The Enzymes of the Glyoxylate Bypass Operon of *Escherichia coli* ML308

BY

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For my parents

CONTENTS

CHAPTER TWO - MATERIALS AND METHODS

LIST OF FIGURES

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Summary

1. The glyoxylate bypass allows Escherichia coli to generate precursors for biosynthesis during growth on acetate. During operation of the bypass there is competition for isocitrate between isocitrate dehydrogenase (ICDH) and isocitrate lyase (ICL). Previous studies had shown that ICDH is regulated by a phosphorylation/dephosphorylation mechanism. However, ICL and its possible contribution to regulation at this branchpoint had not been studied.

2. A quick, reliable procedure was developed for purification of ICL from acetate-grown Escherichia coli ML308. 10-12 mg of pure enzyme could be obtained from 20g wet weight of bacteria. The specific activity was 35-40μmol/min/mg of protein at 37°C.

3. The M_r of the native enzyme was 177 000, determined by gel filtration and 180 000, determined by analytical ultracentrifugation. The subunit M_r was 45 000. This implies that Escherichia coli ICL is a tetramer.

4. The optimum pH for ICL activity was pH 7.3, within the physiological range for Escherichia coli.

5. The kinetics of binding of substrates, products and/or their analogues was studied for the cleavage and the condensation reactions of ICL. The results show that, unlike the enzyme from other sources, ICL from Escherichia coli obeys a random order mechanism in which an enzyme-isocitrate-succinate ternary complex can be formed.

6. The K_m of ICL for D_s-isocitrate was 0.063mM at pH7.3. The K_m was sensitive to changes in pH and also to the presence of inorganic anions such as Cl^- and SO_4^{2-}.

7. Several compounds were found to inhibit ICL but these effects could be ascribed to structural similarities between the inhibitors and the substrates for the enzyme. 2-Oxoglutarate and phosphoenolpyruvate were shown to be succinate analogues and 3-phosphoglycerate was shown to be a glyoxylate analogue. The intracellular concentration of 3-phosphoglycerate suggests that this compound may inhibit ICL significantly in intact cells. However, none of the effects observed could be attributed to the existence of an allosteric regulatory site.

8. There was no evidence that ICL is a phosphorylated molecule nor that ICDH kinase/phosphatase can affect ICL.

9. A recombinant plasmid carrying an 11 kilobase ClaI-ClaI fragment of genomic DNA which complements an aceA (ICL) mutation was constructed by Dr.E.M.T. El-Mansi. Measurement of enzyme activities in crude cell extracts and in vitro transcription-translation experiments showed that this plasmid (pEM9) encodes the structural genes of the glyoxylate bypass operon, namely malate synthase A (MS-A), ICL and ICDH kinase/phosphatase and in that order.

10 ICL was purified from the overexpressing strain KAT-1/pEM9 following the procedure developed for ICL from Escherichia coli ML308 up to the phenyl-Sepharose step. ICL from both sources was identical by peptide-mapping, K_m, NH_2-terminal sequence and amino acid composition. The pI of both ICLs was 4.4 as determined by chromatofocusing on a Fast Protein

Liquid Chromatography Mono P column.

11. Overexpression of MS-A in Escherichia coli KAT-1/pEM9 meant that it could be distinguished from MS-B and a purification was developed for MS-A from Escherichia coli KAT-1/pEM9. The N-terminal amino acid sequence of MS-A was determined.

12. ICDH kinase/phosphatase from Escherichia coli KAT-1/pEM9 was subjected to limited proteolysis by trypsin. Proteolysis proceeded in two stages in which the first cleavage product had lost its ICDH phosphatase activity but retained its ICDH kinase activity. The second cleavage product was completely devoid of both kinase and phosphatase activities. These results could mean that the active sites for ICDH kinase and ICDH phosphatase are different.

ABBREVIATIONS

Abbreviations used in this thesis are those recommended by the Biochemical Society, London, except for those listed below:

BSA	Bovine serum albumin
BCIG	5-Bromo-4-chloro-indoxyl-β-galactoside
DEAE	Diethylaminoethyl
DTT	DL-Dithiothreitol
FPLC	Fast Protein Liquid Chromatography
ICDH	Isocitrate dehydrogenase
ICL	Isocitrate lyase
LDH	Lactate dehydrogenase
MS	Malate synthase
NBRF	National Biomedical Research Foundation
PEP	Phosphoenolpyruvate
PAGE	Polyacrylamide gel electophoresis
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
TEMED	NNN'N'-Tetramethylethylenediamine

CHAPTER ONE - INTRODUCTION

1.1. The glyoxylate bypass in Escherichia coli

1.1.1. Introduction

In common with many other microorganisms, Escherichia coli can utilize acetate or fatty acids as sole source of carbon and energy (e.g. Kornberg, 1966; Klein et al., 1971). Both provide C atoms for entry into the tricarboxylic acid cycle (TCA cycle) in the form of acetyl-CoA.

After entry into the cell, fatty acids are either directly incorporated into complex lipids or catabolized. Fatty acid degradation occurs by the cyclic β-oxidation and thiolytic cleavage of fatty acids, yielding several moles of acetyl-CoA (e.g. Overath et al., 1969).

Two mechanisms exist to activate acetate to acetyl-CoA. In one, acetyl-CoA synthetase (acetate: CoA ligase [AMP-forming]; EC. 6.2.1.1.) catalyses the acetylation of CoA concomitant with the cleavage of ATP to AMP and inorganic pyrophosphate (Chou and Lipmann, 1952; Berg, 1956). In the other, two enzymes catalyse (i) the conversion of acetate to acetyl phosphate, with cleavage of ATP to ADP, and (ii) the transfer of the acetyl moiety from acetyl phosphate to CoA with liberation of inorganic phosphate (Kaplan and Lipmann, 1948; Stadtman and Barker, 1950). Acetate kinase (ATP acetate phosphotransferase; EC 2.7.2.1.), encoded by the ack gene, catalyses reaction (i), and phospho-transacetylase (acetyl-CoA:orthophosphate acetyltransferase; EC 2.3.1.8.), encoded by the pta gene, catalyses reaction (ii) (Levine et al., 1980). Acetyl phosphate has been implicated as the energy source for transport systems utilizing periplasmic binding proteins (Hong et al.,1979). Studies with ack and pta mutants suggest that the acetate kinase and phosphotransacetylase are required for E. coli to grow optimally on acetate as a carbon source and to incorporate acetate under catabolite-repressing growth conditions. Furthermore, they

suggest that the acetyl-CoA synthetase provides these organisms with an alternative route for conversion of exogenous acetate to acetyl-CoA (Brown et al., 1977).

1.1.2. The glyoxylate bypass

Acetyl-CoA is used for synthesis of higher fatty acids and other compounds and is catabolized by the TCA cycle. However, since each turn of the TCA cycle involves the loss of two carbon atoms as CO_2, no net assimilation of carbon for biosynthesis from acetyl-CoA can occur by this means. Therefore, growth on acetate and fatty acids requires the operation of a separate anaplerotic pathway to replenish necessary intermediates for cellular biosynthesis. This is accomplished by the glyoxylate bypass (Figure 1.1.). The glyoxylate bypass short-circuits the TCA cycle prior to CO_2 release, thereby permitting net synthesis of one molecule of C_4-acid from two molecules of acetyl-CoA.

In E. coli, the two unique enzymes of the glyoxylate bypass, isocitrate lyase (ICL) and malate synthase A (MS-A), are maximally induced during growth on acetate or fatty acids (Kornberg, 1966; Vanderwinkel and De Vliegher, 1968).

Isocitrate lyase (ICL) (threo-D_s-isocitrate glyoxylate-lyase; EC 4.1.3.1.) is the first enzyme of the glyoxylate bypass and it catalyses the reversible aldol cleavage of isocitrate into glyoxylate and succinate (Figure 1.2a.).

Malate synthase (MS) (EC 4.1.3.2.) (Wong and Ajl, 1956) catalyses the condensation of glyoxylate with acetyl-CoA to form malate (Figure 1.2b.). E. coli possesses two MS activities; MS-A is the one which serves an anaplerotic function during growth on acetate. A second isoenzyme , MS-B is induced during growth on glycolate (Falmagne et al., 1965).

The intermediates of the TCA cycle that are used directly as

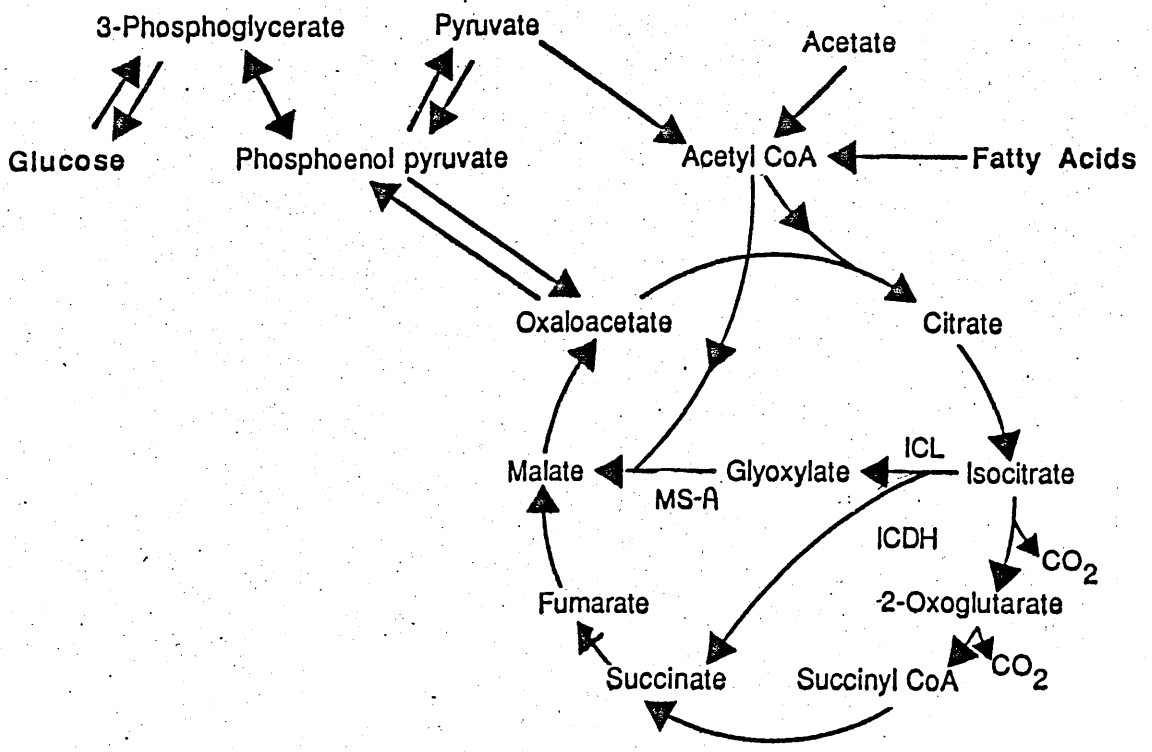
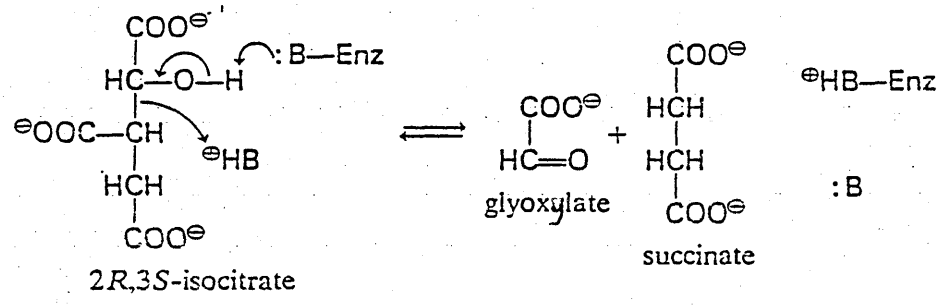


Figure 1.1. Glyoxylate bypass and related reactions in *E. coli*

The abbreviations are: ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase; MS-A, malate synthase A.

a.



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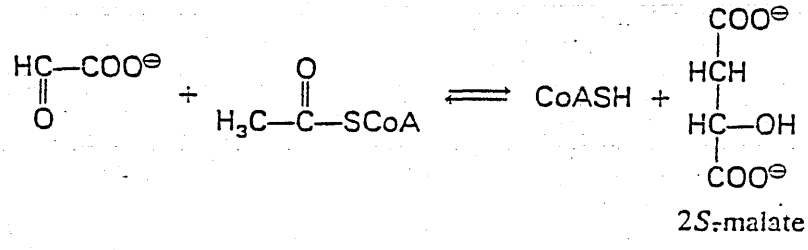


Figure 1.2a. Isocitrate lyase catalyses the reversible aldol cleavage of isocitrate to form succinate and glyoxylate.

1.2b. Malate synthase catalyses the condensation of glyoxylate with acetyl-CoA to form malate.

biosynthetic precursors (oxaloacetate, 2-oxoglutarate, succinyl-CoA) can be synthesised from the products of the glyoxylate bypass, succinate and malate. In addition, pyruvate and phosphoenolpyruvate (PEP) are required and these can be obtained as shown in Figure 1.1.

1.1.3. The ace operon

The genes for ICL (aceA) and MS-A (aceB) map at 90 min on the E. coli chromosome and comprise part of an operon (Maloy and Nunn, 1982). (This is discussed further in Chapter 4; also see Figure 1.3.) Repression of the ace operon is under the control of the fadR and iclR genes which operate in a trans-dominant and synergistic manner at the level of transcription (Maloy and Nunn, 1981;1982). The fadR gene product has been identified as a protein of M_r 29 000 (DiRusso and Nunn,1985). It is thought that the products of the iclR and fadR genes are repressor proteins that can prevent transcription of the ace operon. The fadR gene also regulates expression of the fad regulon and is thought to be responsible for regulatory interactions amongst fatty acid degradation, unsaturated fatty acid biosynthesis and acetate metabolism (Nunn, 1986).

The metabolite(s) responsible for induction of expression of the ace operon have not been identified. Duckworth (1981) grew E. coli strains, with various specific mutational blocks in the TCA cycle, on different carbon sources. She showed that induction of ICL occurs only if the carbon source can be metabolized through the TCA cycle beyond succinate. Metabolism of acetate through both the TCA cycle and the glyoxylate bypass was necessary for full induction of ICL. Changes in the concentrations of acetyl-CoA and pyruvate in cells adapting to a new carbon source did not correlate with the induction of ICL.

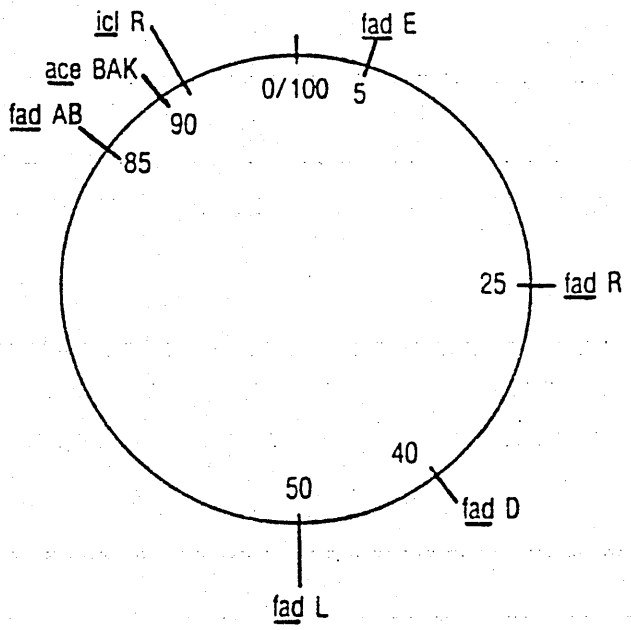


Figure 1.3. Genetic linkage map of E. coli K12 showing the location of ace (glyoxylate bypass) and fad (fatty acid degradation) structural and regulatory genes. Adapted from the revised linkage map of Bachmann and Low (1983).

1.2. The control of the branchpoint at isocitrate in *Escherichia coli*

1.2.1. The first evidence implicating phosphorylation of ICDH

E. coli has no subcellular compartments such as glyoxysomes so in the presence of the glyoxylate bypass enzymes, there is direct competition for isocitrate between ICL and the TCA cycle enzyme isocitrate dehydrogenase (ICDH). The properties of these two enzymes considered in isolation do not indicate how this competition might be controlled. *E. coli*, like most bacteria (Ragland *et al.*,1966) contains only an NADP-linked ICDH. Unlike the NAD-linked ICDH of eukaryotes, this enzyme is not regulated allosterically. There is no evidence for allosteric control of ICL either. However, the first clues pointing to the existence of a different type of control system were obtained, somewhat fortuitously, during a study of the levels of TCA cycle enzymes in *E. coli*.

During growth of *E. coli* on glucose, acetate is excreted into the medium. Holms and Bennett (1971) found that *E. coli* ML308 could adapt to grow on this acetate after exhaustion of glucose and that during adaption the specific activity of ICDH declined, whereas the activities of two other TCA cycle enzymes, malate dehydrogenase and 2-oxoglutarate dehydrogenase, did not. After exhaustion of the acetate, the specific activity of ICDH rose again. During growth on acetate, ICDH activity could be increased by addition of pyruvate; the activity dropped again after exhaustion of pyruvate (Bennett and Holms, 1975). The activity changes were independent of protein synthesis and Bennett and Holms (1975) had the prescience to suggest that reversible covalent modification of ICDH might be involved.

Garnak and Reeves (1979) were the first to show that in *E. coli* strains, ICDH becomes inactivated and phosphorylated on serine residues after the addition of acetate to cultures grown to stationary

phase on limiting glucose or glycerol. However, the quantitative relationship between phosphorylation and activity was not clear until the relevant kinase and phosphatase were isolated.

We regard this as a microbial test system of protein phosphorylation which can be manipulated under laboratory conditions. Extensive kinetic and mechanistic studies have shown, however, that there are unusual features which set it apart from most known phosphorylation systems in higher organisms. Nimmo (1984) has reviewed the regulation of this metabolic branchpoint.

1.2.2. Components of the system

In 1982, LaPorte and Koshland reported that the enzymes responsible for the phosphorylation and dephosphorylation of ICDH in *E. coli* comprise a bifunctional protein. The kinase can phosphorylate one serine residue per ICDH subunit, this inactivates the dehydrogenase completely. The phosphatase releases inorganic phosphate which causes full reactivation of ICDH (LaPorte and Koshland, 1983; Nimmo *et al.*,1984).

1.2.3. Molecular mechanisms of the inactivation of ICDH

ICDH can be almost totally inactivated by phosphorylation of a single serine residue per subunit (Borthwick *et al.*,1984b). This enzyme inactivation contrasts with most of the eukaryotic enzymes regulated by phosphorylation which are allosteric proteins and phosphorylation frequently results in changes in affinity of the target enzyme for a substrate or an allosteric effector (Cohen,1983).

Fluorescence titration experiments with NADPH (Garland and Nimmo, 1984) and the behaviour of ICDH during Procion Red Sepharose chromatography (Borthwick *et al.*, 1984a) suggest that phosphorylated ICDH is unable to bind its coenzyme $NADP^+$. Binding of $NADP^+$ to, or phosphorylation of, active ICDH both reduce the sensitivity to

proteolysis. Garland and Nimmo (1984) have therefore proposed that phosphorylation and binding of $NADP^+$ to active ICDH elicit similar conformation changes, possibly triggered by interaction of a negative charge (on $NADP^+$ or the ser-P group) with a positive charge at the coenzyme binding site (Figure 1.4.). This hypothesis is consistent with the kinetic mechanism of ICDH; that is, compulsory order, steady state, with $NADP^+$ binding first (Nimmo, 1986).

1.2.4. Phosphorylation of ICDH in intact cells

In microorganisms, patterns of growth and concomitant changes in enzyme activity, protein phosphorylation and *in vivo* fluxes can be monitored simultaneously during the course of batch fermentations and in response to changes in the external medium. By labelling cells with $^{32}P_i$ it has been shown that the phosphorylation state of ICDH *in vivo* is inversely related to its activity. In cells grown on acetate some 80% of ICDH molecules are phosphorylated. The inactive phosphorylated ICDH and the active ICDH can be separated on Procion Red Sepharose. Purified ^{32}P-labelled inactive ICDH was found to be phosphorylated at a single serine on each subunit, identical to that which can be phosphorylated by ICDH kinase/phosphatase *in vitro* (Borthwick *et al*, 1984b). This showed that the reversible inactivation is due entirely to the ICDH kinase/phosphatase. Hence the regulatory properties of this enzyme are crucial.

1.2.5. Role and control of the phosphorylation of ICDH

Bennett and Holms (1975) suggested that the role of inactivation of ICDH is to facilitate flux through the glyoxylate bypass. Our ideas about how this happens have come from studies of ICL, ICDH and the kinase/phosphatase. It is believed that in *E. coli* the K_m of ICL is much higher than that of ICDH (Bautista *et al*., 1979; Walsh and

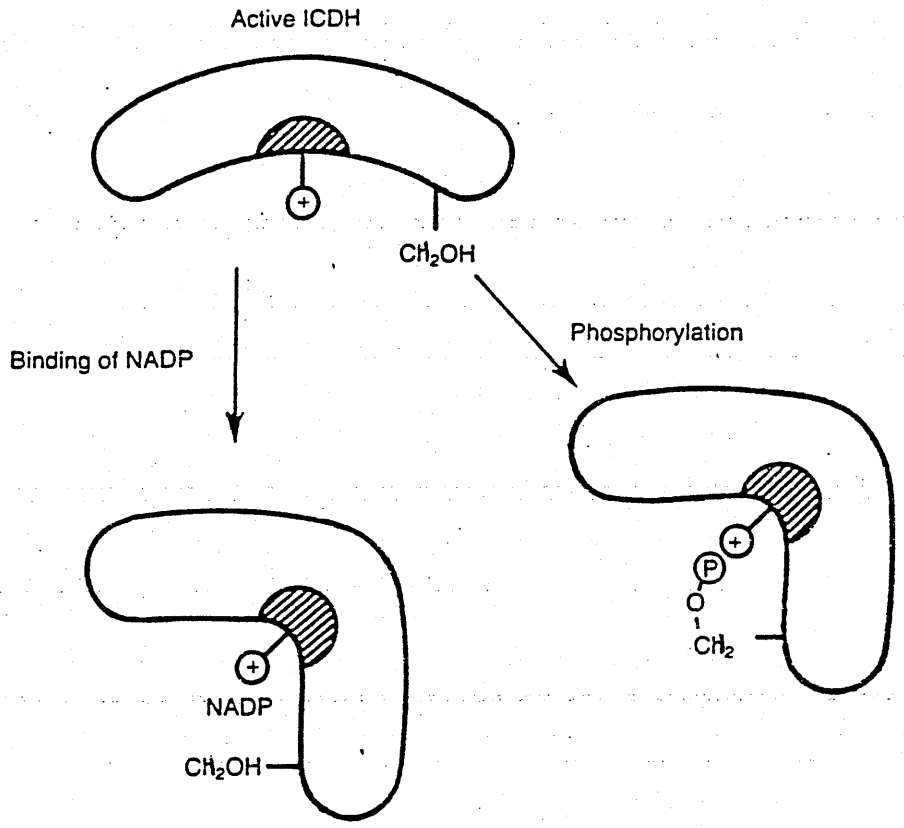


Figure 1.4. Hypothetical scheme for the inactivation of ICDH by phosphorylation, taken from Nimmo (1984). The hatched area represents the coenzyme binding site and the CH_2OH group is the side chain of the phosphorylated serine residue. The positive sign at the surface of the enzyme represents a positively charged group (or groups) that interact with the negatively charged phosphate groups of $NADP^+$.

Koshland, 1984) and our current view is that the phosphorylation of ICDH renders ICDH rate-limiting in the TCA cycle during growth on acetate, thus increasing the intracellular concentration of isocitrate to a level high enough to sustain the flux through ICL needed for biosynthesis (Nimmo and Nimmo, 1984). In agreement with this idea, El-Mansi et al.(1985) found that the intracellular level of isocitrate is much higher in cells grown on acetate than in cells grown on glycerol or glucose. They also showed that addition of pyruvate to cultures growing on acetate, known to cause reversible dephosphorylation and activation of ICDH, causes a transient doubling of the concentration of isocitrate.

Phosphorylation of ICDH has no direct dependence on ICL activity and can occur in mutants devoid of a functional glyoxylate bypass (Reeves and Malloy, 1983). Rather, the degree of phosphorylation of ICDH is determined by the kinetic behaviours of the opposing ICDH kinase and ICDH phosphatase activities. Both activities are sensitive to fluctuations in the concentrations of a large number of metabolic stimuli (Nimmo and Nimmo, 1984; LaPorte and Koshland, 1983). Figure 1.5. lists the metabolites which can simultaneously activate the phosphatase and inhibit the kinase. Isocitrate gives sigmoidal inhibition of the kinase but the other effects are hyperbolic. ATP is anomalous in that it is a substrate for the kinase and an activator of the phosphatase. In addition, the phosphatase is strongly inhibited by NADPH but not by other nicotinamide nucleotides. It is perhaps surprising that the product of β-oxidation of fatty acids, NADH, has no effect here. Compared with growth on acetate, it is to be expected (although it has not been tested) that during growth on fatty acids flux through the glyoxylate bypass would be increased with respect to flux through the TCA cycle. Perhaps the integration of β-oxidation, the glyoxylate bypass and the TCA cycle is achieved by the influence of NADPH on energy charge and in turn by the effect of ADP and AMP on ICDH

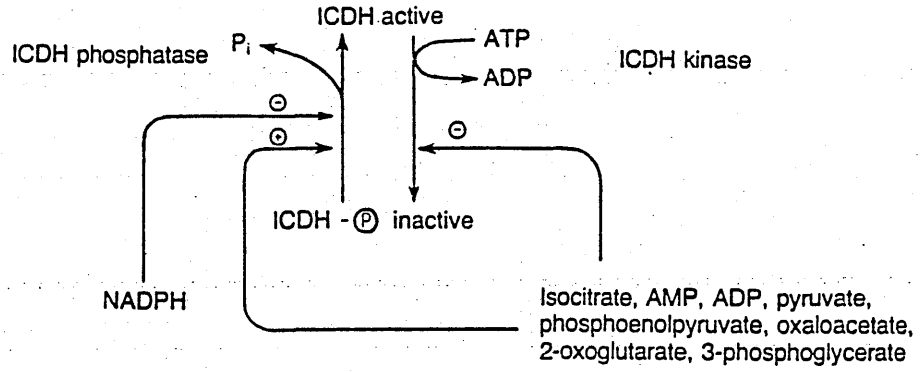


Figure 1.5. Control of the phosphorylation state of ICDH + and - represent stimulatory and inhibitory effects respectively. Both ICDH kinase and ICDH phosphatase activities are associated with a single bifunctional protein. Figure taken from Nimmo (1984).

kinase/phosphatase activities.

Nimmo and Nimmo (1984) have suggested that the effectors of ICDH kinase/phosphatase most likely to be significant in vivo are isocitrate, PEP, 3-phosphoglycerate, AMP, ADP and NADPH, since each of these has a significant effect at concentrations that are thought to occur in intact E. coli (Lowry et al., 1971; Bautista et al., 1979; Morikawa et al., 1980; El-Mansi et al., 1985). Thus, for example, the activation of ICDH observed in E. coli during anaerobiosis (El-Mansi et al.,1985) may be mediated via changes in energy charge. Increased phosphorylation of ICDH upon addition of deoxyglucose or methyl α-glucoside to cultures of S. typhimurium which had been trained on acetate and then starved (Wang and Koshland, 1982) could have resulted from a decrease in the level of PEP. A decrease in PEP would be expected since these compounds enter the cell via the phosphotransferase system.

In addition to the sensitivity of both ICDH kinase and ICDH phosphatase activities to many metabolities, LaPorte and Koshland (1983) have demonstrated that control of ICDH by reversible phosphorylation may be the first in vivo case of "zero-order ultrasensitivity" (Goldbeter and Koshland, 1981). Their analysis refers to a situation in which one or both of the interconverter enzymes of a covalent modification are close to saturation with the target protein, i.e. in the zero-order region. Then the steady state level of the modification can be more sensitive to an effector binding to the "zero-order" convertor enzyme(s) even if that effector behaves in a Michaelis-Menten manner.

The phosphorylation system can respond to variations in the levels of ICDH in vivo. LaPorte et al. (1985) have compared the levels of ICDH activity and phosphorylation in strains containing either one or multiple copies of the ICDH gene. They showed that the phosphorylation system can compensate for changes in the cellular level of ICDH in excess of 10-fold by increasing the phosphorylation state of ICDH while

maintaining a nearly constant level of active, unphosphorylated ICDH during growth on acetate.

1.3. The bifunctional nature of ICDH kinase/phosphatase

Multifunctional enzymes, especially those that catalyse consecutive reactions of a metabolic pathway, are well known. ICDH kinase/phosphatase is a member of one sub-set of these enzymes that has only been recognised recently, namely, bifunctional regulatory enzymes bearing opposing activities.

1.3.1. Other bifunctional regulatory enzymes

There are four other examples of physical association of two reciprocal regulatory activities. The enzymes responsible for the adenylation and deadenylation of glutamine synthase in *E. coli* reside on the same polypeptide chain (Caban and Ginsburg, 1976). The uridylyltransferase and uridylyl-removing enzyme have also been reported to be physically associated (Garcia and Rhee, 1983). Rat liver 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase which catalyze the synthesis and breakdown of fructose-2,6-bisphosphate also comprise a bifunctional protein (El-Maghrabi *et al.*, 1982a; El-Maghrabi *et al.*, 1982b), apparently with two distinct active sites (Sakakibara *et al.*, 1984). More recently, pyruvate, phosphatase dikinase-regulatory protein from maize, has been shown to be a bifunctional kinase/phosphatase. It is probably unique in using ADP as phosphate donor (Burnell and Hatch, 1985).

The phenomenon of bifunctional organisation of opposing activities is therefore found in animals, plants and microorganisms and all examples involve systems which play major roles in the regulation of intermediary metabolism. This provokes speculation about possible regulatory advantages of such bifunctionality.

Several benefits have been attributed to the multifunctional organisation of enzymes which catalyse consecutive metabolic reactions. The connection of active sites may confer advantages by such means as substrate channelling, protection of unstable intermediates and enhancement of catalytic activity at individual sites (discussed in Hardie and Coggins, 1986). There are few well-authenticated examples of these properties and none would be important where reciprocal activities are concerned.

An example of a bifunctional enzyme which does not catalyse consecutive reactions is the E. coli aspartokinase I-homoserine dehydrogenase I; both activities are subject to feedback inhibition by threonine and share a single threonine-binding site (Cohen and Dautry-Varsat, 1980).

1.3.2. Possible advantages of bifunctional organisation of ICDH kinase/phosphatase

One essential feature of all the examples of bifunctionality described in Section 1.3.1., is that they are regulated by multiple signals of mainstream metabolites rather than by a single signal such as cAMP or Ca^{2+}.

Conceptually, it seems a neat economy that for any one effector both activities could be controlled from a single site. This would provide simultaneous regulation of both activities; opposing activities on any one protein molecule would be prevented from being active simultaneously. But since the intracellular concentration of ICDH kinase/phosphatase is low in relation to the intracellular concentrations of its effectors (i.e. there is no obvious need to save on binding sites) it is not clear that two monofunctional enzymes would be regulated any less effectively in this situation.

Moreover, Nimmo and Nimmo (1984) have shown that the phosphatase

is sensitive to much lower concentrations of pyruvate and oxaloacetate than is the kinase, but the reverse is true for isocitrate. This suggests that these compounds may each bind to more than one site on ICDH kinase/phosphatase.

In bacteria, co-ordinated expression at the level of transcription can occur either in operon systems or by common control of scattered genes in regulons (Maas and Clark, 1964). However having both functions covalently associated and carried by a single gene provides a simple mechanism which ensures a constant ratio of kinase protein to phosphatase protein. Perhaps there is an advantage in retaining the constant ratio of kinase to phosphatase in the present-day bifunctional system, to simplify the simultaneous regulation by multiple effectors. But were there any selective pressures to cause this bifunctional system to arise in the first place?

Clearly, we need information about the primary and tertiary structures of bifunctional regulatory enzymes before we can deduce anything about the evolutionary and functional significance of their structures. How many active and regulatory sites are there? Why is ICDH phosphatase activated by ATP? If there are two distinct active sites, are there any clues, in the sequence, as to how putative gene duplication and fusion events (LaPorte and Koshland, 1982; Nimmo et al.,1984) might have occurred?

ICDH kinase/phosphatase is an excellent candidate for such a structural investigation. First, the structural gene has been cloned (LaPorte et al.,1985; Chapter 4 of this thesis). Second, in the future we expect to add to our accumulated knowledge of the molecular details and bacterial physiology of the system, by engineering and studying the behaviour of mutants with altered regulatory properties or with two monofunctional activities.

1.4. The physiological role and distribution in nature of the glyoxylate bypass

The glyoxylate bypass has a role, under various nutritional conditions and at certain times of the life-cycle, in many organisms; bacteria, fungi, yeast, algae, protozoa, metazoa and plants but never in higher animals which are unable to use fats for biosynthesis (Cioni *et al.*, 1981). There are different regulatory patterns for the synthesis and degradation of ICL and MS according to the physiological role in any particular organism. The glyoxylate bypass is especially prominent in germinating seeds of higher plants where it allows stored fats to be converted rapidly to the precursors needed for biosynthesis in growing tissues. In accordance with this function, ICL and MS levels reach a peak of activity in the early days of germination and decline thereafter (e.g. in *Ricinus communis*, Gonzales and Beevers, 1976). Various authors have reported data on the possible effects of hormonal control (e.g. Martin and Northcote, 1982), light (Kagawa *et al.*, 1973), O_2 supply (Slack *et al.*, 1977), fatty acids and carbohydrates (Vanni *et al.*, 1977) on *de novo* synthesis and on degradation of ICL and MS.

In nematodes, the glyoxylate bypass operates to convert lipids to carbohydrates during embryogenesis (e.g. Barrett *et al.*, 1970) but is also found in the adult stage of some worms (e.g. *Fasciola hepatica*, Prichard and Schofield, 1969).

In algae, the bypass enzymes are synthesized during interruption of photosynthesis by limiting Co_2 or darkening (e.g. *Chlorella fusca*, Syrett, 1966) but only when acetate is the sole carbon source (John and Syrett, 1968).

In fungi, the glyoxylate bypass is necessary for the germination of spores when stored fat is used as the major source of carbon (e.g. *Neurospora crassa*, Flavell and Woodward, 1971) and also during growth on C_2 compounds (Flavell and Woodward, 1970) and alkanes (which may first

be converted to fatty acids) e.g. in Candida tropicalis (Lebeault et al.,1970).

1.4.1.Control of the branchpoint at isocitrate

Control of ICDH by phosphorylation during operation of the glyoxylate bypass has been investigated only in the past few years in E. coli and Salmonella typhimurium (Wang and Koshland, 1982). The data of Bennett and Holms (1975) suggest that such a system also operates in other enteric bacteria. The bacterium Acinetobacter calcoaceticus is unusual in that it contains two isoenzymes of ICDH. Adaption of Acinetobacter calcoaceticus to growth on acetate is accompanied by an increase in the proportion of isoenzyme-II, which can be allosterically stimulated by glyoxylate, and a concomitant decrease in isoenzyme-I (Reeves et al.,1986). The effect of these activity changes on carbon flux has yet to be assessed. Whether protein phosphorylation of ICDH operates in Acinetobacter calcoaceticus, or indeed, elsewhere in nature, has not yet been investigated. However, many organisms have another strategy which overcomes the problem of competition between ICDH and ICL for isocitrate, namely compartmentation.

Figure 1.6. shows a possible scheme for the intracellular distribution of all the enzymes involved in the conversion of lipids to carbohydrates in plants. The enzymes needed for the β-oxidation of fatty acids and for the glyoxylate cycle are localized in the glyoxysomes (Cooper and Beevers, 1969; Huang and Beevers, 1973; Beevers, 1979). The competing TCA cycle and glyoxylate cycle enzymes are therefore kept separate. Compartmentation of ICL and MS in organelles has also been reported in species of nematodes (e.g. Caenorhabditis elegans, Patel and McFadden, 1977), algae (e.g. Euglena gracilis, Collins and Merrett, 1975) and fungi (e.g. Neurospora crassa, Kobr et al., 1969).

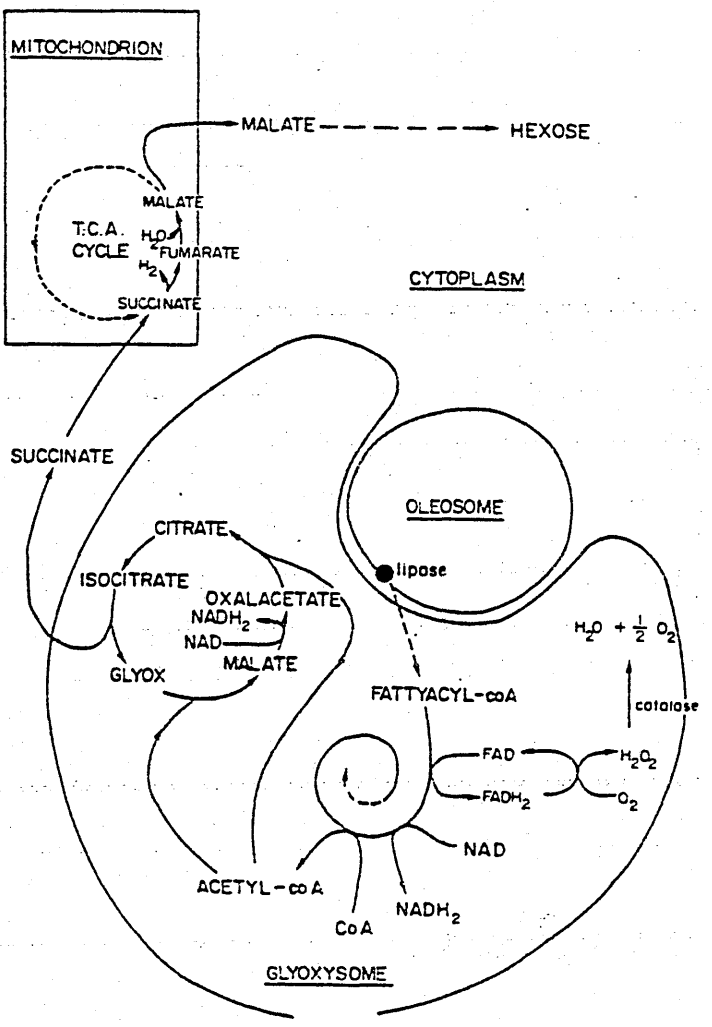


Figure 1.6. Intracellular localization of the processes involved in lipids to carbohydrates conversion in higher plants (taken from Cioni et al.,1981). Oleosomes are intracellular lipid droplets.

Table 1.1. Comparison of some properties of ICL from various sources

Source	Number of isoenzymes	pH optimum (and buffers)	Number of subunits	Subunit M_r	M_r	References
Clorella pyrenoidosa		7.25 (Phosphate)			170 000	John & Syrett (1967)
Pseudomonas indigofera		7.7	4	48 000	206 000-220 000	Shiio et al. (1965)
Neurospora crassa	2	6.8	4	67 000	270 000	Flavell & Woodward (1971) Johanson et al. (1974a) Johanson et al. (1974b)
Turbatrix aceti	5	7.6 (Phosphate & Tris)	4	123 000	480 000	Reiss & Rothstein (1974)
Ascaris lumbricoides	1	7.4			214 000	Colonna & McFadden (1975)
Casenorhabditis eleaans	3	7.7			256 000	Colonna & McFadden (1975)
Linum usitatissinnum	2	7.5 (Phosphate & Tris)	4	67 000	264 000	Lamb et al. (1978)
Cucumis satirus		7.4 (HEPES)	4	64 000	260 000	Frevert & Kindl (1978)
ermophilic bacillus		8 (Tris & Imidazole)	4	48 000	180 000	Chell et al. (1978)
Lupinus	2-5?	7.5		45 000	175 000	
Citrullus vulgaris	4	(Imidazole)		64 000	277 000	Vanni et al. (1979) Jameel et al. (1984)

In the protozoon Tetrahymena pyriformis, ICL and MS are located in microbodies but the enzymes which complete the glyoxylate cycle are probably in the mitochondria (Muller et al., 1968). Thus the glyxoylate cycle functions by the co-operation of mitochondrial enzymes with a flux of succinate, malate and isocitrate between these organelles. The mechanisms which may regulate these fluxes are unknown.

1.4.2. The properties of ICL

Although the glyoxylate bypass is known in many organisms, study of purified ICL has been reported from only one or two representatives of each of the phyla concerned. The most-studied ICLs are from the bacterium Pseudomonas indigofera (McFadden et al.,1968), from the alga Chlorella pyrenoidosa (John and Syrett, 1967), fungus Neurospora crassa (Johanson et al.,1974), nematodes Turbatrix aceti (Reiss and Rothstein, 1974) and Caenorhabditis elegans (Colonna and McFadden, 1975) and from some plants e.g. Cucumis sativus (Frevert and Kindl, 1978). All these ICLs are specific for threo-D_s-isocitrate and require Mg^{2+} and a reducing agent for optimal activity.

M_r and quaternary structure ICL from all the sources that have been studied are tetrameric and composed of identical subunits (see Table 1.1.). There appear to be two classes with different subunit M_r values,i.e. those of M_r 45 000-48 000 and those of M_r 64 000-67 000. The only exception is the ICL from Turbatrix aceti with a subunit M_r of 123 000. In view of the fact that the subunits of ICL from other nematodes are much smaller, this figure should, perhaps, be reinvestigated.

Isoenzymes Several studies have shown more than one form of ICL in the same cell extract. In some cases, this is probably due to the activity of proteases during preparation of extracts. In Pseudomonas MA (Bellion and Woodson, 1975), Bacterium 5H2 (Cox and Zatman, 1973) and Yersinia

pestis (Hillier and Charnetzky, 1981)there may be one form of ICL which has an anaplerotic function in the glyoxylate bypass and another isoenzyme associated with growth on C_1 compounds (e.g. growth of Pseudomonas MA on methylamine-acetate). In Neurospora crassa there is one glyoxysomal ICL and a second mitochondrial ICL whose synthesis seems to be induced by high levels of mitochondrial isocitrate (Flavell and Woodward, 1971).

Kinetics and reaction mechanism. ICL shows Michaelis-Menten kinetics in both the cleavage and condensation reaction.

For the Neurospora crassa enzyme (Johanson et al.,1974), PEP at low concentrations gave uncompetitive inhibition with respect to isocitrate and succinate gave mixed inhibition. This led the authors to propose a compulsory order mechanism with succinate released before glyoxylate. However, higher concentrations of PEP gave mixed inhibition and for succinate, slope and intercept replots were non-linear. This might suggest breakdown of the compulsory order of binding and release of substrates at high concentrations.

For the Pseudomonas indigofera enzyme (Williams et al.,1971), PEP and itaconate gave competitive inhibition with respect to succinate and uncompetitive inhibition with respect to glyoxylate. This again suggests a compulsory mechanism with glyoxylate binding first. Succinate seemed to be a mixed inhibitor with respect to isocitrate, but at high concentrations, slope and intercept replots were non-linear suggesting that binding was no longer ordered. Indeed, direct binding of ^{14}C-succinate to free enzyme has been observed (Rittenhouse and McFadden, 1974) and Schloss and Cleland (1982) observed competition between succinate and the reaction-intermediate analogue 3-nitropropionate which indicated that succinate could interact with the enzyme in the absence of glyoxylate. Both Williams et al.(1971) and Schloss and Cleland (1982) favoured a "random kinetic mechanism with a

preferred pathway".

Examination of the condensation reaction shows that ICL must abstract the α-hydrogen, a hydrogen that is very non-acidic, with a pK_a value estimated to be 24, in order to generate the requisite nucleophile for attack on glyoxylate. The enzyme mechanism for activation of this hydrogen remains obscure. The presence of an essential C-terminal histidine residue has been reported for ICL from Neurospora crassa (Johanson et al.,1974), Pseudomonas indigofera (McFadden et al.,1968) and flax (Khan et al.,1982). It is plausible that this histidine residue could play some part in polarising the α-carbon-hydrogen bond of succinate in catalysis. The presence of essential sulphydryl groups has been reported in ICL, isolated from Pseudomonas indigofera (McFadden, 1969), a thermophilic Bacillus sp. (Chell et al.,1977) and flax, Linum usitatissimum (Kahn et al.,1977). Therefore, cysteine residues also seem to play a part in catalysis.

Fine controls Much attention has been focussed on possible regulation of ICL, in various species, because "it is the first enzyme of the anaplerotic glyoxylate bypass" and as such has been classically considered a likely candidate for feedback regulation by end-product metabolites.

Activation of ICL by precursors or metabolites has never been reported. On the other hand, ICL can be inhibited by various metabolites. Many authors have ascribed physiological significance to inhibition of ICL by a) the reaction products, succinate and glyoxylate (e.g.Williams et al.,1971); b) intermediates of the glyoxylate cycle and TCA cycle, e.g.succinate, 2-oxoglutarate, fumarate, oxaloacetate and malate (e.g.Ozaki and Shiio,1968); and c) distant products of the glyoxylate bypass, e.g. PEP (Ashworth and Kornberg, 1963). However, in no case is there evidence of allosteric inhibition of ICL and many of these effects can be explained by structural similarities to the

substrates.

1.5. Aims and scope of this thesis

This introduction has described how in *E. coli* ICDH activity is controlled by a reversible phosphorylation mechanism catalysed by the bifunctional ICDH kinase/phosphatase. The K_m of ICL for isocitrate was thought to be much higher than that of ICDH and it was proposed that during growth on acetate inactivation of ICDH (by phosphorylation) permits isocitrate to rise to a level that can allow flux through the glyoxylate bypass. When the work described in this thesis was initiated it was not clear whether regulation of ICL was also physiologically important. The first objective of this project was therefore to purify ICL from *E. coli* ML308 and to study the regulatory properties of ICL systematically in order to complete our picture of control of carbon flux at the level of isocitrate.

During the course of this work, Dr. E.M.T. El-Mansi constructed a recombinant plasmid, carrying a fragment of *E. coli* ML308 genomic DNA, which complemented an *aceA* (ICL) mutation. The work described in Chapters 4 and 5 developed from the necessity to characterize the enzymes expressed by this plasmid.

As it happened, this plasmid carried the genes for all three enzymes of the glyoxylate bypass operon, namely MS-A (*aceB*), ICL (*aceA*) and ICDH kinase/phosphatase (*aceK*). Thus,this clone afforded the first chance to purify and characterize MS-A and this became a second strand of my work. *E. coli* ML308 expresses such low levels of ICDH kinase/phosphatase that in the past purification has yielded just enough protein to detect on a silver-stained gel (Nimmo *et al.*, 1984). Purification of ICDH kinase/phosphatase from Dr. El-Mansi's clone provided enough protein to allow me to start tackling the questions of topology of this bifunctional protein.

CHAPTER TWO - MATERIALS AND METHODS

2.1. Materials

[γ-^{32}P] ATP (3000 Ci/mmol) was from Amersham, Bucks, U.K.

Acrylamide, ammonium sulphate (enzyme grade), HCl (Aristar), 2-mercaptoethanol, N,N'-methylenebisacrylamide, nitroblue tetrazolium, phenazine methosulphate, N,N,N',N'-tetramethylenediamine, ATP, lactate dehydrogenase (pig heart), NAD^+ (free acid), NADH (disodium salt), $NADP^+$ (disodium salt), NADPH (disodium salt), phosphoenolpyruvate and 3-phosphoglycerate were from Boehringer Corp. (London), Lewes, Sussex, U.K.

Staphylococcus aureus V8 protease was obtained from Miles, Slough, U.K.

Nutrient broth (CM1) and nutrient agar No.1 (CM3) were from Oxoid Ltd.,Basingstoke, Hants, U.K.

Blue dextran, DEAE-Sephacel, FPLC chromatofocusing (Mono P) and ion-exchange (Mono Q) pre-packed columns, low M_r standard proteins for SDS-PAGE, phenyl-Sepharose, Polybuffer PB74, Sephadex G-50 and G-25 superfine and Sephacryl S-300 superfine were obtained from Pharmacia, Milton Keynes, Bucks., U.K.

Acetyl-CoA, ADP (disodium salt), ampicillin, benzamidine hydrochloride, bovine serum albumin (for Bradford's protein determination), Bicine, Coomassie Brilliant Blue G250, dithiothreitol, glyoxylate, DL-isocitrate (trisodium salt), Mes, Mops, 2-oxoglutarate, phenylmethylsulphonyl fluoride (PMSF), protamine sulphate (salmon, grade II), soybean trypsin inhibitor and Tris were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

DEAE-cellulose (Whatman DE52) was purchased from Uniscience Ltd. (Cambridge U.K.).

Chymotrypsin and trypsin (treated with L-1-tosylamido-2-

phenylethyl chloromethyl ketone) were obtained from Worthington Biochemical Corp.,Freehold, NJ 07728, U.S.A.

Dimethyl suberimidate was a gift from Prof. J.R. Coggins.

All other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of the highest grade obtainable.

METHODS

2.2. General biochemical methods

2.2.1. pH measurements were made with a Radiometer pH probe calibrated at room temperature.

2.2.2. Conductivities were measured at 4°C with a Radiometer conductivity meter, type CDM2e (Radiometer, Copenhagen, Denmark).

2.2.3. Glassware and plastics were washed in 'Haemo-sol' solutions (Alfred Cox (Surgical) Ltd., U.K.), rinsed with tap water and then distilled water and dried in an oven.

For protein chemistry experiments, glassware was soaked overnight in concentrated nitric acid, rinsed thoroughly with distilled water and dried in an oven. The clean glassware was stored in a seal-top plastic box and handled with disposable plastic gloves. Pipette tips were taken from a newly-opened bag.

2.2.4. Protein estimations were done by the method of Bradford (1976). Bovine serum albumin was used as standard. Concentrations of BSA solutions were determined assuming that a 1mg/ml solution has an A_{280} of 0.67. Protein concentrations of the fractions eluted from columns were monitored at 280nm.

2.2.5. Dialysis membranes (Scientific Instruments Centre Ltd., London), were boiled for 15 min in 1% (w/v) EDTA, pH7, stored in 20% (v/v) ethanol and then rinsed in distilled water prior to use.

2.2.6. Concentrations of protein samples.

Samples of 10 to 60ml were concentrated by vacuum dialysis. Samples of less than 10ml were concentrated using Centricon 30 microconcentrators (Amicon Ltd., Stonehouse, U.K.).

2.2.7. Preparation of chromatographic media.

Sephadex G-50 superfine was swollen and poured according to the manufacturer's instructions.

DEAE-cellulose (Whatman DE52), DEAE-Sephacel ,phenyl-Sepharose and Sephacryl S-300 superfine were obtained pre-swollen, resuspended in starting buffer and poured. DEAE-cellulose was adjusted to the correct pH before equilibrating in starting buffer. Phenyl-Sepharose was regenerated, after use, by washing with 24% (v/v) ethanol and then distilled water.

Blue Sepharose CL-6B (freeze-dried powder) was swollen in starting buffer (200ml per gram) for 15 min and poured. The column was washed with 0.5M NaCl then with 6M urea before equilibrating for use. All columns were poured at room temperature then moved to a 4°C cold room. Columns were packed at higher flow rates than would be used during a run. They were stored in 0.02% (w/v) sodium azide and equilibrated with starting buffer before use.

2.2.8. Lyophilisation

Samples were frozen in a suitable vessel (allowing space for bumping at the sample surface) by dipping into methanol/dry ice. Tops were sealed with Nescofilm punctured with a needle. Samples were placed in a dessicator connected to a Flexi-dry (FTS Systems Inc., Stone Ridge, N.Y., U.S.A.) and vacuum was maintained by a high vacuum pump (Javac PTY Ltd., U.K.).

2.3. Microbiological techniques

2.3.1 Baterial strains

Escherichia coli ML308 (ATCC 15224) was originally obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). It has been maintained in Glasgow for 20 years. It has the genetic configuration $i^-z^+y^+a^+$ for the *lac* operon. Because defective repressor is produced, synthesis of the products of the *lac* operon (β-galactosidase, β-galactoside permease and thiogalactoside transacetylase) is constitutive. The identity of the organism can therefore be checked by assaying for β-galactosidase which is present during growth on all media. In other respects the strain is regarded as wild-type.

Escherichia coli strains LE392 (a K12 derivative) and KAT-1 (an *aceB*$^+$ *aceA*$^-$ mutant derived from LE392 by transposon Tn*10* insertion (Maloy and Nunn, 1982) were from Dr. W.D. Nunn (University of California, Irvine, U.S.A). The plasmid-bearing strains KAT-1/pEM9 and KAT-1/pEM901 were constructed by Dr. E.M.T. El-Mansi (see Chapter 4). Growth of these strains required the supplements described in Methods 2.3.3.f).

2.3.2. Storage of bacteria

Organisms were maintained in nutrient broth, stored at 4^{o}C. Subcultures were made at 6 to 12 monthly intervals. Samples were checked for homogeneity microscopically and, in the case of *E. coli* ML308, by plating out on nutrient agar containing 10μg/ml 5-bromo-4-chloro-indoxyl-β-galactoside (BCIG agar). A cell constitutive for β-galactosidase produces a blue colony on BCIG agar, so a homogeneous culture of *lac*-constitutive cells produces only blue clones.

2.3.3. Media

a) Nutrient broth was prepared from Oxoid dehydrated granules, dispensed in 10ml aliquots into Universals and sterilised by autoclaving at 15 p.s.i.

b) Nutrient agar 15g agar was dissolved in 1000ml nutrient broth, sterilised at 15 p.s.i., poured into petri dishes in a filtered-air cabinet and allowed to solidify. Plates were stored at 4°C.

c) BCIG agar BCIG (2mg/ml in dimethylformamide) was added to nutrient agar to a final concentration of 10µg/ml. Plates were stored at 4°C.

d) Defined media for specifically pre-inducing inocula

This was prepared as 3 components:

I. PNS contained 66.7mM potassium dihydrogen phosphate (9.07g/l KH_2PO_4) and 16.7mM ammonium sulphate (2.2g/l $(NH_4)_2SO_4$) adjusted to pH7 with NaOH. 60ml volumes in 250ml conical flasks were autoclaved at 15 p.s.i.

II. $FeSO_4$ contained 0.8mM ferrous sulphate (0.22g/l $FeSO_4.7H_2O$) adjusted to pH2 with HCl and autoclaved at 15 p.s.i.

III. Carbon source (at 2.5 times final concentration required) plus 1.25mM magnesium sulphate (0.3g/l $MgSO_4.7H_2O$). 40 ml volumes were autoclaved at 15 p.s.i.

Complete defined medium was prepared by adding 40ml of III and 1.25 ml of II to 60ml of I.

e) Defined media for large-scale growth

This was prepared as 4 separate components:

I. 3850ml of 67mM potassium dihydrogen phosphate (9.12g/l KH_2PO_4) adjusted to pH7 with KOH, autoclaved at 15 p.s.i. in a 10 litre round bottom flask with a plugged 10ml pipette inserted through a cotton-wool bung.

II. MgNS contained 40mM magnesium sulphate (9.84g/l $MgSO_4.7H_2O$) and 800mM ammonium sulphate (105.6g/l $(NH_4)_2SO_4$), autoclaved, in 50ml volumes, at 15 p.s.i.

III. $FeSO_4$ 0.8mM ferrous sulphate (0.22g/l $FeSO_4.7H_2O$), adjusted to pH2 with HCl, autoclaved in 50ml volumes at 15 p.s.i.

IV. Carbon source 3.2M sodium acetate or 1.6M glycerol, autoclaved in 50ml volumes, at 15 p.s.i.

Complete defined medium was prepared by adding 50ml each of II, III and IV to I, to give 4000 ml.

f) Specific supplements to defined media

E. coli strains LE392, KAT-1, KAT-1/pEM9 and KAT-1/pEM901 required addition of vitamin B_{12} (to 1µg/ml from a 0.5mg/ml stock solution) and methionine (to 50µg/ml from a 25mg/ml stock solution) to all media. KAT-1/pEM9 and KAT-1/pEM901 were always grown in the presence of ampicillin (at 100µg/ml from a 10mg/ml, pH7, stock solution).

g) Sterilisation

All media, with the exception of the components listed below, were autoclaved by steam at 109°C for the appropriate times as determined by Prof. C.A. Fewson of this Department. Sterilisation was verified by Browne's tubes (Albert Browne Ltd., U.K.) unless volumes were greater than 2 litres. Because of their probable heat lability the following compounds were adjusted to pH7 and sterilised by filtration through 0.22µm pore-sized Millex GV filters (Millipore Ltd., U.K.) into sterile bottles: ampicillin, methionine and vitamin B_{12}.

Glass pipettes for inoculations were sealed in Kraft Paper and heated to 160°C in an oven, overnight.

2.3.4 Growth of bacteria

a) Preparation of specifically pre-induced inocula

100ml of complete defined medium (Methods 2.3.3.d) and 2.3.3.f))

was inoculated with 6 drops of a nutrient broth culture and grown on an orbital shaker at 37°C. 1ml was inoculated into another 100ml of identical medium (2nd passage). A 3rd passage was grown similarly and stored at 4°C for up to 24 hours before use. Growth times depended on the carbon source, but were generally overnight and stopped in mid log-phase.

b) Large-scale batch cultures

Four 4000ml flasks of defined medium (Methods 2.3.3.e) and 2.3.3.f)), at 37°C, were inoculated with 3rd passage culture to the required cell density. Cultures were grown at 37°C on a 4-place stirrer (Harvey et al., 1968). Stirring magnets were turned, by horse-shoe magnets under the flasks, fast enough to break the vortex in the flask, causing bubbles to form. Compressed air was passed through a cotton-wool plug, at a rate of 400ml/hr.

The mean generation times were as follows (time in min):

E. coli strain	Carbon source	
	40mM acetate	20mM glycerol
ML308	110	90
KAT-1	no growth	90
KAT-1/pEM9	170	90
KAT-1/pEM901	no growth	90

The desired inocula size and required time of growth to late log-phase were predicted from these values.

c) Measurement of growth

Optical densities of bacterial suspensions were estimated by measuring the OD_{420} relative to water in 1cm light-path cuvettes using a Unicam SP500 spectrophotometer (Pye Unicam Instruments Ltd., U.K.)

equipped with a Gilford photoelectric detector and digital read-out. If necessary, samples were diluted 1 in 10 with the appropriate medium to give an OD_{420} less than 0.5.

d) Harvesting procedure

Cells were harvested by centrifugation at 6000g for 15 min at 4°C (MSE Mistral 6L with a 6 x 750ml rotor). To harvest 16 litres of culture, the same six centrifuge pots were used for 5 runs. The accumulated pellets were resuspended in chilled Buffer A (Methods 2.5.1,) and then centrifuged at 30 000g for 10 min at 4°C (MSE 18 with an 8 x 50ml rotor). The pellets were weighed (wet weight) and stored at -20°C.

2.3.5. Disruption of bacteria

a) Ultrasonic disruption

A sample of culture (2.5ml) was added to 2.5ml of chilled BSA (10mg/ ml) in 0.15M NaCl in a 2-dram vial within a brass holder (Holms and Bennett, 1971) surrounded by an ice-water slurry. The sample was ultra-sonicated (Dawe Soniprobe type 1130A from Dawe Instruments Ltd., London) for three 30 sec periods alternating with two 30 sec cooling periods.

b) French pressure cell disruption

The thawed bacterial pellets (Methods 2.3.4.d)) were resuspended gradually in 2 volumes of Buffer A (Methods 2.5.1) at 4°C and disrupted by 2 passages through the French pressure cell (Cat.no. 4-3398A, American Instruments Company, Maryland, U.S.A.) at a pressure of 98MPa (14 300 lb/in^2). The pressure cell was cooled in ice before use.

2.4. Enzyme assays

2.4.1 Instrumentation and micropipetting.

Spectrophotometric assays were done at 37°C in semi-micro quartz cuvettes (1cm path, 1ml). The instrument used throughout was a thermostatted Unicam SP500 spectrophotometer (Pye Unicam Instruments Ltd., U.K.) equipped with a Gilford photoelectric detector and slave chart-recorder.

Adjustable Finnpipettes (Finnpipette Ky, Pulti 9, SF-00810, Helsinki 81, Finland) were used for 10µl-1000 l volumes. 0.5-50 µl volumes were dispensed with micro-syringes (Scientific Glass Engineering Ply Ltd., North Melbourne, Australia 3051).

Assay mixes were allowed to equilibrate then the component to initiate the reaction was added, and mixed in, on a plumper (Calbiochem, Los Angeles, U.S.A.).

2.4.2 Assays

a) Isocitrate lyase (method 1)

In crude extracts, during purification and in some steady-state kinetic experiments, isocitrate lyase was assayed, at 340nm and 37°C, by coupling the formation of glyoxylate to the oxidation of NADH with lactate dehydrogenase (Warren, 1970). Each cuvette contained in a final volume of 1ml, 50mM Mops-NaOH pH 7.3, 5mM $MgCl_2$, 1mM EDTA, 5mM DL-isocitrate, 0.2mM NADH, 0.1mg/ml pig heart lactate dehydrogenase and extract. At early stages of the purification NADH oxidase activity was measured and then the ICL reaction was initiated by addition of DL-isocitrate. NADH oxidase was eliminated during acid treatment (step d) of purification scheme).

b) Isocitrate lyase (method 2)

In some steady-state kinetic experiments, isocitrate lyase was assayed using a modification of the procedure of Dixon and Kornberg

(1959). Each cuvette contained in a final volume of 1ml, 50mM Mops-NaOH pH 7.3, 5mM $MgCl_2$, 1mM EDTA, DL-isocitrate (variable), 4mM phenylhydrazine-HCl. The reaction was started by the addition of enzyme and the change in A_{334} was monitored. The phenylhydrazine-HCl was stored at -20°C in the dark.

(c) Isocitrate lyase (method 3)

The condensation reaction was measured by coupling the formation of isocitrate to the reduction of $NADP^+$ with ICDH (from E. coli ML308; Methods 2.5.6.). ICDH is well suited to this purpose because it has a low K_m for isocitrate (Bautista et al., 1979; Nimmo, 1986) and the formation of 2-oxoglutarate is thermodynamically favourable (Ochoa, 1945). Each cuvette contained in a final volume of 1ml, 50mM Mops-NaOH pH7.3, 5mM $MgCl_2$, 1mM EDTA, succinate (variable), glyoxylate (variable), 0.4mM $NADP^+$, 6µg ICDH and isocitrate lyase.

For each of these isocitrate lyase assay methods, the linearity of rate dependent on protein concentration was checked. The maximum amount of enzyme used in the assays was then set, so that the change in A_{340}, or A_{334}, was not greater than 0.2/min for a 1ml assay.

d) Isocitrate dehydrogenase was assayed by the reduction of $NADP^+$. The mixture contained in a final volume of 1ml, 0.15M Tris-HCl pH 7.5, 0.5mM $MnCl_2$, 0.4mM $NADP^+$, 2.5mM DL-isocitrate and enzyme. The reaction was initiated by the addition of enzyme.

e) Isocitrate dehydrogenase kinase was assayed by measuring the rate of inactivation of ICDH (Nimmo et al., 1984). 15µl of assay "cocktail" (25mM Bicine-NaOH pH9.0, 10mM ATP, 20mM $MgCl_2$, 1mM DTT, 6.0µM active ICDH, 2% (v/v) glycerol) was incubated at 37°C with 15µl ICDH kinase diluted in 50mM Mops-NaOH pH 7.3, 1mM EDTA, 1mM DTT. 1µl samples of the incubation were withdrawn at intervals and assayed for ICDH activity.

The amount of kinase was adjusted so that the inactivation of ICDH was linear with time for at least 10 min. 1mU ICDH kinase is the amount of enzyme required to inactivate 1nmol ICDH subunits/min.

f) Isocitrate dehydrogenase phosphatase was assayed by monitoring the release of ^{32}P from ICDH (Nimmo et al., 1984) that had been phosphorylated in vitro using [γ-^{32}P]ATP (see below). The following components were incubated in microfuge tubes at 37°C in a final volume of 40µl: 50mM Mops-NaOH pH 6.7, 12.5mM $MgCl_2$, 0.5mM ATP, 0.5mM phosphoenolpyruvate, 5% (v/v) glycerol and ICDH phosphatase. The reactions were started by the addition of ^{32}P-ICDH. After a suitable time (5-30 min) the reactions were terminated by the addition of 10µl 100mg/ml BSA and 0.2ml of 5% (w/v) TCA. After 10 min at 0°C the tubes were centrifuged at 12 000g for 2 min and 0.2ml portions of the supernatant were counted in 2ml portions of Ecoscint (National Diagnostics, Somerville, New Jersey, U.S.A.) in a Beckman LS 8100 scintillation counter. Dephosphorylation of ICDH was linear with time provided that less than 30% of the ^{32}P was released. 1mU ICDH phosphatase catalyses the release of 1nmol of phosphate from phosphorylated ICDH/min.

ICDH phosphatase was also assayed in some experiments by measuring reactivation of phosphorylated ICDH. The incubation was as above and 1µl samples were withdrawn at intervals and assayed for ICDH activity.

^{32}P-ICDH was prepared as described by Nimmo et al. (1984). The following were incubated at 37°C: 32mM Bicine-NaOH pH 9.0, 0.64mM DTT, 15.5µM active ICDH, 10mM $MgCl_2$, 0.5mM [γ-^{32}P]ATP (50-100 Ci/mol) and 0.25-0.50 mU/ml ICDH kinase. The reaction was started by the addition of kinase and continued until the ICDH activity had fallen to less than 0.5% of the original value. The reaction was stopped by the addition of EDTA to a final concentration of 10mM. The ^{32}P-ICDH was separated from ATP by gel filtration at room temperature through a column of Sephadex G-50 (3cm

x 15cm) equilibrated in Mops-NaOH pH 7.3, 1mM EDTA, 1mM DTT. Protein was dialysed overnight against 50mM Mops-NaOH pH 7.3, 1mM EDTA, 1mM DTT, 40% (v/v) glycerol and stored at -20°C.

g) Malate synthase (Method 1)

This method was based on that of Dixon and Kornberg (1959). The decrease of A_{232} upon breakage of the thio-ester bond of acetyl CoA was measured. The reaction contained, in 1ml, 66mM potassium dihydrogen phosphate pH 7.4, 6mM $MgCl_2$ and 0.12mM acetyl CoA. Enzyme was added and the A_{232} due to the presence of acetyl CoA hydrolase was recorded. Glyoxylate was added to 1mM and the initial A_{232} measured. The molar extinction coefficient for cleavage of the thio-ester bond of acetyl CoA was assumed to be 4.5×10^3 (Stadtman, 1957). This method could only be used with purified enzyme or during the purification of malate synthase A from E. coli KAT-1/ pEM9 (which had a high specific activity of malate synthase) due to the presence of acetyl CoA hydrolase in E. coli.

h) Malate synthase (Method 2)

This method was used for crude extracts (Flavell and Fincham, 1968). Cell extract was added to 66mM potassium phosphate pH 7.4, 6mM $MgCl_2$, 0.08mM acetyl CoA and 0.3mM glyoxylate in a total volume of 0.3ml. A control had no glyoxylate. The reaction was terminated by the addition of 0.5µmole of 5,5'-dithiobis(2-nitrobenzoic acid), DTNB, which reacts with CoA to form the yellow compound thionitrobenzoic acid. The increase in A_{412} dependent on the presence of glyoxylate is proportional to the CoA formed by malate synthase activity. Method 1 was used during the purification of malate synthase A from E. coli KAT-1/pEM9 and gave a lower specific activity in the crude extract than did Method 2. Possibly the fact that the K_m for glyoxylate is greater than 100µM (recent results; H.G. Nimmo) contributed to this and, in any case, high background interference by acetyl CoA hydrolase made measurement of malate synthase in crude extracts very difficult, whichever assay method

was used.

i) Glycerol kinase was assayed by coupling the formation of glycerol-1-phosphate to the reduction of $NADP^+$ with glycerophosphate dehydrogenase. The following were added to each cuvette, to a final volume of 1ml: 0.9ml of 2mM $MgCl_2$ in 1M hydrazine/0.2M Glycine pH to 9.8 with KOH, 25µl of 75mM ATP, 25µl of 20mM $NADP^+$ and 70µg rabbit muscle glycerophosphate dehydrogenase in 10µl and extract. The background increase in A_{340} was measured and then the glycerol kinase reaction was initiated by addition of 25µl of 0.1M glycerol.

2.4.3. Standardisation of solutions

Substrate stock solutions were standardised as follows:-

Isocitrate Enough isocitrate to give an A_{340} of approx. 0.3 was added to the components of an ICDH assay (Methods 2.4.2.d)). The reaction was allowed to go to completion and the change in A_{340} was measured. The absorption coefficient of NADPH at 340nm is 6.22×10^3 $M^{-1}.cm^{-1}$.

Succinate was measured indirectly by the procedure just described for isocitrate, except that ICL and excess (2µmoles) glyoxylate, instead of isocitrate were added to the $NADP^+$ and ICDH mixture.

Glyoxylate was measured in a similar way, but with excess succinate (100µmoles) instead of glyoxylate in the mixture.

2.4.4. Methods for steady-state kinetic studies

The continuous assays described in Methods 2.4.2. a), b) and c) were used with appropriate changes of buffer and of substrate concentration. Volumes less than 50µl were added with micro-syringes. Identical results were obtained whether assays were initiated by addition of enzyme or substrate(s). As a routine, enzyme was added last. Chart recorder and monochromator settings were not altered during the course of individual experiments.

Volumes of substrate(s), inhibitor and buffer required for each data point were read off a prepared chart and assays were done in random order.

Individual data points were plotted on double reciprocal primary plots. Lines were fitted by eye, and secondary replots were constructed from the resulting data. K_m and V_{max} values, from primary plots, were also checked with the Enzpack computer program (Williams, 1985) which uses the direct-linear method (Cornish-Bowden and Eisenthal, 1974).

2.5 Methods developed for purification of enzymes

2.5.1 Purification buffers

Buffer A: 10mM potassium phosphate pH6.5, 0.5M KCl, 2mM $MgCl_2$, 1mM benzamidine, 1mM DTT, 1.2mM PMSF.

Buffer B_1: 50mM Mops-NaOH pH 7.3, 1mM EDTA, 1mM benzamidine, 1mM DTT, 1.2mM PMSF.

Buffer B_2: Buffer B_1 without PMSF.

Buffer B_3: Buffer B_1 with 40% (v/v) glycerol and 5mM $MgCl_2$

Buffer C: 50mM Bicine-NaOH pH 9, 1mM benzamidine, 0.05% (v/v) 2-mercaptoethanol.

Benzamidine and DTT were stored as 1M solutions at -20°C and added just before use. PMSF was dissolved in ethanol (20mg/ml) and added just before use.

2.5.2 Purification of ICL from *E. coli* ML308

Steps a) to f) were performed at 0-4°C

a) Preparation of crude extract

20-30g of *E. coli* ML308 grown to the end of log phase ($OD_{420} \simeq 3.2$, after overnight growth on 40mM acetate) were passed through the French Pressure cell twice (Methods 2.3.5.a)), in 2 volumes of Buffer A. After the first passage the material was extremely viscous due to unsheared

DNA, but the second passage through the pressure cell resulted in a marked decrease in viscosity. The resulting material was centrifuged at 30 000g for 20 min at 4°C (MSE 18 with an 8 x 50ml rotor) and the supernatant was decanted through glass wool to give the crude extract.

b) Protamine sulphate treatment

Protamine sulphate (100mg/ml in buffer A) was added dropwise with stirring to give a ratio of 0.3mg protamine sulphate per mg of protein. The suspension was stirred for 15 min and centrifuged at 40 000g for 10 min. The supernatant was retained.

c) Ammonium sulphate fractionation

Powdered $(NH_4)_2SO_4$ was added gradually to give 30% saturation (175g/l), while the pH of the solution was maintained in the range pH 6.5-7.3 by the addition of 5M NH_3. The solution was stirred for 30 min and centrifuged at 40 000g for 10 min and the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernatant to give 45% saturation (278g/l) and the solution was stirred and centrifuged as before. The pellet was dissolved in a small volume of Buffer B_1.

d) Acid treatment

The dissolved pellet was gradually brought to pH4.5 by the addition of 1M acetic acid with stirring. The resulting precipitate was removed by centrifugation at 100 000g for 15 min (Beckman L8-M Ultracentrifuge with a Ti 50 rotor). The supernatant was decanted and returned, gradually, to pH 7.3 by addition of 1M KOH with stirring.

e) Gel filtration on Sephacryl S-300 superfine

The acid-soluble material was loaded onto a 2.2cm x 71cm column of Sephacryl S-300 superfine, equilibrated in Buffer B_1. The flow rate was 10ml/hr and 3ml fractions were collected. Fractions containing ICL activity, up to and including the fraction after the peak, were pooled. Later ICL-containing fractions were discarded (Figure 3.1.).

f) Phenyl-Sepharose chromatography

Powdered $(NH_4)_2SO_4$ was added to the pooled fractions from step e) to bring the concentration to 0.6M. The solution was then loaded onto a 10ml column of phenyl-Sepharose equilibrated in 0.6M $(NH_4)_2SO_4$ in Buffer B_1. The column was washed with this buffer, then a gradient (total volume 100ml) of 0.6 to 0M $(NH_4)_2SO_4$ in Buffer B_1 was applied. The flow rate was 30ml/hr and 2.5ml fractions were collected. The enzyme pool from this step was dialysed for 4 hours against 2 x 2 litres buffer B_2.

g) FPLC ion-exchange chromatography on Mono Q

This was carried out at room temperature. One quarter of the enzyme pool from step f) was applied to a Mono Q column equilibrated with Buffer B_2. The enzyme was eluted with a gradient of 0 to 0.5M NaCl in Buffer B_2 (see Figure 3.3.). The flow rate was 1ml/min and 0.5ml fractions were collected. After 3 more runs, enzyme purity was assessed with SDS-PAGE and homogeneous material was pooled.

h) Storage

Pooled enzyme was dialysed overnight into Buffer B_3 and stored at -20°C.

The isolation of ICL took 2 days. The gel filtration column was run overnight and steps f) to h) were carried out on the second day.

2.5.3 Purification of ICL from *E. coli* KAT-1/pEM9

The purification described in section 2.5.2. was followed with 2 changes:

i. A 20ml phenyl-Sepharose column was used with a 150ml, 0.6 to 0M $(NH_4)_2SO_4$, gradient in buffer B_1.

ii. Ion exchange chromatography on Mono Q was not required; instead the enzyme pool from phenyl-Sepharose chromatography was concentrated by vacuum dialysis and then dialysed into buffer B_3.

2.5.4. Purification of malate synthase A from *E. coli* KAT-1/pEM9

All steps were performed at 0-4°C.

a) Preparation of crude extract

A crude extract of acetate-grown *E. coli* KAT-1/pEM9 was prepared, as in 2.5.2.a).

b) Protamine sulphate treatment

This was as described in 2.5.2.b).

c) Ammonium sulphate fractionation

Powdered $(NH_4)_2SO_4$ was added gradually to give 50% saturation (312g/l) while the pH of the solution was maintained in the range pH 6.5 -7.3 by the addition of 5M NH_3. The solution was stirred for 30 min and centrifuged at 40 000g for 10 min. The supernatant was discarded. The pellet was dissolved in a small volume of buffer B_2.

d) Gel filtration on Sephacryl S-300 superfine

The dissolved pellet was run on Sephacryl S-300 superfine as described in 2.5.2.e). Malate synthase was eluted from the column just after ICL.

e) DEAE-Sephacel chromatography

The pooled enzyme was loaded onto a column of DEAE-Sephacel (3cm x 8cm) equilibrated in Buffer B_1. The column was washed with this buffer; then a gradient (total volume 350ml) of 0 to 0.5M NaCl in Buffer B_1 was applied. The flow rate was 100 ml/hr and 10 ml fractions were collected. Fractions of highest activity were pooled.

f) Storage

Pooled enzyme was dialysed overnight into Buffer B_3 and stored at -20°C.

The isolation of malate synthase A took 2 days.

2.5.5 Purification of ICDH from E. coli ML308

Active ICDH was purified from glycerol-grown cells by the method of Borthwick et al. (1984).

a) and b) Preparation of crude extract and protamine sulphate treatment

This was as described in 2.5.2.a) and 2.5.2.b).

c) Ammonium sulphate fractionation

Powdered $(NH_4)_2SO_4$ was added gradually to give 50% saturation while the pH of the solution was maintained in the range pH6.3-6.5 by the addition of 5M NH_3. The solution was stirred for 30min and centrifuged at 40 000g for 10min and the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernatant to give 60% saturation and the solution was stirred and centrifuged as before; the pellet was discarded. $(NH_4)_2SO_4$ was added to the supernate to give 75% saturation and the solution was stirred and centrifuged as before. The pellet was dissolved in a small volume of buffer B (40mM potassium citrate, 2mM $MgCl_2$ pH6.5).

d) Ion-exchange chromatography

The dissolved pellet was desalted into buffer B on a column of Sephadex G-25. The material was then loaded onto a column of DEAE-cellulose (1 X 10cm) equilibrated in buffer B. The column was washed with this buffer at a flow rate of 100ml/h until the absorbance at 280nm of the effluent was lower than 0.1. The column was then washed with 100mM potassium phosphate, 5mM potassium citrate, 2mM $MgCl_2$, pH6.5. ICDH activity was eluted in a sharp peak; fractions with an activity greater than 10 µmol/min/ml were pooled and dialysed overnight against buffer D (10mM potassium citrate, 2mM $MgCl_2$ pH6.5 containing 10% v/v glycerol).

e) Chromatography on Procion Red Sepharose

The dialysed material was loaded onto a column of Procion Red Sepharose (1 X 10cm) equilibrated in buffer D at a flow rate of 48ml/h. The column was washed exhaustively with buffer D until the absorbance at

280nm of the effluent was below 0.05. The column was then washed with buffer D containing 0.5mM $NADP^+$. The ICDH activity was eluted as a sharp peak with the $NADP^+$ front; fractions containing an activity of more than 5µmol/min/ml were pooled, concentrated by vacuum dialysis and dialysed against buffer E (10mM potassium phosphate, 5mM potassium citrate, 2mM $MgCl_2$ pH6.5 containing 40% glycerol. Steps a) to c) of this procedure were carried out as rapidly as possible at 0-4°C. Steps d) to e) were carried out at room temperature. The procedure was completed in 48h. The enzyme was stored in buffer E at -20°C.

2.5.6. Purification of ICDH kinase/phosphatase from E. coli KAT-1/pEM9

The purification scheme was developed by Dr. H.G. Nimmo and was based on that devised for purification of ICDH kinase/phosphatase from E. coli ML308 (Nimmo et al.,1984).

a) and b) Preparation of crude extract and protamine sulphate treatment

This was as described in 2.5.2.a) and 2.5.2.b)

c) Ammonium sulphate fractionation

The fraction precipitating between 30 and 45% was collected as described in 2.5.2. The precipitate was redissolved in a small volume of buffer B_1 containing 150mM NaCl. It was desalted into this buffer by gel filtration on a column of Sephadex G-25 (2.2 X 50cm).

d) Ion-exchange chromatography at pH7.3

The desalted material was loaded onto a column of DEAE-cellulose (3.3 X 6cm) equilibrated in the same buffer. The column was washed with this buffer until the A_{280} of the eluate was zero. The enzyme was then eluted with buffer B_1 containing 250mM NaCl. The flow rate was 200ml/h and fractions of 10ml were collected and assayed for ICDH phosphatase. The active fractions were pooled and dialysed overnight into buffer B_1 containing 100mM NaCl.

e) Chromatography on blue dextran Sepharose

The dialysed material was loaded on to a column of blue dextran Sepharose (2.2 X 12cm) equilibrated in buffer B_1 containing 100mM NaCl. The column was washed with this buffer until the A_{280} of the eluate was zero and was then washed in the same buffer containing 1mM NAD^+. The enzyme was then eluted with buffer B_1 containing 400mM NaCl. The flow rate was 60ml/h and 5ml fractions were collected. A solution of 10% (w/v) Lubrol PX was added to the fractions to give a final concentration of 0.05% (w/v). The fractions were assayed for ICDH phosphatase and the active fractions were pooled and concentrated by vacuum dialysis to approximately 1ml.

f) Ion-exchange chromatography at pH7.3 on Mono Q

The concentrated material was loaded on to an FPLC Mono Q column in buffer B_1 containing 0.05% (w/v) Lubrol PX.The flow rate was 1ml/min and 0.5ml fractions were collected. The column was developed with increasing concentrations of NaCl in buffer B_1; 0-0.23M over 4min, then 0.23-0.39M over the next 20min. ICDH kinase/phosphatase was eluted at a NaCl concentration of approx. 0.27M NaCl. The most active fractions were pooled.

g) Ion-exchange chromatography at pH9.0 on Mono Q

The pooled fractions were diluted 1 in 4 in 50mM Bicine-NaOH pH9.0, 0.05% (w/v) Lubrol PX and were loaded on to a Mono Q column equilibrated in this buffer containing 100mM NaCl. The column was washed in this buffer and then developed with a linear gradient of 100-400mM NaCl over 30min. The flow rate was 1ml/min and 0.5ml fractions were collected. The pH of the most active fractions was reduced to 7.3 by the addition of 200µl of 0.5M Mops-NaOH pH7.3. These fractions were pooled and concentrated to 200µl.

h) Gel filtration on an FPLC Superose 12 column

The concentrated material was loaded on to a Superose 12 column

equilibrated in buffer B_1 containing 0.05% Lubrol PX. The flow rate was 0.2ml/min and 0.5ml fractions were collected. Active fractions were pooled and dialysed into 50mM Mops-NaOH pH7.3, 1mM EDTA, 1mM dithiothreitol, 1mM benzamidine hydrochloride, 0.05% (w/v) Lubrol PX, 40% (v/v) glycerol and stored at -20°C.

2.6 Polyacrylamide gel electrophoresis techniques

2.6.1 Non-denaturing PAGE

This was carried out in 7% (v/v) polyacrylamide gels at pH 8.0 (Davis, 1964).

The following stock solutions were stored at 4°C:

A. 3.0M Tris-HCl pH 8.0, 0.25% (v/v) TEMED.

B. 28% (w/v) acrylamide, 0.74% (w/v) bisacrylamide.

C. 10mM Tris/72mM glycine.

"Gel monomer" was prepared by mixing A, B and H_2O in the ratio 1:2:1. An equal volume of ammonium persulphate (1.4mg/ml) was prepared. Solutions were degassed separately and then they were mixed and poured into the custom-built casting box. Slab gels (19cm x 9.5cm x 0.15cm) were poured and a well-forming template was inserted until the gel had polymerised.

Electrophoresis buffer was solution C diluted 5-fold with H_2O and containing 0.1% (v/v) 2-mercaptoethanol. The gel was pre-electrophoresed for 30 minutes at 45mA at 4°C. 5µl of tracking dye (0.02% (w/v) bromophenol blue in 20% (v/v) glycerol) was layered into each well and current was switched on until the dye had just penetrated the gel. The samples (less than 50µl in 20% (v/v) glycerol) were loaded and electrophoresis was performed at 45mA, at 4°C, until the tracking dye approached the bottom of the gel. Pieces of wire were used to mark the dye front.

2.6.2. Staining for ICL activity in non-denaturing gels

Gels were soaked at 4°C in 4 changes of 50mM Mops-NaOH pH7.3, 1mM EDTA, 5mM $MgCl_2$ over 1 hour. The gels were then transferred to the staining solution: 50mM Mops-NaOH pH7.3, 1mM EDTA, 5mM $MgCl_2$, 5mM DL-isocitrate, 50mM NAD^+, 10µg/ml pig heart lactate dehydrogenase, 550µM phenazine methosulphate, 55µM nitroblue tetrazolium. This was kept in the dark, at 37°C, until ICL activity was visible as a purple precipitate of formazan.

2.6.3 SDS-PAGE (discontinuous system)

Protein samples were analyzed by discontinuous slab gel electrophoresis in the presence of SDS according to Laemmli (1970).

Glass plates were separated by 0.8mm or 1.5mm spacers and sealed with 0.8% (w/v) agarose.

Gels were prepared from the volumes (mls) of stock solutions shown on the table on page 47. The acrylamide, Tris buffer and H_2O were mixed and degassed; SDS, ammonium persulphate and TEMED were added and the gel was poured into the cast immediately. Isopropanol was layered onto the separating gel and rinsed off upon polymerisation. Then a stacking gel was made up and poured around a well-forming template. The wells were rinsed after polymerisation. Electrophoresis buffer consisted of 25mM Tris/192mM glycine, pH 8.8, 0.1% (w/v) SDS.

Protein samples were mixed with at least an equal volume of sample buffer, comprising 50mM Tris-HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue and 1% (v/v) 2-mercaptoethanol and were immediately placed in a boiling water bath for 2 minutes.

Electrophoresis was carried out at a constant current of 50mA for 1.5mM gels or 30mA for 0.8mm gels.

Stock solution	Stacking gel	Separating gel 8%	10%	15%
30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide	1.5	8	10	15
1.5M Tris-HCl,pH8.8	-	7.5	7.5	7.5
0.5M Tris-HCl, pH 6.8	3.75	-	-	-
H_2O	9.45	14.05	12.05	7.05
10% (w/v) SDS	0.15	0.3	0.3	0.3
10% (w/v) ammonium persulphate (freshly made)	0.15	0.15	0.15	0.15
TEMED	0.01	0.01	0.01	0.01

2.6.4 Staining gels for protein

Routinely, gels were stained in 0.1% (w/v) Coomassie Brilliant Blue G250, 50% (v/v) methanol, 10% (v/v) acetic acid for 1 hour at 45°C and destained in several changes of 10% (v/v) methanol, 10% (v/v) acetic acid at 45°C.

A more sensitive method was silver staining, based on the method of Wray et al. (1981). Gels were soaked for 2 or 3 days in 3 changes of 50% (v/v) methanol. Staining solution was prepared by adding solution A

(0.8g $AgNO_3$ in 4ml H_2O) to solution B (1.4ml 14.8M NH_4OH plus 21ml 0.36% (w/v) NaOH) dropwise, stirring vigorously. Distilled water was added to give 100ml. The gel was gently agitated in staining solution for 8 minutes and then rinsed for 1 hour with 6 changes of distilled water. The stain was developed by immersing the gel in a solution comprising 2.5ml 1% (w/v) citric acid and 0.25ml 38% (v/v) formaldehyde in 500ml distilled water. Staining was stopped by washing the gel immediately in distilled water.

2.6.5 M_r determination by SDS-PAGE

M_r values were obtained from SDS gels, after Coomassie Blue staining, by scanning on an LKB 2202 Ultrascan laser densitomer. The electrophoretic mobilities (R_f values) were calculated from:

$$R_f = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracker dye}}$$

The following standard proteins were used for calibration: phosphorylase b, 94 000 (Serry et al., 1967); bovine serum albumin 67 000 (Castellino and Barker, 1968); ovalbumin, 43 000 (Castellino and Barker, 1968); carbonic anhydrase, 30 000 (Reynaud et al., 1971); trypsin inhibitor, 20 100 (Koide and Ikenaka, 1973); α-lactalbumin, 14 400 (Brew et al., 1967).

2.6.6 Peptide mapping by gel electrophoresis

The method of Cleveland et al.(1977) was used. The protein bands of interest were sliced out of a Coomassie Blue-stained gel. The gel chips were rinsed in Cleveland buffer (0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS) for 30 min and were then loaded into the slots of a 15% SDS gel (Methods 2.6.3). Each chip was overlaid with 10µl of Cleveland buffer containing 20% glycerol and then 20µl of protease in Cleveland buffer containing 10% glycerol and 0.01% bromophenol blue.

Electrophoresis was carried out until the tracking dye had reached the end of the stacking gel. The current was switched off for 30 min to allow digestion to occur. The current was switched on again until the tracking dye had reached the bottom of the gel. The gel was stained with silver stain (Methods 2.6.4.).

2.7 Further analysis of proteins

2.7.1 Sedimentation equilibrium centrifugation

The native M_r of *E. coli* ICL was estimated by sedimentation equilibrium centrifugation performed in a Beckman Model E Analytical Ultracentrifuge equipped with a AN-G-T-I rotor and 12mm double sector charcoal filled Epon cells with quartz windows.

The benzamidine was removed by dialysis against 50mM Mops-NaOH pH 7.3, 1mM EDTA, 1mM DTT to reduce background A_{280}. Centrifugation was carried out at 8000 r.p.m. for 20 hours at 20°C. An A_{280} scan was made, then the protein was pelleted at 30 000 r.p.m. for 60 mins and the background absorbance was measured.

The M_r was calculated using the equation:

$$M_r = \frac{2RT}{\omega^2 (1-\bar{v}\rho)} \times \frac{d \ln c}{d r^2}$$

R is the gas constant (8.314 x 10 erg/degree/mol)

T is the absolute temperature in degrees K (293K)

ω is the angular velocity in radians/sec (837.74 rads/sec)

$\bar{v}$ is the partial specific volume in ml/g

ρ is the density of the solvent in g/ml (0.7066g/ml)

c is the concentration of the protein in mg/ml at a distance, r, in cm from the axis of rotation

The partial specific volume, v, of ICL was calculated from the amino acid composition by the method of Schachman (1957) and was found to be 0.73 ml/g.

2.7.2 Native M_r determination by gel filtration

Native M_r values were determined using a Sephacryl S-300 superfine column (75cm x 1.6cm) calibrated with the following proteins:

Protein	Native
Ferritin	450 000
Catalase	240 000
Fumarase	194 000
Aldolase	158 000
Bovine serum albumin	68 000 (monomer)
Carbonic anhydrase	30 000
Chymotrypsinogen A	25 000
Cytochrome C	12 500

The column was equilibrated and run in 100mM potassium phosphate buffer, pH 7.5, 2mM DTT at 20ml/hr. Fractions of approximately 2ml were collected.

2.7.3 Analysis of amino acid composition

a) Performic acid oxidation

Solutions were made with filtered distilled water.

Samples of protein (about 0.9mg) were dialysed, at 4°C, for 4 days against 4 x 5l of 0.5% (w/v) ammonium bicarbonate. Equal portions were placed in 9 hydrolysis tubes (16mm x 150mm), lyophilised, resuspended in H_2O and lyophilised again. It was necessary to carry out subsequent steps in the hydrolysis tubes because performic acid-oxidised ICL was very insoluble, sticking to glass vessels and making subdivisions and transfers impossible. This meant that removal of performic acid (usually done by diluting in a large volume of water and lyophilising several times) was very slow, taking several days.

Performic acid was prepared by mixing 95 volumes of formic acid

with 5 volumes of 30% (v/v) hydrogen peroxide and incubating at -5°C for 120 min in a methanol/water/dry ice mixture (Hirs, 1967). 1ml of performic acid was added to each hydrolysis tube and the tubes were left on ice for 4 hours. The samples were then resuspended in 3ml H_2O and again dried *in vacuo* over NaOH pellets. This washing was carried out twice. The samples were then freeze-dried 10 times from 3ml H_2O. The walls of the tubes were coated with protein as was shown by addition of Bradford's reagent to one tube.

b) Internal standardisation

DL-norleucine (25nmoles) was added to each tube and lyophilised.

c) Acid hydrolysis

1ml of 6M Aristar HCl containing 0.1% (v/v) 2-mercaptoethanol was added to each tube and duplicates were incubated *in vacuo* at 110°C for 24, 48, 72 and 96 hours.

d) Analysis

Duplicate analyses were carried out on an LKB 4400 amino acid analyser, operated by Mr. J. Jardine.

2.7.4 N-terminal amino acid sequencing

a) Reduction and carboxymethylation

30 to 100 nanomoles of native protein were dialysed against 3 changes of 10mM Tris-HCl, pH 8.2 over 48 hours. The dialysed material was lyophilised in a 2-dram vial, resuspended in distilled H_2O and lyophilised again. The dried sample was dissolved in 2ml of 0.1M Tris-HCl, pH 8.2, 8M urea (recrystallised), 2mM DTT and incubated in the dark, under N_2, at room temperature for 60 min. The solution was then made 15mM in iodoacetic acid (from a fresh 0.5M stock solution in 0.1M Tris-HCl, pH 8.2), flushed with N_2 again and incubated in the dark for a further 60 minutes. The reaction was stopped by the addition of DTT to a final concentration of 20mM. The solution was gel filtered on a

Sephadex G50 superfine column (1cm x 30cm) into 10% (v/v) formic acid. The absorbance at 280nm was measured and protein-containing fractions were pooled, diluted to 5% formic acid and lyophilised over NaOH pellets in a dessicator.

b) Liquid-phase sequencing

This was carried out in collaboration with Professor J.E. Fothergill and Mr. B. Dunbar at the SERC-funded protein sequencing facility, Aberdeen University. Automated Edman degradation of reduced and carboxymethylated protein was performed, using a Beckman liquid-phase sequenator (Model 890C) operated by Mr. B. Dunbar. The anilinothiazoline products were converted to phenylthiohydantoins by treatment with a 1M HCl solution containing 1% (v/v) ethanethiol according to the general procedure of Beckman Instruments. The phenylthiohydantoins were identified and quantitated by HPLC on a Waters 5µm "Resolve" C_{18} reverse phase column with a pH 5 sodium acetate-acetonitrile buffer system.

2.7.5. Digestion of ICDH kinase/phosphatase with trypsin

10µl aliquots of ICDH kinase/phosphatase (approx. 40µg/ml) in 50mM Mops-NaOH pH7.3, 1mM DTT, 1mM EDTA, 40% glycerol were incubated at 32°C. Varying amounts of trypsin in 1mM HCl (stored at -20°C at 1mg/ml, then diluted 1/50) were added and at various times an excess of soybean trypsin inhibitor was added to stop the digestion. Trypsin inhibitor was added before trypsin for zero-time reactions.

All samples were kept at 32°C for the duration of the experiment, then were removed to ice.

Aliquots of the digested enzyme were assayed for ICDH kinase and ICDH phosphatase and analysed on SDS-PAGE.

CHAPTER THREE - ISOCITRATE LYASE FROM ESCHERICHIA COLI ML308

3.1. Purification of ICL from acetate-grown E. coli ML308

ICL was purified from E. coli ML308 as described in Methods 2.5.2. The following points are noteworthy :-

3.1.1. Cell breakage

The French pressure cell (Methods 2.3.5b)), which can hold up to 45ml, was used in order to obtain high specific activities of ICL in a crude extract. Two passages through the pressure cell gave most effective release of enzyme. Three passages gave a lower specific activity of ICL and no better yield in the filtered crude extract. Perhaps this was due to increased release of protein from membranes.

3.1.2. Enzyme stability - DTT and protease inhibitors

1mM DTT improved stability of ICL in crude extracts. However, partial purification studies showed that ICL was not fully stable even in the presence of DTT. Fractions purified by ammonium sulphate fractionation, Sephacryl S-200 superfine and DEAE-Sephacel chromatography were run on SDS-gels. The intensity of a band of $M_r \simeq 45\,000$ (as well as several minor bands) correlated with ICL activity. It was also noticed that a band of $M_r \simeq 41\,000$, present in ICL-containing fractions, increased in relative intensity with time if fractions were stored. These two bands were analysed by peptide mapping by gel electrophoresis (Methods 2.6.6.), using trypsin, chymotrypsin and V8 protease. The patterns were similar and it was judged that the M_r 41 000 band was a degradation product of the M_r 45 000 band. Subsequently, 1mM benzamidine and 1.2mM PMSF (the maximum which will dissolve in aqueous solutions) as well as 1mM DTT were added to all buffers (Methods 2.5.1.). These additions solved the problem of enzyme stability and gave increased yield and specific activity of ICL in the crude extract.

3.1.3. Removal of an M_r 80 000 contaminant

Using a combination of S-200 superfine gel filtration and either phenyl-Sepharose chromatography or ion-exchange on the FPLC Mono Q column (Methods 2.5.2.g)) it was relatively easy to obtain enzyme which showed two major bands on SDS-PAGE, one of M_r 45 000 and one of $M_r \simeq 80$ 000, and several minor bands. Only the M_r 45 000 band exactly correlated with ICL activity. Various strategies failed to remove the M_r 80 000 contaminant. These included chromatofocusing, phosphocellulose chromatography and Mono Q with a pH gradient. The suspicion that there was some type of association between ICL and the M_r 80 000 contaminant was considered. However, direct enzyme assay showed that no malate synthase enzyme was present and a cross-linking experiment with dimethyl suberimidate gave no indication that the M_r 80 000 component was physically associated with ICL.

Gel-filtration on Sephacryl S-300 superfine gave acceptable resolution between ICL and the M_r 80 000 protein. SDS-PAGE of fractions from this column showed that the M_r 80 000 protein was eluted after the ICL activity but there was overlap. Judicious pooling at this stage was crucial. Generally the ICL-containing fractions up to and including the fraction after the peak were pooled. The profile from an S-300 superfine column is shown in Figure 3.1. Fractions 47-54 were pooled. A little of the M_r 80 000 could be tolerated in the ICL pool, in which case it was eluted just at the beginning of the ICL peak from the final Mono Q step (Figure 3.3.) and could be observed on SDS-PAGE. Too much and it would be spread through all the ICL-containing fractions from the Mono Q column.

3.1.4. Binding of ICL to phenyl-Sepharose

During the development of this step the following observations were made (all $(NH_4)_2SO_4$ solutions were made up in Buffer B_1) :-

Table 3.1. Purification of ICL from E. coli ML308

ICL was measured by Method 2.4.2.a).

Step	Protein (mg)	Enzyme activity (µmol/min)	Specific activity (µmol/min/mg)	Yield %	Purification factor
Crude extract	1650	1250	0.76	100	1.0
Protamine suphate	1468	1223	0.83	98	1.1
$(NH_4)_2SO_4$ 30-45%	461	1096	2.4	88	3.2
Acid soluble	217	920	4.2	74	5.5
Sephacryl S-300	72	792	11.0	63	14.5
phenyl-Sepharose	19	531	27.9	42	36.7
Mono Q	12	450	37.5	36	49.3

i. ICL was bound to phenyl-Sepharose in 1M or in 0.75M $(NH_4)_2SO_4$ and was eluted when the $(NH_4)_2SO_4$ concentration was reduced to 0.4M but not to 0.5M.

ii. When bound in 0.75M $(NH_4)_2SO_4$, ICL was not eluted by a decreasing gradient from 0.75M to 0.3M $(NH_4)_2SO_4$. Neither was ICL eluted in a series of concentration steps of 50mM each, decreasing from 0.75M to 0M $(NH_4)_2SO_4$.

iii. When the column was equilibrated in 0.5M $(NH_4)_2SO_4$, 80% of ICL activity did not bind.

iv. When the column was equilibrated in 0.6M $(NH_4)_2SO_4$, ICL bound to the column and was eluted by a decreasing gradient from 0.6 to 0M $(NH_4)_2SO_4$. The resulting profile is depicted in Figure 3.2. and this is the method which was incorporated into the purification procedure.

These findings suggests that ICL can bind to phenyl-Sepharose in more than one way and with different affinities depending on the $(NH_4)_2SO_4$ concentration.

3.1.5. The final procedure

The procedure finally adopted is described in Methods 2.5.2. Details of a typical purification of ICL from E. coli ML308 are given in Table 3.1. the procedure was reproducible although final yield depended on the choice of fractions pooled from the S-300 gel filtration column. It was carried out 5 times. As a result ICL was purified 47-60(4) fold over the crude extract in 25-47%(4) yield of activity. The purification from cell breakage through to final dialysis (Methods 2.5.2.) took only 2 days.

When dialysed into Buffer B_3 (Methods 2.5), at least 95% of original activity was retained after six months at -20°C. DTT was important in stabilising activity. With ICL which had been stored at

Figure 3.1. Gel filtration of ICL from E. coli ML308 on Sephacryl S-300 superfine

(step (e) of purification scheme) Enzyme from step (d) was loaded onto a 2.2cm X 71cm column of Sephacryl S-300 superfine. The flow rate was 10ml/h and 3ml fractions were collected. Fractions containing ICL activity, up to and including the fraction after the peak (fractions 47-54) were pooled.

●, A_{280}: ■, ICL activity.

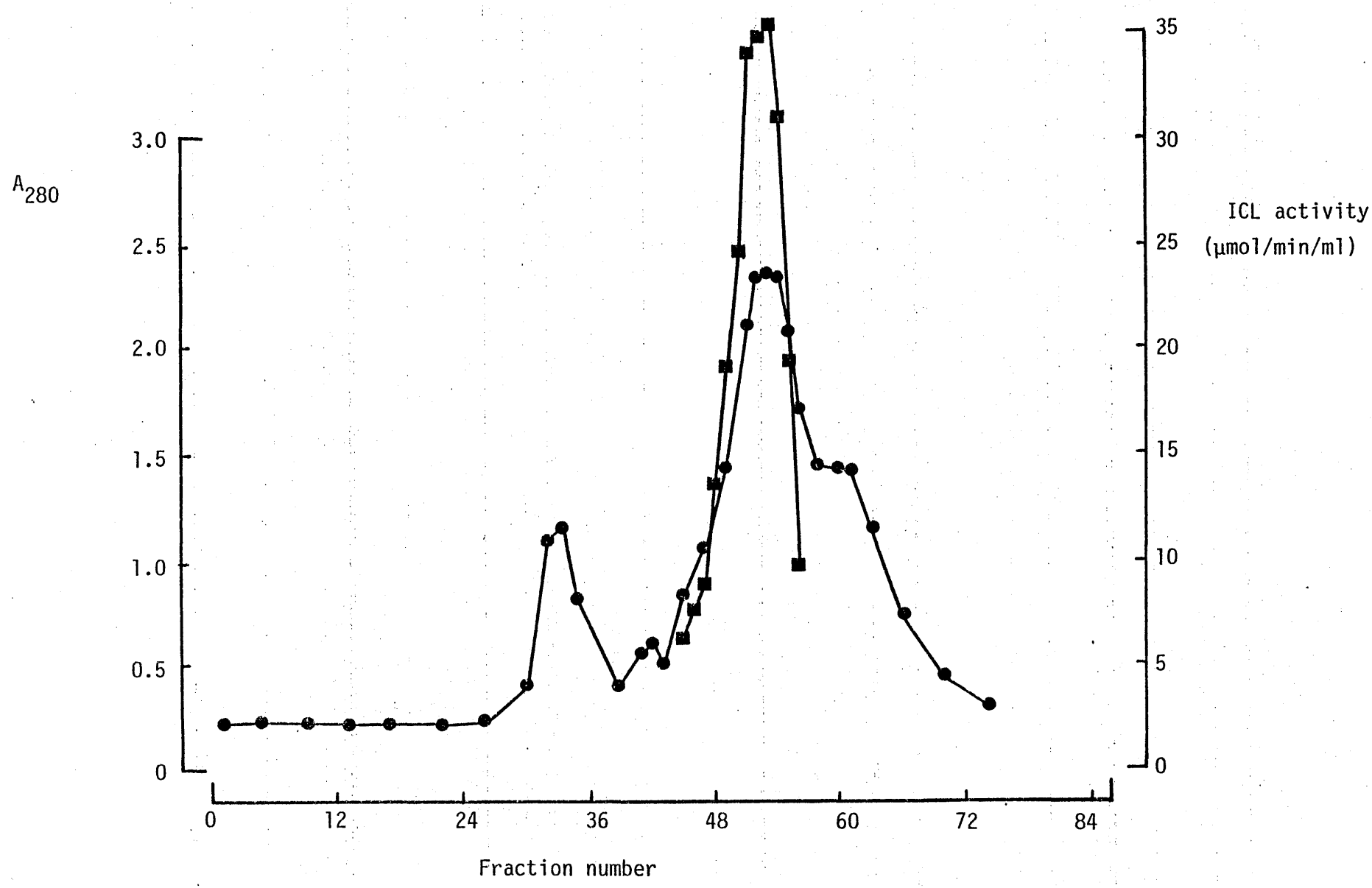

A_{280}
3.0
2.5
2.0
1.5
1.0
0.5
0
35
30
25
20
15
10
5
0
ICL activity
(µmol/min/ml)
0
12
24
36
48
60
72
84
Fraction number

Figure 3.2. Chromatography of ICL from E. coli ML308 on phenyl-Sepharose

(step (f) of purification scheme) $(NH_4)_2SO_4$ was added to the enzyme pool from step (e), to 0.6M, and this was loaded onto a 10ml column of phenyl-Sepharose equilibrated in 0.6M $(NH_4)_2SO_4$ in buffer B_1. The column was washed in this buffer, then a gradient (total volume 100ml) of 0.6 to 0M $(NH_4)_2SO_4$ was applied. The flow rate was 30ml/h and 2.5ml fractions were collected. ●, A_{280}: ■, ICL activity:— —,conductivity.

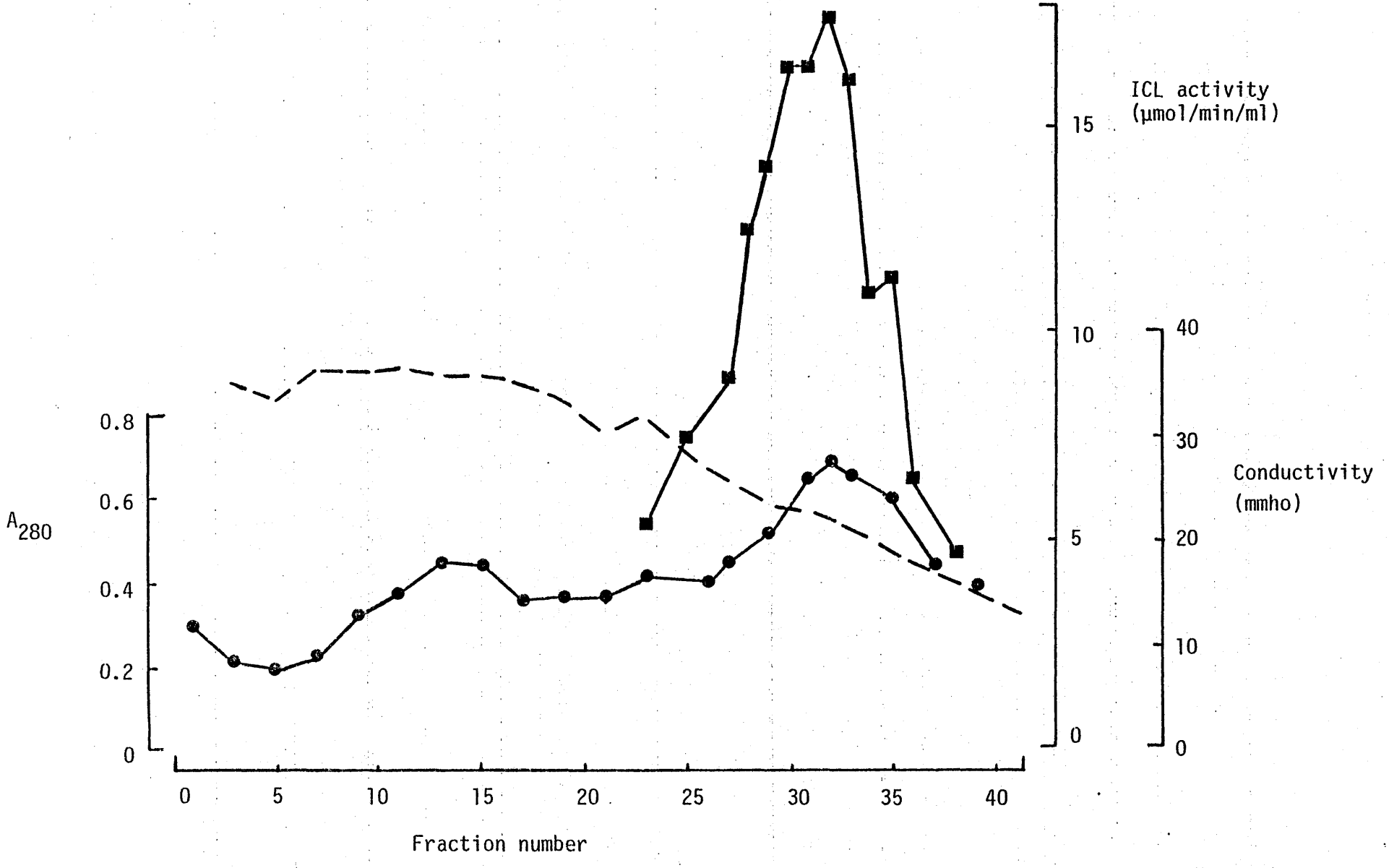
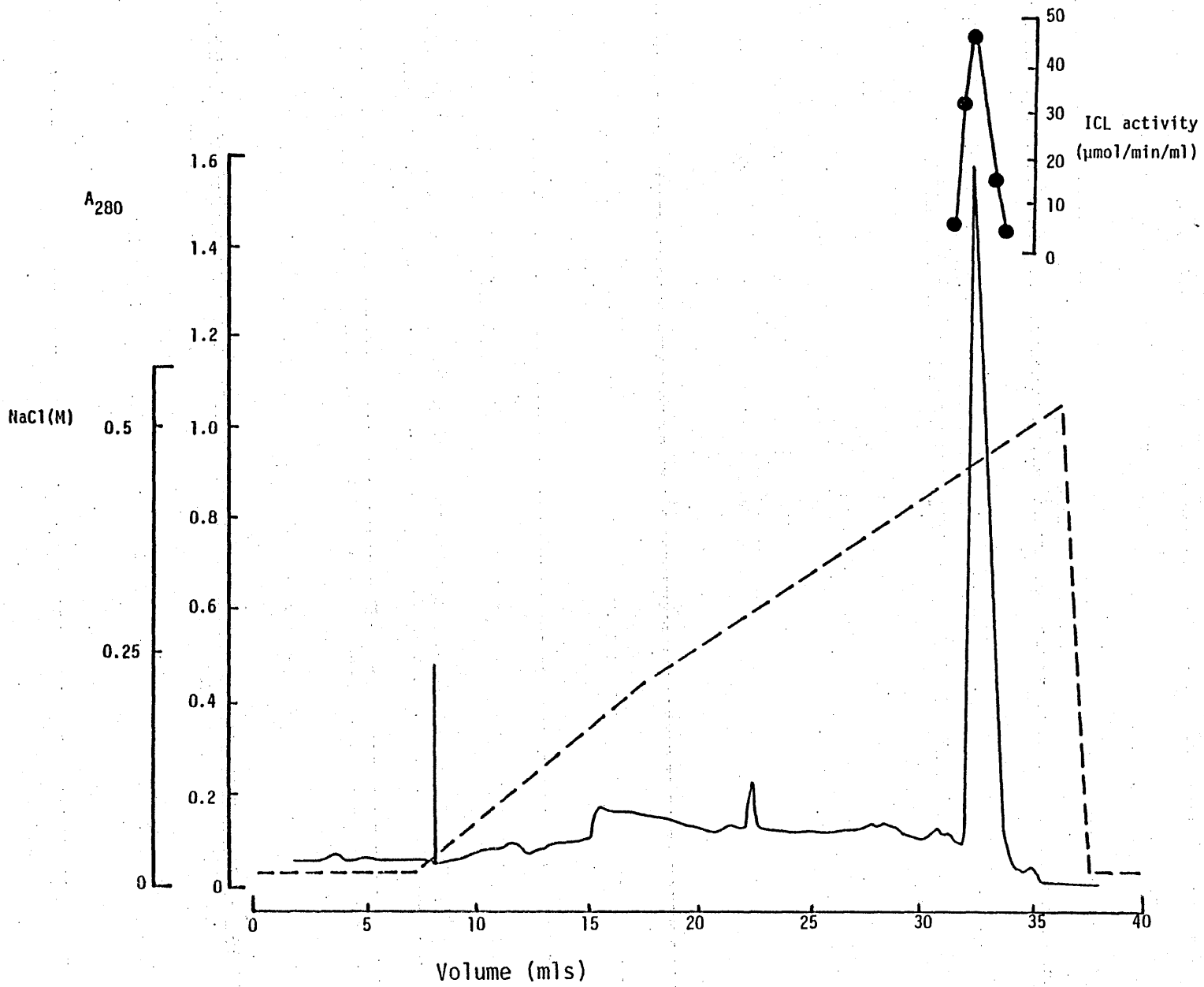

A_{280}
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
Fraction number
ICL activity
(µmol/min/ml)
15
10
5
0
Conductivity
(mmho)
40
30
20
10
0

Figure 3.3. Chromatography of ICL from E. coli ML308 on Mono Q

(step (g) of purification scheme) Enzyme from step (f) was dialysed for 4 hours against 2 X 2 litres of buffer B_2. One quarter was applied to a Mono Q column equilibrated with buffer B_2. The flow rate was 1ml/min and 0.5ml fractions were collected. After three more runs, enzyme was dialysed overnight against buffer B_3 before long-term storage at $-20^{o}C$.


A280
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
NaCl(M)
0.5
0.25
0
50
40
30
20
10
0
ICL activity
(µmol/min/ml)
0
5
10
15
20
25
30
35
40
Volume (mls)

-20°C for some months, DTT acted as an activator in the assays, presumably by generating essential free sulphydryl groups on the enzyme. However if fresh DTT (1mM) was added to the stored enzyme there was no further need to include DTT in the assay mixtures. Protease inhibitors were included to reduce the possibility of proteolytic degradation during long term storage.

The purification of ICL was monitored by SDS-PAGE and a gel is shown in Figure 3.4. The final pool from the Mono Q column gave a single band when stained with Coomassie Blue or silver stain (Methods 2.6.4)

When purified ICL was analysed on non-denaturing 7% (w/v) polyacrylamide gels and stained for protein, a single band was observed (Figure 3.5.). Gels stained for ICL activity also gave a single band, with the same mobility. However, the activity stain took at least two hours to develop, by which time the background was quite dark and the gel could not be photographed successfully. A sample of ICDH run on the same gel was activity stained successfully in a few minutes. The problem with ICL staining could have been slow diffusion of LDH into the gel or, perhaps, inactivation of ICL in the gel running buffer. However, despite this problem, it was possible to observe that ICL activity was seen only in the presence of isocitrate.

3.2. M_r

SDS-PAGE was used to determine the subunit M_r value of the purified enzyme. A typical standard curve of the electrophoretic mobility relative to Bromophenol Blue (R_f) against log M_r is shown in Figure 3.6. Using 5 batches of enzyme, the subunit M_r was determined to be 44670 $\pm$ 460 (7). *E. coli* ICL therefore belongs to the class of ICLs which have a smaller subunit M_r (see Table 1.1.).

The native M_r was determined to be 177 000 (duplicates agreed

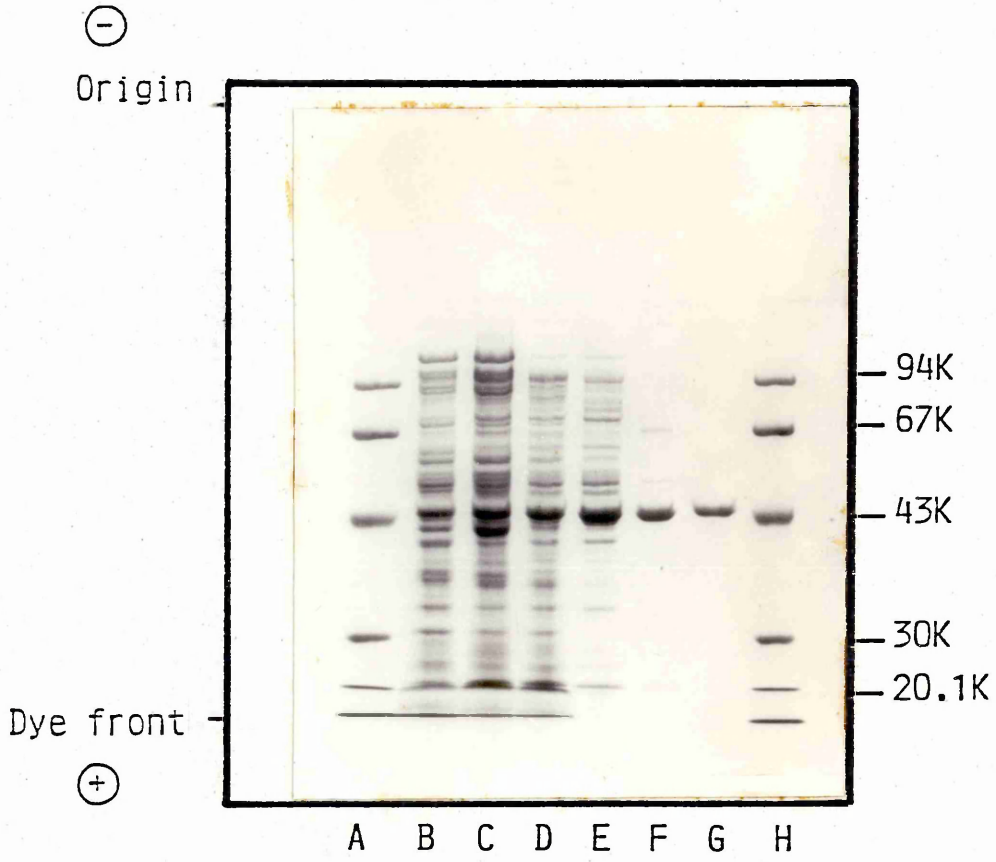


Figure 3.4. Purification of ICL from E. coli ML308 - This 10% polyacrylamide gel run in the presence of SDS monitors the purification of ICL. Tracks A and H, M_r standard proteins (Methods 2.6.5.); track B; crude extract; track C, 30 - 45% ammonium sulphate fraction; track D, acid soluble protein; track E, enzyme eluted from Sephacryl S-300 superfine column; track F, enzyme eluted from phenyl-Sepharose column; track G, enzyme eluted from Mono Q column.

Figure 3.5. Non-denaturing PAGE of purified ICL from *E. coli* ML308

A 7% polyacrylamide slab gel of purified ICL is shown. The gel was prepared and electrophoresed as described in Methods 2.6.1.

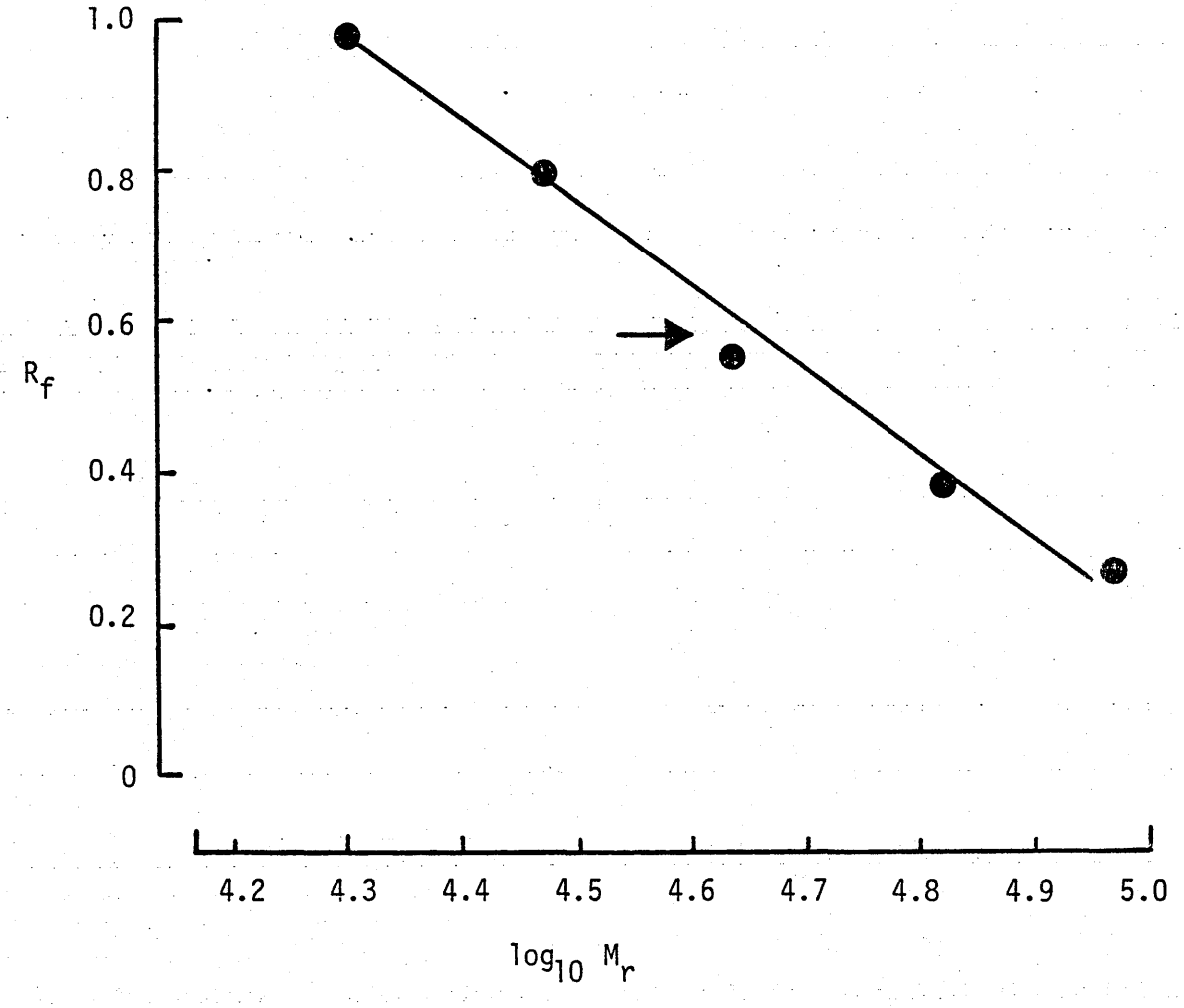


Figure 3.6. Plot of mobility against subunit M_r of standards on SDS-PAGE Analysis was performed on a 10% slab gel as described in Methods 2.6.5. The arrow indicates the average mobility of the E. coli ML308 ICL subunits. This corresponds to an M_r value of 45 000.

Table 3.2.

Effect of $MgCl_2$ concentration on ICL activity

Final conc. of added Mg^{2+} (mM)	ICL activity (% of maximum)
0	0
0.1	14
1.0	81
5.0	100
10.0	93

exactly) by gel filtration on Sephacryl S-300 superfine (Methods 2.7.2.). The standard curve of V_e-V_o/V_t against log native M_r for proteins of known native M_r is shown in Figure 3.7. A single sedimentation equilibrium run on an analytical ultracentrifuge (see Figure 3.8.) gave a value of 180 000 for the native M_r. These results suggest that the active form of the enzyme is a tetramer.

Further physiochemical characterisation of ICL from E. coli ML308 is presented in Chapter 5.

3.3 Effects of Mg^{2+} and pH

In 50mM Mops-NaOH, pH7.3, 1mM EDTA enzyme activity was depressed at $MgCl_2$ concentrations below 1mM or above 5mM (Table 3.2.). 5mM $MgCl_2$ was used for all subsequent assays.

The pH-activity profile (Figure 3.9.) indicated maximal activity at pH7.3.

3.4 The effect of substrate concentration

The substrate of ICL is threo-D_s-isocitrate (eg.Sprecher et al., 1964). L_s-isocitrate is neither a substrate nor an inhibitor of ICL (eg.McFadden and Howes, 1963) so DL-isocitrate was used in all experiments. Concentrations of isocitrate recorded in plots and tables refer to the D_s-isomer only.

Lineweaver-Burk double reciprocal plots of the rate of glyoxylate formation versus the threo-D_s-isocitrate concentration (eg.Figure 3.10.) were linear, conforming to Michaelis-Menten kinetics, and extrapolated to a K_m of 0.063 ± 0.006mM(4) at pH7.3, 0.032 ± 0.005mM(3) at pH6.8 and 0.007mM(1) at pH6.3. The pH6.8 value is close to the value of 0.018mM determined by Ashworth and Kornberg (1963) who studied partially

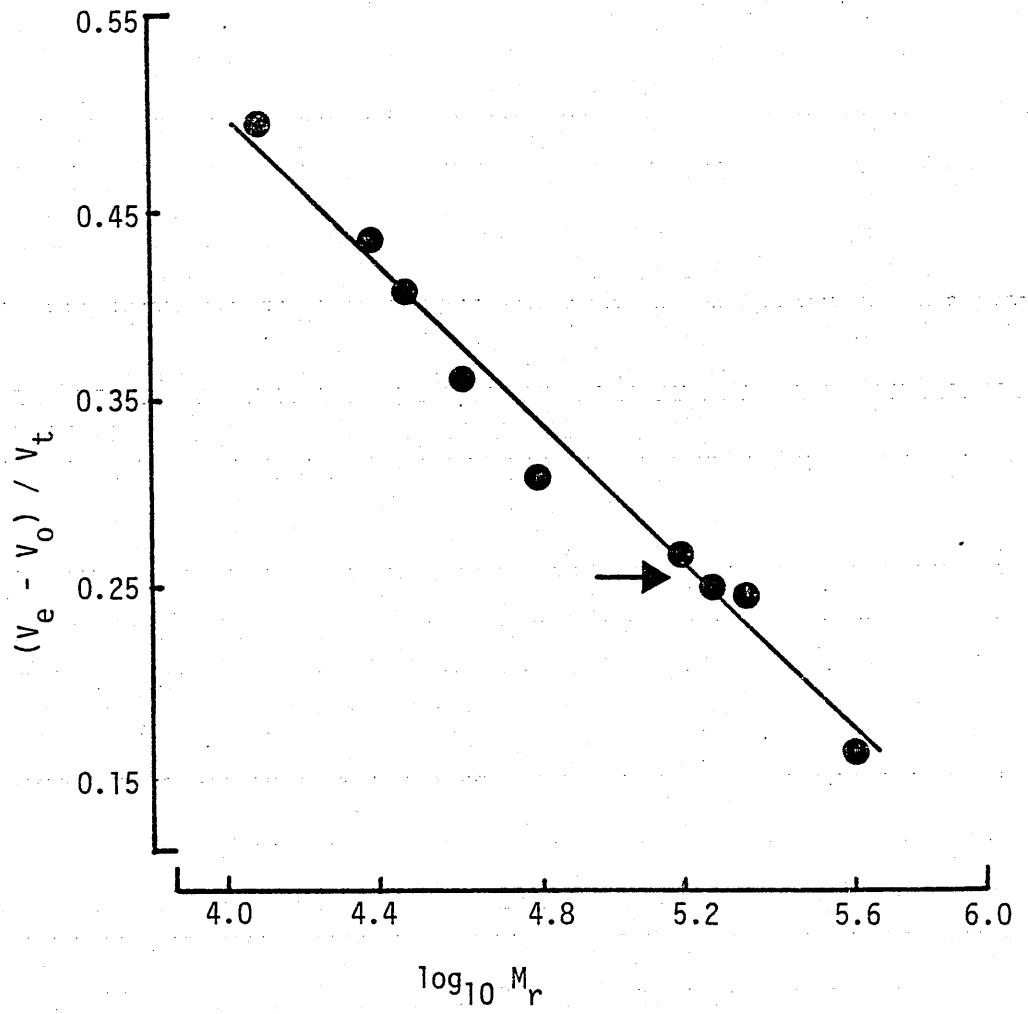


Figure 3.7. Plot of $(V_e - V_o)/V_t$ against $\log_{10} M_r$ of standards on a Sephacryl S-300 superfine column The arrow indicates the $(V_e - V_o)/V_t$ of native ICL from E. coli ML308. This corresponds to an M_r of 177 000. Further details are given in Methods 2.7.2.

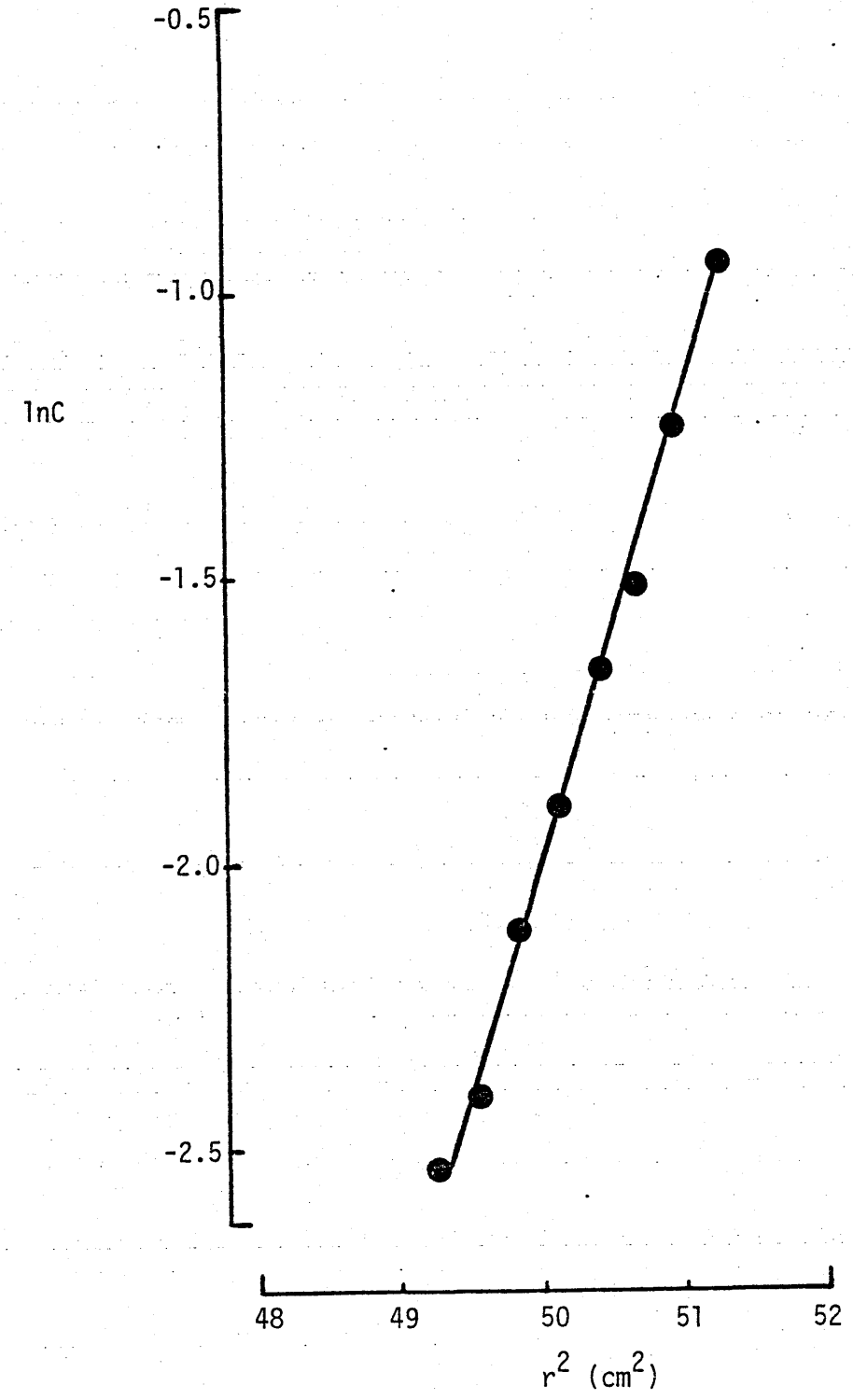


Figure 3.8. Sedimentation-equilibrium ultracentrifugation of purified ICL from E. coli ML308 For full experimental details see Methods 2.7.1. The M_r calculated from the slope was 180 000.

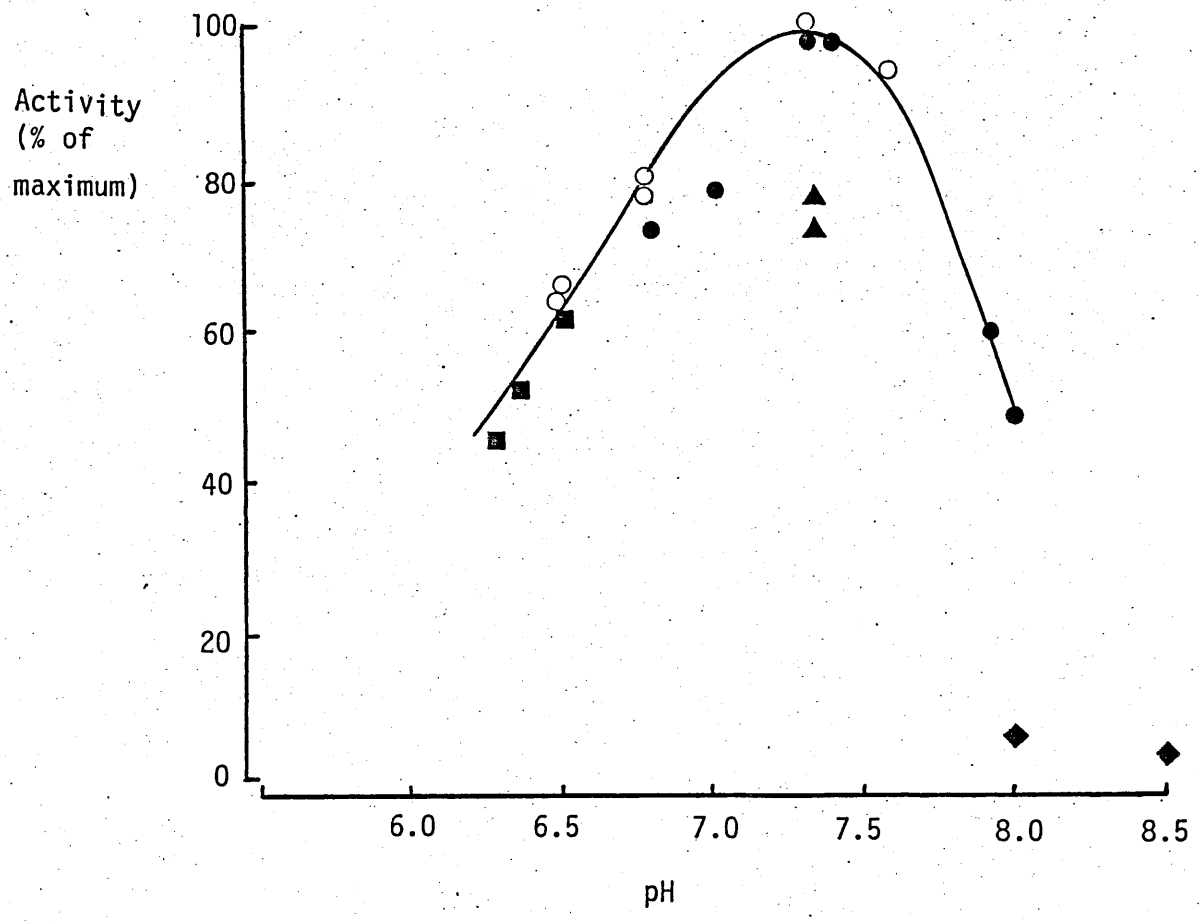


Figure 3.9. Effect of pH on the activity of purified ICL from E. coli ML308 The assays were carried out as described in Methods 2.4.2.a) and 2.4.2.b).■●▲◆, LDH-coupled assay; ○, phenylhydrazine-coupled assay. The buffers were ■, 50mM NaOH-Mes (pH 6.0-7.0); ○ ● 50mM NaOH-Mops (pH6.5-8.0); ◆ 50mM NaOH-Bicine (pH 7.5-9.0) and ▲, 50mM phosphate buffer. Each assay also contained 5mM $MgCl_2$, 1mM EDTA and 5mM DL-isocitrate.

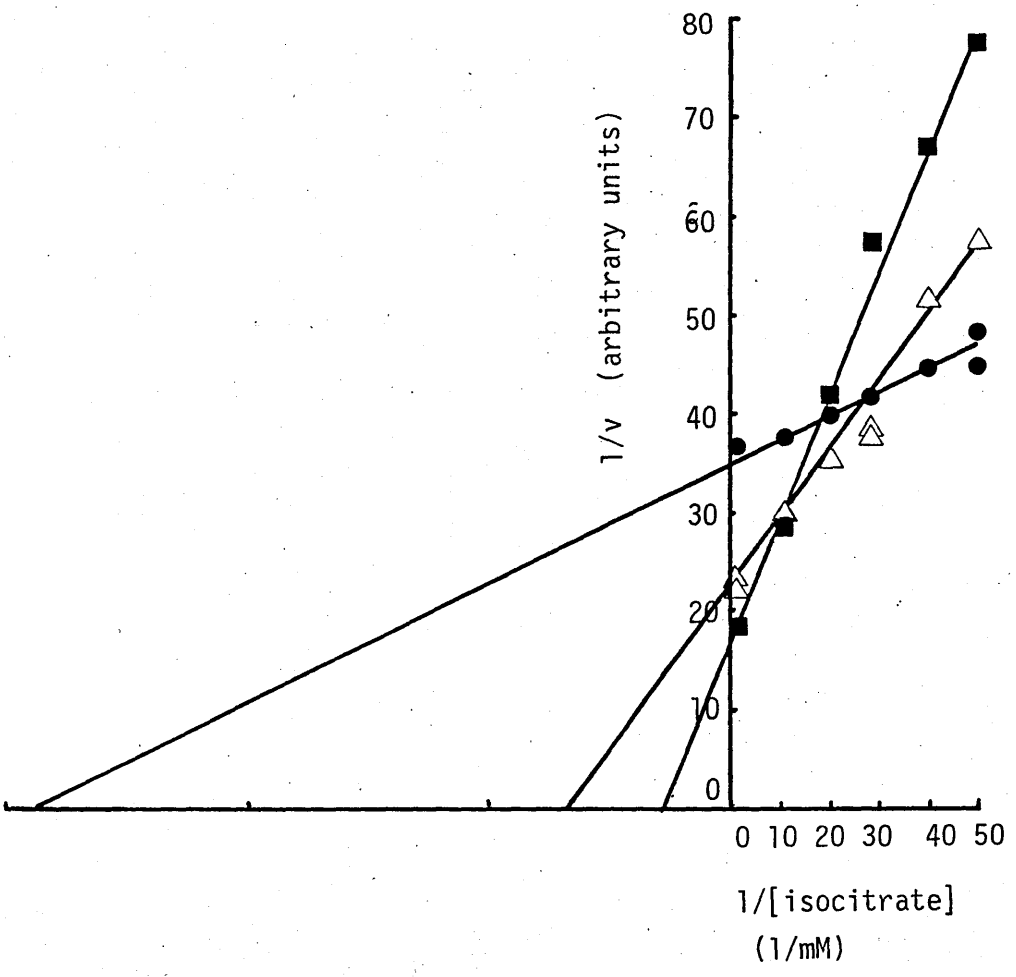


Figure 3.10.

Double-reciprocal plots showing the effect of [D_s-isocitrate] on the activity of ICL from E. coli ML308 The assays were carried out at pH6.3 (●), pH6.8 (△) and pH7.3 (■) and the K_m values, from this and similar plots, were 0.007mM(1), 0·032±0.005mM(3) and 0·063±0.006mM(4), respectively. The LDH-coupled assay (Methods 2.4.2.a)) was used in this particular experiment, but the K_m values given here include some which were obtained by the phenylhydrazine-coupled assay (Methods 2.4.2.b)).

purified ICL from E. coli Bm.

These values are considerably lower than the 3mM reported by Bautista et al. (1979) (who did not describe the assay method) and the value of 0.604mM reported by Walsh and Koshland (1984). Walsh and Koshland (1984) used the stopped phenylhydrazine assay described by Roche et al. (1970) but in the presence of 200mM KCl and 60mM 2-mercaptoethanol.

When ICL from E. coli ML308 was assayed in this buffer using the continuous phenylhydrazine method (2.4.2.b)) a K_m of 0.6mM was obtained.

3.5 The effect of 2-mercaptoethanol and Cl^- ions

Because of these vast discrepancies in K_m values, I examined the effect, on ICL, of the 2-mercaptoethanol and KCl used in Walsh and Koshland's (1984) assay.

When ICL activity was measured by either coupling with LDH (Methods 2.4.2.a)) or phenylhydrazone formation (Methods 2.4.2.b)) 2-mercaptoethanol had the effect of increasing the lag period of the assay, up to 20 mins for 60mM 2-mercaptoethanol. The lag periods depended only on 2-mercaptoethanol concentration and not on ICL concentration. The specific activities as judged by the steady state rates were not affected by 2-mercaptoethanol concentration. This suggests that glyoxylate interacts reversibly with 2-mercaptoethanol to form an adduct that is not a substrate for LDH and that does not react with phenylhydrazine.

KCl was found to be a competitive inhibitor of ICL. NaCl had an identical effect. As shown in the Lineweaver-Burk plot in Figure 3.11. and the slope replot shown in the inset, Cl^- is a non-linear competitive inhibitor with the replot being concave upwards. This implies that Cl^- ions can interact in more than one way at the active site.

Inhibition of ICL by Cl^- ions probably explains why ICL activity

Figure 3.11. Inhibition of ICL by KCl with respect to isocitrate

A. Double-reciprocal plot of the initial rates against 1/[isocitrate] at a series of fixed [KCl]: 0mM (●), 100mM (□), 200mM (■) and 300mM (△).

B. Replot of the slopes (▲) as a function of [KCl].

The LDH-coupled assay was used (Methods 2.4.2.a)). NaCl gave essentially identical results (data not shown).

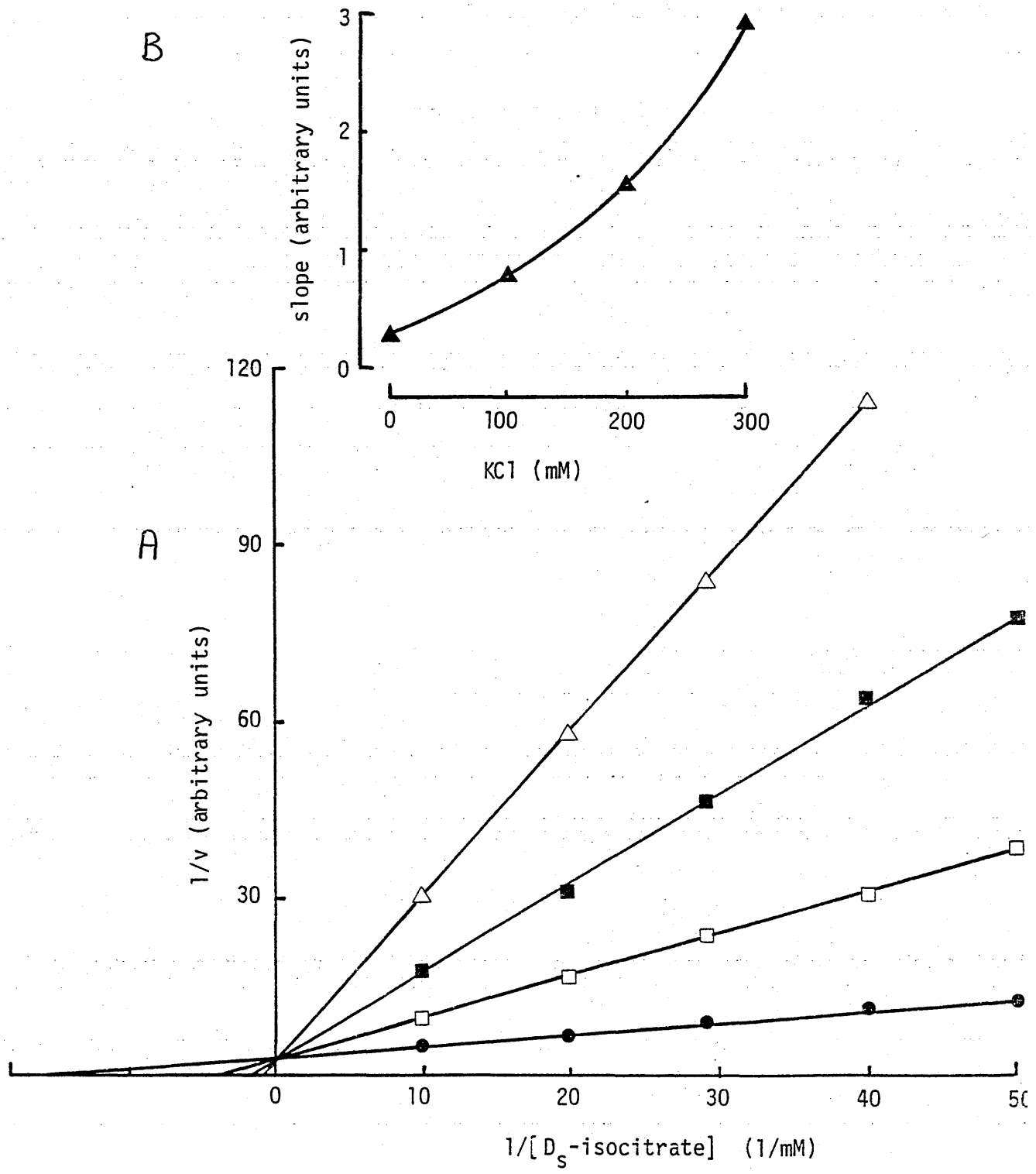

B
slope (arbitrary units)
3
2
1
0
0
100
200
300
KCl (mM)
A
120
90
60
30
1/v (arbitrary units)
0
10
20
30
40
50
1/[D_s-isocitrate] (1/mM)

Table 3.3 Kinetic constants obtained with ICL at pH7.3

CONDENSATION REACTION

K_m of glyoxylate - 0.125mM

K_m of succinate - 0.59mM

a) Inhibition with respect to glyoxylate

Glycolate (competitive)	K_i=0.16mM
PEP (non-competitive)	K_i=1.06mM

b) Inhibition with respect to succinate

Glycolate (non-competitive)	K_i=0.26mM
PEP (competitive)	K_i=0.48mM

CLEAVAGE REACTION

K_m of isocitrate - 0.063mm

Inhibition with respect to isocitrate

Succinate (non-competitive)	K_i=1.125mM
PEP (non-competitive)	K_i=1.04mM

declined at $MgCl_2$ concentrations greater than 5mM (section3.5.).

Here in Glasgow, E. coli are grown in a medium containing SO_4^{2-} and PO_4^{3-}/HPO_4^{2-} (Methods 2.3.3.d) and e)). I found that SO_4^{2-} was a linear competitive inhibitor of E. coli ICL with a K_i of about 20mM (data not shown). No data is available on the internal inorganic anion composition of E. coli.

Johanson et al. (1974) found that ICLs from both N. crassa and P. indigofera were competitively inhibited by the inorganic anions SO_4^{2-}, HPO_4^{2-}, Cl^- and NO_3^-. Their data, and mine, is compatible with the masking of adjacent sites by one divalent anion and the multiple binding of the monovalent Cl^- to these sites. Johanson et al. (1974) suggested that these sites may contain Mg^{2+} and may constitute the glyoxylate sub-site. An alternative explanation is that the enzyme contains three binding sites for carboxylic acid groups and that Cl^- ions can interact with these sites independently but only one SO_4^{2-} can be bound at any one time. Anions of different sizes, e.g. the series F^-, Cl^-, Br^-, I^-, and with different charges, could be used as part of an investigation into the electrostatic nature of the active site.

3.6. The kinetic mechanism of ICL

This section will show the effects of varying substrate concentrations plus some product and dead-end inhibition studies. All K_m and K_i values are summarised in Table 3.3.

Particular attention is drawn to the use of the term "non-competitive inhibitor" to mean one which changes the slope and vertical axis intercept, but not the horizontal axis intercept, of a Lineweaver-Burk plot (Dixon and Webb, 1979).

a) Condensation reaction

The velocity of the ICL-catalysed condensation reaction was followed at various glyoxylate and succinate concentrations and in the

presence of substrate analogues by coupling to ICDH (see Methods 2.4.2.c) and 2.5.5.). In the ICL assay buffer used here (50mM Mops-NaOH pH7.3, 1mM EDTA, 1mM DTT and 5mM $MgCl_2$) ICDH had 75% activity compared with that in its usual assay buffer (Borthwick et al., 1984a) which contains $MnCl_2$. The assays were initiated by the addition of ICL; the results were identical when reactions were initiated with glyoxylate or succinate.

Linear double-reciprocal plots were obtained when glyoxylate and succinate were varied at fixed concentrations of the other substrate (Figures 3.12. and 3.13.). In both cases all the lines intersected at the x-axis.

Since the assay method precluded use of isocitrate as a product inhibitor, dead-end inhibitors were sought. Glycolate was chosen as a likely analogue of glyoxylate. It did indeed show competitive inhibition with respect to glyoxylate at a sub-saturating concentration of succinate (Figure 3.14.). Glycolate gave non-competitive inhibition with respect to succinate (Figure 3.15.).

Phosphoenolpyruvate (PEP) gave non-competitive inhibition with respect to glyoxylate (Figure 3.16.), and competitive inhibition with respect to succinate (Figure 3.17.). PEP is therefore acting as an analogue of succinate.

The simplest explanation for these results is a) that succinate and glyoxylate bind randomly at equilibrium to the enzyme, and b) that binding at the glyoxylate site does not affect the affinity at the succinate site and vice-versa.

Examination of the structures (Figure 3.18.) shows why glycolate and PEP behave as analogues of glyoxylate and succinate respectively.

b) Cleavage reaction

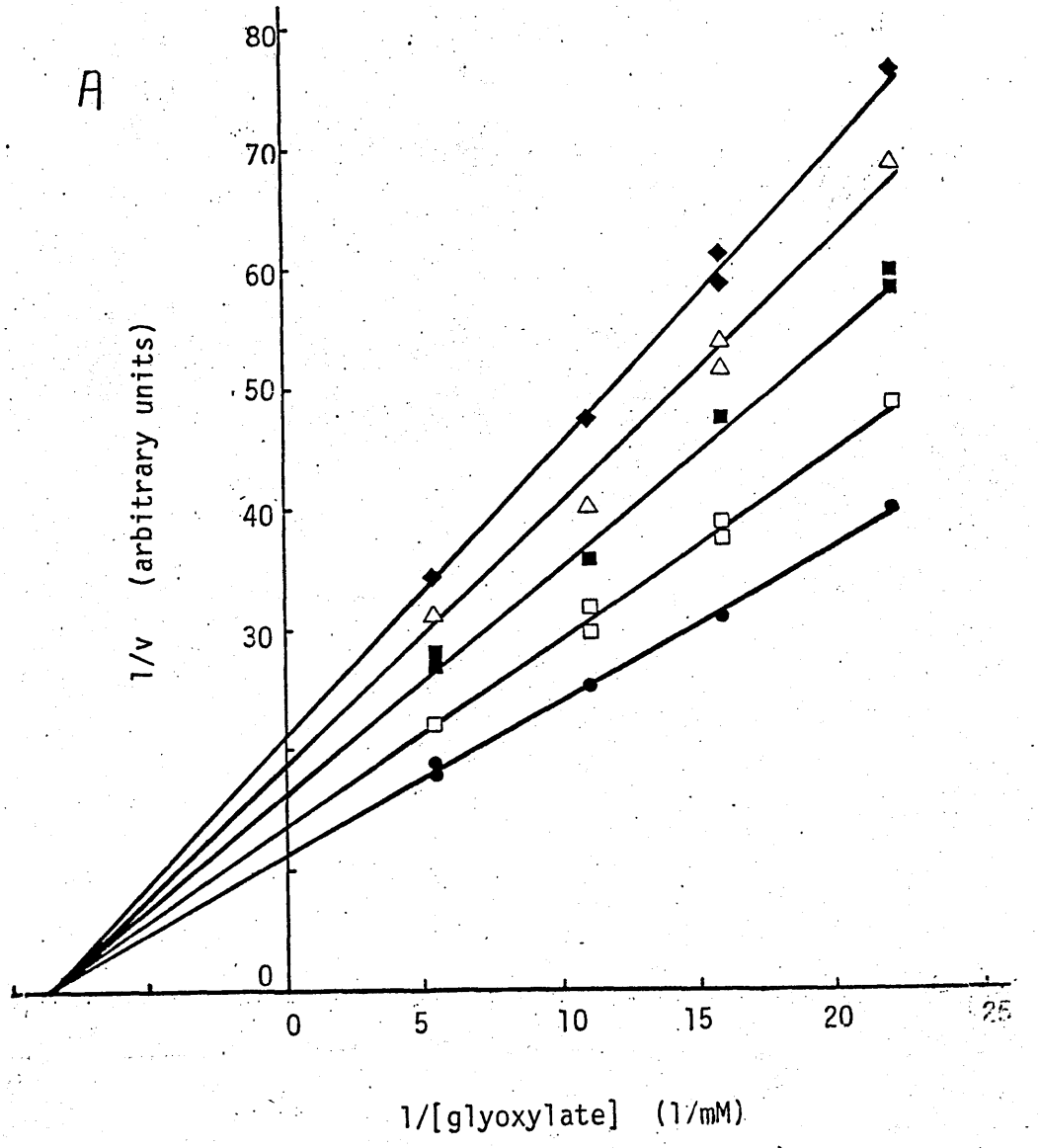
Double-reciprocal plots against isocitrate were linear. Succinate and PEP gave non-competitive inhibition with respect to isocitrate

Figure 3.12. Plots of initial rates of the condensation reaction of ICL: [glyoxylate] varied

A. Double-reciprocal plot of the initial rates against 1/[glyoxylate] at a series of fixed [succinate]: 0.39mM (◆), 0.48mM (△), 0.63mM (■), 0.96mM (□) and 1.93mM (●).

B. Replot of slopes (■) and intercepts (●) as a function of 1/[succinate].

ICL was assayed as described in Methods 2.4.2.c).


A
80
70
60
50
40
30
0
1/v (arbitrary units)
0
5
10
15
20
25
1/[glyoxylate] (1/mM)

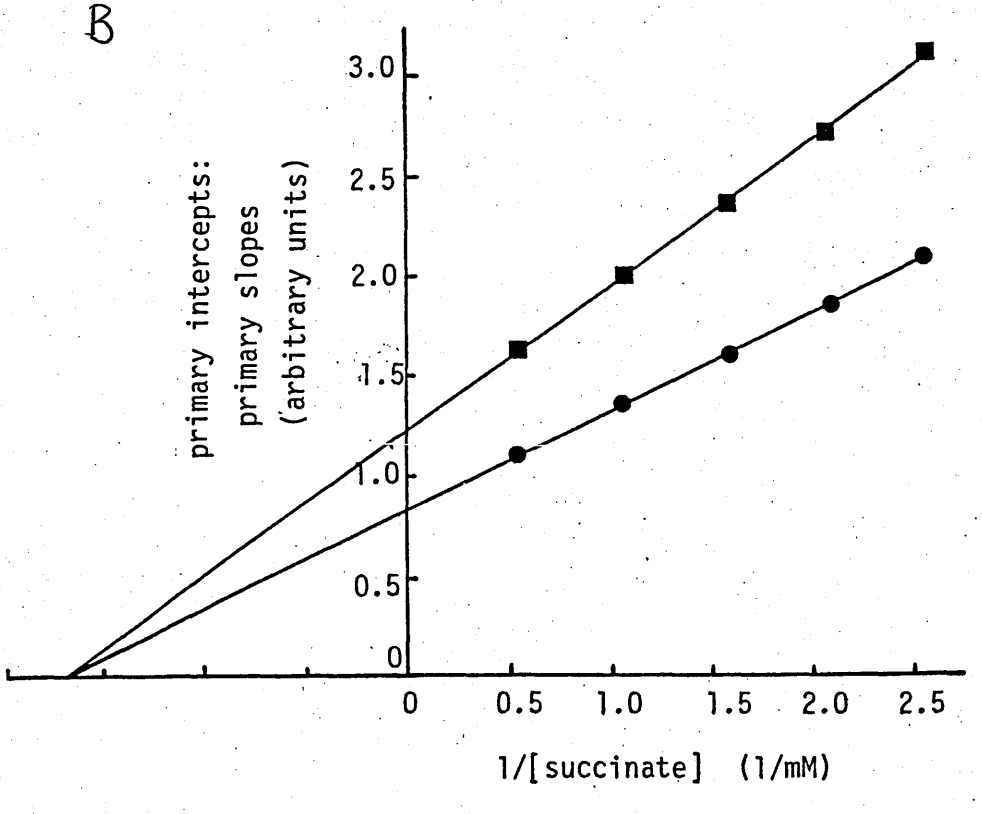
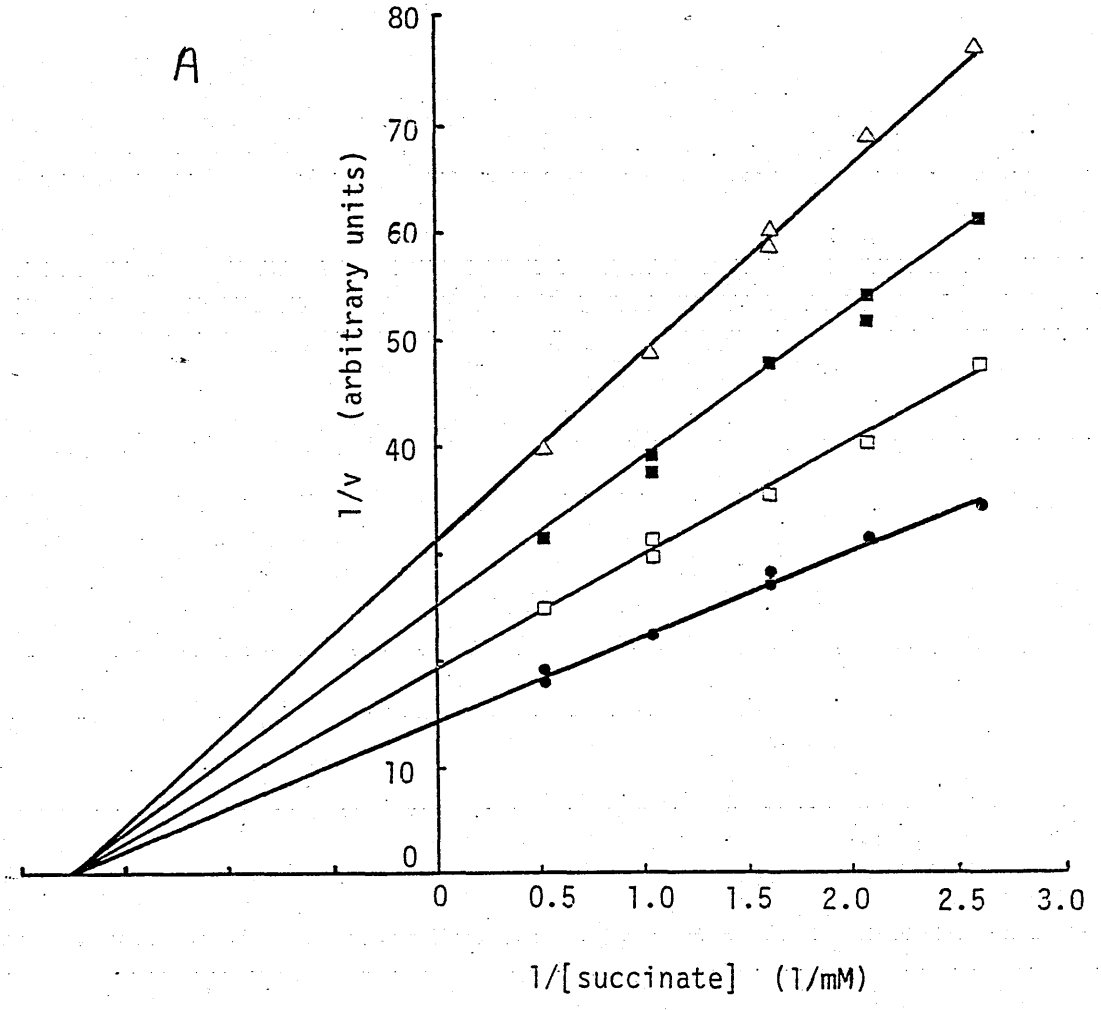

B
3.0
2.5
2.0
1.5
1.0
0.5
0
primary intercepts:
primary slopes
(arbitrary units)
0
0.5
1.0
1.5
2.0
2.5
1/[succinate] (1/mM)

Figure 3.13. Plots of initial rates of the condensation reaction of ICL: [succinate] varied

A. Double-reciprocal plot of the initial rates against 1/[succinate] at a series of fixed [glyoxylate]: 0.045mM (△), 0.063mM (■), 0.09mM (□) and 0.18mM (●).

B. Replot of slopes (●) and intercepts (■) as a function of 1/[glyoxylate].

ICL was assayed as described in Methods 2.4.2.c).


A
80
70
60
50
40
10
0
1/v (arbitrary units)
0
0.5
1.0
1.5
2.0
2.5
3.0
1/[succinate] (1/mM)

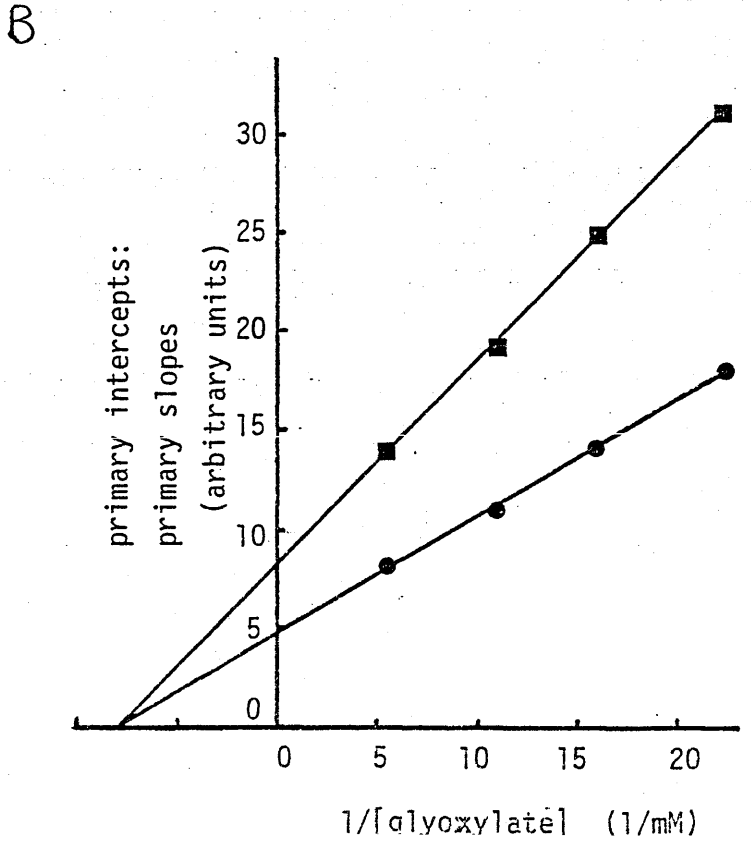
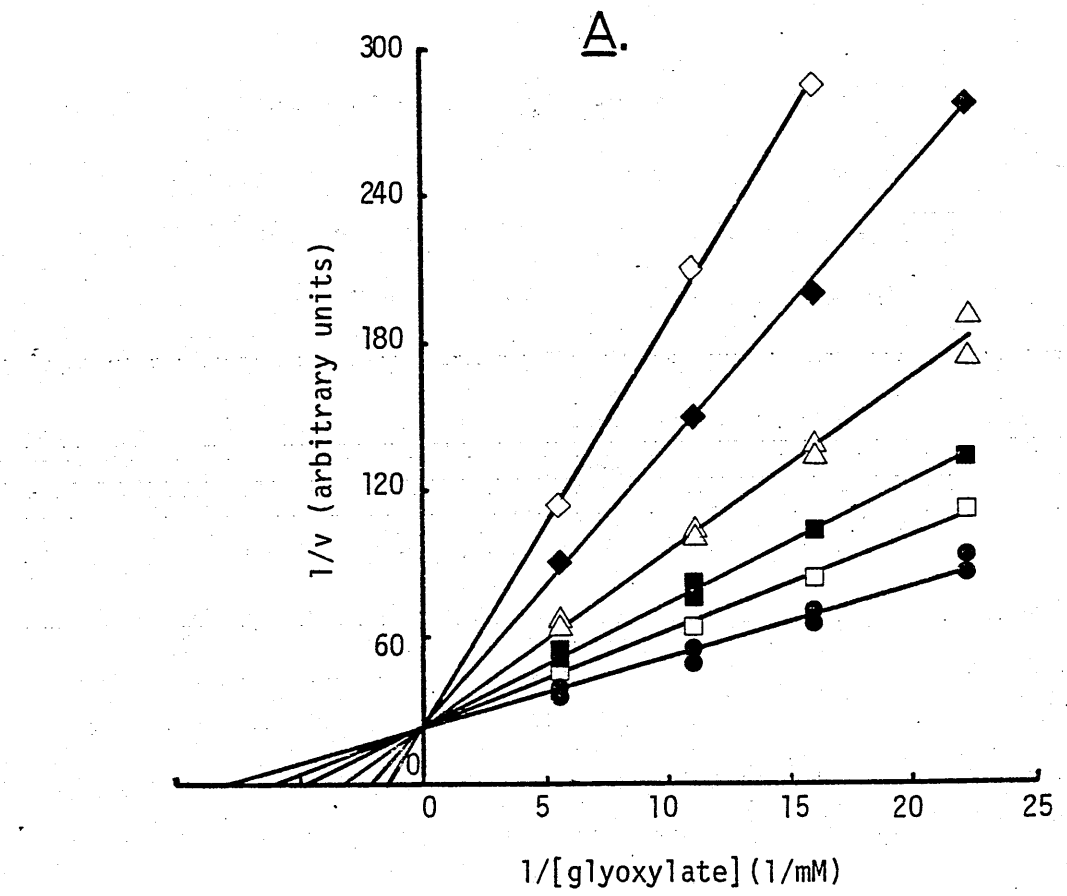

B
30
25
20
15
10
5
0
primary intercepts:
primary slopes
(arbitrary units)
0
5
10
15
20
1/[glyoxylate] (1/mM)

Figure 3.14. Inhibition of ICL by glycolate with respect to glyoxylate

A. Double-reciprocal plots of the initial rates against 1/[glyoxylate] at a series of fixed [glycolate]; 0mM (●), 0.06mM (□), 0.12mM (■), 0.24mM (△), 0.48mM (◆) and 0.72mM (◇). The [succinate] was fixed at 0.39mM.

B. Dixon plot of the same data. The glyoxylate concentrations were: 0.045mM (△), 0.063mM (■), 0.09mM (□) and 0.18mM (●). The K_i value for glycolate, from this plot, was 0.16mM.

The assay was that described in Methods 2.4.2.c).


A.
300
240
180
120
60
0
1/v (arbitrary units)
0
5
10
15
20
25
1/[glyoxylate] (1/mM)

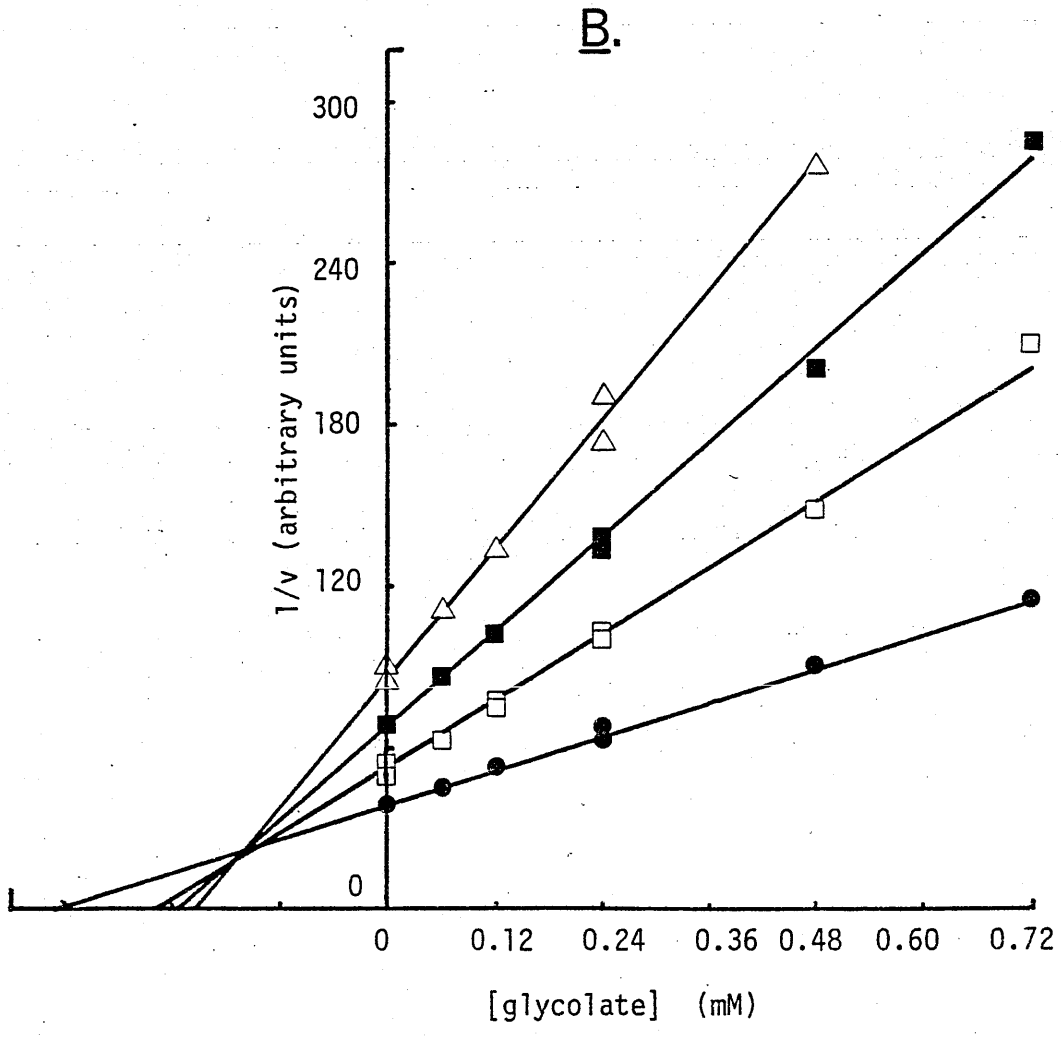
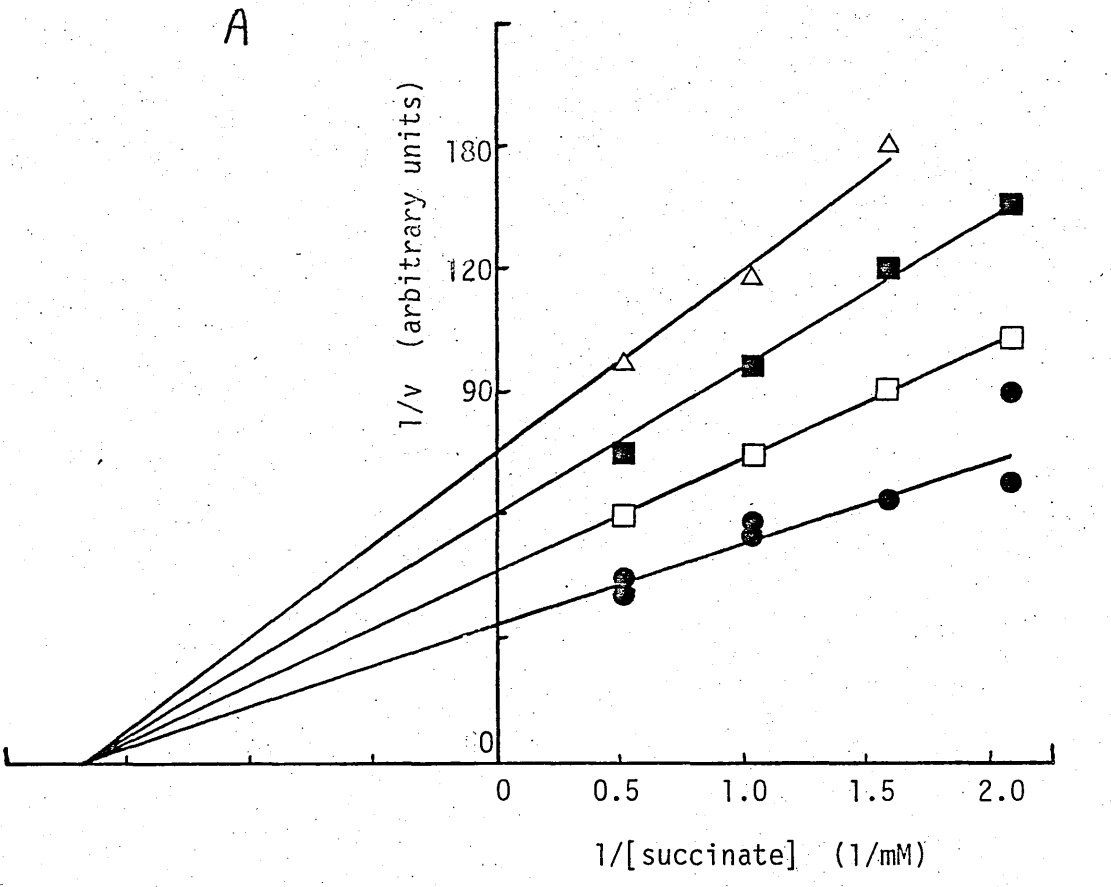

B.
300
240
180
120
0
1/v (arbitrary units)
0
0.12
0.24
0.36
0.48
0.60
0.72
[glycolate] (mM)

Figure 3.15. Inhibition of ICL by glycolate with respect to succinate

A. Double-reciprocal plot of the initial rates against 1/[succinate] at a series of fixed [glycolate]; 0mM (●), 0.011mM (□), 0.022mM (■) and 0.033mM (△). The [glyoxylate] was fixed at 0.045mM.

B. Dixon plot of the same data. The succinate concentrations were: 0.48mM (△), 0.63mM (■), 0.97mM (□) and 1.93mM (●).

The assay was that described in Methods 2.4.2.**c**).


A
1/v (arbitrary units)
180
120
90
0
0
0.5
1.0
1.5
2.0
1/[succinate] (1/mM)

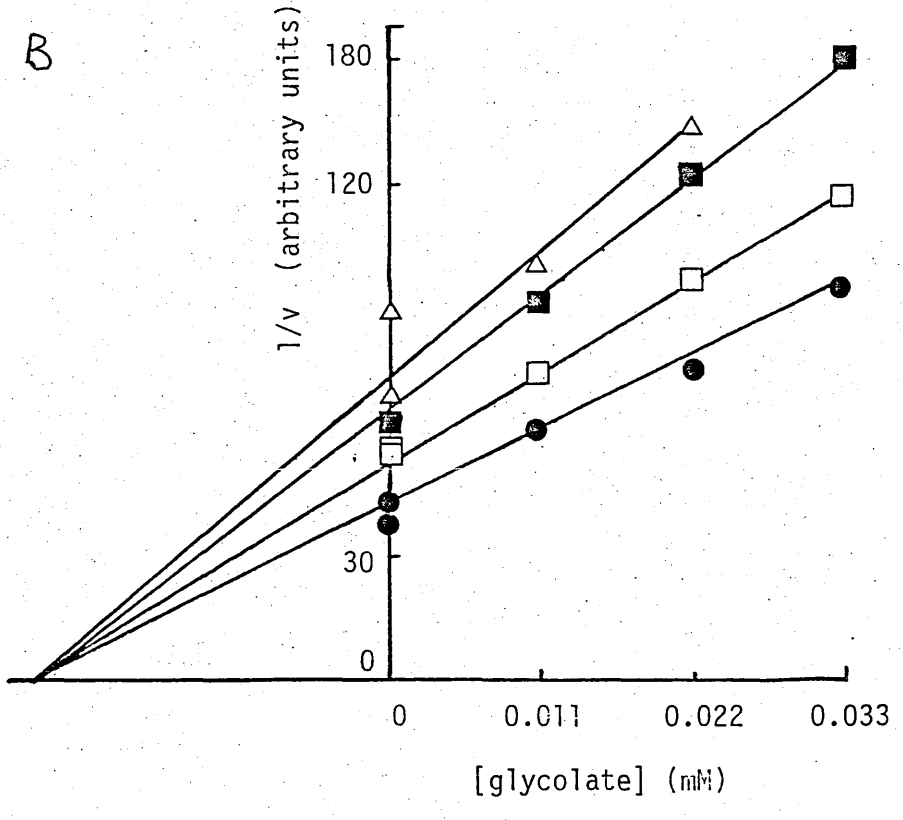
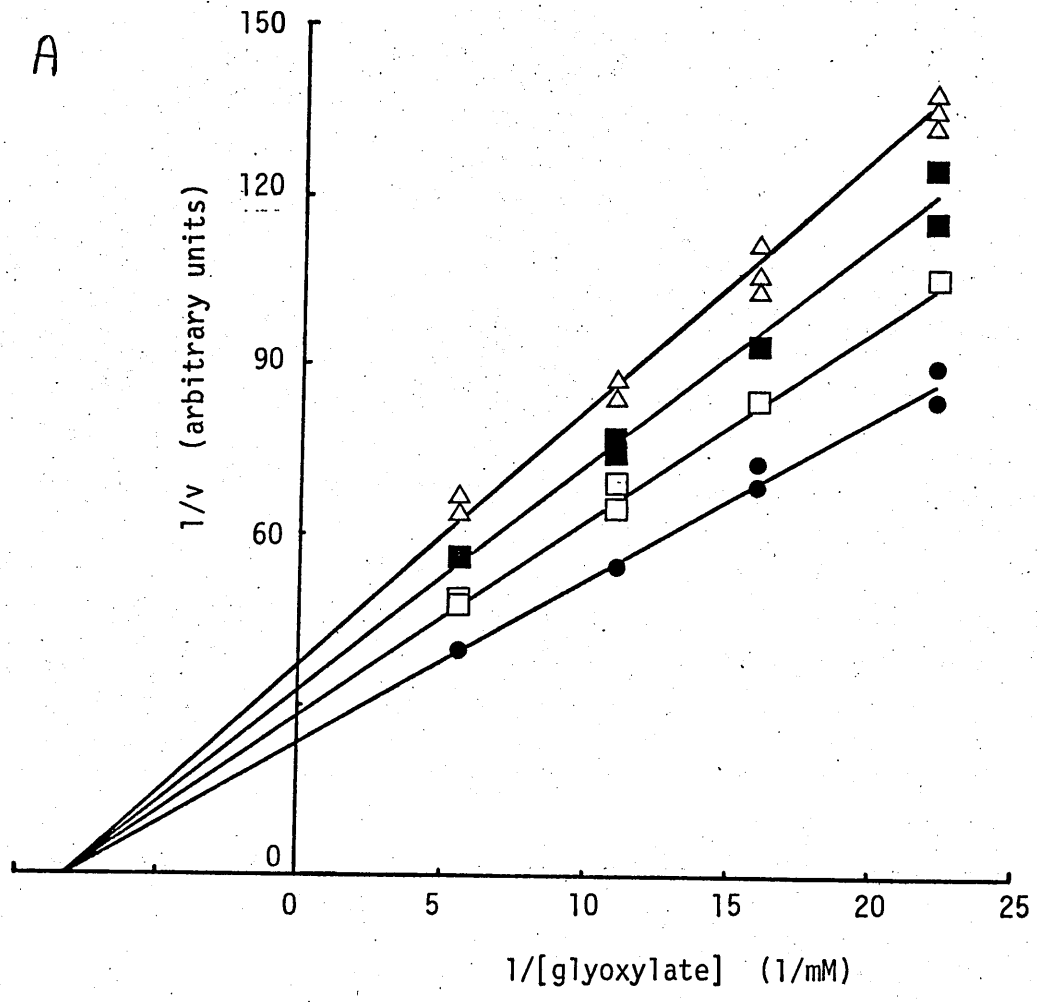

B
1/v (arbitrary units)
180
120
30
0
0
0.011
0.022
0.033
[glycolate] (mM)

Figure 3.16. Inhibition of ICL by PEP with respect to glyoxylate

A. Double-reciprocal plot of the initial rates against 1/[glyoxylate] at a series of fixed [PEP]: 0mM (●), 0.2mM (□), 0.4mM (■) and 0.6mM (△). The [succinate] was fixed at 0.39mM.

B. Replot of the same data. The glyoxylate concentrations were: 0.045mM (△), 0.063mM (■), 0.09mM (□) and 0.18mM (●).

ICL was assayed as described in Methods 2.4.2.c).


A
150
120
90
60
0
1/v (arbitrary units)
0
5
10
15
20
25
1/[glyoxylate] (1/mM)

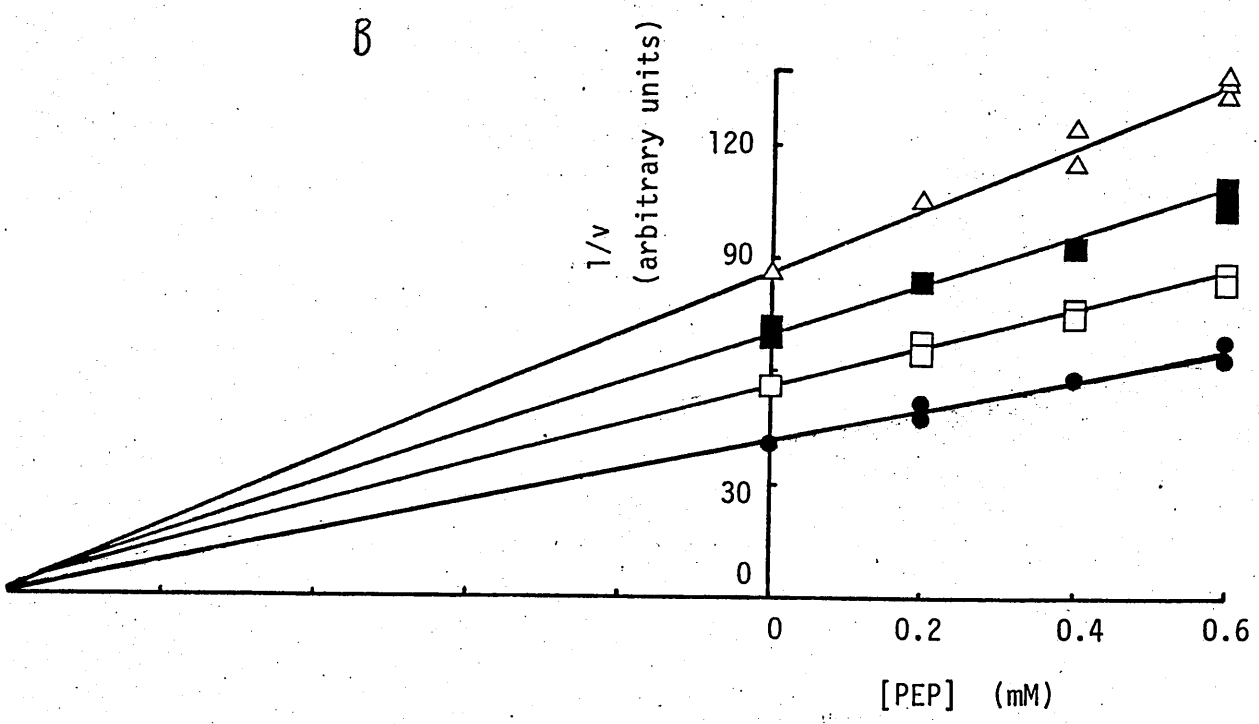
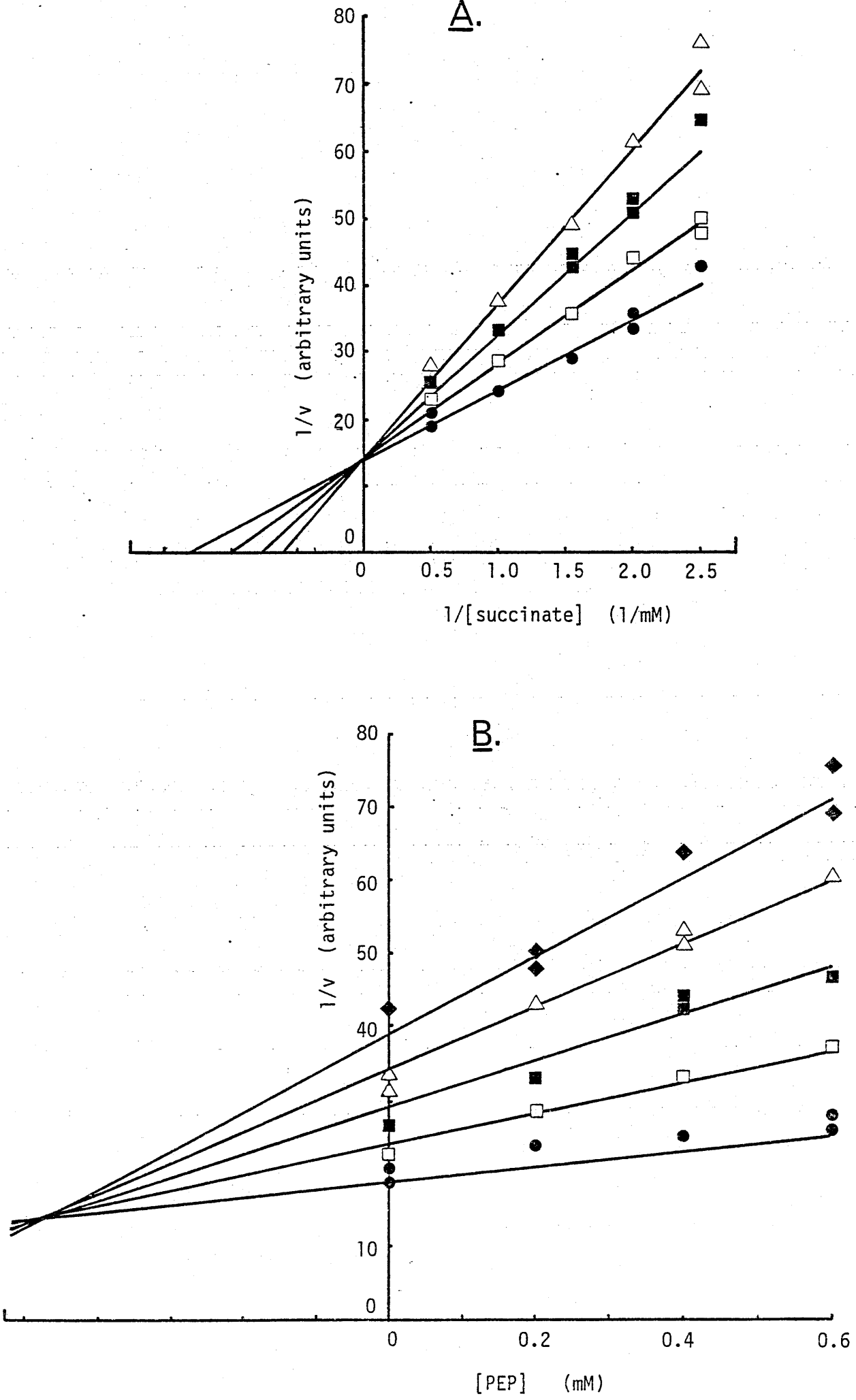

B
120
90
30
0
1/v (arbitrary units)
0
0.2
0.4
0.6
[PEP] (mM)

Figure 3.17. Inhibition of ICL by PEP with respect to succinate

A. Double-reciprocal plot of the initial rates against 1/[succinate] at a series of fixed [PEP]; 0mM (●), 0.2mM (□), 0.4mM (■) and 0.6mM (△).

B. Dixon plot of the same data. The succinate concentrations were: 0.39mM (◆), 0.48mM (△), 0.63mM (■), 0.97mM (□) and 1.93mM (●).

The assay was that described in Methods 2.4.2.**c**).


A.
80
70
60
50
40
30
20
0
1/v (arbitrary units)
0
0.5
1.0
1.5
2.0
2.5
1/[succinate] (1/mM)
B.
80
70
60
50
40
10
0
1/v (arbitrary units)
0
0.2
0.4
0.6
[PEP] (mM)

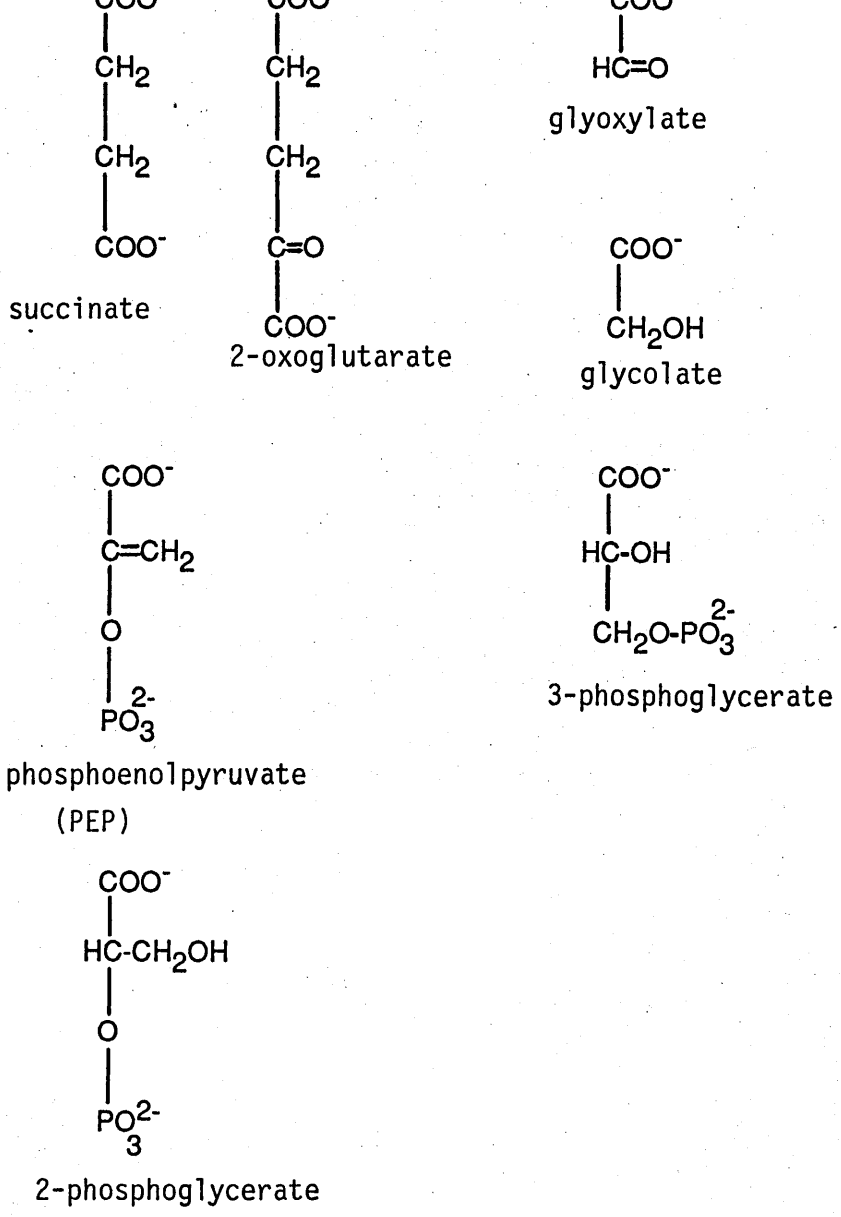


Figure 3.18. Structures of succinate and its analogues, 2-oxoglutarate, PEP and 2-phosphoglycerate and glyoxylate and its analogues, glycolate and 3-phosphoglycerate

(Figure 3.19. and 3.20.) These patterns are not consistent with a simple random order release of products which would give rise to competitive inhibition in both cases. However, the observed data can be accommodated in a random order equilibrium mechanism by postulating the existence of a ternary enzyme-isocitrate-succinate complex, but not the analogous enzyme-isocitrate-glyoxylate complex, as shown in Figure 3.21. If this postulate is correct, saturating isocitrate would not be able to displace succinate completely from the enzyme. Thus, succinate would affect both the slope and the vertical intercept of double reciprocal plots against isocitrate. On the other hand, saturating isocitrate would displace glyoxylate completely, so only slope effects would be observed.

3.7. Inhibition of ICL by potentially regulatory metabolites

The Introduction to this thesis describes how a number of metabolites are thought to affect the phosphorylation state of ICDH because they can modulate the activities of ICDH kinase/phosphatase. It is probable that pyruvate, PEP, NADPH, the adenine nucleotides and especially isocitrate are the most important effectors *in vivo* (Nimmo *et al.*,1984; El-Mansi *et al.*,1985). Intermediates in metabolism might regulate flux through the diverging glyoxylate and TCA pathways by interacting with ICL as well as ICDH kinase/phosphatase. Therefore the effects upon ICL of various metabolites were examined. ICL was assayed at 37^{o}C by the phenylhydrazine method (Methods 2.4.2.b)) and/or by the LDH-coupled assay (Methods 2.4.2.a)).

Ashworth and Kornberg (1963) found that ICL was strongly inhibited by PEP at pH6.8. They suggested that allosteric control of ICL is exerted by variations in the intracellular concentration of PEP.

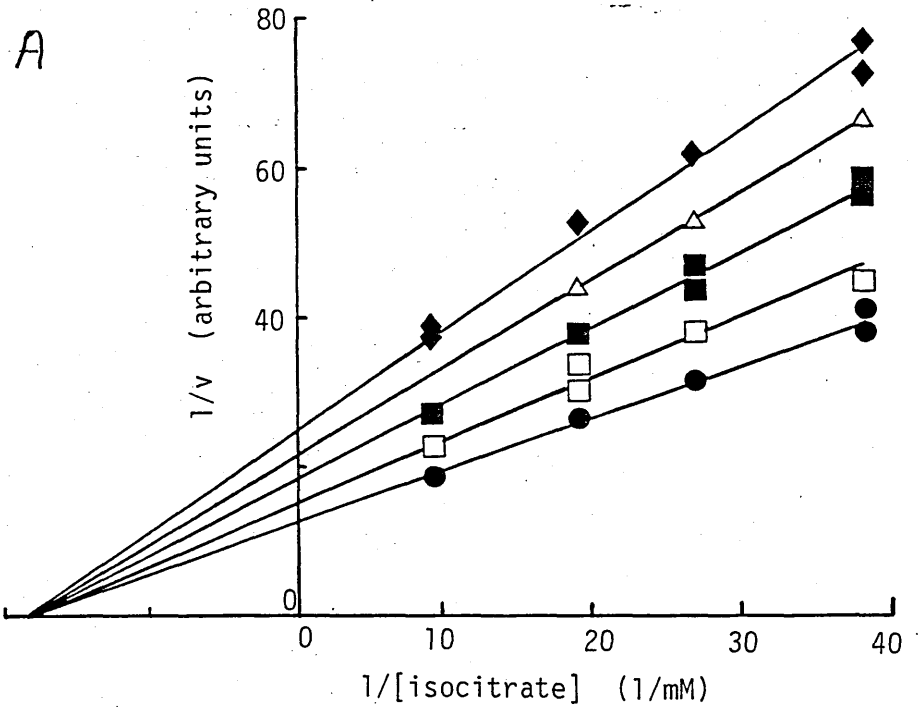
In agreement with Ashworth and Kornberg (1963), PEP was found to be a non-competitive inhibitor with respect to isocitrate, with a K_i of

Figure 3.19. Inhibition of ICL by succinate with respect to isocitrate at pH7.3

A. Double-reciprocal plot of the initial rates against 1/[isocitrate] at a series of fixed [succinate]: 0mM (●), 0.48mM (□), 0.96mM (■) and 1.44mM (△).

B. Dixon plot of the same data. The isocitrate concentrations were: 0.047mM (△), 0.063mM (■), 0.088mM (□) and 0.18mM (●).

ICL was assayed as described in Methods 2.4.2.b).


A
80
60
40
0
1/v (arbitrary units)
0
10
20
30
40
1/[isocitrate] (1/mM)

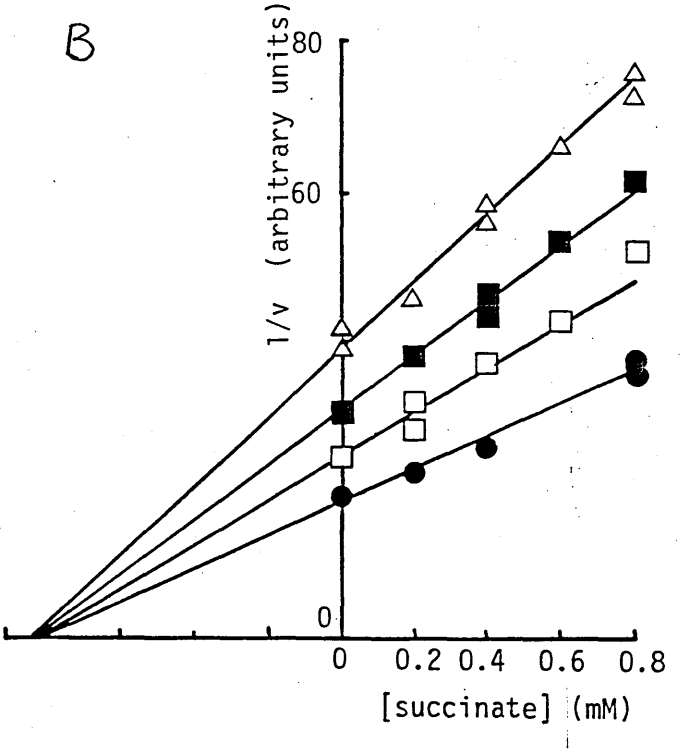
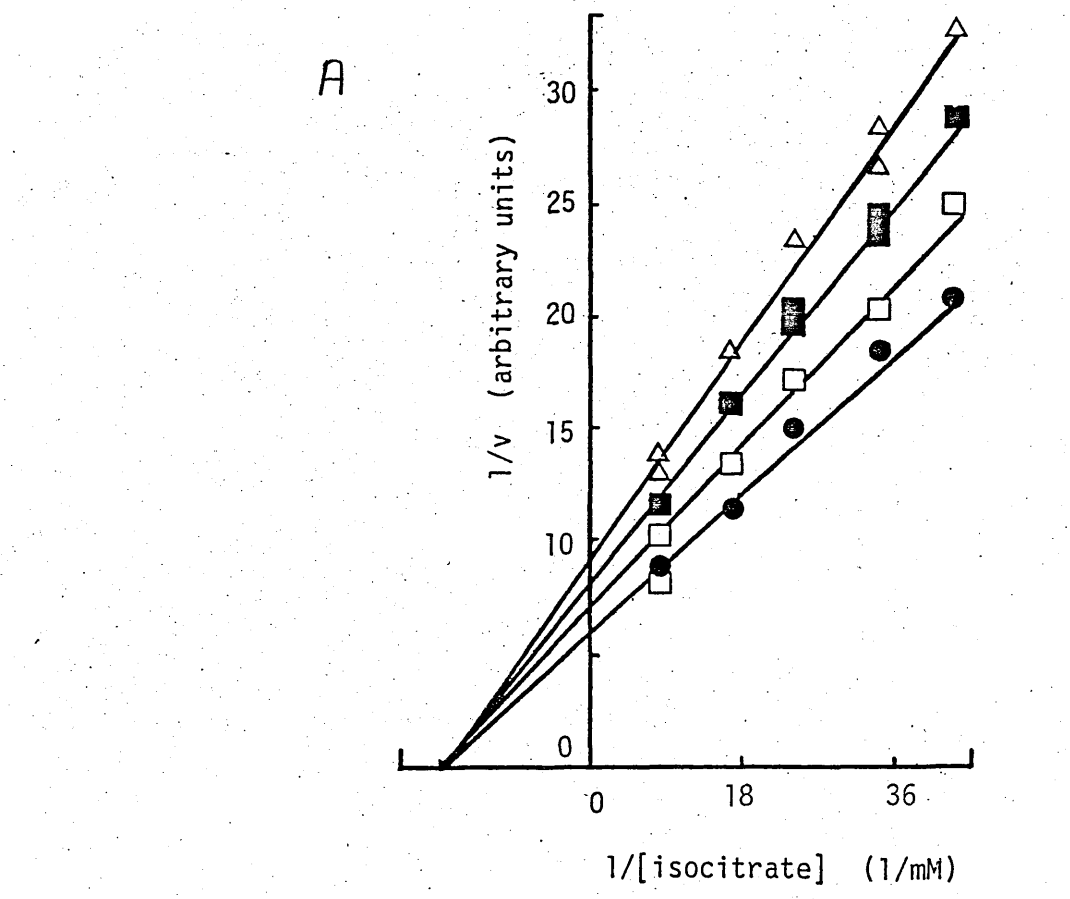

B
80
60
0
1/v (arbitrary units)
0
0.2
0.4
0.6
0.8
[succinate] (mM)

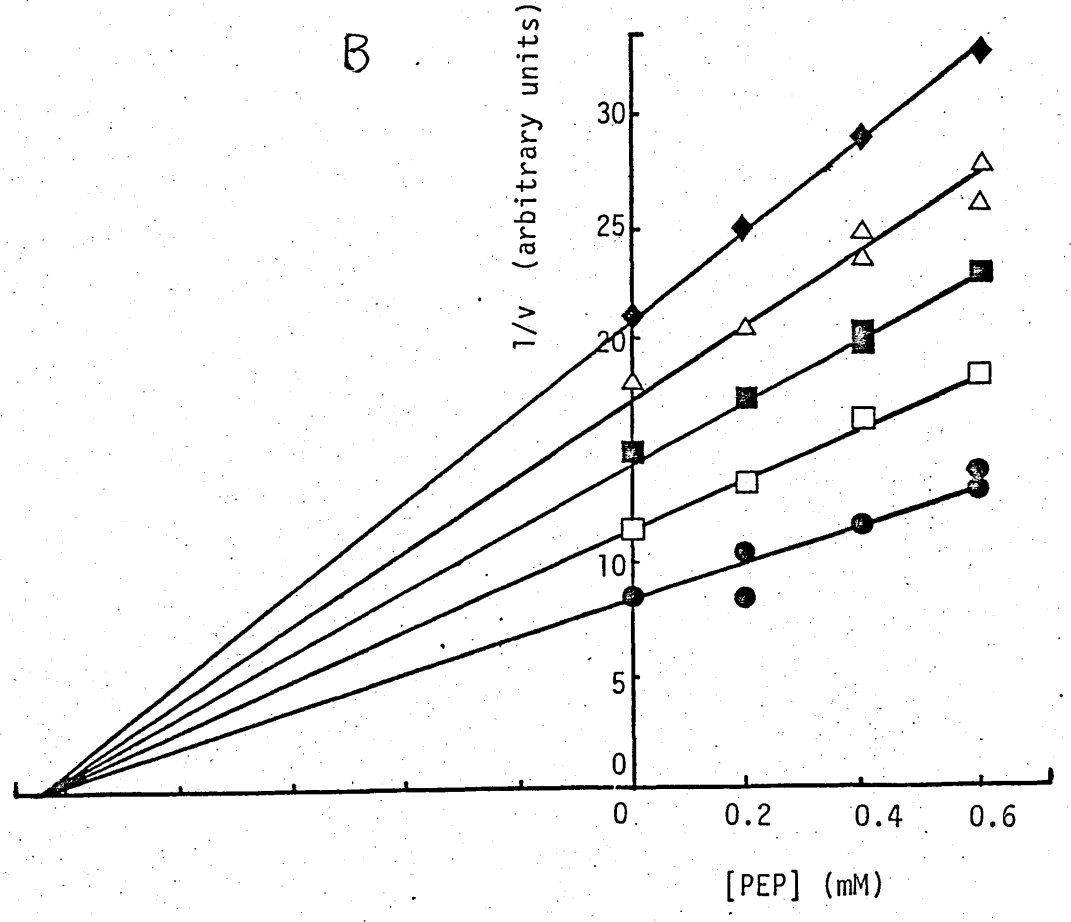
Figure 3.20. Inhibition of ICL by PEP with respect to isocitrate at pH7.3

A. Double-reciprocal plot of the initial rates against 1/[isocitrate] at a series of fixed [PEP]; 0mM (●), 0.2mM (□), 0.4mM (■) and 0.6mM (△).

B. Dixon plot of the same data. The isocitrate concentrations were: 0.021mM (◆), 0.026mM (△), 0.037mM (■), 0.053mM (□) and 0.105mM (●). The K_i value for PEP, from this plot, was 1.04mM.

The assay was that described in Methods 2.4.2.b).


A
1/v (arbitrary units)
30
25
20
15
10
0
0
18
36
1/[isocitrate] (1/mM)


B
1/v (arbitrary units)
30
25
20
10
5
0
0
0.2
0.4
0.6
[PEP] (mM)

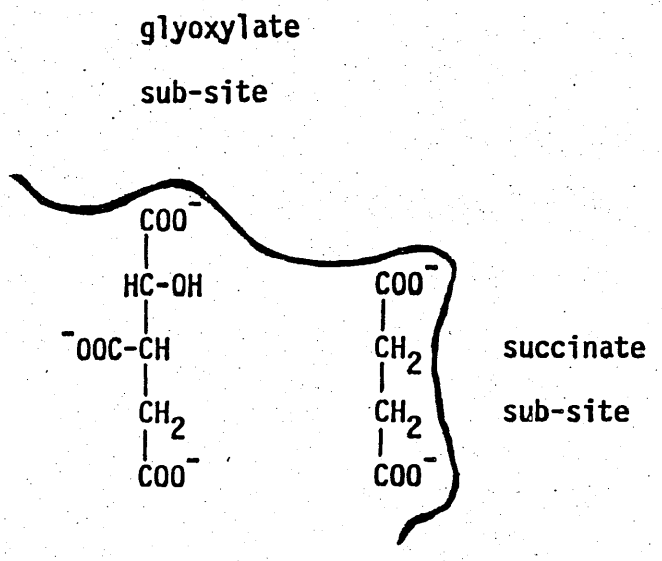


Figure 3.21a. Postulated ternary enzyme-isocitrate-succinate complex No suggestion about the orientation of the isocitrate and succinate is intended

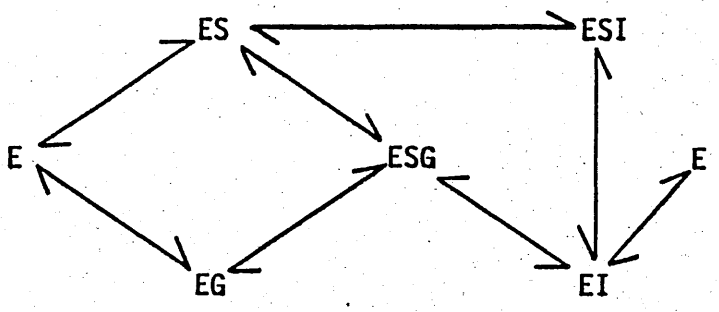


Figure 3.21b. Proposed random order mechanism E, enzyme; S, succinate; G, glyoxylate; I, D_s- isocitrate.

0.1mM (see Figure 3.22.) at pH6.8. However, the inhibition was very pH dependent and at the more physiological pH of 7.3, the K_i for PEP was ten-fold higher at 1.04mM (Figure 3.20.). Figure 3.23. shows the variation in K_i of PEP with pH. PEP binds at the same site as succinate (see section 3.6.) and succinate also shows pH dependence in its inhibition with respect to isocitrate, with a K_i of 1.12mM at pH7.3 (Figure 3.19.) and 0.3mM at pH6.8 (data not shown).

At 0.15mM D_s-isocitrate, several metabolites gave less than 10% inhibition when present at 1.5mM. These were glutamate, aspartate, citrate, ATP, GTP, ADP, AMP, $NADP^+$, NADH, NAD^+, fumarate, malate, fructose-6-phosphate, and fructose-1,6-bisphosphate.

LaPorte and Koshland (1983) showed that 3-phosphoglycerate is another compound which affects the phosphorylation state of ICDH. A kinetic analysis showed that 3-phosphoglycerate is a competitive inhibitor of ICL, with respect to isocitrate, with a K_i of 0.8mM at pH7.3 (Figure 3.24) and 0.36mM at pH6.8. 2-oxoglutarate was a non-competitive inhibitor of ICL with a K_i of 1.35mM at pH 7.3 (Figure 3.25.). Oxalate and oxaloacetate are known to be competitive inhibitors, acting as glyoxylate analogues, of ICL from watermelon (Jameel et al., 1984) and Pseudomonas indigofera (Rogers and McFadden, 1977) but these compounds were not tested.

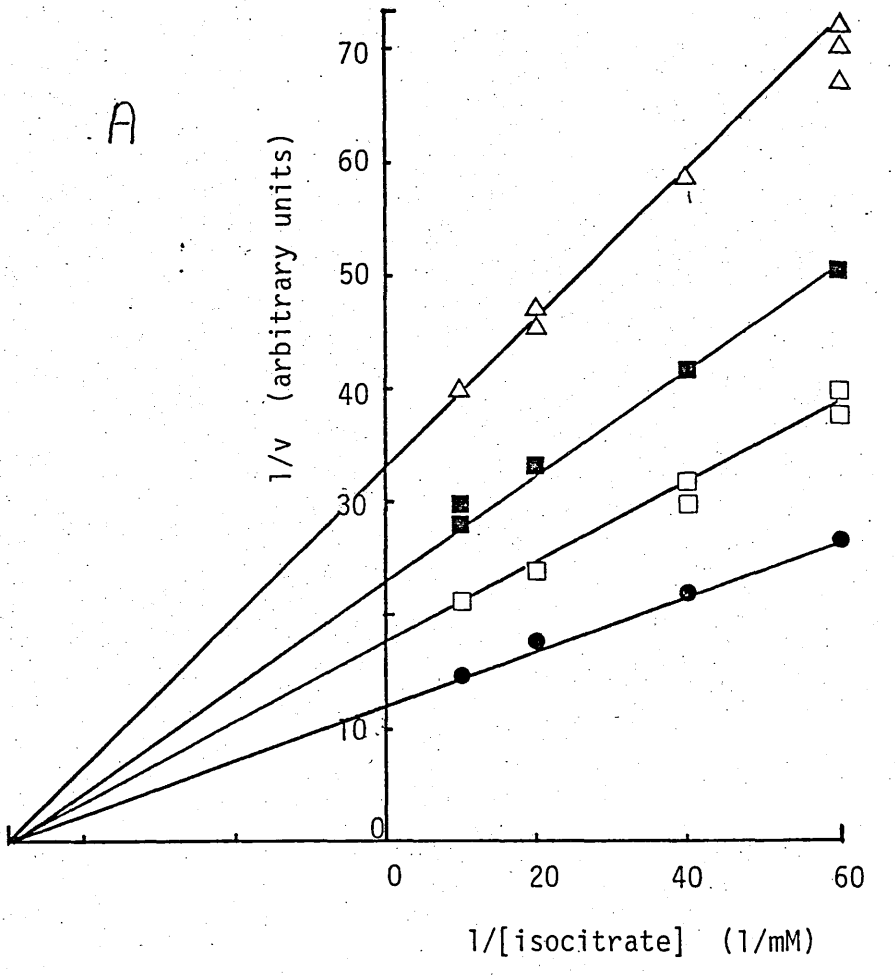
Pyruvate interferes with both ICL assays. In theory, ICL could be assayed by measuring the formation of succinate using succinate dehydrogenase. This would allow the effect of pyruvate to be tested. Unfortunately, it is very difficult to purify active succinate dehydrogenase to act as a coupling enzyme.

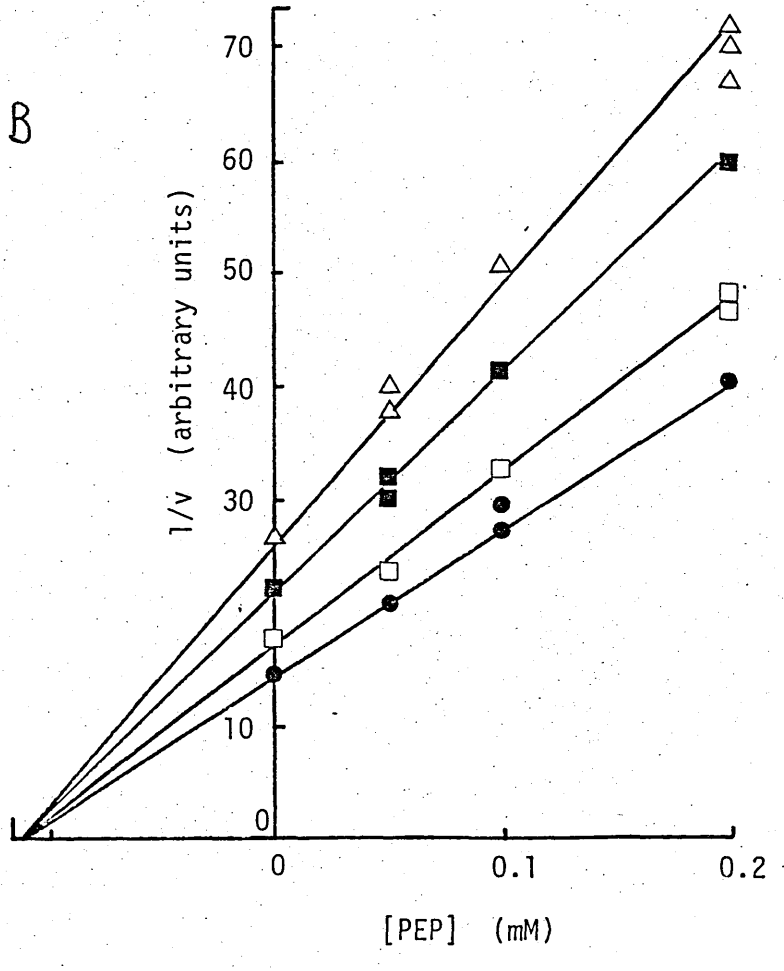
Figure 3.22. Inhibition of ICL by PEP with respect to isocitrate at pH6.8

A. Double-reciprocal plot of the initial rates against 1/[isocitrate] at a series of fixed [PEP]: 0mM (●), 0.05mM (□), 0.1mM (■) and 0.2mM (●).

B. Dixon plot of the same data. The isocitrate concentrations were: 0.017mM (△), 0.025mM (■), 0.05mM (□) and 0.1mM (●).

ICL was assayed as described in Methods 2.4.2.b).


A
70
60
50
40
30
10
0
1/v (arbitrary units)
0
20
40
60
1/[isocitrate] (1/mM)


B
70
60
50
40
30
10
0
1/v (arbitrary units)
0
0.1
0.2
[PEP] (mM)

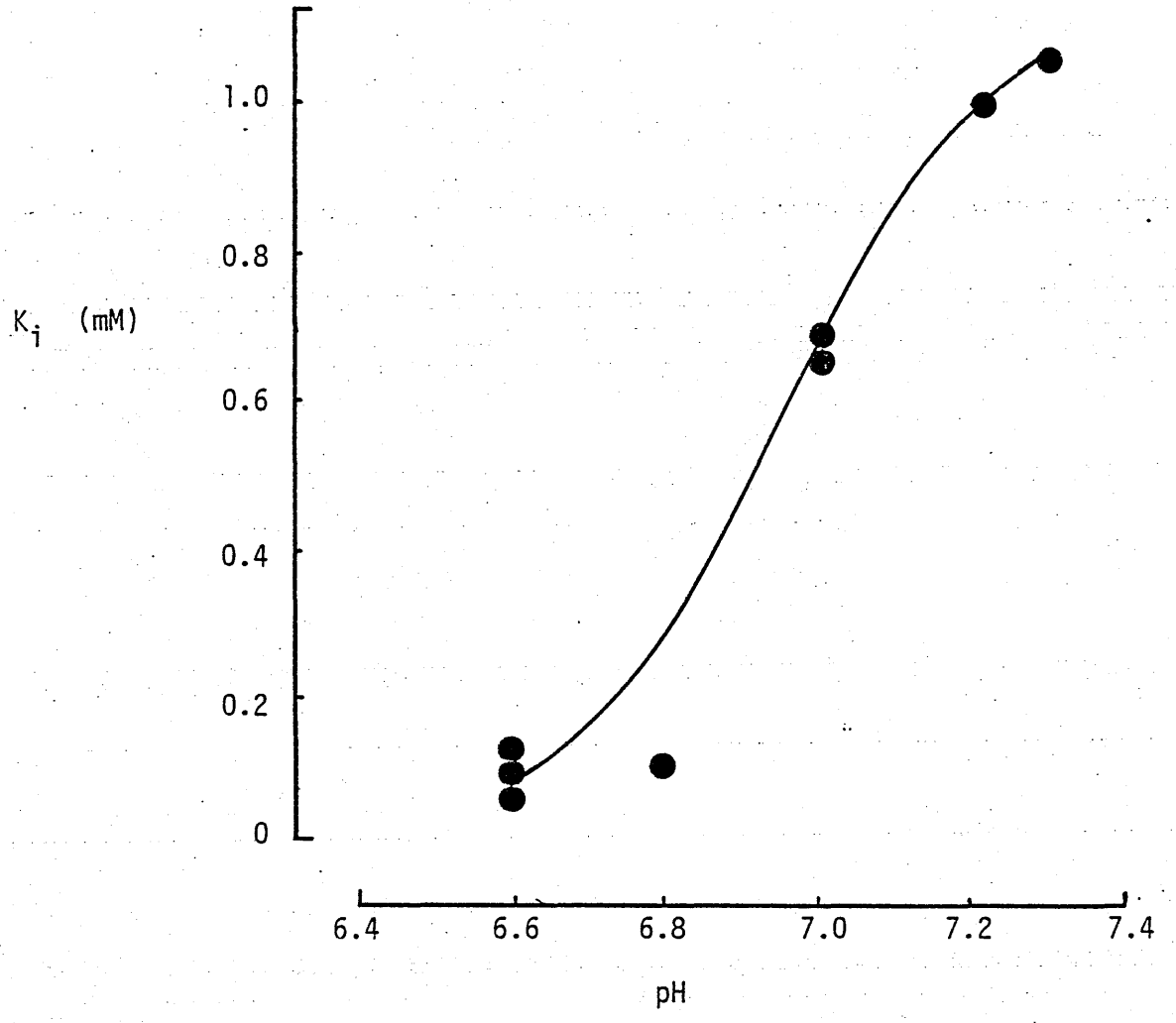


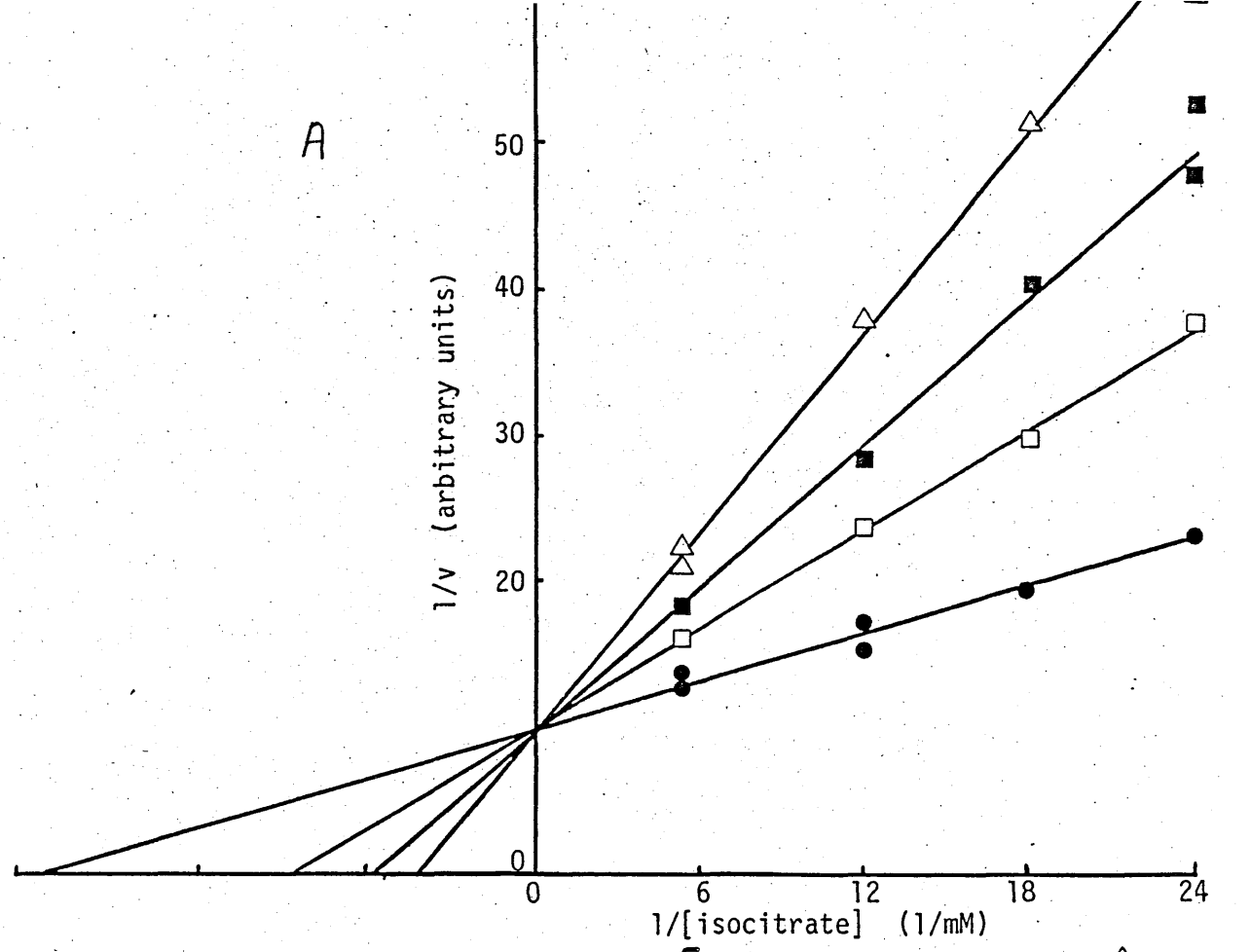
Figure 3.23. The pH dependence of the inhibition of ICL by PEP with respect to isocitrate

Figure 3.24. Inhibition of ICL by 3-phosphoglycerate with respect to isocitrate at pH7.3

A. Double-reciprocal plot of the initial rates against 1/[isocitrate] at a series of fixed [3-phosphoglycerate]: 0mM (●), 0.9mM (□), 1.8mM (■) and 2.7mM (△).

B. Dixon plot of the same data. The isocitrate concentrations were: 0.042mM (△), 0.055mM (■), 0.083mM (□) and 0.189mM (●).

ICL was assayed as described in Methods 2.4.2.b).


A
1/v (arbitrary units)
0
20
30
40
50
0
6
12
18
24
1/[isocitrate] (1/mM)

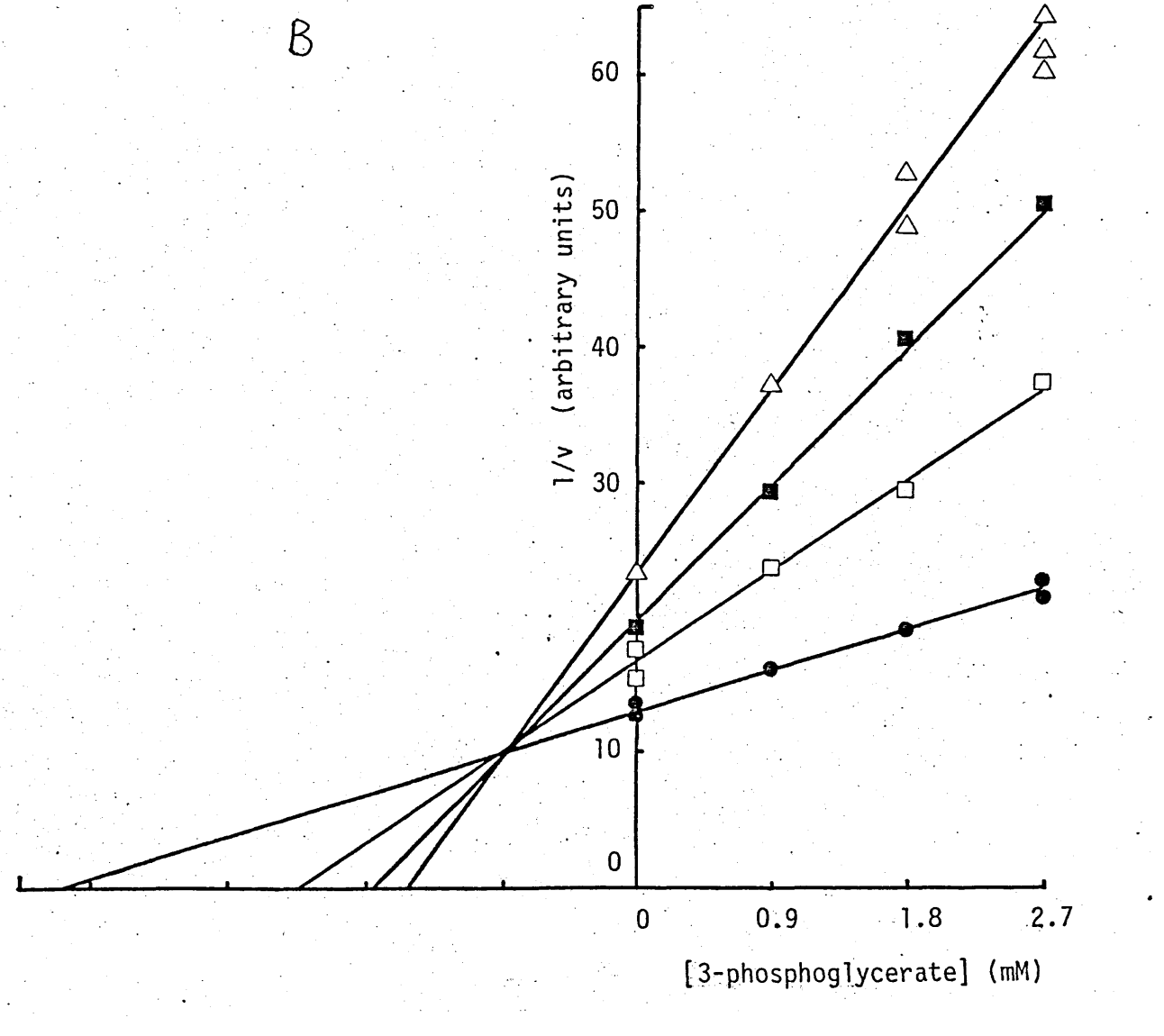
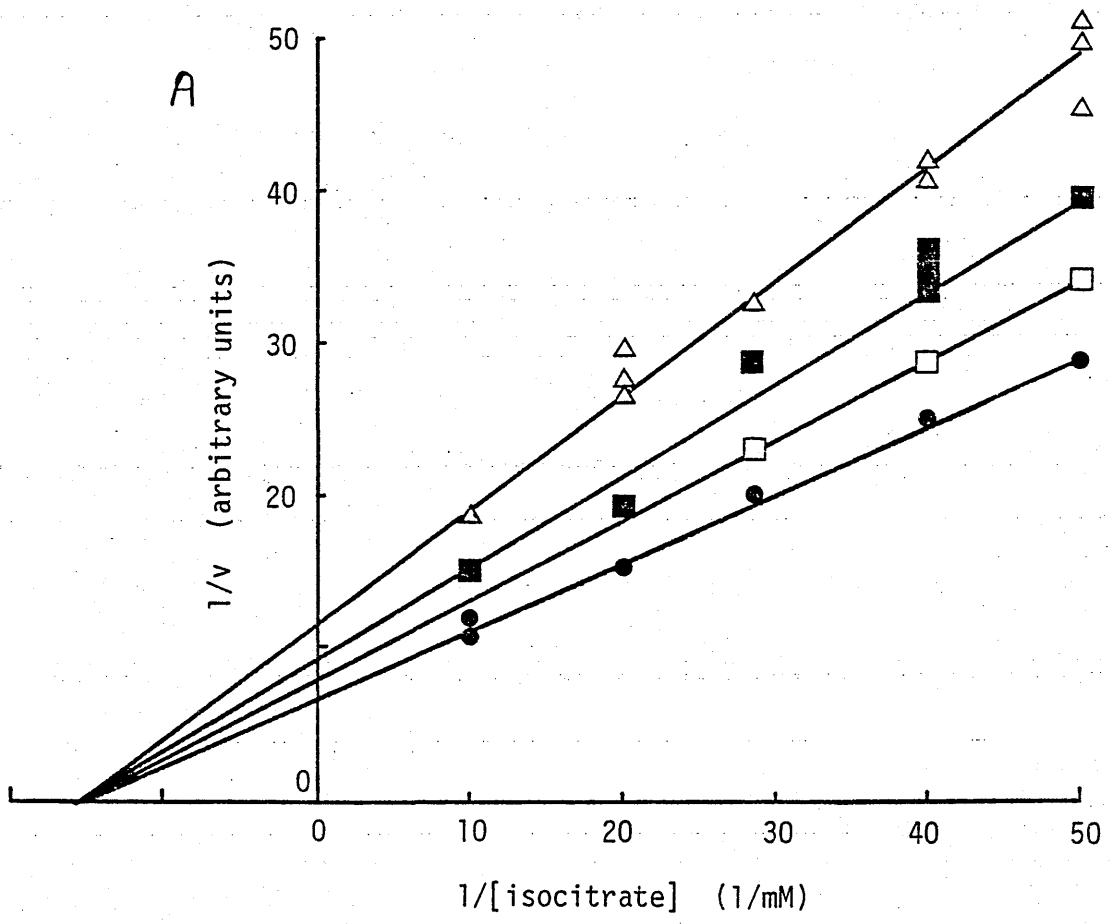

B
1/v (arbitrary units)
0
10
30
40
50
60
0
0.9
1.8
2.7
[3-phosphoglycerate] (mM)

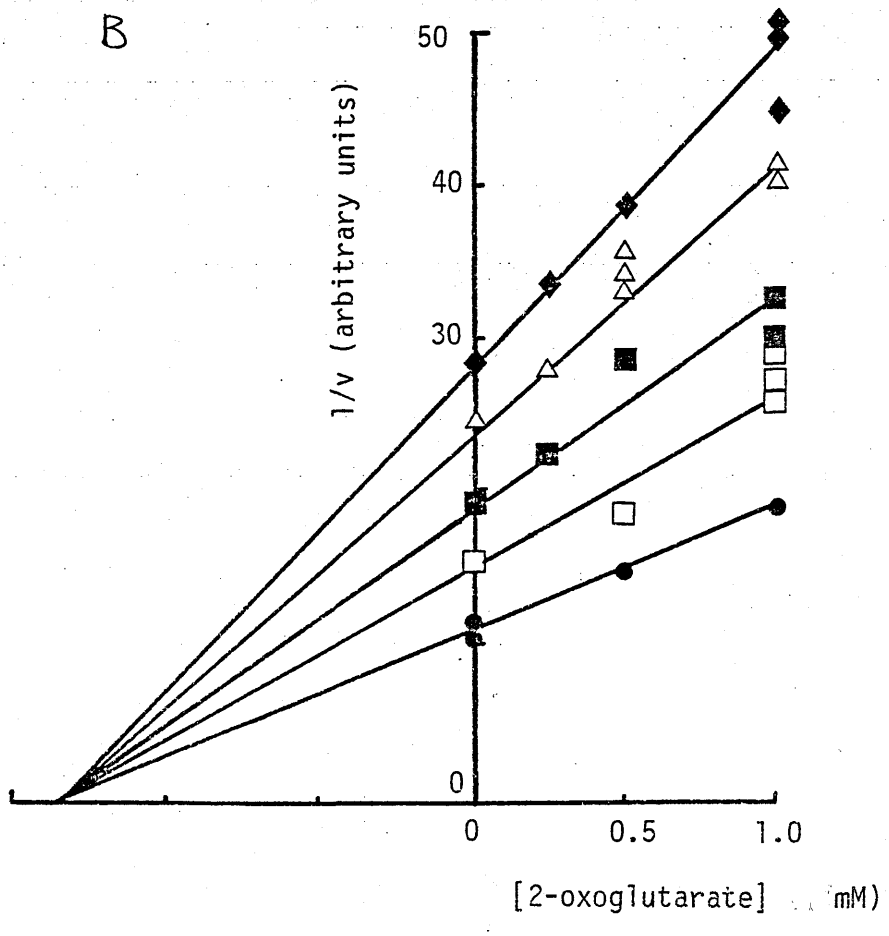
Figure 3.25. Inhibition of ICL by 2-oxoglutarate with respect to isocitrate at pH7.3

A. Double-reciprocal plot of the initial rates against 1/[isocitrate] at a series of fixed [2-oxoglutarate]: 0mM (●), 0.25mM (□), 0.5mM (■) and 1.0mM (△).

B. Dixon plot of the same data. The isocitrate concentrations were: 0.02mM (◆), 0.025mM (△), 0.035mM (■), 0.05mM (□) and 0.1mM (●).

The LDH-coupled assay was used (Methods 2.4.2.a)).


A
50
40
30
20
0
1/v (arbitrary units)
0
10
20
30
40
50
1/[isocitrate] (1/mM)


B
50
40
30
0
1/v (arbitrary units)
0
0.5
1.0
[2-oxoglutarate] mM)

3.8. Discussion

Enzyme stability

Persistent early problems with loss of enzyme activity were overcome by the combination of DTT and protease inhibitors. The increased activity and enhanced stability of the enzyme obtained upon addition of DTT suggest the presence of important sulphydryl groups in the enzyme.

ICLs from *Chlorella pyrenoidosa* (John and Syrett, 1967), *Neurospora crassa* (Johanson *et al.*, 1974), *Pseudomonas indigofera* (Rao and McFadden, 1965) and *Citrullis vulgaris* (watermelon) (Jameel *et al.*, 1984) all require thiol compounds for maximal activity.

3-Bromopyruvate irreversibly inactivates ICL from *Pseudomonas indigofera* by alkylating a cysteine residue at the active site (Roche *et al.*, 1970). 3-Bromopyruvate also inactivates the *Neurospora crassa* enzyme; the substrate, D_s-isocitrate and products protect against this.

The protease inhibitors were selected to cover a wide spectrum of protease types. Benzamidine is a specific, competitive inhibitor of proteases with a trypsin-like specificity and PMSF binds irreversibly to serine proteases. These increased stability of ICL in crude extracts, suggesting that such proteolysis may occur.

Quaternary structure

E. coli ICL is a tetramer, as are the ICLs from all other sources that have been studied to date. The subunit M_r of 45 000 puts *E. coli* ICL in the smaller subunit class compared with ICL from other sources (see Table 1.1.).

Physiological pH

The pH optimum for *E. coli* ICL of 7.3 is in the pH range tolerated within the cell. Regulation of the cytoplasmic pH in bacteria has been

reviewed by Booth (1985). He showed that E. coli is very sensitive to internal pH variation. If the internal pH is dropped to 7.2, by titration with a weak acid, there is 50% growth inhibition and there is almost complete inhibition of growth if the internal pH falls to pH6.8. The relevant kinetic properties are therefore those at pH7.3.

The kinetic mechanism of ICL

The mechanism shown in Figure 3.21b. is different from that reported for the ICL from Pseudomonas indigofera. Both Williams et al.(1971) and Schloss and Cleland (1982) reported that the condensation reaction has a "random kinetic mechanism but with a preferred pathway in which glyoxylate binds first". This preferred order mechanism would predict that PEP should give uncompetitive inhibition with respect to glyoxylate, that succinate should give mixed inhibition with respect to isocitrate and that reciprocal plots against succinate should become non-linear in the presence of glycolate. The data presented in section 3.6. shows very clearly that the ICL of E. coli does not obey such a compulsory order mechanism. The one unusual feature of the proposed random order mechanism (Figure 3.21b.) is the postulated existence of a ternary enzyme-isocitrate-succinate complex. This indicates that isocitrate may interact with the enzyme preferentially at the glyoxylate sub-site. Isocitrate seems to be able to bind at the glyoxylate sub-site even when the succinate sub-site is filled, but not at the succinate sub-site when the glyoxylate sub-site is filled. This prediction could perhaps be tested by direct binding studies, although the rather low affinities involved would make this difficult.

The kinetic results for the binding of succinate and glyoxylate, with respect to isocitrate, for the flax enzyme are very similar to these reported here for E. coli ICL. However, the authors (Khan and McFadden, 1982) favoured an ordered reaction mechanism.

Table 3.4. K_m for isocitrate of ICL from various sources

K_m (D_s-isocitrate)	Source	Reference
0.086mM at pH7.6	*Azotobacter vinelandii*	Kennedy & Dilworth, 1963
0.085mM at pH8	*Pseudomonas indigofera*	Schloss & Cleland,1982
0.056mM at pH7.7	*Pseudomonas indigofera*	Rogers & McFadden,1976
0.013mM at pH6.8	*Pseudomonas indigofera*	John & Syrett,1967
0.023mM at pH6.8	*Chlorella pyrenoidosa*	John & Syrett,1967
0.056mM at pH6.8	*Neurospora crassa*	Johanson *et al.*,1974
0.063mM at pH7.3 0.032mM at pH6.8	*Escherichia coli* ML308	This thesis

K_m of *E. coli* ICL

As discussed in Chapter 1, the role of the phosphorylation of ICDH seems to be to maintain the intracellular isocitrate at a level high enough to sustain the necessary flux through the glyoxylate bypass to give biosynthetic precursors. Evaluation of the K_m for isocitrate under physiological conditions is therefore important in the context of understanding the role of ICL in control of flux through the glyoxylate bypass. Unfortunately, this is very difficult because there is no good information about the ionic composition of the interior of an *E. coli* cell and because I have shown here that anions affect the K_m of ICL for isocitrate (section 3.5.).

The D_s-isocitrate K_m values obtained here are of the same order of magnitude as K_m values obtained for ICL from several other organisms. These are shown in Table 3.4. and were determined using continuous spectophotometric assays.

Walsh and Koshland (1984) determined a K_m value of 0.604mM D_s-isocitrate for *E. coli* ICL. They also made *in vivo* measurements of net flux through the major steps in acetate metabolism. Rates were assigned based on the ^{13}C NMR spectrum of intracellular glutamate in cells growing on [^{13}C]-acetate, measured rates of substrate incorporation into end products, the constituent composition of *E. coli* and a series of conservation equations which described the system at steady state during growth on acetate. But I have shown (section 3.5.) that the K_m value they determined for ICL (0.604mM) was inflated by an order of magnitude due to the inclusion of 200mM KCl in their assays. Walsh and Koshland (1984) found that *in vivo* flux measurements compared well with theoretical calculations based on their kinetic constants and certain assumptions about the intracellular concentrations of substrates (in particular isocitrate and $NADP^+$). This agreement may just be fortuitous, firstly because the K_m value they used for ICL may not be appropriate and

also because binding of isocitrate to enzyme binding sites was not taken into account. Thus the effective K_m of ICL in intact cells is not known with any certainty. My data show that it must be at least 0.063mM and is probably somewhat higher. This is much higher than the K_m of ICDH for isocitrate (Nimmo, 1986) and also than the intracellular concentration of isocitrate in cells growing on glucose or glycerol (El-Mansi et al., 1985). Thus the hypothesis for the control of the glyoxylate bypass/TCA cycle, outlined in Chapter 1 and above, is still tenable. One point of uncertainty, however, is whether changes in isocitrate concentration in the range 0.5-1.0mM, seen on addition of pyruvate to E. coli growing on acetate (El-Mansi et al., 1985), would affect flux through ICL. If the working K_m of ICL in intact cells really is about 60μM, an increase in isocitrate concentration from 0.5 to 1.0mM would have the sole effect of restricting flux through the TCA cycle by promoting the phosphorylation of ICDH by ICDH kinase/phosphatase. I return to this important consideration, with regard to inhibition of ICL by metabolites, in the last section of this discussion.

PEP as a regulator of ICL?

With a variety of metabolic mutants, Kornberg (1966) presented evidence which suggested that phosphoenolpyruvate (PEP) can control the synthesis of ICL and can also regulate its activity. Ashworth and Kornberg (1963) found that ICL was strongly inhibited by PEP at pH6.8 (K_i=0.13mM), but not by pyruvate. They suggested that allosteric control is exerted by variations in intracellular concentration of PEP and that this was an example of end-product inhibition.

However, the intracellular concentration of PEP in E. coli growing on acetate is thought to be about 0.25mM and it is not increased by the addition of glucose (Lowry et al.,1971) which reduces the requirement for flux through the glyoxylate bypass. Rather, the PEP levels decline upon

glucose addition.

Moreover, I found that the inhibition of ICL by PEP, with respect to isocitrate,is pH dependent. At the physiological pH of 7.3 (Booth, 1985) the K_i for PEP (1.04mM) is ten-fold higher than that at pH6.8 (0.1mM). The kinetic analysis of PEP inhibition shows that PEP interacts with the succinate-binding site of E. coli ICL rather than at an allosteric site as suggested by Ashworth and Kornberg (1963).

These factors demonstrate that PEP is not a physiologically significant allosteric effector of ICL.

The variation of the K_i for PEP with pH resembles a titration curve with a pK_a of about 6.9. This is higher than the pK of ionization of the phosphate group of PEP (6.38; Kiessling, 1934) so it is unlikely that pH is affecting inhibition through this ionisation. The effect of pH on the K_i of succinate, with respect to isocitrate, seems to be similar to that of PEP. This very stongly suggests ionisation of a group on the enzyme. ICL from other organisms is inhibited by PEP and in Chlorella pyrenoidosa (John and Syrett, 1968), Pseudomonas indigofera (Roche et al.,1970) and Neurospora crassa (Rogers and McFadden, 1977) the inhibition is also very pH dependent.

Other potential regulators of ICL

Of a variety of other plausible feedback inhibitors, amino acids and nucleoside phosphates gave low or no inhibition. 3-Phosphoglycerate, which is one of the compounds that affects the phosphorylation state of ICDH (LaPorte and Koshland, 1983), gave competitive inhibition of ICL with respect to isocitrate. This implies that 3-phosphoglycerate binds at the glyoxylate sub-site. 3-Phosphoglycerate is structurally analogous to the hydrated form of glyoxylate (Figure 3.18.). 2-Oxoglutarate is a non-competitive inhibitor of ICL with respect to isocitrate and probably binds at the succinate sub-site. Evidently, the oxo-carboxyl grouping of

2-oxoglutarate does not bind to the glyoxylate site. This could either be because the oxo group of 2-oxoglutarate is not hydrated, thus implying that the hydrated form of glyoxylate is the true substrate or resembles the transition-state intermediate, or, perhaps, steric hindrance prevents binding of 2-oxoglutarate to the glyoxylate sub-site.

By analogy with the structures of succinate and glyoxylate (hydrated form) and their analogues (Figure 3.18.) it can be predicted that compounds such as 2-phosphoglycerate, glyphosate (the herbicide Round-Up) and phosphonopropionic acid would be expected to bind at the succinate-binding site.

The physiological significance of the inhibition of ICL by metabolites that can interact with the succinate or glyoxylate sub-sites is difficult to assess in the absence of accurate information about *in vivo* levels of metabolites and knowledge of fluxes through the glyoxylate bypass and TCA cycle under different conditions.

However, we do know that the intracellular concentration of 2-oxoglutarate during growth on acetate is 0.01mM (Lowry *et al.*,1971). This is much lower than the K_i of 1.35mM for 2-oxoglutarate inhibition of ICL at pH7.3 and so 2-oxoglutarate inhibition is unlikely to be physiologically significant.

The intracellular concentration of 3-phosphoglycerate, however, has been reported to be 2.5mM, during growth on acetate (LaPorte and Koshland, 1983). In the presence of 2.5mM 3-phosphoglycerate, the apparent K_m of ICL would be raised from 0.063mM to 0.26mM at pH7.3. In fact, the working K_m of ICL during growth on acetate is likely to be greater than 0.26mM due to the presence of sulphate and other anions or inhibitors of ICL in the cell.

Walsh and Koshland (1984) showed that the carbon flux through ICDH in cells growing on acetate is at the maximal catalytic capacity of the enzyme. In *E. coli* ML308 growing on acetate, the maximal catalytic

capacities of ICDH and ICL are similar (Holms and Nimmo; unpublished results). The partition of flux at the ICL/ICDH branchpoint has been calculated to be 1:2 (Holms,1986) and 1:2.6 (Walsh and Koshland,1984). That is, during growth on acetate, flux through ICL is about half of that through ICDH. This means that ICL must actually be half-saturated *in vivo* and the K_m of ICL in intact cells must be close to the intracellular concentration of isocitrate during growth on acetate, that is 0.57mM (El-Mansi *et al.*,1985).

These considerations support the proposal that the rise in intracellular isocitrate concentration, which results from phosphorylation of ICDH, is necessary to allow sufficient flux through ICL for cellular biosynthesis (Nimmo, 1984). Also, the doubling of isocitrate concentration caused by addition of pyruvate to *E. coli* growing on acetate (El-Mansi *et al.*,1985) will actually affect the flux through ICL.

CHAPTER FOUR - LOCATION, CLONING AND OVEREXPRESSION OF THE GLYOXYLATE BYPASS OPERON FROM ESCHERICHIA COLI ML308

4.1. Introduction

Expression of the glyoxylate bypass enzymes, isocitrate lyase (ICL) and malate synthase A (MS-A), is normally induced only when E. coli is grown on acetate or fatty acids (Kornberg, 1966; Vanderwinkel and De Vliegher, 1968). Repression of the ace operon is under the control of the iclR and fadR gene products which are thought to be repressor proteins that can prevent transcription (Maloy and Nunn, 1981;1982). Thus expression of the operon is derepressed during growth on acetate. The molecular details of this joint regulation have not yet been examined. The glyoxylate shunt enzymes also appear to be regulated by catabolite repression.

Since E. coli ML308 expresses only low amounts of ICDH kinase/phosphatase (Nimmo et al.,1984) studies of the structural organisation of the protein were being hampered by the very low amounts of purified material that could be produced. We therefore wished to locate, clone and overexpress the gene encoding the kinase/phosphatase.

We also wished to clone the ace operon from E. coli ML308 to facilitate isolation of large amounts of ICL and identification and isolation of MS-A. It was hoped that, in the long term, this work would facilitate elucidation of the molecular details of the regulation of the ace operon.

When this work started the location of the ICDH kinase/phosphatase gene was unknown. However, the activity of ICDH kinase/phosphatase to phosphorylate and inactivate ICDH and allow flux through the glyoxylate bypass is only required during growth on acetate and so it seemed likely that its structural gene might be part of or co-regulated with the glyoxylate bypass operon.

The work described in this chapter was initiated by Dr. E.M.T. El-Mansi and Dr. H.G. Nimmo and was carried out in collaboration with them. In general, they carried out cloning and *in vitro* transcription-translation experiments. I did cell-growth experiments, enzyme assays and purified the enzymes which allowed interpretation of the *in vitro* transcription-translation experiments. During the course of this study LaPorte *et al.*(1985) worked along similar though not identical lines. They showed that the ICDH kinase/phosphatase gene of *E. coli* K12, which they termed *aceK*, mapped in the *ace* operon downstream from *aceB* and *aceA*. LaPorte and Chung (1985) then subcloned the *aceK* gene from a plasmid containing the *E. coli* K12 glyoxylate bypass operon.

4.2. Cloning of the glyoxylate bypass operon from *E. coli* ML308 and construction of subclones

Plasmid pCTS, a derivative of pBR322, was obtained from Dr. W.D. Nunn (University of California, Irvine, U.S.A.). pCTS contains a 10 kilobase insert of *E. coli* K12 DNA encoding the glyoxylate bypass operon structural genes, *aceA* and *aceB* (W.D. Nunn,personal communication).A ^{32}P-labelled probe was prepared from pCTS by nick-translation and was used to probe *E. coli* ML308 genomic DNA. The probe hybridised with only a single ClaI fragment. A ClaI digest of *E. coli* ML308 DNA was therefore cloned into pAT153 and was used to transform *E. coli* KAT-1. *E. coli* KAT-1 is an *aceA* mutant and is therefore unable to grow on acetate (Maloy and Nunn, 1982). KAT-1 is also tetracycline resistant by virtue of a Tn*10* insertion in *aceA*, so recombinants were selected in minimal medium containing acetate and ampicillin. Two identical transformants able to grow on acetate were obtained and one, KAT-1/pEM9 was used for further experiments.

Three fragments of the insert in pEM9 were subcloned in pAT153. The resulting plasmids, pEM901, pEM902 and pEM903, were used to

Table 4.1. Specific activities of enzymes in crude extracts of *E. coli* strains

Malate synthase was assayed by Method 2.4.2.d).

Enzyme	Isocitrate Lyase ($\mu mol.min^{-1}.mg^{-1}$)		Malate Synthase ($\mu mol.min^{-1}.mg^{-1}$)		ICDH Kinase ($nmol.min^{-1}.mg^{-1}$)	
Carbon Source	Glycerol	Acetate	Glycerol	Acetate	Glycerol	Acetate
Strain						
ML308	0.04	0.67	0.12	1.26	0	0.027
LE392	0.24	0.81	0.48	1.39	0.010	0.036
KAT-1	0	ng	0.21	ng	0	ng
KAT-1/pEM9	1.49	5.44	2.70	9.14	0.080	0.490

ng, no growth. Values are representative of several experiments.

transform E. coli KAT-1. The ampicillin-resistant transformants grew on glycerol but pEM901, pEM902, and pEM903 could not transform E. coli to growth on acetate. Partial restriction maps of the insets in pEM9, pEM901, pEM902 and pEM903 are depicted in Figure 4.1.

4.3. A comparison of specific activities of enzymes in crude extracts of E. coli strains

The specific activities of ICL,MS-A and ICDH kinase in extracts of E. coli ML308, LE392, KAT-1 and KAT-1/pEM9 grown on glycerol or acetate are shown in Table 4.1. Since ICDH kinase cannot be assayed satisfactorily in crude extracts, the enzyme was first partially purified by protamine sulphate treatment and fractionation with $(NH_4)_2SO_4$ (Nimmo et al., 1984). Proteins that precipitated between 25% and 45% saturation were collected and dissolved in 50mM Mops-NaOH pH7.3, 1mM EDTA, 1mM DTT, 1mM benzamidine. No ICDH kinase or ICDH phosphatase activity was detected in E. coli KAT-1 grown on glycerol (Table 4.1.). Addition of acetate (40mM) for periods up to 18h elicited only barely detectable levels of activity (Dr E.M.T.El-Mansi and also see section 4.6.4.). Strain KAT-1/pEM9 overexpresses ICDH kinase 13 to 18-fold relative to either ML308 or LE392. The over-expression factors for ICL and MS-A are slightly lower. These factors are lower than would be expected from the copy number of pAT153 (Twigg and Sherratt,1980). However it was later found (Figure 4.2. ; section 4.4.) that in E. coli KAT-1/pEM9 grown on acetate, ICL and MS-A together comprise a large part of the total cell protein and this may restrict the degree of over-expression available. These results suggest that pEM9 carries the aceA and aceB genes.They also suggest that pEM9 bears the structural gene for ICDH kinase/phosphatase, though since the KAT-1 chromosome has an intact aceK gene other interpretations are possible. For example, ICL or MS-A protein could enhance expression of the chromosomal aceK gene.

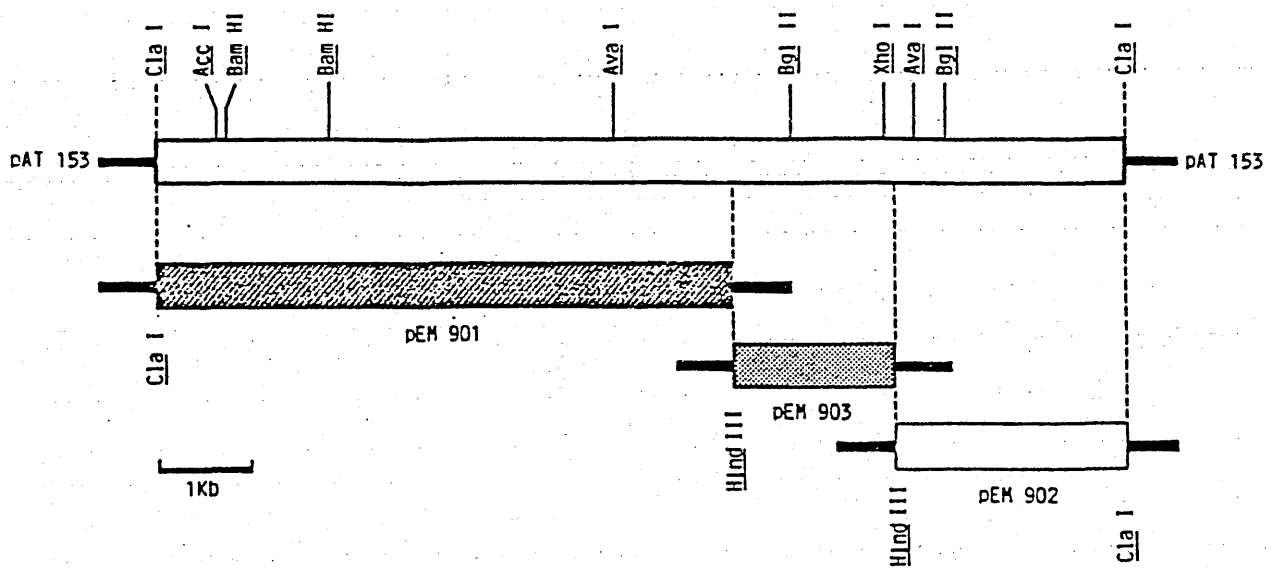


Figure 4.1. Restriction map of the insert in pEM9 (Dr. E.M.T. El-Mansi)

A ClaI-HindIII double digest of pEM9 gave three genomic fragments. These were isolated from a low melting point agarose gel and subcloned in pAT153

The results also show that the activities of all three enzymes in each of strains ML308, LE392 and KAT-1/pEM9 are higher during growth on acetate than on glycerol. Since KAT-1 is *iclR*$^+$ (Maloy and Nunn, 1982) the results do not test the possibility that pEM9 also carries the *icIR* gene (which is downstream of the *ace* operon itself).

4.4. SDS-PAGE of extracts of *E. coli* strains

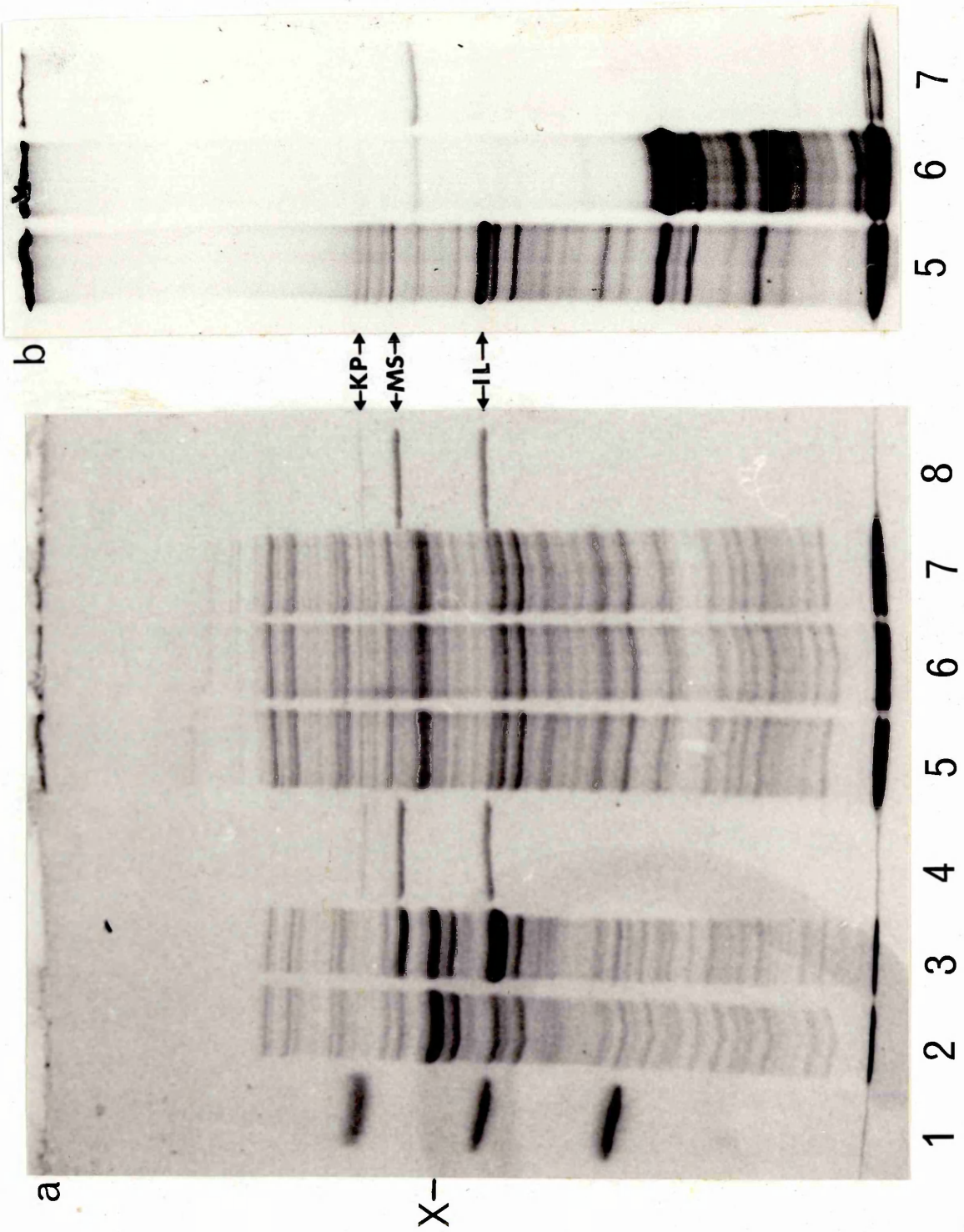
Figure 4.2. shows the polypeptides present in crude supernatants of *E. coli* strains LE392 and KAT-1/pEM9 grown on acetate. Homogeneous preparations of ICL, MS-A and ICDH kinase/phosphatase (see Chapter 5 for details of purification) were mixed and run on parallel tracks. Bands corresponding to ICL and MS-A are overproduced in *E. coli* KAT-1/pEM9. No band corresponding to the kinase/phosphatase can be seen on the gel. A prominent band, marked **X**, is seen in extracts of *E. coli* LE392 and KAT-1/pEM9 grown on acetate but not in these strains grown on glycerol. It does not appear to be any more expressed in KAT-1/pEM9 than in LE392. The bulk of this protein **X** was found to precipitate between 50 and 70% saturation with $(NH_4)_2SO_4$. It seemed likely that this was a protein involved in acetate metabolism. The enzymes which convert acetate to acetyl-CoA; acetyl-CoA synthetase or acetate kinase and phosphotransacetylase were all found to precipitate below 45% $(NH_4)_2SO_4$ saturation. Moreover, it is known that the levels of acetate kinase and phosphotransacetylase in extracts of wild-type *E. coli* do not vary with different carbon sources (Brown *et al.*, 1977). PEP carboxykinase is another candidate for protein **X** which would be worth investigating.

4.5. In vitro transcription-translation: proteins encoded by pEM9 and pEM901

Dr. E.M.T. El-Mansi and Dr. H.G. Nimmo investigated which genes were present in pEM9, pEM901, pEM902, and pEM903 by carrying out *in*

Figure 4.2. Proteins encoded by pEM9

(a) Shows a polyacrylamide gel stained for protein. Track 1, marker proteins bovine serum albumin, *E. coli* ICDH and pig heart lactate dehydrogenase (M_r values 68 000, 45 000 and 35 000 respectively). Tracks 2 and 3, crude supernatants from *E. coli* LE392 (track 2) and KAT-1/pEM9 (track 3); 20µg of protein each. Tracks 4 and 8, homogeneous marker proteins; ICDH kinase/phosphatase (1µg), malate synthase and isocitrate lyase (2µg each). Tracks 5 - 7, products from transcription-translation *in vitro* (incubation time 60 min) of pEM9 (1µg, track 5), pAT153 (2.5µg, track 6) and no DNA (track 7). (b)Shows an autoradiograph (48h exposure) of tracks 5 - 7. The arrows indicate the mobilities of ICDH kinase/phosphatase (KP), malate synthase (MS) and isocitrate lyase (IL).


b
5
6
7
←KP→
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a
1
2
3
4
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6
7
8
X

vitro transcription-translation experiments. I prepared homogeneous ICL and MS-A and helped to purify ICDH kinase/phosphatase, as described in Chapter 5. These proteins were used as markers on SDS-PAGE to allow identification of proteins synthesised in the in vitro transcription-translation experiments. Figures 4.2. and 4.3. show that several polypeptides were expressed from pEM9 and pEM901 but not from pAT153 or in a "no DNA" control. Since the insert in pEM9 is only 11 kilobases (see Figure 4.1.), it is likely that several of these bands result from proteolysis or premature termination of transcription or translation. The most heavily labelled polypeptide expressed from both pEM9 and pEM901 comigrated with ICL, and another prominent band expressed by both plasmids comigrated with MS-A. pEM9, but not pEM901, directed synthesis of a band which comigrated with ICDH kinase/phosphatase. This was the least mobile band observed with either plasmid and it is therefore very unlikely that it resulted from proteolysis or premature termination. This suggested that pEM9 carries the aceK gene but pEM901 does not. This is consistent with the work of LaPorte et al. (1985) and LaPorte and Chung (1985) which showed the order of genes in the glyoxylate bypass operon to be aceBAK. The aceA and aceB genes clearly lie within the 6.7 kilobase Cla I-Hind III fragment shown in Figure 4.1. This Hind III site presumably lies within the aceK gene or in the aceA-aceK intergenic region. The nucleotide squence of this region is currently being determined in Glasgow(Dr. I. Varela, personal communication).

4.6. ICDH kinase/phosphatase from E. coli KAT-1/pEM901

The plasmid pEM901 was unable to express full-length ICDH kinase/phosphatase (Figure 4.3.). The strain KAT-1/pEM901 grew extremely poorly, if at all, on acetate. Signs of slight growth were seen, in a small-scale batch culture (Methods 2.3.4.a)) after several days but after this time there may have been airborne contamination. It

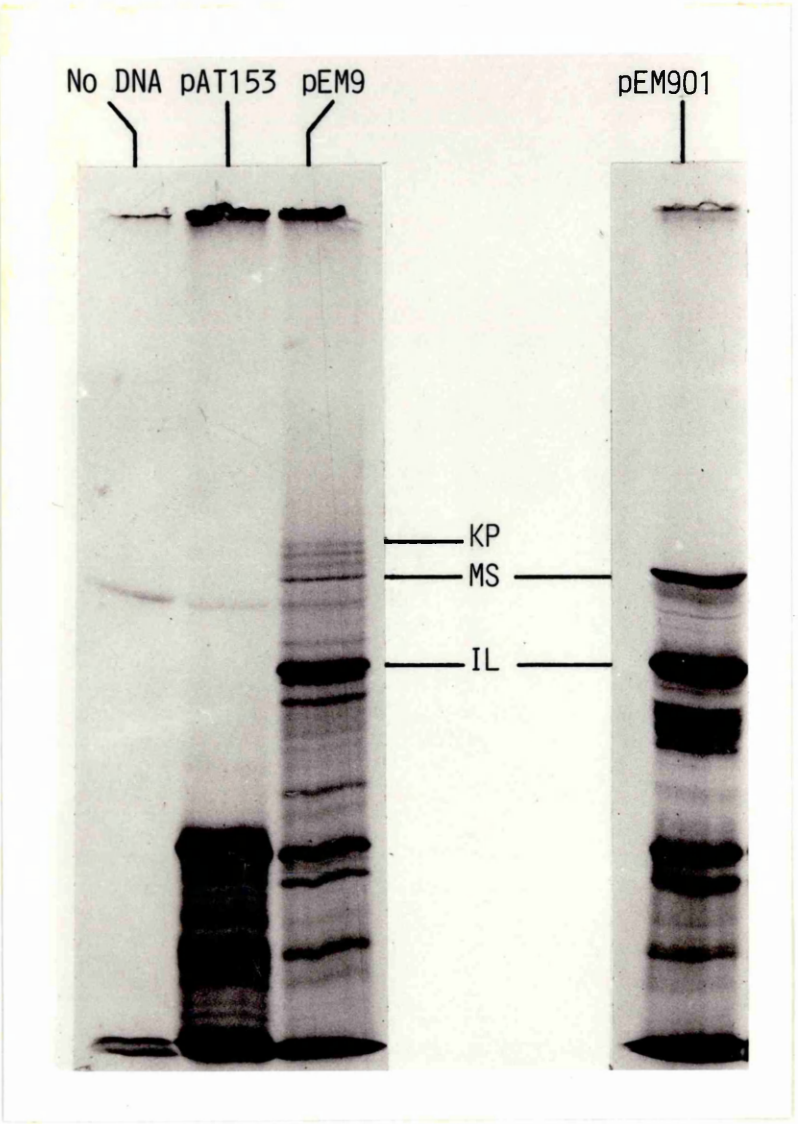


Figure 4.3. Proteins encoded by pEM9 and pEM901

This shows an autoradiograph (48h exposure) of products from a separate transcription-translation experiment in vitro using pEM9 (2µg) (track 1) and pEM901 (2µg) (track 2). The bars indicate the mobilities of ICDH kinase/phosphatase (KP), malate synthase (MS) and isocitrate lyase (ICL).

therefore seemed that expression of the aceK gene was essential for growth on acetate. This was not entirely expected. pEM901 carries the ICL (aceB) and MS-A (aceA) genes. One would anticipate that E. coli KAT-1/pEM901 would be able to grow on acetate by virtue of the overexpression of these two enzymes. That is, a high concentration of ICL in KAT-1/pEM901 would be able to compete to some extent with active ICDH, compensating for the lack of ICDH kinase/phosphatase and allowing sufficient carbon to be diverted via the glyoxylate bypass to permit growth. Another possibility is that DNA downstream from aceK is required for growth on acetate.

It was not certain whether the HindIII site at the end of the pEM901 insert fell within the aceK gene or in the aceA-aceK intergenic region. The former possibility was of considerable interest. It is not yet determined whether ICDH kinase/phosphatase has two distinct sites per monomer, one catalysing the kinase reaction and the other catalysing the phosphatase reaction, or one active site per monomer which catalyses both reactions. We were interested in the possibility of pEM901 expressing an incomplete ICDH kinase/phosphatase molecule, perhaps carrying only one of the activities. The in vitro transcription-translation experiment suggested that incomplete kinase/phosphatase might well be made.

4.6.1. Induction of glyoxylate bypass enzymes by challenge of glycerol-grown cells with acetate

E. coli KAT-1/pEM901 would not grow on acetate. However, when KAT-1/pEM901 was grown on glycerol (to an A_{420} of about 3.5) and then challenged with 40mM acetate for 2-3 hours, ICL and MS activities were detected.

Crude extracts (Methods 2.3.5.b)) of E. coli KAT-1/pEM901 grown on glycerol and challenged with acetate had specific activities of 2.4 µmol/ min/mg of protein for ICL and 3.9 µmol/min/mg of protein for MS. These values are intermediate between those of crude extracts of E. coli KAT-1/pEM9 grown on glycerol or grown on acetate (see Table 4.1.) and so the ace operon had been induced under these conditions. The acetate challenge was not continued for more than 3 hours because there was evidence of cell breakage in the stirring cultures at high cell densities. Cultures became increasingly yellow and cloudy with a pungent smell. This happened several times when cultures of E. coli KAT-1/pEM9 or KAT-1/pEM901 were left stirring after the log-phase of growth. Contamination checks proved negative and small-scale plasmid preparations and restriction enzyme digests (under the guidance of Dr. K. Duncan) showed that the expected plasmids were present.

4.6.2. Initially only ICDH phosphatase could be detected in E. coli KAT-1/pEM901

I investigated whether the acetate-challenged E. coli KAT-1/pEM901 contained either ICDH kinase or ICDH phosphatase. ICDH kinase cannot be assayed in crude extracts (Nimmo et al.,1984) so a protamine sulphate treatment was followed by $(NH_4)_2SO_4$ fractionation. Proteins precipitating between 0 and 45%, 45 and 55%. and 55 and 70% $(NH_4)_2SO_4$ saturation were collected. ICDH phosphatase was assayed by measuring release of ^{32}P from ^{32}P-ICDH (Methods 2.4.2.f)). ICDH kinase was assayed by measuring inactivation of ICDH (Methods 2.4.2.3e)). ICDH phosphatase activity was found in the 0 to 45% $(NH_4)_2SO_4$ fraction. However, no ICDH kinase activity could be detected in any fraction.

I then decided to purify the ICDH phosphatase activity, following the conventional ICDH kinase/phosphatase purification procedure (Methods 2.5.5.) as far as it proved feasible.

4.6.3. Evidence that *E. coli* KAT-1/pEM901 expresses normal ICDH kinase/phosphatase

The protein from a 0 to 50% $(NH_4)_2SO_4$ fraction was desalted on Sephadex G-50 and then fractionated by chromatography on DE52 and Blue Dextran Sepharose as described in Methods 2.5.5. ICDH phosphatase activity was detectable after the first step but not after the second, probably because Blue Sepharose chromatography always results in considerable dilution of the enzyme. The enzyme activity could well have been below the limits of detectability. In the purification of ICDH kinase/phosphatase from *E. coli* ML308 (Nimmo *et al.*,1984) or *E. coli* KAT-1/pEM9 (Section 5.3.) the enzyme is eluted from Blue Sepharose quite reproducibly and is characteristically retarded from a peak of A_{280}. By comparing the A_{280} profiles, the corresponding fractions in this experiment were pooled and concentrated by vacuum dialysis or were bound to and eluted from an FPLC Mono Q column at pH7.3 (as in Methods 2.5.5.) Surprisingly, both ICDH kinase and ICDH phosphatase activities were detected in the concentrated enzyme. Measurement of the ICDH kinase and ICDH phosphatase activities revealed that the ratio of the two activities was the same as for normal ICDH kinase/phosphatase. The enzyme from *E. coli* KAT-1/pEM901 was examined on SDS-PAGE. A sample of partially purified ICDH kinase/phosphatase (purified by Dr. H.G. Nimmo) of comparable activity was run in parallel for comparison. As can be seen in Figure 4.4. the *E. coli* KAT-1/pEM901 enzyme pool was by no means homogeneous but a band of the expected intensity and of the same subunit M_r as pure ICDH kinase/phosphatase could be detected.

We concluded that although the plasmid pEM901 could not express ICDH kinase/phosphatase, this enzyme could be expressed from the chromosomal *aceK* gene despite the facts that the chromosomal *aceA* gene carries a Tn*10* insertion and the *aceK* gene is downstream of *aceA* in the

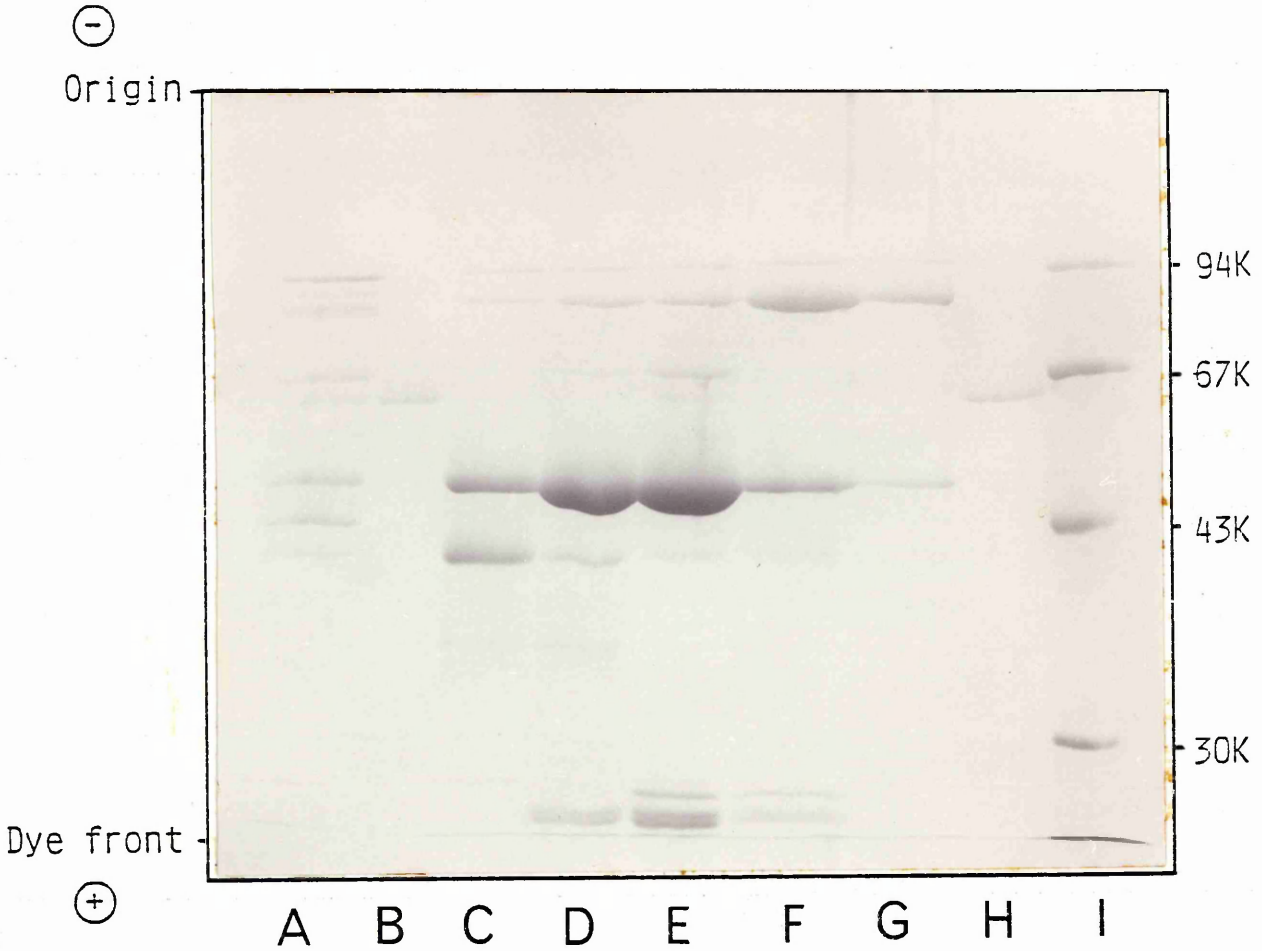


Figure 4.4. Partially purified ICDH kinase/phosphatase from E. coli KAT-1/pEM9 This 10% polyacrylamide gel run in the presence of SDS shows fractions across the peak of ICDH kinase/phosphatase activity eluted from a Mono Q column run at pH7.3 (as in Methods 2.5.5.). Track A, partially purified ICDH kinase/phosphatase from E. coli KAT-1/pEM9, of similar activity to that in track E; tracks B and H, pure ICDH kinase/phosphatase from E. coli KAT-1/pEM9; tracks C - G, consecutive Mono Q fractions containing ICDH kinase/phosphatase from E. coli KAT-1/pEM901, of which track E is the peak fraction; track I, M_r standard proteins (Methods 2.6.5.).

same operon (LaPorte et al.,1985). This was tested by going through the same growth, acetate challenge and purification procedure with E. coli KAT-1. Both ICDH kinase and ICDH phosphatase activities were found in the final concentrated pool from the Blue Sepharose column. The ratio of ICDH kinase to ICDH phosphatase was normal.

ICDH kinase/phosphatase from E. coli KAT-1/pEM901 and KAT-1 could not be properly assayed until partial purification had been carried out. The intracellular concentration of ICDH kinase/phosphatase in these cells could therefore not be determined accurately. But I estimate that the kinase and phosphatase specific activities in these cells,grown on glycerol and challenged with 40mM acetate for 3 hours, must be lower than in E. coli ML308 grown on acetate and much lower than in E. coli KAT-1/pEM9 grown on acetate (see Table 4.1.).

4.6.4.The apparent inhibition of ICDH kinase

The reasons for the inability to detect ICDH kinase in extracts from E. coli until after Blue Sepharose chromatography were investigated. This inability is referred to below as inhibition without implying anything about the mechanism involved.The following facts were determined :-

i. The enzyme pooled from the DE52 chromatography inhibited the kinase activity of purified ICDH kinase/phosphatase unless it was boiled for 10 minutes. This suggested that a protein was responsible for the inhibition.

ii. The inhibition was time-dependent. That is, the longer the delay between adding the "DE pool" and ICDH kinase/phosphatase to an assay, the greater the inhibition.

iii. Inhibition was relieved by addition of excess ATP (30mM) to the assay.

iv. Glycerol kinase (assayed as in Methods 2.4.2.i)) was present in the DE pools and the wash-through pools from the Blue Sepharose column but not in the 0.4M NaCl pool from the Blue Sepharose column.

Since the ICDH used as substrate in the ICDH kinase assays was stored in glycerol, it was very likely that destruction of the ATP, by glycerol kinase was responsible for the interference in the assays. *E. coli* KAT-1/pEM901 and KAT-1 had been grown on glycerol as sole carbon source (before challenge with 40mM acetate) and in this medium all carbon must be channelled into the cell through glycerol kinase (e.g. Kistler and Lin, 1971). These cells therefore contain very high levels of glycerol kinase activity whereas cells grown on acetate do not.When the substrate, ICDH, was dialysed out of glycerol and used for assay of authentic ICDH kinase using "DE pool" to inhibit there was still some interference, so perhaps some other kinase was also involved in the inhibition of ICDH kinase in those assays.

4.7. Discussion

The work described in this chapter has shown that we have constructed a strain that overexpresses the enzymes encoded by the glyoxylate bypass operon of *E. coli* ML308. The overexpression obtained here facilitated identification and purification of MS-A and characterization of ICL and ICDH kinase/phosphatase. This work is reported in Chapter 5.

The results presented here are consistent with previous studies on the order of genes in the glyoxylate bypass operon (Maloy and Nunn, 1982; LaPorte *et al.*, 1985) and subcloning of the *aceK* gene (LaPorte and Chung, 1985). The *aceA* and *aceB* genes clearly lie within the 6.7 kilobase ClaI-HindIII fragment shown in Figure 4.1. This HindIII site presumably lies within the *aceK* gene or in the *aceA-aceK* intergenic

region. Strains ML308, LE392 and KAT-1/pEM9 all express ICDH kinase/phosphatase at a level some 500- to 1000-fold lower than ICL or MS-A. The molecular mechanisms responsible for this difference are unknown. While the cellular amount of ICDH kinase/phosphatase may be regulated in part at the level of degradation, it seems likely that some feature of the nucleotide sequence in the intergenic region results in a step-down in the level of expression. Investigation of this would be of great interest since we would like to be able to manipulate the expression of all enzymes involved at the branchpoint between the TCA cycle and the glyoxylate bypass. The nucleotide sequence of the aceA-aceK intergenic region is currently being determined in Glasgow (Dr. I. Varela; personal communication).

Another point of note from measurement of the specific activities of enzymes in crude extracts (Table 4.1.) is that the degree of repression of the ace operon during growth on glycerol is greater in strain ML308 than in LE392 which is a K12 strain. Variation in the regulatory patterns between different strains of E. coli has been observed, for example, for the arginine biosynthetic enzymes. The arg enzymes are repressed under non-inducing conditions in the E. coli K12 and W strains, but are constitutive or slightly inducible in the B strain (Jacoby and Gorini , 1967, 1969; Karlström and Gornini, 1969). The arg-regulation phenotype of E. coli B can be altered to that of the K12 strain by a single point mutation (Jacoby and Gorini , 1969). Since biochemists have concentrated on a small number of strains, the full extent of, and any general trends in, inter-strain variation of control patterns is unknown.

Expression of ICDH kinase/phosphatase in E.coli KAT-1 and KAT-1/pEM901

There are three main possibilities which could explain why ICDH kinase/phosphatase can be expressed from the chromosomal aceK gene of

E. coli KAT-1 despite the facts that the chromosomal aceA gene carries a Tn10 insertion and that the aceK gene is downstream of aceA in the same operon. First, there may be low level read-through from the natural ace promoter. A second possibility is the presence of an additional promoter between aceA and aceK. Third, transcription of aceK could be directed from pOUT, the outward-directed Tn10 promoter which can initiate transcription of adjacent chromosomal material (Simons et al., 1983).

These possibilities could, perhaps, be distinguished because Tn10-promoted transcription of aceK would be independent of the carbon source. On the other hand, transcription initiated from the natural ace promoter would exhibit normal repression except during growth on acetate. If there is a separate promoter between aceA and aceK this would give rise to monocistronic aceK transcripts. It is not unreasonable to suggest the existence of such a promoter, because there may be circumstances (perhaps growth on fatty acids) where the cell requires a different ratio of the aceK gene product to the aceB and aceA gene products than is observed during growth on acetate. One precedent for such a system concerns the gene lpd encoding the lipoamide dehydrogenase component (E3) of the pyruvate dehydrogenase and oxoglutarate dehydrogenase complexes of E. coli. This gene lies at the downstream end of the aceEF operon but can also be expressed from its own promoter. Most of the E3 for the pyruvate dehydrogenase complex is synthesized from aceEF-lpd read-through transcripts whereas most of the E3 for the oxoglutarate dehydrogenase complex is synthesized from lpd transcripts (Spencer and Guest, 1985).

The observation that E. coli KAT-1/pEM901 is capable of synthesizing ICDH kinase/phosphatase again raises the question of why this strain is unable to grow on acetate (see section 4.6). Probably the level of expression of the aceK gene is too low to allow proper

regulation at the ICL/ICDH branchpoint. This could be tested by measuring the phosphorylation state of ICDH *in vivo* when acetate is added to the growth medium of cells which have been grown on some other carbon source. Another possibility is that another gene, downstream of *aceK*, is required for growth of *E. coli* KAT-1/pEM901 on acetate.

Potential use of pEM9 to locate the *iclR* gene

Expression of the glyoxylate bypass operon during growth on acetate is controlled by the gene *iclR* which maps downstream of, and in close genetic linkage to, the operon itself (Maloy and Nunn, 1982; Brice and Kornberg, 1968). Cloning of the *iclR* gene is desirable in order to facilitate the identification of the gene product and to study the mechanism by which this gene exerts its control.

Since *E. coli* strain KAT-1 is *iclR*$^+$ (Maloy and Nunn, 1982) and *iclR* is thought to code for a diffusible repressor protein, our results do not test the possibility that pEM9 also carries the *iclR* gene. We know that the *aceK* gene is near the HindIII site equivalent to the end of the 6.7 kilobase pEM901 insert. Whether the pEM9 DNA downstream of this region carries *iclR* could be tested by transformation of an *iclR*$^-$ strain (Maloy and Nunn, 1982) with pEM9. If *iclR* is carried by pEM9, expression of the glyoxylate bypass enzymes in such a transformant would be derepressed only during growth on acetate. If not, expression would be constitutive and in that case, genomic DNA from *E. coli* ML308, downstream of that in the pEM9 insert, could be searched for *iclR*. This would involve preparation of a probe from pEM902 (see Figure 4.1.) which could be used to probe restriction-endonuclease digests of genomic DNA from *E. coli* ML308.

Expression of band "X"

Band "X" (section 4.4 and Figure 4.2.) is a chromosomally-encoded protein which is expressed in E. coli LE392 and E. coli KAT-1/pEM9 only during growth on acetate. It would be interesting to examine whether the expression of band "X" is under the control of iclR, i.e. whether its expression is constitutive in iclR$^-$ mutants of E. coli LE392 (available from Maloy and Nunn, 1982). This protein has not yet been identified but I found that it comprised the majority of protein in a 50-70% $(NH_4)_2SO_4$ fraction from E. coli KAT-1/pEM9 grown on acetate and so it should be very easy to purify if it is identified and proves to be of interest.

Table 5.1 Purification of ICL

Starting material: 12.7g (wet weight) *E. coli* KAT-1/pEM9 grown on acetate. The specific activity of the crude extract from this preparation (*) was lower than usual (5.44 and 6.02 mmol/min/mg for two other preparations)

Step	Volume (ml)	Protein (mg/ml)	ICL activity (μmol/min/ml)	Total activity (μmol/min)	Specific activity (mmol/min/mg)	Purification	Recovery (%)
Crude extract	27	33	132.7	3582	(*) 4.0	1.0	100
$(NH_4)_2SO_4$ 30-45%	4	90	547.5	2190	6.1	1.5	61.1
Acid	5.3	32	373.6	1980	11.7	2.9	55.3
Sephacryl S-300	26	4	63.0	1638	15.8	3.9	45.7
Phenyl-Sepharose	32	1.2	42.8	1370	35.7	8.9	38.2

CHAPTER FIVE - THE ENZYMES OF THE GLYOXYLATE BYPASS FROM THE OVEREXPRESSING STRAIN ESCHERICHIA COLI KAT-1/pEM9

5.1. ICL from E. coli KAT-1/pEM9

5.1.1. Purification

Details of the purification of ICL from the overexpressing E. coli strain KAT-1/pEM9 described in Chapter 4, are given in Table 5.1. The purification scheme was that employed for ICL from E. coli ML308 (Methods 2.5.2.) except that the last step, Mono Q ion-exchange chromatography, was not required. An attempt to amalgamate the early stages of the purification of ICL and ICDH kinase/phosphatase (Methods 2.5.5.) in order to economise on cells was not successful. The A_{280} and activity profiles from the final phenyl-Sepharose column are shown in Figure 5.1.

A polyacrylamide gel showing the protein at each stage of the purification is shown in Figure 5.2. The purification was carried out twice and homogeneous enzyme, as judged by polyacrylamide gel electrophoresis under native conditions (not shown) and in the presence of SDS (Figure 5.2.), was obtained in 38.2% and 31% overall yield.

5.1.2. Comparison of ICL from E. coli KAT-1/pEM9 and E. coli ML308

a) Peptide mapping

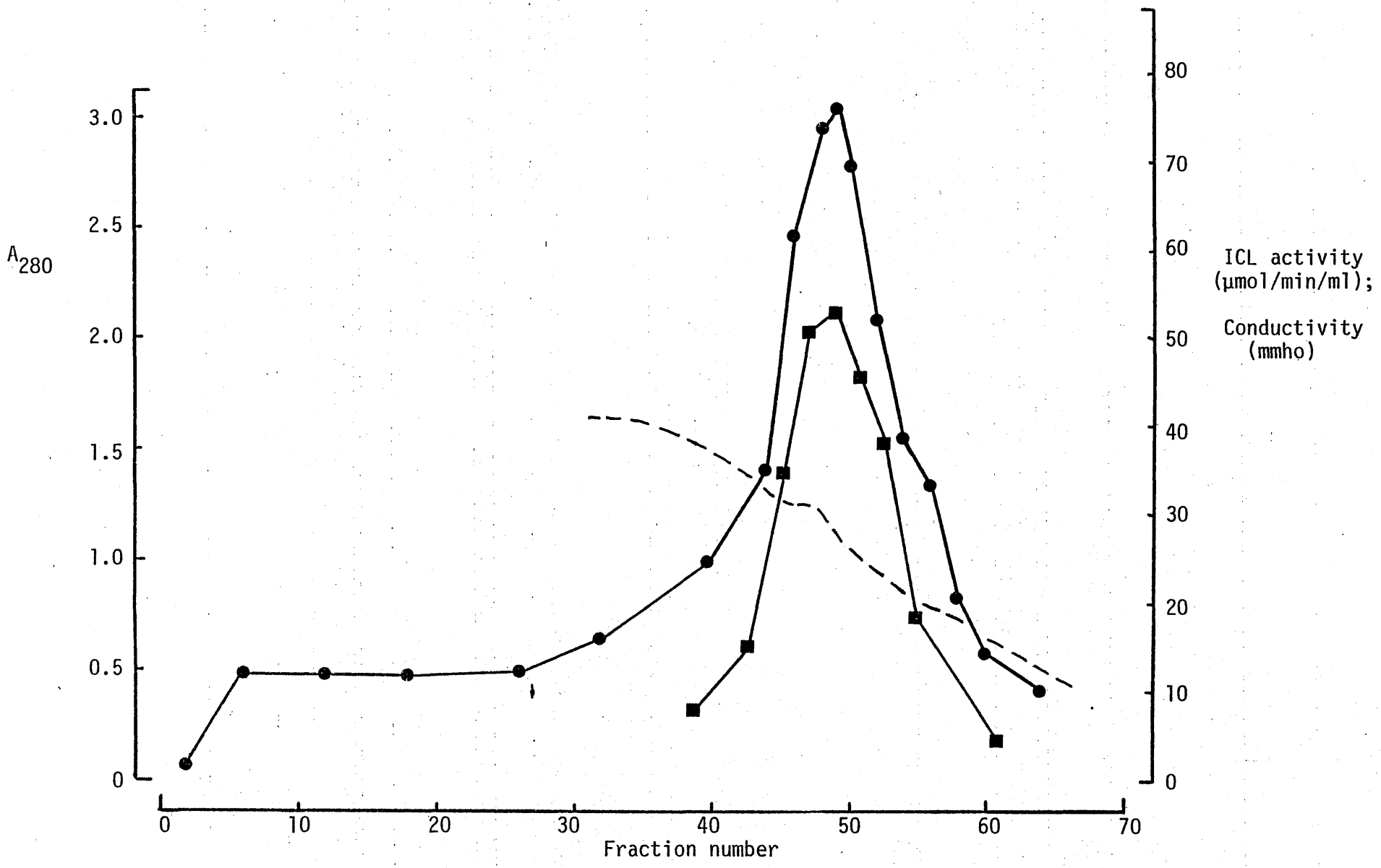
Figure 5.3. shows that the patterns and band intensities were identical when ICL from E. coli ML308 or from E. coli KAT-1/pEM9 was digested with either chymotrypsin or S. aureus V8 protease. This is strong evidence that the two enzymes are identical in primary structure.

b) Amino acid compositions

Table 5.2. shows the amino acid compositions of ICL from E. coli ML308 and E. coli KAT-1/pEM9.

Figure 5.1. Chromatography of ICL from E. coli KAT-1/pEM9 on phenyl-Sepharose

(step (f) of purification scheme) $(NH_4)_2SO_4$ was added to the enzyme pool from step (e), to 0.6M, and this was loaded onto a column of phenyl-Sepharose (20ml) equilibrated in 0.6M $(NH_4)_2SO_4$ in buffer B_1, The column was washed with this buffer, then a gradient (total volume 150ml) of 0.6M to 0M $(NH_4)_2SO_4$ was applied. The flow rate was 30ml/h and 2.5ml fractions were collected. ● ,A_{280}; ■,ICL activity; — -, conductivity.


A_{280}
3.0
2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
Fraction number
80
70
60
50
40
30
20
10
0
ICL activity (µmol/min/ml);
Conductivity (mmho)

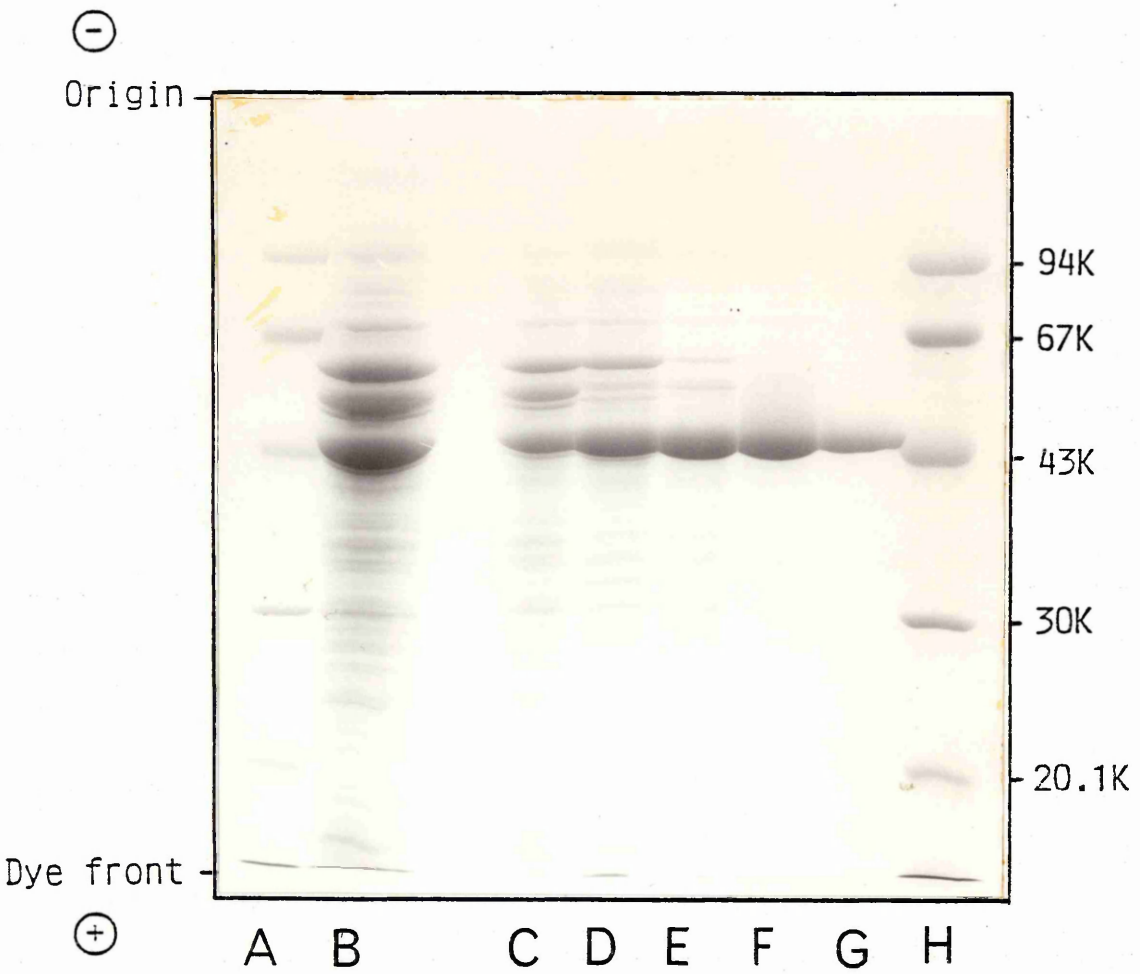


Figure 5.2. Purification of ICL from E. coli KAT-1/pEM9 This 10% polyacrylamide gel run in the presence of SDS monitors the purification of ICL. Tracks A and H, M_r standard proteins (Methods 2.6.5.); tracks B and C, crude extract; track D, 30 - 45% ammonium sulphate fraction; track E, acid soluble protein; track F, enzyme eluted from Sephacryl S-300 superfine column; track G, enzyme eluted from phenyl-Sepharose column.

Amino acid	E. coli ML308 Mean ± S.D. % (X)	E. coli KAT-1/pEM9 Mean ± S.D. % (X)
CysAH	1.49± 0.09 (7)	1.33± 0.12 (6)
Asp	9.3 ± 0.24 (7)	9.23± 0.23 (6)
MetSO	4.67± 0.77 (7)	4.02± 0.66 (6)
Thr	6.81 - (2)	6.78 - (2)
Ser	6.40 - (2)	6.45 - (2)
Glu	12.13± 1.42 (7)	13.25± 1.00 (6)
Pro	4.50± 0.54 (4)	4.47± 0.06 (3)
Gly	9.30± 0.28 (7)	9.28± 0.27 (6)
Ala	13.84± 0.57 (7)	13.47± 0.62 (6)
Val	6.60± 0.22 (4)	6.4 ± 0.20 (3)
Ile	5.08± 0.17 (4)	4.9 ± 0.10 (3)
Leu	8.41± 0.23 (7)	8.48± 0.30 (6)
Tyr	1.57± 0.58 (7)	1.40± 0.42 (6)
Phe	4.84± 0.13 (7)	4.83± 0.37 (6)
His	1.81± 0.10 (7)	1.85± 0.14 (6)
Lys	5.13± 0.22 (6)	5.05± 0.15 (6)
Arg	3.90± 0.10 (3)	4.05± 0.13 (4)

Table 5.2. Amino acid composition of ICL from E. coli ML308 and E. coli KAT-1/pEM9 Generally, with the exceptions mentioned below, the values from the 24h, 48h and 72h hydrolysates were averaged to give the amino acid contents. The values for serine and threonine were obtained by linear extrapolation to zero-time hydrolysis. The values for isoleucine and valine, which are slowly released during hydrolysis, were obtained from the results after 72h hydrolysis. Cysteine was determined as cysteic acid and methionine was determined as its sulphone in the performic acid-oxidised enzyme. Tryptophan is destoyed during acid hydrolysis and was not determined. Thus, the values are expressed as percentages of the amino acids determined, with tryptophan nominally given a value of 0%.

Figure 5.3. One-dimensional peptide maps of wild type E. coli ICL and the overproduced ICL Samples were digested with chymotrypsin (CT) or with S. aureus V8 protease (V8) and electrophoresed as described in Methods 2.6.6. The 15% polyacrylamide gel, run in the presence of SDS, was stained for protein by the silver method (Methods 2.6.4.).

	E. coli ML308		E. coli KAT-1/pEM9	
Sequence number	PTH amino acid	nmoles recovered	PTH amino acid	nmoles recovered
1	-	-	Met	30.3
2	Lys	156	Lys	33.6
3	Thr	41	Thr	15.3
4	Arg	79	Arg	36
5	Thr	48	Thr	15.3
6	Gln	127	Gln	36
7	Gln	129	Gln	45
8	Asn	99	Asn	33
9	Glu	102	Glu	42
10	Glu	104	Glu	33
11	Leu	60	Leu	19.2
12	Gln	99	Gln	15.6
13	Lys	60	Lys	9.6
14	Glu	99	Glu	16.5
15	Trp	41	Trp	9
16	Thr	25	Thr	4.5
17	Gln	77	Gln	11.1
18	Pro	29	Pro	6.6
19	Arg	12	Arg	9
20	Trp	13	Trp	4.2
21	Glu	24.5	Glu	11.7
22	Gly	19.5	Gly	8.4
23	-	-	-	-
24	Thr	5.5	Thr	2.4
25	Arg	5	Arg	4.6
26	Pro	7.5	Pro	3.2
27	Tyr	6.5	Tyr	3.4
28	Ser	1.5	-	-
29	Ala	9	Ala	4
30	Glu	10	Glu	4
31	Asp	8	Asp	3.8
32	Val	6.5	Glu/Val	-
33	Lys	3.9		
34	Lys	4.8		
35	-	-		
36	Arg	4.5		
37	Pro	3.9		

Table 5.3. NH_2-terminal sequences of purified ICL from E. coli ML308 and E. coli KAT-1/pEM9

A first run of ICL from E. coli ML308 gave Met instead of Arg at positions 4. 19 and 25.

c) pI values

Figure 5.4. shows the profiles of A_{280} and ICL activity after chromatofocusing on an FPLC Mono P column. The pI value for ICL from E. coli ML308 or E. coli KAT-1/pEM9 was between 4.4 and 4.5. However, the recovery of ICL and specific activity was less than 10% in both cases. During purification the pH could not be reduced below 4.5 without causing irreversible inactivation of ICL (Methods 2.5.2.d)). These two facts therefore raise doubt about whether the pI values obtained from Mono P chromatofocusing represent native or denatured ICL.

d) K_m for isocitrate

The K_m values for isocitrate at pH 7.3 are essentially identical for ICL from E. coli ML308 (0.063±0.006mM(3)) or from E. coli KAT-1/pEM9 (0.059mM). The LDH-coupled assay was used (Methods 2.4.2.a)) for determination of the K_m for ICL from E. coli KAT-1/pEM9.

e) NH_2-terminal sequence analysis

Table 5.3. lists the NH_2-terminal amino acid sequences of ICL from E. coli ML308 and E. coli KAT-1/pEM9 obtained by automatic protein sequencing of the intact proteins (Methods 2.7.4.). Degradation cycle 23 of each analysis showed no peaks at all on HPLC, monitored at A_{269}.

Collectively, these studies show that the overproduced ICL is chemically and enzymatically indistinguishable from the wild-type enzyme.

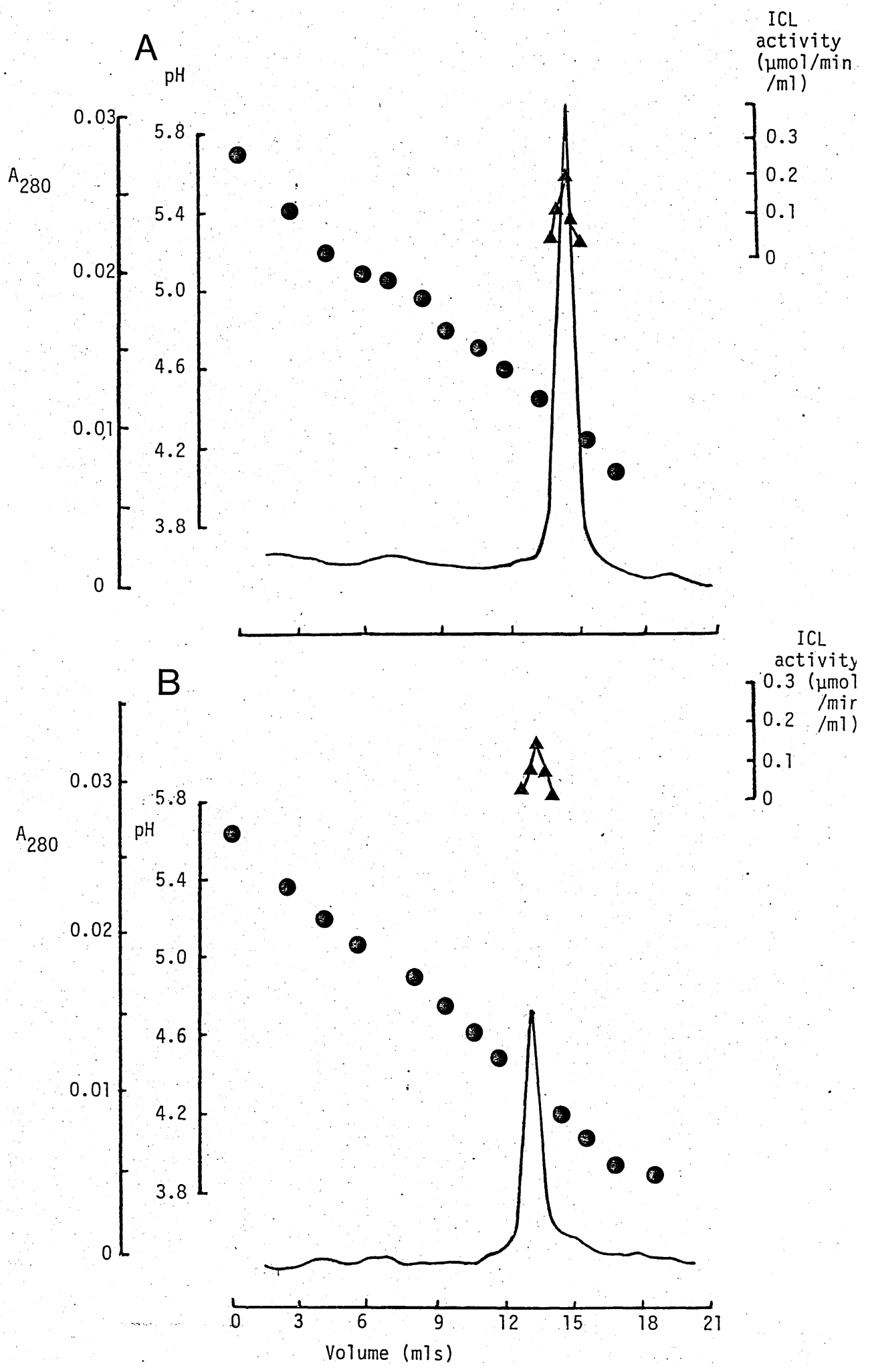
5.1.3. Phosphorylation of ICL

Robertson et al. (1987) recently proposed that active ICL from E. coli is phosphorylated. They found that a protein, in a crude extract phosphorylated in vitro with [γ-^{32}P]-ATP, comigrated with active ICL on 2-dimensional gel electrophoresis. Surprisingly, the putative phosphorylated amino acid was acid-labile and therefore could not be

Figure 5.4. Determination of isoelectric point

Purified ICL (0.1mg) in 20mM piperidine/HCl pH5.7 (0.5ml) was applied to an FPLC Mono P chromatofocusing column (20cm X 0.5cm) pre-equilibrated in the same buffer. Protein was eluted with Polybuffer PB74 diluted 1 to 8 with water and adjusted to pH3.6 with HCl. The flow rate was 1ml/min and 0.75ml fractions were collected. The fractions were assayed for ICL activity (Methods 2.4.2.a)). The A_{280} (—) was monitored and the pH value (●) of fractions measured (Methods 2.2.1.).

A, ICL from *E. coli* ML308; **B**, ICL from *E. coli* KAT-1/pEM9.


A
pH
ICL activity (µmol/min /ml)
A_{280}
0.03
0.02
0.01
0
5.8
5.4
5.0
4.6
4.2
3.8
0.3
0.2
0.1
0
B
ICL activity
0.3 (µmol
/mir
0.2 /ml)
0.1
0
A_{280}
pH
0.03
0.02
0.01
0
5.8
5.4
5.0
4.6
4.2
3.8
0
3
6
9
12
15
18
21
Volume (mls)

serine, threonine or tyrosine.

The NH_2-terminal amino acid sequence of ICL (section 5.1.3.e)) was compared with an archived data bank (National Biomedical Research Foundation) of amino acid sequences in collaboration with Drs. A. Coulson and J. Collins at the Department of Molecular Biology, Edinburgh University. A match was seen between ICL from E. coli and citrate synthase (EC.4.1.3.7.), which also catalyzes an aldol condensation, from baker's yeast (Figure 5.5.). There was no homology with E. coli citrate synthase. Evaluation of the significance of this homology must await complete sequencing of the E. coli aceA gene. However, it was noticed that in this match the unknown residue 23 in ICL corresponds to a serine in citrate synthase.

Since cycle 23 of sequence runs with ICL showed no peaks at all on HPLC, we felt it possible that it was a phosphorylated serine residue. The phenylthiohydantoin derivative of phosphoserine does not absorb at 269nm and so is not detected in many HPLC systems.

Because of these observations, I looked for alkali-labile phosphate in purified ICL from E. coli KAT-1/pEM9. After NaOH hydrolysis of up to 1mg of purified ICL, no phosphate was detected. KH_2PO_4 was used as standard. The sensitivity of the method would have allowed detection of about 0.1 phosphate groups/subunit.

ICDH kinase/phosphatase, in the presence of ATP and Mg^{2+}, cannot phosphorylate ICL (Dr. I. Varela, personal communication) and has no effect on ICL activity.

Thus we have no convincing evidence that active ICL is phosphorylated.

5.2. MS-A from E. coli KAT-1/pEM9

5.2.1. Purification

Measurement of activity showed that E. coli KAT-1/pEM9

		* * * * * * * * * * * *	
citrate synthase	113	E I Q R E L P K A E G S T E P	127
ICL	10	E L Q K E W T Q P R W E G X T E P	26

Figure 5.5. Alignment of the amino acid sequence homology between the NH_2-terminal sequence of E. coli ICL and citrate synthase from baker's yeast Homologies are marked by *. The single-letter code of amino acid residues has been used: A,Ala; E,Glu; G,Gly; I,Ile; K,Lys; L,Leu; P,Pro; Q,Gln; R,Arg; S,Ser; T,Thr; W,Trp; X,unknown.

Table 5.4 Purification of MS-A

Starting material: 18g (wet weight) E. coli KAT-1/pEM9 grown on acetate. Malate synthase was assayed by Method 2.4.2.e).

Step	Volume (ml)	Protein (mg/ml)	MS-A activity (mol/min/ml)	Total activity (mol/min)	Specific activity (mmol/min/mg)	Purification	Recovery (%)
Crude extract	36	28	50.4	1814	1.8	1	100
$(NH_4)_2SO_4$ 0-50%	8.1	52	182	1474	3.5	1.9	81.3
Sephacryl S-300	54	5.9	23	1242	3.9	2.2	68.5
DEAE-Sephacel (Total)	76	1.1	12.4	942	11.3	6.3	51.9
DEAE-Sephacel (Fractions 46-49)	37	1.9	20.9	773	11.0	6.1	42.6

overproduced MS-A (Table 4.1.).

In order to allow interpretation of the *in vitro* transcription/translation experiments described in Chapter 4, I originally set out to identify MS-A as a band on SDS-PAGE. I then carried on to develop a purification for the enzyme. The final procedure is described in Methods 2.5.4. Details of the purification of MS-A from *E. coli* KAT-1/pEM9 are given in Table 5.4. and the A_{280} and activity profiles from the final DE52 ion-exchange column are shown in Figure 5.6.

An SDS gel which monitors the purification procedure is shown in Figure 5.7. It appears that the Sephacryl S-300 superfine gel filtration chromatography provides little purification and could perhaps be omitted.

Not all of the final MS-A-containing fractions were homogeneous so only peak fractions (Figure 5.6.) were pooled.

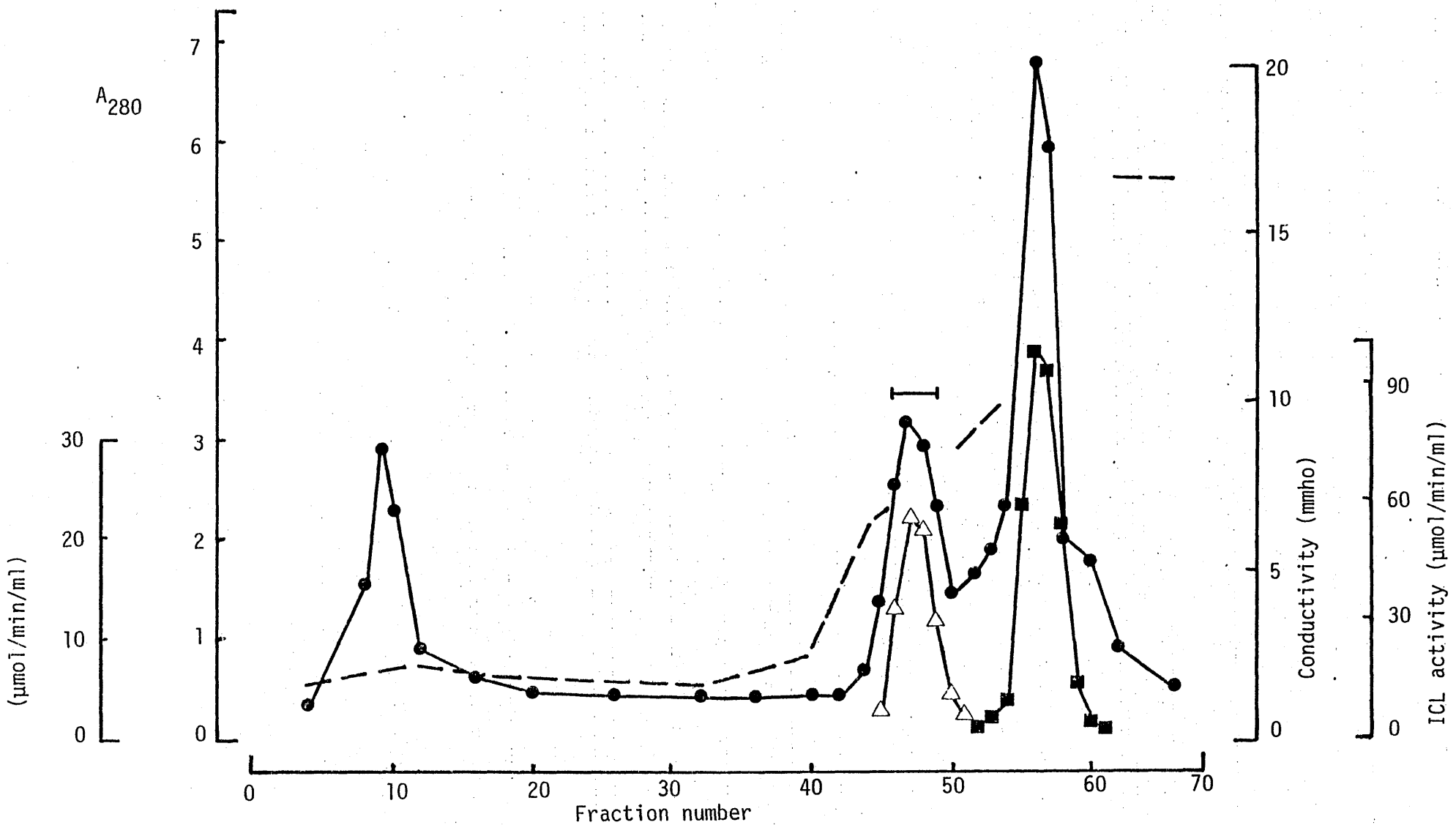
E. coli has two malate synthase isozymes; MS-B predominates in cells grown on glycolate (Vanderwinkel and De Vlieghere, 1968) and MS-A predominates in cells grown on acetate. The malate synthase purified here was identified as MS-A because it was overexpressed in *E. coli* KAT-1/pEM9 which carries multiple copies of the genes *aceB* and *aceA*; compare track B of Figure 3.4. and track B of Figure 5.7. KAT-1 has a functional *aceB* gene so it is expected that a small proportion of the purified enzyme is MS-A expressed from the KAT-1 chromosome.

5.2.2. NH_2-terminal sequence analysis

Table 5.5. shows the NH_2-terminal amino acid sequence of MS-A from *E. coli* KAT-1/pEM9. After degradation cycle 15, background on HPLC increased rapidly and no more residues could be identified. This suggests that the protein broke up. Perhaps MS-A has many bonds that are particularly sensitive to acid hydrolysis.

Figure 5.6. Chromatography of MS-A from *E. coli* KAT-1/pEM9 on DEAE-Sephacel

(step (e) of purification scheme) Enzyme from step (d) was loaded onto a column of DEAE-Sephacel (3cm X 8cm) equilibrated in buffer B_1. The column was washed with this buffer; then a gradient (total volume 350ml) of 0M to 0.5M NaCl in buffer B_1 was applied. The flow rate was 100ml/h and 10ml fractions were collected. Fractions 46-49 were pooled (⊢—⊣). ●,A_{280}; △,MS-A activity; ■ ICL activity;— —,conductivity.


A_{280}
7
6
5
4
3
2
1
0
30
20
10
0
(µmol/min/ml)
20
15
10
5
0
Conductivity (mmho)
90
60
30
0
ICL activity (µmol/min/ml)
0
10
20
30
40
50
60
70
Fraction number

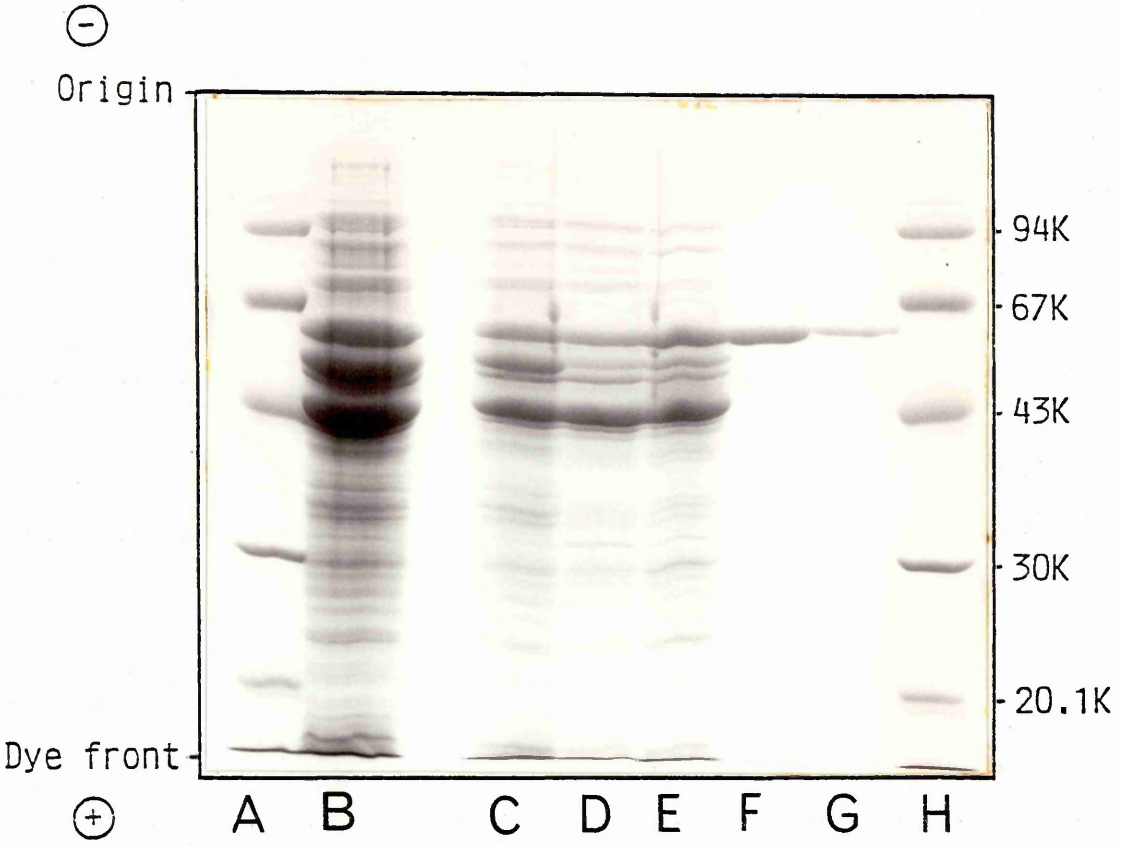


Figure 5.7. Purification of MS-A from E. coli KAT-1/pEM9 This 10% polyacrylamide gel run in the presence of SDS monitors the purification of MS-A. Tracks A and H, M_r standard proteins (Methods 2.6.5.); tracks B and C, crude extract; track D, 0 - 50% ammonium sulphate fraction; track E, enzyme eluted from Sephacryl S-300 superfine column; tracks F and G, enzyme eluted from DEAE-Sephacel column.

Sequence number	PTH amino acid	nmoles recovered
1	-	-
2	Glu	45
3	Gln	36
4	His	51
5	Thr	9.3
6	Thr	10.5
7	Thr	9.6
8	Asp	37.5
9	Glu	40.2
10	Leu	27
11	Ala	29.1
12	Phe	38.1
13	Thr	10.2
14	-	-
15	Met	28.8

Table 5.5. NH_2-terminal sequence of MS-A

5.3. Discussion - ICL and MS-A from *E. coli* KAT-1/pEM9

a) NH_2-terminal sequences

Knowledge of the NH_2-terminal sequences obtained from analysis of ICL and MS-A will be useful in future work on the corresponding DNA sequences because this will facilitate identification of the start of the protein-coding regions of the genes. Sub-cloning of pEM9, in preparation for DNA sequencing, has been started, in Glasgow, by Dr. I. Varela.

b) Crystallographic studies of ICL

ICDH and ICL both bind isocitrate. They carry out very different reactions and have quite different affinities for isocitrate. This is functionally significant in that phosphorylation of ICDH seems to have been selected as a mechanism to increase the intracellular concentration of isocitrate and thus facilitate flux through ICL. This consideration raises two points. First, one can speculate as to why development of the ability to grow on acetate involved selection of the phosphorylation of ICDH rather than the evolution of an ICL with a lower K_m for isocitrate. Perhaps something about the chemistry of the ICL reaction precludes high-affinity binding of isocitrate. Second, it is intriguing that two enzymes can bind the same metabolite quite specifically with markedly different affinities. How do the two sites differ?

These questions could be answered by establishing the 3-dimensional structure of the two enzymes. Attempts to crystallize the two enzymes are under way, in collaboration with Dr. L. Sawyer (ICDH) and Dr. D. Rice (ICL). In a further project, protein sequencing of ICDH will be carried out in Glasgow with the primary aim of studying the effects of phosphorylation on conformation of ICDH. A secondary aim is to identify and mutate the isocitrate binding site of ICDH. Similar studies of ICL could be attempted once the 3-dimensional structure of the enzyme is known. It would be very interesting to look at the

effects of ICL and ICDH isocitrate-binding mutants, on carbon flux in intact cells.

5.4. ICDH kinase/phosphatase from E. coli KAT-1/pEM9

5.4.1. Purification

The procedure outlined in Chapter 2 was devised by Dr. H.G. Nimmo and allowed isolation of 5-10µg of homogeneous protein from 30g (wet weight) of cells. This represented a yield of ≃5% and a purification factor of ≃9000 from the ammonium sulphate precipitate.

5.4.2. Physical analysis of ICDH kinase/phosphatase

a) Introduction

The cloning of the glyxoylate bypass operon from E. coli ML308 and the development of a purification providing about 10µg of homogeneous ICDH kinase/phosphatase meant that at last the questions raised in Chapter 1, about the topology of the bifunctional ICDH kinase/phosphatase, could be tackled. In the first instance, does ICDH kinase/phosphatase have one or two active sites?

I used limited proteolysis to investigate the structure of ICDH kinase/phosphatase. Interdomain regions are often more accessible to proteolytic attack than the globular domains. Limited proteolysis has sometimes been used to isolate fragments containing single functions from multifunctional proteins (e.g. Coggins and Boocock, 1986; Evans, 1986; McCarthy et al.,1983).

b) Limited proteolysis with trypsin

Limited proteolysis of purified ICDH kinase/phosphatase with trypsin, as described in Methods 2.7.5., was carried out twice with similar results. Accurate protein estimation of the small amounts of pure ICDH kinase/phosphatase available was difficult. Therefore, appropriate ratios of trypsin to ICDH kinase/phosphatase had to be

worked out empirically before each time-course experiment. Only one experiment is described here.

Figure 5.8 depicts the time course of digestion with trypsin and concomitant change in ICDH kinase and ICDH phosphatase activities. It is apparent that ICDH phosphatase is inactivated much more rapidly than ICDH kinase. This result could be interpreted as an indication that the active sites are different.

SDS-PAGE patterns of these samples, taken at the indicated times of digestion, are shown in Figure 5.9. The tryptic digestion causes reduction of the subunit M_r of the enzyme in two steps. Disappearance of phosphatase activity correlates with a reduction in subunit M_r which can just be resolved on this 10% polyacrylamide gel. The smallest fragment is devoid of kinase activity. This was verified in another experiment.

Incubation of ICDH kinase/phosphatase with carboxypeptidase A or with aminopeptidase M had no effect on activity but there was not enough pure ICDH kinase/phosphatase to allow me to follow the progress of digestion by SDS-PAGE.

c) Future experiments

There are several important experiments which should be tackled as soon as sufficient protein is available. Subcloning the aceK gene from E. coli ML308 into a high-level expression vector should provide enough ICDH kinase/phosphatase for a complete structural analysis.

Trypsin digestion of ICDH kinase/phosphatase on a larger scale would allow quantitation of the protein fragments on the gel by scanning densitometry. This would allow correlation of changes in amounts of protein fragments with changes in activity . The effects on digestion of the presence of ligands, use of other proteases, NH_2-terminal sequencing of fragments and study of the kinetic characteristics of

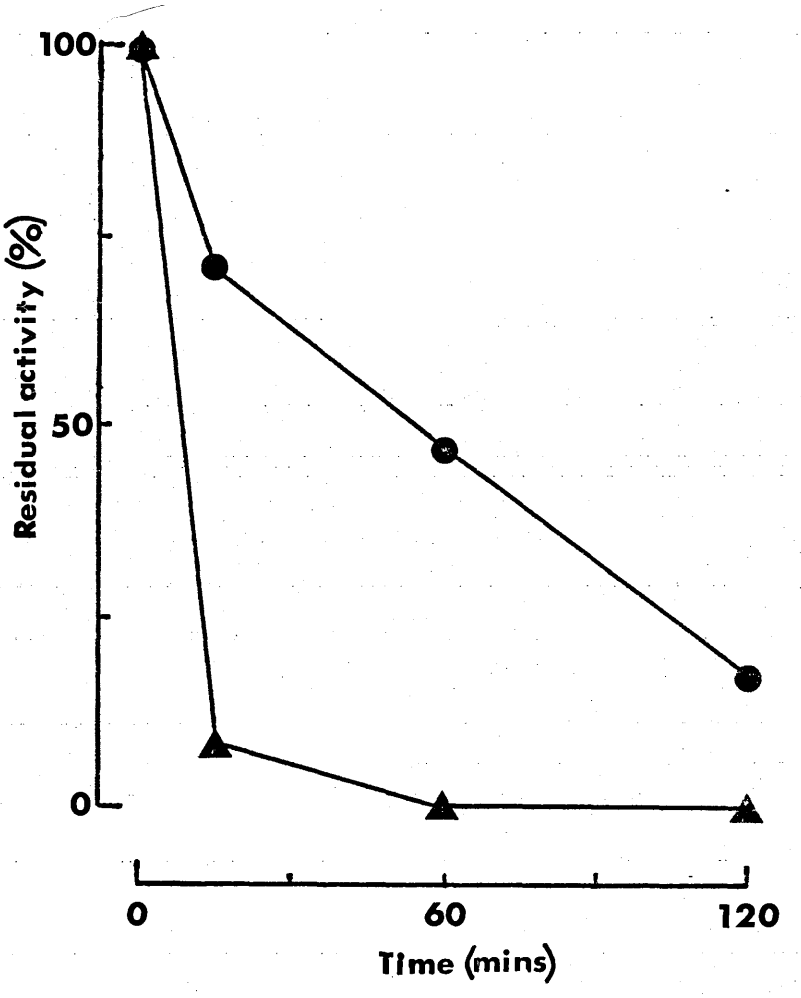


Figure 5.8. Kinetics of inactivation of ICDH kinase/phosphatase by trypsin digestion Four aliquots of ICDH kinase/phosphatase (approx. 0.4µg in 10µl) were digested with trypsin (0.04µg in 2µl) at 30°C. At the times indicated, soybean trypsin inhibitor (12µg in 12µl) was added. 1µl of each solution was assayed for ICDH kinase (Methods 2.4.2.e)) and 5µl for ICDH phosphatase (Methods 2.4.2.f)). The remainder of each incubation (about 17µl) was analysed by SDS-PAGE (see Figure 5.9.). ▲, ICDH phosphatase; ●, ICDH kinase. Further details are in Methods 2.7.5.

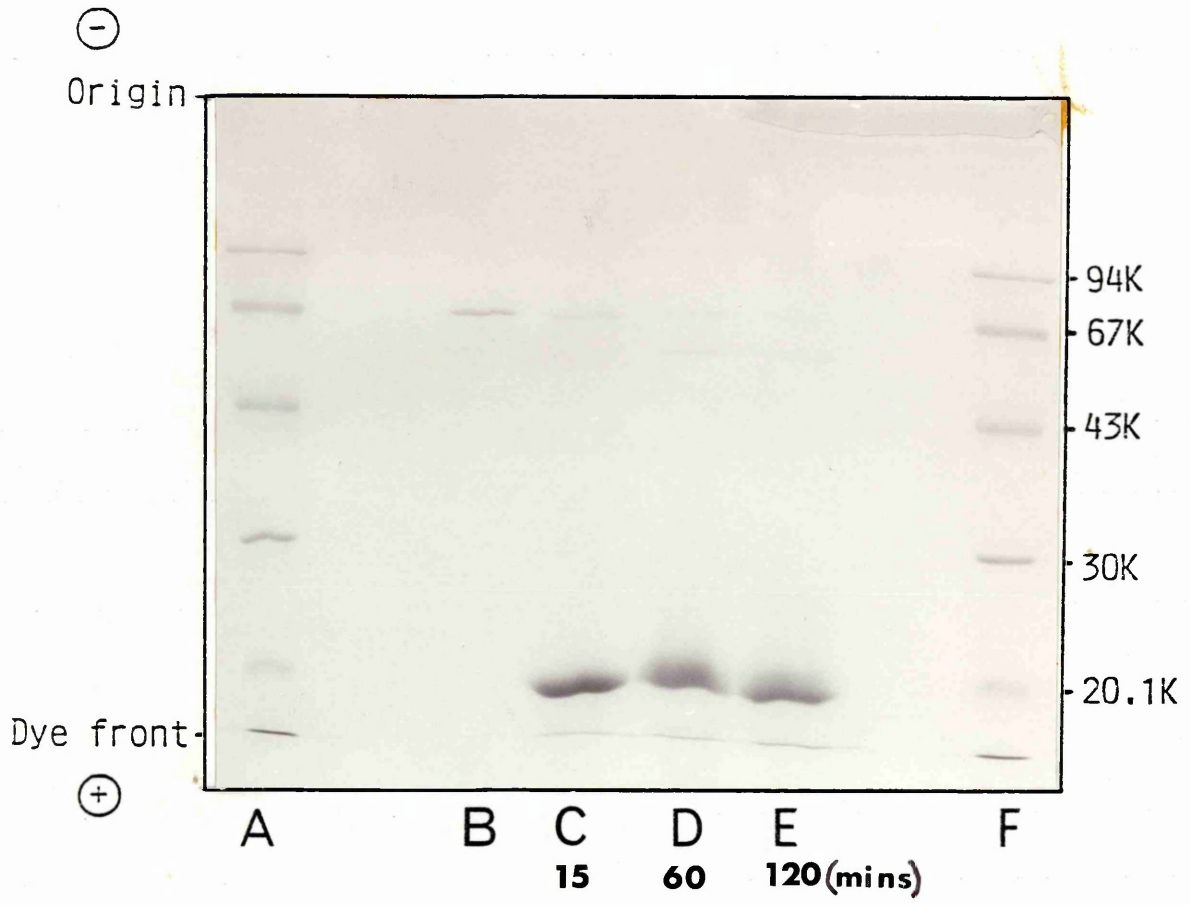


Figure 5.9. SDS-PAGE of limited proteolysis of ICDH kinase/phosphatase by trypsin The experiment is the same one shown in Figure 5.8. Tracks A and F, M_r standard proteins (Methods 2.6.5.); track B, purified ICDH kinase/phosphatase - in this particular experiment, too much of the zero-time material described in Methods 2.7.5. was used up in assays and a sample of purified ICDH kinase/phosphatase was substituted on this gel. The remaining tracks show incubations of the times indicated. Further details are given in Methods 2.7.5.

active fragments will give increasingly accurate data on the location of active sites.

Determination of the native M_r of digested ICDH kinase/phosphatase is a priority. It is possible that the two subunits do not function independently and that losses of activity upon trypsin digestion are due to destruction of subunit binding sites.

In Glasgow, Dr. I. Varela is using another approach to investigate the structure of ICDH kinase/phosphatase that of photoaffinity labelling with 8-azido-[α-^{32}P]-ATP in the presence and absence of ATP followed by isolation and sequencing of labelled peptides.

The aceK gene has now been sequenced (D. LaPorte; personal communication) but the data are not yet available. It will therefore soon become clear whether the bifunctional kinase/phosphatase arose from a gene duplication and fusion. In the long term, it may prove possible to investigate the advantages of bifunctional regulatory enzymes compared to monofunctional ones by judicious engineering of the aceK gene.

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