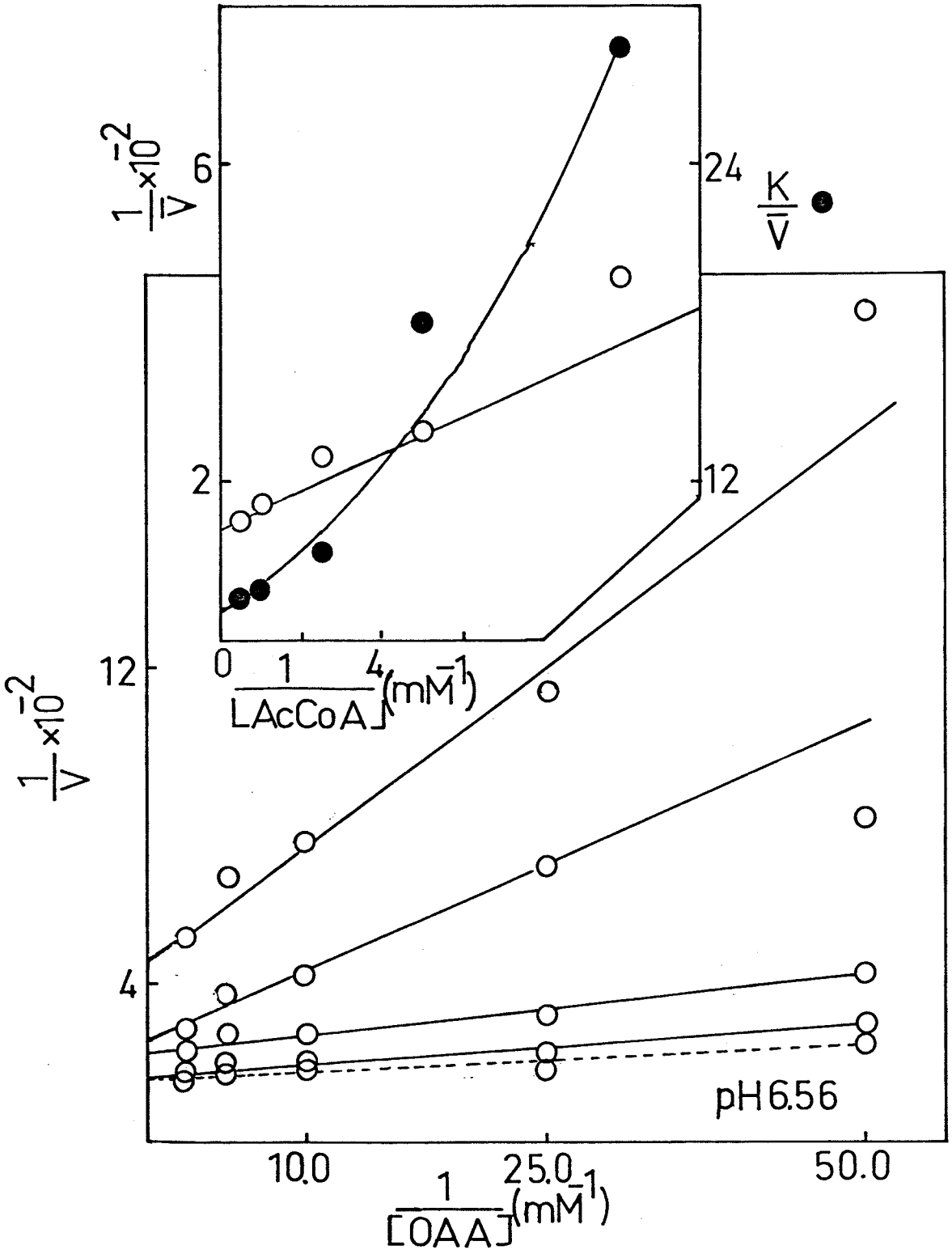

1/V x10^-2
6
2
12
4
24
K/V
12
0
1
4
1/[AcCoA] (mM^-1)
pH6.56
10.0
25.0
50.0
1/[OAA] (mM^-1)

FIGURE 12a: Lineweaver-Burk plots (see caption to Figure 11a). Double reciprocal plots of velocity against OAA concentration at several fixed levels of acetyl-CoA, pH 7.72. Acetyl-CoA concentration: 0.10, 0.20, 0.40, 1.00 and 2.00 mM from top to bottom.

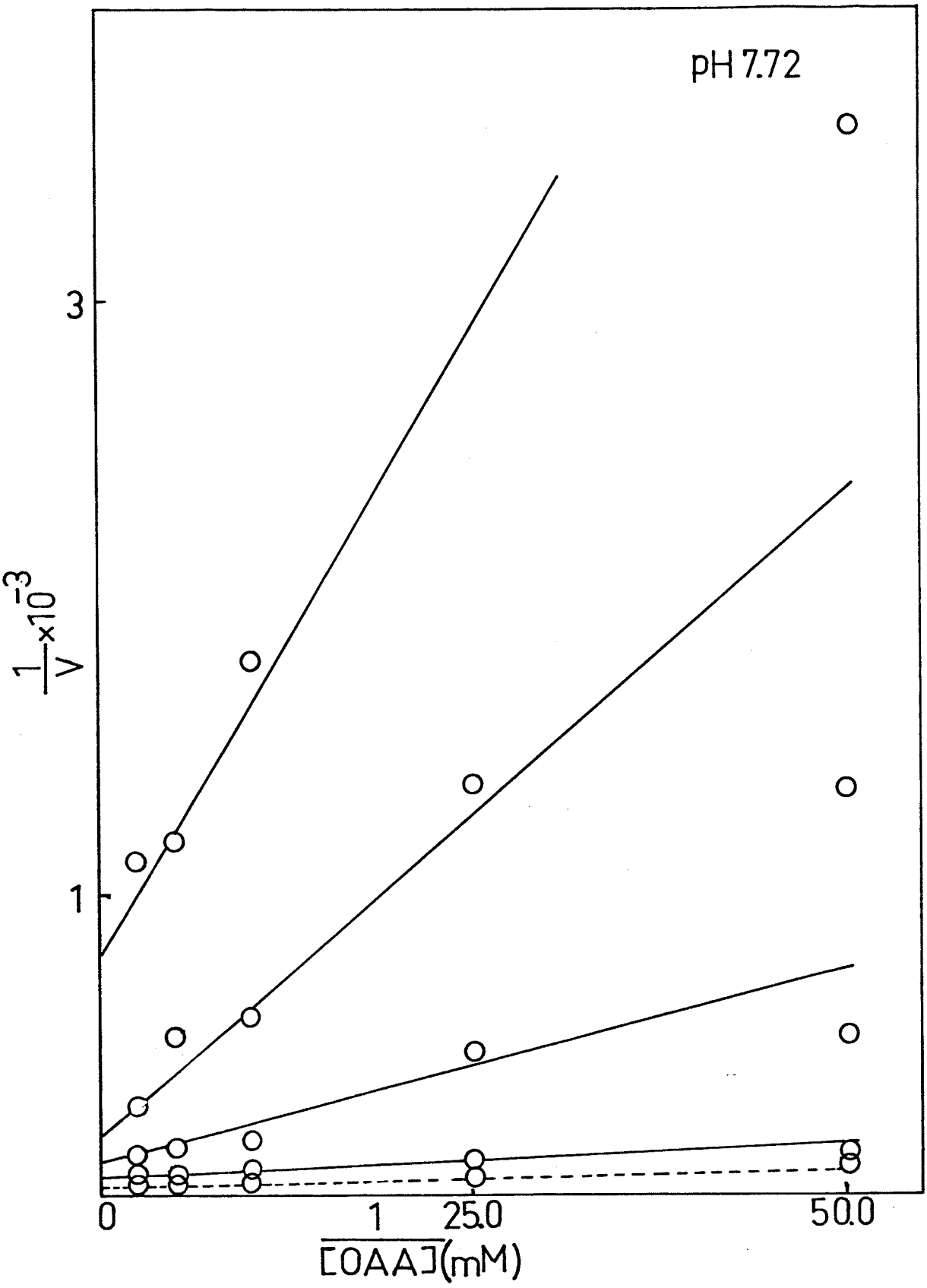

pH 7.72
3
1
$\frac{1}{V} \times 10^{-3}$
0
1
25.0
50.0
$\frac{1}{[OAA]}$(mM)

FIGURE 12b: Lineweaver-Burk plots (see caption to Figure 11a). Double reciprocal plots of velocity against acetyl-CoA concentration at several different levels of OAA concentration, pH 7.72. OAA concentrations: .02, .04, 0.10, 0.20 and 0.40 mM from top to bottom.

Inset: Intercepts ($I/\bar{V}$) and slopes ($K/\bar{V}$) of double reciprocal plots versus reciprocal of acetyl-CoA concentration.

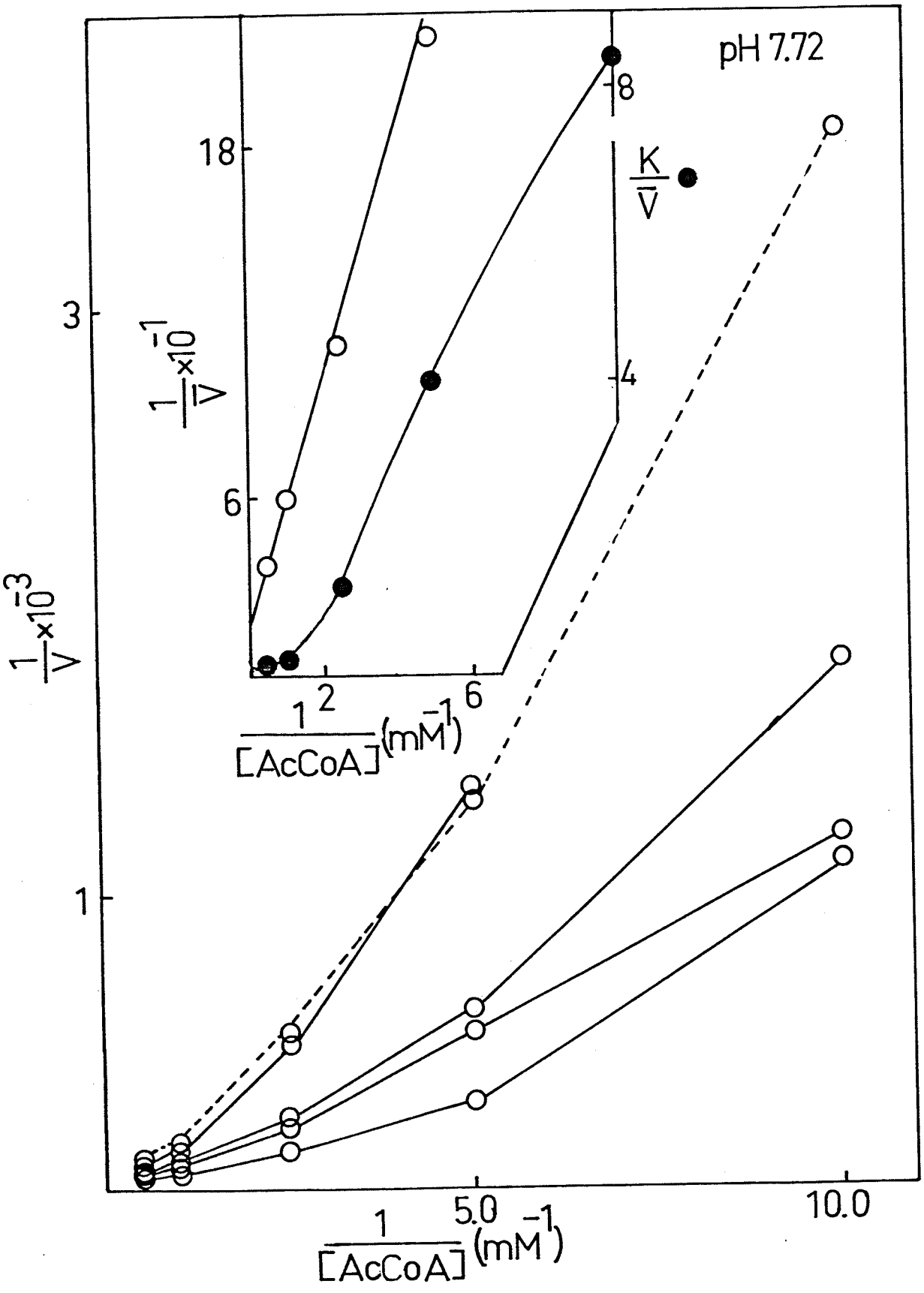

pH 7.72
18
8
K/V
3
1/V ×10^-1
4
6
1/V ×10^-3
1
2
6
1/[AcCoA] (mM^-1)
5.0
10.0
1/[AcCoA] (mM^-1)

FIGURE 13a: Lineweaver-Burk plots (see caption to Figure 11a). Double reciprocal plots of velocity against OAA concentration at several fixed levels of acetyl-CoA, pH 8.37. Acetyl-CoA concentration: 0.20, 0.40, 1.00, 2.00 and 4.00 mM from top to bottom.

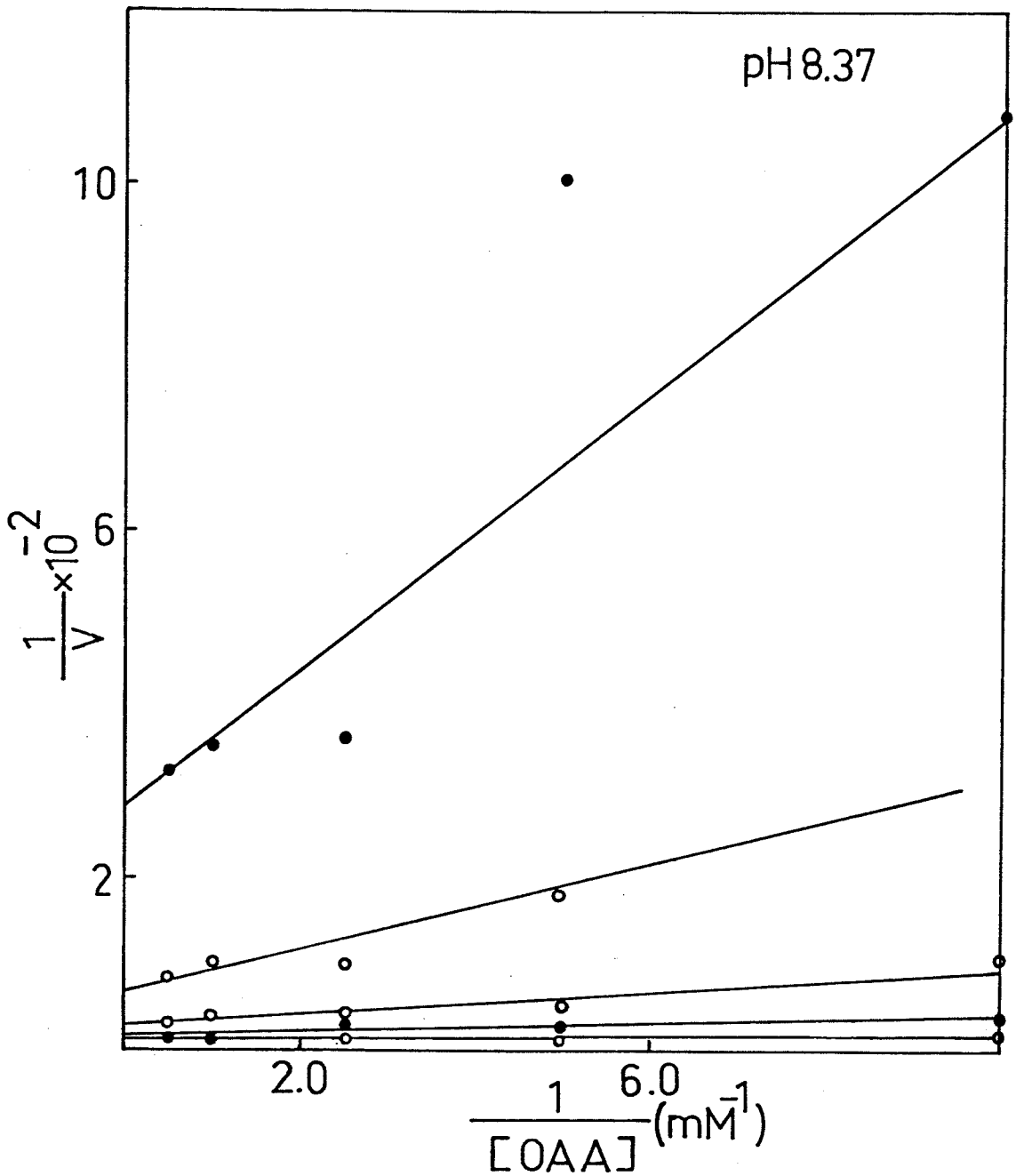
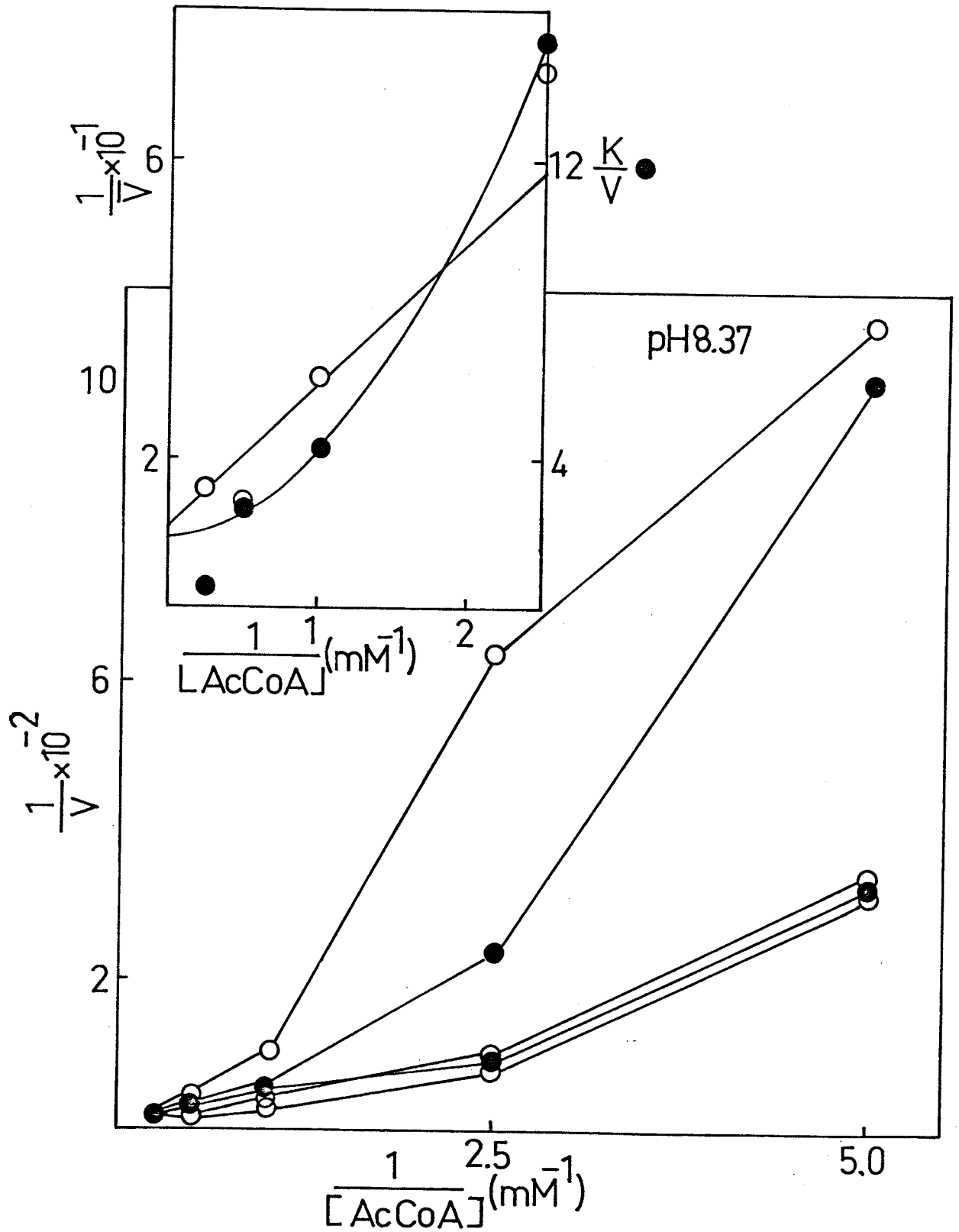

pH 8.37
10
6
2
$\frac{1}{V} \times 10^{-2}$
2.0
6.0
$\frac{1}{[OAA]}$ (mM^{-1})

FIGURE 13b: Lineweaver-Burk plots (see caption to Figure 11a). Double reciprocal plots of velocity against acetyl-CoA concentration at several fixed levels of OAA, pH 8.37. OAA concentrations: 0.10, 0.20, 0.40, 1.00 and 2.00 mM from top to bottom.

Inset: Intercepts ($I/\bar{V}$) and slopes ($K/\bar{V}$) of double reciprocal plots versus reciprocal of acetyl-CoA concentration.


pH8.37
$\frac{1}{V} \times 10^{-2}$
$\frac{1}{V} \times 10^{-1}$
$\frac{K}{V}$
$\frac{1}{[AcCoA]}(mM^{-1})$
$\frac{1}{[AcCoA]}(mM^{-1})$
10
6
2
12
4
2
6
2
2.5
5.0

appears to decrease if nearly saturating concentrations of OAA are used. The slopes and intercepts of the Lineweaver-Burk plots, which correspond to apparent Michaelis constants, K_m^{app}, and apparent maximum velocity, $\bar{V}_{max}^{app}$, were plotted versus the reciprocals of the concentrations of the second substrate to determine the true kinetic constants (insets of Figures 11b, 12b and 13b). These slope replots also displayed cooperativity for AcCoA binding to the enzyme as expected. From these plots, values of $\bar{V}_{max}^{true}$ and K_m^{true} for acetyl-CoA were obtained (Table III), while, because of curvature of the replots versus (1/[acetyl-CoA]), insufficient kinetic parameters were available to obtain true kinetic constants of OAA. KCl behaves like an allosteric activator of E.coli citrate synthase and thus the K_m values for both substrates increased substantially in its absence. These K_m values also seem to increase with an increase in pH.

Hill plots for acetyl-CoA saturation, at pH's 6.56, 7.72 and 8.34, were obtained in order to determine if higher concentrations of OAA have any effect on the cooperativity (Figures 14 and 15). These plots indicate that the Hill's n values are not affected by OAA concentrations. While Hill's values at pH's 7.72 and 8.34 indicate positive cooperativity, no cooperativity was seen at pH 6.56. It appears from these data that OAA concentration does not affect the acetyl-CoA binding at any of the concentrations studied.

$\bar{V}_{max}$ values in the presence and in the absence of KCl were calculated from V_i values and plotted versus pH. The curves obtained in the presence and in the absence of KCl are of similar shape (Figure 16B) in contrast to the simple plots of V_i versus pH (Figure 16A).

TABLE III

Kinetic Constants for Citrate Synthase as a Function of pH

[E][1] p moles/ml	pH[2]	V_{max}^{true} ΔA/min	K_{cat}[3] sec^{-1}	K_m AcCoA mM
52.87	6.56	$(6.98\pm.31)\times10^{-3}$	9.78	0.20±0.03
48.06	7.72	$(5.81\pm.38)\times10^{-2}$	44.80	2.46±.25
52.87	8.37	$(9.38\pm2.22)\times10^{-2}$	65.72	12.40±.5

[1]The enzyme concentration was calculated from the velocity of an assay under standard conditions (0.1M KCl, 0.1 mM OAA, 0.1 mM AcCoA, 1 mM EDTA, 0.05 mM DTNB and 20 mM Tris-HCl, pH 7.8) assuming that specific activity to be 60 μmoles of product formed per minute per mg of enzyme (60 μmoles/min/mg) for a freshly prepared enzyme sample. E.coli citrate synthase has a subunit M. Wt. of 47,000 according to Tong and Duckworth (1975).

[2]pH is the average of the initial and the final pH of the assay mixture.

[3]By definition $V_{max} = K_{cat}[E]$. The value of V_{max} was determined graphically and since the enzyme concentration can be determined from a standard assay

$$K_{cat} = \frac{V_{max}}{[E]}$$

FIGURE 14A: Hill plots for the steady state kinetic data in Figures 11a and 11b. The symbols used ●, □, ○, ○, △ and ○ correspond to 0.01, 0.02, 0.04, 0.1, 0.2 and .4 mM OAA respectively.

B: Hill plots for the steady state kinetic data in Figures 12a and 12b. The symbols used ●, □, ⊖, △ and ○ correspond to 0.02, 0.04, 0.10, 0.20 amd 0.40 mM OAA respectively.

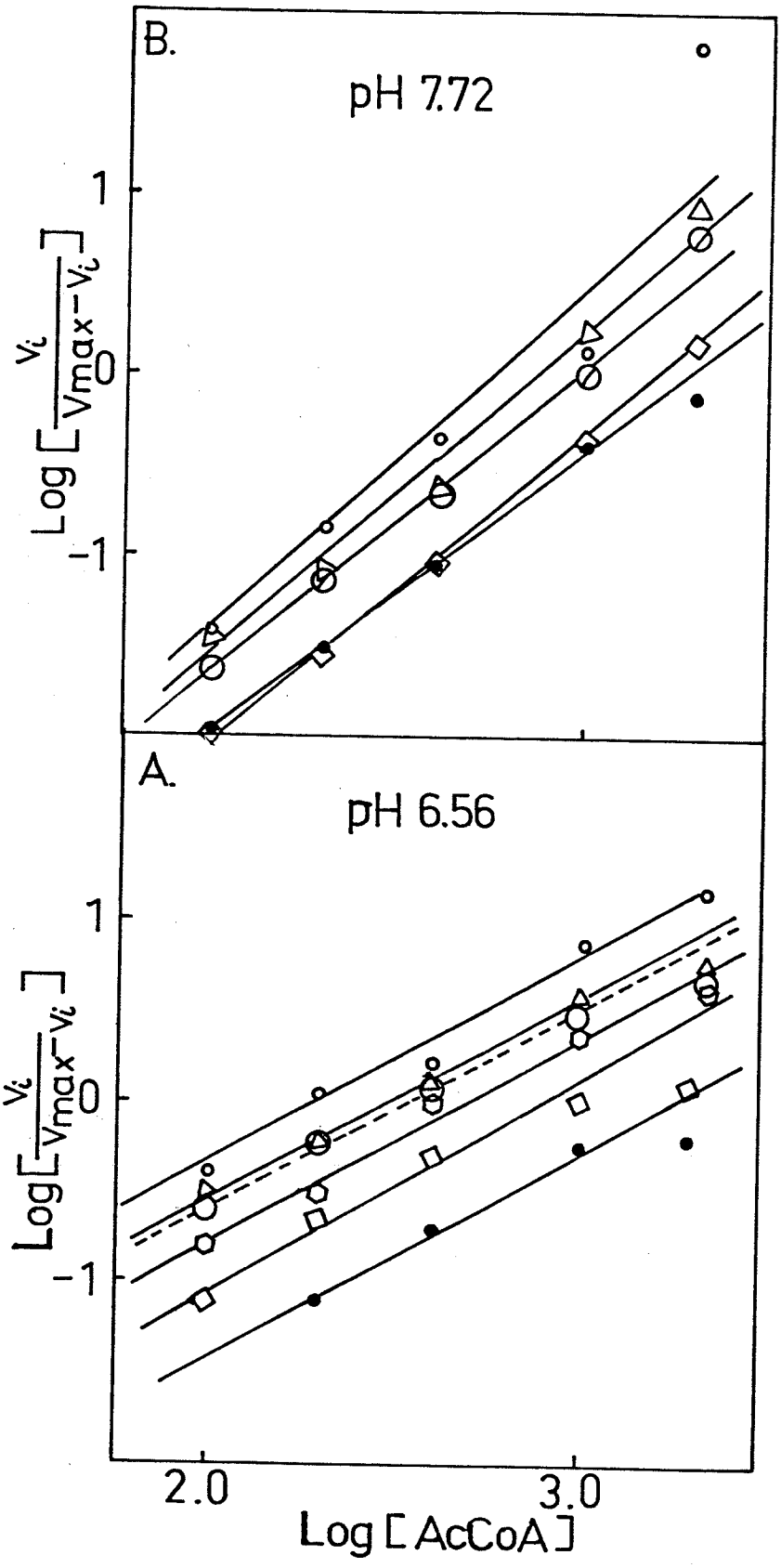

B.
pH 7.72
1
0
-1
Log [Vi / (Vmax - Vi)]
A.
pH 6.56
1
0
-1
Log [Vi / (Vmax - Vi)]
2.0
3.0
Log [AcCoA]

FIGURE 15: Hill plots for the steady state kinetic data in Figures 13a and 13b. The symbols used ○, □, △, ⊕ and ● correspond to .1, .2, .4, 1.0 and 2.0 mM of OAA respectively. Data for .0.4 and 1.0 mM OAA concentrations were displaced ½ and 1 log units respectively.

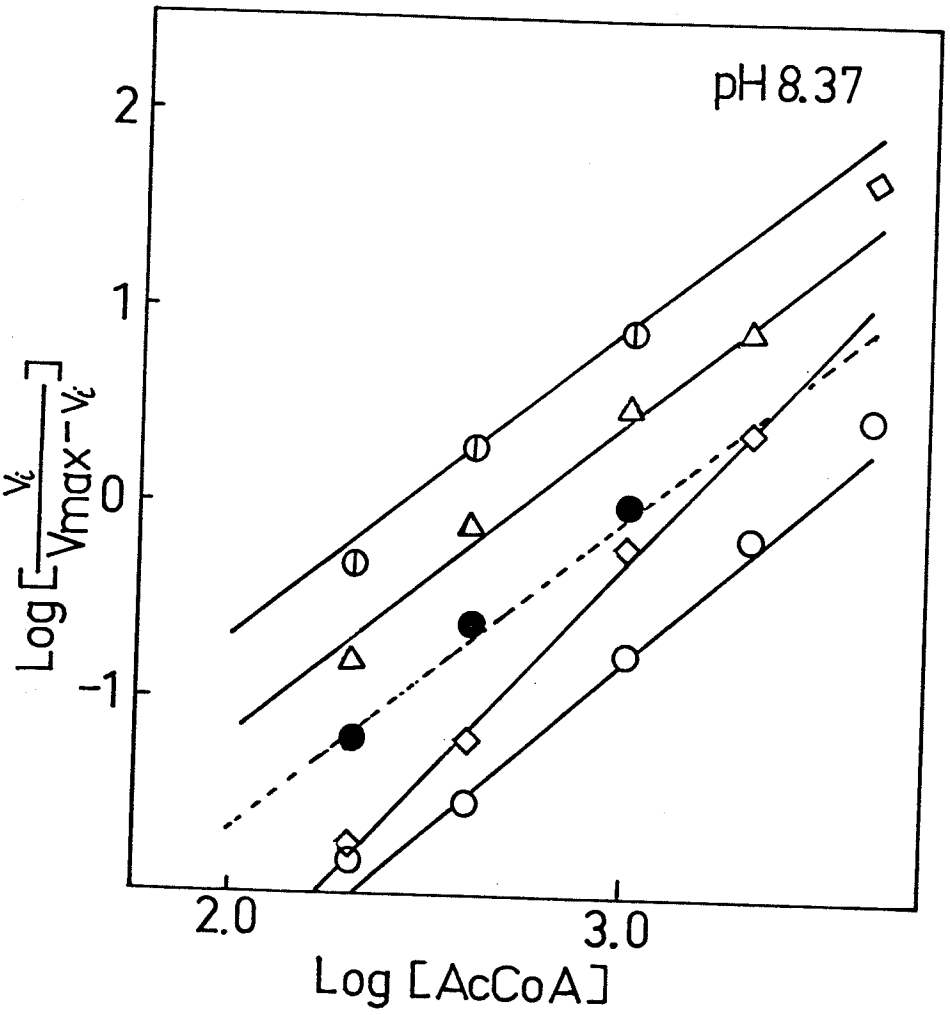
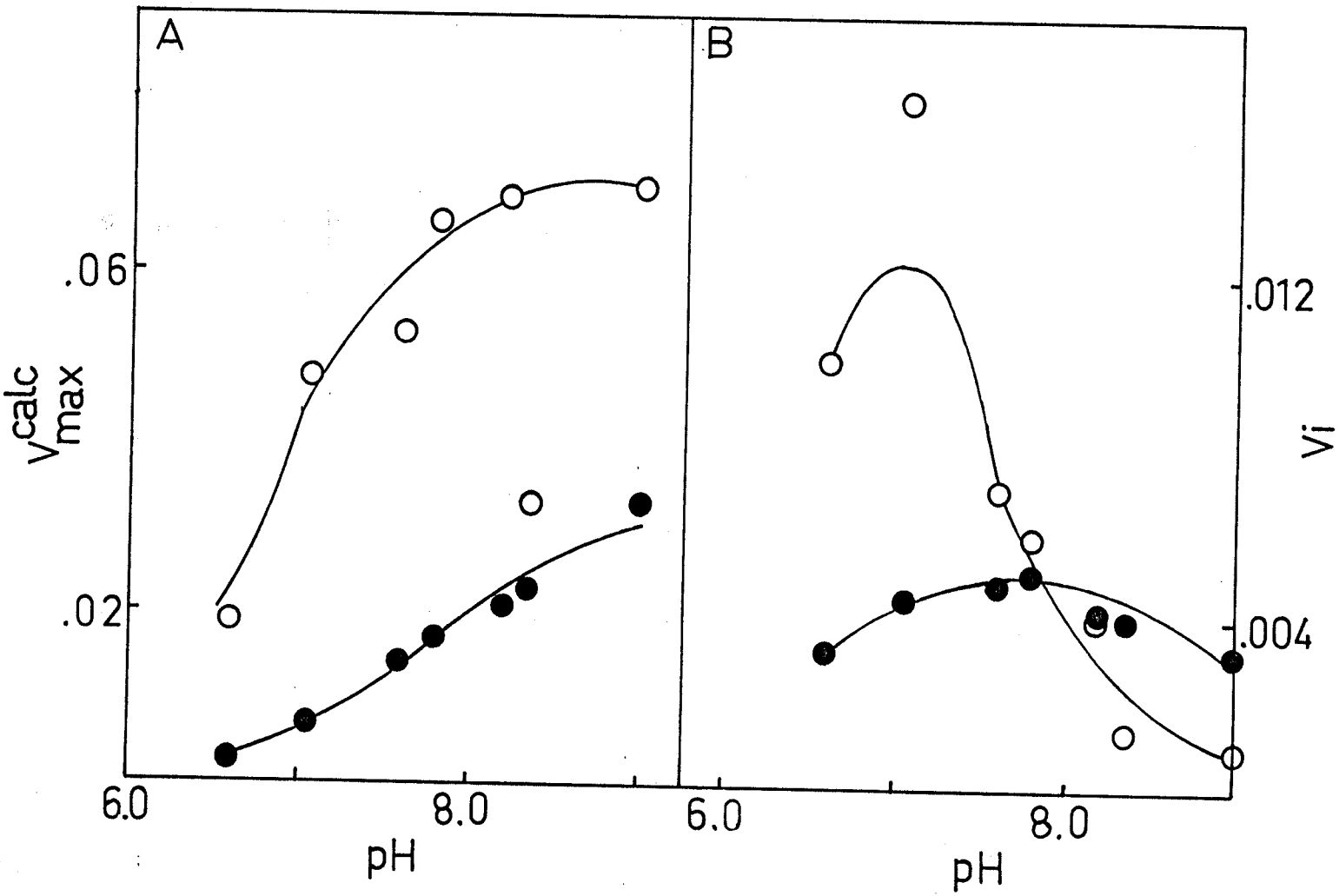

pH 8.37
2
1
0
-1
Log [Vi / (Vmax - Vi)]
2.0
3.0
Log [AcCoA]

Figure 16A: Effect of pH on the V_{max}. Values of V_{max} were calculated from plot of $\frac{V^{true}_{max}}{Vi}$ against pH (data used in presence of KCl was from Bell (1978) and without KCl see Table III).

B: pH rate profiles in presence of KCl (●-●) and in absence of KCl (○-○). In absence of KCl, velocity was measured at [AcCOA]=[OAA]= 0.4 M at 232 nm using same amount of enzyme for all the pH's studied. In presence of KCl, velocity was measured by standard assays at 412 nm using same amount of enzyme for all the pH's studied. The pH was measured before and after the reaction. The amount of enzyme used in the absence of KCl was 40 times greater than in the presence of KCl.


A
B
.06
.02
V_{max}^{calc}
.012
.004
Vi
6.0
8.0
pH
6.0
8.0
pH

DISCUSSION

DISCUSSION

The knowledge of a specific chemical cleavage mechanism, together with the amino acid composition of a protein, allows for the prediction of the number of peptides expected to be generated by a particular chemical cleavage reaction. E.coli citrate synthase contains six or seven cysteine and sixteen methionine residues per subunit as reported by Tong and Duckworth (1975). Wright and Sanwal (1971) had reported from their amino acid composition, peptide mapping experiments and SDS gel electrophoresis results that E.coli citrate synthase is composed of identical subunits. This has been further verified by Tong and Duckworth (1975) and from their SDS gel electrophoresis and sedimentation equilibrium study results they have also reported that the subunit molecular weight of E.coli citrate synthase is about 47,000 Daltons. The N-terminal sequence results give the best indication that subunits are identical (Table I).

Chemical cleavage of proteins with cyanogen bromide is specific for methionine residues at acid pH's if sulfhydryl functions have been blocked (Gross and Witkop, 1962 and Steers et al., 1965). In the studies reported here, citrate synthase was carboxymethylated with ^{14}[C]IAA and then cleaved with cyanogen bromide. Provided that the cleavage went to completion, seventeen peptides should be generated, up to six of which should contain ^{14}[C] from IAA. Amino acid analysis of the cyanogen bromide cleavage products revealed no detectable methionine which would indicate that the cleavage went to completion. This observation ruled out the possibility of the presence of Met-Ser and Met-Thr linkages. If these dipeptide sequences were present in the sequence, cyanogen bromide should have given an incomplete scission at methionine residues (Schroeder

et al., 1969 and Waxdal et al., 1968).

The first fractionation procedure by which I attempted to separate the cyanogen bromide peptide mixture was gel filtration on Sephadex G-75 which has a fractionation range of 3,000-70,000 Daltons for globular proteins. This gel should be suitable for resolving the larger peptides from the mixture. Given the molecular weight 47,000 for the citrate synthase subunit and sixteen cleavage points for the cyanogen bromide reaction, the average molecular weight of the peptides should be about 3,000. There were four peaks resolved on the Sephadex G-75 gel on the basis of ninhydrin reactive material and radioactivity. Following concentration on the rotary evaporator, three of the four peptide mixtures (CNBr I, CNBr II and CNBr IV) precipitated and could not be redissolved in any ionic solvent routinely used for peptide purification. The third (CNBr III) peptide mixture was soluble in 30% acetic acid.

CNBr I migrated as a single major band of molecular weight about 9,000 Daltons on SDS gels and as a major doublet on urea gels. The amino acid composition of this peptide (Table II) is in close agreement with its molecular weight as determined by SDS gel electrophoresis. There was a single detectable threonine as determined by N-terminal analysis. These observations lead to the conclusion that CNBr I is a relatively pure peptide, but the sequence analysis may prove to be difficult as preliminary experiments indicated that the N-terminal amino acid was blocked, resulting in low yields during the coupling reaction on the amino acid sequenator. This blockage seemed to have occurred under the storage conditions used to store it over a long period of time. The UV spectrum of CNBr I showed an absorption maximum at 274 nm but not at 280 nm. This indicates the

presence of tyrosine and the absence of tryptophan in the CNBr 1.

On urea gel electrophoresis, CNBr III revealed the presence of 8-10 peptides of the seventeen which were expected. Attempts to further purify this peptide mixture by gel filtration met with no success.

The differential solubility in two phases of the CNBr III peptides may be enough to allow the separation of these peptides by counter current distribution. The solvent system used in the purification was 2-butanol:0.5M acetic acid:10% TCA (40:50:1 V/V) and CNBr III had a partition coefficient of 1.6 between the upper and the lower phase. There was little resolution obtained after 95 transfers, although lowering the partition coefficient by adjusting the amount of TCA and using a larger number of transfers may have led to the separation of some of the peptides. The procedure was not repeated with a large number of transfers in consideration of the time and the amount of solvent that would be necessary for the experiment.

Cation exchange on SP-Sephadex under strongly acidic conditions did not resolve the peptides in CNBr III. In fact no peptides bound to the column at all.

Failure of the above methods to purify the peptides in CNBr III is probably due to the aggregation of the peptides under the conditions used. Peptide aggregates will move as a single entity during gel filtration. The failure of CNBr III to bind to SP-Sephadex seems to indicate that the basic functions of the peptides are not exposed. It is also possible that during counter current distribution aggregates were being distributed between the two phases rather than individual peptides.

Chemical modification of CNBr III peptides with citraconic anhydride

as reported by Atassi and Habeeb (1969) was used in an attempt to solve these aggregation difficulties. Haberland and Smith (1977) have used maleylation of peptides while Cossar-Gheerbrant et al. (1977) have used citraconylation of peptides to alleviate solubility and aggregation problems. The citraconylated CNBr III mixture was resolved into six peaks on a DE-52 anion exchange column (Figure 5). Amino acid analyses of these peaks gave amino acid ratios, after normalizing to homoserine lactone, near integral numbers which is one indication of the purity of these peptides. The amount of homoserine lactone recovered during amino acid analysis of CNBr III5c was very low and it is likely that this peptide is a C-terminal peptide. Neither of the two peptides, CNBr III4b and CNBr III5c, gave significant amino acids when submitted to ten cycles of the Edman degradation in the sequenator. This low yield during several successive cycles on the sequenator indicates that the amino-terminal residues are blocked in these peptides.

N-terminal analysis is one criterion which was used in the work presented here to check the purity of the peptides, although some difficulties were encountered which may have been due to blocked N-terminal residues. Blockage of amino-terminal residues of course also interferes with attempts at sequence determination of peptides by Edman degradation as it prevents the coupling reaction.

The most frequently observed blocking in proteins, and peptides derived from these proteins, occurs by the acylation of α-amino groups with simple carboxylic acids or with the γ-carbonyl group of glutamic acid via intramolecular cyclization of amino terminal glutamine (Konigsberg and Steinman, 1977).

Following citraconylation of CNBr III peptides, the peptide mixture was dissolved in ammonium acetate buffer, pH 8.0. At neutral or mildly acidic pH, amino terminal S-carboxymethyl cysteine may cyclize to form 6-membered cyclic amides (Doolittle, 1972; Smyth and Utsumi, 1967 and Press, 1967). Any peptide in CNBr III mixture with S-carboxymethyl cysteine as the amino terminal residue will become blocked. Under these conditions amino-terminal threonine and serine may also become blocked by migration of acyl groups from the hydroxy to the α-amino functions (Edman, 1970 and Konigsberg, 1967). Asn-Gly sequences either at the amino terminal or adjacent to the amino terminal residue may react under many of the conditions used in the work presented here to result in a blocked amino terminal (Konigsberg and Steinman, 1977).

In addition to this, aldehydes and some unknown factors may also cause blockage of amino terminal residues; these may have been present as impurities in the solvents used or may have been formed under storage conditions. Peptide mixtures were stored for long periods of time under acidic conditions during attempts to isolate these peptides and this may have resulted in blockage of amino terminal residues.

It is evident from the work presented here that the isolation and purification of peptides is a formidable task. However, it is hoped that these studies will lend some insight to the direction that further attempts to obtain the complete sequence of E.coli citrate synthase should take.

Steady State Kinetics

In the absence of KCl, which is an allosteric activator of E.coli citrate synthase, acetyl-CoA saturation curves display cooperativity, which is not observed when 100 mM KCl is present in the assay vessel (Faloona and Srere, 1969; Wright and Sanwal, 1971 and Duckworth and Tong, 1976). Although the cooperativity superficially appears to decrease when higher concentrations of OAA are used, the Hill's n values obtained from Hill plots (Figures 14 and 15) show no effect on cooperativity as OAA concentrations are varied. The data obtained at pH 6.56 show no cooperativity in acetyl-CoA saturation curves. These observations seem to indicate that cooperativity in the binding of the acetyl-CoA molecule to the enzyme is independent of binding of the OAA molecule.

We know from NADH binding studies that NADH inhibition increases with a decrease in pH (Duckworth and Tong, 1976). This could be explained by the hypothesis that the allosteric equilibrium ($R \rightleftharpoons T$) changes with pH. At lower pH, the $R \rightleftharpoons T$ would shift towards T state. If this is true, then, according to the allosteric theory (Monod et al., 1965) at lower pH the enzyme will have lower affinity for the substrate and as a result, there will be an increase in its K_M. The results reported in this thesis show that there is actually a decrease in the K_M for acetyl-CoA with a decrease in pH and that there is no cooperativity in acetyl-CoA saturation at the lower pH value tested, 6.56. These results are contrary to those expected according to the above hypothesis, and suggest that the stronger NADH inhibition at lower pH is not the result of changes in the L value. The observation of no cooperativity at pH 6.56 seems to indicate that the enzyme is in R state at that pH, which is consistent with the high affinity for acetyl-CoA.

The trend in K_{cat} values obtained here is similar to the one Bell (1978) observed in the presence of KCl. The K_{cat} values increase with an increase in pH. The shapes of the $V_{max}^{calc.}$ versus pH plots (Figure 16b) in presence and in the absence of KCl are similar; this indicates that the enzyme might follow the same reaction mechanism under both conditions.

Suggestions for Further Studies:

Of the seventeen possible peptides which could be obtained, one was purified to the point necessary for sequence analysis, and five more obtained after citraconylation seemed to be pure, although blockage of amino terminal residues prevented sequence analysis. Difficulties with aggregation and solubility of peptides has so far prevented the isolation and purification of other peptides.

The work presented in this thesis indicates that citraconylation of peptides immediately after cyanogen bromide cleavage will prevent most or all of the solubility and aggregation difficulties. As there is no necessity to use strongly acidic conditions (or denaturing solvents) to solubilize citraconylated peptides, there is a greater ease of handling. Gel filtration and ion exchange chromatography in buffers like ammonium bicarbonate can be performed to isolate and purify the peptides. Citraconylation itself blocks amino groups and this will prevent blockage of amino terminal residues by other agents, and since these peptides can easily be decitraconylated, the only possible blockage of amino terminal residue will occur during amino acid sequence determination itself.

BIBLIOGRAPHY

BIBLIOGRAPHY

Atassi, M. Z. and Habeeb, A. F. S. A. (1972) "Methods in Enzymology", Vol. 25 (C. H. W. Hirs and S. N. Timasheff, eds.), p. 546, Academic Press, New York.

Bell, A. W. (1978) M.Sc. Thesis, University of Manitoba.

Chou, P. Y. and Fasman, G. D. (1974) Biochemistry 13, 22.

Chou, P. Y. and Fasman, G. D. (1978) Ann. Rev. Biochem. 47, 251.

Cornish-Bowden, A. (1978) J. Theor. Biol. 74, 155.

Cossar-Gheerbrant, P., Sibilli-Weill, L., Briley, P. A., Chalvignac, M., Bras, G. L. and Cohen, G. N. (1978) Biochim. Biophys. Acta 535, 206.

Craig, L. C. (1960) "Analytical Methods of Protein Chemistry", Vol. 1 (P. Alexander and R. J. Block,eds.), p. 122, Pergamon Press, New York.

Danson, M. J. and Weitzman, P. D. J. (1973) Biochem. J. 135, 513.

Dayhoff, M. O., Barker, W. C. and Hardman, J. K. (1972) "Atlas of Protein Sequence and Structure" Vol. 5 (M. O. Dayhoff, ed.) p. 53, National Biomedical Research Foundation, Washington, D.C.

Doolittle, R. F. (1972) "Methods in Enzymology" Vol. 25 (C. H. W. Hirs and S. N. Timasheff, eds.) p. 231, Academic Press, New York.

Duckworth, H. W. and Tong, E. K. (1976) Biochemistry 15, 108.

Edman, P. (1970) "Protein Sequence Determination" (S. Needleman, ed.) p. 211, Springer-Verlag, Berlin and New York.

Faloona, G. R. and Srere, P. A. (1969) Biochemistry 8, 4497.

Flavell, R. B. and Fincham , J. R. S. (1968) J. Bacteriol. 95, 1063.

Gottschalk, G. (1968) Eur. J. Biochem. 5, 346.

Gray, W. R. (1972) "Methods in Enzymology" Vol. 25 (C. H. W. Hirs and S. N. Timasheff, eds.) p. 121, Academic Press, New York.

Haberland, M. E. and Smith, E. L. (1977) J. Biol. Chem. 252, 8196.

Higa, A. I., Massarini, E. and Cazzulo, J. J. (1978) Can. J. Microbiol. 24, 215.

Hirs, C. H. W., Moore, S. and Stein, W. H. (1956) J. Biol. Chem. 219, 623.

Hirs, C. H. W., Moore, S. and Stein, W. H. (1960) J. Biol. Chem. 235, 633.

Johnson, M. J. (1960) "The Enzymes" (2nd Ed.) Vol. 3 (P. D. Boyer, H. Lardy and K. Myrback, eds.) p. 407, Academic Press, New York.

Johansson, C. J. and Pettersson, G. (1974a) Eur. J. Biochem. 42, 383.

Johansson, C. J. and Pettersson, G. (1974b) Eur. J. Biochem. 46, 5.

Kobr, M. J., Vanderhaeghe, F. and Combépine, G. (1969) Biochem. Biophys. Res. Comm. 37, 640.

Köller, W. and Kindl, H. (1977) Arch. Biochem. Biophys. 181, 236.

Konigsberg, W. H. (1967) "Methods in Enzymology" Vol. 11 (C. H. W. Hirs, ed.) p. 576, Academic Press, New York.

Konigsberg, W. H. and Steinman, H. M. (1977) "The Proteins" (3rd Ed.) Vol. 3 (H. Neurath and R. L. Hill, eds.) p. 1, Academic Press, New York.

Leboy, P. S., Cox, E. C. and Flaks, J. G. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 1367.

Monod, J., Wyman, J. and Changeux, J. P. (1965) J. Mol. Biol. 12, 88.

Morse, D. and Duckworth, H. W. (1980) Can. J. Biochem., in press.

Press, E. M. (1967) Biochem. J. 104, 30c.

Sadar, M. H., Queen, A. and Duckworth, H. W. (1977) Can. J. Biochem. 55, 714.

Sarkissian, I. V. (1970) Biochem. Biophys. Res. Comm. 40, 1385.

Schwartz, R. M. and Dayhoff, M. O. (1978) "Atlas of Protein Sequencing and Structure" Vol. 5, Supplement 3 (M. O. Dayoff, ed.) p. 29, National Biomedical Research Foundation, Washington, D.C.

Schroeder, W. A., Shelton, J. B. and Shelton, J. R. (1969) Arch. Biochem. Biophys. 130, 551.

Singh, M., Brooks, G. C. and Srere, P. A. (1970) J. Biol. Chem. 245, 4636.

Smyth, D. and Utsumi, S. (1967) Nature 216, 332.

Srere, P. A. (1972) Curr. Top. Cell. Reg. 5, 283.

Srere, P. A., Brazil, G. and Gonen, L. (1963) Acta. Chem. Scand. 17, s129.

Steers, E. Jr., Craven, G. R. and Anfinsen, C. B. (1965) J. Biol. Chem. 240, 2478.

Talgoy, M. M., Bell, A. W. and Duckworth, H. W. (1979) Can. J. Biochem. 57, 822.

Tong, E. K. and Duckworth, H. W. (1975) Biochemistry 14, 235.

Waxdal, M. J., Konigsberg, W. H., Henley, W. and Edelman, G. M. (1968) Biochemistry 7, 1959.

Weber, K., Pringle, J. R. and Osborn, M. (1972) "Methods in Enzymology" Vol. 26 (C. H. W. Hirs and S. N. Timasheff, eds.) p. 3, Academic Press, New York.

Weitzman, P. D. J. (1969) "Methods in Enzymology" Vol. 13 (J. M. Lowenstein, ed.) p. 22, Academic Press, New York.

Weitzman, P. D. J. and Danson, M. (1976) Curr. Top. Cell. Reg. 10, 161.

Weitzman, P. D. J. and Jones, D. (1968) Nature 219, 270.

Wilkinson, G. N. (1961) Biochem. J. 80, 32A.

Woods, K. R. and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 369.

Wright, J. (1970) Ph.D. Thesis, University of Manitoba.

Wright, J. and Sanwal, B. D. (1971) J. Biol. Chem. 246, 1689.