STUDIES ON CITRATE SYNTHASE

by

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A thesis submitted in partial fulfilment of the regulations governing the Ph.D. degree at the University of Leicester.

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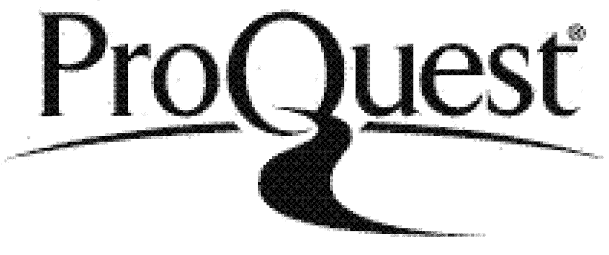
July, 1977



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To my parents and Jean.

ACKOWLEDGMENTS

I wish to express the deepest gratitude to my supervisor, Dr. P.D.J. Weitzman, for his help, guidance and encouragement throughout this work.

I thank Professors H.L. Kornberg FRS and W.V. Shaw in whose department this work was carried out and the Science Research Council for the award of a Research Studentship.

I am indebted to Professor H.L. Kornberg FRS and Dr. J.R. Guest for the gift of some of the bacterial strains used in this work.

I am also grateful to Mr. J.R. Parkin for his expert help with the analytical ultracentrifugation, and similarly to Ms. J. Hunt with the electron microscopy. My gratitude is also owed to the Computer Laboratory for the extensive use of their facilities.

Finally I would like to thank all my friends and colleagues in this university who have helped my research with useful comments and suggestions and whose companionship has made my stay in Leicester such an enjoyable experience.

ABSTRACT.

I The mode of action of a number of inhibitors of citrate synthase has been studied. The results suggest that α-oxoglutarate acts as an allosteric inhibitor of the enzyme from Gram negative facultatively anaerobic bacteria, but as an isosteric inhibitor of the enzyme from other sources. Similarly, NADH has been shown to be a powerful allosteric inhibitor of citrate synthases from Gram negative bacteria, but an isosteric inhibitor of the enzyme from Gram positive bacteria and eucaryotes. Other nucleotides have been shown to act only as isosteric inhibitors of citrate synthases from all sources examined.

II Techniques have been developed which facilitate the rapid determination of the regulatory properties of a citrate synthase and its molecular size. The striking correlation between these properties and the Gram reaction of bacteria is discussed. It has been proposed that the rapid techniques described here could be of value in bacterial taxonomic studies and for bacteriological identification.

III A citrate synthase deficient mutant of Escherichia coli has been isolated using a penicillin enrichment technique. A method has been developed which allows for the direct selection of citrate synthase deficient mutants by virtue of their intrinsic resistance to fluoroacetate. Two citrate synthase deficient strains of Acinetobacter lwoffi have been isolated using this method.

IV A number of revertants, which have regained citrate synthase activity, were isolated from these citrate synthase deficient strains. A comparative study of the molecular, catalytic and regulatory properties of these enzymes has been carried out and possible structure-function relationships have been discussed.

V Using revertant strains of E. coli which produce citrate synthases with regulatory properties different to those of the enzyme from the wild type organism, an attempt has been made to investigate the physiological significance of this altered regulatory behaviour of the enzyme.

Revertants which have a citrate synthase which is not inhibited by α-oxoglutarate (an allosteric inhibitor of the enzyme from E. coli wild type) appear to overproduce and excrete this (or a related) compound under certain growth conditions. Such a finding does suggest that the α-oxoglutarate inhibition of citrate synthase has a physiological role in the regulation of this enzyme in this organism.

ABBREVIATIONS

Most of the abbreviations used in this thesis are those recommended in the Biochemical Society Publication "Policy of the Journal and Instructions to Authors (Biochem. J. (1973) 131, 1-20).

Non-Standard Abbreviations

DTNB; 5,5'-dithiobis-(2-nitrobenzoic acid)

EMS; Ethyl methane sulphonate

'Tris buffer'; 20mM-Tris-HCl (pH 8.0), 1mM EDTA

All temperatures are indicated by (^{o}) and are in ^{o}Centigrade.

CONTENTS

INTRODUCTION.

The Position of the Tricarboxylic Acid (TCA) Cycle in Metabolism.

Metabolic pathways may be subdivided into two major categories, one being responsible for catabolism (or degradation) and the other for anabolism (or biosynthesis). A catabolic pathway accomplishes the breakdown of compounds to yield energy whereas an anabolic reaction sequence is responsible for the building-up of components required for biosynthesis. The reactions of the TCA cycle are represented in Fig. 1 and it can be seen that the cycle fulfils both catabolic and anabolic functions. Davis (1961) proposed the term amphibolic to describe a pathway with both degradative and biosynthetic roles and the TCA cycle is clearly an example of such an amphibolic sequence.

The catabolic role of the cycle was the first to be realised when Krebs and Johnson (1937) suggested that the cycle was utilised by animal tissues during the terminal stage of carbohydrate metabolism. More generally it is now known that this metabolic cycle serves for the final oxidation of all major foodstuffs in respiring organisms (Kornberg, 1959; Krebs & Lowenstein, 1960). The cycle is able to achieve the complete oxidation of acetyl moieties to carbon dioxide and reduced pyridine nucleotide which may be re-oxidised via the electron transport chain with the concomitant production of energy in the form of ATP.

The anabolic role was proposed by Krebs et al. (1952) who suggested that, in addition to the provision of energy, the cycle also serves to supply biosynthetic precursors for many cell components. Tracer studies by Roberts et al. (1953) confirmed this function, aspartate and glutamate being the most important metabolites produced by the cycle.

With few exceptions all aerobic and facultatively anaerobic chemo-organotrophs have a complete functional TCA cycle. Obligate chemolitho-trophs and anaerobic chemo-organotrophs have an incomplete cycle. It is

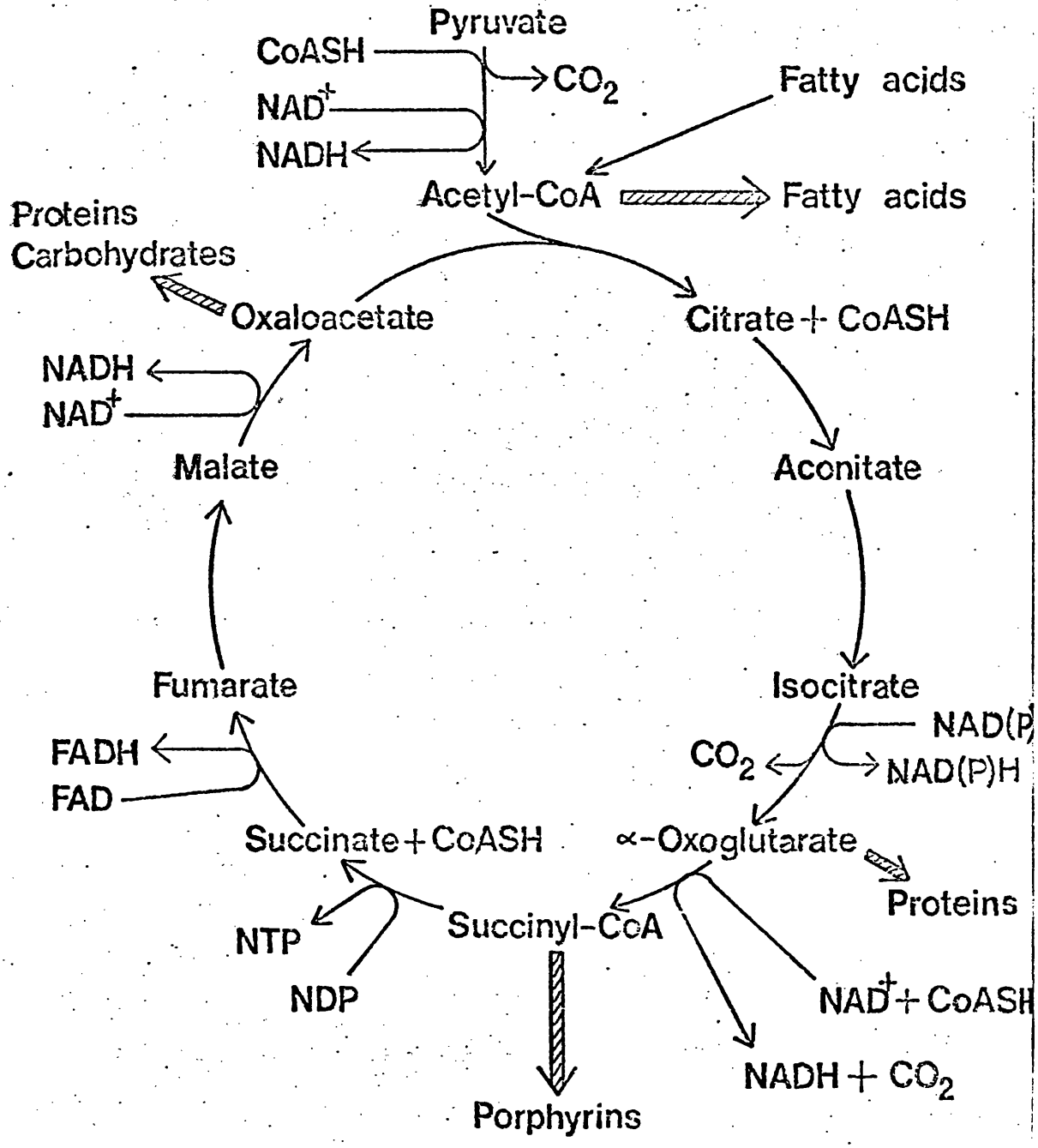


Figure 1. The role of the tricarboxylic acid cycle in the production of energy and cell constituents

particularly interesting to note that when grown anaerobically, facultative anaerobes (e.g. Escherichia coli) are unable to derive ATP from reduced pyridine nucleotides and thus the energy-producing role of the cycle is lost. Under these conditions, α-oxoglutarate dehydrogenase is absent (Amarasingham & Davis, 1965) but the anabolic functions are maintained by a non-cyclic forked pathway (Fig. 2).

The Position of Citrate Synthase in the TCA Cycle.

Citrate synthase has been found in animal tissues, bacteria and yeasts (Stern & Ochoa, 1951), fungi (Ramakrishnan & Martin, 1954) and plants (Desphande & Ramakrishnan, 1959) while its apparent absence from aerobic organisms has only been reported in a few bacteria, Lactobacillus plantarum, Streptococcus faecalis and Gemella haemolysans (Weitzman & Danson, 1976).

The enzyme occupies a prominent position in the TCA cycle as it is the only enzyme which catalyses the formation of a carbon - carbon bond and thereby effects the entry of carbon into the cycle in the form of acetyl moieties in the following reaction,

$$\text{Oxaloacetate}^{2-} + \text{acetyl-CoA} + H_2O = \text{citrate}^{3-} + \text{CoASH} + H^+$$

It may therefore be considered the 'initial' enzyme of the cycle.

Importance of Citrate Synthase in the Regulation of the TCA Cycle.

The cycle occupies a central role in many metabolic processes and must be under strict control. As citrate synthase may be considered the initial enzyme of the cycle, its control should make a considerable contribution to the overall control of the cycle. Indeed, the fact that cycle intermediates do not accumulate and that addition of such intermediates produces an increased rate of oxidation led Krebs and Lowenstein (1960) to conclude that the rate limiting step of the cycle is the

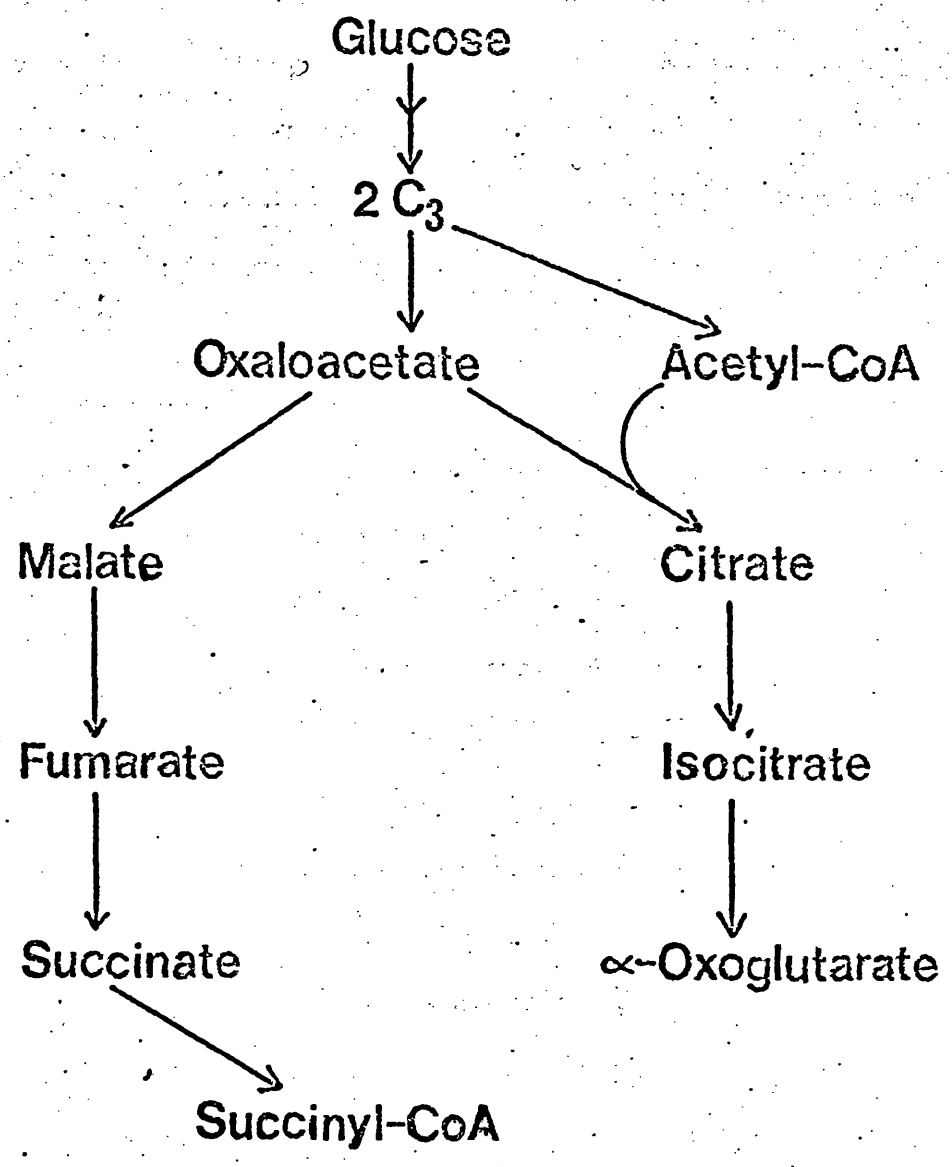


Figure 2. Modified tricarboxylic acid cycle in E.coli
Scheme proposed by Amarasingham & Davis (1965) for E.coli growing under anaerobic conditions.

synthesis of citrate and that primary control of the cycle must be exerted on citrate synthase, the enzyme responsible for this synthesis. It must be emphasised that other enzymes of the cycle may also be regulated but, as citrate synthase is responsible for the introduction of carbon, the control of this enzyme will regulate the flux of carbon through the cycle. It is therefore not surprising that the molecular, catalytic and regulatory properties of citrate synthase have been intensively investigated (Srere, 1971; Weitzman & Danson, 1976) and it is an extension of such studies that is reported here.

Mechanisms for Regulating Citrate Synthase.

The control mechanisms which operate on citrate synthase may be divided into three categories.

1. Genetic control, that is the control of gene expression. Natural selection will operate against an organism which produces a large excess of unnecessary enzyme and an organism therefore regulates the amount of enzyme produced according to the environmental conditions. The level of citrate synthase (and indeed of all TCA cycle enzymes) in bacteria varies considerably when growth occurs on different carbon sources (Gray, Wimpenny & Mossman, 1966; Flechtner & Hanson, 1969; Eidels & Preiss, 1970). Indeed, it has been suggested that the level of citrate synthase in a cell is a measure of its oxidative capacity (Srere, 1969).

2. Control by regulation of the supply of substrates for the enzyme. To ensure that the overall rate of a sequence of reactions should be constant, Dixon and Webb (1964) have proposed that the concentration of substrates must be adjusted to be in the region of the K_m values for their enzymes. It is clear that an alteration in the availability of either oxaloacetate or acetyl-CoA may play an important role in the

control of this enzyme.

3. The enzyme may also be controlled by effector molecules which interact with the enzyme and result in inhibition or activation. Interest in metabolic regulation has largely centred on this method of control of enzyme activity. In general, anabolic pathways are controlled by negative feedback mechanisms whereby the ultimate end-product of the pathway inhibits the activity of the initial enzyme (Umbarger, 1956). In contrast, catabolic sequences are often controlled by molecules which indicate a low-energy state acting as activators of the initiating enzyme. An example of such a case is the activation of the degradative threonine deaminase of E. coli by AMP. However, as citrate synthase is the initial enzyme of an amphibolic pathway, the control of this enzyme would be expected to be particularly complex (Sanwal, 1970); the control mechanisms taking account of both the anabolic and catabolic roles of the enzyme. Indeed, a great variety of effectors have been shown to regulate citrate synthase activity, and the nature of this control has predictbly been found to be complex.

Regulation of Citrate Synthase by Effectors.

It must be emphasised that all reported studies on the properties of citrate synthase have been obtained using in vitro cell-free extracts or in situ toluenised cell suspensions and therefore the in vivo significance of the findings remains uncertain.

Hathaway and Atkinson (1965) demonstrated that ATP inhibits yeast citrate synthase and proposed that ATP, being an ultimate end-product of the TCA cycle, acts as a feedback inhibitor of this enzyme. This finding has prompted a comprehensive study of the effect of this and other nucleotides on a wide variety of citrate synthases (Weitzman & Danson, 1976).

ATP inhibits citrate synthase from eucaryotes (Lee & Kosicki, 1967; Moriyama & Srere, 1971) and Gram positive bacteria (Flechtner & Hanson, 1969) and, to a much lesser extent, that from Gram negative bacteria (Weitzman, 1966b). In addition to this ATP inhibition, a number of other nucleotides inhibit citrate synthase, albeit weakly (Weitzman & Danson, 1976).

Citrate synthase from E. coli is relatively insensitive to ATP and in a search for an alternative regulator Weitzman (1966a,b) found that NADH, a direct end-product of the TCA cycle, acts as a very powerful inhibitor of this enzyme. The specificity of the inhibition is clearly demonstrated by the fact that NADPH, $NADP^+$ or NAD^+ do not inhibit this enzyme. That the NADH inhibition is allosteric was shown by the fact that it may be abolished by high salt concentration or pH (Weitzman, 1966b).

In a comprehensive study of a range of bacteria Weitzman and Jones (1968) found that Gram positive bacterial citrate synthases are unaffected by NADH but that all Gram negative bacterial citrate synthases examined are powerfully inhibited by this compound. Furthermore, the Gram negative bacterial enzymes could be differentiated by the finding that whereas the inhibitory effect of NADH on the enzyme from facultative anaerobes was unaffected by AMP, the inhibition of the enzyme from strict aerobes was completely overcome by AMP. This effect may be the response of the enzyme to the low-energy signal of increasing AMP concentration.

In addition to this correlation between the Gram reaction and regulatory sensitivity (Weitzman & Jones, 1968) a further correlation has been established between the Gram reaction of bacteria and the molecular weight of their citrate synthases (Weitzman & Dunmore, 1969a). It was found that the mol. wt. of citrate synthases from Gram positive bacteria and eucaryotic sources is approximately 100,000, whereas citrate synthases from Gram negative bacteria are considerably larger with a

mol. wt. of approximately 250,000.

Citrate synthases are also inhibited by α-oxoglutarate. The eucaryotic enzyme is only weakly inhibited (Srere, 1971) but E. coli citrate synthase is powerfully inhibited by this compound. This inhibition of the E. coli enzyme is abolished by high salt or pH and is therefore probably allosteric (Wright et al., 1967). Weitzman and Dunmore (1969b) showed that α-oxoglutarate is a powerful inhibitor of the citrate synthases from other Gram negative facultative anaerobes and proposed a rationale for this inhibition in terms of the metabolic pathway operative in such organisms. Fig. 2 shows the forked pathway which exists under anaerobic conditions (Amarasingham & Davis, 1965) and the absence of α-oxoglutarate dehydrogenase results in α-oxoglutarate being a direct end-product of the pathway and it therefore acts as a feedback inhibitor of citrate synthase in these organisms. Other bacteria which lack α-oxoglutarate dehydrogenase have citrate synthases which are powerfully inhibited by α-oxoglutarate; examples of such organisms are blue green bacteria (Taylor, 1973; Lucas & Weitzman, 1975) and strictly autotrophic thiobacilli (Taylor et al.,1969).

All inhibitors of citrate synthase which have been examined are structural analogues of one or other substrate of the enzyme; α-oxoglutarate is an analogue of oxaloacetate and ATP, NADH and other nucleotides are analogues of acetyl-CoA. The possible mechanism of action of these inhibitors is therefore twofold; they may either be bound at the catalytic site resulting in inhibition competitive with the substrate or they may be bound at a distinct, allosteric site.

Various treatments have been shown to abolish the specific NADH inhibition of Gram negative bacterial citrate synthases (Weitzman & Danson, 1976) and the enzymes from Gram negative facultative anaerobes have similarly been desensitised to α-oxoglutarate inhibition (Danson & Weitzman, 1973). Such observations accord with the view that these

inhibitions are allosteric. Attempts to abolish ATP and other nucleotide inhibitions of citrate synthase have not been successful and therefore it is uncertain whether these inhibitors act allosterically or at the catalytic site in an isosteric manner. Similarly uncertainty also exists concerning the mechanism whereby α-oxoglutarate inhibits the enzyme from all but the Gram negative facultative anaerobes.

Aspects of Citrate Synthase Requiring Further Investigation.

A full understanding of the molecular processes involved in catalysis and its regulation requires knowledge of the chemical structure of the enzyme, the functional groups participating in the binding of ligands (substrates and effectors) and the interactions leading to modulation of activity. Although no single technique can provide this knowledge, information may be integrated from a variety of chemical, physical and biochemical methods. Studies carried out in Dr. P.D.J. Weitzmans' laboratory involve a number of techniques with the aim of gaining further knowledge of the molecular processes of catalysis and regulation for citrate synthase and the present work is a part of these investigations into structure-function relationships of this enzyme.

Two techniques which have been extensively used to explore the relationship between enzyme structure and function are those of chemical modification and genetical modification. The success of chemical modification relies on the reaction between a variety of chemical reagents and certain amino acids within a protein. The consequence of such treatment is often the loss of essential groups in the protein resulting in changes in the molecular properties. Chemical modification methods have several limitations. The non-specificity of most reagents may prevent the definite and unambiguous implication of particular amino

acids in enzyme functions. Furthermore, suitable modification methods are only available for a limited range of the twenty amino acids commonly found in proteins; therefore the role played by the unreactive amino acids cannot be established. Even amino acids for which modification methods are available may be situated in the protein structure such that they are inaccessible to the modifying reagent and therefore their role in enzyme functions cannot be determined. After modification the enzyme may be heterogeneous, consisting of unreacted native enzyme and a mixture of modified enzymes, and it may thus be very difficult to interpret the observed changes in molecular properties. However, using this technique a comparative study of native and modified protein has been made for a number of citrate synthases and various functional groups have been implicated in the activity and regulation of these enzymes (Weitzman & Danson, 1976).

To further the understanding of the structure-function relationships in citrate synthase the present work has employed the second method of investigation, i.e. the study of genetically altered enzymes. Although this method has provided useful information for other enzyme systems, it has not hitherto been applied to citrate synthase.

Whereas chemical modification involves the *in vitro* treatment of a protein, genetical modification is achieved by selecting 'mutant' organisms which have an alteration in the DNA sequence (or gene) coding for a protein. Such mutants will produce a protein, in this case citrate synthase, with a primary structure different from that produced by the wild type organism and this alteration in structure may result in changes in the molecular properties of the protein. This method of obtaining modified proteins *in vivo* has several advantages over the chemical, *in vitro*, treatments. Among the advantages are that all amino acids are

susceptible to changes regardless of their reactivity or position within the protein and also that the enzyme synthesised by such a mutant will be homogeneous, thus simplifying the interpretation of observations.

A further advantage of the genetical modification method is that some mutants may produce citrate synthases with altered molecular properties. A study of such mutants may therefore greatly help in the assessment of the *in vivo* physiological significance of the properties observed *in vitro*. For example, the isolation of mutants which produce citrate synthases with altered regulatory properties would be invaluable in proposing the physiological role of the *in vitro* observations of regulation by effectors.

The present work is therefore concerned with the isolation of mutants of *E. coli* (a Gram negative facultative anaerobe) and of *Acinetobacter lwoffi* (Gram negative strict aerobe) which produce citrate synthases with properties different from those of the corresponding wild type organisms. Comparative studies of the molecular properties of these enzymes have been undertaken. Furthermore, using the same mutants, an attempt has been made to establish the *in vivo* significance of the molecular properties observed *in vitro*. In addition, the mechanism of inhibition of citrate synthase by a variety of effectors has been investigated in order to establish whether these inhibitors act isosterically or allosterically.

MATERIALS AND METHODS.

Chemicals

Citrate synthase (pig heart), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart), oxaloacetic acid, Coenzyme A and NADH were from Boehringer Corp. (London) Ltd., London W.5, U.K.; catalase (bovine liver), glutamic dehydrogenase (bovine liver), ATP, ADP, AMP, NADPH, $NADP^+$, NAD^+, α-oxoglutarate, D-cycloserine, ethyl methane sulphonate (EMS) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were from Sigma London Chemical Co., Kingston-upon-Thames, Surrey, U.K.; Sephadex G-10 and G-200 were obtained from Pharmacia (Great Britain) Ltd., London, U.K.; protamine sulphate was from Koch-Light Laboratories Ltd., Colnbrook, U.K.; ampicillin and carbenicillin were from Beecham Research Laboratories, Brentford, U.K. and 'API 50 Enterobacteriaceae' microtube systems were from API Products, Rayleigh, Essex, U.K. All other chemicals used were the finest grade commercially available.

Preparation of Acetyl-CoA and Bromoacetyl-CoA

Acetylation of CoASH was carried out as described by Stadtman (1957). Bromoacetyl-CoA was prepared by transacylation from bromoacetylthiophenol to CoASH by the method of Chase and Tubbs (1969) and, before use, was passed through a small column of Sephadex G-10.

Preparation of Fluoro-oxaloacetate

Fluoro-oxaloacetate was prepared by transesterification of diethyl fluoro-oxaloacetate (Kun et al., 1958) which had been synthesised by the method of Rivett (1953).

Determination of Acetyl-CoA and Bromoacetyl-CoA Concentrations

A sample of acetyl-CoA or bromoacetyl-CoA was mixed with 0.1mM DTNB and 0.2mM oxaloacetate in 20mM Tris-HCl buffer, pH 8.0, 1mM EDTA (hereafter referred to as 'Tris buffer') in a total reaction volume of 1.0ml. Pig heart citrate synthase (10μg) was added and the reaction allowed to proceed to completion. The concentration of acetyl-CoA or bromoacetyl-CoA was calculated from the observed absorption change at 412nm due to the

thionitrobenzoate anion (E_m = 1.36 x 10^4) produced during the reaction.

Determination of Oxaloacetate and Fluoro-oxaloacetate Concentrations

A sample of oxaloacetate or fluoro-oxaloacetate was mixed with 0.1mM DTNB and 0.15mM acetyl-CoA in 'Tris buffer' in a total reaction volume of 1.0ml. Pig heart citrate synthase (10µg) was added and the reaction allowed to proceed to completion. The concentration of oxaloacetate or fluoro-oxaloacetate was calculated from the observed absorption change at 412nm as above.

Organisms Used in this Investigation

The strains of E. coli used in this work are listed in Table 1 which shows the auxotrophic markers they carry, and hence indicates their growth requirements. The abbreviations used for genetic markers are those of Taylor and Trotter (1972).

Other organisms used in the work were Acetobacter xylinum (NCIB 8246), Acinetobacter lwoffi 4B (laboratory strain), Arthrobacter globiformis (NCIB 8602), Bacillus megaterium (laboratory strain), Brevibacterium linens (ATCC 9174), Cellulomonas rossica (NCIB 8074), Kurthia zopfii C5 (laboratory strain), Pseudomonas aeruginosa (NCIB 8295), Pseudomonas fluorescens D1086 (laboratory strain), Pseudomonas iodinum (NCIB 8179) and Saccharomyces cerevisiae (Bakers' yeast).

Maintenance of Organisms

The organisms used were routinely stored at 4° on nutrient agar slopes supplemented with thymine and methionine (each at 50µg/ml) and glutamate (200µg/ml). All strains were maintained by periodic subculture and were checked before use for the possession of the appropriate genetic markers, and for the absence of contaminants, by testing their growth on selective media. Organisms in frequent use were also stored as single colony isolates on nutrient agar plates.

Table 1: Strains of *E. coli* used in this present work.

Strain	Markers	Reference
K1.1	Hfr *thy metB pps* str^s	Brice and Kornberg (1967).
K1.1.4	Hfr *thy metB pps* str^s *gltA*	From H.L. Kornberg.
K1.1.4.R1-R40	Hfr *thy metB pps* str^s $gltA^{rev}$	This work.
K2.1	F^- *his arg(HBCE) thr leu galP mgl pps* str^r	Brice and Kornberg (1967).
K2.1.4	F^- *his arg(HBCE) thr leu galP mgl pps* str^r *gltA*	From H.L. Kornberg.
K2.1.4.R1-R25	F^- *his arg(HBCE) thr leu galP mgl pps* str^r $gltA^{rev}$	This work.
AB1621	F^- *ara lac gal thi* str^r	Ashworth *et al.* (1965).
AB1623	F^- *ara lac gal thi* str^r *gltA*	Ashworth *et al.* (1965).
AB1623.R1-R37	F^- *ara lac gal thi* str^r $gltA^{rev}$	This work.
AB259	Hfr *lac* str^s T_6^s	Ashworth *et al.* (1965).
AB259-CS1	Hfr *lac* str^s T_6^s *gltA*	This work.
AB259-CS1R1-R18	Hfr *lac* str^s T_6^s $gltA^{rev}$	This work.

s = sensitive.

r = resistant.

rev = revertant.

Media

Organisms were grown in either nutrient broth or the basal salts medium of Ashworth and Kornberg (1966) with a carbon source at a concentration of 10mM, and was supplemented with growth factors where necessary (50µg/ml for amino acids and thymine and 1µg/ml for vitamin B_1).

Media and supplements were sterilised by autoclaving at 15lb/in^2 for 20 min except where this would have led to decomposition or alteration of the media in which case sterilisation was achieved by membrane filtration.

Solid medium was prepared by including 1.5% (w/v) agar before autoclaving.

Growth of Organisms

Liquid cultures were inoculated with a single colony from a pure culture of the organism on a nutrient agar plate. Cultures were grown in Erlenmeyer flasks in a rotary shaker thermostatically controlled at the optimum growth temperature for each organism. To ensure that the cultures were well aerated, the volume of liquid medium never exceeded 20% of the volume of the containing vessel.

Anaerobic cultures were grown in Erlenmeyer flasks completely filled with growth medium and sealed from the atmosphere by a rubber bung. These cultures were placed on a shelf in an incubation cabinet set at the optimum growth temperature for the organism.

To measure the growth rate of a strain on any particular carbon source an inoculum culture was grown in glycerol medium. This culture was harvested, washed with 0.9% saline solution and transferred to the test medium to give an initial cell density of about 5×10^7/ml. The growth rate was monitored by measuring the increase in absorption of the bacterial suspension at 550nm in a Unicam SP600 spectrophotometer using 3ml glass cuvettes of 1cm light path. Under these conditions an absorbance of 1.0

represents 0.4mg dry mass/ml for E. coli (Fig. 3) and 0.46mg dry mass/ml for A. lwoffi (Fig. 4).

Enzyme Assays

Definition of an Enzyme Unit

Unless otherwise stated, one enzyme unit is that which catalyses the utilisation of 1μmole substrate /min or the production of 1μmole product/ min.

Citrate Synthase

Two methods were used to assay this enzyme.

Method 1

Citrate synthase was routinely assayed spectrophotometrically at 412nm and 25° by the method of Srere et al. (1963). Unless otherwise stated, assay mixtures contained 0.2mM oxaloacetate, 0.15mM acetyl-CoA and 0.1mM DTNB, all in 'Tris buffer'. The reaction was started by the addition of enzyme to give a total volume of 1.0ml. The molar extinction coefficient of the thionitrobenzoate anion, produced during the reaction of DTNB with the liberated CoASH, is 1.36×10^4 at pH 8.0 and 412nm (Ellman, 1959).

Method 2

Some citrate synthases are inactivated by DTNB and to study such enzymes the continuous polarographic assay described by Weitzman (1969b) was used. The reaction vessel contained 0.2mM oxaloacetate and 0.15mM acetyl-CoA in 'Tris buffer'. Oxygen was expelled from the system by bubbling nitrogen through the reaction vessel for a few minutes. The reaction was started by the addition of enzyme to bring the total volume to 1.0ml; the rate of appearance of anodic current at -0.3 volt was monitored. This current is produced by the appearance of CoASH in the reaction vessel, and a calibration of the mercury electrode showed that 1.0mM CoASH produced a wave of magnitude 1.7μA at this potential.

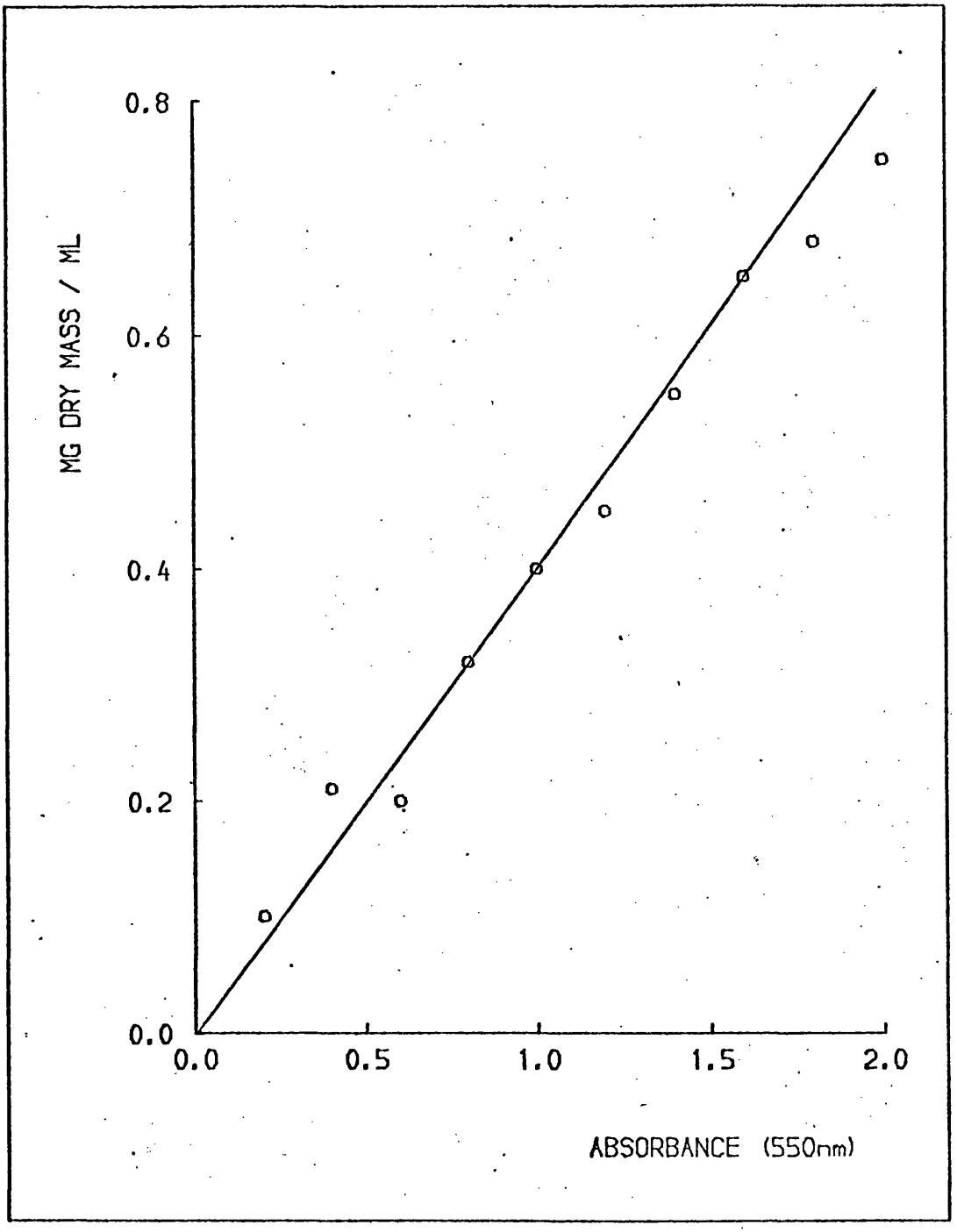


Fig. 3: Relationship between dry weight (mg dry mass/ml) and absorbance (at 550nm) of a suspension of E. coli.

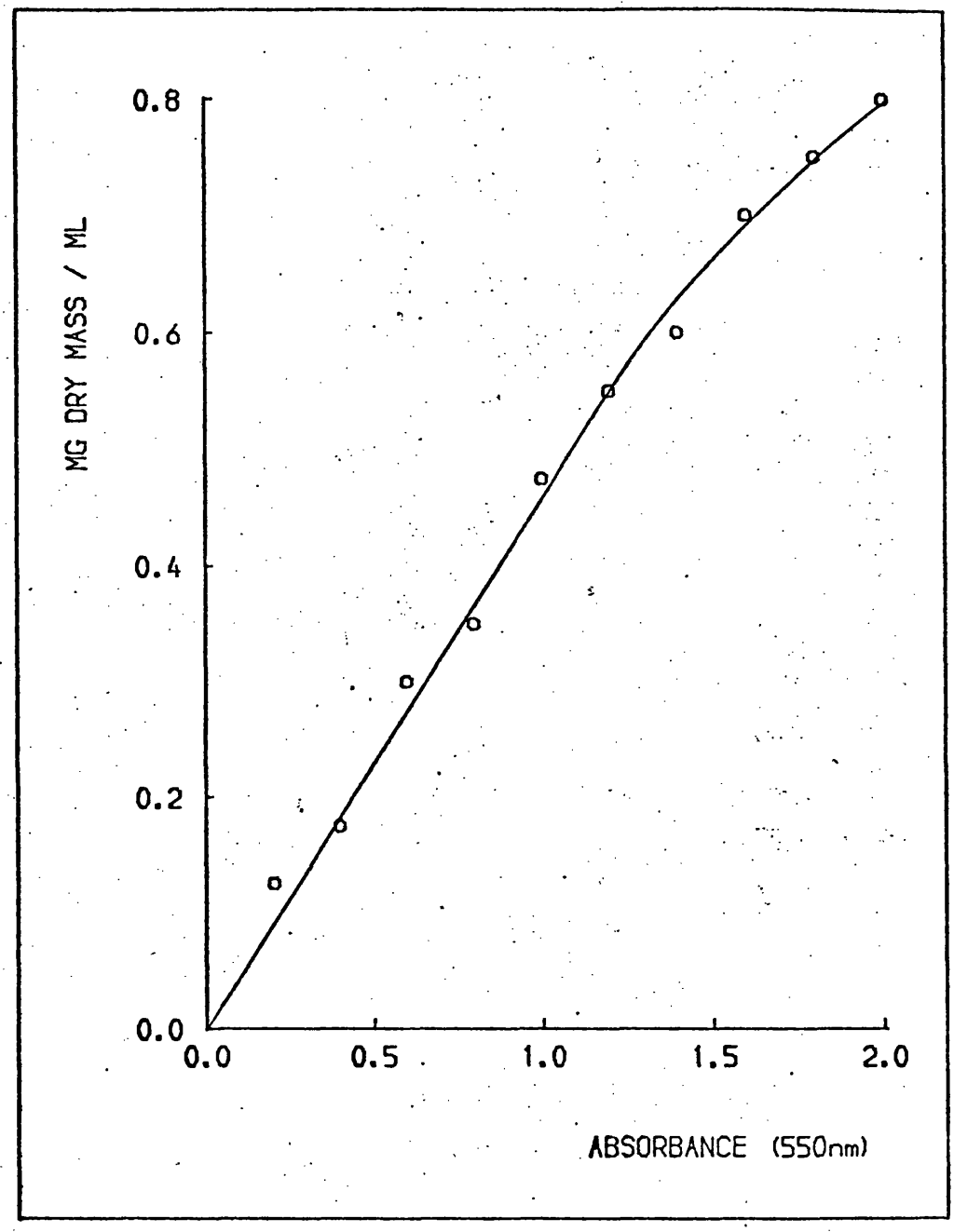


Fig. 4: Relationship between dry weight (mg dry mass/ml) and absorbance (at 550nm) of a suspension of A. lwoffi.

α-Oxoglutarate Dehydrogenase

This enzyme was assayed spectrophotometrically at 25° by following the formation of NADH at 340nm. Reaction mixtures contained 0.1M Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 0.1mM thiamine pyrophosphate, 2.5mM cysteine hydrochloride, 0.1mM CoASH, 0.5mM NAD^+ and 5mM α-oxoglutarate. The reaction was started by the addition of enzyme to give a final volume of 1ml.

Pyruvate Dehydrogenase

This enzyme was assayed spectrophotometrically at 25° by following the formation of NADH at 340nm. Reaction mixtures contained 0.1M Tris-HCl, pH 8.0, 10mM $MgCl_2$, 1mM EDTA, 0.1mM thiamine pyrophosphate, 2.5mM cysteine hydrochloride, 0.1mM CoASH, 0.5mM NAD^+ and 5mM pyruvate. The reaction was started by the addition of enzyme to give a total volume of 1ml.

Lactate Dehydrogenase

This enzyme was assayed spectrophotometrically at 340nm and 25° by following the disappearance of NADH. Reaction mixtures, in a total volume of 1.0ml, contained 0.2mM NADH, 0.2mM pyruvate and enzyme all in 'Tris buffer'. The E_m of NADH at 340nm is 6.2×10^3.

Catalase

Catalase is a coloured protein and its concentration was determined by measuring its absorbance at 400nm (Weitzman & Dunmore, 1969a).

Aconitase

Aconitase was assayed by measuring the rate of formation of cis-aconitate from citrate at 240nm (Racker, 1950). Reaction mixtures contained 50mM phosphate buffer, pH 7.4, and 30mM citrate. The reaction was initiated by the addition of enzyme to give a total volume of 1.0ml. An enzyme unit was that which produced an absorbance change of 0.001/min.

Isocitrate Dehydrogenase

This enzyme was assayed by the determination of $NADP^+$ reduction at 340nm. (Kornberg, 1955). Reaction mixtures contained 40mM phosphate buffer, pH 7.0, 3mM $MgCl_2$, 1.5mM $NADP^+$ and 0.2mM DL-isocitrate.

Malate Dehydrogenase

This enzyme was assayed by an adaptation of the method described by Ochoa (1955). Reaction mixtures contained 0.15mM NADH and 0.2mM oxaloacetate in 'Tris buffer'. Enzyme was added to give a final reaction volume of 1.0ml and the disappearance of NADH was followed at 340nm.

Estimation of Protein Concentration

The concentration of protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as a protein standard.

Determination of α-Oxoglutarate Concentration

A sample of solution containing α-oxoglutarate was mixed with 0.2mM NAD^+ and 5mM NH_4Cl in 'Tris buffer' in a total reaction volume of 1ml. Glutamic dehydrogenase (2μg) was added and the reaction allowed to proceed to completion. The concentration of α-oxoglutarate was calculated from the observed change in absorption at 340nm using a value of 6.2×10^3 for the molar extinction coefficient of NADH.

Determination of Glutamate Concentration

A sample of solution containing glutamate was mixed with 0.2mM NADH in 'Tris buffer' in a total reaction volume of 1ml. Glutamic dehydrogenase was added and the reaction allowed to proceed to completion. The concentration of glutamate was calculated from the observed change in absorption at 340nm using a value of 6.2×10^3 for the molar extinction coefficient of NADH.

Determination of Pyruvate Concentration

A sample of solution containing pyruvate was mixed with 0.2mM NADH in 'Tris buffer' in a total reaction volume of 1.0ml. Lactate dehydrogenase

(10μg) was added and the reaction allowed to proceed to completion. The concentration of pyruvate was calculated from the observed change in absorption at 340nm using a value of 6.2×10^3 for the molar extinction coefficient of NADH.

In Situ Examination of Bacterial Citrate Synthase

Three nutrient plates were patched identically with overnight cultures of the bacteria to be studied. Each strain was inoculated onto a $1cm^2$ region of each plate, up to five strains per plate. After incubation at the optimum growth temperature for the particular bacteria had allowed good growth (usually overnight), a 7cm circular Whatman No. 1 filter paper was pressed onto each plate so that most of the growth was transferred to the paper. The papers were then placed individually in glass Petri dishes containing a mixture of toluene and ethanol (1:4 v/v) and allowed to stand for 5 min at 25°. Each paper was transferred to a glass beaker containing 250ml 'Tris buffer' at 4° and stirred gently for 2h by means of a magnetic stirrer. This latter procedure was found to remove malate dehydrogenase from the bacterial cells without significant loss of citrate synthase activity (P.D.J. Weitzman, personal communication); the presence of malate dehydrogenase would otherwise interfere with the subsequent examination of the effect of NADH on citrate synthase activity (Weitzman, 1966a).

After draining off the excess solution the three papers of each set were placed in individual Petri dishes and flooded with 5ml of one of the following reaction mixtures: (i) 0.15mM acetyl-CoA, 1.0mM oxaloacetate and 0.1mM DTNB in 'Tris buffer'; (ii) as (i) but also containing 1.0mM NADH; (iii) as (i) but also containing 3.0mM ATP.

As in the spectrophotometric assay of citrate synthase, the CoASH produced on enzymic cleavage of acetyl-CoA reacts with the DTNB to form the intensely yellow thionitrobenzoate anion. The appearance of an

intense yellow colour in the patches of bacteria on the paper treated with solution (i) is a demonstration of citrate synthase activity. Whether or not NADH or ATP are inhibitory may be assessed readily by visual comparison of the corresponding patches on the three filter papers.

In addition, the effect of AMP on NADH inhibition was assessed by flooding a fourth paper (prepared as the other three) with a reaction mixture the same as (ii) above, but also containing 1.0mM AMP. Similarly, the effect of α-oxoglutarate on enzyme activity was established by flooding a paper with a reaction mixture as (i) above but also containing 1.0mM α-oxoglutarate.

The yellow thionitrobenzoate produced during the assay gradually diffused from the patches of bacterial growth on the filter papers. No more than five strains were tested on each plate and the patches were at least 2cm away from each other.

Rapid Determination of Molecular Size of Citrate Synthase

The method used by Weitzman and Dunmore (1969a) was modified in the following way. The dimensions of the Sephadex G-200 column were reduced to 0.9 x 25.0cm and a small syringe needle was attached to the outflow. The resolving power of this size column was tested by applying 0.08ml of a mixture of Dextran Blue (0.1mg), lactate dehydrogenase (0.01 units) and citrate synthase (0.01 units) from either B.megaterium or E. coli, all dissolved in 'Tris buffer' also containing 10mM $MgCl_2$ and 5% (w/v) sucrose. The eluent was 'Tris buffer' containing 10mM $MgCl_2$. As the Dextran Blue emerged from the column the collection of single drop fractions was started. These were allowed to fall into 1.5ml semi-micro disposable plastic cuvettes and stored at 4° until assay.

A total of 24 drops was collected over a period of about 15 min and alternate fractions were assayed for either lactate dehydrogenase or citrate synthase.

To determine the molecular size of a number of different citrate synthases, the bacteria were grown in 10ml of nutrient broth in 100ml flasks as previously described. The cells were harvested by centrifugation and resuspended in 0.5ml 'Tris buffer' containing 10mM $MgCl_2$. After disruption with an MSE 100W ultrasonic disintegrator and microprobe for a total of 1 min (10sec bursts interrupted by short cooling periods), cell-free extracts were prepared by centrifugation in a bench centrifuge. Lactate dehydrogenase, Dextran Blue and sucrose were added to each extract (see quantities above) and one of these mixtures applied to the column. This was allowed to enter the Sephadex and further extracts were added at 50 drop intervals. In this way it was possible for the column to carry up to four extracts simultaneously.

Partial Purification of Citrate Synthase

The method used was similar to that described by Weitzman (1969a)

(1) Preparation of Cell-Free Extracts

Cells were grown in liquid medium, centrifuged, washed and resuspended in 'Tris buffer'. This suspension was disrupted by sonication with an MSE 100W ultrasonic disintegrator operated at full power for a total of 1 min sonication time per ml of suspension by applying intermittent bursts of sonication with intervals of cooling. This and all subsequent steps were carried out at 4°. Cell debris was removed by centrifugation at 25,000g for 30 min.

(2) Removal of Nucleic Acid

Nucleic acid was removed from the supernatant by the drop-wise addition of a 2% (w/v) aqueous solution of protamine sulphate (0.8mg protamine sulphate per 10mg protein) and the mixture was stirred for 15 min. The precipitated nucleic acid was removed by centrifugation at 25,000g for 20 min.

(3) Ammonium Sulphate Fractionation

Finely ground ammonium sulphate was added to the stirred supernatant to give 45% saturation, the mixture stirred for 20 min and the precipitate removed by centrifugation as above. Further additions of ammonium sulphate were made to the supernatant to increase the saturation by 10% steps and the precipitates collected for each fraction by centrifugation. Each precipitate was dissolved in 'Tris buffer' and assayed for enzyme activity.

(4) Gel filtration on Sephadex G-200

To the ammonium sulphate fraction containing the greatest amount of enzyme was added sufficient sucrose to achieve a final concentration of 10% (w/v) and this solution was applied to a column of Sephadex G-200 (30.0 x 2.5cm) equilibrated with 'Tris buffer'. Elution with this same buffer was carried out at a flow rate of 10-20ml/h and about 60 x 2ml fractions were collected and assayed for enzyme activity.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis at pH 8.9 was carried out using 7% polyacrylamide gels prepared in 9.0 x 0.5cm glass tubes (Davis, 1964). 0.05ml of sample solution (1mg protein per ml) was coloured with 0.001% (w/v) bromophenol blue, made 10% (w/v) in sucrose and applied to the gels. The sample was allowed to enter the gel at 0.5mA/gel and electrophoresis continued at 2.0mA/gel until the marker dye had travelled about 8cm and the gels were removed from the tubes.

The citrate synthase band (or bands) were located by cutting the gel into 1mm slices; each slice was homogenised in 1.0ml 'Tris buffer', with a glass rod, and enzyme activity in these solutions was determined by assay Method 1. In this way R_f values for the active enzymes were calculated.

Analytical Ultracentrifugation

The sedimentation velocity of citrate synthase was determined using

an MSE analytical ultracentrifuge. The method used was an active-enzyme centrifugation technique to determine the sedimentation coefficient of the active-enzyme-substrate complex (Cohen & Mire, 1971).

A substrate solution containing acetyl-CoA (approx. 50x the K_m value), oxaloacetate (approx. 50x the K_m value) and 5.0mM DTNB all in 'Tris buffer' was dispensed into a Vinograd centrifuge cell. Enzyme (15µl) was placed in the side-well of the cell and transfer to the surface of the substrate solution occurred at about 10,000rev/min. Enzyme activity was followed by measuring the increase in absorption at 412nm and the boundary positions (r_b) could be estimated from the derivative curves of the scanner traces. The sedimentation coefficient was evaluated from a linear regression analysis of $\log_e(r_b)$ against time; the values obtained were corrected to the density and viscosity of water at 20° ($s_{20,w}$ values) as described by Bowen (1970).

Electron Microscopy of Bacterial Cell Walls

Bacterial cultures were grown in 500ml of nutrient broth for 24h at 30°. The growth was harvested by centrifugation at 25,000g for 10min. The cells were resuspended in 20ml of growth medium, prefixed in glutaraldehyde, fixed with osmium tetroxide, embedded in agar and finally dehydrated in ethanol and embedded in'Araldite' as described by Glauert and Thornley (1966). Thin sections were cut on an LKB ultratome equipped with a glass knife. These sections were collected on 300-mesh copper grids coated with Formvar, stained for 30 min with undiluted lead citrate stain (Reynolds, 1963) and examined using an AEI 802 electron microscope.

Preparation of Toluenised Suspensions

An overnight culture of bacteria was centrifuged and resuspended in 'Tris buffer' to a density of 0.1mg wet wt./ml. This suspension was heated to 37° and treated with a toluene/ethanol mixture as desribed by Weitzman and Hewson (1973). In this method 0.05ml of toluene/ethanol (1:4 v/v) was added per ml of suspension, vigorously mixed and incubated for 5 min at 37°.

The suspension was then cooled in ice and the toluene removed by three cycles of centrifugation and resuspension in fresh 'Tris buffer'. The resulting suspension of permeabilised cells was assayed directly for enzyme activity.

Method of Mutagenesis

Mutagenesis of bacteria was achieved by the use of the alkylating agent ethyl methane sulphonate (EMS) as in the method described by Hopwood (1970). A sample (1ml) of an overnight culture in nutrient broth was transferred to fresh medium. This culture was grown for 2h, centrifuged and resuspended in 10ml of 100mM phosphate buffer, pH 7.0. EMS was added to this suspension to give a final concentration of 1% (v/v) and incubated for 20 min at 37°. After centrifugation the cells were resuspended in fresh medium (20ml), grown overnight to allow recovery and this culture was used as a source of mutated bacteria.

Isolation of Citrate Synthase Deficient Strains by Penicillin Enrichment

Citrate synthase deficient strains of E. coli are auxotrophic for glutamate (Ashworth et al., 1965). Therefore, the isolation of a number of glutamate-requiring mutants should include some which are citrate synthase deficient. The method used here to isolate glutamate auxotrophs is based on the double-cycle penicillin enrichment procedure of Molholt (1967). A mutated culture of either E. coli or A. lwoffi (prepared as described above) was grown overnight in minimal medium containing succinate (10mM) and glutamate (1mM). This overnight culture was transferred to fresh succinate-glutamate medium to a density of 10^6 cells/ml and incubated for 3h at 37°. The cells were washed in succinate (10mM) minimal medium and resuspended in it and incubated for 3h at 37°. Ampicillin (20μg/ml) was then added and lysis of prototrophs occurred over an incubation period of 1h. The cells were centrifuged, resuspended in succinate-glutamate medium and allowed to grow to 10^8 cells/ml. This culture was again washed in succinate medium and

treated with ampicillin as previously described. At any point in this procedure, a culture reaching 2.0 x 10^8 cells/ml was diluted by a factor of five with the same medium.

The resulting culture, enriched with respect to auxotrophs, was serially diluted, plated onto nutrient agar plates and incubated overnight. The plates with about 100 colonies were replicated onto succinate and succinate-glutamate plates by the replica plating technique of Lederberg and Lederberg (1952). However, whereas the original replicating method described by these workers involved the use of velvet pads for replication, in the present work a few sheets of 12.5cm Whatman No. 1 filter papers were substituted for the velvet.

Using this technique mutants could be isolated which although unable to grow on succinate alone, would grow on succinate-glutamate medium. These glutamate auxotrophs were grown in nutrient broth and cell-free extracts were prepared which were tested for citrate synthase activity.

In addition to this procedure, glutamate auxotrophs of A. lwoffi were also prepared by antibiotic enrichment using a mixture of D-cycloserine (2.0mg/ml) and carbenicillin (2.0mg/ml) in place of the ampicillin in the above method.

Isolation of Citrate Synthase Deficient Strains by Selection for Resistance to Fluoroacetate

A mutated culture of A. lwoffi , grown overnight in nutrient broth, was transferred to 100ml of minimal medium containing fluoroacetate (1mM) and glutamate (10mM) and grown to stationary phase. This culture was diluted and plated onto fluoroacetate-glutamate plates (concentrations as above) at 100cells/plate and incubated for three days. The colonies were replicated, as previously described, onto acetate, succinate and glutamate minimal media plates. Mutants were isolated which were unable to grow on acetate or succinate as sole carbon source but did grow on glutamate. Cell-free extracts

of these mutants were tested for citrate synthase activity.

Bacterial Conjugation

Conjugation between Hfr and F^- strains was carried out as described by Miller (1972). The mating process was interrupted by diluting a sample (0.5ml) of the mating culture 20-fold in 0.9% (w/v) saline and agitating with a vortex mixer for 1 min. Samples and serial dilutions of this suspension were spread onto selective media and nutrient agar. Counter-selection against the Hfr donor strains was achieved by including streptomycin sulphate (100µg/ml) in the selective plates.

Phage (P1) Mediated Transduction

The propagation and subsequent use of transducing phage during phage mediated transduction were as described by Miller (1972). The host range mutant, P1 Kc (Lennox, 1955) was used as this plaques well with E. coli.

Transformation in A. lwoffi

5.0ml of an overnight culture of A. lwoffi was centrifuged, resuspended in 0.3ml of solution containing sodium lauryl sulphate (0.5% w/v), sodium citrate (0.015M) and sodium chloride (0.15M). To obtain a DNA preparation this suspension was incubated for 1h at 60°. A patch ($1cm^2$) of recipient strain was inoculated onto a nutrient agar plate; a sample (0.01ml) of the DNA preparation was spread onto this patch. After 7h the growth was transferred from the nutrient agar plate onto a selective plate on which the recipient can only grow by mutating or by receiving a portion of donor DNA by the transformation mechanism.

Computer Assisted Identification of Bacteria

A bacterial suspension was made in peptone water and this was used to inoculate the API 50 Enterobacteriaceae microtube system according to the manufacturer's instruction. This microtube method is a rapid method whereby the growth characteristics of a bacterium can be assessed. The results obtained were analysed by the identication program of Friedman et al.(1973),

adapted by Mr M. J. Sackin (Department of Microbiology, Leicester University), by comparison with data compiled by Bascomb et al.(1973). This program selects the most probable identity for the bacterium and expresses quantitatively the relative probability for the identification of a particular bacterium.

Double Immunodiffusion (Ouchterlony Technique)

A solution containing 'Tris buffer' and 1.5% (w/v) agar was sterilised by autoclaving and 30ml aliquots were poured into Petri dishes and then allowed to cool.

Seven wells were cut in the agar, one central well surrounded by six equally spaced outer wells. Antiserum, which had been raised against the purified citrate synthase from wild type A. lwoffi, was placed in the central well and the cell-free extracts of the test bacteria were placed in the outer wells. Several wet paper tissues were placed in an air-tight box, the Petri dishes placed on these tissues and the box sealed. In this way a constant humid atmosphere was maintained. The box was placed at 26° overnight and the presence of cross-reacting material in the extracts was determined visually by examining the plates for the presence of white precipitation lines between the central and outer wells.

Data Handling

The determination of K_m values was carried out by entering enzyme velocity measurements at different substrate concentrations into program I (Appendix I).

Values of $S_{0.5}$ were similarly determined using computer program II (Appendix I).

Determination of the K_i value for an enzyme inhibitor was performed using computer program III (Appendix I).

The best straight line fit for the data was assessed using program IV (Appendix I).

Furthermore, all these programs gave error statistics for the results and have been modified in the present work to give graphical representation of the data where appropriate.

RESULTS.

Section A - Investigation of the Mechanism of Inhibition of Citrate Synthase

In vitro studies have shown that citrate synthase is inhibited by α-oxoglutarate and a variety of nucleotides. These inhibitions may have a physiological role, regulating the activity of the enzyme *in vivo*. However, these compounds are all structural analogues of the substrates and therefore the mechanisms of these inhibitions has been investigated to ascertain whether they are exerted isosterically or allosterically. Several methods have been used to study the inhibition of different citrate synthases by a number of these inhibitors.

Mechanism of Nucleotide Inhibition

Figs. 5-10 are double reciprocal plots showing the dependence of the activity of pig heart citrate synthase on acetyl-CoA concentration in the presence and absence of a number of nucleotides (ATP, ADP, AMP, NADPH, NADH and $NADP^+$ respectively). It was found that all these inhibitors were strictly competitive with the substrate acetyl-CoA. Furthermore, replots of the slopes of these lines against the concentration of the nucleotide inhibitor gave a linear relationship in each case (Fig. 11). K_i values were determined by fitting the data to program III (Appendix I) and these are given in Table 2. Similar studies were carried out on partially purified citrate synthases from the yeast *S. cerevisiae*, the Gram positive bacterium *B. megaterium* and the Gram negative bacteria *A. lwoffi* and *Ps. aeruginosa*. The inhibition of *A. lwoffi* citrate synthase by NADH was not strictly competitive with acetyl-CoA (Fig 12), but in all other cases studied the inhibitors acted competitively with this substrate. K_m values for acetyl-CoA were determined by fitting the data to program I (Appendix I) and the K_i values for the nucleotide inhibitors (program III) for each enzyme are given in Table 2.

It was found that the order of effectiveness of the nucleotide inhibitors was ATP $>$ ADP $>$ AMP $>$ NADPH $>$ NADH $>$ $NADP^+$ except for the Gram negative bacterial enzymes, in which case NADH was by far the most powerful inhibitor of the

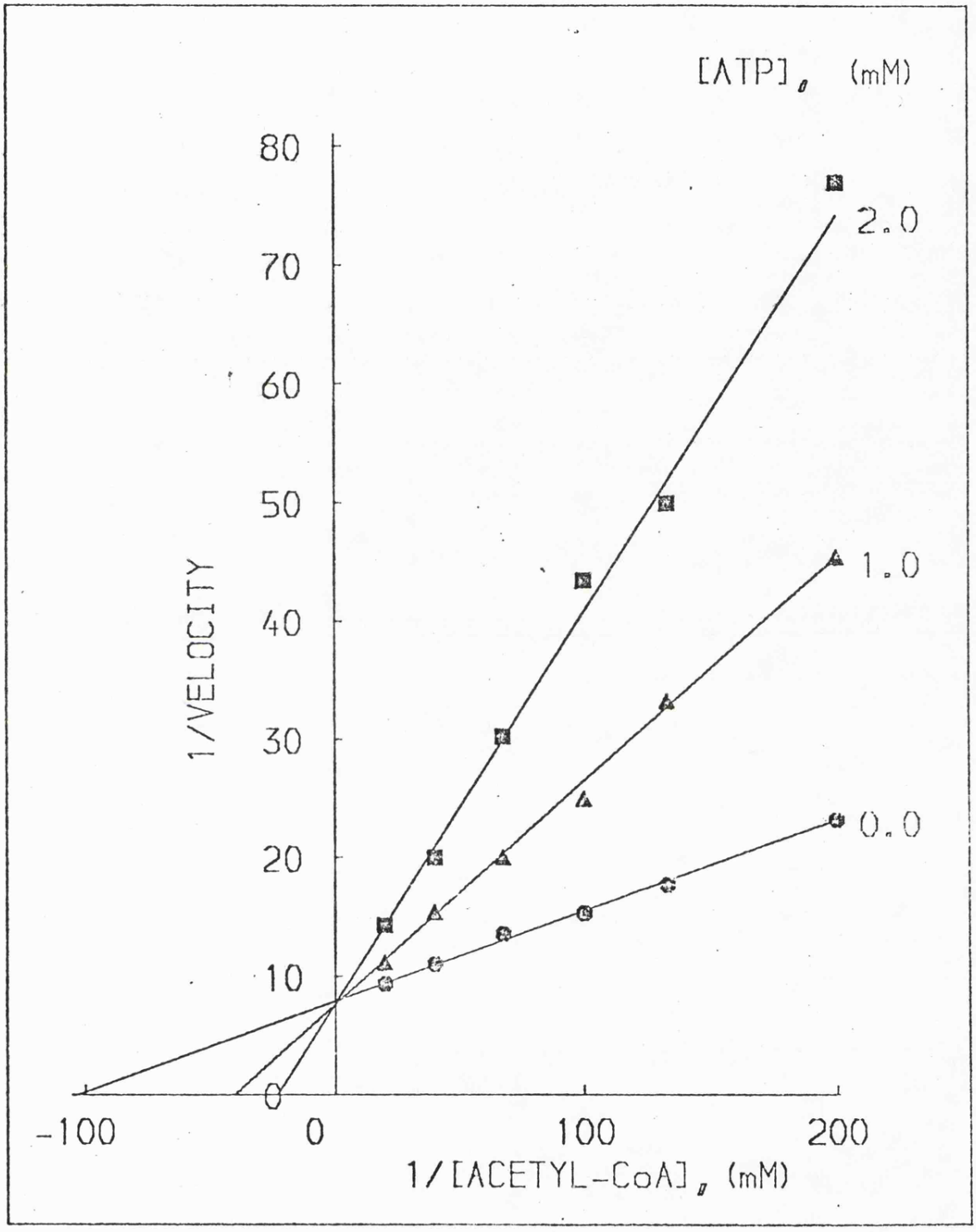


Fig. 5: Double reciprocal plots of the rate dependence of pig heart citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of ATP

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

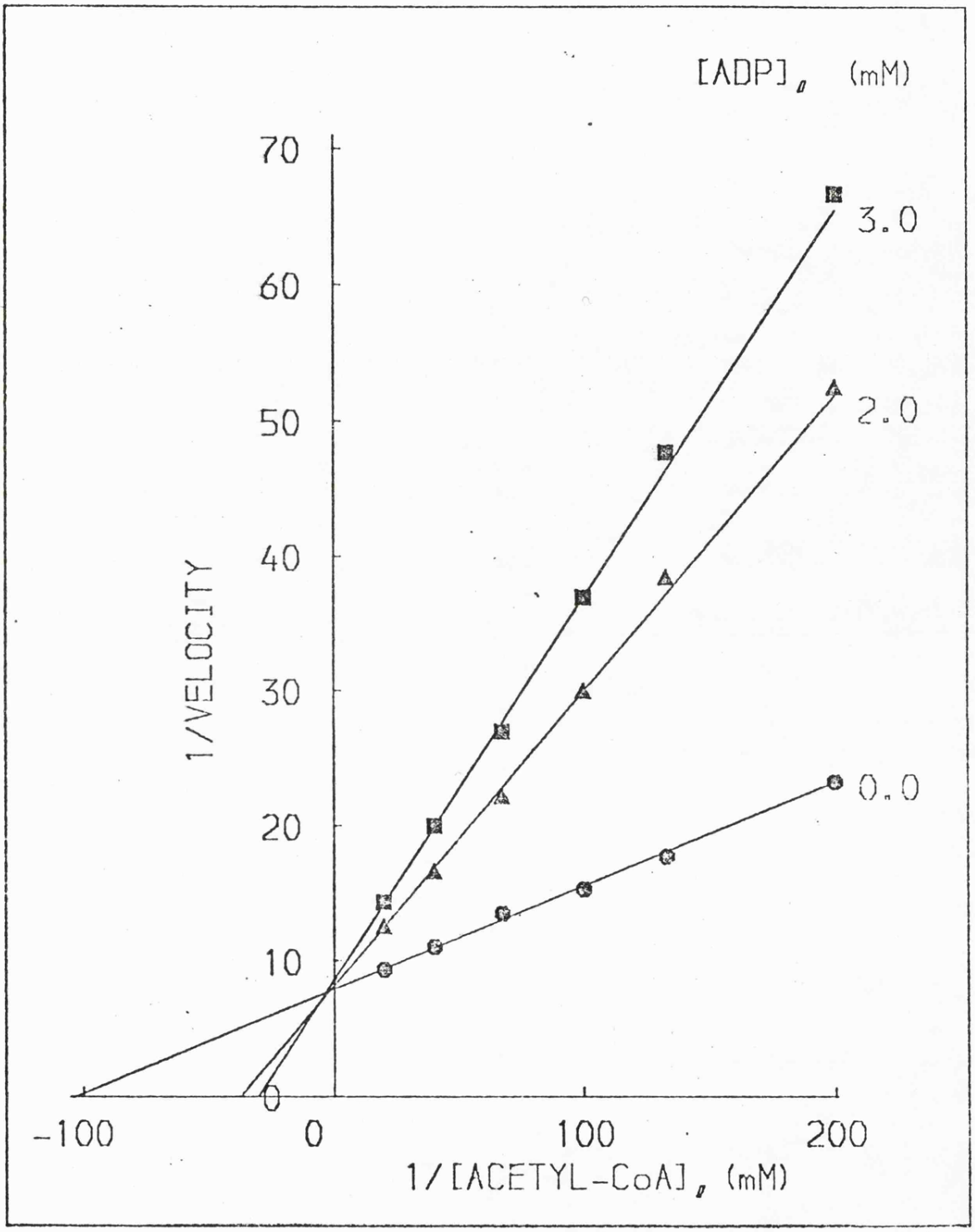


Fig. 6: Double reciprocal plots of the rate dependence of pig heart citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of ADP.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

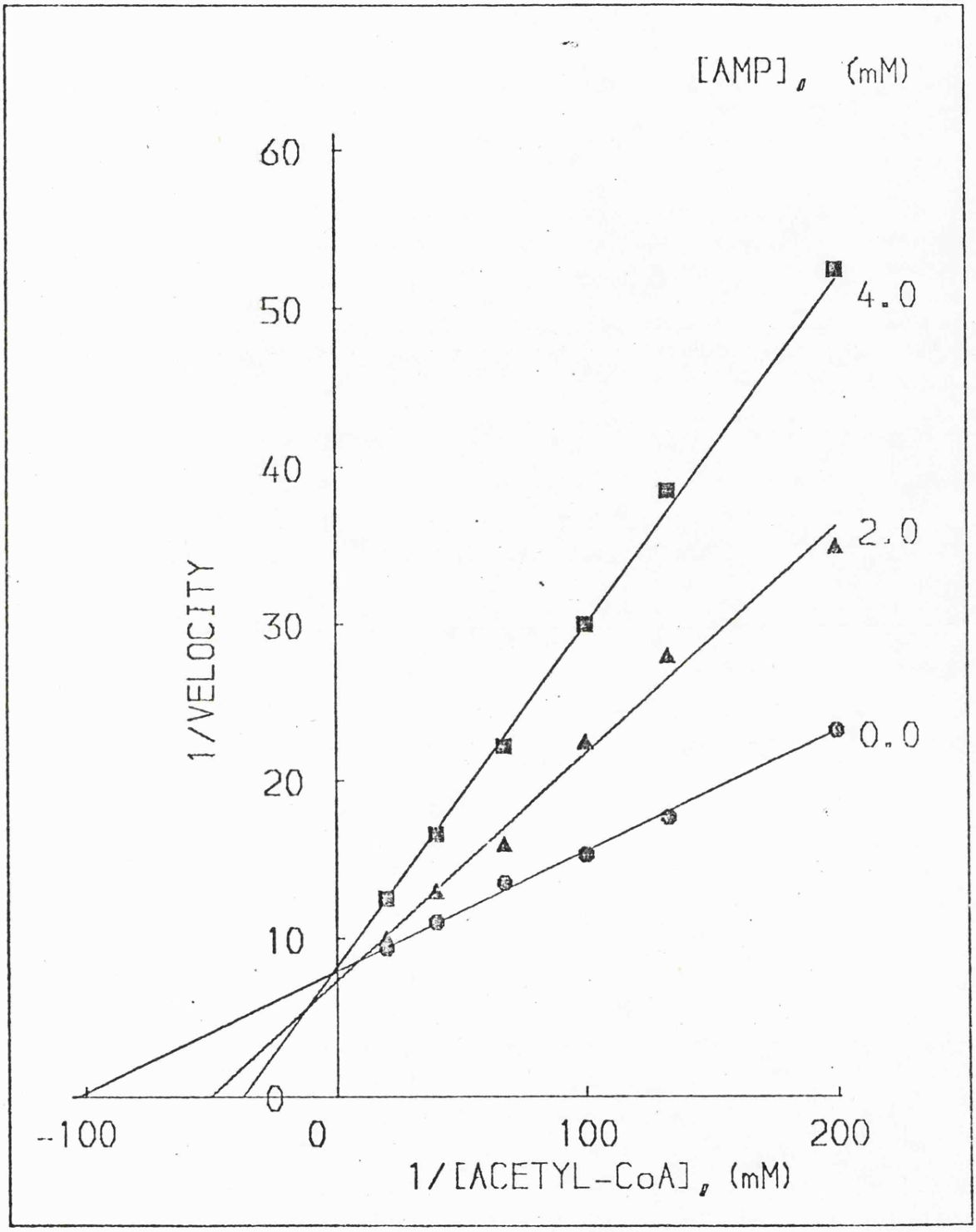


Fig. 7: Double reciprocal plots of the rate dependence of pig heart citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of AMP.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

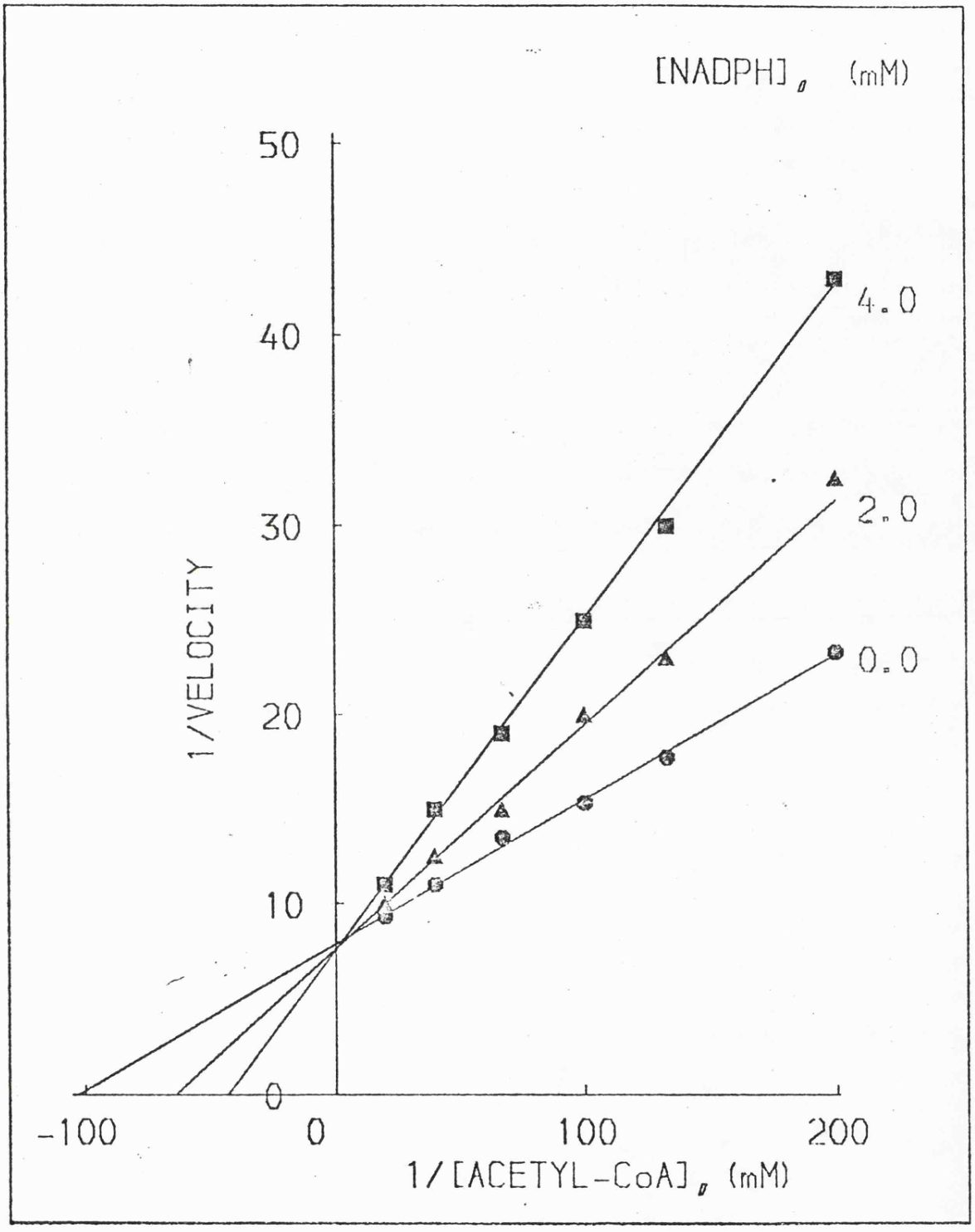


Fig. 8: Double reciprocal plots of the rate dependence of pig heart citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of NADPH.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

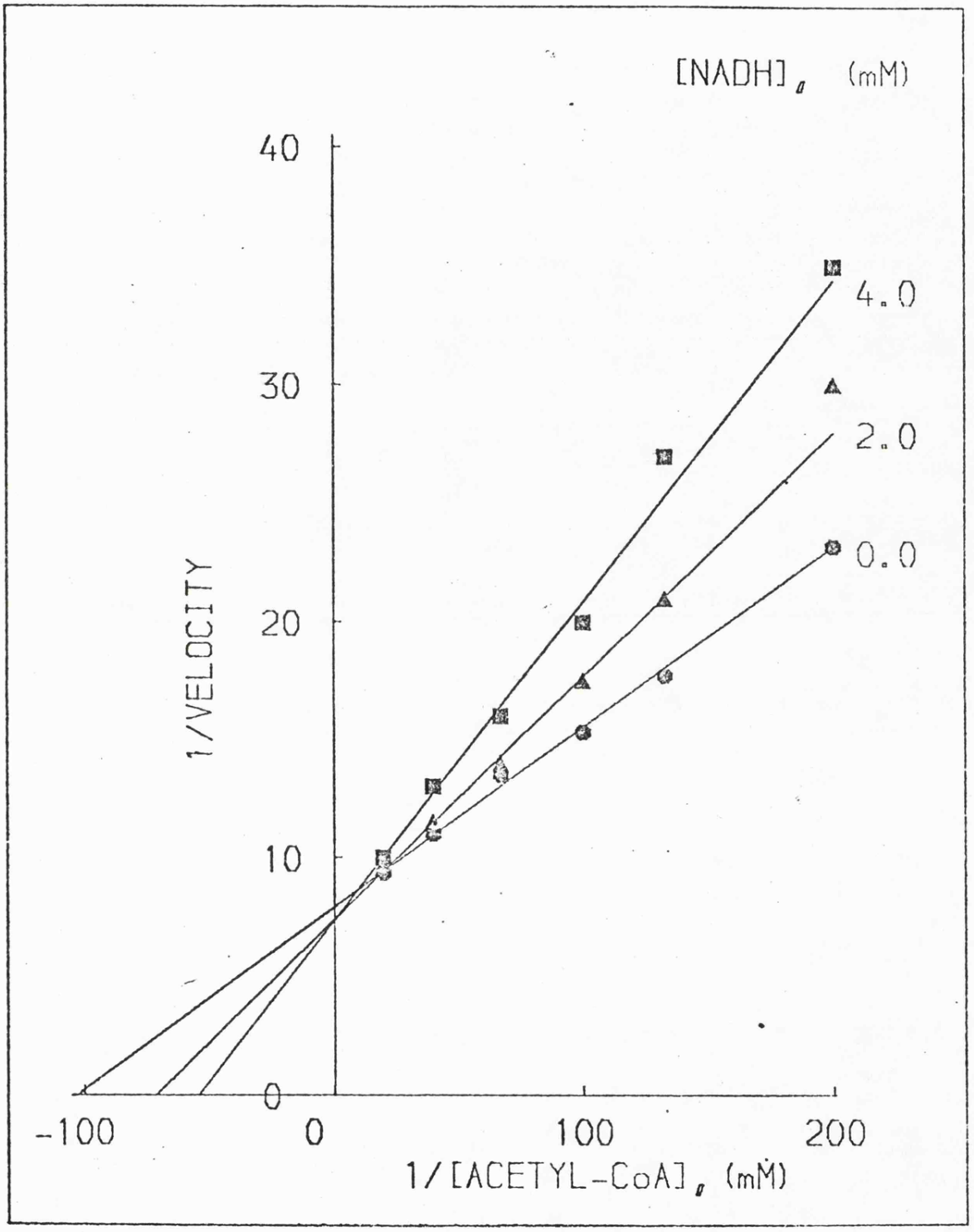


Fig. 9: Double reciprocal plots of the rate dependence of pig heart citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of NADH.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

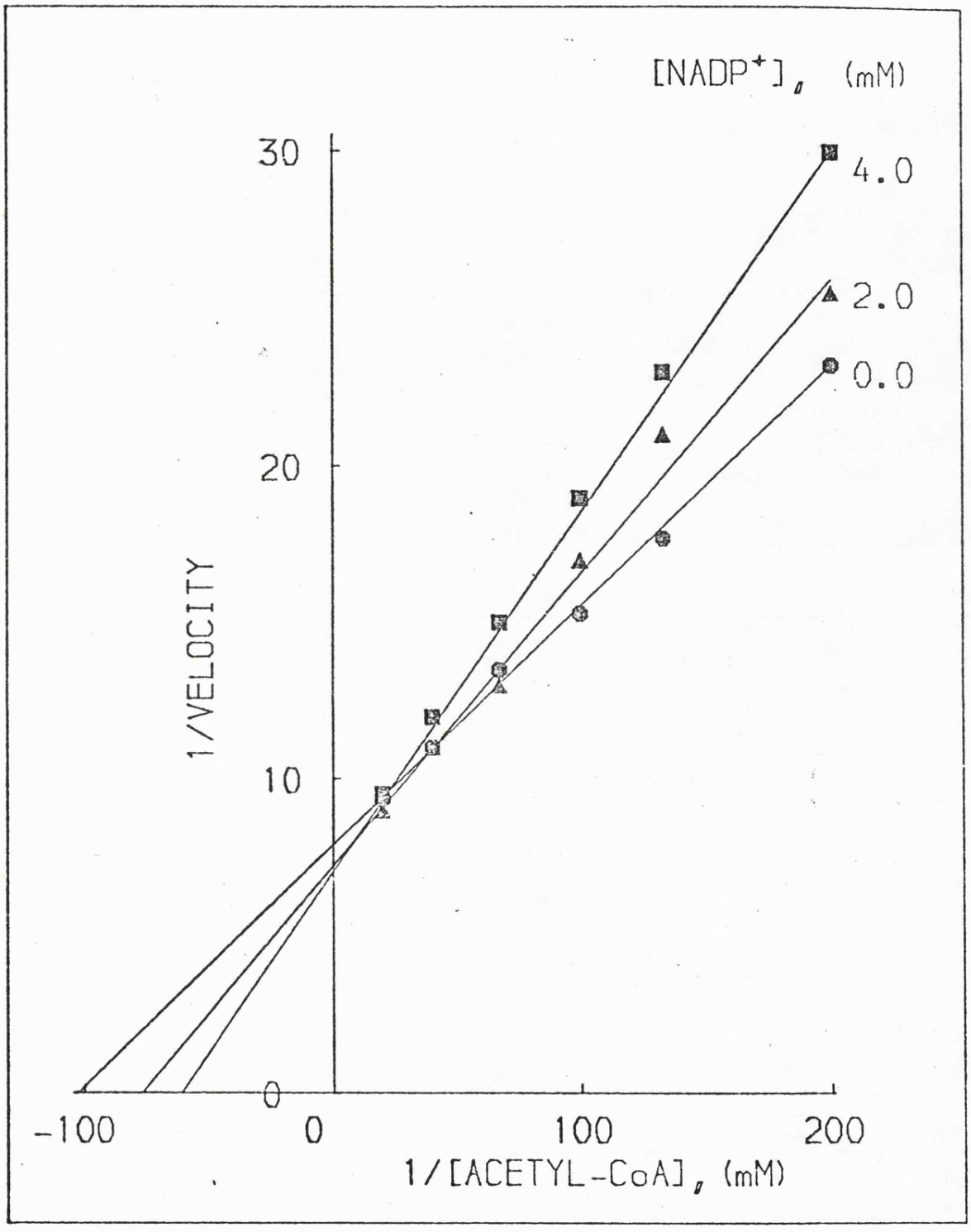


Fig. 10: Double reciprocal plots of the rate dependence of pig heart citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of $NADP^+$.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

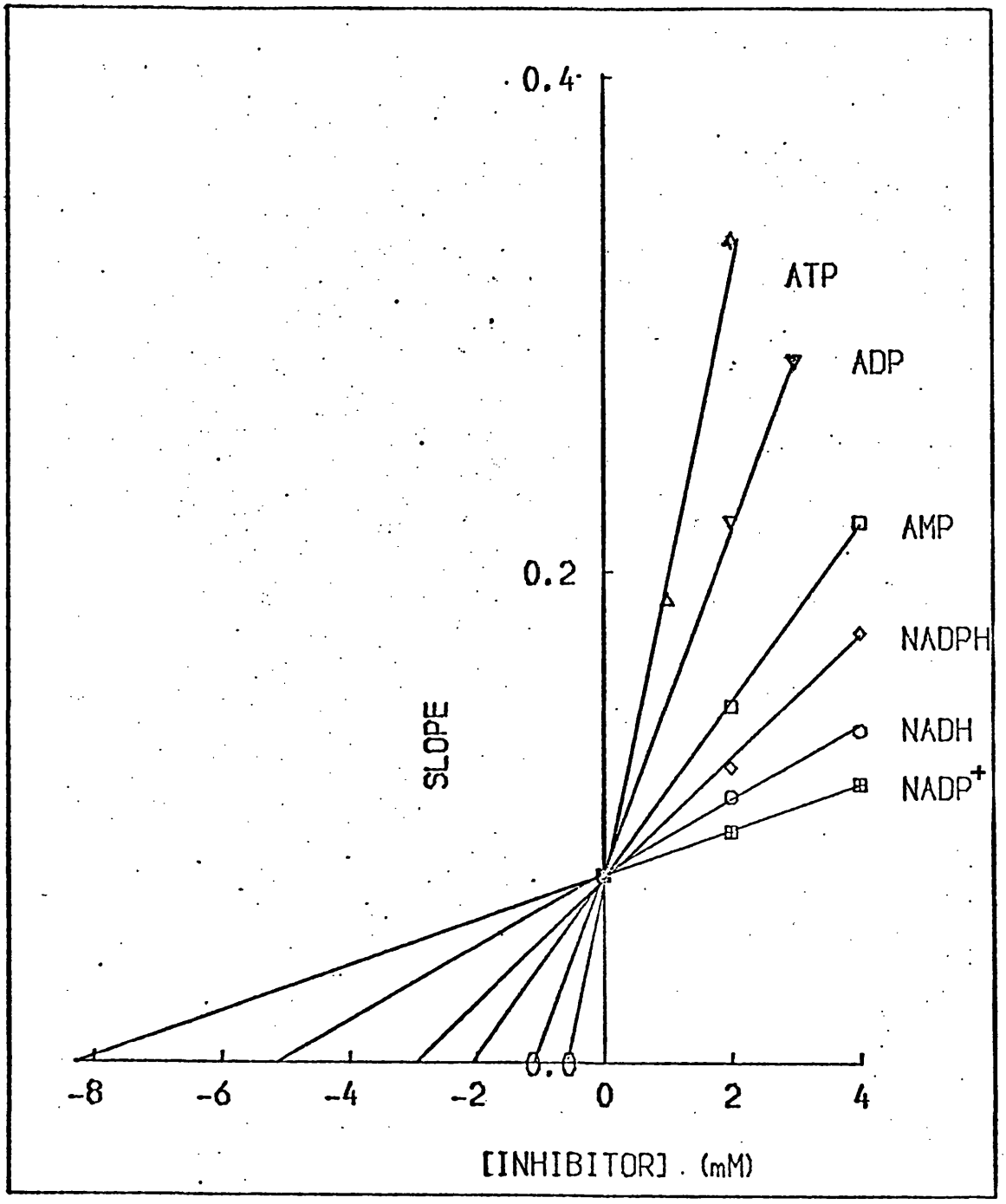


Fig. 11: Replots of the slopes against inhibitor concentration for the nucleotide inhibitors of pig heart citrate synthase.

This graph is a replot of the data given in Figs. 5-10.

Table 2: The relationship between the K_m value for acetyl-CoA and the K_i values for a number of nucleotide inhibitors for a variety of citrate synthases.

		K_i value for inhibitor (mM)					
Enzyme source	K_m acetyl-CoA (μM)	ATP	ADP	AMP	NADPH	NADH	$NADP^+$
S. cerevisiae	3.2 ± 0.2	0.17 ± 0.01	0.31 ± 0.02	0.67 ± 0.04	1.0 ± 0.1	1.6 ± 0.1	2.7 ± 0.2
Pig heart	9.8 ± 0.6	0.55 ± 0.04	0.92 ± 0.06	2.0 ± 0.1	2.9 ± 0.2	5.1 ± 0.3	8.2 ± 0.9
B. megaterium	29.4 ± 2.6	1.6 ± 0.1	2.5 ± 0.2	6.2 ± 0.5	8.4 ± 0.7	12.4 ± 1.0	-
Ps. aeruginosa	50.3 ± 3.8	2.8 ± 0.2	4.6 ± 0.3	9.7 ± 1.0	15.3 ± 1.1	0.08 ± 0.003	-
A. lwoffi	75.6 ± 6.9	4.4 ± 0.3	7.3 ± 0.8	15.6 ± 1.1	-	0.04 ± 0.005[a]	-

a. This inhibition is not strictly competitive.

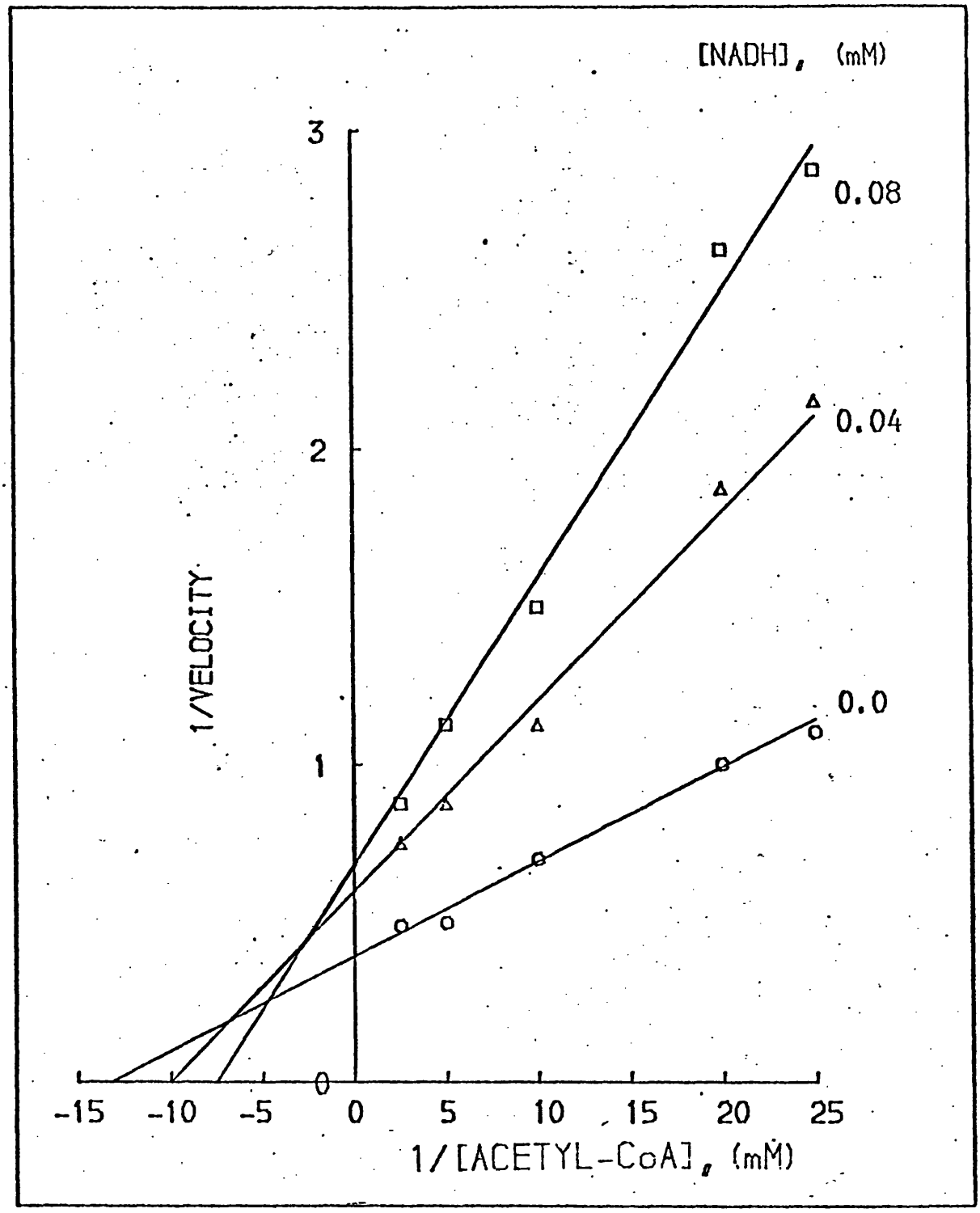


Fig 12: Double reciprocal plots of the rate dependence of A. lwoffi wild type citrate synthase activity on the concentration of acetyl-CoA both in the presence and absence of NADH.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

series. Furthermore, with the exception of this latter case, there was a direct relationship between the K_m value for acetyl-CoA and the K_i values for each inhibitor (Fig. 13).

It has been shown that the NADH inhibition of E. coli citrate synthase is abolished in the presence of 0.1M KCl (Weitzman, 1966a). Similarly, Ps. aeruginosa enzyme may be desensitised to NADH inhibition by incubation with 0.1mM DTNB for 5min prior to assay (Weitzman & Hewson, in preparation). The results of a detailed analysis of the effect of these desensitisations on the enzymes are given in Table 3. It was found that whereas these treatments abolished the sensitivity of the enzymes to NADH, the activity and sensitivity to ATP remained relatively unchanged. These results are consistent with the view that NADH inhibition of the Gram negative bacterial enzyme is an allosteric phenomenon.

Further investigations into the site of action of nucleotide inhibitors were made using the method of multiple-inhibition analysis (Yonetani & Theorell, 1964). This method involves the examination of the inhibition produced by two inhibitors, each competitive with the same substrate.

Three different citrate synthases were chosen for study. Pig heart enzyme is typical of that from eucaryotic sources, B. megaterium enzyme is representative of Gram positive bacterial citrate synthases and Ps. aeruginosa enzyme is typical of that from Gram negative species. Multiple-inhibition was studied using the three inhibitors bromoacetyl-CoA, NADH and ATP, all of which act strictly competitively with acetyl-CoA for each enzyme examined. Bromoacetyl-CoA was used as it will act as a substrate, albeit a poor one, for citrate synthase; its inhibitory action may therefore reasonably be assumed to result from competition with acetyl-CoA for the active site. The inhibitors were used in the pairs ATP + bromoacetyl-CoA and ATP + NADH. In each case, the concentration of one inhibitor was varied at different fixed concentrations of the other. The reciprocal of the

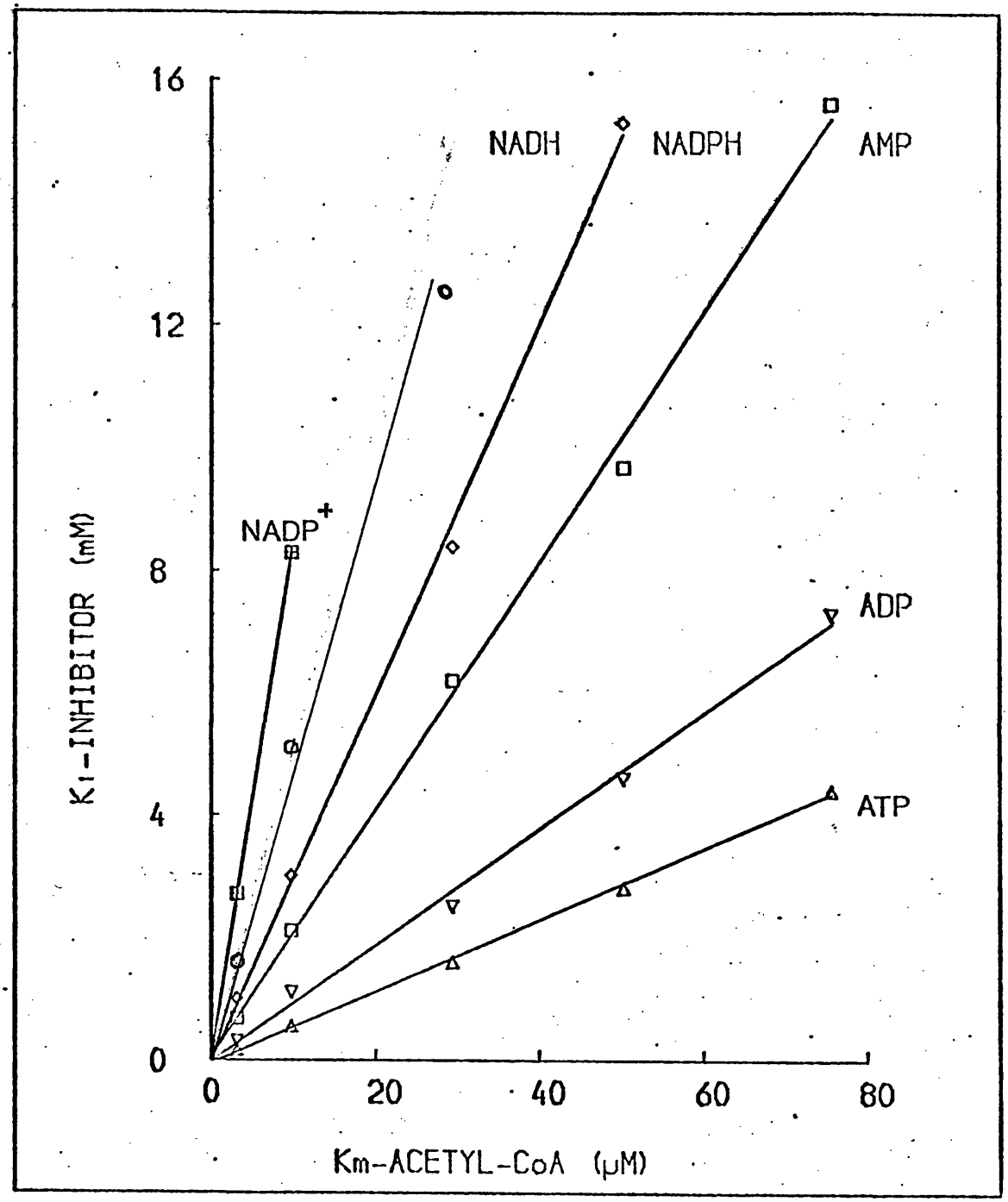


Fig. 13: The relationship between Km value for acetyl-CoA and the Ki values for nucleotide inhibitors for a number of citrate synthases

This is a graphical representation of the data presented in Table 2.

Table 3: The effect of 'desensitisation' treatments on the K_m (or $S_{0.5}$) value for acetyl-CoA and the K_i values for ATP and NADH for *E. coli* and *Ps. aeruginosa* citrate synthases.

Enzyme source	K_m (or $S_{0.5}$) acetyl-CoA (μM)	K_i ATP (mM)	K_i NADH (mM)
E. coli	420. ± 80.	23.3 ± 3.1	0.04 ± 0.004
E. coli (desensitised)[a]	105.1 ± 9.2	5.8 ± 0.3	42.0 ± 7.1
Ps. aeruginosa	50.3 ± 3.8	2.8 ± 0.2	0.08 ± 0.003
Ps. aeruginosa (desensitised)[b]	63.2 ± 3.9	3.7 ± 0.3	25.2 ± 3.1

a. The enzyme was assayed in the presence of 0.1M KCl.

b. The enzyme was incubated with 0.1mM DTNB for 5 min prior to assay.

measured enzyme rate was plotted against the concentration of the varaiable inhibitor.

As Yonetani and Theorell (1964) have shown, parallel line plots indicate that the inhibitors interact at the same site on the enzyme whereas different sites of action are indicated by a pattern of intersecting lines.

Fig. 14 shows that parallel line plots were obtained in all cases with the pig heart and B. megaterium enzymes suggesting that ATP, NADH and bromoacetyl-CoA all interact with the same site. In view of the likely identity of the bromoacetyl-CoA site it may be concluded that the probable site of action of both NADH and ATP, for both these enzymes, is at the acetyl-CoA, or active, site.

For the Gram negative bacterial citrate synthases, typified by Ps. aeruginosa enzyme, the parallel line plots (Fig. 15) again showed that ATP and bromoacetyl-CoA have a common binding site. However, the pattern of intersecting lines obtained with the pair of inhibitors, ATP and NADH, clearly indicates that these inhibitors act at different sites on the enzyme. The result strongly supports the view that NADH is an allosteric inhibitor of Gram negative bacterial citrate synthase.

As mentioned earlier, treatment of the Ps. aeruginosa enzyme with DTNB results in the loss of sensitivity to NADH (Weitzman & Hewson, in preparation). Multiple-inhibition analysis of this desensitised enzyme gave only parallel line plots (Fig. 15) indicating that the residual, low-level NADH inhibition was exerted at the same site as ATP and bromoacetyl-CoA, i.e. at the active site. Therefore, loss of the allosteric site revealed weak isosteric inhibition of Gram negative bacterial enzyme by NADH.

Mechanism of α-Oxoglutarate Inhibition

α- Oxoglutarate inhibits many citrate synthases. This inhibition has been found to be strictly competitive with the substrate oxaloacetate. The sensitivity of a number of citrate synthases to this compound was examined

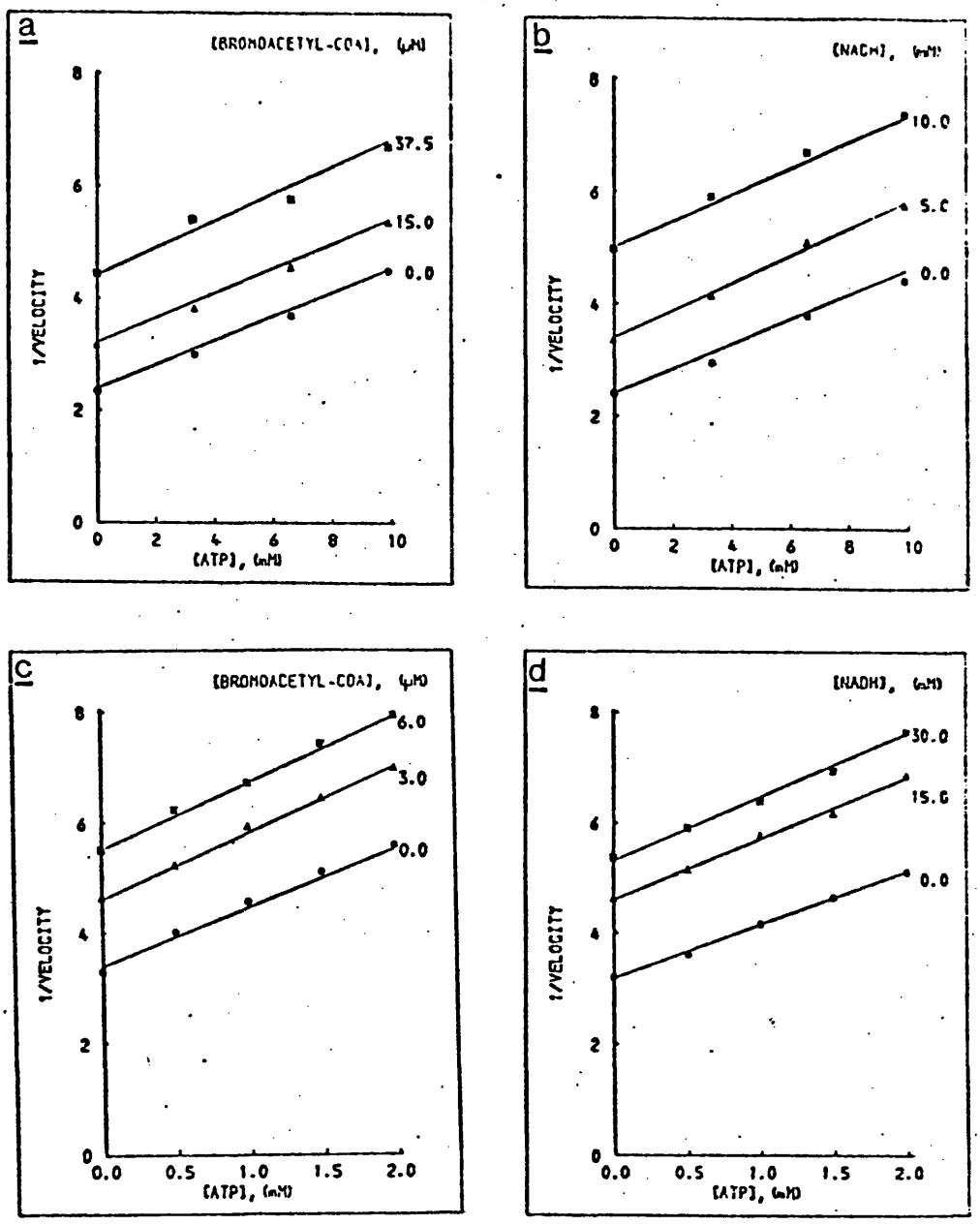


Fig. 14: Multiple inhibition analysis of citrate synthases.

The enzyme rates (in arbitrary units) were determined using assay method 1.

a. Inhibition of pig heart citrate synthase in the presence of ATP and bromoacetyl-CoA.
b. Inhibition of pig heart citrate synthase in the presence of ATP and NADH.
c. Inhibition of B. megaterium citrate synthase in the presence of ATP and bromoacetyl-CoA.
d. Inhibition of B. megaterium citrate synthase in the presence of ATP and NADH.

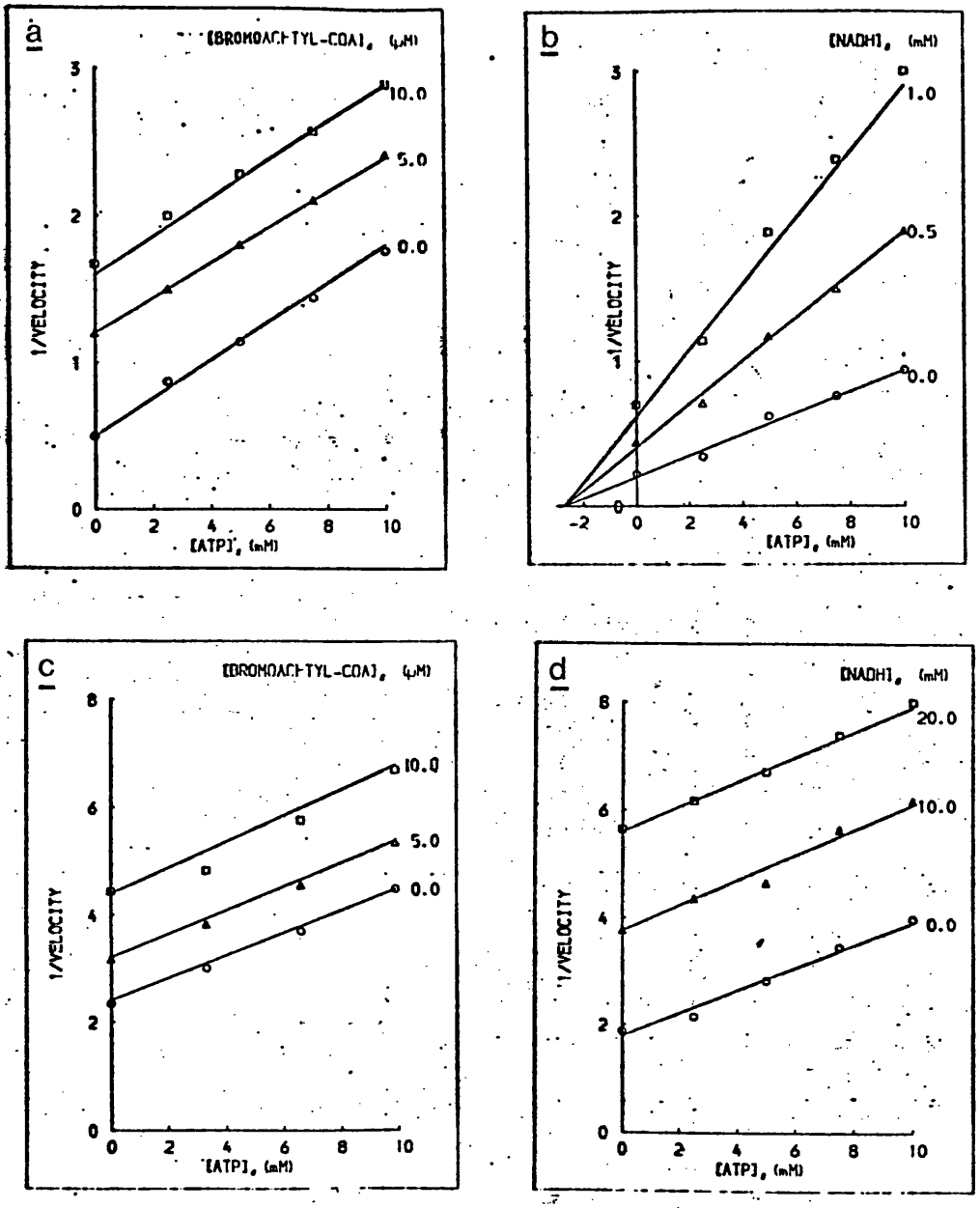


Fig. 15: Multiple inhibition analysis of citrate synthases.

The enzyme rates (in arbitrary units) were determined using assay method 1.

a. Inhibition of Ps. aeruginosa citrate synthase in the presence of ATP and bromoacetyl-CoA.

b. Inhibition of Ps. aeruginosa citrate synthase in the presence of ATP and NADH.

c. Inhibition of Ps. aeruginosa citrate synthase (treated with DTNB) in the presence of ATP and bromoacetyl-CoA.

d. Inhibition of Ps. aeruginosa citrate synthase (treated with DTNB) in the presence of ATP and NADH

and a list of K_m values for oxaloacetate and K_i values for α-oxoglutarate is given in Table 4.

Desensitisation studies have shown that the α-oxoglutarate inhibition of the citrate synthases from Gram negative facultative anaerobes is overcome by the presence of 0.1M KCl (Wright et al., 1967; Weitzman & Dunmore, 1969b). The effect of 0.1M KCl on the K_m (or $S_{0.5}$) value for oxaloacetate and the K_i value for α-oxoglutarate for E. coli citrate synthase is shown in Table 5. It is clear that although KCl has little effect on the K_m (or $S_{0.5}$) value for oxaloacetate, the inhibition produced by α-oxoglutarate is markedly reduced. These findings are therefore in agreement with the view that α-oxoglutarate inhibition of the citrate synthases from facultative anaerobes is allosteric.

To gain further knowledge of the site of action of α-oxoglutarate on a range of citrate synthases, the method of multiple-inhibition analysis has again been employed.

Multiple-inhibition was studied using the two inhibitors α-oxoglutarate and fluoro-oxaloacetate. Both of these compounds were demonstrated to be competitive with oxaloacetate for each enzyme examined. Fluoro-oxaloacetate was used as it is a very poor substrate for citrate synthase and its inhibitory action may therefore be assumed to be a result of its competition with oxaloacetate for the active site. Three different citrate synthases were studied - pig heart enzyme, typical of eucaryotes, B. megaterium enzyme, representative of Gram positive bacteria and E. coli enzyme, typical of Gram negative facultative anaerobes.

The parallel line plots of Fig. 16-17 show that α-oxoglutarate and fluoro-oxaloacetate both act at the same site on the pig heart and B. megaterium enzymes. In view of the likely identity of the fluoro-oxaloacetate site, as the active site, it may be concluded that α-oxoglutarate inhibition of these enzymes is probably isosteric.

Table 4: The relationship between the K_m (or $S_{0.5}$) value for oxaloacetate and the K_i value for α-oxoglutarate for a variety of citrate synthases.

Enzyme source	K_m (or $S_{0.5}$) oxaloacetate (μM)	K_i α-oxoglutarate (mM)
S. cerevisiae	2.1 ± 0.2	2.4 ± 0.3
A. lwoffi	3.7 ± 0.2	3.0 ± 0.4
Pig heart	5.3 ± 0.4	5.8 ± 0.6
B. megaterium	10.7 ± 0.9	9.3 ± 1.0
E. coli	48.2 ± 6.6	0.04 ± 0.003

Table 5: The effect of 'desensitisation' treatment on the K_m (or $S_{0.5}$) value for oxaloacetate and the K_i value for α-oxoglutarate for *E. coli* citrate synthase.

Enzyme source	K_m (or $S_{0.5}$) oxaloacetate (μM)	K_i α-oxoglutarate (mM)
E. coli	48.2 ± 6.6	0.04 ± 0.003
E. coli (desensitised)[a]	42.3 ± 4.1	35.8 ± 5.2

a. The enzyme was assayed in the presence of 0.1M KCl.

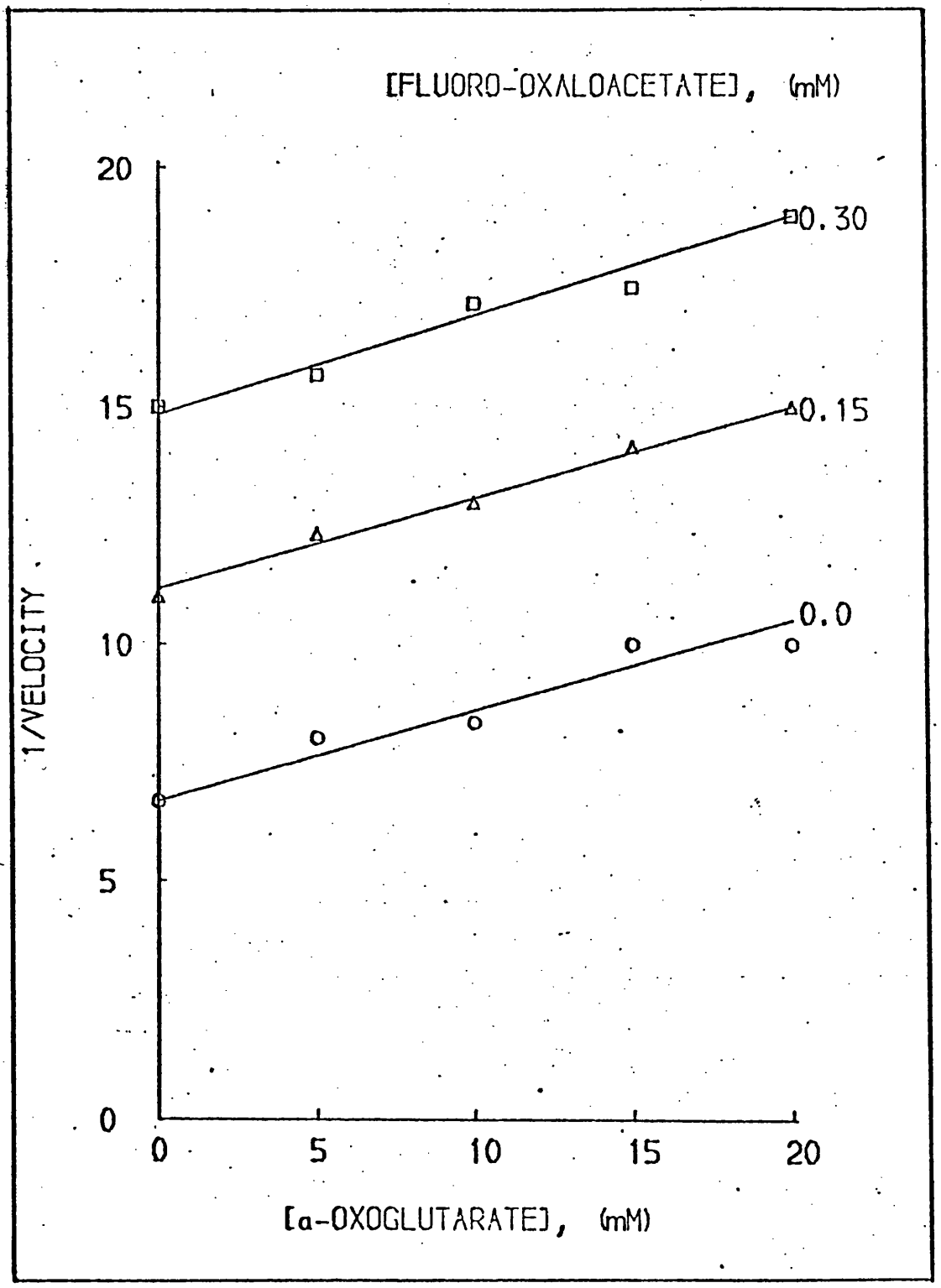


Fig. 16: Multiple inhibition analysis of pig heart citrate synthase in the presence of α-oxoglutarate and fluoro-oxaloacetate.

The enzyme activities (in arbitrary units) were determined using assay method 1.

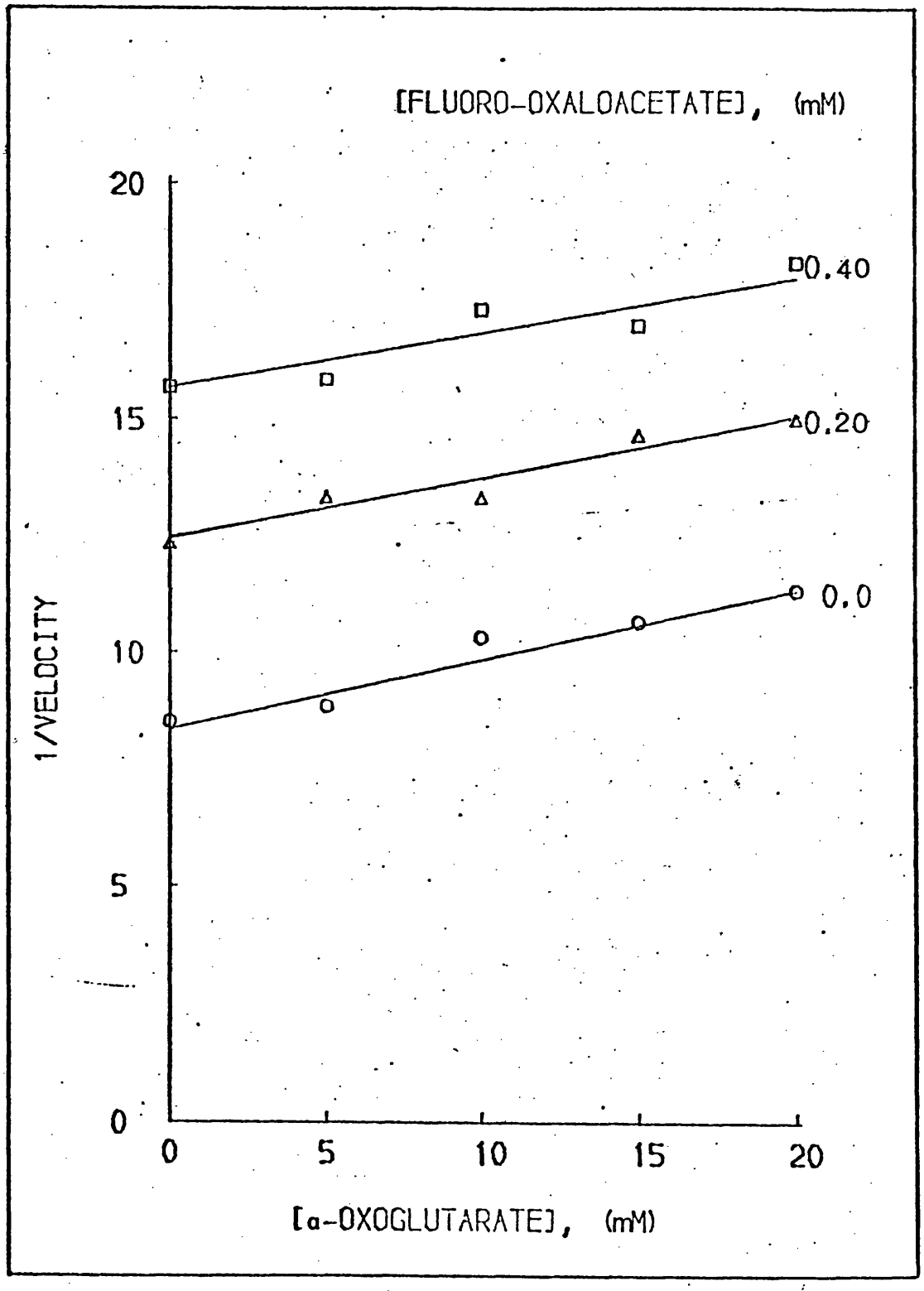


Fig. 17: Multiple inhibition analysis of B. megaterium citrate synthase in the presence of α-oxoglutarate and fluoro-oxaloacetate.

The enzyme activities (in arbitrary units) were determined using assay method 1.

On the other hand, the non-identity of the fluoro-oxaloacetate and α-oxoglutarate sites for the E.coli enzyme can be seen from the intersecting pattern of lines obtained in Fig. 18. This finding supports the view that α-oxoglutarate is an allosteric inhibitor of the enzymes from the Gram negative facultative anaerobes.

In the presence of 0.1M KCl the powerful α-oxoglutarate inhibition of E. coli citrate synthase is not observed. Multiple inhibition analysis was carried out in the presence of 0.1M KCl and only a parallel line plot was observed (Fig. 19). This result indicates that when the allosteric site is modified, α-oxoglutarate acts as a weak isosteric inhibitor as it does for the other enzymes examined.

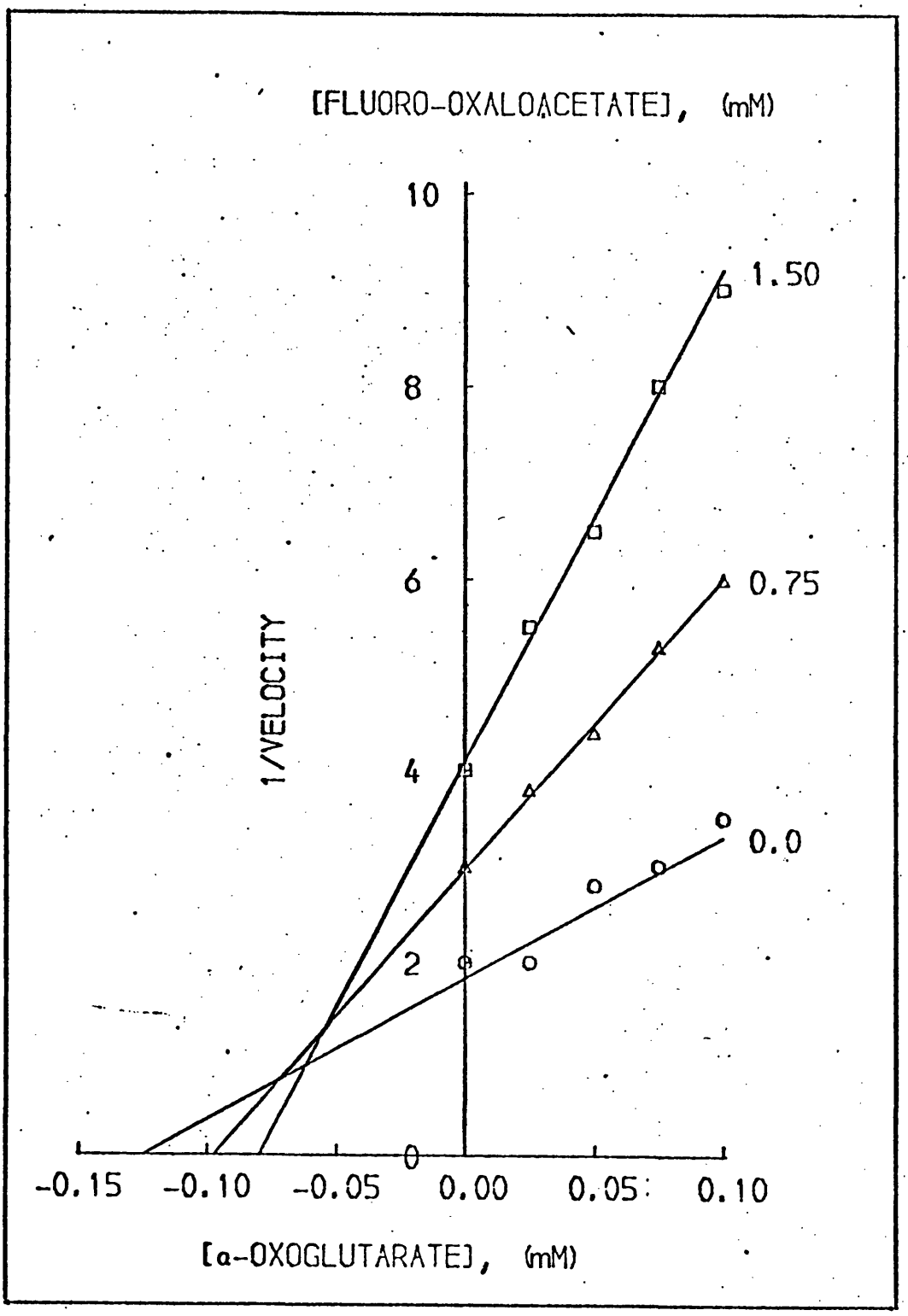


Fig. 18: Multiple inhibition analysis of E. coli citrate synthase in the presence of α-oxoglutarate and fluoro-oxaloacetate.

The enzyme activities (in arbitrary units) were determined using assay method 1.

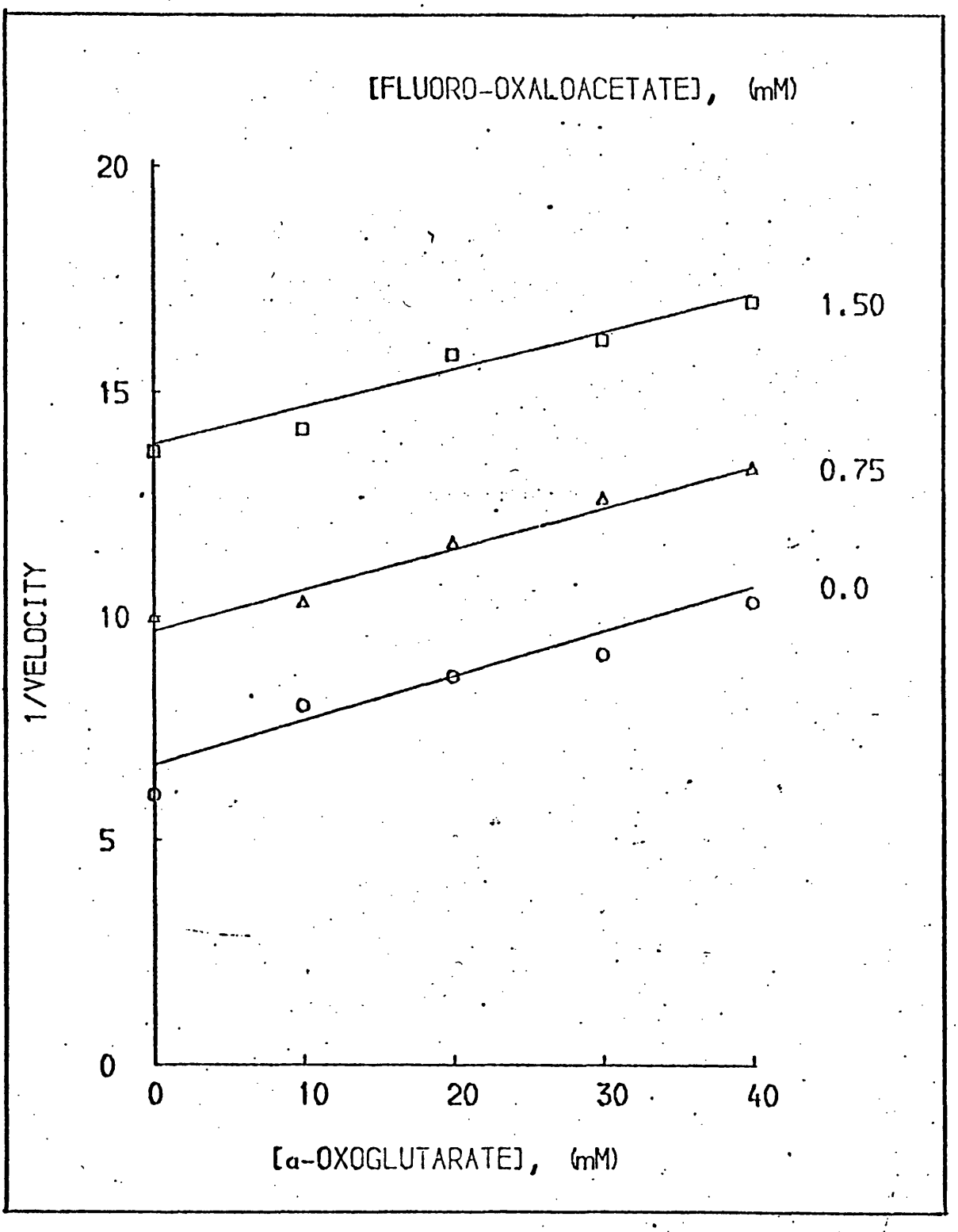


Fig. 19: Multiple inhibition analysis of E. coli citrate synthase in the presence of α-oxoglutarate and fluoro-oxaloacetate.

The enzyme activities (in arbitrary units) were determined using assay method 1. All assays were carried out in the presence of 0.1M KCl.

Section B - Rapid Techniques for Scanning the Regulatory Properties and Molecular Size of Citrate Synthase

These rapid techniques were developed for use in scanning the properties of citrate synthases from a large number of organisms. The procedures are outlined in the Methods Section and the reliability of these methods was assessed using a number of test organisms. The properties of the citrate synthases from the test organisms had been established using the method described by Weitzman and Jones (1975); these properties were compared with those obtained by employing the rapid techniques (Harford, Jones & Weitzman, 1976).

Screening of Regulatory Properties

This method has been tested by observing the inhibitory effect of ATP and NADH on citrate synthases from a wide range of organisms. The results, presented in Table 6, show that the observations from the rapid technique are in complete agreement with those of the conventional method, thereby establishing the reliability of this very quick qualitative determination of regulatory behaviour.

Screening of Molecular Size

The method employs the use of a comparatively small column of Sephadex G-200 thereby greatly reducing the time required to determine the molecular size of a citrate synthase. Weitzman and Dunmore (1969a) have shown that the 'large' citrate synthase has a molecular weight greater than lactate dehydrogenase and should therefore be eluted from the column before this marker enzyme. Conversely, the 'small' citrate synthases should be eluted from the column after lactate dehydrogenase.

The resolution of the small column was tested by applying a mixture of lactate dehydrogenase and either a 'large' citrate synthase, from *E. coli*, or a 'small' citrate synthase, from *B. megaterium*. The results shown in Fig. 20 demonstrate quite clearly that the column was able to resolve

Table 6: Comparison of some of the characteristics of citrate synthases by conventional techniques and micromethods.

	Conventional techniques[a]			Micromethods		
	Inhibition			Inhibition		
Bacteria	NADH	ATP	Molecular size	NADH	ATP	Molecular size
Ar. globiformis	-	+	small	-	+	small
B. megaterium	-	+	small	-	+	small
Br. linens	-	+	small	P	P	small
C. rossica	+	-	large	+	-	large
E. coli	+	-	large	+	-	large
K. zopfii	-	+	small	-	+	small
Ps. iodinum	-	+	small	-	+	small
Ps. fluorescens	+	-	large	+	-	large

a. Methods from Weitzman and Jones (1968) and weitzman and Dunmore (1969a).

P. Pigment production masked the reaction.

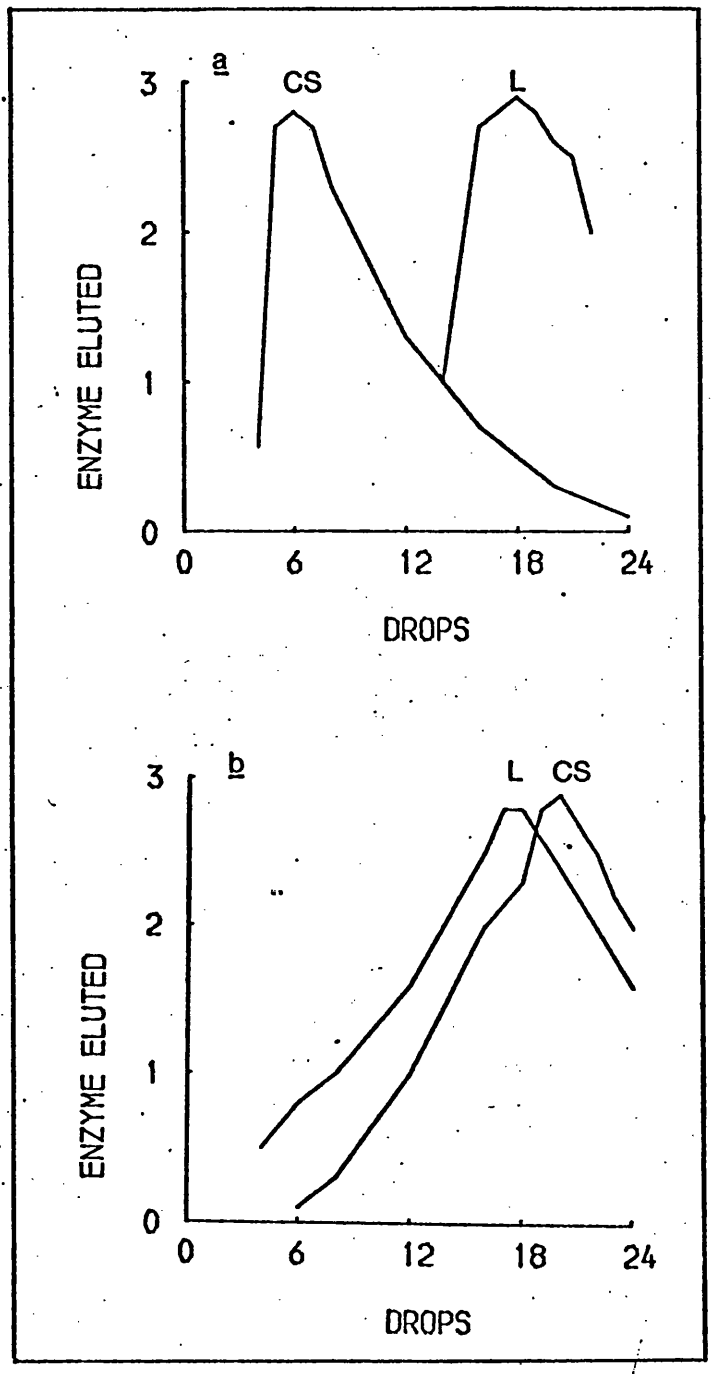


Fig. 20: Elution profile of citrate synthase and lactate dehydrogenase on a small column of Sephadex G-200.

a. Typical 'large' citrate synthase (E. coli).

b. Typical 'small' citrate synthase (B. megaterium).

CS, citrate synthase; L, lactate dehydrogenase.

the positions of these two enzymes relative to lactate dehydrogenase.

The use of this method to examine the molecular sizes of citrate synthases, from a range of organisms, gave results in absolute agreement with those obtained by the conventional method using a much larger column of Sephadex G-200 (Table 6). The reliability of this small column for assessing the molecular size of citrate synthase was thus confirmed.

Section C - Isolation of Bacterial Mutants Producing Structurally Altered Citrate Synthases

The organisms chosen for this work were E. coli and A. lwoffi. The first step in the isolation of strains producing citrate synthases with altered structure was to select for citrate synthase deficient mutants. The subsequent isolation of revertants which had regained citrate synthase activity may yield some strains which produce a structurally altered enzyme. It was decided that by determining the molecular properties of the citrate synthases produced by the revertant strains, an organism may be found which produced such an enzyme.

The properties most readily assessed were sensitivity to inhibitors and molecular size. The methods for determining these properties have hitherto involved a partial purification of the enzyme and have been time-consuming in practice (Weitzman & Jones, 1968; Weitzman & Jones, 1975). As it was necessary to scan large numbers of revertants to identify any which produced altered citrate synthase, the rapid screening techniques were employed to determine the regulatory behaviour and molecular size of the enzymes.

Isolation of Citrate Synthase Deficient Mutants by Penicillin Enrichment

Cultures of E. coli (AB259) and A. lwoffi were mutated by treatment with EMS and glutamate auxotrophs were selected by penicillin enrichment as desribed in the Methods Section. Analysis of cell-free extracts of these auxotrophs resulted in the successful isolation of an E. coli mutant which possessed 1% of the citrate synthase activity of the wild type organism. This citrate synthase deficient mutant was termed AB259-CS1.

Examination of the cell-free extracts of the A. lwoffi glutamate auxotrophs were disappointing as they showed that all of these mutants possessed a level of citrate synthase activity similar to that of the wild type. Therefore, a more direct selection method was devised to obtain the

citrate synthase deficient strains of this organism.

Isolation of Citrate Synthase Deficient Mutants Using Fluoroacetate

A method was devised for selection of citrate synthase deficient mutants, which relies on the fact that these may be resistant to the toxic effect of fluoroacetate by virtue of their inability to metabolise this compound to fluorocitrate. The procedure is given in the Methods Section, and the feasibility of the technique was established by testing the resistance of the citrate synthase deficient strains of E. coli to fluoroacetate.

It was found that when plated onto medium containing succinate (10mM), glutamate (1mM) and fluoroacetate (1mM), the wild type strains of E. coli (AB1621, K1.1, K2.1 and AB259) were all killed by the presence of the fluoroacetate. However, citrate synthase deficient strains (AB1623, K1.1.4, K2.1.4 and AB259-CS1) all grew on this medium (Table 7); they were therefore resistant to fluoroacetate.

The method was employed for A. lwoffi (Methods Section). A large number of mutants were isolated which grew on the glutamate-fluoroacetate medium. From these mutants a number were obtained which although able to grow on glutamate medium were unable to grow on either acetate or succinate. Analysis of the cell-free extracts of these mutants showed that several had an undetectable level of citrate synthase. Two of these citrate synthase deficient strains were used for further study; these were termed 4B-CS1 and 4B-CS2.

Studies on the Citrate Synthase Deficient Strains of E. coli and A. lwoffi

The comparative levels of citrate synthase activity in the wild type organisms, E. coli AB1621 and A. lwoffi, and the citrate synthase deficient strains of these organisms are given in Table 8. The E. coli and A. lwoffi citrate synthase deficient strains all required glutamate, α-oxoglutarate or proline in the medium for growth.

Studies on the E. coli citrate synthase deficient strains showed that

Table 7: Growth of strains of E. coli in the presence of fluoroacetate.

Strain	Presence of citrate synthase	Growth in the presence of fluoroacetate[a]
AB1621	+	-
AB259	+	-
K1.1	+	-
K2.1	+	-
AB1623	-	+
AB259-CS1	-	+
K1.1.4	-	+
K2.1.4	-	+

a. Growth medium consisted of sucoinate (10mM), glutamate (1mM) and fluoroacetate (1mM).

Table 8: Comparative levels of citrate synthase activity in strains of *E. coli* and *A. lwoffi*.

Strain	Specific activity[a] of citrate synthase (Enzyme units/mg protein)
E. coli	
AB1621 (wild type)	0.91
AB1623	0.003
AB259-CS1	0.001
K1.1.4	0.005
K2.1.4	0.005
A. lwoffi	
4B (wild type)	1.46
4B-CS1	0.002
4B-CS2	0.011

a. The specific activity was determined for cell-free extracts of cultures grown in nutrient broth.

when grown in medium containing glucose (10mM) and glutamate (0.1mM) these mutants produced and excreted a high concentration (approx. 2mM) of pyruvate into the growth medium. Fig. 21 shows the growth of strain AB1623 in this medium; growth was monitored by following the change in absorption at 550nm, and pyruvate concentration in the medium was determined by assaying aliquots with lactate dehydrogenase. The corresponding wild type strain, AB1621, did not produce a detectable level of pyruvate in the growth medium. The pyruvate accumulation by the citrate synthase deficient strains is probably a direct result of the lack of this enzyme.

Isolation of Citrate Synthase Revertants

Citrate synthase deficient strains of A. lwoffi and E. coli were grown overnight in nutrient broth. These cultures were plated onto succinate minimal medium at 5×10^8 cells/plate. Colonies which grew had lost their glutamate requirement; all of these revertants were found to have regained citrate synthase activity. Table 9 shows the reversion frequency of each strain occurring both spontaneously and after mutation by EMS treatment.

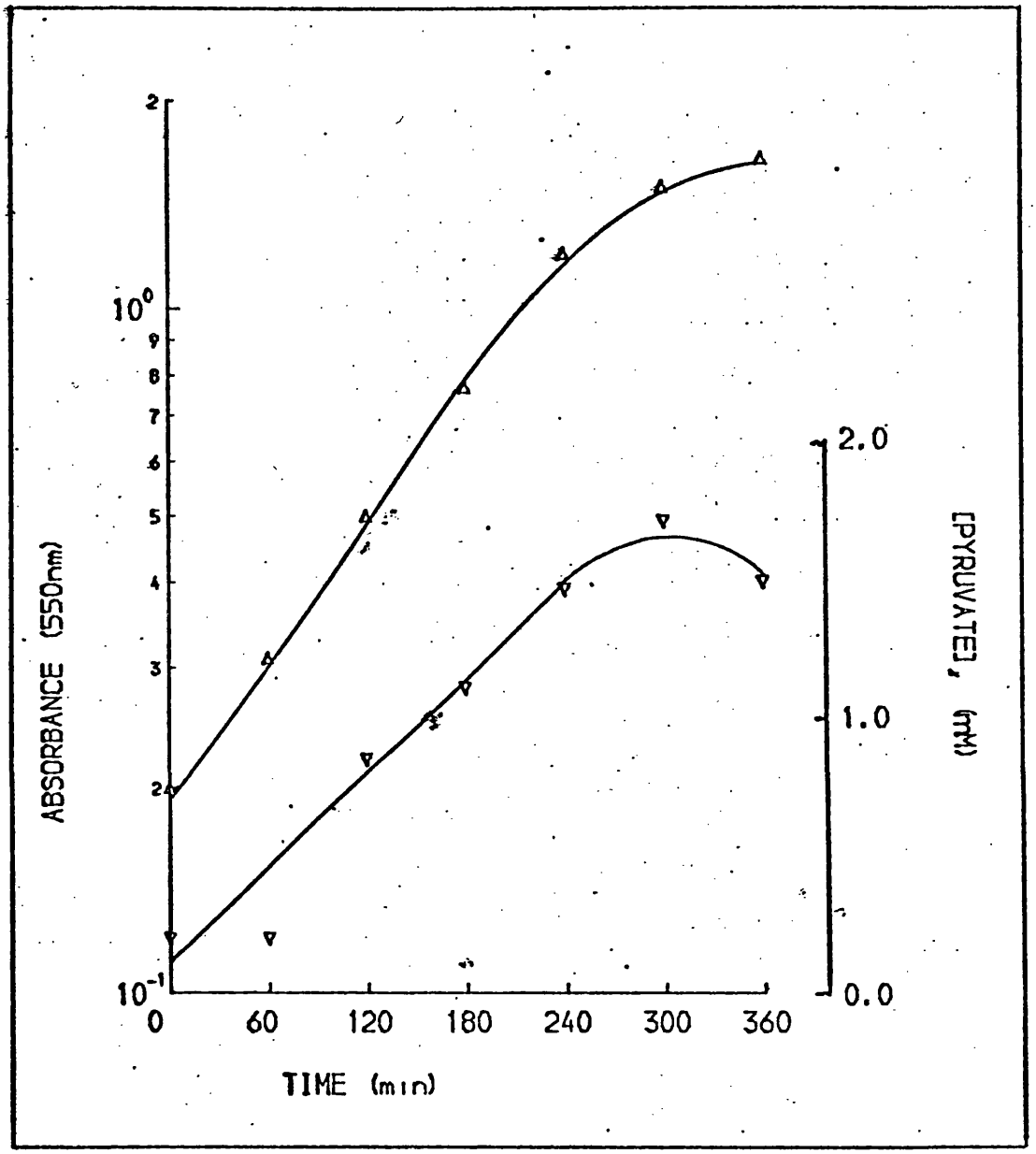


Fig. 21: Pyruvate accumulation during growth on glucose by the citrate synthase deficient strain of E. coli (AB1623).

Growth of the culture was monitored (△) and the pyruvate concentration (▽) was determined by using lactate dehydrogenase, as described in the Methods Section.

Table 9: Rate of reversion of the citrate synthase deficient strains of E. coli and A. lwoffi.

Citrate synthase deficient strain	Rate of reversion [a]	
	Spontaneous	EMS treated
E. coli		
AB1623	6.2×10^{-7}	4.1×10^{-5}
AB259-CS1	4.3×10^{-7}	6.0×10^{-5}
K1.1.4	7.9×10^{-7}	5.2×10^{-5}
K2.1.4	8.6×10^{-7}	3.8×10^{-5}
A. lwoffi		
4B-CS1	8.4×10^{-8}	6.3×10^{-6}
4B-CS2	1.3×10^{-8}	7.8×10^{-5}

a. Reversion was tested by plating the strains onto succinate minimal medium.

Section D - The Properties of E. coli Revertant Citrate Synthases

The rapid examination techniques, described earlier, were used to determine the regulatory properties and molecular sizes of the large number of citrate synthases from the E. coli revertants; the results are presented in Table 10. Citrate synthase from wild type E. coli is powerfully inhibited by α-oxoglutarate and NADH and has a molecular weight of about 230,000. It can be seen that the enzymes from the revertant strains fall into three types.

Type E1 enzyme, produced by Type E1 revertants, had regulatory properties and a molecular size similar to those of the wild type enzyme. This type of enzyme was produced by revertants K1.1.4.R1-R3, K2.1.4.R1, AB259-CS1R1 and AB1623.R1-R5.

Type E2 enzyme, produced by Type E2 revertants, had a molecular size similar to that from wild type E. coli, but had lost sensitivity to NADH and α-oxoglutarate inhibition. This type of enzyme was produced by revertants AB1623.R6-R8.

Type E3 enzyme, produced by Type E3 revertants, had similarly lost regulatory sensitivity to NADH and α-oxoglutarate. However, these enzymes had a molecular size of the 'small' type. This type of enzyme was by far the most common, being produced by revertants K1.1.4.R4-R40, K2.1.4.R2-R25, AB259-CS1R2-R18 and AB1623.R9-R37.

A detailed study was made of the molecular properties of representatives of each of these enzyme types. These properties were compared with wild type E. coli (AB1621) citrate synthase. Revertant AB1623.R1 was chosen as a representative of the strains producing Type E1 enzyme, AB1623.R6 of those producing Type E2 and AB1623.R9 and K1.1.4.R7 as representative of the strains producing Type E3 enzyme.

Table 10: The properties of the citrate synthases from the revertants of the E. coli citrate synthase deficient strains as determined using the rapid techniques.

Parent strain	Revertant strain	Inhibition		Molecular size
		NADH	α-oxoglutarate	
AB1621 (wild type)	-	+	+	large
AB1623	AB1623.R1-R5	+	+	large
	AB1623.R6-R8	-	-	large
	AB1623.R9-R37	-	-	small
AB259-CS1	AB259-CS1R1	+	+	large
	AB259-CS1R2-R18	-	-	small
K1.1.4	K1.1.4.R1-R3	+	+	large
	K1.1.4.R4-R40	-	-	small
K2.1.4.	K2.1.4.R1	+	+	large
	K2.1.4.R2-R25	-	-	small

Section E - Confirmation of the Identity of the Revertant Strains

The strains AB1623.R1, AB1623.R6, AB1623.R9 and K1.1.4.R7 were examined and gave the following results. All strains were found to be non-sporing, Gram negative rods when viewed under oil-emersion at 1000x magnification. The Gram reaction of each was confirmed by examination of the cell wall structure by electron microscopy at 40,000x magnification. It can be seen from Fig. 22 that the strain K1.1.4.R7 showed the cell wall structure typical of Gram negative bacteria. Similar results were obtained for the other strains.

The revertants were all methyl red test positive, Voges-Proskauer test negative, produced indole at 44^{o} (Eijkman test) and were unable to utilise citrate as a carbon source. These results identified the strains as probably E. coli. Further examination using API 50 (Enterobacteriaceae) microtube tests, followed by computer assisted identification, confirmed their identity.

Finally, the parent strain AB1621 was auxotrophic for vitamin B_1; this property was also exhibited by AB1623.R1, R6 and R9. Similarly the parent strain K1.1 was auxotrophic for methionine and thymine; these properties were shared by K1.1.4.R7. Therefore it may be concluded that AB1623.R1,R6 and R9 are all derived from the original wild type strain AB1621. Similarly, K1.1.4.R7 is a mutant of the wild type K1.1.

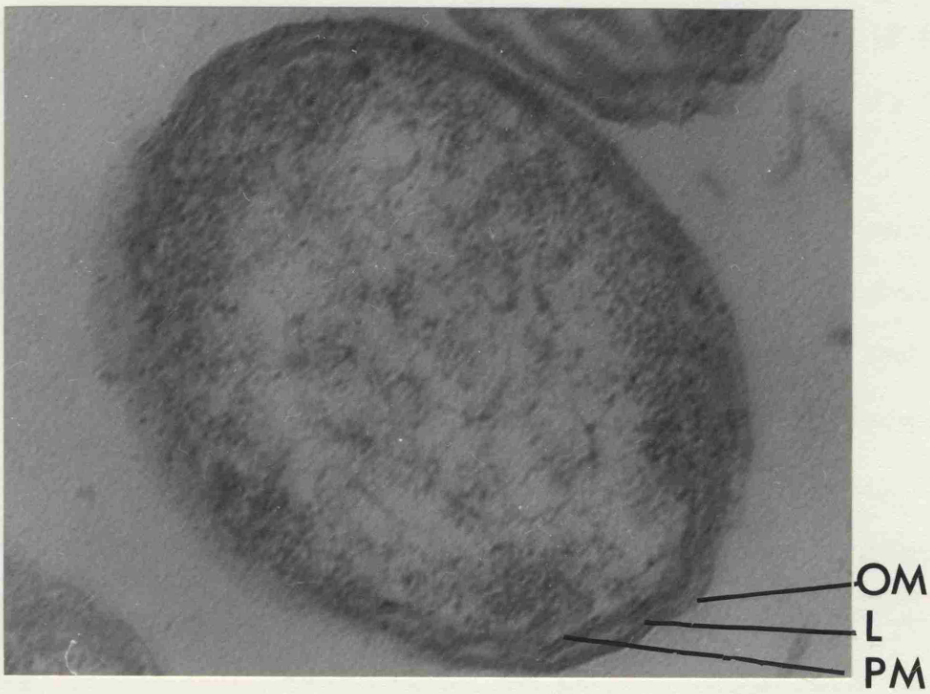
Fig. 22: Electron micrograph showing the typical cell wall architecture of Gram negative bacteria.

Type E3 revertant (~~AB1623.R9~~ K1.1.4.R7) at x 4 0,000.

OM, outer membrane of cell wall.

L, intermediate layers of cell wall.

PM, plasma membrane.


OM
L
PM

Section F - Mapping of the Gene Locus for Citrate Synthase in the *E. coli* Revertants

Interrupted mating experiments were carried out to map the approximate position of the citrate synthase gene. The donor strain used in this experiment was AB259, which is an Hfr strain (*ara*$^+$ *gal*$^+$ *gltA*$^+$). This strain was mated with AB1623 (*ara*$^-$ *gal*$^-$ *gltA*$^-$ F$^-$) and the time of entry of the markers was established (Fig. 23). It was found that the gltA (citrate synthase) lesion mapped at a position almost co-incident with the gal marker.

The revertants AB1623.R6 and R9, although they had regained citrate synthase activity, were unable to grow on acetate as sole carbon source (Table 19). Therefore, by using these strains as recipients and strain AB259 as donor, the entry of the gltA marker could be determined by the ability of the recombinants to grow on acetate. It can be seen (Figs. 24-25) that the gltA marker again maps with the gal marker. Furthermore, the citrate synthases of the recombinant strains had the properties of the wild type enzyme (i.e. from AB259). The gaining of one type of citrate synthase accompanying the loss of the other clearly suggests that they are at the same locus, and therefore that the gene coding for the revertant enzymes maps at the same position as that coding for the enzyme in the wild type organism.

More precise mapping of this gene locus was carried out using phage P1-mediated transduction. Strain AB1623, and therefore AB1623.R6 and R9, was found to be resistant to P1 infection; this finding was confirmed by other workers (R.A.Cooper, personal communication; J.R.Guest, personal communication). Therefore phage lysates of strains K1.1.4 (*gal*$^+$ *gltA*$^-$) and K1.1.4.R7 (*gal*$^+$ *gltA*^{rev}) were prepared. These lysates were used to infect strain K2.1. (*gal*$^-$ *gltA*$^+$). Selection of recombinants of the K2.1 strain, which had gained the ability to utilise galactose, showed that the

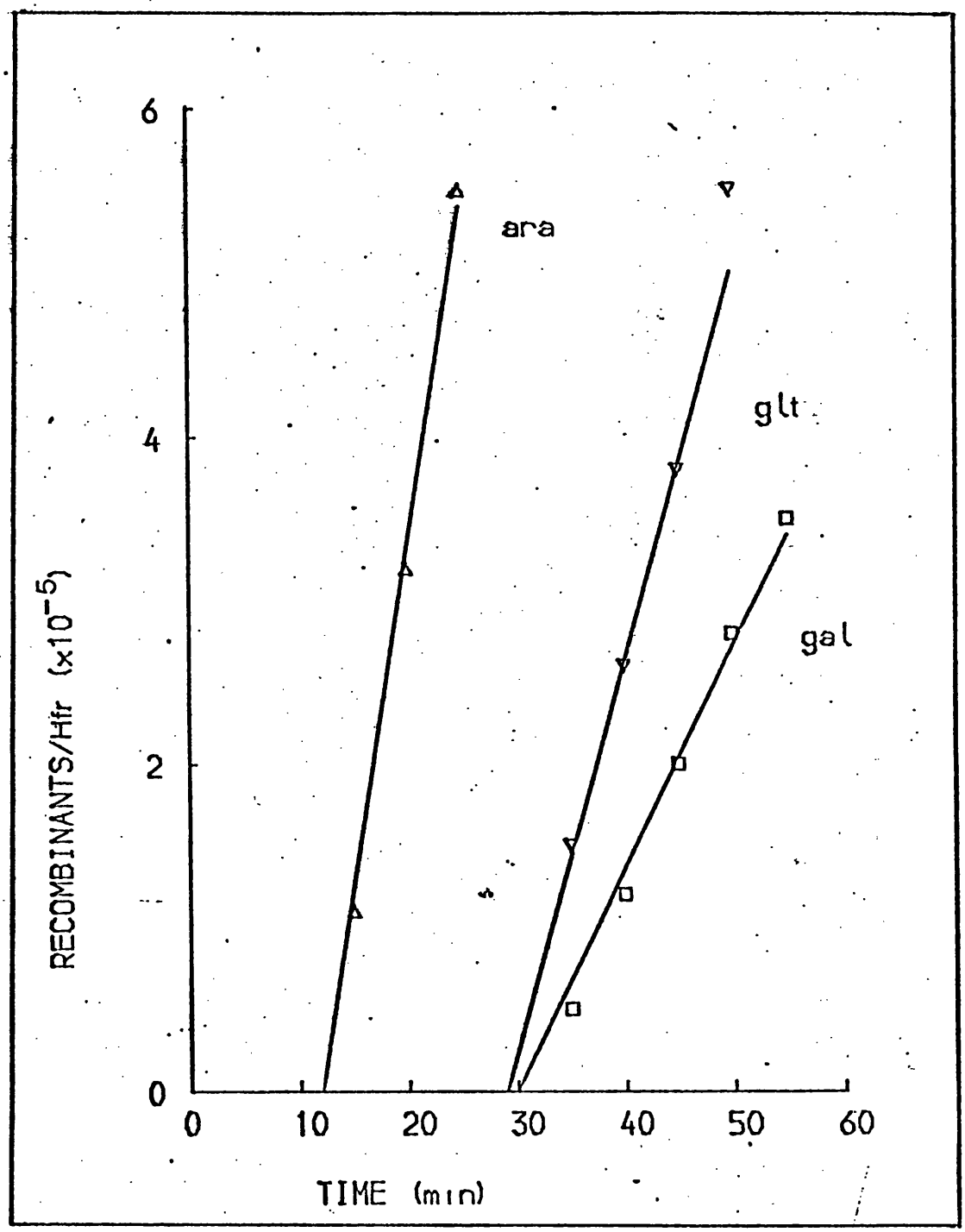


Fig. 23: Kinetics of transfer at 37° to AB1623 of the ara, glt and gal markers by AB259.

Time plotted on the abscissa is the interval between the commencement of mating and its interruption.

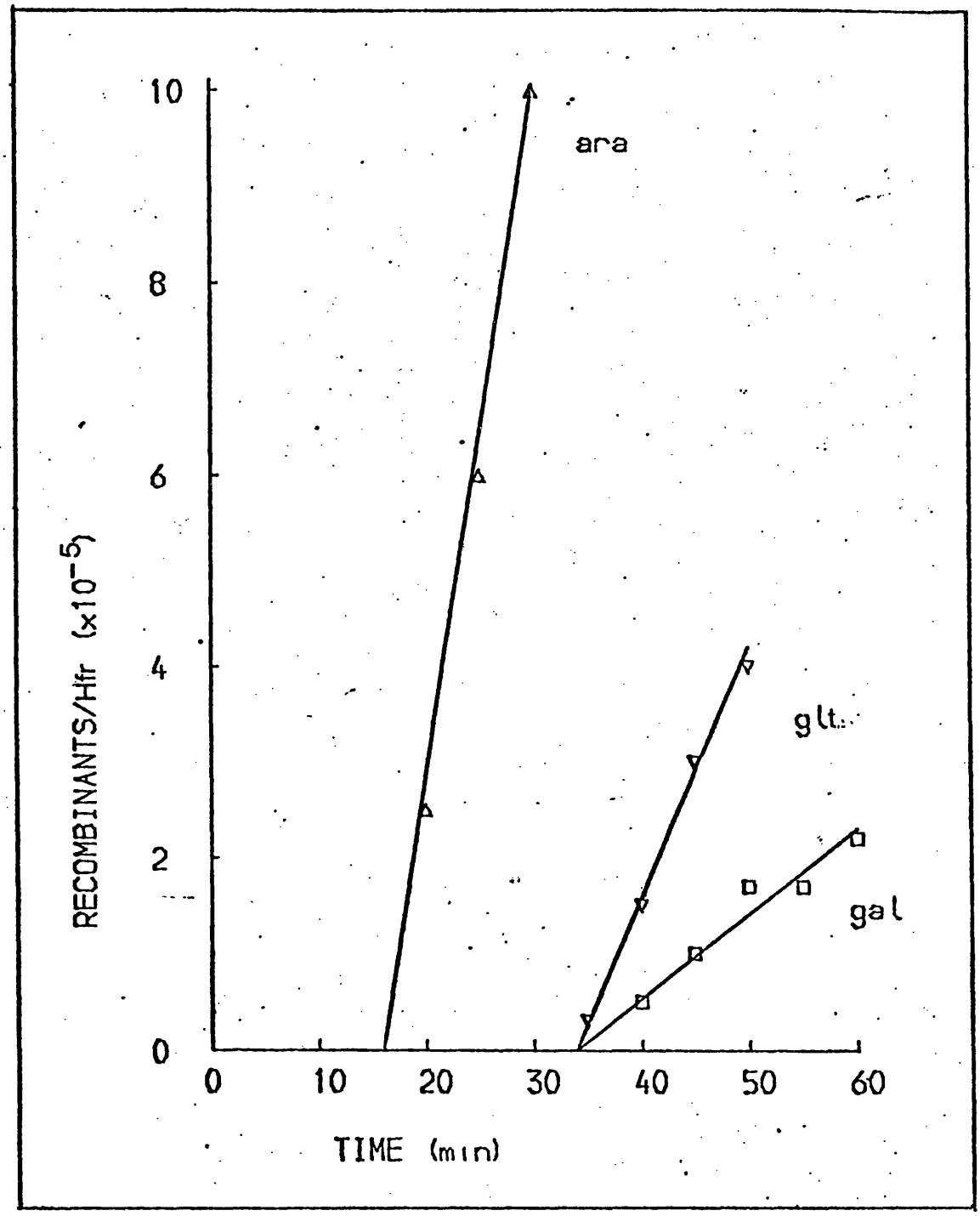


Fig. 24: Kinetics of transfer at 37° to AB1623.R6 of the ara, glt and gal markers by AB259.

Time plotted on the abscissa is the interval between the commencement of mating and its interruption.

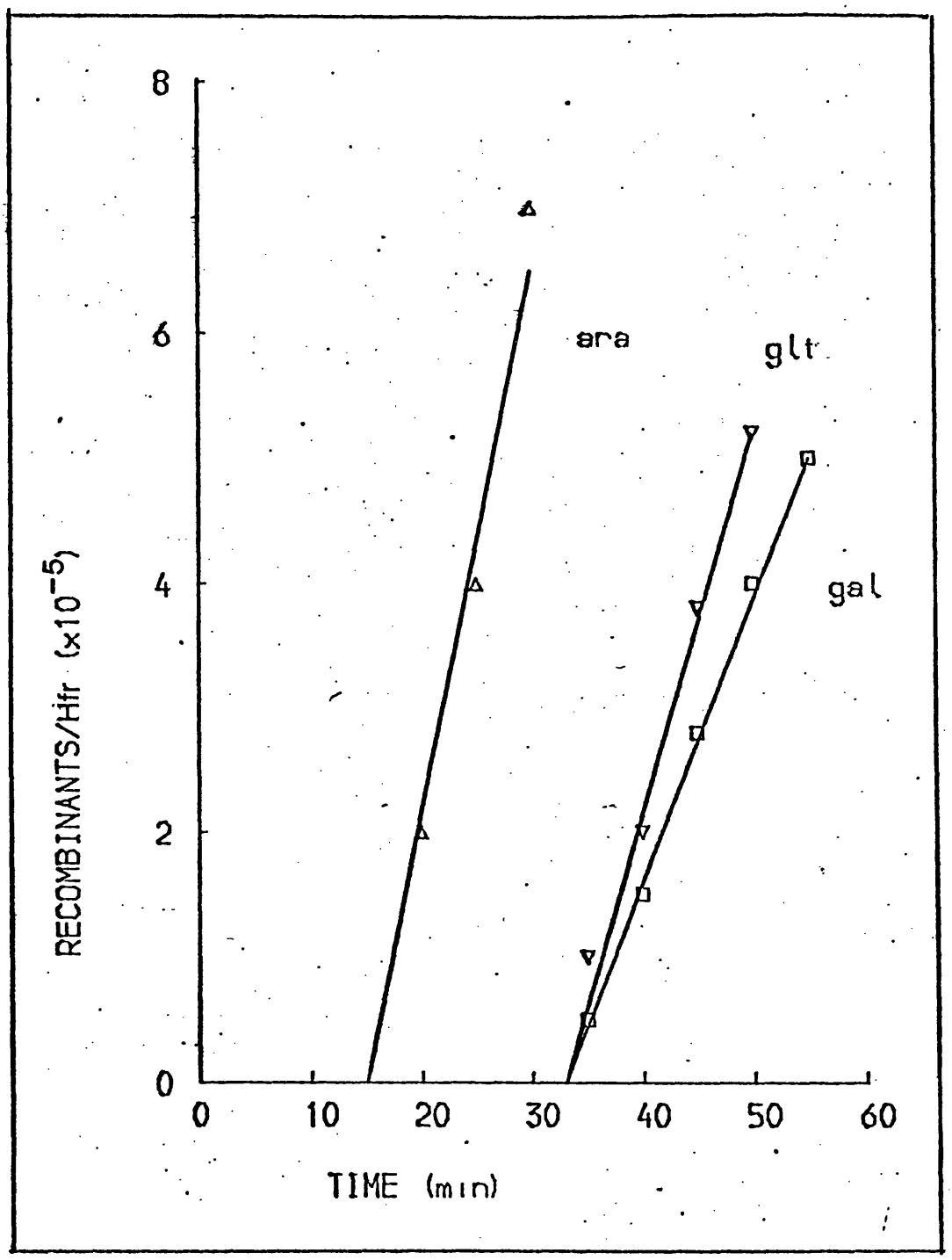


Fig. 25: Kinetics of transfer at 37° to AB1623.R9 of the *ara*, *glt* and *gal* markers by AB259.

Time plotted on the abscissa is the interval between the commencement of mating and its interruption.

gltA$^-$ property was 22% co-transducible with the *gal* marker (400 recombinants tested). Similarly, it was found that the *gltA*^{rev} marker (identified by an inability to grow on acetate as sole carbon source) was 20% co-transducible with the gal marker (150 recombinants tested).

The results of both the interrupted mating experiments and the phage mediated transduction experiments clearly demonstrate that the genetic locus coding for citrate synthase in AB1623.R6 and R9 and K1.1.4.R7 is the same as that coding for the enzyme in the wild type *E. coli*.

Section G - Comparison of the Molecular Properties of the *E. coli* Revertant Citrate Synthases

The properties of Type E1, E2 and E3 citrate synthases were compared with those of the enzyme from wild type *E. coli*, *B. megaterium*, pig heart and *Ac. xylinum*. The latter organism is a Gram negative bacterium which produces a citrate synthase of the 'large' type, but this is not sensitive to NADH inhibition, having regulatory properties very similar to those of Gram positive bacteria and eucaryotes (Swissa & Benziman, 1976).

The enzymes were partially purified by ammonium sulphate fractionation and passage through a column of Sephadex G-200. The dependence of enzyme activity on acetyl-CoA concentration was determined by assay method 1 (Figs. 26-27) and the dependence on oxaloacetate was determined by assay method 2 (Figs. 28-29). For each enzyme the K_m (or $S_{0.5}$) values for both substrates were determined and are given in Table 11.

Double reciprocal plots of the dependence of enzyme activity on acetyl-CoA concentration in the presence and absence of a variety of nucleotides (ATP, ADP, AMP, NADPH, NADH and $NADP^+$) showed that, as in the case of the pig heart enzyme (Figs. 5-10), the nucleotides were strictly competitive inhibitors with respect to acetyl-CoA; the K_i values for each inhibitor is given in Table 11. Similar studies showed that α-oxoglutarate acts as an inhibitor of all the enzymes in a strictly competitive manner with respect to the substrate oxaloacetate; the K_i values for this inhibitor are also given in Table 11.

A comparison was made of the mol. wt. of each enzyme by fitting data to the equation

$$\text{mol.wt.} = \frac{6\pi\eta N a^o s^o}{(1 - \bar{v}\rho)}$$

where η (viscosity) = 0.01poise, N = Avogadros' Number (6.023×10^{23}),

Table 11: A comparison of some of the catalytic, regulatory and molecular properties of citrate synthases from a number of different organisms and from the E. coli revertant types.

	Source of citrate synthase							
					E. coli revertant types			
Enzyme property	Pig heart	B. megaterium	Ac. xylinum	E. coli[a]	Type E1	Type E2	Type E3[b]	Type E3[c]
Molecular weight (approx.)	100,000	100,000	250,000	230,000	230,000	230,000	110,000	110,000
Substrate dependences	hyperbolic	hyperbolic	hyperbolic	sigmoidal	sigmoidal	hyperbolic	hyperbolic	hyperbolic
K_m (or $S_{0.5}$) acetyl-CoA (μM)	9.8 ± 0.6	29.4 ± 2.6	15.3 ± 1.1	420. ± 80.	360. ± 70.	30.3 ± 2.1	10.4 ± 0.6	10.9 ± 1.1
K_m (or $S_{0.5}$) oxaloacetate (μM)	5.3 ± 0.4	10.7 ± 2.6	9.6 ± 0.8	48.2 ± 6.6	41.3 ± 5.1	15.3 ± 0.8	11.8 ± 1.0	10.9 ± 0.7
K_i ATP (mM)	0.55 ± 0.04	1.6 ± 0.1	0.76 ± 0.05	23.3 ± 3.1	20.7 ± 2.6	1.7 ± 0.2	0.61 ± 0.05	0.58 ± 0.04
K_i ADP (mM)	0.92 ± 0.06	2.5 ± 0.2	1.5 ± 0.09	-	-	2.2 ± 0.3	1.2 ± 0.09	0.90 ± 0.05
K_i AMP (mM)	2.0 ± 0.1	6.2 ± 0.5	3.2 ± 0.02	-	-	6.4 ± 0.6	1.8 ± 0.1	2.3 ± 0.2
K_i NADPH (mM)	2.9 ± 0.2	8.4 ± 0.7	4.8 ± 0.6	-	-	9.4 ± 1.1	2.7 ± 0.3	3.1 ± 0.3
K_i NADH (mM)	5.1 ± 0.3	12.4 ± 1.0	7.6 ± 0.6	0.04 ± 0.004	0.06 ± 0.004	13.7 ± 2.1	4.8 ± 0.4	5.3 ± 0.6
K_i $NADP^+$ (mM)	8.2 ± 0.9	-	-	-	-	25.6 ± 7.3	7.6 ± 1.2	9.1 ± 1.8
K_i α-oxoglutarate (mM)	5.8 ± 0.6	9.3 ± 1.0	8.1 ± 0.4	0.04 ± 0.003	0.07 ± 0.01	17.3 ± 0.9	9.3 ± 0.7	9.0 ± 1.2

a. E. coli wild type strain AB1621.

b. Type E3 revertant (AB1623.R9).

c. Type E3 revertant (K1.1.4.R7).

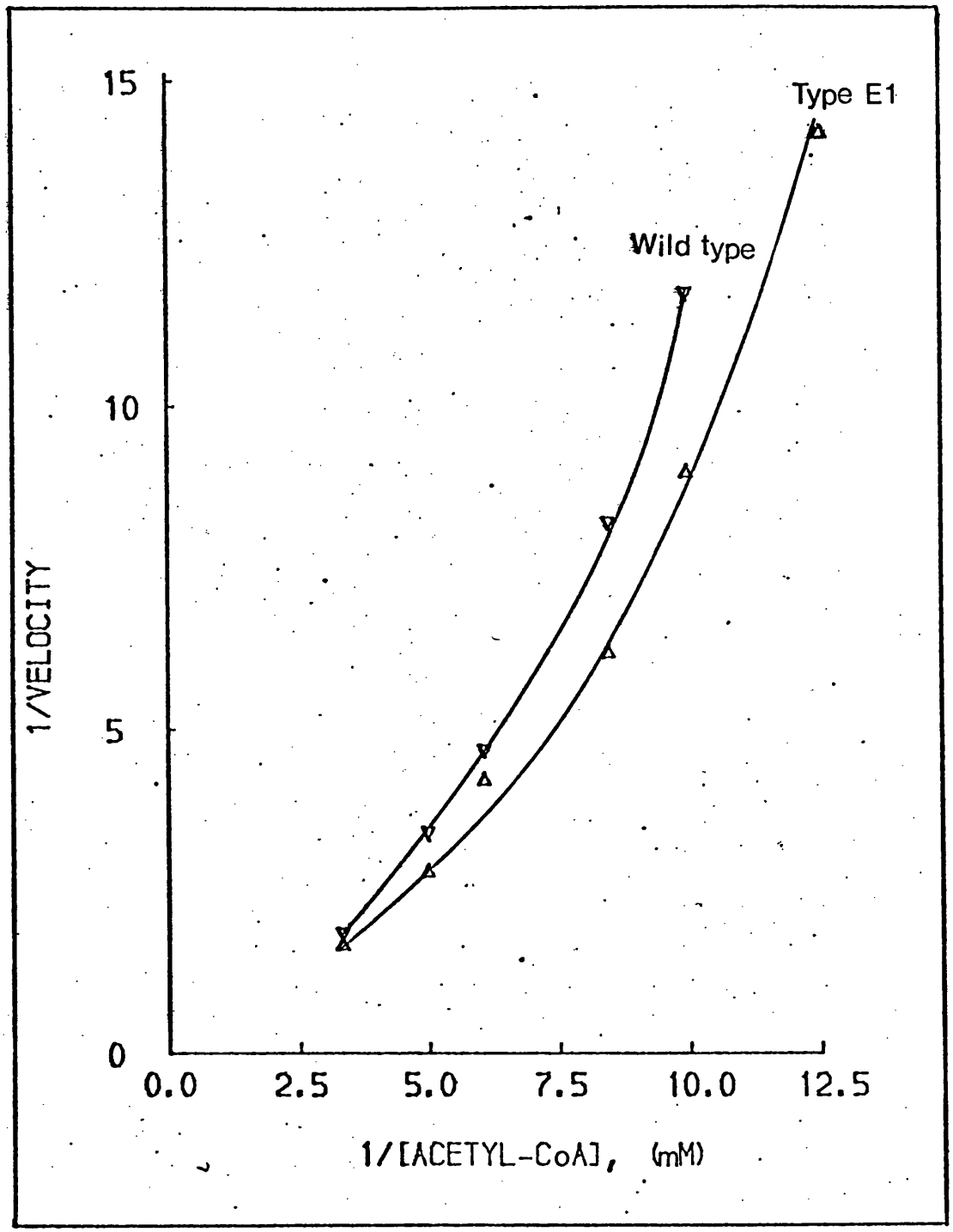


Fig. 26: Double reciprocal plots of the rate dependences of E. coli wild type and Type E1 citrate synthases on the concentration of acetyl-CoA.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

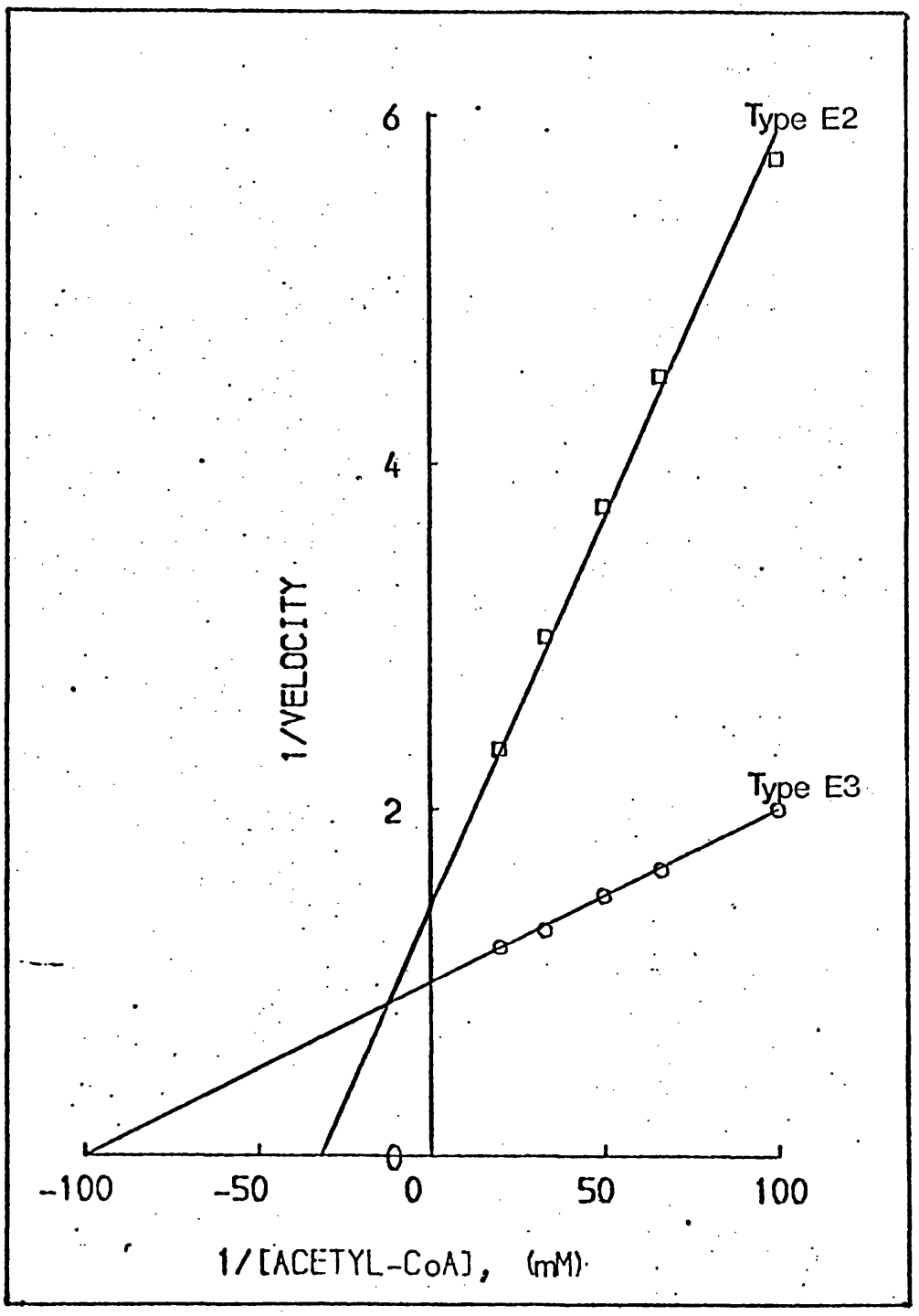


Fig. 27: Double reciprocal plots of the rate dependences of Type E2 and E3 citrate synthases on the concentration of acetyl-CoA.

Enzyme activity (in arbitrary units) was measured using assay method 1 at a fixed oxaloacetate concentration of 0.2mM.

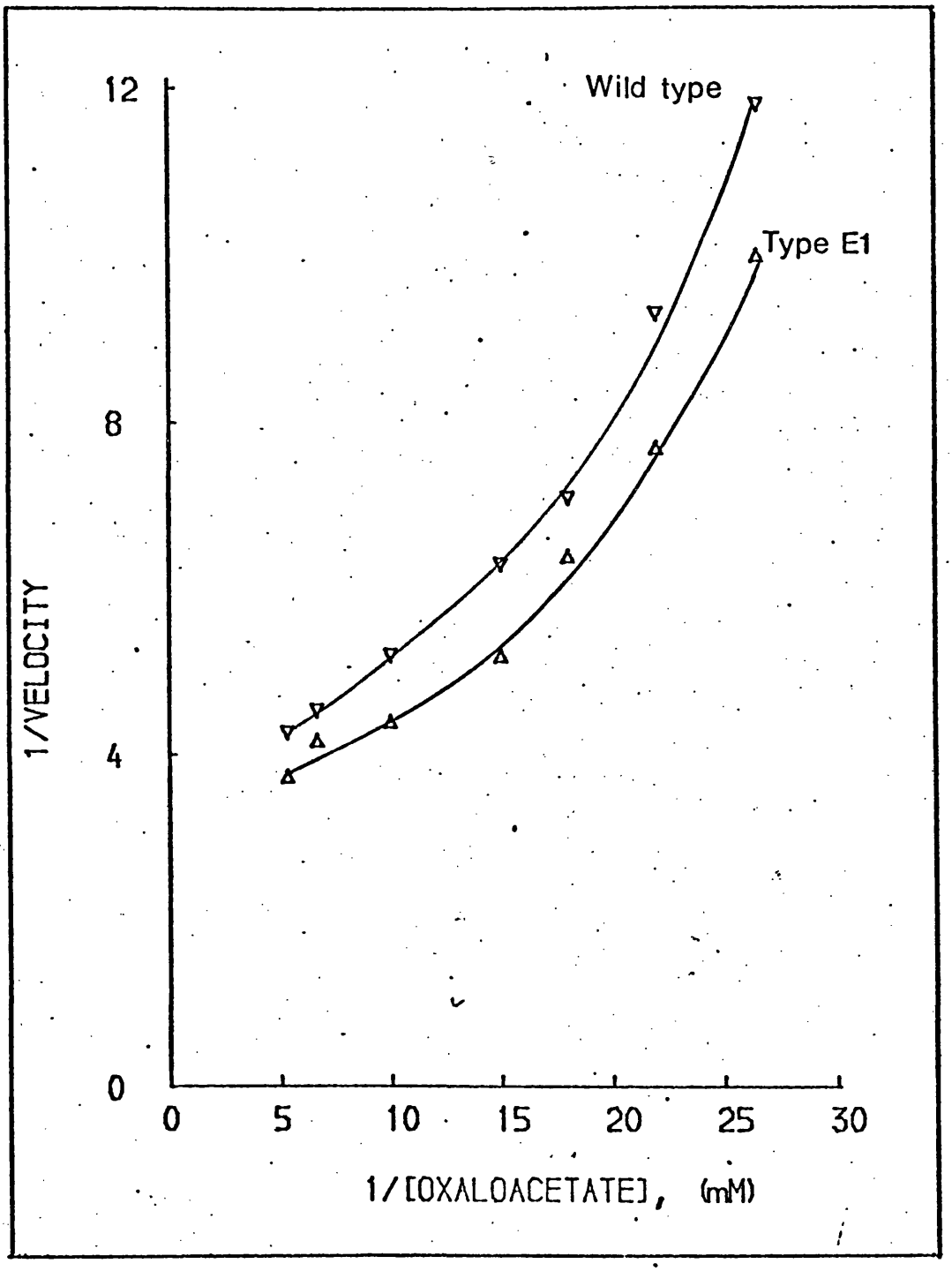


Fig. 28: Double reciprocal plots of the rate dependences of E. coli wild type and Type E1 citrate synthases on the concentration of oxaloacetate.

Enzyme activity (in arbitrary units) was measured using assay method 2 at a fixed acetyl-CoA concentration of 0.15mM.

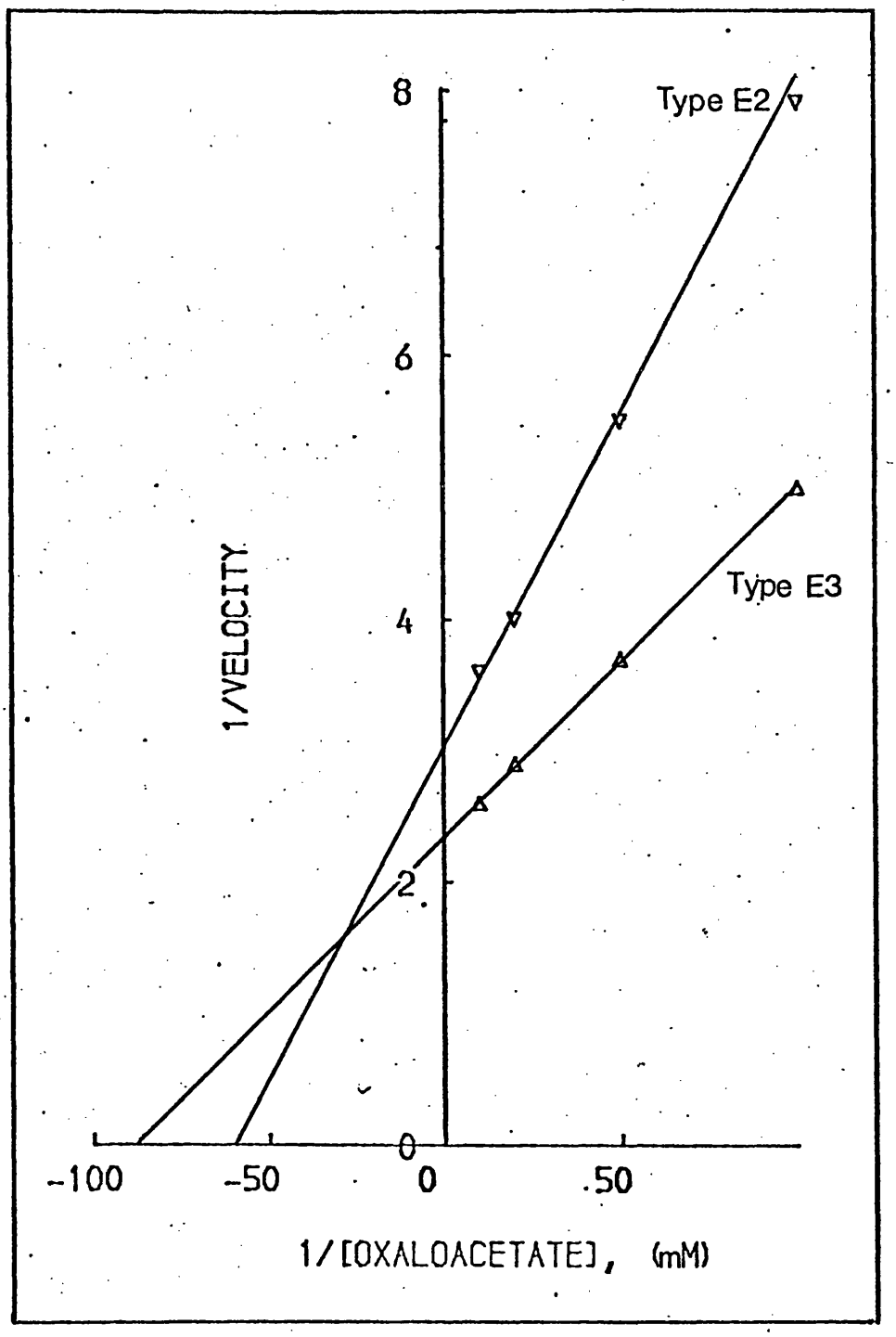


Fig. 29: Double reciprocal plots of the rate dependences of Type E2 and E3 citrate synthases on the concentration of oxaloacetate.

Enzyme activity (in arbitrary units) was measured using assay method 2 at a fixed acetyl-CoA concentration of 0.15mM.

a^o (Stoke's radius) was determined for each enzyme by gel filtration in the presence of marker proteins of known a^o value, s^o (Sedimentation coefficient) was determined by active-enzyme centrifugation of the partially purified enzyme, $\bar{v}$ (partial specific volume) was approximately 0.73ml/g for citrate synthase, as determined by Danson (1974) and ρ (density) was about 1.0.

This equation was derived from a combination of the Stoke's equation:

$$f_o = 6 \pi \eta a^o$$

(where f_o is the frictional coefficient of the molecule), and the Svedberg equation:

$$\frac{\text{mol.wt.}}{N} = \frac{f_o \; s^o}{(1 - \bar{v}\rho)}$$

by substituting for f_o.

Table 12 lists values for $\bar{v}$, s^o, mol.wt. and a^o for the proteins used as molecular weight markers, i.e. catalase, wild type E. coli citrate synthase, lactate dehydrogenase and malate dehydrogenase.

The partially purified enzyme extracts were each mixed with the marker proteins listed above and applied to a column of Sephadex G-200. The elution profiles of the proteins were of two types. Fig. 30 shows the gel filtration profile of a 'large' citrate synthase and Fig. 31 illustrates the profile of a 'small' enzyme. Fig. 32 shows the relationship between the Stoke's radius (a^o) value and the elution volume from the column. From this calibration, the Stoke's radii of a variety of citrate synthases have been determined and the values are presented in Table 13. The sedimentation coefficient (s^o) value and the calculated molecular weight are also given

Table 12: Comparative data for the physical properties of the marker enzymes used during gel filtration.

Enzyme	$\bar{v}$ (ml/g)	a° (m)	s° (sec)	mol.wt.	References
Catalase	0.72	51.38×10^{-8}	11.3×10^{-13}	244,000	Sumner and Gralén (1935)
E. coli (wild type) citrate synthase	0.73	55.28×10^{-8}	9.9×10^{-13}	230,000	Danson (1974)
Lactate dehydrogenase	0.72	45.51×10^{-8}	7.3×10^{-13}	140,000	Pesce *et al.* (1964)
Malate dehydrogenase	0.73	32.98×10^{-8}	4.4×10^{-13}	61,000	Siegel and Englard (1961)

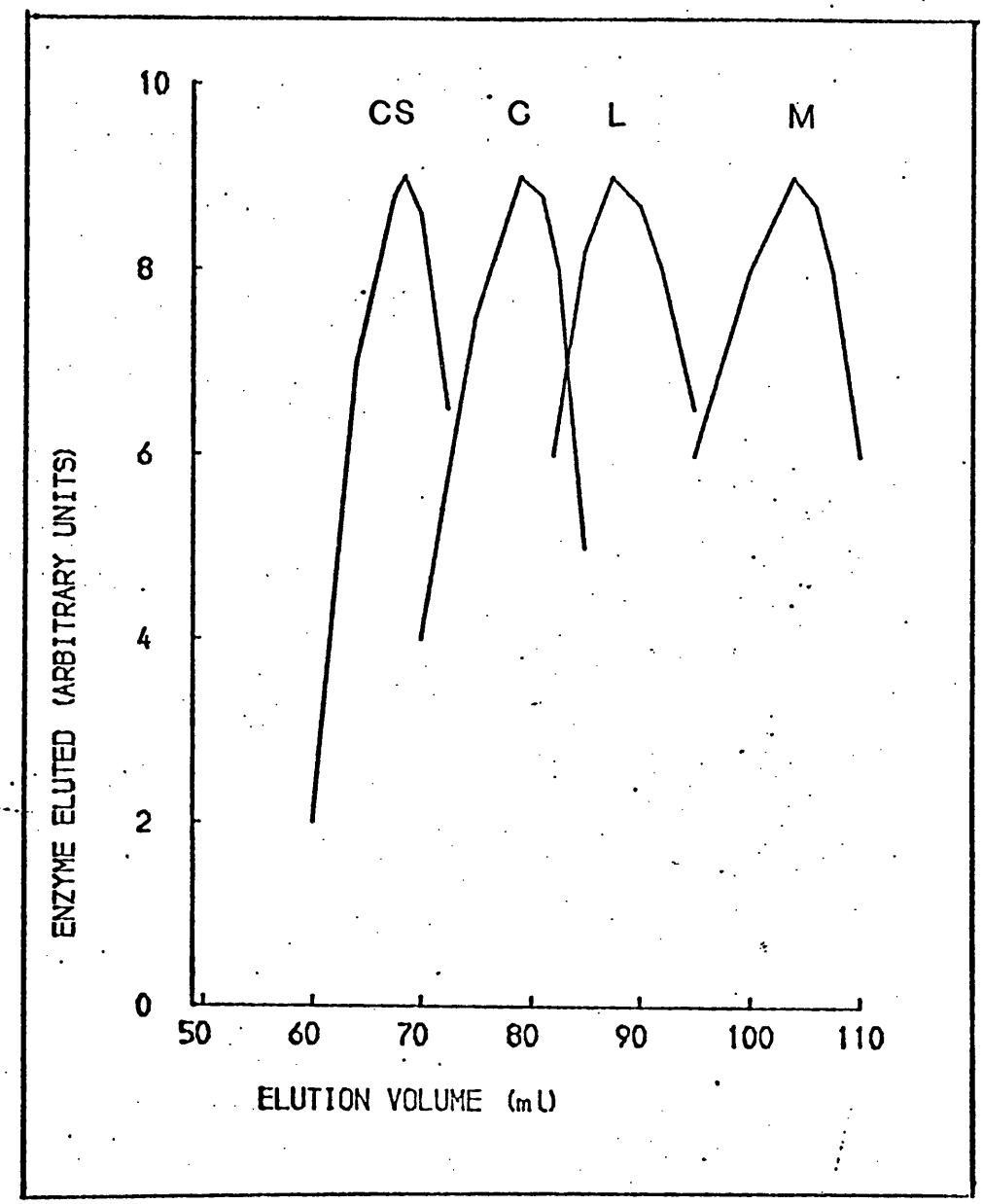


Fig. 30: Elution of a 'large' citrate synthase (from E. coli wild type) and marker enzymes on gel filtration.

A mixture (2ml) of citrate synthase (CS), catalase (C), lactate dehydrogenase (L) and malate dehydrogenase (M) was run on a column of Sephadex G-200 (2.5cm x 35.cm), using 'Tris buffer' to elute and collecting 1ml fractions.

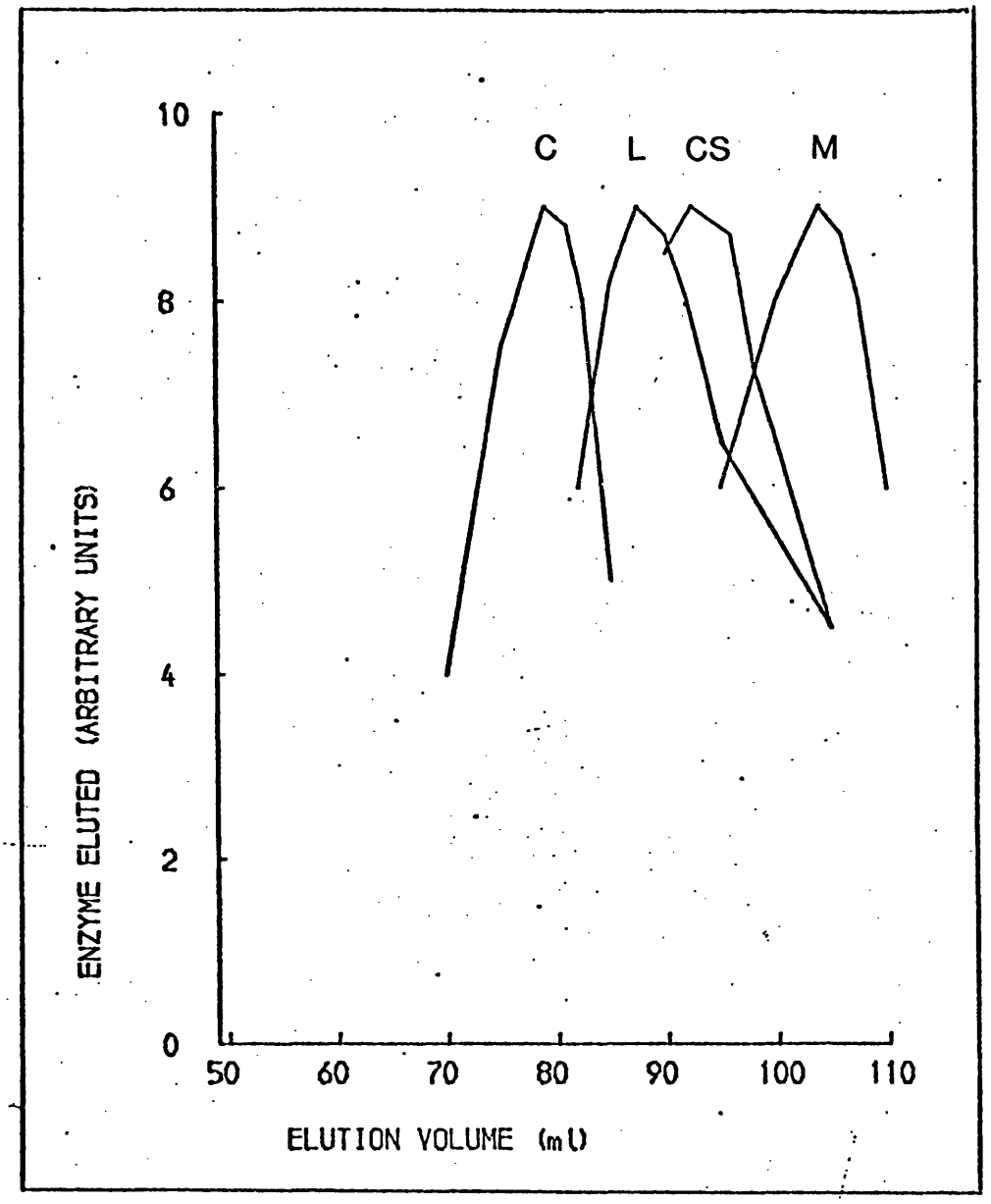


Fig. 31: Elution of a 'small' citrate synthase (from Type E3 revertant) and marker enzymes on gel filtration.

A mixture (2ml) of citrate synthase (CS), catalase (C), lactate dehydrogenase (L) and malate dehydrogenase (M) was run on a column of Sephadex G-200 (2.5cm x 35.cm), using 'Tris buffer' to elute and collecting 1ml fractions.

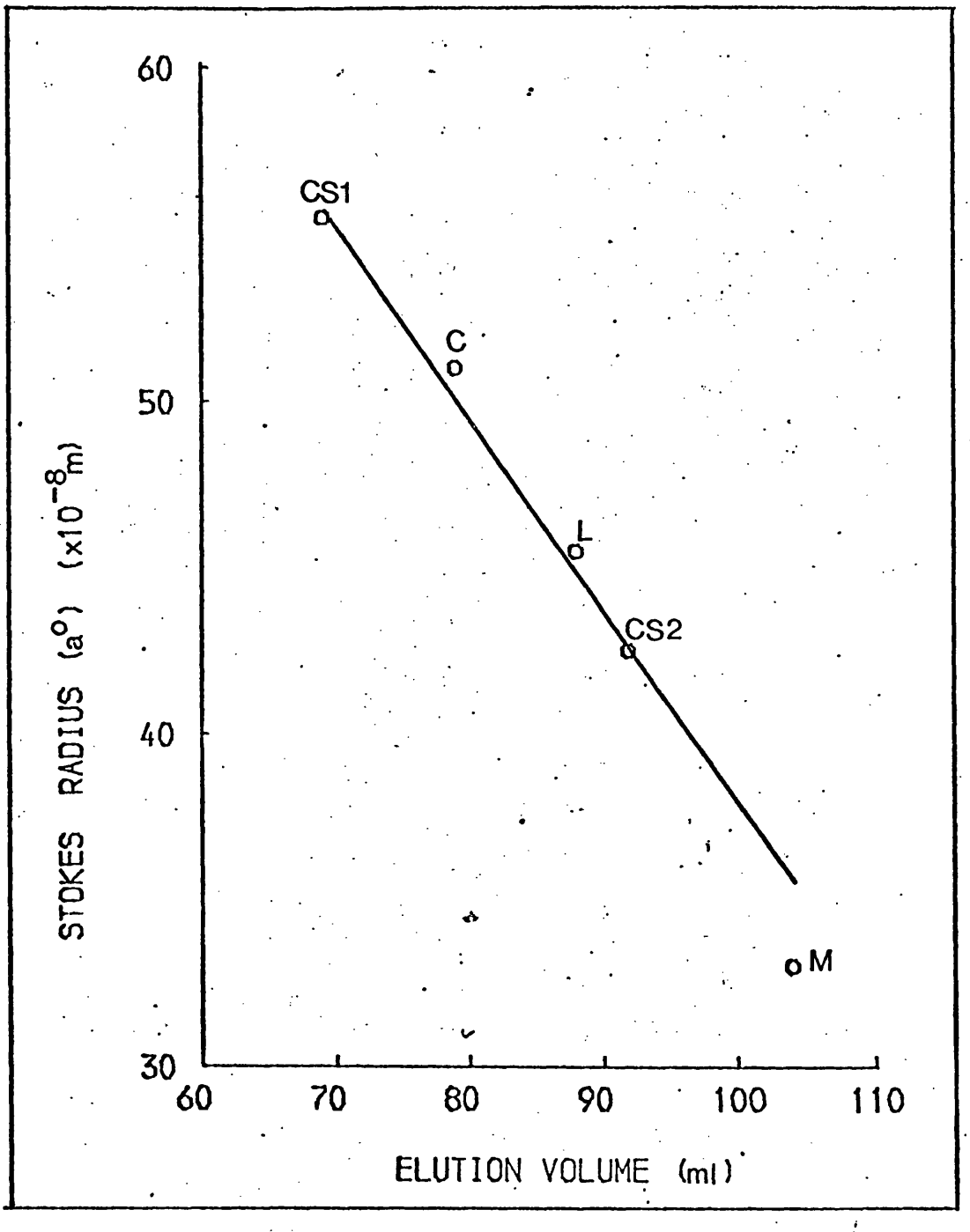


Fig. 32: Relationship between the Stokes' radii of proteins and their comparative elution volumes from a Sephadex G-200 gel filtration column.

The proteins used were 'large' citrate synthase (CS1) from *E. coli* wild type, catalase (C), lactate dehydrogenase (L), 'small' citrate synthase (CS2) from Type E3 revertant and malate dehydrogenase (M).

Table 13: Determination of the molecular weights of citrate synthases from a number of organisms and from the E. coli revertant strains.

Enzyme source	a^o ($x10^{-8}$ m)[a]	s^o ($x10^{-13}$ sec)[b]	mol. wt.
Pig heart	46.1	5.02	97,000
B. megaterium	48.0	5.10	102,800
Ac. xylinum	56.2	10.93	258,000
E. coli (wild type)	55.3	9.90	230,000
Type E1 revertant	56.1	10.2	240,000
Type E2 revertant	54.	10.7	242,700
Type E3 revertant[c]	48.	4.77	96,200
Type E3 revertant[d]	47.	4.8	94,800

a. Determined by gel filtration in the presence of marker enzymes.

b. Determined by active-enzyme ultracentrifugation.

c. Revertant (AB1623.R9).

d. Revertant (K1.1.4.R7).

in this table.

The thiol-blocking reagent DTNB has been shown to inactivate wild type E. coli citrate synthase (Weitzman, 1966a; Danson & Weitzman, 1973). Figs. 33-34 show the rate of inactivation of a range of citrate synthases by this compound, when used at a concentration of 0.1mM. It was found that although many of the enzymes were rapidly inactivated by this treatment, the presence of 0.2mM oxaloacetate completely protected the enzymes from inactivation by DTNB. Table 14 gives comparative rates of inactivation by DTNB treatment of each enzyme in the presence and absence of oxaloacetate.

It has been shown that polyacrylamide gel electrophoresis, at pH 8.9, of wild type E. coli citrate synthase reveals three protein bands. Elution of the protein from these bands, followed by assay for activity at pH 8.0, has shown that all three bands contain citrate synthase activity. Similar active-enzyme polyacrylamide gel experiments were carried out on a variety of citrate synthases. As described in the Methods Section, each gel was cut into thin sections and these were assayed for enzyme activity at pH 8.0. Fig. 35 shows the activity profile obtained using wild type E. coli enzyme. Similar profiles were obtained for each of the E. coli revertant citrate synthases. However, the enzyme from Ac. xylinum, B. megaterium and pig heart gave only a single active band. The R_f values for the active-enzyme bands for each enzyme have been calculated and are presented in Table 15.

Type E1 Enzyme

The in vitro molecular properties of this enzyme were identical to those of the enzyme from wild type E. coli. Both enzymes have a molecular weight of about 230,000 (Table 13), show sigmoidal substrate dependences (Figs. 26,28) with $S_{0.5}$ values of about 400μM for acetyl-CoA and 40μM for oxaloacetate (Table 11). The effect of all inhibitors studied was the same for the two enzymes and, furthermore, the powerful inhibition by NADH and α-oxoglutarate was abolished in the presence of 0.1M KCl for both enzymes.

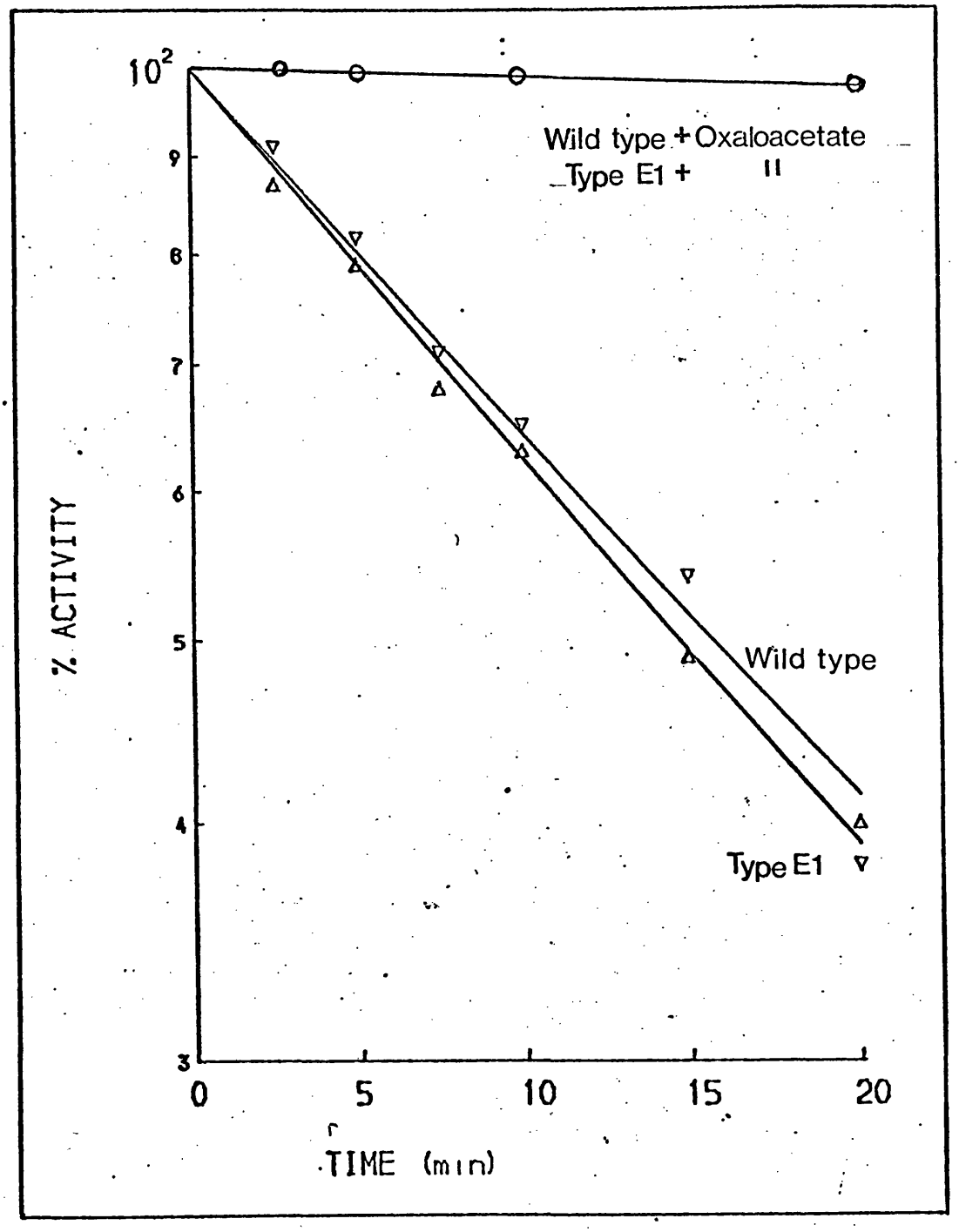


Fig. 33: Inactivation of E. coli wild type and Type E1 citrate synthases by incubation with DTNB.

Time course of incubation of these enzymes with 0.1mM DTNB.
Activity was measured using assay method 1.
Protection was afforded by the presence of 0.2mM oxaloacetate.

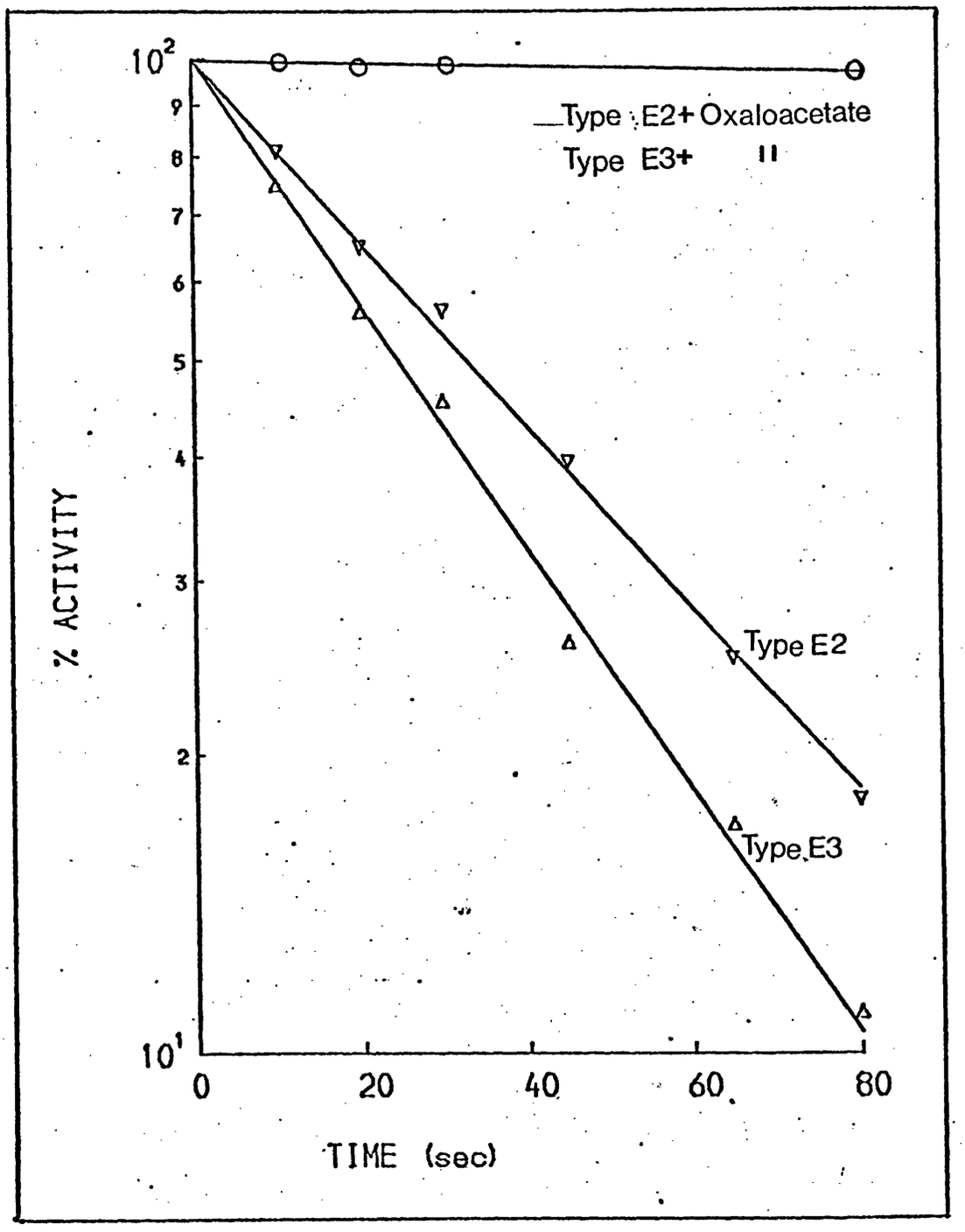


Fig. 34: Inactivation of Type E2 and E3 citrate synthases by incubation with DTNB.

Time course of incubation of these enzymes with 0.1mM DTNB.
Activity was measured using assay method 1.
Protection was afforded by the presence of 0.2mM oxaloacetate.

Table 14: Rate constants for the inactivation of E. coli wild type and revertant citrate synthases resulting from treatment with DTNB.

Enzyme source	Rate constant of inactivation (min^{-1})	
	Control	+Oxaloacetate (0.2mM)
E. coli wild type	0.029 ± 0.001	0.002
Revertant Type E1	0.041 ± 0.001	0.002
Revertant Type E2	1.77 ± 0.02	0.007
Revertant Type E3[a]	2.25 ± 0.03	0.008
Revertant Type E3[b]	2.31 ± 0.03	0.009

a: AB1623.R9.

b: K1.1.4.R7.

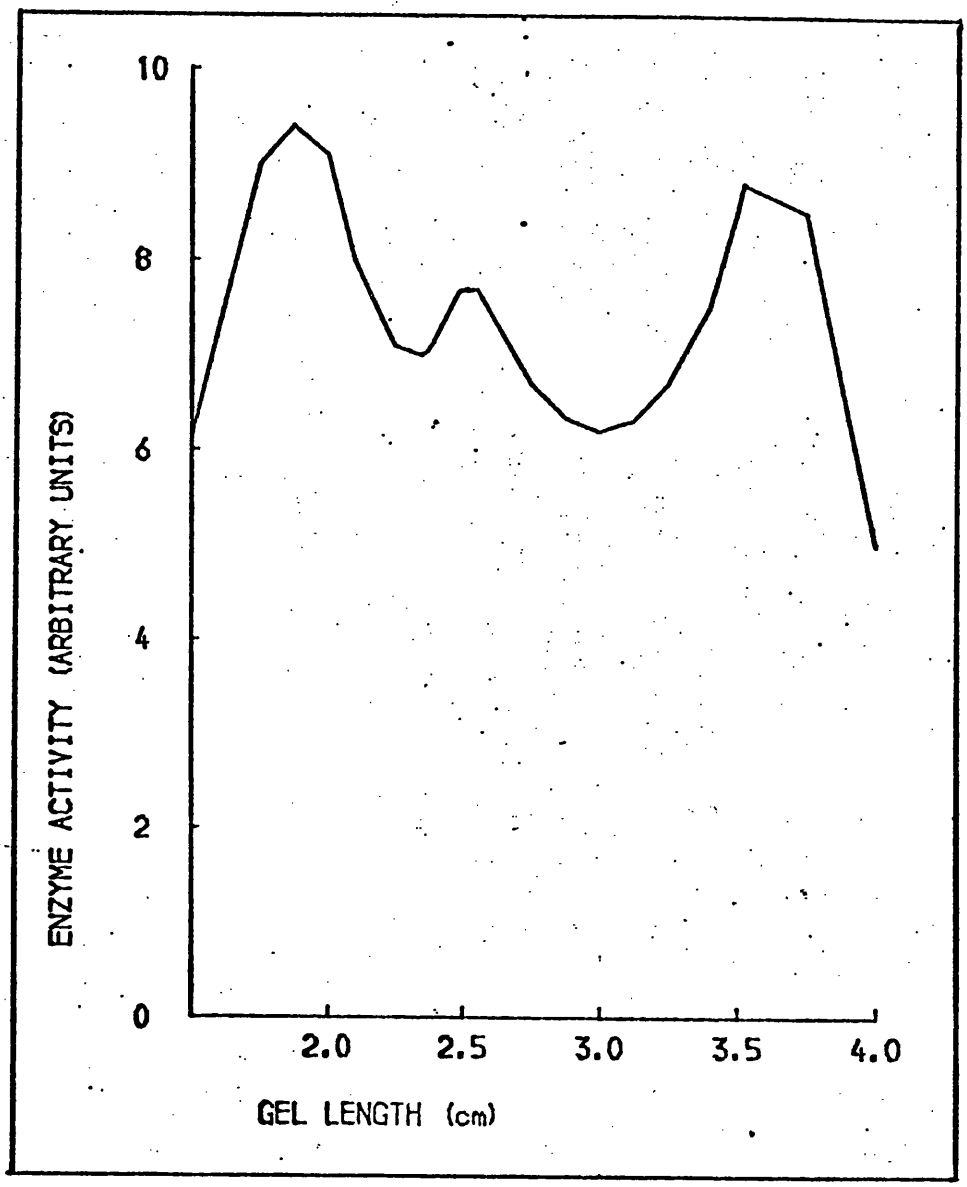


Fig. 35: 'Active enzyme' polyacrylamide gel electrophoresis of *E. coli* wild type citrate synthase.

The gel was cut into thin slices and the enzyme activity in each slice was determined using assay method 1.

The marker dye had travelled a total distance of 8.0cm.

The two enzymes were inactivated by DTNB at a similar rate (Table 14). The results of the polyacrylamide gel electrophoresis of the enzymes showed that they both exist as at least three molecular forms at pH 8.9 and, furthermore, the comparative R_f values of these three molecular species are very similar (Table 15).

An examination was made of the *in situ* properties of the enzymes using cells made permeable with toluene. The properties examined included the determination of substrate dependences, $S_{0.5}$ values for acetyl-CoA and oxaloacetate and the K_i values for NADH, ATP and α-oxoglutarate. The results of these investigations were in complete agreement with the *in vitro* observations (Table 16).

Type E2 Enzyme

The molecular weight of this enzyme was about 230,000 (Table 13) and the pattern of three enzyme forms on polyacrylamide gel electrophoresis at pH 8.9, was very similar to that observed with the enzyme from wild type *E. coli* (Table 15). Apart from these findings, all other properties examined were very different from those of the wild type enzyme. The striking correlation between the properties of Type E2 enzyme and those of *B. megaterium*, pig heart, or especially *Ac. xylinum* citrate synthase can be clearly seen from Table 11. Unlike the enzyme from wild type *E. coli*, Type E2 citrate synthase showed hyperbolic substrate dependences (Figs. 27,29) with a low K_m value of 30μM for acetyl-CoA and 15μM for oxaloacetate (Table 11). Furthermore, the inhibitory effects of the nucleotides and α-oxoglutarate were very different from the wild type enzyme, having K_i values for each inhibitor in close agreement with those of the *B. megaterium*, pig heart and *Ac. xylinum* enzymes (Table 11). NADH and α-oxoglutarate were not powerful inhibitors of this enzyme. Moreover, the very low level of inhibition that was observed in the presence of these two compounds was not abolished by 0.1M KCl, and the results obtained by multiple-inhibition

Table 15: The determined R_f values of a number of citrate synthases from a variety of organisms and from the E. coli revertants, using polyacrylamide gel electrophoresis.

Enzyme source	Number of enzyme species	R_f values
Pig heart	1	0.21
B. megaterium	1	0.28
Ac. xylinum	1	0.23
E. coli (wild type)[a]	3	0.23 0.30 0.45
Type E1 revertant	3	0.26 0.37 0.48
Type E2 revertant	3	0.20 0.29 0.40
Type E3 revertant[b]	3	0.28 0.38 0.53
Type E3 revertant[c]	3	0.21 0.29 0.45

a. E. coli wild type (AB1621).
b. Revertant (AB1623.R9).
c. Revertant (K1.1.4.R7).

Table 16: Comparison of the in vitro and in situ properties of the E. coli wild type and revertant citrate synthases.

Property	Conditions	Source of citrate synthase: E. coli wild type	E. coli revertants: Type E1	Type E2	Type E3[a]	Type E3[b]
K_m (or $S_{0.5}$) acetyl-CoA (μM)	in vitro	420. ± 80.	360. ± 70.	30.3 ± 2.1	10.4 ± 2.1	10.9 ± 1.1
	in situ	490. ± 120.	510. ± 100.	35.6 ± 2.4	9.8 ± 1.1	9.3 ± 0.7
K_m (or $S_{0.5}$) oxaloacetate (μM)	in vitro	48.2 ± 6.6	41.3 ± 5.1	15.3 ± 0.8	11.8 ± 1.0	10.9 ± 0.7
	in situ	55.6 ± 8.0	52.1 ± 5.0	18.2 ± 1.1	12.0 ± 1.8	11.4 ± 1.1
K_i ATP (mM)	in vitro	23.3 ± 3.1	20.7 ± 2.6	1.7 ± 0.2	0.61 ± 0.05	0.58 ± 0.04
	in situ	24.6 ± 3.7	26.4 ± 5.1	2.0 ± 0.2	0.57 ± 0.05	0.57 ± 0.04
K_i NADH (mM)	in vitro	0.04 ± 0.004	0.06 ± 0.004	13.7 ± 2.1	4.8 ± 0.4	5.3 ± 0.6
	in situ	0.03 ± 0.006	0.08 ± 0.009	14.3 ± 1.9	5.6 ± 0.6	5.4 ± 0.6
K_i α- oxoglutarate (mM)	in vitro	0.04 ± 0.003	0.07 ± 0.009	17.3 ± 0.9	9.3 ± 0.7	9.0 ± 1.2
	in situ	0.02 ± 0.002	0.04 ± 0.007	10.6 ± 1.5	6.2 ± 0.5	6.4 ± 0.6

a: AB1623.R9.
b: K1.1.4.R7.

analysis showed that NADH and ATP act at the same site (Fig. 36). These results strongly suggest that the allosteric sites of this enzyme have been lost.

In situ studies, using toluene treated cells, showed similar enzyme properties as those observed in vitro. The substrate dependences were hyperbolic; the K_m value for acetyl-CoA was about 35μM and for oxaloacetate was about 18μM. The K_i values for ATP, NADH and α-oxoglutarate were 2.0mM, 14.3mM and 10.6mM respectively (Table 16).

Type E3 Enzyme

Two enzymes from this category of revertants were investigated. The results of the comparative studies of the molecular properties of these enzymes showed that, far from resembling the citrate synthase from wild type E. coli, they had properties which were superficially indistinguishable from those of the B. megaterium and pig heart citrate synthases. The molecular weight of the Type E3 enzyme was about 110,000 (Table 13). The substrate dependences were hyperbolic (Figs. 27,29); the K_m values for acetyl-CoA (10μM) and oxaloacetate (11μM) were similar to those of the pig heart and B. megaterium enzymes (Table 11). The K_i values for α-oxoglutarate and nucleotide inhibitors were also similar to those of the pig heart and B. megaterium enzymes (Table 11). As with Type E2 enzyme, multiple-inhibition analysis using the inhibitor pair NADH and ATP, showed that parallel line plots were obtained (Fig. 37) indicating that ATP and NADH interact with the same site on the enzyme. These results suggest that the allosteric sites have been lost.

In situ studies, using toluene treated cells, showed that these enzymes have hyperbolic substrate dependences with a K_m value for acetyl-CoA of about 10μM and for oxaloacetate of about 12μM; the K_i values for ATP, NADH and α-oxoglutarate were about 0.6, 5.6 and 6.2 respectively.(Table 16). These results do not differ significantly from those obtained with the in

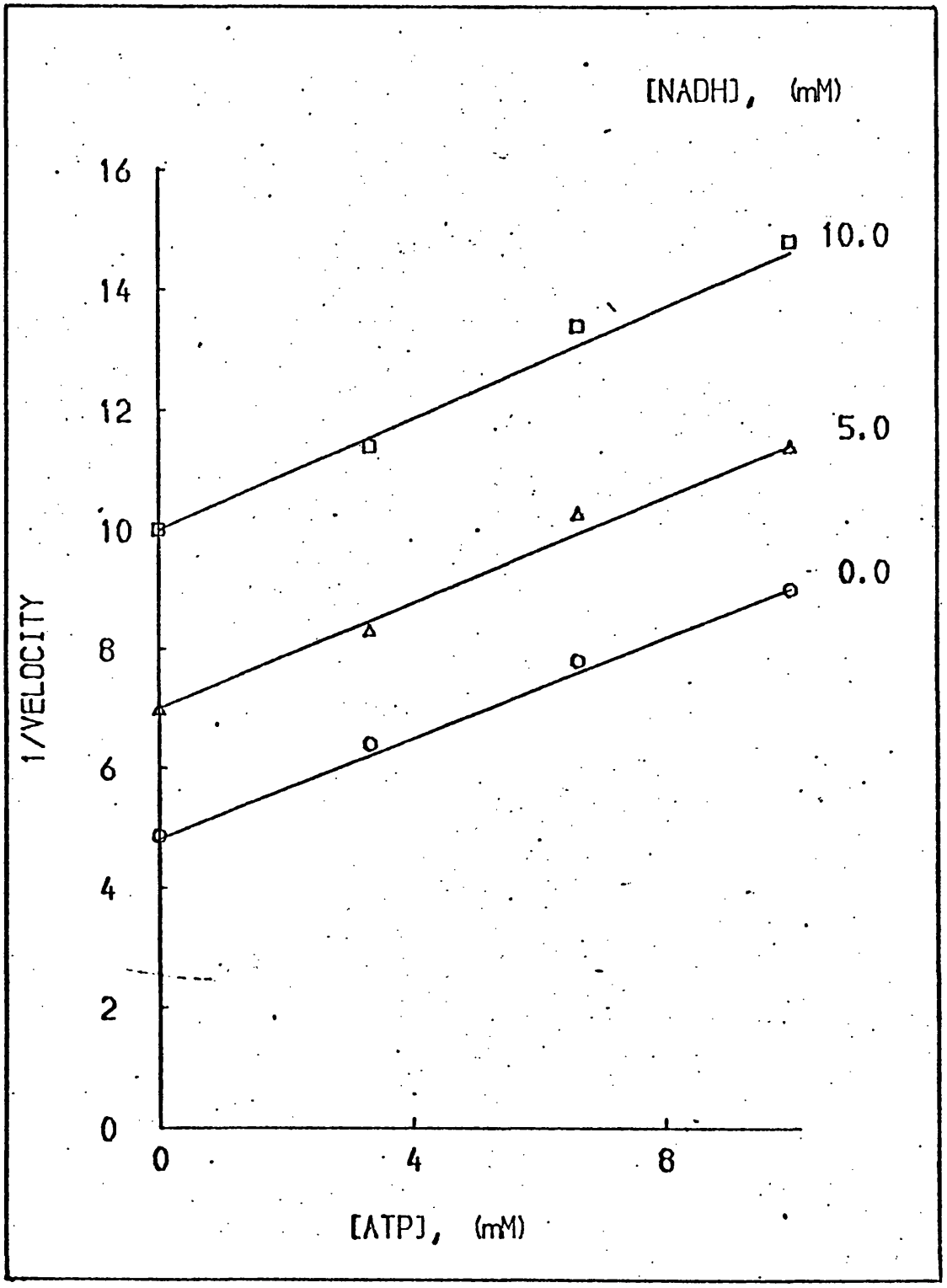


Fig. 36: Multiple inhibition analysis of Type E2 citrate synthase (from AB1623.R6) in the presence of NADH and ATP.

The enzyme activities (in arbitrary units) were determined using assay method 1.

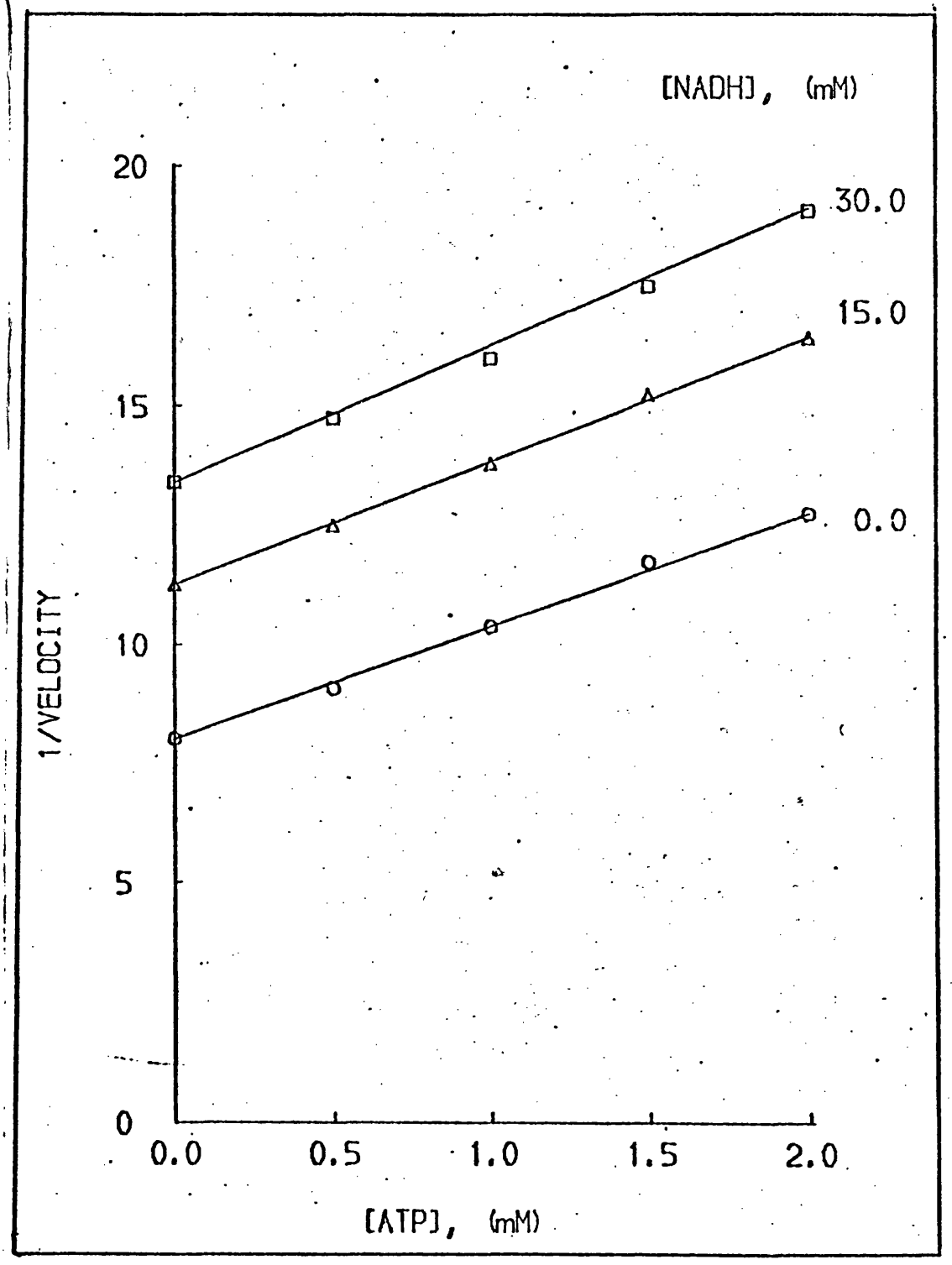


Fig. 37: Multiple inhibition analysis of Type E3 citrate synthase (from AB1623.R9) in the presence of NADH and ATP.

The enzyme activities (in arbitrary units) were determined using assay method 1.

vitro cell-free extracts.

In addition to these comparative studies, some further studies were made of the properties of the Type E3 enzyme. As described in the Methods Section, all *in vitro* studies were performed in 'Tris buffer'. However, it was unexpectedly found that if the revertants containing Type E3 enzyme were disrupted in this buffer additionally containing 1mM oxaloacetate, some differences in molecular properties were observed.

The enzyme extract, prepared in the presence of oxaloacetate, was mixed with the marker proteins catalase and lactate dehydrogenase in 10% sucrose. This mixture was applied to a column of Sephadex G-200 which had previously been equilibrated in 'Tris buffer' containing 1mM oxaloacetate. The resulting elution profile, Fig.38, showed that under these conditions the Type E3 enzyme had an elution profile similar to that of wild type *E. coli* citrate synthase; equivalent to a molecular weight of about 230,000. Therefore, the Type E3 enzyme had a molecular weight of about 230,000 in the presence of oxaloacetate and about 110,000 in the absence of this compound. It is noteworthy that although the molecular weight changes in the presence of oxaloacetate other properties examined remained relatively unaltered (Table 17). A hyperbolic substrate dependence was still observed for acetyl-CoA and the K_m value for this substrate was about 20μM. The K_i values for NADH, ATP and α-oxoglutarate were also unchanged being 12.3, 1.2 and 10.0mM respectively.

Similarly, it was found that when a cell-free extract containing Type E3 enzyme in 'Tris buffer' was incubated at 4° in the presence of 1mM oxaloacetate for 24h, this ligand-induced increase in molecular weight was again observed.

Further studies, substituting 1mM acetyl-CoA, NADH, ATP or α-oxoglutarate for oxaloacetate, did not result in an increase in the molecular weight of the Type E3 enzyme. Similarly, the presence of oxaloacetate or any of these

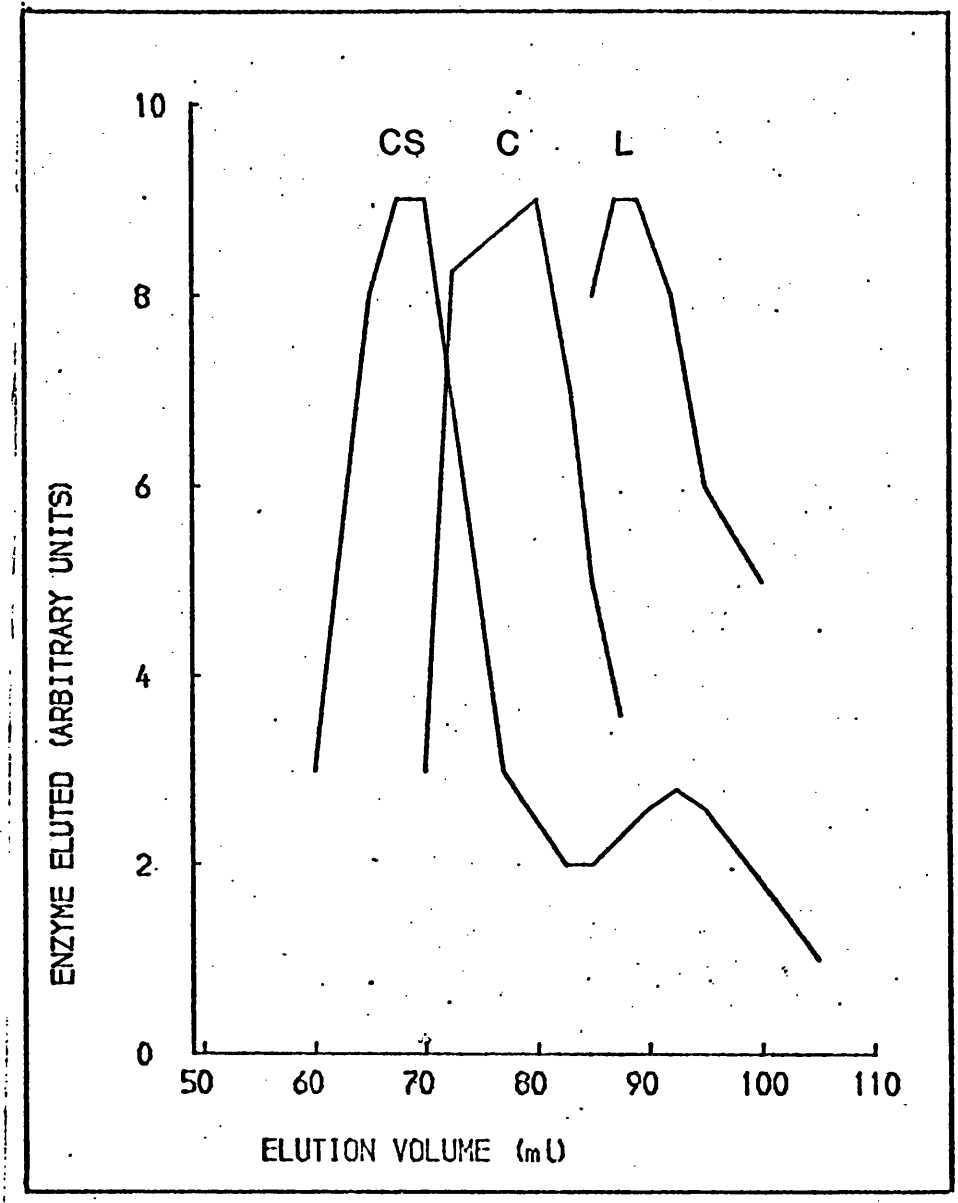


Fig. 38: Elution of Type E3 citrate synthase and marker enzymes on gel filtration in the presence of 1mM oxaloacetate.

A mixture (2ml) of citrate synthase (CS)(prepared in the presence of 1mM oxaloacetate), catalase(C) and lactate dehydrogenase (L) was run on a column of Sephadex G-200 (2.5cm x 35.cm) which had been previously equilibrated in 'Tris buffer' containing 1mM oxaloacetate. This same buffer was used to elute collecting 1ml fractions.

Table 17: Comparison of some of the properties of the Type E3 citrate synthase (from revertant AB1623.R9) in cell-free extracts prepared both in the presence and absence of oxaloacetate.

	Cell-free extract of Type E3 citrate synthase	
Property	+ Oxaloacetate	- Oxaloacetate
Molecular weight[a] (approx.)	230,000	110,000
K_m acetyl-CoA (μM)	20.1 ± 1.3	10.4 ± 0.6
K_i ATP (mM)	1.2 ± 0.1	0.61 ± 0.05
K_i NADH (mM)	12.3 ± 0.9	4.8 ± 0.4
K_i α-oxoglutarate	10.3 ± 1.1	9.3 ± 0.7

a: Determined using Stokes' radius (from gel filtration) and sedimentation coefficient (from active-enzyme centrifugation).

other ligands did not alter the molecular weight of the enzyme from B. megaterium or pig heart.

Thermal Inactivation Studies on E. coli Revertant Citrate Synthases

The thermal inactivation of Type E1, E2 and E3 enzymes was determined in the presence and absence of substrates and a number of inhibitors. The results were compared with those of the thermal inactivation of wild type E. coli (AB1621) citrate synthase.

It was found that the inactivation of the wild type enzyme was markedly reduced in the presence of 1mM substrates or 1mM NADH (Fig. 39). The specificity of the NADH protection was demonstrated by the fact that no similar effect was observed with NADPH, $NADP^+$ or NAD^+ (Fig. 40). Furthermore, it was shown that the specific NADH protection was abolished in the presence of 0.1M KCl (Fig. 40).

Similar results were obtained for the thermal inactivation of Type E1 enzyme; rates of inactivation are given in Table 18. Experiments with Type E2 citrate synthase and the Type E3 enzyme from revertant AB1623.R9, showed that the thermal inactivation of these was not markedly different in the presence of any of the compounds examined (Table 18).

However, it was observed that the rate of thermal inactivation of the Type E3 enzyme from revertant K1.1.4.R7 was greatly increased in the presence of 1mM NADH (Fig. 41). The specificity of this NADH effect was shown by the fact that the presence of NADPH, $NADP^+$ or NAD^+did not alter the rate of inactivation. Furthermore, it was found that the rapid inactivation of the enzyme in the presence of NADH was alleviated by 0.1M KCl (Fig. 42); the rates of inactivation are presented in Table 18.

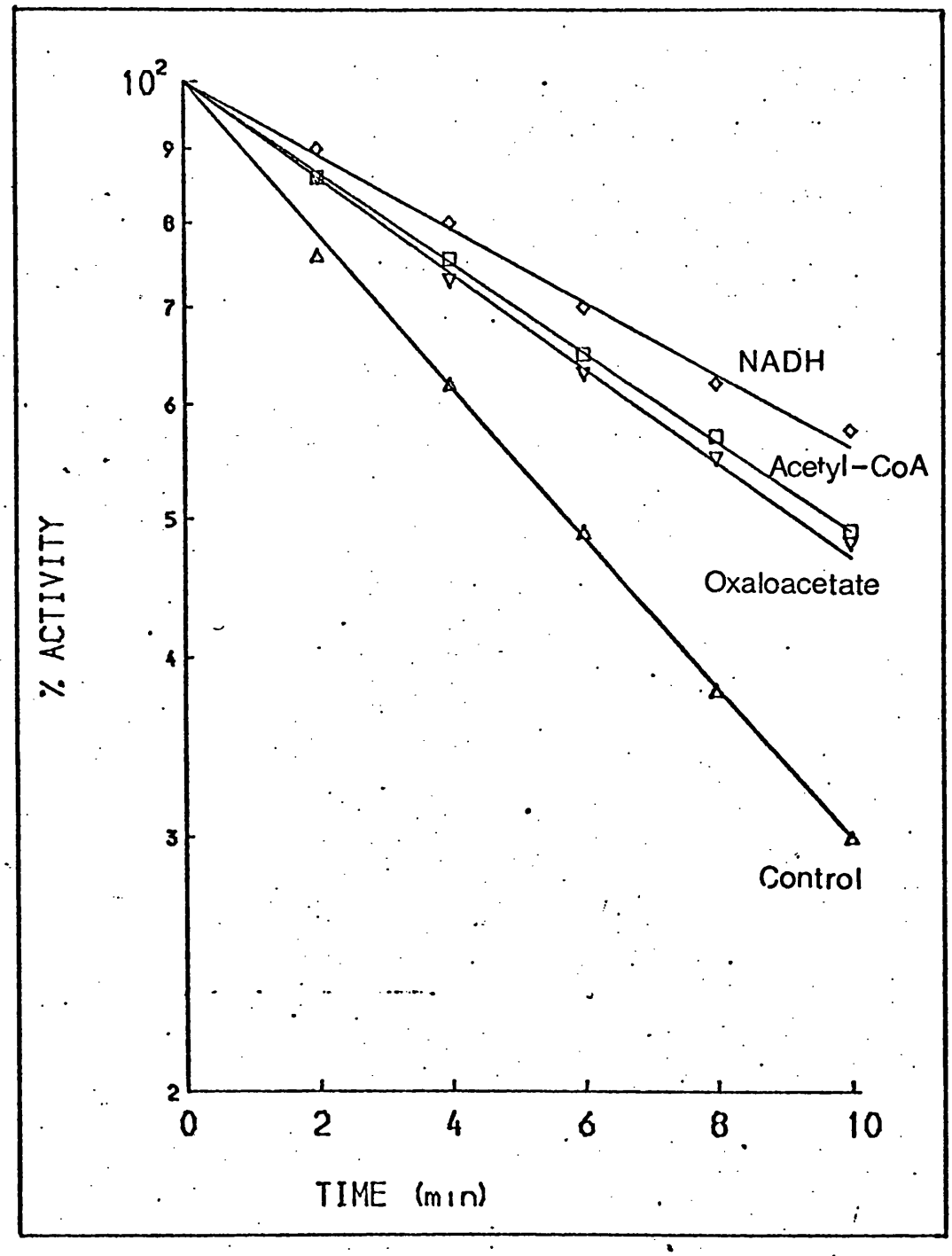


Fig. 39: Thermal inactivation of E. coli wild type citrate synthase in the presence and absence of a number of ligands.

Ligands were used at a concentration of 1mM.
The temperature of incubation was 50°.
Enzyme activities were measured using assay method 1.

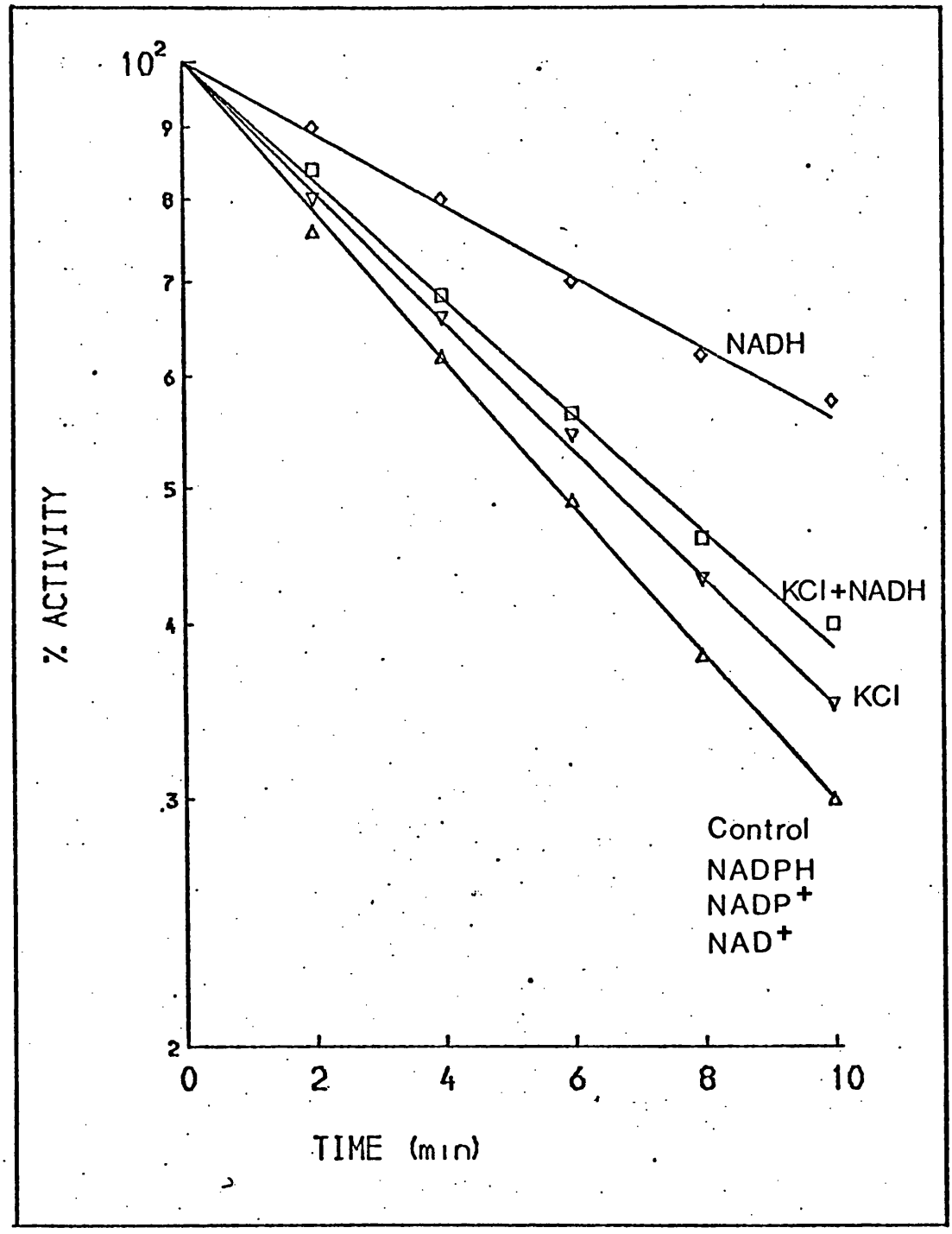


Fig. 40: Thermal inactivation of E. coli wild type citrate synthase in the presence and absence of a number of ligands.

KCl was used at a concentration of 0.1M.
Ligands were used at a concentration of 1mM.
The temperature of incubation was 50°.
Enzyme activities were measured using assay method 1.

Table 18: Rate constants for the thermal inactivation of *E. coli* wild type and revertant citrate synthases in the presence and absence of a number of ligands.

Enzyme source	Temperature (°C)	Rate constant (inactivation/min) in presence and absence of ligands								
		Control	Acetyl-CoA	Oxaloacetate	NADPH	$NADP^+$	NAD^+	NADH	KCl	NADH + KCl
E. coli wild type	50	0.073 ± 0.006	0.049 ± 0.006	0.053 ± 0.006	0.072 ± 0.008	0.074 ± 0.008	0.076 ± 0.006	0.043 ± 0.005	0.067 ± 0.004	0.065 ± 0.006
Revertant Type E1	50	0.079 ± 0.007	0.043 ± 0.007	0.051 ± 0.005	0.083 ± 0.008	0.077 ± 0.009	0.074 ± 0.006	0.031 ± 0.002	0.072 ± 0.006	0.077 ± 0.009
Revertant Type E2	40	0.052 ± 0.003	0.050 ± 0.002	0.046 ± 0.003	0.051 ± 0.004	0.053 ± 0.005	0.054 ± 0.005	0.050 ± 0.003	0.039 ± 0.002	0.037 ± 0.002
Revertant Type E3[a]	35	0.072 ± 0.006	0.068 ± 0.004	0.066 ± 0.002	0.074 ± 0.001	0.076 ± 0.004	0.069 ± 0.006	0.075 ± 0.010	0.059 ± 0.002	0.055 ± 0.006
Revertant Type E3[b]	35	0.073 ± 0.002	0.043 ± 0.004	0.047 ± 0.002	0.071 ± 0.009	0.076 ± 0.002	0.075 ± 0.003	0.193 ± 0.009	0.065 ± 0.004	0.063 ± 0.004

a: AB1623.R9.
b: K1.1.4.R7.

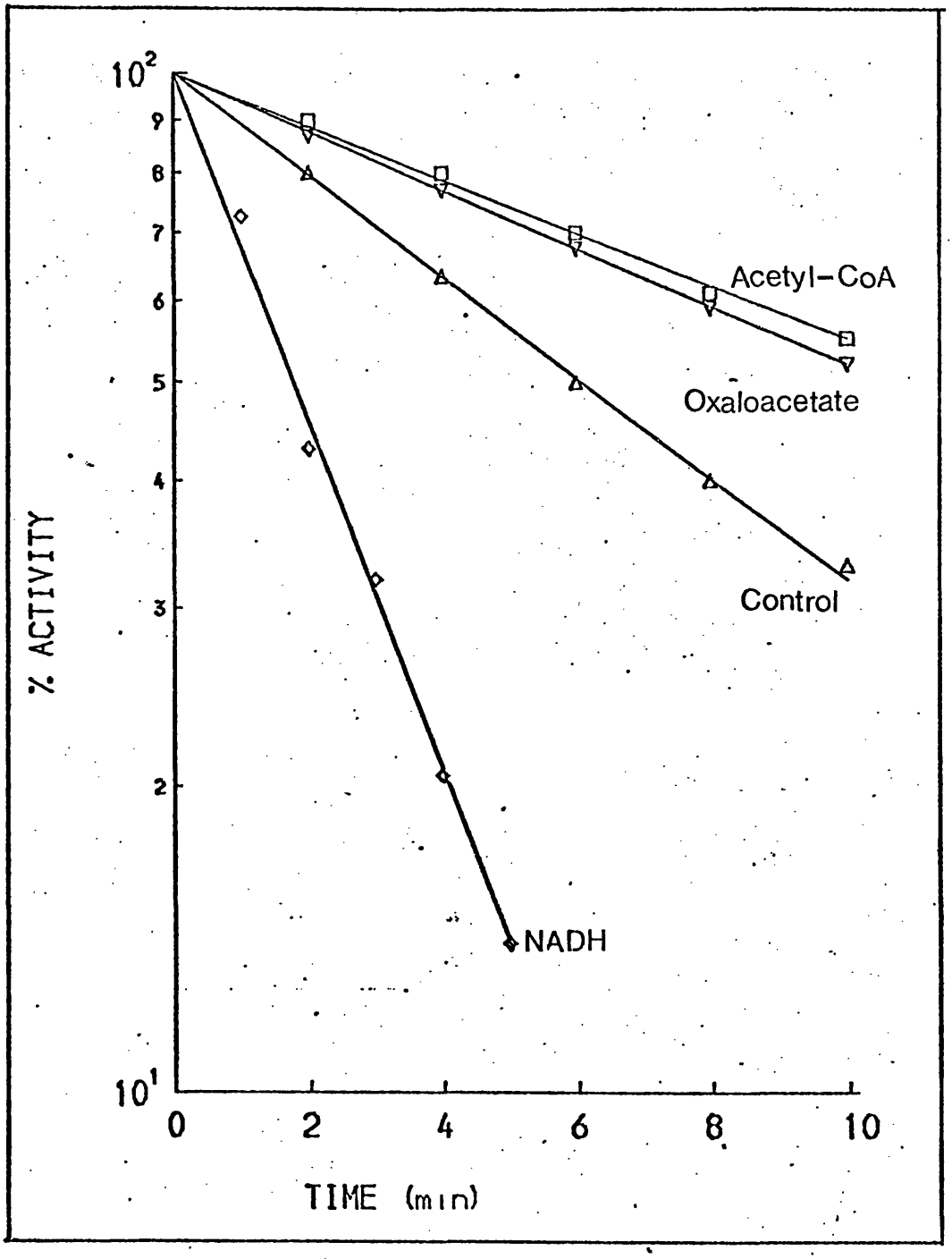


Fig. 41: Thermal inactivation of Type E3 citrate synthase (from K1.1.4.R7) in the presence and absence of ligands.

Ligands were used at a concentration of 1mM.
The temperature of incubation was 35°.
Enzyme activities were measured using assay method 1.

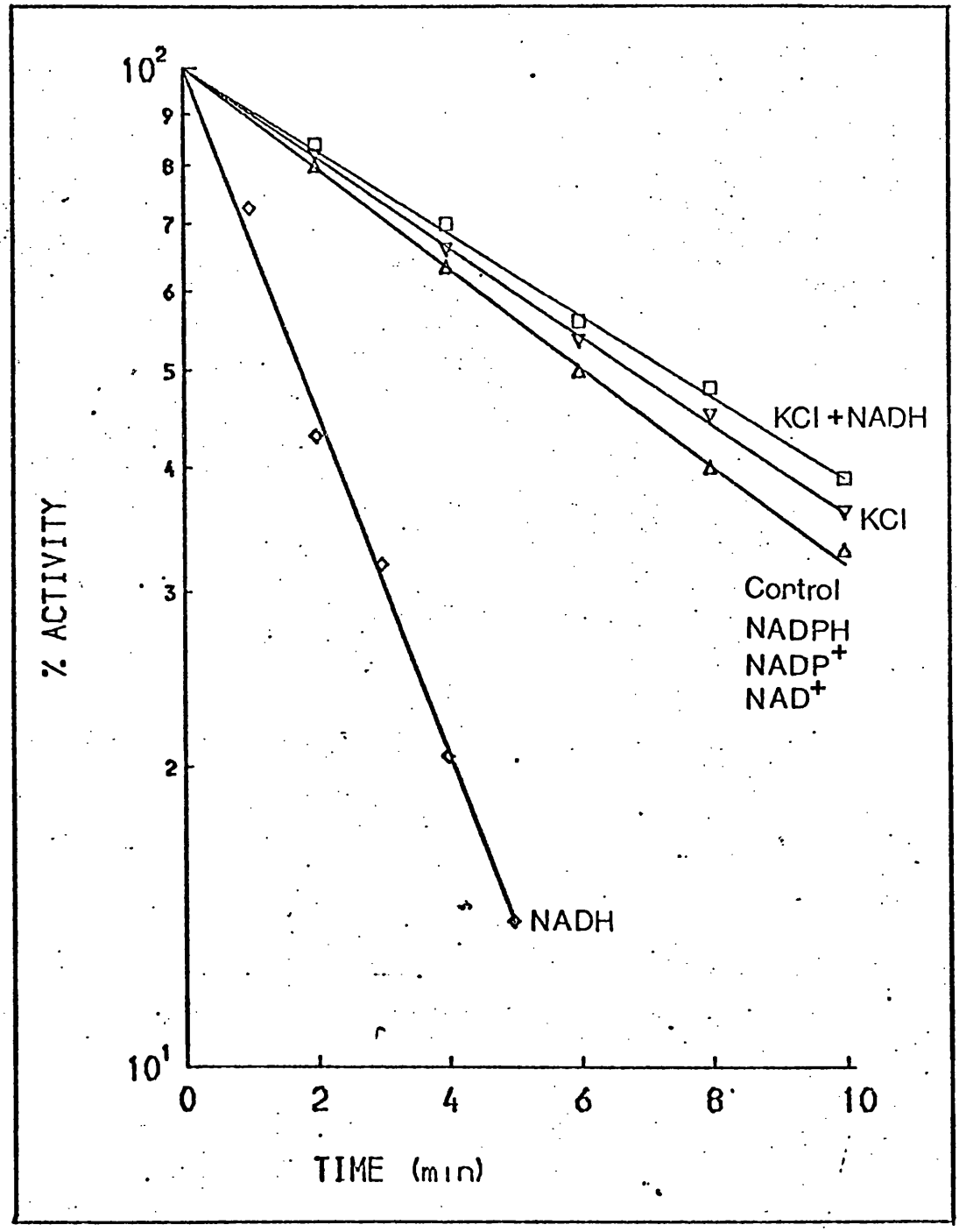


Fig. 42: Thermal inactivation of Type E3 citrate synthase (from K1.1.4.R7) in the presence and absence of ligands.

KCl was used at a concentration of 0.1M.
Ligands were used at a concentration of 1mM.
The temperature of incubation was 35°.
Enzyme activities were measured using assay method 1.

Section H - Comparative Physiology of the *E. coli* Revertants

Representative strains of each group of revertants were inoculated onto solid medium containing a number of different carbon sources. The growth, on each medium, was recorded after 36h and compared with that of the wild type organism (AB1621). It was shown (Table 19) that all revertants were able to grow on glucose, succinate or glycerol. However, only Type E1 and *E. coli* wild type were able to utilise acetate as sole carbon source for growth.

Similar experiments were carried out using liquid media and the same carbon sources as above, each at a concentration of 10mM. Growth of each culture was monitored by measuring the absorbance (at 550nm) of the suspensions at 30min intervals; the doubling time for each culture was calculated and is given in Table 20. Again it was found that Type E2 and E3 revertants grew very poorly on acetate as sole carbon source.

Detection of the Overproduction of Metabolites

Type E2 and E3 revertants have citrate synthases which are insensitive to inhibition by both NADH and α-oxoglutarate. Therefore, if the inhibitory effect of these compounds has physiological significance, such revertants might be expected, under certain suitable conditions, to overproduce these metabolites. It is difficult to devise a test to demonstrate the overproduction of NADH, but the accumulation and excretion of α-oxoglutarate might be more readily determined. An attempt was therefore made to demonstrate such an overproduction.

A suspension of the glutamate-requiring mutant of *E. coli*, AB1623, was grown overnight in nutrient broth. The cells were harvested and then resuspended in 0.9% (w/v) saline to a final concentration of 10^8 bacteria/ml. An aliquot (0.1ml) of this suspension was spread onto a glucose minimal medium plate. Patches of Type E1, E2 and E3 revertants and *E. coli* wild

Table 19: Comparison of growth of *E. coli* wild type and the revertants on a variety of carbon sources on solid medium.

	Carbon source[a]			
Strain	Glucose	Glycerol	Succinate	Acetate
E. coli wild type	+	+	+	+
Revertant Type E1	+	+	+	+
Revertant Type E2	+	+	+	-
Revertant Type E3[b]	+	+	+	-
Revertant Type E3[c]	+	+	+	-

a: Carbon source was added to give a final concentration of 10mM.

b: AB1623.R9.

c: K1.1.4.R7.

Table 20: Comparison of the doubling times of E. coli wild type and the revertants on a variety of carbon sources in liquid medium.

Strain	Doubling time (min) on carbon source[a]			
	Glucose	Glycerol	Succinate	Acetate
E. coli wild type	100	110	130	200
Revertant Type E1	100	100	140	220
Revertant Type E2	210	130	150	1420
Revertant Type E3[b]	320	120	150	2800
Revertant Type E3[c]	360	130	160	3200

a: Carbon source was at a concentration of 10mM.

b: AB1623.R9.

c: K1.1.4.R7.

type were inoculated onto this background lawn of bacteria. After overnight incubation, the patches of bacteria had grown but the lawn remained clear. However, after 3 days of incubation, haloes of growth appeared in the lawn immediately adjacent to the patches of Type E2 and E3 revertants, indicating that these revertants were overproducing a metabolite which cross-fed the glutamate auxotroph lawn. This excreted compound was probably either α-oxoglutarate, glutamate or proline. No cross-feeding was found to occur around the E. coli wild type or Type E1 revertant patches of growth (Fig. 43).

In an effort to identify the compound being overproduced, cultures of Type E1, E2 and E3 revertants and of E. coli wild type were grown on glucose minimal liquid medium (both aerobically and anaerobically) and the 'spent' media, after growth, were examined for the presence of overproduced metabolites. Neither glutamate nor α-oxoglutarate were detected by assay with glutamic dehydrogenase. Similarly, drops of the 'spent' media placed onto a lawn of the glutamate auxotroph, AB1623, did not satisfy the nutritional requirement of this organism. Therefore, it appears that none of these strains overproduces the metabolite in liquid culture.

The observation that growth of citrate synthase deficient strains of E. coli on glucose+glutamate results in the overproduction of pyruvate in the growth medium was described earlier (Fig. 21). Similarly, it was found that, when grown in liquid medium, Type E2 and E3 revertants also accumulated pyruvate. This accumulation did not occur in cultures of Type E1 revertant and E. coli wild type. This result suggests that the citrate synthase of Type E2 and E3 revertants is operating at a sub-optimal rate.

Glutamate Dependence of E. coli Revertants

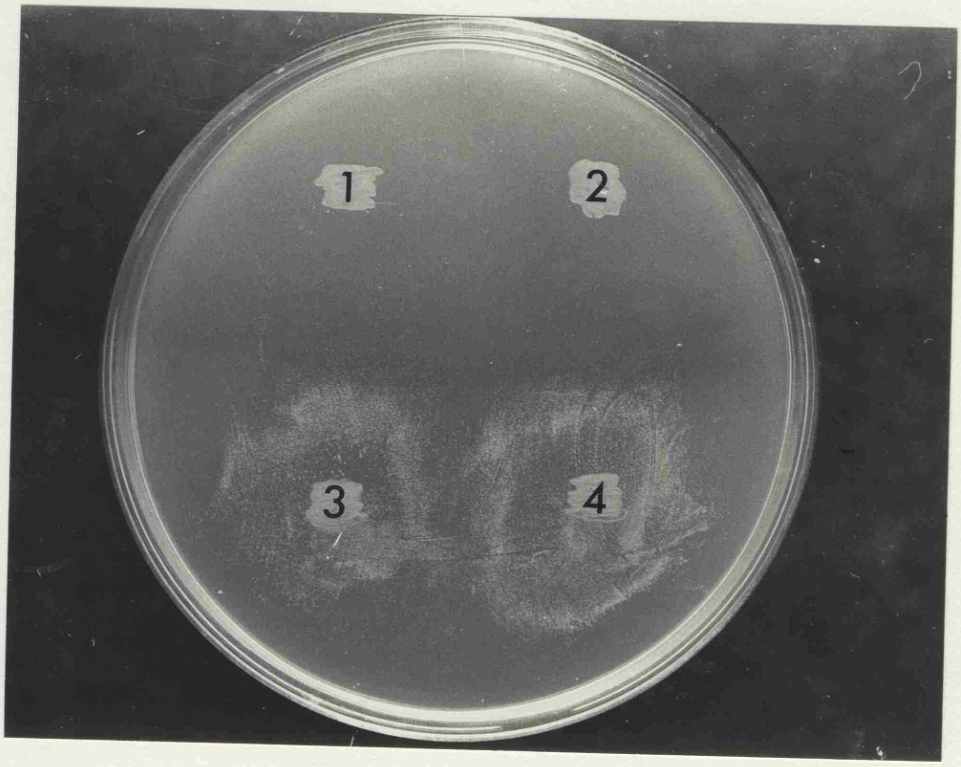
Type E1, E2 and E3 revertants, E. coli wild type and the citrate synthase deficient strain AB1623, were each grown overnight in glucose (10mM) liquid medium which was supplemented with glutamate (1mM). The

Fig. 43: Cross-feeding of a glutamate auxotroph by *E. coli* revertants.

A glutamate auxotroph of E. coli (AB1623) was spread onto the surface of a glucose minimal medium plate (10^7 bacteria/plate). The following strains were patched onto this lawn:

1. *E. coli* wild type.
2. Type E1 revertant.
3. Type E2 revertant.
4. Type E3 revertant.

Growth of the glutamate auxotroph can clearly be seen around Type E2 and E3 revertants (3days incubation at 37°).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cultures were harvested and then washed with 0.9% (w/v) saline. The cell suspensions were inoculated into flasks of glucose medium and growth was followed by measuring the absorbance (at 550nm) of each culture at 30min intervals. It can clearly be seen from Fig. 44 that Type E1 revertants grew at the same rate as the wild type organism, whereas Type E2 and E3 revertants grew at significantly slower rates and AB1623 did not grow at all. However, upon the addition of glutamate, to a final concentration of 1mM, all cultures grew at the same rate, i.e. that of the wild type organism. This result strongly suggests, that far from overproducing glutamate, Type E2 and E3 revertants are unable to synthesise sufficient of this compound to satisfy their own requirements.

Similar results were obtained for cultures grown anaerobically on glucose minimal medium. However, growth of all revertants proceeded at the wild type rate when grown in succinate or glycerol minimal media; the addition of glutamate had no effect on the growth of these cultures.

Determination of Enzyme Levels in the *E. coli* Strains

The overproduction of pyruvate and the under-production of glutamate by Type E2 and E3 revertants, when grown in glucose as sole carbon source, strongly suggests that the citrate synthase activity in these strains is too low to allow sufficient flux of carbon through the TCA cycle to satisfy the requirements for maximal growth rates. It was therefore pertinent to determine enzyme levels in the three revertant types, and to compare these with the levels in the wild type organism.

The three revertant types and *E. coli* wild type were grown in glucose and in succinate minimal media. Cell-free extracts were prepared by sonication and the specific activities of citrate synthase, aconitase, NADP-linked isocitrate dehydrogenase, α-oxoglutarate dehydrogenase, pyruvate dehydrogenase and malate dehydrogenase were determined for each extract. It

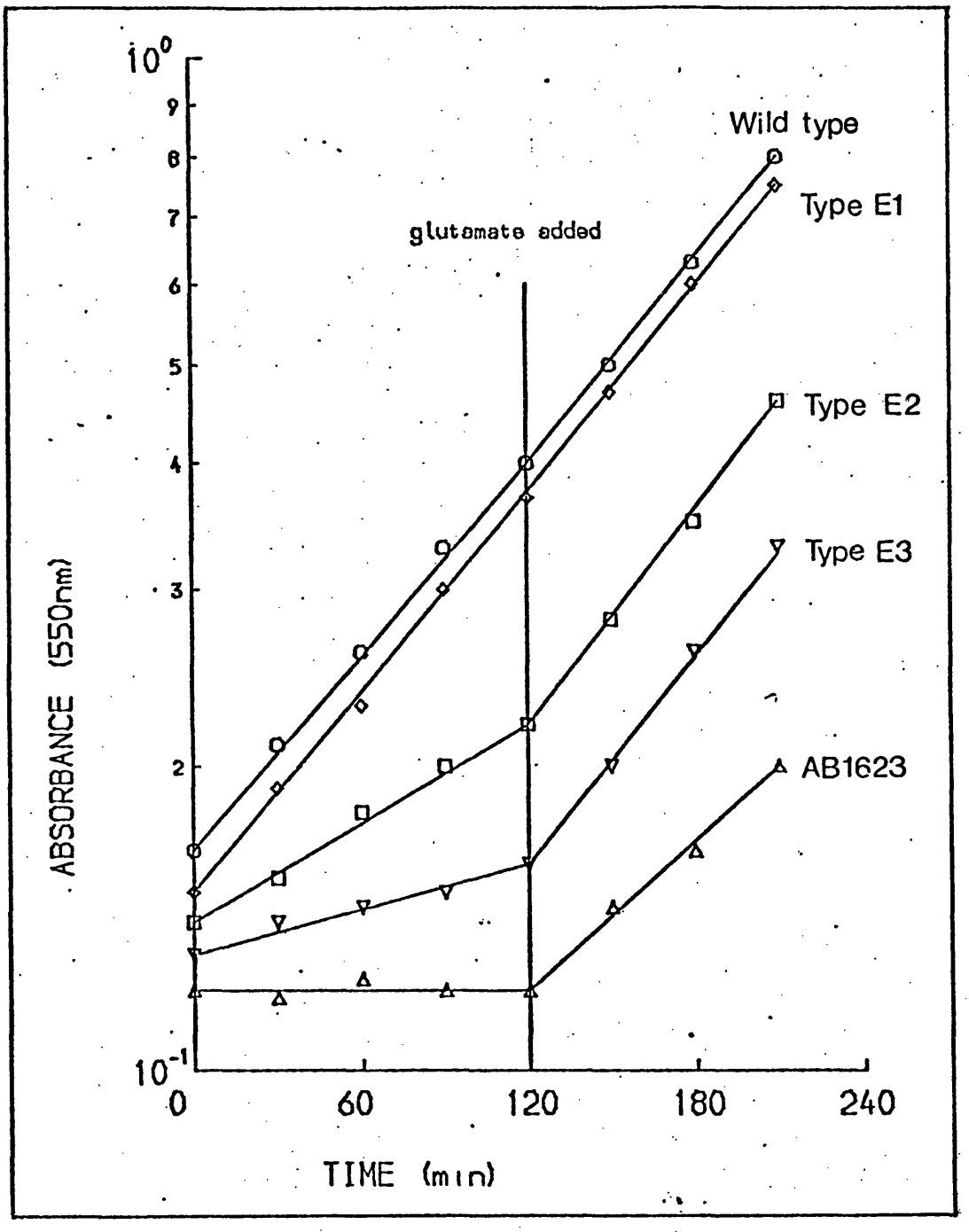


Fig. 44: Demonstration of the glutamate dependence of the Type E1 and E2 revertants, when grown on glucose medium.

Glucose was at a concentration of 10mM.

was found (Table 21) that the levels of all enzymes were far lower in the glucose-grown than the succinate-grown cells. It was also observed that the level of each enzyme, except citrate synthase, was very similar in the three revertants and the E. coli wild type. However, the specific activity of citrate synthase was notably very low in the Type E2 and E3 revertants (Table 21).

The extracts from the glucose-grown cultures were diluted to a concentration of 1mg protein /ml. The dependence of citrate synthase activity on acetyl-CoA concentration was determined for each extract using 0.01ml extract for each assay. In this way a comparative estimation of the citrate synthase activity was determined (Fig. 45). It was found that although the K_m values for acetyl-CoA for the enzymes produced by Type E2 and E3 revertants were much lower than that of the wild type enzyme, the V_{max} values were also strikingly reduced.

Table 21: Comparison of the specific activities of a number of enzymes in cell-free extracts of E. coli wild type and the three E. coli revertant types.

Strain	carbon source	Specific activity[a] citrate synthase	aconitase	isocitrate dehydrogenase	α-oxoglutarate dehydrogenase	malate dehyrogenase	pyruvate dehydrogenase
E. coli wild type	glucose	0.41	0.23	1.13	0.001	14.1	0.01
	succinate	1.07	0.47	1.86	0.06	15.2	0.04
Type E1 revertant	glucose	0.34	0.31	1.21	0.001	11.3	0.02
	succinate	0.91	0.33	2.02	0.05	16.2	0.05
Type E2 revertant	glucose	0.035	0.26	1.07	0.001	13.7	0.02
	succinate	0.098	0.37	1.73	0.04	15.2	0.03
Type E3 revertant[b]	glucose	0.014	0.27	1.16	0.002	13.3	0.01
	succinate	0.045	0.51	2.11	0.06	16.6	0.05

a: expressed in enzyme units/mg.protein.

b: AB1623,R9.

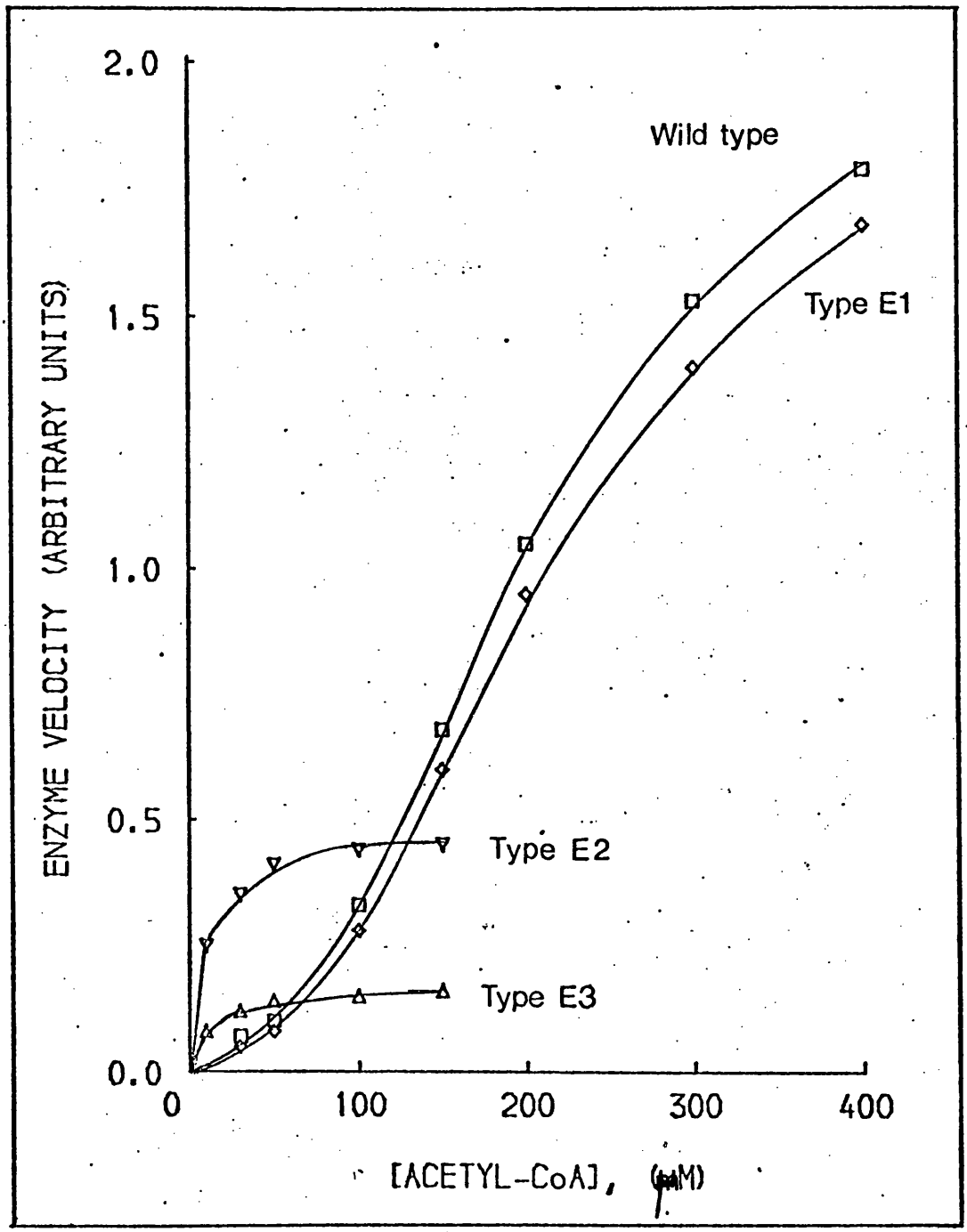


Fig. 45: Comparison of the rate dependences of E. coli wild type and revertant citrate synthases on acetyl-CoA concentration.

Cell-free extracts of each strain were diluted to 1mg protein/ml and 0.02ml of this extract was assayed for citrate synthase activity using assay method 1. In this way a comparative determination was made of the citrate synthase activity in each extract.

Section I - Properties of A. lwoffi Revertant Enzymes

The isolation of A. lwoffi revertants from the citrate synthase deficient strains 4B-CS1 and 4B-CS2, has been previously described. The rapid techniques were used to determine the regulatory properties and molecular size of the citrate synthase from a number of these revertants. The results are presented in Table 22, and they show that the revertant enzymes fall broadly into two types.

A comparative study has been made of the molecular properties of the citrate synthases from a number of these revertants and A. lwoffi wild type enzyme. The properties investigated were molecular weight, K_m values for both substrates, NADH inhibition(and the effect of AMP on this inhibition) and also the effect on enzyme activity of incubating the enzyme with the thiol blocking reagent DTNB.

Type A1 Enzymes

The molecular weight of Type A1 enzymes was determined by gel filtration on Sephadex G-200 in the presence of marker proteins of known molecular weight. Fig. 46 shows a typical elution profile for a Type A1 enzyme; this enzyme has a molecular weight of about 250,000 (Fig. 47), similar to that of A. lwoffi wild type citrate synthase (Table 23).

The substrate dependences of the Type A1 enzyme were found to be hyperbolic with a K_m value of about 75μM for acetyl-CoA and 3μM for oxaloacetate (Table 23). These results are very similar to those obtained with the A. lwoffi citrate synthase.

Under the routine assay conditions for citrate synthase, it was found that A. lwoffi wild type enzyme was about 90% inhibited by the inclusion of 0.5mM NADH. This inhibition was completely overcome by 1mM AMP. It can be seen from Table 23 that Type A1 enzyme has very similar regulatory properties.

Finally, incubation of A. lwoffi wild type citrate synthase with

Table 22: The properties of the citrate synthases from the revertant strains of the A. lwoffi citrate synthase deficient mutants as determined using the rapid techniques.

Parent strain	Revertant strain	Inhibition		Molecular size	Type
		NADH	NADH + AMP		
4B (wild type)	-	+	-	large	-
4B-CS1	4B-CS1.R1-R25	+	-	large	A1
	4B-CS1.R26-R30	-	-	small	A2
4B-CS2	4B-CS2.R1-R36	+	-	large	A1
	4B-CS2.R37-R43	-	-	small	A2

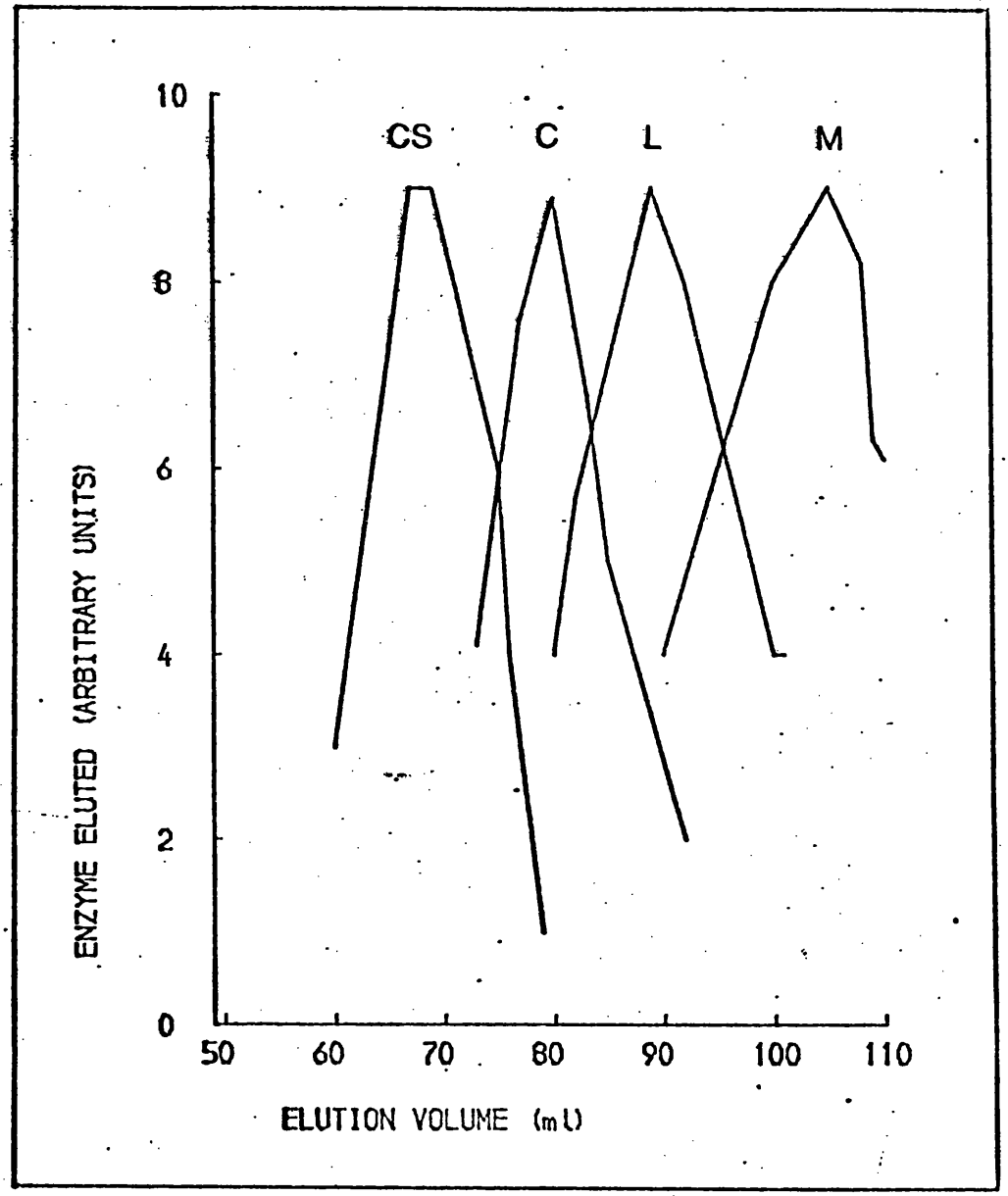


Fig. 46: Elution of a 'large' citrate synthase (from a Type A1 revertant) and marker enzymes on gel filtration.

A mixture (2ml) of citrate synthase (CS), catalase (C), lactate dehydrogenase (L) and malate dehydrogenase (M) was run on a column of Sephadex G-200 (2.5cm x 35.cm), using 'Tris buffer' to elute and collecting 1ml fractions.

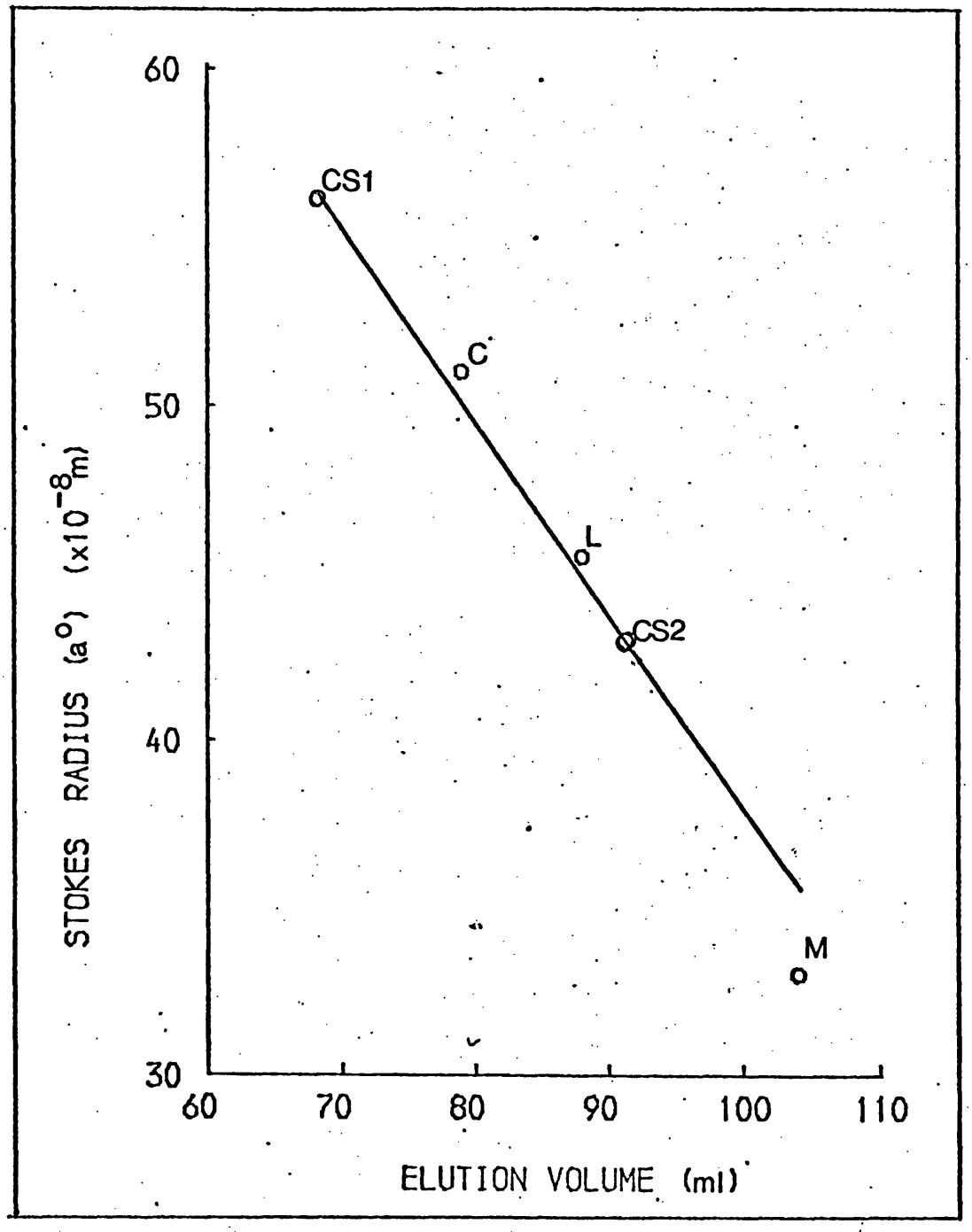


Fig. 47: Relationship between the Stokes' radii of proteins and their comparative elution volumes from a Sephadex G-200 gel filtration column.

The proteins used were 'large' citrate synthase (CS1) from a Type A1 revertant, catalase (C), lactate dehydrogenase (L), 'small' citrate synthase (CS2) from a Type A2 revertant and malate dehydrogenase (M).

The 'large' and 'small' A. lwoffi citrate synthases are slightly larger than their E. coli counterparts. Therefore, the 'large' A. lwoffi enzyme has a molecular weight of about 250,000 and the 'small' of about 110,000-120,000.

Table 23: A comparison of some of the catalytic, regulatory and molecular properties of citrate synthases from A. lwoffi wild type and A. lwoffi revertants.

	Source of citrate synthase		
		A. lwoffi revertants	
Enzyme property	A. lwoffi wild type	Type A1	Type A2
Molecular weight (approx.)	250,000	250,000	110,000
Substrate dependences	Hyperbolic	Hyperbolic	Hyperbolic
K_m acetyl-CoA (μM)	75.6 ± 6.9	73.4 ± 5.8	69.3 ± 4.7
K_m oxaloacetate (μM)	3.7 ± 0.2	3.1 ± 0.2	5.2 ± 0.3
Inhibition produced by 0.5mM NADH	90%	90%	2%
Inhibition produced by 0.5mM NADH + 1mM AMP	3%	5%	5%

0.1mM DTNB at 25° had no effect on enzyme activity over a period of 10min (Fig. 48). However, Type A1 enzymes could be further subdivided into two groups; those which were similarly unaffected by DTNB (Type A1-1) and those which were rapidly inactivated by this compound (Type A1-2) as can be seen from Fig. 48.

Type A2 Enzyme

The elution profile of this enzyme on Sephadex G-200 (Fig. 49) showed that it had a molecular weight of about 110,000; this is approximately half that of the enzyme from A. lwoffi wild type.

Substrate dependences were found to be hyperbolic; the K_m value was about 70μM for acetyl-CoA and about 5μM for oxaloacetate (Table 23). These values are very similar to those of A. lwoffi wild type citrate synthase.

It was found that Type A2 enzymes were only weakly inhibited by NADH, and that AMP had no effect on this inhibition (Table 23). This result strongly suggests that these enzymes have lost their allosteric site for NADH inhibition.

Finally, as with the Type A1 enzymes, the Type A2 citrate synthases could be subdivided into two groups according to their sensitivity to DTNB. One group (Type A2-1) was unaffected by incubation with 0.1mM DTNB for 10min at 25°, whereas the second group (Type A2-2) was rapidly inactivated by this treatment (Fig. 48).

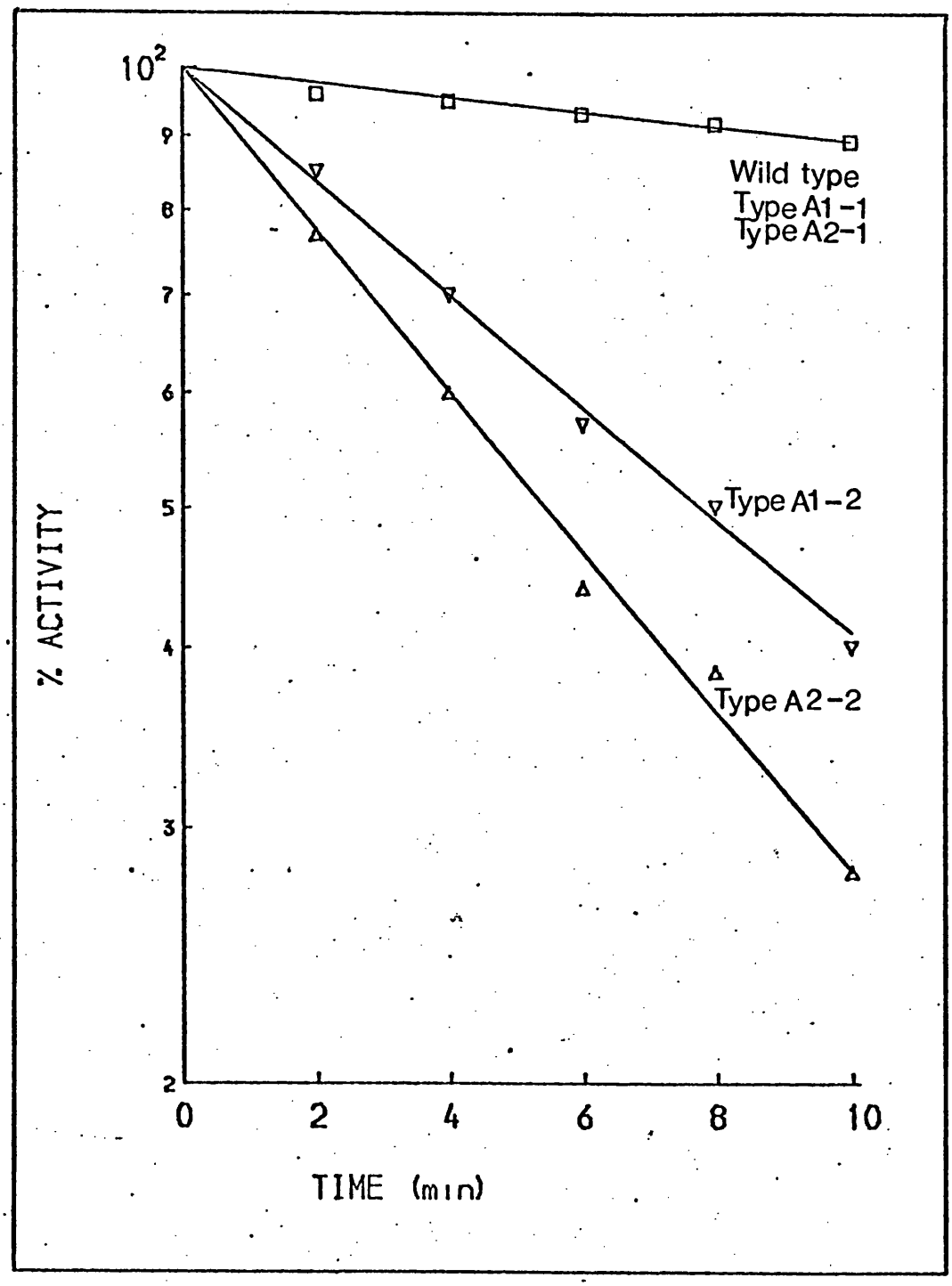


Fig. 48: Inactivation of A. lwoffi wild type and revertant citrate synthases by incubation with DTNB.

Time course of the incubation of these enzymes with 0.1mM DTNB. Activity was measured using assay method 1.

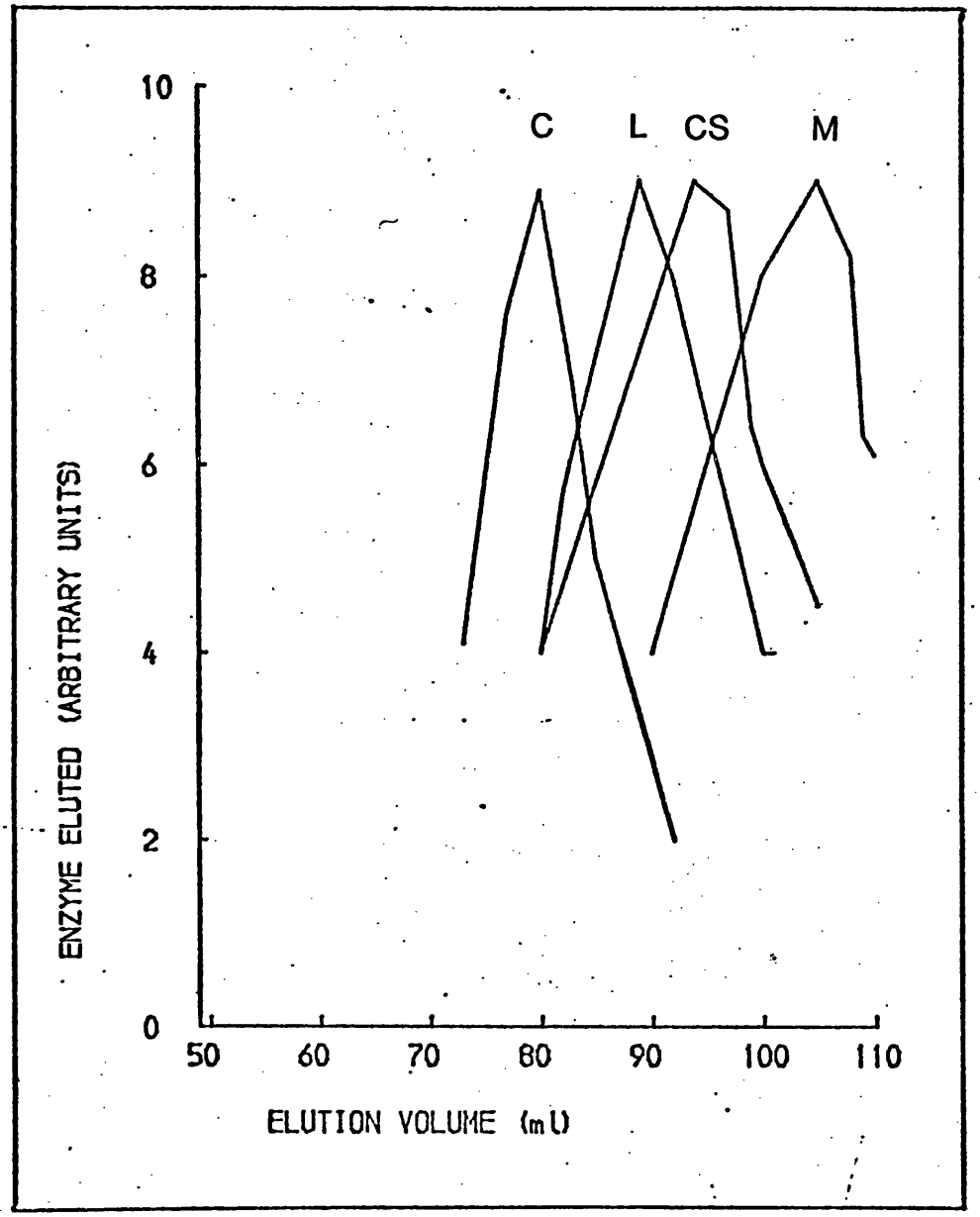


Fig. 49: Elution of a 'small' citrate synthase (from a Type A2 revertant) and marker enzymes on gel filtration.

A mixture (2ml) of citrate synthase (CS), catalase(C), lactate dehydrogenase (L) and malate dehydrogenase (M) was run on a column of Sephadex G-200 (2.5cm x 35.cm), using 'Tris buffer' to elute and collecting 1ml fractions.

Section J - Transformation Studies with *A. lwoffi* 4B-CS1 and 4B-CS2

A DNA preparation of *A. lwoffi* wild type was made as described in the Methods Section. The citrate synthase deficient strains, 4B-CS1 and 4B-CS2, were treated with this DNA preparation and then plated onto succinate minimal medium. Such a procedure was found to enhance markedly the reversion rate of these strains. These results clearly demonstrate that the mutation in the citrate synthase gene of 4B-CS1 and 4B-CS2 was readily repaired by the introduction of transforming DNA.

Similar experiments were carried out in which the transforming DNA preparation was from *Ps. aeruginosa*, *B. megaterium* and *E. coli*. Treatment of the citrate synthase deficient strain, 4B-CS2, with the DNA from *Ps. aeruginosa* produced a great increase in the frequency of its reversion; no such effect was observed for the 4B-CS1 strain when treated with the DNA from *Ps. aeruginosa*. The presence of the DNA from *E. coli* or *B. megaterium* did not increase the reversion rate of either 4B-CS1 or 4B-CS2.

Section K - Immunological Studies with *A. lwoffi* Citrate Synthases

Antiserum containing antibodies raised against purified *A. lwoffi* wild type citrate synthase was used in double diffusion tests. The procedure has been given in the Methods Section, and Fig. 50 shows a typical immunoprecipitation pattern obtained using this procedure. It can be seen that a heavy precipitin line developed between the antiserum and a cell-free extract of *A. lwoffi* wild type. Similar precipitation lines were obtained for the extracts of 4B-CS1 and 4B-CS2, showing that although there was no detectable enzyme activity, the inactive protein is probably present in these strains.

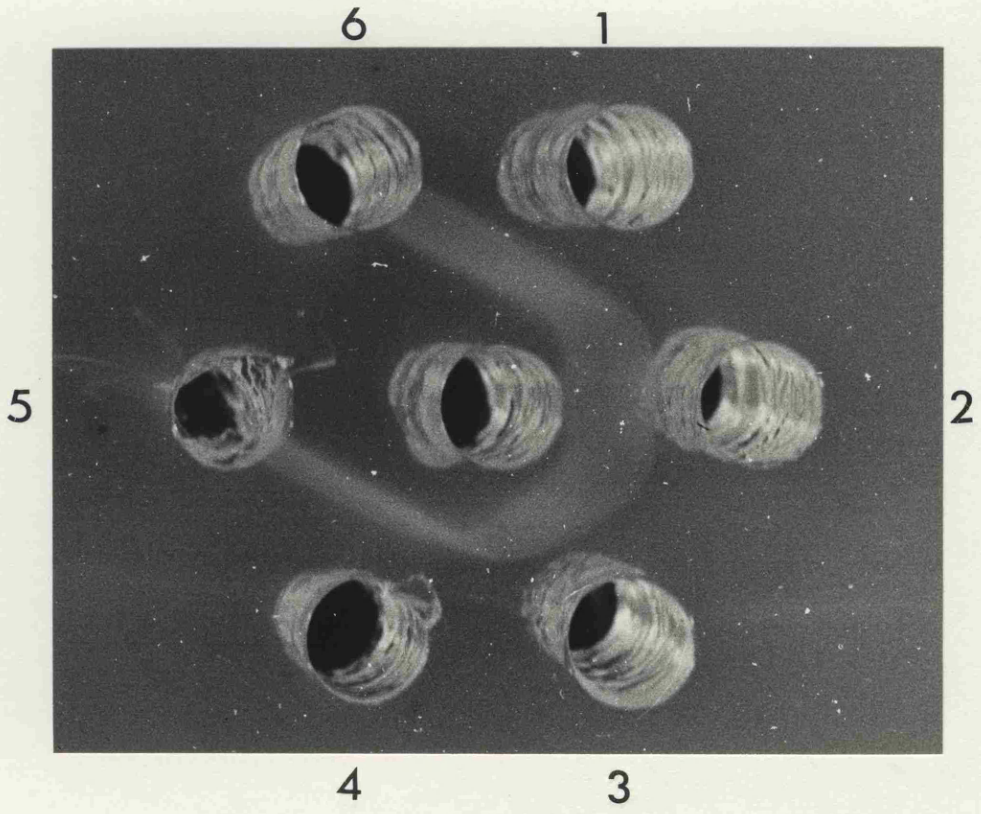
Similar experiments showed that extracts containing Type A1-1, A1-2, A2-1 and A2-2 enzymes also possessed cross-reacting material. Moreover, it was shown that the transformants of 4B-CS1 and 4B-CS2 which had regained citrate synthase activity, after transormation with DNA from *A. lwoffi*, also possessed cross-reacting material.

It was shown that cell-free extracts of *Ps. aeruginosa*, *B. megaterium* and *E. coli* did not contain cross-reacting material; furthermore the transformants of 4B-CS2 which had regained citrate synthase activity after treatment with *Ps. aeruginosa* DNA did not possess any cross-reacting protein.

Fig. 50: Immunodiffusion pattern of antiserum (raised against purified citrate synthase from A. lwoffi) and various cell-free extracts of A. lwoffi strains.

Antiserum was placed in the centre well. The contents of the outer wells were as follows;

1. Cell-free extract of A. lwoffi wild type.
2. Cell-free extract of 4B-CS1.
3. Cell-free extract of 4B-CS2.
4. Cell-free extract of Type A1 revertant.
5. Cell-free extract of Ps. aeruginosa.
6. Cell-free extract of a strain of 4B-CS2 after transformation with Ps. aeruginosa DNA.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DISCUSSION

There is a striking correlation between the Gram reaction of a bacterium and the molecular and regulatory properties of its citrate synthase (Weitzman & Jones, 1968; Weitzman & Dunmore, 1969a; Weitzman & Danson, 1976). Gram positive bacterial citrate synthases, like those from eucaryotic sources, are inhibited by ATP, are unaffected by NADH, have a molecular weight of about 100,000 and are termed 'small' enzymes. On the other hand, Gram negative bacterial citrate synthases are insensitive to ATP, are powerfully inhibited by NADH, have a molecular weight of about 250,000 and are termed 'large' enzymes.

It is difficult to explain this correlation between enzyme properties and the Gram reaction but, with only a few exceptions (Weitzman & Danson, 1976), all bacterial citrate synthases examined follow the pattern outlined above. The division of bacteria into Gram positive and Gram negative types has a significance far beyond that of an empirical staining procedure. Gram negative bacteria are usually very adaptable organisms with highly developed synthetic capabilities enabling them to grow on simple media containing only a carbon source such as glucose, an inorganic nitrogen source and traces of necessary mineral salts. They do not concentrate nutrients and metabolites and consequently have a low cytoplasmic osmolarity. By contrast, Gram positive bacteria have more exacting nutritional requirements. These organisms usually need to supplement their less developed synthetic capabilities with an exogenous supply of amino acids, vitamins and growth factors; consequently these organisms are grown on rich, undefined media in order to meet their requirements. The cytoplasm has a relatively high osmolarity, as these organisms concentrate low molecular weight metabolites such as amino acids and nucleotides. Furthermore, these general biochemical divisions are accompanied by large differences in cell wall architecture. The Gram positive bacteria have a

thick cell wall, of a comparatively simple structure, consisting of a bi-layer of teichoic acid and murein. The cell wall of Gram negative bacteria, although less thick, is very much more complex, having a multilayered architecture consisting of a wide range of different components.

This well-tried division of bacteria on the basis of their Gram reaction is of profound diagnostic and taxonomic importance. However, some groups of bacteria, particularly the sporeformers and those organisms known collectively as the coryneform bacteria, give variable Gram stain according to the age and condition of culture. As a result of this uncertainty in Gram reaction, the classification of many of these bacteria has proved both difficult and controversial. Weitzman and Jones (1975) have proposed that an examination of the molecular size and regulatory properties of citrate synthase may be used as a taxonomic tool in the classification of such bacteria. These workers have studied the properties of the citrate synthase from a number of bacteria of disputed taxonomic position (Jones & Weitzman, 1974; Weitzman & Jones, 1975) and, in each case, their results agree with the current classification of these organisms as assessed by other criteria, such as cell wall structure. Therefore, the value of this test as a taxonomic aid has been fully justified.

Many other tests used by bacterial taxonomists have a similar biochemical basis. These usually involve testing for the presence or absence of a given enzyme. For example, the absence of the enzyme catalase is a characteristic of most strict anaerobes and is also used as a diagnostic feature of the lactic acid bacteria. Similarly, the oxidase test indicates the presence of cytochrome c; the enteric group of bacteria are characteristically oxidase negative. These and many other tests are used routinely in diagnostic bacteriology and in taxonomic studies; the examination of the properties of citrate synthase could be added to this number.

The method employed by Weitzman and Jones (1975) consisted of growing a large volume of bacteria, preparing a cell-free extract, fractionating with ammonium sulphate and carrying out gel filtration on a large column of Sephadex G-200. This pufication successfully removes malate dehydrogenase; the presence of this enzyme would interfere with the examination of the inhibition of citrate synthase by NADH. The molecular size of the citrate synthase is determined from its elution from the column. However, this method is time-consuming and involves the use of equipment which may not always be available in bacteriology laboratories, thereby restricting the widespread use of this technique as a taxonomic tool.

During the course of the present work rapid techniques have been developed to determine the molecular size and regulatory properties of citrate synthase. We have proposed (Harford, Jones & Weitzman, 1976) that these techniques permit the examination of large numbers of bacterial citrate synthases in a short time, using inexpensive routine laboratory equipment.

The molecular size has been determined by using a small column of Sephadex G-200, greatly reducing the necessary loading volume of extract and the time taken to elute the enzyme. The resolution of the column has been shown to be quite acceptable, and as fractions are collected for a period of only 15 min the use of a fraction collector is no longer essential.

The regulatory properties of the enzyme were examined by observing the effectiveness of ATP and NADH as inhibitors of the enzyme within toluenised patches of bacteria on filter papers. It was found that malate dehydrogenase eluted from toluenised cells by washing in a large volume of buffer (P.D.J. Weitzman, personal communication). The method therefore

avoids the need for growing large volumes of bacteria and for undertaking any enzyme purification, thereby greatly reducing the time required to study the regulatory properties of the enzyme. Furthermore, I would propose that, whenever a suitable colorimetric assay procedure is available, this method could be adapted for other enzyme systems when it is necessary to scan large numbers of bacteria, particularly mutant strains, for the presence or absence of an enzyme or of enzyme inhibition by a given effector.

The reliability of the rapid methods was tested by comparing their results with those obtained by the previous method of Weitzman and Jones (1975). Out of a total of 36 bacteria tested, a selection of which has been presented, the results were in complete agreement in 35 cases. These techniques enable the molecular and regulatory properties of citrate synthase to be determined accurately and rapidly, therefore permitting examination of these properties to be used as a routine taxonomic tool.

The one bacterium which could not be studied using these techniques was Br. lineâs. This organism contained a bright yellow pigment which obscured the examination of the colour development and the method may therefore be inapplicable with heavily pigmented bacteria. If necessary, this problem of pigmentation may be overcome by growing the bacterium under conditions which do not favour pigment production or it may be possible to extract the compound from toluenised cells, using a solvent which does not severely inactivate citrate synthase.

Results have been presented which confirm the findings of other invetigators, that the nucleotide inhibition of Gram positive bacterial and eucaryotic citrate synthases is a general non-specific inhibition; in all cases studied (pig heart, S. cerevisiae and B. megaterium) the order of effectiveness of the nucleotide inhibitors is ATP $>$ ADP $>$ AMP $>$ NADPH $>$

NADH > $NADP^+$.

It has been demonstrated that although very much higher concentrations of inhibitors are required, this general non-specific nucleotide inhibition is also found for the enzyme from Gram negative bacteria (A. lwoffi and Ps. aeruginosa). The same order of effectiveness is observed with the exception that NADH acts as an extremely powerful inhibitor of these enzymes. This NADH inhibition is specific; NADPH, NAD^+ or $NADP^+$ do not produce this inhibitory effect.

Weitzman and Danson (1976) have proposed that the non-specific nucleotide inhibition found for all citrate synthases, like the general non-specific di- and tri-carboxylic acid inhibition of fumarase (Massey, 1953), is an isosteric phenomenon resulting from the common structural features shared by the nucleotides and the substrate acetyl-CoA. They showed that there is a relationship between the sensitivity to ATP inhibition and the Km value for acetyl-CoA. The specific NADH inhibition exhibited by all Gram negative bacterial citrate synthases, however, is probably an allosteric mechanism. Considerable evidence has been reported here in support of this hypothesis.

It has been shown that, in all cases studied, the non-specific nucleotide inhibitors compete with the substrate acetyl-CoA. Furthermore, a direct relationship has been demonstrated between the Km value for acetyl-CoA and the Ki values for the various nucleotide inhibitors. It is noteworthy that the specific NADH inhibition of Gram negative bacterial citrate synthases is not always competitive with this substrate (Fig. 12); a finding consistent with the view that this latter inhibition is allosteric.

The relationship between the Km value for acetyl-CoA and the Ki values for the non-specific nucleotide inhibitors, prompted a search of

the literature for values of the Ki for ATP and the Km values for acetyl-CoA for a large number of citrate synthases; these are given in Table 24. Plotting of these values (Fig. 51) shows that there is a striking correlation between the two parameters.

Good correlation between the Km value for acetyl-CoA and the Ki value for NADH was also noted for the eucaryotic and Gram positive bacterial citrate synthases. However, the relationship was not observed for the Gram negative bacterial enzymes indicating that NADH had a different mode of action in these cases.

A variety of treatments of the Gram negative bacterial enzymes greatly decreases the effectiveness of NADH as an inhibitor, whereas the non-specific nucleotide inhibition of ATP remains unaffected. Treatment of Ps. aeruginosa citrate synthase with the thiol-blocking reagent DTNB, abolishes the inhibitory effect of NADH (Weitzman & Hewson, in preparation). The presence of 0.2M KCl has the same effect on the NADH inhibition of the E. coli citrate synthase (Weitzman, 1966a). In this work these findings have been confirmed, and mutants of E. coli and A. lwoffi have been isolated which produce a citrate synthase which has lost sensitivity to NADH while retaining the general non-specific nucleotide inhibition. These findings are consistent with the view that the specific NADH inhibition of the Gram negative bacterial enzyme is allosteric, whereas other nucleotide inhibitors act isosterically.

The final evidence for this hypothesis relies on the method of multiple-inhibition analysis (Yonetani & Theorell, 1964). The interpretation of the results obtained from this kinetic study are as follows. Parallel line plots indicate that the binding of one inhibitor totally excludes the binding of a second. Although it is possible that a large molecule, binding at one site, may sterically prevent the binding of a second

Table 24: Comparison of the K_m-value for acetyl-CoA and the K_i-value for ATP for a number of citrate synthases.

Enzyme source	K_m-acetyl-CoA	K_i-ATP	Reference
S. cerevisiae	2.0	0.1	Hathaway and Atkinson (1965)
Beef heart	2.7	0.2	Jangaard *et al.* (1968)
Rat liver	2.8	0.7	Moriyama and Srere (1971)
S. cerevisiae	3.2	0.17	This work
Maize scutellum mitochondria	4.	0.2	Barbareschi *et al.* (1974)
Rat heart	4.6	0.7	Moriyama and Srere (1968)
Beef liver	5.8	0.3	Jangaard *et al.* (1968)
Lemon fruit	8.	0.4	Bogin and Wallace (1966)
Pig heart	9.8	0.55	This work
E. coli revertant Type E3	10.4	0.61	This work
Pig heart	11.	0.52	Kosicki and Lee (1966)
Pig heart	11.	0.67	Lee and Kosicki (1967)
Ac. xylinum	15.3	0.76	This work
Rat liver	17.	0.55	Shepherd and Garland (1969)
Castor bean endosperm mitochondria	18.	0.8	Axelrod and Beevers (1972)
B. subtilis	20.	0.8	Flechtner and Hanson (1969)
Sea anenome	25.	1.3	Sarkissian and Boatwright (1974)
Maize scutellum glyoxysomes	29.	2.1	Barbareschi *et al.* (1974)
B. megaterium	29.4	1.6	This work
Penicillium spiculisporum	30.	3.	Mahlen (1972)

Enzyme source	K_m-acetyl-CoA	K_i-ATP	Reference
E. coli revertant Type E2	30.3	1.7	This work
White shrimp	35.	2.5	Sarkissian and Boatwright (1974)
Streptomyces aureofaciens	42.5	1.8	Hostalek *et al.* (1969)
Trout 'W' form at 15°	45.	3.	Hochachka and Lewis (1970)
Trout 'C' form at 10°	45.	3.2	Hochachka and Lewis (1970)
E. coli (0.5M cacodylate buffer, pH 6)	50.	2.4	Jangaard *et al.* (1968)
Trout 'W' form at 10°	50.	2.8	Hochachka and Lewis (1970)
Trout 'C' form at 15°	50.	3.2	Hochachka and Lewis (1970)
Ps. aeruginosa	50.3	2.8	This work
Anabaena flos-aquae	60.	2.5	C. Lucas (personal communication)
Ps. aeruginosa (after DTNB treatment)	63.2	3.7	This work
Trout 'W' form at 22°	75.	3.5	Hochachka and Lewis (1970)
A. lwoffi	75.6	4.4	This work
Azotobacter vinelandii	80.	0.75	Flechtner and Hanson (1970)
Nostoc sp	100.	5.5	C. Lucas (personal communication)
Trout 'C' form at 22°	100.	5.5	Hochachka and Lewis (1970)
E. coli (in presence of 0.1M KCl)	105.1	5.8	This work
Trout 'W' form at 30°	110.	6.	Hochachka and Lewis (1970)
Trout 'C' form at 30°	150.	8.	Hochachka and Lewis (1970)
E. coli revertant Type E1	360.	20.7	This work
E. coli	420.	23.3	This work

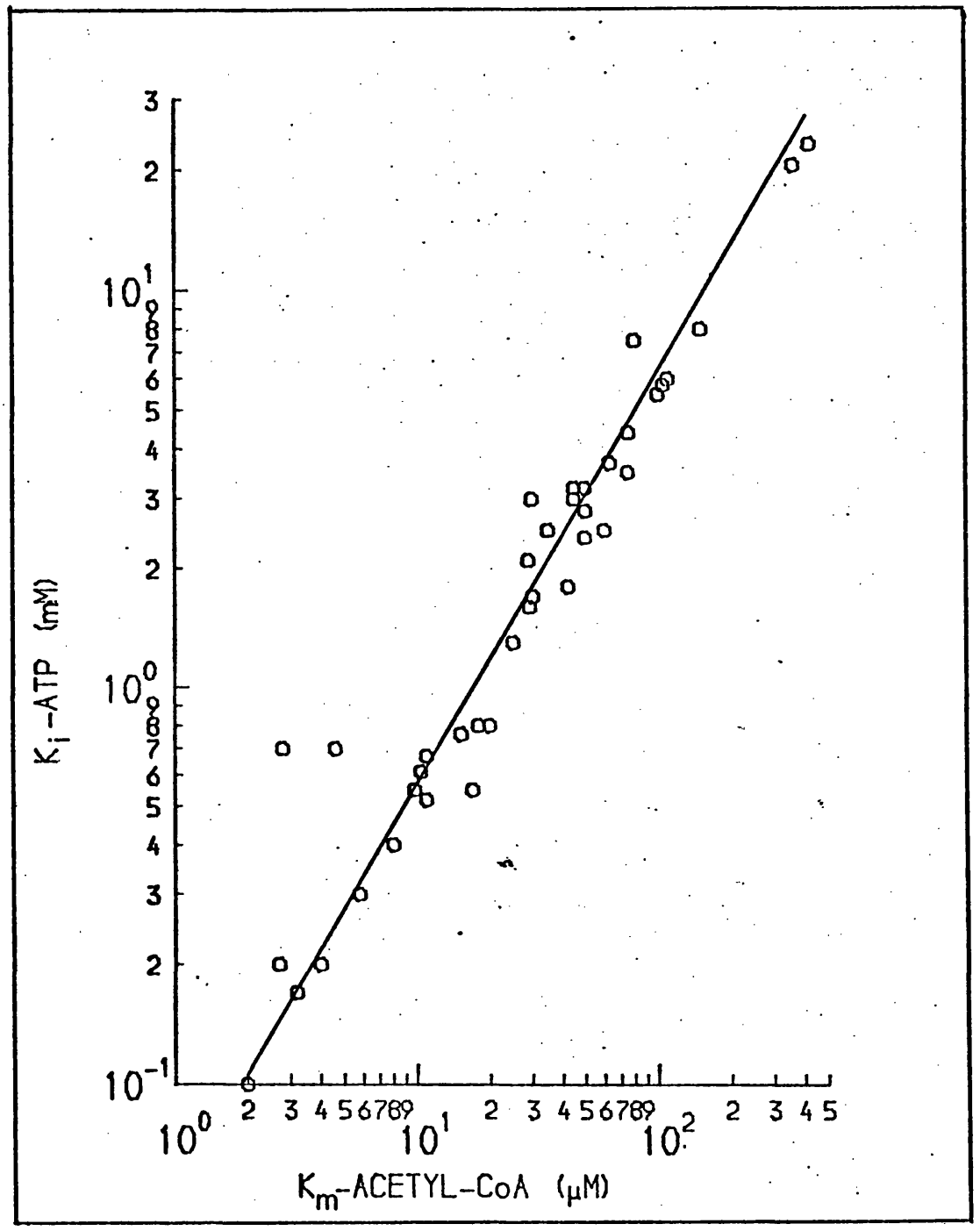


Fig. 51: The relationship between Km value for acetyl-CoA and Ki value for ATP for a number of citrate synthases.

This is a graphical representation of the data presented in Table 24.

inhibitor at an independent site, the inhibitors used in the present work were all relatively small and structurally related; it has been assumed that in this case, parallel line plots indicate that the two inhibitors act at the same site, thereby mutually excluding the binding of each other.

An intersecting pattern of lines indicates that the two inhibitors act at independent sites. This intersecting plot may be of three types. The binding of one inhibitor may partially interfere with the binding of the second (thus increasing its Ki value), or it may enhance the binding of the second (thereby decreasing its Ki value) or, finally, the binding of the two inhibitors may be totally independent and therefore the Ki value of each will be unaffected by the presence of the other.

Using this technique, Harford and Weitzman (1975) have shown that ATP inhibits eucaryotic, Gram positive bacterial and Gram negative bacterial citrate synthases at the same site as the inhibitor bromoacetyl-CoA. NADH also acts at the bromoacetyl-CoA site of the eucaryotic and Gram positive bacterial enzymes, but at a different site in the case of Gram negative bacterial citrate synthases. Furthermore, in this latter case, the Ki value for each inhibitor was unaffected by the presence of the second, thereby showing that the binding of the inhibitors is totally independent. However, after treating the Gram negative bacterial enzyme to abolish the specific NADH inhibitory response, NADH was then found to act at the bromoacetyl-CoA site. Bromoacetyl-CoA can act as a substrate, albeit a poor one, for citrate synthase and it would therefore seem reasonable to assume that this compound acts isosterically at the acetyl-CoA site. This being the case, the results indicate that the specific NADH inhibition of the Gram negative bacterial enzyme is an allosteric effect whereas other nucleotide inhibitions are exerted isosterically.

Clements, Wallace and Keech (1976) have recently claimed that the

method of synthesising bromoacetyl-CoA, as reported by Chase and Tubbs (1969), is unsatisfactory. Clements et al.(1976) found that the product of synthesis reacts chemically with a mixture of oxaloacetate and DTNB at identical rates in the presence or absence of enzyme; they therefore concluded that bromoacetyl-CoA is not the product of this synthesis. The method of Chase and Tubbs (1969) has been used in this present work for preparing bromoacetyl-CoA. In my hands, the product did not react with the assay reagents in the absence of enzyme. Moreover, when used as an inhibitor, the greater the concentration of bromoacetyl-CoA in the assay mixture the greater was the observed inhibition. These findings conflict with the proposals of Clements et al. (1976) as, if the reaction were non-enzymic, the observed rate should increase with increasing bromoacetyl-CoA concentration. However, it was found that the bromoacetyl-CoA rapidly decreased in concentration and, as a result, was always used within 24h of synthesis, over which period of time the compound was reasonably stable. It is therefore possible that the instability of this compound may be responsible for the difference between results obtained by Chase and Tubbs (1969) and in this present work and those obtained by Clements et al. (1976).

In conclusion, the hypothesis that the general non-specific nucleotide inhibition of citrate synthase is an isosteric phenomenon whereas specific NADH inhibition of Gram negative bacterial enzyme is allosteric, seems very well supported by the findings reported here.

It has been found that α-oxoglutarate acts as a powerful inhibitor of citrate synthase from E. coli and other Gram negative facultative anaerobes (Wright et al., 1967; Weitzman & Dunmore, 1969b), and that this inhibition is overcome in the presence of 0.1M KCl, indicating an allosteric effect. In the present work, mutants of E. coli have been isolated which produce

citrate synthases which have lost their sensitivity to α-oxoglutarate, thereby providing further evidence for the view that α-oxoglutarate acts as an allosteric inhibitor.

Finally, the plots from the multiple-inhibition analysis show that the weak α-oxoglutarate inhibition observed with citrate synthases from eucaryotes (pig heart) and Gram positive bacteria (B. megaterium) is exerted at the same site as fluoro-oxaloacetate inhibition. As this fluoro- derivative can act as a substrate for citrate synthase, it would seem reasonable to assume that its inhibitory action is at the active site. In contrast, the powerful α-oxoglutarate inhibition of E. coli citrate synthase is exerted at a different site from that of fluoro-oxaloacetate and is therefore allosteric. Moreover, it has been shown that when examining the inhibition of the E. coli enzyme in the presence of 0.1M KCl, the residual weak α-oxoglutarate inhibition is exerted isosterically, at the fluoro-oxaloacetate, or active, site.

The success of multiple inhibition analysis in the study of the mechanism of inhibitions has prompted other workers in this laboratory to apply the method to other inhibitors and enzymes. Lucas and Weitzman (1977) have shown that succinyl-CoA acts as an allosteric inhibitor of citrate synthases from the blue-green bacteria and Hall and Weitzman (1977) have shown that the NADH binding site of A. lwoffi α-oxoglutarate dehydrogenase is distinct from the α-oxoglutarate binding site. Although other investigators have used this technique with benefit (Northrop & Cleland, 1974), in view of the success of the procedure in this laboratory, it is surprising that the method of multiple-inhibition analysis has not found more widespread application in the study of inhibition mechanisms.

Structure-function relationships for citrate synthase have been proposed from the results of in vitro studies; the physiological

significance of regulatory behaviour has been proposed on the basis of these investigations. A different approach has been adopted in the present work in which mutants have been isolated which produce structurally altered citrate synthases. A study of the properties of these enzymes could reveal valuable information concerning structure-function relationships and, as these enzymes are produced *in vivo*, a study of the physiology of these mutants may provide evidence for the *in vivo* significance of the enzyme properties observed *in vitro*.

A similar approach has been successfully employed by other workers. For example, Cohen (1969) described the isolation of a mutant of *E. coli* which lacked the lysine-sensitive aspartokinase. A number of revertants were selected and some of these produced an enzyme with altered regulatory properties; such revertants were found to overproduce,and excrete,lysine into the growth medium. Similarly, the first enzyme of histidine biosynthesis, catalysing the formation of N-1-(5'-phosphoribosyl)-ATP from ATP and phosphoribosyl pyrophosphate, was shown to be allosterically inhibited by histidine under *in vitro* conditions (Martin,Ames & Garry, 1961). Moyed (1961) isolated mutants of *E. coli* which grew in the presence of the histidine analogue, 2-thiazolealanine. These mutants were found to produce an enzyme which was no longer sensitive to inhibition by histidine and it was found that they overproduced and excreted histidine into the growth medium. These mutants provide good evidence for the allosteric nature of the histidine inhibition as it was demonstrated that the inhibitor site could be lost without affecting activity. Furthermore, the overproduction of histidine by such mutants strongly indicates that histidine inhibition of this enzyme does have a physiological role in the regulation of histidine biosynthesis. It was hoped that a similar result might be obtained with mutants of citrate synthase.

Before discussing the results obtained using the citrate synthase mutants, it is pertinent to discuss the strategy for their isolation. As no procedure could be envisaged that would directly select for mutants with structurally altered, but nonetheless active, citrate synthase, it was necessary to adopt a two stage approach.

The first stage was to isolate mutants which had an altered citrate synthase gene, resulting in the production of inactive citrate synthase. The nature of this alteration, or mutation, was preferably a single base substitution, as this would lead to minimal alteration in the amino acid sequence of the protein and would also be the easiest form of mutation from which to obtain revertants. The subsequent selection of revertants, which again produced an active citrate synthase, relied on the fact that a second mutation in the citrate synthase gene may lead to a second change in the amino acid sequence of the protein, and this may compensate for the initial alteration thereby restoring activity. However, this enzyme would probably have a slightly altered amino acid sequence compared with that of the parent enzyme; this change in structure may be accompanied by changes in the molecular and regulatory properties of the enzyme.

The organisms used in this part of the work were E. coli and A. lwoffi. These bacteria were chosen because the citrate synthases which they produce have been intensively studied in this and other laboratories. Only Gram negative bacterial citrate synthases have been shown to have allosteric binding sites and this makes their study more challenging. A. lwoffi, a strict aerobe, has a citrate synthase which is allosterically inhibited by NADH, and this inhibition is overcome by AMP (Weitzman & Jones, 1968). E. coli is a facultative anaerobe and produces a citrate synthase which is allosterically inhibited both by NADH and α-oxo-glutarate.

It was first necessary to isolate mutants of each organism deficient in citrate synthase activity. Such mutants of E. coli have been successfully isolated by other workers (Ashworth, Kornberg & Nothmann, 1965) by selection for glutamate auxotrophs; some of these strains have been used in the present work. The selection method relies on the fact that a strain lacking citrate synthase activity is unable to grow on glucose medium, unless supplemented with glutamate, proline or α-oxoglutarate. The presence of penicllin kills only growing bacteria. By growing a culture of E. coli on glucose minimal medium, in the presence of penicillin, it was possible to enrich the population with respect to auxotrophs as these were able to withstand the bactericidal effect of the penicillin. The surviving population was screened by replica plating to identify the glutamate auxotrophs and some of these were found to lack citrate synthase activity. A similar method was used in the present work, glutamate auxotrophs being enriched by penicillin treatment of an E. coli culture in succinate minimal medium. A mutant of E. coli was successfully isolated which lacked citrate synthase activity.

When this approach was used in an attempt to isolate similar citrate synthase deficient mutants of A. lwoffi it was not successful. As a strict aerobe, the dependence of this organism on the TCA cycle is greater than is the case for E. coli and this may, in part, explain the failure. Moreover, it was found that the penicillin enrichment technique was not very successful with this organism as most of the wild type strain also survived the treatment. Further attempts were made to increase the lethal effect of this antibiotic, by increasing the penicillin concentration and by using a variety of different penicillin derivatives, but these changes did not improve the enrichment procedure. An alternative technique was therefore developed which would directly select for citrate synthase

deficient mutants. To do this, growth conditions had to be devised in such a way as to favour the growth of mutants which lack citrate synthase activity compared with the growth of the wild type organism.

The method devised relied on the lethal effect of fluoroacetate on many forms of life. Peters (1952) showed that fluoroacetate is metabolised by the cell to fluorocitrate. Fluorocitrate is a powerful competitive inhibitor of the enzyme aconitase (Peters, 1955) thus effectively blocking the TCA cycle at this step and causing an accumulation of citrate.

One result of this inhibition may be that the organism is unable to satisfy its requirement for TCA cycle intermediates, resulting in the cessation of growth. However, the presence of glutamate in the growth medium did not overcome the lethal effect of fluoroacetate on E. coli wild type. The toxicity of fluoroacetate may therefore be the result of an accumulation of citrate and fluorocitrate, which in turn may act as inhibitors of other enzymes, e.g. fluorocitrate has been shown to inhibit succinic dehydrogenase activity in vitro (Kun, 1969).

Lakshmi and Helling (1976) have reported that isocitrate dehydrogenase deficient strains of E. coli grow only very slowly on glucose plus glutamate medium. However, fast growing colonies may readily be isolated and, when analysed, these fast growing mutants were found to lack citrate synthase activity in addition to the original isocitrate dehydrogenase deficiency. These workers explained the results by the fact that isocitrate dehydrogenase deficient strains produce a build-up of citrate which is toxic to the cell; the citrate synthase deficient strains cannot produce citrate and therefore this toxic synthesis is overcome.

Therefore, whatever the toxic effect of fluoroacetate, citrate synthase deficient mutants may be unaffected by the presence of this compound, being unable to convert it to fluorocitrate. Indeed, it has been shown that when

grown on a mixture of succinate, glutamate and fluoroacetate (conditions which completely inhibit the growth of wild type E. coli) citrate synthase deficient strains of this organism grow normally. Two of the fluoroacetate resistant mutants of A. lwoffi that were selected were found to lack citrate synthase activity. Following the success of this selection procedure, other investigators in this laboratory have applied this method to obtain further citrate synthase deficient mutants. Harford and Weitzman (in preparation) have proposed that this method could be used to obtain citrate synthase deficient mutants of a wide variety of bacteria and even of eucaryotic organisms. There are some organisms however, e.g. Ps. aeruginosa, which has a detoxification mechanism whereby it is able to hydrolyse this compound rendering it harmless; therefore it may not be possible to select for citrate synthase deficient mutants of such organisms by this method.

The mutations giving rise to the citrate synthase deficient strains of E. coli and A. lwoffi may each be in one of two possible loci. The mutation may either be in the structural gene of citrate synthase, resulting in the production of an inactive enzyme, or it may be in a regulatory gene, thereby preventing the synthesis of the otherwise active enzyme. The present work relied on the isolation of mutants which produce a structurally altered, inactive enzyme; regulatory mutants being of no direct interest, it was necessary to characterise each mutant.

Antibody, raised against purified citrate synthase from wild type A. lwoffi was found to cross react with material in the crude extracts of the citrate synthase deficient strains of this organism. This cross-reacting material strongly suggests the presence of inactive citrate synthase protein in these extracts, and that the mutation lies in the structural gene of these mutants (Perrin, Jacob & Monod, 1960). Antibody

against purified E. coli citrate synthase was not available, therefore the method of immuno-diffusion could not be used for testing the mutants of this organism. However, if the locus of a mutation was in a regulatory gene, the structural gene being unaffected, revertants which had regained citrate synthase activity would be expected simply to repair this regulatory defect, and the structure and properties of the citrate synthase produced by all these revertants would be identical to those of the enzyme from the wild type organism. It is clear that this is not the case for any of the citrate synthase deficient strains of E. coli or A. lwoffi, as the enzyme produced by some of the revertants of each of these mutants exhibited very different properties from those of the wild type organism, indicating that mutations had indeed occurred in the structural gene in all cases.

In order to estimate the likely extent of the change in amino acid sequence of the active citrate synthase from the revertant strains, it was necessary to establish the nature of these mutations be they base substitution, frame shift or deletion. Deletion mutants do not revert to produce active enzymes (Hayes, 1970). As revertants which had regained enzyme activity could readily be isolated from all the citrate synthase deficient strains, the mutations were unlikely to be the result of deletions. The presence of alkylating agents has been shown to greatly enhance the frequency of reversion of base substitutions but to have no effect on the reversion of frame shifts (Whitfield, Martin & Ames, 1966). Ethyl methane sulphonate is an example of such an agent, and it was found that the frequency of reversion of all the citrate synthase deficient mutants was greatly increased in the presence of this compound. These findings strongly indicate that each citrate synthase deficient mutant is the result of one, or possibly more, base substitutions in the structural

gene for the enzyme.

Revertants, which had regained citrate synthase activity were easily isolated as the selection procedure involved the isolation of prototrophic organisms from a background of glutamate-requiring auxotrophs. All revertants were found to have regained citrate synthase activity.

Many revertants were isolated from the citrate synthase deficient strains of both organisms; some of these revertants produced enzymes with altered properties. Before a direct comparison of the molecular properties of these enzymes was undertaken, it was necessary to demonstrate that the enzymes were not the product of a contaminating source. This contamination could take one of two forms; either at the bacterial or gene level. For example, the revertant producing Type E2 enzyme could be a contaminating Acetobacter, and that producing Type E3 or A2 enzyme could be a Gram positive organism. Contamination at the gene level could be achieved if E. coli or A. lwoffi possess more than one citrate synthase gene. Under laboratory growth conditions it may be that only the gene coding for the 'large' wild type enzyme is expressed. However, when this gene is mutated to produce inactive enzyme, subsequent mutation may alter a second gene to produce the 'latent' citrate synthase. Both of these contamination possibilities were investigated.

All of the A. lwoffi revertants were shown to be Gram negative rods. Growth characteristics of the A. lwoffi revertants compared well with those of the wild type organism and, using API identification tests, it was shown with 99.97% confidence that the revertants were derived from A. lwoffi wild type. This identification was further supported by the results from the immuno-diffusion tests, from which it was found that the revertant citrate synthases reacted with antiserum raised against the purified enzyme fom A. lwoffi wild type. The conclusion drawn from

these observations was that the revertants were all from the original A. lwoffi strain. Furthermore, the immuno-diffusion results show that the enzymes produced by these revertants must have a number of structural features in common with the wild type enzyme, thereby indicating that the gene coding for citrate synthase in the revertants is the same as that coding for the enzyme in the wild type organism.

The E. coli revertants were all shown to be Gram negative rods; this finding was demonstrated by the conventional Gram staining procedure and by electron microscopy of the cell wall. Although the electronmicrographs are a little astigmatic, the characteristic multilayered architecture of Gram negative bacteria could be clearly seen. API tests, followed by computer assisted identification, showed that with 99.95% confidence the revertants were strains of E. coli. Furthermore, the fact that the E. coli revertants shared the same auxotrophic requirements as the parent organisms, clearly demonstrated that they were derived from the original E. coli wild type strains.

The citrate synthase gene (glt A) has been mapped in E. coli, in a position close to the galactose genes. Bacterial conjugation experiments have shown that the gene coding for the citrate synthase in the E. coli revertants also lies very close to the galactose marker. Furthermore, phage-mediated transduction experiments showed that the gene locus coding for Type E3 enzyme was completely indistinguishable from the gene locus of E. coli wild type citrate synthase. Moreover, , all attempts to isolate a recombinant strain producing both a wild type and a Type E2 or E3 enzyme were unsuccessful, thereby indicating that the presence of one gene totally excluded the other; such would be the case when both genes were at the same locus.

Having established that the citrate synthase produced by the revertants

was not derived from a contaminating source, a comparative study was made of the molecular properties of the revertant enzymes with those of the citrate synthases from naturally occurring organisms.

The enzymes used in this work were from pig heart (eucaryotic), B. megaterium (Gram positive bacterium), E. coli and A. lwoffi (Gram negative bacteria) and Ac. xylinum. This final organism is also a Gram negative bacterium, but one which produces a citrate synthase which, although of the 'large' type, is atypical in that it is not inhibited by NADH.

A. lwoffi wild type citrate synthase has been intensively studied in this and other laboratories, and the results reported in the present work are in good agreement with previous findings.

The enzyme has a molecular weight of about 250,000 and has been shown by Johnson and Hanson (1974) to consist of four monomeric units, each with a molecular weight of about 60,000. Only the tetrameric association has been shown to possess catalytic activity (Weitzman & Danson, 1976). The enzyme had a relatively high Km for acetyl-CoA, was insensitive to ATP, but was powerfully inhibited by NADH; this latter inhibition was overcome by the presence of AMP, as originally observed by Weitzman and Jones (1968).

Chemical modification studies have suggested that histidine may be important for both catalysis and regulation by NADH (Iredale & Weitzman, in preparation). Studies with the thiol-blocking reagent DTNB did not implicate a sulphydryl group in either function, but inactivation with $HgCl_2$ did implicate this group in activity (P.D.J. Weitzman, personal communication). It may well be that while the sulphydryl group is accessible for $HgCl_2$, the protein structure protects this group from the much larger reagent, DTNB.

The A. lwoffi revertants isolated produced citrate synthases of two types. The first, Type A1, had properties very similar to the wild type

citrate synthase. However, the second type of revertant produced an enzyme with a much lower molecular weight of about 100,000, presumably a dimeric association of subunits. The catalytic and regulatory properties investigated showed that the enzyme had a Km value for acetyl-CoA similar to that of the wild type enzyme, was insensitive to ATP and, furthermore, was not inhibited by NADH.

In view of the chemical modification results achieved with the wild type enzyme, the fact that some of the revertant citrate synthases were inactivated by treatment with DTNB was not altogether surprising. As previously discussed, although the wild type enzyme is not affected by DTNB, nonetheless, sulphydryl groups have been implicated in activity by treatment with other reagents. Therefore, the revertant enzymes which are inactivated by DTNB may have a slightly altered structure rendering the sulphydryl groups more accessible to this compound.

The E. coli wild type citrate synthase has also been intensively investigated in a number of laboratories. The active enzyme has a molecular weight of about 230,000 and consists of four identical subunits, each having a molecular weight of about 60,000 (Danson & Weitzman, 1973). Many molecular forms have been reported for the enzyme. Wright and Sanwal (1971) have demonstrated that, between pH 8.0 and 9.5, the enzyme exists as a monomer , tetramer and octamer, all forms existing in a dynamic equilibrium. Tong and Duckworth (1975) have also shown that dimeric and hexameric forms are favoured under certain conditions. In spite of this diversity in molecular species, only the tetrameric association has been shown to possess catalytic activity (Wright & Sanwal, 1971; Danson & Weitzman, 1973).

In the present work, the molecular and regulatory properties of E. coli wild type citrate synthase have been studied, and the results obtained

are in very good agreement with those of other workers. Substrate dependences were sigmoidal with high $S_{0.5}$ values for both acetyl-CoA and oxaloacetate. The enzyme was insensitive to ATP but powerfully inhibited by both NADH and α-oxoglutarate, these latter inhibitions being overcome by the presence of 0.1M KCl. The enzyme was rapidly inactivated by DTNB, implicating a sulphydryl group in activity; this inactivation was completely overcome by the presence of 0.1mM oxaloacetate. This DTNB inactivation made determination of enzyme rate, at low oxaloacetate concentration, very difficult. Therefore, when determining the $S_{0.5}$ value for oxaloacetate, the polarographic assay procedure was employed (Weitzman, 1969b), thereby obviating the need for DTNB.

Active enzyme centrifugation of the wild type enzyme showed that the tetrameric form was active. Polyacrylamide gel electrophoresis showed that, at pH 8.9, there were three molecular forms of the enzyme; presumably the monomer, tetramer and octamer observed by Wright and Sanwal (1971) and Danson and Weitzman (1973).

The E. coli revertant citrate synthases fell into three distinct groups.

Type E1: 'large', with kinetic and regulatory characteristics similar to wild type E. coli enzyme.

Type E2: 'large', but otherwise like the 'small' citrate synthase in kinetic and regulatory properties.

Type E3: 'small', with kinetic and regulatory properties typical of 'small' citrate synthase.

It is remarkable that this family of enzyme types in fact resembles those occurring naturally in diverse bacteria - i.e. E. coli, Acetobacter and Gram positive bacteria respectively. Whereas the Type E1 enzymes show sigmoidal substrate dependences, a high $S_{0.5}$ value for acetyl-CoA, are

insensitive to ATP but are sensitive to allosteric inhibition by NADH and α-oxoglutarate, the TypeE2 and E3 enzymes show hyperbolic substrate dependences, a low Km value for acetyl-CoA, are sensitive to ATP inhibition but insensitive to allosteric inhibition by NADH and α-oxoglutarate.

Some of the properties of all E. coli mutant citrate synthases were similar to those of the wild type enzyme. It was found, using polyacrylamide gel electrophoresis, that Type E1, E2 and E3 enzymes all existed as three distinct molecular forms at pH 8.9. Although it was not possible to determine the molecular weight of each enzyme species, these three forms may well be the monomer, tetramer and octamer observed for the wild type enzyme. This multiplicity of enzyme forms complicates the interpretation of results for the Type E3 enzyme. At pH 8.0, this enzyme has only one active species, a 'small' enzyme with a molecular weight corresponding to a dimer. However, at pH 8.9, this dimer may not be present, suggesting that at this pH value the dimeric association is not favoured. Furthermore, the evidence suggests the presence of a tetrameric form at pH 8.9; as this form was not observed as an active species at pH 8.0 it must be concluded, that at this pH, either the tetramer was not formed or, that if present, this association of subunits does not result in an active enzyme.

As described earlier, E. coli wild type enzyme is rapidly inactivated when incubated with the thiol-blocking reagent DTNB. The inactivation is prevented in the presence of oxaloacetate, thereby implicating a sulphydryl group as important in the active site. Similar results were obtained for all three revertant enzyme types, but the inactivation was very much more rapid for the Type E2 and E3 enzymes, suggesting that the alteration in protein structure of these citrate synthases results in greater accessibility, or reactivity, of the sulphydryl groups.

The allosteric inhibition of citrate synthase by NADH is restricted to the Gram negative bacterial enzymes (Weitzman & Jones, 1968) which suggests that the NADH site may only be found on the tetrameric species. Thermal inactivation studies were carried out on the Type E1, E2 and E3 enzymes and E. coli wild type citrate synthase to determine whether the NADH site is absent from the Type E2 and E3 enzymes, or whether there are any vestiges of this site, albeit with a deficient transducing system for modulating catalytic activity.

The thermal stability of E. coli wild type citrate synthase, in the presence of effector molecules, paralleled the regulatory behaviour of this enzyme. The allosteric inhibitor, NADH, afforded considerable protection of the enzyme against thermal inactivation. This effect was specific for NADH; NADPH, $NADP^+$ or NAD^+ afforded no such protection. Furthermore, the presence of 0.1M KCl, which abolishes the allosteric inhibition by NADH, was also found to overcome this enhanced thermal stability. Similar studies with Type E3, 'small', citrate synthase, from K1.1.4.R7, showed that the thermal inactivation of this enzyme was markedly increased in the presence of NADH; this effect was specific, NADPH, $NADP^+$ or NAD^+ having little or no effect. Furthermore, the presence of 0.1M KCl was shown to completely overcome this NADH effect. Therefore, it appears that a specific site exists on the dimeric enzyme, and the binding of NADH to this site produces a conformational change in the protein which here results in an increased heat lability of the enzyme. The similarity of the results for the Type E3 enzyme and the E. coli wild type citrate synthase suggests that the allosteric binding site for NADH may still be present on this dimeric enzyme species, but that binding of NADH to this site produces a conformational change which does not manifest itself by inhibition of catalytic activity.

Similar experiments did not indicate the presence of specific binding sites for NADH and α-oxoglutarate on other Type E3 or E2 enzymes. This was either because the binding sites were completely absent from these enzymes, or the binding of these compounds does not significantly affect the thermal stability of these citrate synthases.

It seems peculiar that the 'small' enzyme, at pH 8.0, does not associate into an active tetramer. This finding is particularly interesting in view of the fact that gel electrophoresis of the 'small' enzyme shows what appears to be a tetrameric species. It must therefore be, that at pH 8.0, either the formation of a tetramer is not favoured or, that if the tetramer does exist, it is not catalytically active.

To study the association of the dimeric units into tetramers, ligand-induced association was attempted, using oxaloacetate as the ligand. At pH 8.0, in the presence of oxaloacetate, the dimer was found to associate into an active tetramer; the catalytic and regulatory properties of this enzyme did not differ significantly from the dimer. Furthermore, it was found that this association was reversible; if oxaloacetate was removed by dialysis, the enzyme reassumed its dimeric form.

Ligand-induced association-dissociation has been observed for other enzymes, e.g. CTP synthetase from E. coli has a molecular weight of about 200,000 in the presence of a mixture of ATP, UDP and Mg^{2+} but of about 100,000 in its absence (Levitzki & Koshland, 1970). In fact, these workers have proposed that this phenomenon may serve as a useful property in the purification of such enzymes. A method similar to that which they described was used in the present work in an attempt to purify the Type E3 enzyme (results not reported here). The method involved the passage of a cell-free extract through a Sephadex G-200 column in the presence of oxaloacetate (i.e. with the enzyme in its tetrameric form).

The pooled fractions, containing only 'large' proteins, were collected and ammonium sulphate added to precipitate the protein. This precipitate was resuspended and dialysed to remove oxaloacetate; the enzyme, now in its dimeric form, was again passed through a Sephadex G-200 column, but in the absence of oxaloacetate. In this way, a purification of 100-fold was achieved with a final recovery of 9%.

Of the ligands tested, only oxaloacetate produced this ligand-induced association; ATP, NADH, α-oxoglutarate or acetyl-CoA had no effect on the molecular size of the enzyme.

The subunit composition of a number of citrate synthases has been studied. The 'small' enzymes, from eucaryotes and Gram positive bacteria, are dimers, and the 'large' enzymes, from Gram negative bacteria, are tetramers. The molecular weight of the monomeric subunit of both 'large' and 'small' enzymes has been shown to be about 60,000 (Shepherd & Garland, 1969; Wright & Sanwal, 1971; Danson & Weitzman, 1973). On the basis of the similarity in the molecular weights of all the subunits, it is conceivable that all citrate synthases are composed of very similar monomeric units. Indeed, Srere (1972) has claimed a striking similarity in the amino acid compositions of 'small', mammalian, and 'large', *E. coli* wild type citrate synthases. No obvious similarities were observed by Weitzman and Danson (1976) when they repeated the comparison with their data. However, this result does not discount the possibility that there may be extensive areas of homology in these two enzymes, especially in the functional regions of substrate binding sites, e.g. as found for several aldolases (Gibbons, Anderson & Perham, 1970), and for a number of chloramphenicol acetyl-transferases (W.V. Shaw, personal communication).

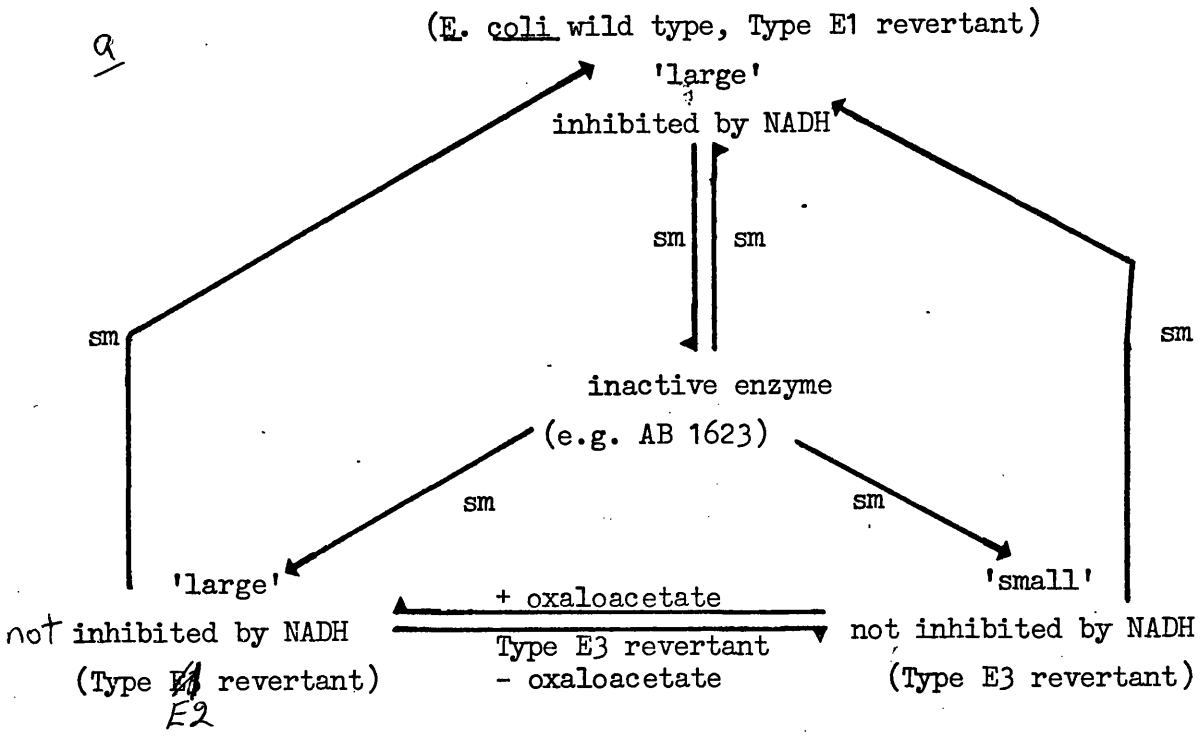
The studies on the revertant enzymes from *A. lwoffi* and *E. coli* show that the tetrameric form of the Gram negative bacterial citrate synthases

is not a pre-requisite for their activity; with only very minor changes in amino acid sequence an active dimer is readily obtained. Moreover, the fact that revertants which produce an active monomer have not been isolated does suggest that at least the dimeric association is necessary for activity; this view is supported by the fact that no naturally occurring monomeric citrate synthase has been reported.

The isolation of revertants, of both A. lwoffi and E. coli, which produce active enzymes that are insensitive to NADH inhibition, further confirms the view that NADH is an allosteric inhibitor of Gram negative bacterial citrate synthases. Similarly, E. coli revertants have been isolated which produce an enzyme that is insensitive to α-oxoglutarate, thereby supporting the view that this compound too is an allosteric inhibitor of the enzyme from Gram negative facultative anaerobes.

E. coli revertants producing Type E2 and E3 enzymes were unable to grow on acetate. The further isolation of mutants of these revertants, which had regained the ability to grow on acetate, showed that the Type E2 and E3 gene could be readily converted to a Type E1 gene.

It appears therefore, that with only minor alterations in the amino acid sequence of the enzyme from E. coli and A. lwoffi, families of enzymes may be obtained with many different properties. The interrelationships of these enzyme forms are illustrated in Fig. 52. The fact that the properties of these enzymes closely resemble those of naturally occurring citrate synthases suggests that this investigation may have paralleled the evolutionary path by which one citrate synthase developed from others. It is clear that a detailed study of the amino acid sequence of naturally occurring, and revertant, enzymes could give valuable information concerning the degree of homology in different citrate synthases, the groups involved in subunit association, allosteric inhibition sites and active sites of



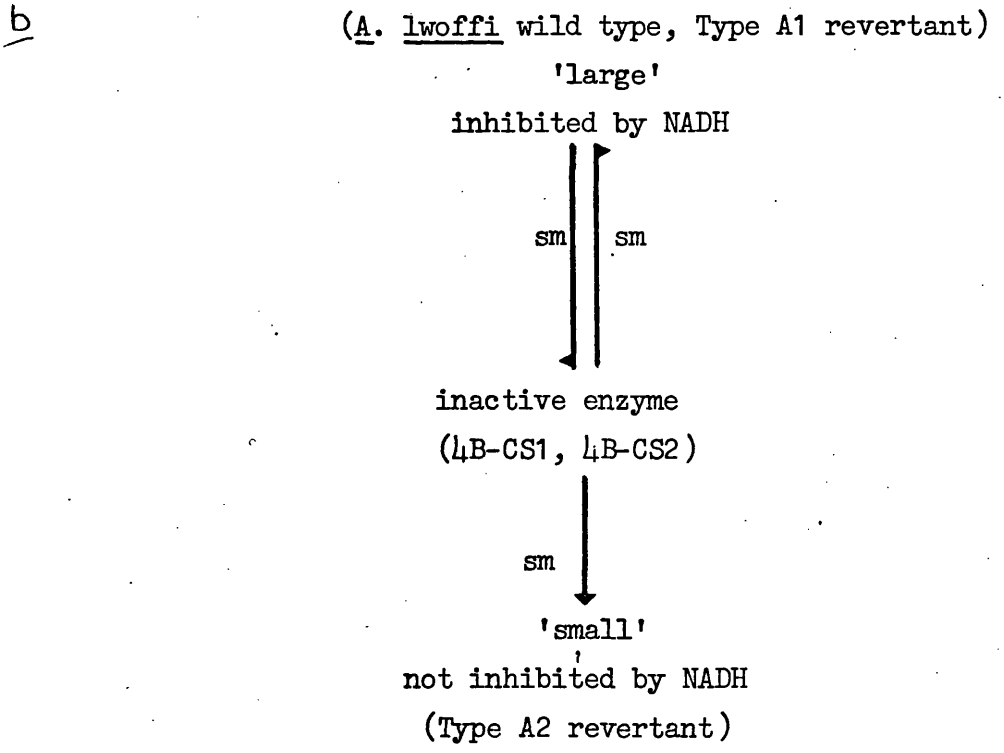


Fig. 52: Interrelationships of the wild type and revertant enzymes.

a. Relationships of E. coli strains
b. Relationships of A. lwoffi strains
sm, spontaneous mutation.

the enzyme.

Further evidence to suggest a high degree of homology in the citrate synthase structure, from different bacteria, was provided by the transformation studies with A. lwoffi. It was found that one citrate synthase deficient strain of A. lwoffi was transformed, at a high frequency, in the presence of Ps. aeruginosa DNA, to regain citrate synthase activity. It was found that the citrate synthase produced by the transformants neither reacted with antibody raised against A. lwoffi wild type citrate synthase, nor did it lose NADH sensitivity when treated with DTNB. The conclusion must be that the Ps aeruginosa DNA has recombined with part of the A. lwoffi citrate synthase gene, resulting in the production of an active enzyme, but one which has similarities to both the Ps. aeruginosa and A. lwoffi citrate synthases. Therefore, some partial DNA sequence homology is indicated between the A. lwoffi and Ps. aeruginosa citrate synthase genes. Transformation of citrate synthase deficient strains of A. lwoffi with DNA from other bacteria has not yet been achieved, however, using this same technique it may be possible to establish further homologies. Furthermore, this same method, or a modification of it, may make it possible to study homologies between the E. coli gene and those of related and unrelated genera.

All investigations so far discussed, have been carried out in vitro using cell-free extracts. The physiological significance of these in vitro observations remains uncertain, and the in vivo significance of the regulatory behaviour, in particular, is a challenging problem.

The inhibition of yeast citrate synthase by ATP (Hathaway & Atkinson, 1965), was interpreted as a negative feedback mechanism for regulating the activity of the initial enzyme of a pathway by an ultimate end-product. The finding that ADP and AMP also inhibited the enzyme, although to a

lesser extent, suggested that it might be the relative concentrations of these inhibitors that determined the activity of citrate synthase *in vivo*; this idea has subsequently been developed into the 'energy charge' concept (Atkinson, 1968).

Many other citrate synthases have been shown to be inhibited by ATP, and also by a number of other nucleotides. This general non-specific nucleotide inhibition is isosteric; the inhibitors acting competitively with acetyl-CoA Isosteric inhibition may often be an artefact of the *in vitro* assay conditions; *in vivo*, the concentration of effector may never reach a level sufficient to cause a significant inhibition. Using permeabilised cells, Weitzman and Hewson (1973) have tried to simulate the *in vivo* conditions of the enzyme in yeast cells; Reeves and Sols (1973) have suggested the term *in situ* to describe such a system. In fact, Weitzman and Hewson (1973) found that the properties of the enzyme *in situ* differed considerably from those of the isolated enzyme; the most important differences being its apparent lack of sensitivity to ATP accompanying an increase in the Km value for acetyl-CoA from 5μM for isolated enzyme to 800μM for that in permeabilised cells. Furthermore, it has been demonstrated that physiological concentrations of Mg^{2+} abolish ATP inbibition of citrate synthase (Kosicki & Lee, 1966); this is probably due to the chelation of the cation by the pyrophoshate group of ATP, resulting in a weakened interaction of the nucleotide with the binding site on the enzyme. In order to assess the significance of ATP inhibition of citrate synthase, Garland *et al.*(1969), using rat liver mitochondria, obtained results consistent with the view that ATP may control citrate synthase activity, whereas Olson and Williams (1971) reached the opposite conclusion, that ATP does not have an important role in the control of this enzyme. However tempting the extrapolation of the *in vitro*

observations of ATP inhibition to the *in vivo* situation, the findings outlined above highlight the difficulty in doing so. Similar difficulties are encountered when attributing physiological significance to the NADH inhibition of the enzyme from Gram negative bacteria, and to the α-oxo-glutarate inhibition of the facultative anaerobes.

The NADH inhibition of Gram negative bacterial citrate synthases has been demonstrated both *in vitro* and *in situ*. This inhibition may be a negative feedback mechanism whereby the initial enzyme is regulated by an end-product of the TCA cycle. The possible physiological significance of this finding is strengthened by the fact that the NADH inhibition is allosteric. It would seem an unlikely coincidence that all Gram negative bacterial citrate synthases would have a distinct site within their structure which specifically recognises NADH, unless there was a physiological significance for this site. However, it has been found that physiological concentrations of KCl overcome this inhibition in some citrate synthases (Weitzman & Danson, 1976), and therefore its *in vivo* significance remains to be confirmed. However, it is an interesting point that 0.1M KCl has been found to inactivate Type E2 and E3 enzymes (results not presented here); the fact that active enzyme can be isolated from the Type E2 and E3 revertants indicates that, *in vivo*, the enzyme is in some way protected from the KCl. This protection may take the form of compartmentalisation of the enzyme or it may be that, *in vivo*, the enzyme has a slightly altered conformation, making it insensitive to KCl. This information does suggest that the citrate synthase of *E. coli* wild type may similarly be protected from KCl, and that, *in vivo*, the KCl does not abolish the NADH inhibition.

Therefore, if NADH inhibition of citrate synthase is physiologically important, the *E. coli* and *A. lwoffi* revertants which produce an enzyme

that is insensitive to this compound should have some change in their metabolism. However, the most likely outcome of this increased flux of carbon through the TCA cycle would be an overproduction of NADH. This overproduction of an energetically important compound could be manifested in any number of ways and would seem a very difficult parameter to assess. Since revertants have been isolated which produce a citrate synthase that is insensitive to NADH, it would appear that this control mechanism is not essential for viability. In the case of the E. coli revertants, it may be suggested that sensitivity to ATP has been substituted for sensitivity to NADH, and that either of these compounds may serve to regulate citrate synthase activity. However, some of the A. lwoffi revertants appear to be insensitive to NADH and ATP but still retain viability and these revertants seem as competent in growth as the wild type organism, suggesting that lack of NADH inhibition, in this bacterium, does not have serious physiological consequences.

It may be argued teleologically, that sensitivity of A. lwoffi wild type citrate synthase to NADH is maintained in the natural population for some purpose. It may be that the lack of this inhibition results in the organism being at a very slight disadvantage compared with the wild type. Although it may not be possible to discern this dysfunction, under laboratory conditions, the selection operating on a natural population would discriminate against such an organism. It may well be that citrate synthase activity is controlled by a number of mechanisms, and this loss of regulation by NADH may be largely compensated by these other controlling factors; this view is supported by Krebs (1970), who emphasised that it is unrealistic to attribute control of this enzyme to any single factor. Randle et al. (1970) have indicated that variation in acetyl-CoA concentration may serve to regulate the activity of the rat heart enzyme.

Similarly, variation in the concentration of oxaloacetate could impose a very significant regulatory effect on the enzyme from rat heart mitochondria (LaNoue et al., 1973). The importance of oxaloacetate concentration for citrate synthase activity was also emphasised by Lehninger (1946) and, more recently, by Lopes-Cardozo and van den Bergh (1972).

Gram negative facultative anaerobes, when grown under anaerobic conditions, or during some stages of aerobic growth on glucose, have a split TCA cycle; the enzyme α-oxoglutarate dehydrogenase being absent, or present at a very low level (Amarasingham & Davis, 1965). Under certain conditions α-oxoglutarate is a direct end-product of the pathway and therefore the finding that this compound allosterically inhibits the citrate synthase from these bacteria suggests a feedback inhibition mechanism. The physiological significance of this inhibition is supported by the finding that, whereas all Gram negative facultatively anaerobic bacterial citrate synthases exhibit this property, the effect is not observed with the enzyme from strict aerobes. Furthermore, the in situ occurrence of this inhibition has been demonstrated using toluene-permeabilised cells of E. coli.

If this inhibition is physiologically important, it would be expected that the revertants of E. coli which produce a citrate synthase which is insensitive to α-oxoglutarate should, under suitable conditions, over-produce and possibly excrete this metabolite.

A microbiological method was developed to detect the presence of α-oxoglutarate, or a similar compound, in the growth medium. Microbiological assay systems have found widespread use, particularly in the pharmaceutical industry, in both the qualitative and quantitative analysis of chemicals. The citrate synthase deficient strain of E. coli, AB1623, required glutamate, or some related compound, for growth on glucose. The over-

production of any of these compounds should manifest itself by cross-feeding this glutamate auxotroph. In this way it has clearly been shown that the Type E2 and E3 revertants do indeed overproduce a compound which will cross-feed the glutamate auxotroph when grown on the surface of glucose minimal medium agar plates. Although this assay system is a very sensitive qualitative technique, it is not possible to adapt this particular method to give quantitative results.

Attempts were made to demonstrate the overproduction of intermediates in liquid culture but these were not successful. A number of results indicated that, although Type E2 and E3 revertants produced citrate synthases which were insensitive to α-oxoglutarate and NADH, the enzyme was 'inefficient' and the revertants were, in effect, leaky mutants.

Citrate synthase deficient strains of E. coli were shown to accumulate pyruvate when grown on glucose. This pyruvate accumulation was probably a direct consequence of the lack of citrate synthase activity, resulting in a build-up of acetyl-CoA which, by inhibiting the action of pyruvate dehydrogenase (Sanwal,1970), leads to pyruvate accumulation. While E. coli wild type and Type E1 revertants did not overproduce pyruvate under these conditions, the Type E2 and E3 revertants did. This result indicates that in these revertants there is a build-up of acetyl-CoA and this is probably due to the 'inefficiency' of the citrate synthase in these strains.

It has been reasoned, and in fact demonstrated, that citrate synthase deficient organisms are resistant to fluoroacetate; this resistance is probably the result of the failure to metabolise fluoroacetate to fluorocitrate. It was found that E. coli wild type and Type E1 revertants were sensitive to this compound whereas the Type E2 and E3 revertants were resistant to its toxic effects. Thus these two revertant types do

not readily metabolise fluoroacetate to fluorocitrate.

The specific activity of citrate synthase from Type E2 and E3 revertants was shown to be very low compared with that of Type E1 and E. coli wild type enzyme. It was also demonstrated that the 'effective level' of activity of the Type E2 and E3 enzymes, at a standard protein concentration, was very low over a range of acetyl-CoA concentrations.

When grown on glucose minimal medium, the growth rate of Type E2 and E3 revertants was far below that of the E. coli wild type. However, the addition of a small amount of glutamate to these cultures increased the growth rate to that of the wild type. This result, in conjunction with the observation of relative enzyme levels, pyruvate accumulation and resistance to fluoroacetate, suggests that far from overproducing α-oxoglutarate (or a related compound), the Type E2 and E3 revertants, in liquid culture, cannot satisfy their own requirement for this compound. Therefore it may be for this reason that the overproduction was not observed in liquid culture.

In liquid culture, all cells metabolise and grow until the carbon source is exhausted, or some other factor prevents further growth. However, when a colony is formed on solid medium, the cells at the centre of growth are not dividing but simply metabolising; only a small percentage of cells on the periphery are replicating. Under these conditions it is possible that the non-growing cells of Type E2 and E3 revertants metabolise glucose through their 'uncontrolled' citrate synthase. As these cells are not undergoing active division, the α-oxoglutarate produced may not be required and therefore is excreted into the growth medium, whereupon it is utilised by the glutamate auxotrophs.

This proposal would account for the occurrence of cross-feeding of

the glutamate auxotroph on solid, but not in liquid, medium. The reason why cross-feeding was not observed until the third day of incubation may be because the proportion of non-growing cells in the revertant colony has to reach a high level to produce sufficient α-oxoglutarate.

In conclusion, a number of strains of E. coli and A. lwoffi have been isolated which produce citrate synthases with altered properties. These properties have been intensively studied and some important structure-function relationships have been indicated. A more detailed study of the proteins should give information concerning subunit association and allow the identification of important groups or structural features for catalysis and allosteric regulation by NADH and α-oxoglutarate. The study of the physiological roles of the in vitro observations concerning the revertant enzymes has made some progress but much further work remains to be undertaken in this area. Although it has been demonstrated that E. coli revertants which produce an enzyme which is insensitive to α-oxoglutarate do overproduce this, or a related compound, it is clear that many problems are far from solved and that the in vivo significance of many properties remains unconfirmed.

No work has been attempted with the A. lwoffi revertants producing citrate synthases which lack sensitivity to NADH. If , as suggested, the consequence of the loss of this property is slight it may be possible, using mixed populations of these revertants and A. lwoffi wild type, in a continuous culture fermenter, to show a selective advantage for the wild type organism over a period of time.

The work with Type E2 and E3 revertants has shown that these are probably 'leaky' mutants producing a very inefficient enzyme which is insensitive to allosteric inhibition by NADH and α-oxoglutarate. This inefficient enzyme may indicate why the lack of NADH sensitivity in E.

coli did not result in a serious physiological imbalance. Indeed, it may be speculated that a revertant producing a more active enzyme which was not regulated by NADH may not be viable, thus explaining why such mutants were not isolated in the present work. However, this is purely speculative and more work is necessary to devise a selection for such revertants; these should prove more suitable for testing the importance of the allosteric inhibitions and thereby clarify some of the findings.

When this project was first undertaken, the feasibility of the genetical approach was untried. The isolation of a citrate synthase deficient strain of a strict aerobe had not been reported and such a strain may not have been viable. The method used to obtain revertants having citrate synthases with altered properties had not been used in this system and may have proved unsuccessful. The fact that citrate synthase deficient mutants of A. lwoffi were obtained, and that revertants of E. coli and A. lwoffi were isolated which possessed a citrate synthase with altered properties fully justifies the original approach. I think that the mutants obtained in this work should prove valuable in further studies on citrate synthase and, that the success of the method should encourage other workers in this laboratory to embark on a similar study using different organisms and mutants.

APPENDIX I.

The four programs listed below were originally obtained from Professor W.W. Cleland, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin. The programs have been modified slightly to make them more suitable for the present work.

Program I - (GRAPH) was used when reciprocal plots of initial velocity vs. substrate concentration were linear; the apparent K_m value was computed.

Program II - (SIGM) was used in the calculation of the $S_{0.5}$ value for a substrate in cases where reciprocal plots of initial velocity vs. substrate concentration were non-linear.

Program III - (COMPG) was used in the calculation of the K_i value for a competitive inhibitor of an enzyme.

Program IV - (LINE) was used in the calculation of the least squares fit of data to a straight line.

In addition a graphical representation of the data was obtained from each program using a Culham Laboratories Ghost Package. An example of how this was achieved can be seen from the Subroutine GPLOT of the Program GRAPH.

During the present work I have adapted a number of programs used in the department to give graphical output whenever this is required. These programs are being used for undergraduate teaching, in theoretical studies of protein synthesis and in the prediction of protein secondary structure from amino acid sequence. All graphs presented in this thesis were produced using the computer graph plotting facilities.

PROGRAM I

```
      PROGRAM GRAPH (INPUT,OUTPUT,TAPE7=INPUT,TAPE2=OUTPUT)
     DIMENSION V(20), A(20), W(20), S(3,4), Q(3), SM(3), SS(3),
    2RECV1(20,8), RECV2(20,8), RECA(20,8), RECA1(20,8), NC(8),
    3RECK(8), Y(20), SV1(20), SV2(20),PHRASE(8)
     WRITE (2,100)
     WRITE (2,119)
 119 FORMAT (38H THIS IS THE DATA SET FOR GRAPH NO   1)
100  FORMAT(35H FIT TO HYPERBOLA V=VMAX*A/(K+A)   ///)
  11 FORMAT (I3, 17X, 42H                                                )
300  FORMAT(//     )
     KOUNT=1
     JJ = 0
     LL=1
     KK=1
     NGRAPH=0
      CALL PAPER(1)
  14 PRINT(2,*)"ENTER THE NUMBER OF POINTS"
     READ (7,*) NP
     IF (NP) 99,99,12
  12 PRINT(2,*)"ENTER ONE SUBSTRATE CONC. & ONE VELOCITY"
     IF (NP.GT.100) NGRAPH = 1
     IF (NP.GT.100) NP=NP-100
     M = 1
     N = 2
     P = NP-N
     N1 = N+1
     N2 = N+2
     GO TO 2
  15 READ (7,*) A(I), V(I)
     W(I) = 1.0
     Q(1) = V(I)**2/A(I)
     Q(2) = V(I)**2
     Q(3) = V(I)
     GO TO 13
  16 CK = S(1,1) / S(2,1)
     JJ = JJ+1
     NC(JJ)=NP
      WRITE(2,300)
     WRITE (2,11)  JJ
     NT = 0
     M = 2
     GO TO 2
  17 D = CK + A(I)
     Q(1) = A(I) / D
     Q(2) = Q(1) / D
     Q(3) = V(I)
     GO TO 13
  18 CK = CK - S(2,1) / S(1,1)
     NT = NT + 1
     IF (NT-5) 2,21,21
  21 S2 = 0
     DO 22 I=1,NP
  22 S2 = S2 + (V(I) - S(1,1)*A(I) / (CK+A(I) ))**2 * W(I)
     S2 = S2 / P
```

continued.

PROGRAM I (cont.)

```
      S1 = SQRT  (S2)
      SL = CK / S(1,1)
      VINT = 1. / S(1,1)
      VK = 1. / SL
      DO 10 J=2,N1
      DO 10 K=1,N
 10   S(K,J) = S(K,J) * SM(K) * SM(J-1)
      SEVT = S1 * SQRT ( S(1,2) )
      SECK = S1 * SQRT ( S(2,3) ) / S(1,1)
      SEVI=SEVT/S(1,1)**2
      S(1,3) = S1*SQRT (CK**2*S(1,2) + S(2,3) + 2.*CK*S(1,3) )
      SESL = S(1,3) / S(1,1)**2
      SEVK = S(1,3) / CK**2
      WCK = 1. / SECK**2
      WV=1./SEVT**2
      WSL = 1. / SESL**2
      WVI = 1. / SEVI**2
      WVK = 1. / SEVK**2
      WRITE (2,30)  CK, SECK, WCK
      WRITE (2,31)  S(1,1),SEVT,WV
      WRITE (2,32)  SL, SESL, WSL
      WRITE (2,33)  VINT, SEVI, WVI
      WRITE (2,34)  VK, SEVK, WVK
      WRITE (2,35)  S2, S1
 30   FORMAT (7H K   = ,F12.6,13H S.E.(K)   = ,F11.6,5H W = ,E14.5 )
 31   FORMAT (7H V   = ,F12.6,13H S.E.(V)   = ,F11.6,5H W = ,E14.5 )
 32   FORMAT (7H K/V = ,F12.6,13H S.E.(K/V) = ,F11.6,5H W = ,E14.5 )
 33   FORMAT (7H 1/V = ,F12.6,13H S.E.(1/V) = ,F11.6,5H W = ,E14.5 )
 34   FORMAT (7H V/K = ,F12.6,13H S.E.(V/K) = ,F11.6,5H W = ,E14.5 )
 35   FORMAT (12H VARIANCE = ,E14.5,10H  SIGMA = ,F12.7// )
      WRITE(2,104)
 104  FORMAT(  70H     V          S        BEST V      1/V        1/S       RE
     2ST 1/V           )
      DO 50 I=1,NP
      Y(I) = S(1,1)*A(I) / (A(I)+CK)
      RECV1(I,JJ)=1./V(I)
      RECV2(I,JJ)=1./Y(I)
      RECA(I,JJ)=1./A(I)
      RECA1(I,JJ)=RECA(I,JJ)
      SV1(I) = A(I) / V(I)
      SV2(I) = A(I) / Y(I)
 105  FORMAT(9F10.5)
  50  WRITE (2,105) V(I),A(I),Y(I),RECV1(I,JJ),RECA(I,JJ),
     2RECV2(I,JJ)
      RECK(JJ)=1./CK
      IF (NGRAPH.EQ.1) CALL GPLOT (JJ,NC,RECA,RECA1,RECV1,RECV2,
     2RECK,KOUNT,PHRASE,NGRAPH)
      GO TO 14
C   MATRIX SOLUTION SUBROUTINE
   2  DO 3 J=1,N2
      DO 3 K=1,N1
   3  S(K,J) = 0
      DO 4 I=1,NP
      GO TO (15,17), M
  13  DO 4 J =1,N1
      DO 4 K=1,N
   4  S(K,J) = S(K,J) + Q(K)*Q(J)*W(I)
      DO 5 K=1,N
   5  SM(K) = 1. / SQRT (S(K,K) )
      SM(N1) = 1.0
      DO 6 J=1,N1
      DO 6 K=1,N
   6  S(K,J) = S(K,J) * SM(K) * SM(J)
      SS(N1) = -1.0
      S(1,N2) = 1.0
```

continued.

PROGRAM I (cont.)

```
      DO 8 L = 1,N
      DO 7 K = 1,N
 7    SS(K) = S(K,1)
      DO 8 J = 1,N1
      DO 8 K= 1,N
 8    S(K,J) = S(K+1,J+1) - SS(K+1) * S(1,J+1) / SS(1)
      DO 9 K=1,N
 9    S(K,1) = S(K,1) * SM(K)
      GO TO (16,18), M
99    WRITE (2,36)  JJ
36    FORMAT (23H PROGRAM COMPLETED FOR ,I4, 6H LINES  )
      CALL GREND
      STOP
      END
      SUBROUTINE GPLOT (N,NPN,X,X1,Y,Y1,R,K,PHRASE,NGRAPH)
       DIMENSION NPN(8), X(20,8), Y(20,8), X1(20,8), Y1(20,8), R(8)
     2,X1MAX(8), Y1MAX(8)
      YMAX=0.0
      YTMAX=0.0
      XMAX = 0.0
      RMAX=0.0
      DO 1 J=1,N
      NP=NPN(J)
      Y1MAX(J)=0.0
      DO 2 I=1,NP
      IF(X(I,J).GT.XMAX)XMAX=X(I,J)
      IF (Y(I,J).GT.YMAX) YMAX=Y(I,J)
      IF (Y1(I,J).GT.Y1MAX(J)) Y1MAX(J)=Y1(I,J)
    2 CONTINUE
      IF (Y1MAX(J).GT.YTMAX) YTMAX=Y1MAX(J)
      IF (R(J).GT.RMAX) RMAX=R(J)
    1 CONTINUE
      IF (YMAX.GT.YTMAX) YTMAX=YMAX
      RAX=RMAX
      YAX =YTMAX
      YFAC = 10./YAX
      DO 100 J=1,N
      NP=NPN(J)
      DO 200 I=1,NP
      Y(I,J)=Y(I,J)*YFAC
  200 Y1(I,J)=Y1(I,J)*YFAC
  100 Y1MAX(J)=Y1MAX(J)*YFAC
      YAX=YAX*YFAC
      L=1
      M=1
      NN=1
      D = 1. / (12.5*2.54)
      CALL DENSTY (2)
      CALL PSPACE (2.*D,25.*D,4.*D,26.*D)
      XM = XMAX /10**6
      LCOUNT = 1
   97 XM = XM * 10.
      IF(XM.GT.10)GOTO 107
      LCOUNT = LCOUNT +1
       GOTO 97
  107 IF(XM.GT.90)XAX=100.
      DO 108 I = 10,80,10
      IX = 100 -I
  108 IF(XM.LT.IX)XAX=IX
      IF(XM.LT.15.)XAX=15.
```

continued.

PROGRAM I (cont).

```
      XAX = XAX * 10**6/(10**LCOUNT)
      YAX=10.
      CALL CTRMAG (12)
      LX=5
      LY=5
      CALL MAP(-RAX,(XAX+.001),0.0,(YAX+.001))
      CALL AXESSI ((XAX)/LX, (YAX)/LY)
      CALL CTRSET (4)
      DO 22 J=1,N
      NP=NPN(J)
      NOC=49+J
      DO 21 I=1,NP
   21 CALL PLOTNC (X(I,J), Y(I,J),NOC)
      CALL POINT (-R(J),0.)
   22 CALL JOIN (XMAX,Y1MAX(J))
      CALL PSPACE (0.*D,30.*D,0.*D,30.*D)
      CALL MAP (0.,30.,0.,30.)
      CALL BORDER
      CALL CTRSET (1)
      PRINT (2,*) "IF SUBSTRATE IS ACETYL-COA(1), OXALOACETATE(2)"
      READ (7,83) J
   83 FORMAT (I1)
      IF (J.EQ.1) GOTO 37
      CALL PCSEND (20.,1.5,20H 1/[OXALOACETATE], (20)
      GOTO 43
   37 CALL PCSEND (20.,1.5,12H 1/[ACETYL-C,12)
      CALL CTRSET (2)
      CALL TYPENC (25)
      CALL CTRSET (1)
      CALL TYPECS (5HA], (,5)
   43 CALL CTRSET (2)
      CALL TYPENC (23)
      CALL CTRSET (1)
      CALL TYPENC (23)
      CALL TYPENC (49)
      XFAC = 23./(RAX+XAX)
      RDIS=(RAX*XFAC)-1.
      CALL CTRORI (1.)
      CALL PCSEND (RDIS,20.,11H 1/VELOCITY,11)
      K=K+1
      WRITE (2,119) K
      CALL FRAME
      N=0
      CALL CTRORI (0.0)
      NGRAPH = 0
  119 FORMAT (35H1 THIS IS THE DATA SET FOR GRAPH NO,I4)
   23 RETURN
      END
```

PROGRAM II

```
      PROGRAM SIGM (INPUT,OUTPUT,TAPE7=INPUT,TAPE 8= OUTPUT)
      DIMENSION V(20), A(20), S(4,5), Q(4,20), SM(4), SS(4),
     2RECV1(20,10), RECV2(20,10), RECA(20,10), NA(10), CIV(10)
     3,SHALF(6), Y(20), RECA1(20,6), RECF(6)
   11 FORMAT(13,I17,46H                                              )
    1 FORMAT(2F10.5)
      CALL DARRAY(2000,1000,1000,1000)
      JJ = 1
   14 READ(7,11) NP, NO
      NGRAPH =0
      IF (NP.LE.0) GO TO 99
      IF (NP.LT.100) GO TO 12
      NGRAPH=1
      NP=NP-100
      CALL PAPER (1)
   12 N = 1
      N = 3
      P = NP-N
      N1= N+1
      N2= N+2
      GO TO 2
   15 READ(7,1) V(I), A(I)
      Q(1,I) = V(I)**2/A(I)**2
      Q(2,I) = V(I)**2/A(I)
      Q(3,I) = V(I)**2
      Q(4,I) = V(I)
      GO TO 13
   16 CA = S(1,1) / S(3,1)
      CB = .5*S(2,1) / S(3,1)
      JJ = JJ+1
      NA(JJ)=NP
      WRITE(2,11) JJ,NP
      NT = 0
      M = 2
      GO TO 2
   17 D   = CA + 2.*CB*A(I) + A(I)**2
      Q(1,I) = A(I)**2/D
      Q(2,I) = A(I)**2/D**2
      Q(3,I) = A(I)**3/D**2
      Q(4,I) = V(I)
      GO TO 13
   18 CA = CA - S(2,1)/S(1,1)
      CB = CB - S(3,1)/(2.*S(1,1))
      NT = NT+1
      IF(NT-5) 2, 87, 87
   87 S2 = 0
      CV2= S(1,1)
      C1V= 1./S(1,1)
      DO 82 I=1,NP
   82 S2 = S2+(V(I)-CV2*A(I)**2/(CA+2.*CB*A(I)+A(I)**2))**2
      S2 = S2/D
      S1 = SQRT(S2)
      DO 10 J=2,4
      DO 10 K=1,3
   10 S(K,J) = S(K,J)*SM(K)*SM(J-1)
      SEA = S1*SQRT(S(2,3))/ S(1,1)
      SEB = .5*S1*SQRT(S(3,3)) /S(1,1)
      SEV = S1*SQRT(S(1,1))
      SE1V= SEV/S(1,1)**2
      WV  = 1./SEV**2
      W1V = 1./SE1V**2
      WRITE(2,37) CA, SEA
      WRITE(2,38) CB, SEB
      WRITE(2,39) CV2, SEV, WV
      WRITE(2,40) C1V, SE1V, W1V
   37 FORMAT(6H   A = ,F12.8,15H    S.E.(A)    = , F11.8)
   38 FORMAT(6H   B = ,F12.8,15H    S.E.(B)    = , F11.8)
   40 FORMAT(6H 1/V= ,F12.8,15H    S.E.(1/V) = , F11.8,6H   W = ,E14.5 )
   39 FORMAT(6H   V = ,F12.8,15H    S.E.(V)    = , F11.8,6H   W = ,E14.5 )
      CIV(JJ)=C1V
      NA1=NA(JJ)
      NA2=NA(JJ)+2
      CB=CB*2
      FUN =SQRT(CB**2+4*CA)
      X1=  (-CB+FUN ) /(-2)
      X2 = (-CB-FUN) / (-2)
      WRITE (2,65) X1,X2
   65 FORMAT (2F10.5)
      IF (X1.GT. .) SHALF(JJ)=X1
      IF (X2.GT. .) SHALF(JJ)=X2
      IF  (X1.LT.0..AND.X2.LT.0.) SHALF(JJ)=0.
```

continued.

PROGRAM II (cont).

```
      RECV2(2,JJ)=CIV(JJ)
      RECA(2,JJ)= 0.
      RECA1(2,JJ) =0.
      RECV2(1,JJ) =1.
      RECF(JJ) = 1./SHALF(JJ)
      RECA(1,JJ) = -1./SHALF(JJ)
      RECA1(2,JJ) =-1./SHALF(JJ)
      II=3
      DO 50 I=1,NA1
      Y(I) = CV2*A(I)**2 / (CA+2*CB*A(I)+A(I)**2)
      II=I+2
      RECA(I,JJ)=1./A(I)
      RECV1(I,JJ)=1./V(I)
      RECV2(II,JJ)=1./Y(I)
      RECA1(II,JJ)=RECA(I,JJ)
   50 CONTINUE
      KA=0
      RECF(JJ)=1.
      IF (IGRAPH.EQ.1) CALL GPLOT (JJ,NA,RECA,RECA1,RECV1,RECV2,RECF,KA)
      GO TO 14
C     MATRIX SOLUTION SUBROUTINE
    2 DO 3 J=1,NP
      DO 3 K=1,N1
    3 S(K,J) = 0
      DO 4 I=1,MP
      GO TO (13,17), M
   13 DO 4 J =1,N1
      DO 4 K=1,N
    4 S(K,J) = S(K,J) + Q(K,I)*Q(J,I)
      DO 5 K=1,N
    5 SM(K) = 1. / SQRT (S(K,K) )
      SM(NL) = 1.
      DO 6 J=1,NL
      DO 6 K=1,N
    6 S(K,J) = S(K,J) * SM(K) * SM(J)
      SS(NL) = -1.0
      S(1,N2) = 1.0
      DO 8 L = 1,N
    7 SS(K) = S(K,1)
      DO 8 J = 1,N1
      DO 8 K= 1,N
    8 S(K,J) = S(K+1,J+1) - SS(K+1) * S(1,J+1) / SS(1)
      DO 9 K=1,N
    9 S(K,1) = S(K,1) * SM(K)
      GO TO (16,18), M
   18 WRITE (2,56)  JJ
   56 FORMAT (23H PROGRAM COMPLETED FOR ,I4, 6H LINES  )
      CALL GREND
      STOP
      END
```

PROGRAM III

```
      PROGRAM COMPG  (INPUT,OUTPUT, TAPE7=INPUT, TAPE8=OUTPUT)
      DIMENSION V(50) , A(50), CI(50), S(5,6), Q(5,50),SM(5),
     2SS(5), CKI(10), RECV1(50,10), RECV2(50,10), RECA(50), Y(50),
     3CIM(50,10), SL(10), NC(10), SLOPE(50,10), SLOP2(50,10)
      WRITE(2,100)
      WRITE (2,119)
  119 FORMAT (35H THIS IS THE DATA SET FOR GRAPH NO   1)
  100 FORMAT(46H FIT TO     Y = V*A/(K(1+I/KI) + A)          ///)
   11 FORMAT(13,I17,46H                                          )
    1 FORMAT (2F10.5,10X,F10.5)
  300 FORMAT(//    )
  400 FORMAT(/35H ITERATIVE VALUES OF KN,VMAX,AND KI        )
      KOUNT=1
      JJ = 0
      LL=1
   14 READ(7,11) NP, NO
      NGRAPH = 0
      IF (NP-100) 51,51,50
   50 NP=NP-100
      NGRAPH=1
   51 IF(NP) 99,99,12
   12 M = 1
      N = 3
      P = NP-N
      N1= N+1
      N2= N+2
      MT=1
      JJ = JJ+1
      GO TO 2
   15 READ(7,1)  V(I), A(I), CI(I)
      CIM(1,JJ)=CI(1)
      IF (I.GT.1) GO TO 40
   20 Q(1,I) = V(I)**2/A(I)
      Q(2,I) = V(I)**2*CI(I)/A(I)
      Q(3,I) = V(I)**2
      Q(4,I) = V(I)
      GO TO 13
   40 IF (CIM(MT,JJ).NE.CI(I)) MT=MT+1
      CIM(MT,JJ)= CI(I)
      GO TO 20
   16 CV = 1. / S(3,1)
      CK = S(1,1) / S(3,1)
      CKIS = S(1,1) / S(2,1)
      WRITE(2,300)
      WRITE(2,11) JJ, NP
      WRITE(2,400)
      NT = 0
      M  = 2
      GO TO 2
   17 D  = (1. + CI(I)/CKIS)*CK / A(I) + 1.
      Q(1,I) = 1. / D
      Q(2,I) = (1. + CI(I)/CKIS) / A(I) / D**2
      Q(3,I) = CI(I) / A(I) / D**2
      Q(4,I) = V(I)
      GO TO 13
   18 CV = S(1,1)
      CK = CK - S(2,1)/S(1,1)
      CKIS = CKIS*(1. + S(3,1)*CKIS/S(1,1)/CK     )
      WRITE(2,1) CK, CV, CKIS
      NT = NT+1
      IF(NT-J) 2, 87, 87
   87 S2 = 0
      WRITE(2,300)
      DO 82 I=1,NP
   82 S2 =S2+(V(I)-CV/((1.+CI(I)/CKIS)*CK/A(I)+1.))**2
      S2 = S2/P
      S1 = SQRT(S2)
      DO 10  J=2,M1
      DO 10  K=1,M
   10 S(K,J) = S(K,J)*SM(K)*SM(J-1)
      SEV = S1 * SQRT(S(1,2))
      SEK = S1 * SQRT(S(2,3)) / S(1,1)
      SEKIS=S1 * CKIS**2*SQRT(S(3,4)) / S(1,1) / CK
      WV = 1. / SEV**2
      WK = 1. / SEK**2
      WKIS=1. / SEKIS**2
      WRITE(2,43) CK, SEK, WK
      WRITE(2,39) CV, SEV, WV
      WRITE(2,37) CKIS, SEKIS, WKIS
   43 FORMAT(7H    K = ,F12.6 ,15H    S.E.(K)    = ,F11.6,7H    W = ,E14.6 )
   37 FORMAT(7H KIS = ,F12.6 ,15H    S.E.(KIS) = ,F11.6,7H    W = ,E14.6 )
```

continued.

PROGRAM III (cont.)

```
      89  FORMAT(7H   V = ,F12.6 ,15H   S.E.(V)   = ,F11.6,7H   W = ,E14.6 )
          WRITE(2,44) S2
      44  FORMAT(13H VARIANCE = ,E14.5   //)
          WRITE(2,102)
     102  FORMAT( 6HO    A          I          V       BEST V      1/A        1/
     2V        BEST 1/V          )
          CKI(JJ)=CKIS
          MT=1
          SL(1) = CK*(1. + CI(1) /CKIS) /CV
          SLOPE(MT,JJ)=SL(1)
          DO 55 I=1,NP
          Y(I) = CV*A(I) / (CK*(1.+CI(I)/CKIS) + A(I) )
          RECA(I) = 1. / A(I)
          RECV1(I,JJ) = 1./V(I)
          RECV2(I,JJ) = 1./Y(I)
          SL(I) =  CK*(1. + CI(I)/CKIS) / CV
          IF(SLOPE(MT,JJ).NE.SL(I)) MT=MT+1
          SLOPE(MT,JJ)=SL(I)
          SLOP2(MT,JJ)=SLOPE(MT,JJ)
     103  FORMAT(5F14.5)
      55  WRITE (2,103) A(I), CI(I), V(I), Y(I), RECA(I), RECV1(I,JJ),
         2RECV2(I,JJ)
         3,SL(I)
          NC(JJ)=MT
          IF (NGRAPH.EQ.1) CALL GPLOT (NC,CIM,SLOPE,SLOP2,CKI,JJ,
         2KOUNT)
          GO TO 14
C     MATRIX SOLUTION SUBROUTINE
       2  DO 3 J=1,N2
          DO 3 K=1,N1
       3  S(K,J) = 0
          DO 4 I=1,NP
          GO TO (13,17), M
      13  DO 4 J =1,N1
          DO 4 K=1,N
       4  S(K,J) = S(K,J) + Q(K,I)*Q(J,I)
          DO 5 K=1,N
       5  SM(K) = 1. / SQRT (S(K,K) )
          SM(N1) = 1.0
          DO 6 J=1,N1
          DO 6 K=1,N
       6  S(K,J) = S(K,J) * SM(K) * SM(J)
          SS(N1) = -1.0
          S(1,N2) = 1.0
          DO 9 L = 1,N
          DO 7 K = 1,N
       7  SS(K) = S(K,1)
          DO 8 J = 1,N1
          DO 8 K= 1,N
       8  S(K,J) = S(K+1,J+1) - SS(K+1) * S(1,J+1) / SS(1)
          DO 9 K=1,N
       9  S(K,1) = S(K,1) * SM(K)
          GO TO (16,18), M
      99  CALL GPLOT (NP,CIM,SLOPE,SLOP2,CKI,JJ,KOUNT)
          WRITE (2,36) JJ
      36  FORMAT (23H PROGRAM COMPLETED FOR ,I4, 6H LINES          )
          STOP
          END
```

PROGRAM IV

```
      PROGRAM LINE (INPUT,OUTPUT,TAPE7=INPUT,TAPE2=OUTPUT)
C        LEAST SQUARE FIT TO STRAIGHT LINE WITH ERRORS 3-24 -62 CLELAND
         DIMENSION V(100), A(100), W(100)
      WRITE(2,100)
  100    FORMAT (25H FIT TO LINE Y=A*X+B                ///  )
   11 FORMAT(I3,E17.4,4H                                          )
    1    FORMAT (F10.5, F10.5, F10.5)
         JJ=0
   14 READ(7,11) NP,NO
         IF (NP) 99,99,12
   12    S1=0
         S2=0
         S3=0
         S4=0
         PP=0
         DO 2 I=1,NP
        READ(7,1) V(I), A(I), W(I)
      WRITE(2,1) V(I), A(I)
   17    IF (W(I)) 15, 15, 16
   15    W(I)=1.
   16    S1 =S1 + W(I)*A(I)
         S2=S2 + W(I)*A(I)**2
         S3 = S3+ W(I)*V(I)*A(I)
         S4 = S4 + W(I)*V(I)
    2    PP=PP + W(I)
         DE = PP*S2 - S1**2
          AL = PP*S3 - S1*S4
         BE = S2*S4 -S1*S3
         AA = AL/DE
         B = BE/DE
         VH = B/AA
         RB = 1./    B
         AB =AA/B
         S = 0
         DO 3 I=1, NP
    3    S = S+ (V(I) -AA*A(I) - B)**2*W(I)
         P = NP-2
         S = S/P
         SS = SQRT   (S/DE)
         VA = SS*SQRT (PP)
         VB = SS*SQRT (S2)
         VRB = VB/B**2
         VBA = SS*SQRT (S2+VH**2*PP + 2.*VH*S1)/AA
          VAB = VBA*AB**2
         JJ = JJ+1
   40 FORMAT(25H                SLOPE =   A   =, F12.9, 13H    S.E.(A)   =,F12.7 )
   41 FORMAT(25H VERT. INTERCEPT=   B   =, F12.8, 13H    S.E.(B)   =,F12.7 )
   42 FORMAT(25H  HOR. INTERCEPT= B/A =, F10.6, 13H    S.E.(B/A)=,F12.7 )
   43 FORMAT(25H                  1/B =, F10.6, 13H    S.E.(1/B)=,F12.7 )
   44 FORMAT(25H                  A/B =, F13.6, 13H    S.E.(A/B)=,F12.7 )
   45 FORMAT(12H VARIANCE =   ,E14.6       ///)
      WRITE(2,11) JJ, NP
      WRITE(2,40) AA, VA
      WRITE(2,41)   B , VB
      WRITE(2,42) VH, VBA
      WRITE(2,43) RB, VRB
      WRITE(2,44) AB, VAB
      WRITE(2,45) S
       CALL STATFIT(V,A,NP)
         GO TO 14
   36    FORMAT (23H PROGRAM COMPLETED FOR ,I4, 6H LINES       )
   99 WRITE(2,36) JJ
         STOP
         END
```

BIBLIOGRAPHY.

Amarasingham, C.R. & Davis, B.D. (1965)
J. Biol. Chem. 240, 3664-3668

Ashworth, J.M. & Kornberg, H.L. (1966)
Proc. Roy. Soc. Ser. B 165, 179-188

Ashworth, J.M., Kornberg, H.L. & Nothman, D.L. (1965)
J. Mol. Biol. 11, 654-657

Atkinson, D.E. (1968)
In "Metabolic Roles of Citrate" (T.W. Goodwin, ed.), pp23-40
Academic Press, New York.

Axelrod, B. & Beevers, H. (1972)
Biochim. Biophys. Acta 256, 175-178

Barbareschi, D., Longo, G.P., Servettaz, O., Zulian, T. & Longo, C.P. (1974)
Plant Physiol. 53, 802-807

Bascomb, S., Lapage, S.P., Curtis, M.A. & Wilcox, W.R. (1973)
J. Gen. Microbiol. 77, 291-315

Bogin, E. & Wallace, A. (1966)
Biochim. Biophys. Acta 128, 190-192

Bowen, T.J. (1970)
In "An Introduction to Ultracentrifugation", p.34. Wiley-Interscience,
London.

Brice, C.B. & Kornberg, H.L. (1967)
Proc. Roy. Soc. Ser.B 168, 281-292

Chase, J.F.A. & Tubbs, P.K. (1969)
Biochem. J. 111, 225-235

Clements, P.R., Wallace, J.C. & Keech, D.B. (1976)
Analyt. Biochem. 72, 326-331

Cohen, G.N. (1969)
Curr. Top. Cell. Regul. 1, 183-231

Cohen, R. & Mire, M. (1971)
Eur. J. Biochem. 23, 267-275

Danson, M.J. (1974)
Ph.D. Thesis, University of Leicester

Danson, M.J. & Weitzman, P.D.J. (1973)
Biochem. J. 135, 513-524

Davis, B.D. (1961)
Cold Spring Harbor Symp. Quant. Biol. 26, 1-10

Davis, B.J. (1964)
Ann. N.Y. Acad. Sci. 121, 404-427

Desphande, W.M. & Ramakrishnan, C.V. (1959)
J. Biol. Chem. 234, 1929-1930

Dixon, M. & Webb, E.C. (1964)
In "Enzymes", p.551. Academic Press Inc., New York.

Eidels, L. & Preiss, J. (1970)
J. Biol. Chem. 245, 2937-2945

Ellman, G.L. (1959)
Arch. Biochem. Biophys. 82, 70-77

Flechtner, V.R. & Hanson, R.S. (1969)
Biochim. Biophys. Acta 184, 252-262

Flechtner, V.R. & Hanson, R.S. (1970)
Biochim. Biophys. Acta 222, 253-264

Friedman, R.B., Bruce, D., MacLowry, J. & Brenner, V. (1973)
Amer. J. Clin. Pathol. 60, 395-403

Garland, P.B., Shepherd, D., Nicholls, D.G., Yates, D.W. & Light, P.A. (1969)
In "Citric Acid Cycle" (J.M. Lowenstein, ed), pp.163-212.
Dekker, New york.

Gibbons, I., Anderson, P.J. & Perham, R.N. (1970)
FEBS Letters 10, 49-53

Glauert, A.M. & Thornley, M.J. (1966)
J. Roy. Microbiol. Soc. 85, 449-453

Gray, C.T., Wimpenny, J.W.T. & Mossman, M. (1966)
Biochim. Biophys. Acta 117, 33-41

Hall, E.R. & Weitzman, P.D.J. (1977)
Biochem. Biophys. Res. Commun. 74, 1613-1617

Harford, S., Jones, D. & Weitzman, P.D.J. (1976)
J. Appl. Bacteriol. 41, 465-471

Harford, S. & Weitzman, P.D.J. (1975)
Biochem. J. 151, 455-458

Hathaway, J.A. & Atkinson, D.E. (1965)
Biochem. Biophys. Res. Commun., 20, 661-665

Hayes, W. (1970)
In "The Genetics of Bacteria and their Viruses", 2nd Edition, pp.217-219.
Blackwell Scientific Publications, Oxford and Edinburgh.

Hochachka, P.W. & Lewis, J.K. (1970)
J. Biol. Chem. 245, 6567-6573

Hopwood, D.A. (1970)
In "Methods in Microbiology" (J.R. Norris & D.W. Ribbons, eds.) Vol. 3A, pp. 363-434. Academic Press, London and New York

Hostalek, Z., Ryabushko, T.A., Cudlin, J. & Vanek, Z. (1969)
Folia Microbiol. 14, 121-127

Jangaard, N.O., Unkeless, J. & Atkinson, D.E. (1968)
Biochim. Biophys. Acta 151, 225-235

Johnson, D.E. & Hanson, R.S. (1974)
Biochim. Biophys. Acta 350, 336-354

Jones, D. & Weitzman, P.D.J. (1974)
Int. J. Syst. Bacteriol. 24, 113-117

Krebs, H.A. (1970)
Advan. Enzyme Regul. 8, 335-353

Krebs, H.A., Gurin, S. & Eggleston, L.V. (1952)
Biochem. J. 51, 614-627

Krebs, H.A. & Johnson, W.H. (1937)
Enzymologia 4, 148-156

Krebs, H.A. & Lowenstein, J.M. (1960)
In "Metabolic Pathways", 2nd Edition (D.M. Greenberg, ed), 1, 129-203. Academic Press, London and New York.

Kornberg, A. (1955)
In "Methods in Enzymology" (S.P. Colowick & N.O. Kaplan, eds.) 1, 705-707. Academic Press Inc., New York.

Kornberg, H.L. (1959)
Ann. Rev. Microbiol. 13, 49-78

Kosicki, G.W. & Lee, L.P.K. (1966)
J. Biol. Chem. 241, 3571-3574

Kun, E. (1969)
In "Citric Acid Cycle" (J.M. Lowenstein, ed), pp.297-339.
Dekker, New York.

Kun, E., Grassetti, D.R., Fanshier, D.W. & Featherstone, R.M. (1958)
Biochem. Pharmacol. 1, 207-212

Lakshmi, T.M. & Helling, R.B. (1976)
J. Bacteriol. 127, 76-83

LaNoue, K.F., Walajtys, E.I. & Williamson, J.R. (1973)
J. Biol. Chem. 248, 7171-7183

Lederberg, J. & Lederberg, E,M. (1952)
J. Bacteriol. 63, 399-406

Lehninger, A.L. (1946)
J. Biol. Chem. 164, 291-306

Lennox, E.S. (1955)
Virology 1, 190-206

Lee, L.P.K. & Kosicki, G.W. (1967)
Biochim. Biophys. Acta 139, 195-198

Levitzki, A. & Koshland, D.E.Jnr. (1970)
Biochim. Biophys. Acta 206, 473-475

Lopes-Cardozo, M. & van den Bergh, S.G. (1972)
Biochim. Biophys. Acta 283, 1-15

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951)
J. Biol. Chem. 63, 47-48

Lucas, C. & Weitzman, P.D.J. (1975)
Trans. Biochem. Soc. 3, 379-381

Lucas, C. & Weitzman, P.D.J. (1977)
Arch. Microbiol. (in press)

Mahlen, A. (1972)
Eur. J. Biochem. 29, 60-66

Martin, R.G., Ames, B.N. & Garry, B.J. (1961)
Federation Proc. 20, 225.

Massey, V. (1953)
Biochem. J. 53, 67-71

Miller, J.H. (1972)
In "Experiments in Molecular Genetics", Cold Spring Harbor Laboratory, New York.

Moriyama, T. & Sanwal, P.A. (1971)
J. Biol. Chem. 246, 3217-3223

Molholt, B. (1967)
Micob. Genet. Bull. 27, 8-9

Moyed, H.S. (1961)
Cold Spring Harbor Symp. Quant. Biol. 26, 323-329

Northrop, D.B. & Cleland, W.W. (1974)
J. Biol. Chem. 249, 2928-2931

Ochoa, S. (1955)
In "Methods in Enzymology" (S.P. Colowick & N.O. Kaplan, eds.) 1, 735-739.
Academic Press Inc., New York.

Olson, M.S. & Williamson, J.R. (1971)
J. Biol. Chem. 246, 7794-7803

Perrin, D., Jacob, F. & Monod, J. (1960)
C. R. Acad. Sci. (Paris) 251, 155-157

Pesce, A., McKay, R.H., Stolzenbach, F., Cahn, R.D. & Kaplan, N.O. (1964)
J. Biol. Chem. 239, 1753-1761

Peters, R.A. (1952)
Proc. Roy. Soc. B 139, 143-170

Peters, R.A. (1955)
Bull. John Hopkins Hosp. 97, 21-42

Racker, E. (1950)
Biochim. Biophys. Acta 4, 211- 214

Ramakrishnan, C.V. & Martin, S.M. (1954)
Can. J. Biochem. Physiol. 32, 434-439

Randle, P.J., England, P.J. & Denton, R.M. (1970)
Biochem. J. 117, 677-695

Reeves, R.E. & Sols, A. (1973)
Biochem. Biophys. Res. Commun. 50, 459-466

Reynolds, E.S. (1963)
J. Cell. Biol. 17, 208-212

Rivett, D.E.A. (1953)
J. Chem. Soc. p.3710

Roberts, R.B., Cowie, D.B., Britten, R., Bolton, E. & Abelson, P.H. (1953)
Proc. Nat. Acad. Sci. 39, 1013-1026

Sanwal, B.D. (1970)
Bacteriol. Rev. 34, 20-39

Sarkissian, I.V. & Boatwright, D.T. (1974)
Comp. Biochem. Physiol. B 49, 325-333

Shepherd, B. & Garland, P.B. (1969)
Biochem. J. 114, 597-610

Siegel, L. & Englard, S. (1961)
Biochim. Biophys. Acta 54, 67-76

Srere, P.A. (1969)
Biochem. Med. 3, 61-72

Srere, P.A. (1971)
Advan. Enzyme Regul. 9, 221-233

Srere, P.A. (1972)
Curr. Top. Cell. Regul. 5, 229-283

Srere, P.A., Brazil, H. & Gonen, L. (1963)
Acta Chem. Scand. 17, S129-S134

Stadtman, E.R. (1957)
In "Methods in Enzymology" (S.P. Colowick & N.O. Kaplan, eds.) 3, 931-932

Stern, J.R. & Ochoa, S. (1951)
J. Biol. Chem. 191, 161-172

Sumner, J.B. & Gralen, J. (1935)
J. Biol. Chem. 125, 33-36

Swissa, M. & Benziman, M. (1976)
Biochem. J. 153, 173-179

Taylor, A.L. & Trotter, C.D. (1972)
Bacteriol. Rev. 36, 504-524

Taylor, B.F. (1973)
Arch. Microbiol. 92, 245-249

Taylor, B.F., Hoare, D.S. & Hoare, S.L. (1969)
Bacteriol. Proc. pp.63-64

Tong, E.K. & Duckworth, H.W. (1975)
Biochem. 14, 235-241

Umbarger, H.E. (1956)
Science 123, 848

Weitzman, P.D.J. (1966a)
Biochem. J. 101, 44c-45c

Weitzman, P.D.J. (1966b)
Biochim. Biophys. Acta 128, 213-215

Weitzman, P.D.J. (1969a)
In "Methods in Enzymology" (S.P. Colowick & N.O. Kaplan, eds.) 13, 22-25

Weitzman, P.D.J. (1969b)
In "Methods in Enzymology" (S.P. Colowick & N.O. Kaplan, eds.) 13, 365-368

Weitzman, P.D.J. & Danson, M.J. (1976)
Curr. Top. Cell. Regul. 10, 161-204

Weitzman, P.D.J. & Dunmore, P. (1969a)
Biochim. Biophys. Acta 171, 198-200

Weitzman, P.D.J. & Dunmore, P. (1969b)
FEBS Letters 3, 265-267

Weitzman, P.D.J. & Hewson, J.K. (1973)
FEBS Letters 36, 227-231

Weitzman, P.D.J. & Jones, D. (1968)
Nature 219, 270-272

Weitzman, P.D.J. & Jones, D. (1975)
J. Gen. Microbiol. 89, 187-190

Whitfield, H.J.Jnr., Martin, R.G. & Ames, B.N. (1966)
J. Mol. Biol. 21, 335-355

Wright, J.A., Maeba, P. & Sanwal, B.D. (1967)
Biochem. Biophys. Res. Commun. 29, 34-38

Wright, J.A. & Sanwal, B.D. (1971)
J. Biol. Chem. 246, 1689-1699

Yonetani, T. & Theorell, H. (1964)
Arch. Biochem. Biophys. 106, 243-251

I The mode of action of a number of inhibitors of citrate synthase has been studied. The results suggest that α-oxoglutarate acts as an allosteric inhibitor of the enzyme from Gram negative facultatively anaerobic bacteria, but as an isosteric inhibitor of the enzyme from other sources. Similarly, NADH has been shown to be a powerful allosteric inhibitor of citrate synthases from Gram negative bacteria, but an isosteric inhibitor of the enzyme from Gram positive bacteria and eucaryotes. Other nucleotides have been shown to act only as isosteric inhibitors of citrate synthases from all sources examined.

II Techniques have been developed which facilitate the rapid determination of the regulatory properties of a citrate synthase and its molecular size. The striking correlation between these properties and the Gram reaction of bacteria is discussed. It has been proposed that the rapid techniques described here could be of value in bacterial taxonomic studies and for bacteriological identification.

III A citrate synthase deficient mutant of Escherichia coli has been isolated using a penicillin enrichment technique. A method has been developed which allows for the direct selection of citrate synthase deficient mutants by virtue of their intrinsic resistance to fluoroacetate. Two citrate synthase deficient strains of Acinetobacter lwoffi have been isolated using this method.

IV A number of revertants, which have regained citrate synthase activity, were isolated from these citrate synthase deficient strains. A comparative study of the molecular, catalytic and regulatory properties of these enzymes has been carried out and possible structure-function relationships have been discussed.

V Using revertant strains of E. coli which produce citrate synthases with regulatory properties different to those of the enzyme from the wild type organism, an attempt has been made to investigate the physiological significance of this altered regulatory behaviour of the enzyme.

Revertants which have a citrate synthase which is not inhibited by α-oxoglutarate (an allosteric inhibitor of the enzyme from E. coli wild type) appear to overproduce and excrete this (or a related) compound under certain growth conditions. Such a finding does suggest that the α-oxoglutarate inhibition of citrate synthase has a physiological role in the regulation of this enzyme in this organism.