Enzyme organization of the citric acid cycle

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ENZYME ORGANIZATION OF

THE CITRIC ACID CYCLE

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for my mother and father

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ABSTRACT

Conventional cellular extraction procedures may not be conducive to the maintenance of fine intracellular organization. The belief that the cytoplasm of the cell is unstructured and composed of freely diffusing enzymes and metabolites has been superseded by the view of it as a highly organized and ordered system. Within this system, enzymes catalysing sequential steps of a metabolic pathway may be compartmentalized by specific associations, to the functional and energetic advantage of the cell.

It now emerges that enzymes which have formerly been described as soluble, by virtue of their sedimentation characteristics on cell disruption, show evidence of dynamic organization. Such compartmentation has been described in the glycolytic sequence and was investigated in this study for the enzymes of the citric acid cycle.

A multienzyme cluster containing the activities of five of the citric acid cycle enzymes has been isolated from bacterial cells by the adoption of an extraction procedure judged to be least drastic to the components of the cell. The cluster has been demonstrated to be a physical entity by inactivation and immunochemical studies and sucrose density gradient centrifugation. Two of the other enzymes of the cycle, STK and OGDH, showed a specific interaction and, through OGDH, demonstrated an association with the five-enzyme cluster. The isolatable cluster exhibits a crypticity of enzyme

activity which may be indicative of compartmentation of the cycle enzymes.

The presence and stability of the cluster was shown to be dependent on the physical environment surrounding the enzymes; conditions aimed to be a reflection of the internal cellular milieu yielded a greater proportion of cluster. The citric acid cycle cluster may be located on or adjacent to the surface of the inner membrane of the bacterium. The form of association of the enzymes indicated that dynamic compartmentation may occur.

The findings from studies on bacteria were extrapolated to an examination of mitochondria from eukaryotic cells for the display of any similar enzyme associations.

Electron microscopic examination of the citric acid cycle cluster revealed particles of 20 - 30 nm in diameter, consistent with the estimated molecular weight of the cluster of 2.5×10^6.

The significance and implications of these results are discussed.

ABBREVIATIONS

Buffer systems

MET 8	Tris-EDTA buffer + 2 mM $MgCl_2$, pH 8.0
TAG	Tris-acetate buffer + 20% v/v glycerol

DMDBP	dimethyl 3,3'-dithiobis-propionimidate-2HCl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
PBS	phosphate buffered saline
PEG	polyethylene glycol
TEMED	N,N,N',N'-tetramethylethylenediamine

All other abbreviations used in this thesis are those recommended in the Biochemical Society Publication "Policy of the Journal and Instructions to Authors" (Biochem. J. 1984 **217**, 1).

Definition of Terms

Uml^{-1} are defined as µmole $min.^{-1}ml^{-1}$.

µmolar absorption coefficient is defined as absorbance of a 1 mol/litre solution in a 1 cm light-path.

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CHAPTER 1

INTRODUCTION

A Full Circle

The major portion of the protein content of an average cell is comprised of constituent enzymes of that cell. The previous view of the cell as a bag composed of enzymic proteins and freely diffusing metabolites uniformly dispersed in an aqueous solution has all but been abandoned in contemporary cellular biology (Welch, 1977). The move has been towards a unifying hypothesis viewing cellular metabolism as ordered in space and time, with the cell composed of highly organized and intricately regulated systems of multienzyme sequences.

In the history of enzymology, the vitalist concept of the past century that enzyme catalysis is somehow associated with living cells was superseded by the identification of discrete enzymes as protein catalysts. The analysis of these proteinaceous catalysts required homogeneous uncontaminated enzyme preparations involving elaborate purification and upon which much of the knowledge of modern enzymology is based. However, although this reductionist approach explains much about enzyme action *in vitro* it fails to establish how they work *in vivo*. The basic properties of individual enzymes remain; but superimposed on this is the recognition of order and organization in enzyme systems.

It appears that the concept of cellular metabolism has come full circle and it is now recognized that the behaviour of enzymes

in the cell is that which is allowed or dictated by the environment *in vivo*.

Historical Perspectives

"Life is a web, not a jigsaw puzzle' (Weiss, 1976).

The ideas underlying the modern concept of cellular metabolism, of enzymes operating within a framework of spatio-temporal structure, are not new. Over eighty years ago the cell was regarded "from the metabolic point of view as a congeries of enzymes, a colloidal honeycomb of katalytic agents, as many in number as there are cell functions, and each capable of being isolated and made to do its particular work alone *in vitro*" (Blackman, 1905). Peters (1930) hypothesized that all the enzymic proteins in the cell form a three-dimensional network or mosaic extending throughout the cell, this *in vivo* structure being readily disrupted by harsh extraction techniques. The activities of the enzymes could be controlled by the mosaic itself. Reactions could proceed simultaneously in various parts of the cell whilst the mosaic could act as a whole to perturbing stimuli.

An extension of this forms the basis of the hypothesis that the extensive cytoskeleton of most cells functions in part to serve as a two-dimensional matrix onto which enzyme molecules can be assembled into ordered multienzyme units (De Duve, 1959).

Whole cell centrifugation studies with *Euglena gracilis* (Kempner and Miller, 1969) and *Neurospora crassa* (Zalokar, 1960) have provided evidence that soluble forms of enzymes may be

artifacts of cell disruption. Organelles within the cells were stratified according to their densities. The "soluble" phase isolated on centrifugation contained no enzyme activity. It was concluded that since these cells were capable of recovery, no free or unbound protein existed *in vivo*.

With the development of gentler extraction methods enzymes catalysing multiple sequential reactions have been isolated as soluble multifunctional enzyme systems, as particulate membrane-bound systems or distinct multienzyme complexes. Many multienzyme systems, for example the glycolytic pathway, originally thought to exist freely in the cytoplasm can now be extracted as physically associated components (Mowbray and Moses, 1976; Gorringe and Moses, 1980).

A current view of the living cell holds each enzyme as part of a multienzyme system, corresponding to components of a metabolic pathway. This, in turn, is the component of an even larger network, which finally builds up to what is displayed on the metabolic maps of classical biochemistry. This complex system existing in the cell is closely involved with a highly intricate and organized cytoskeletal framework.

A New Look at the Cell

The cytoplasm of the cell is nowadays regarded as being highly structured. It contains a complex framework of proteins which form a cytomatrix upon which many metabolic processes may take place. The proteins exist in a number of forms, microtubules, actin, tubulin, intermediate filaments as well as the microtrabeculae, a

system which may form a network between all the other cytoskeletal components.

Until recently cell water has been regarded as having the same properties as pure water; however, evidence now exists to sustain a belief in water with physical properties significantly altered as the result of proximity to cellular surfaces and interactions with them (Clegg, 1984). The effect of the surface on the water is thought to decrease with distance from that surface. Two phases of water are now thought to exist: the bulk phase and the water of hydration, which is adsorbed to macromolecular components of the cell such as proteins, carbohydrates and the lipids of membranes. The water of hydration is not immobile; when compared to bulk water it is more ordered, has reduced mobility and altered solvent properties.

Fulton (1982) has described the cytoplasm as "crowded" with high concentrations of proteins. Under such conditions proteins do not behave as in ideal solutions. The volume occupied by proteins affects the activity of the other proteins in solution, in effect crowding them into a small space with less freedom of movement. This tends to force proteins into component configurations, favouring self-associations and hetero-associations and can affect enzyme catalytic activities, if either the configuration or the association of the protein is driven by the high concentration of proteins. Thus actual enzyme activity in the cytoplasm is a function not only of which enzyme is present, but also of the concentration of all other proteins.

The emerging model of the cytoplasm of the cell is that of a compact complex latticework held together by high affinity interactions and by the non-ideal behaviour of proteins under physiological conditions. The latticework is coated with water of hydration, likely to be the phase most important in normal metabolic activities.

Srere (1984) addressed the question of why enzymes are so big since the actual proportion of amino acids involved in the catalytic active site is relatively small. A long polypeptide chain may be needed to ensure that the required site is formed and that the protein structural requirements are met. However, the outside surfaces of proteins help to locate the enzyme in the cell and to specify its interactions with other related enzymes. This indicates that larger proteins have enough surface for multiple interactions with other cellular components. For proteins to associate they must have surface areas complementary to each other, by sharing the same Van der Waals envelope over some area and by the proper positioning of polar groups.

In Vivo vs. *In Vitro*

Two phenomena have been identified by Sies (1980) as distinguishing the living cell from a system *in vitro*. In the cell, steady state conditions prevail, compared to the open metabolic system *in vitro*. In the former, enzymes do not operate under conditions of saturation and the enzymes and reactants are often present in stoichiometric amounts. The existence of cellular compartmentation results in an unequal distribution of components between the various compartments of the cell.

Conventional methods of cell disruption are not conducive to the maintenance of fine associations between enzymes themselves and between enzymes and other cellular components. Removal of enzymes from their natural milieu places them under very unnatural conditions as regards pH, ionic strength, enzyme and substrate conditions and the presence of other proteins.

Organization of Multienzyme Systems

Many if not most enzymes in a living cell function in structured states; membrane-bound, aggregated in solid-state assemblies as in mitochondria or in gel-like surroundings as probably exist in the cytoplasm. Much structured organization may be discerned by light and electron microscopic observations, but also there are examples of metabolic pools of a given metabolite shown by kinetic evidence, for which there is no morphological counterpart.

Levels of organization are emerging in the cytosol, for example between functionally related groups of macromolecules, such that the cytosol is not just a random mixture of proteins, nucleic acids and other components. Certain groups of enzymes are known to form definite multienzyme complexes, e.g. fatty acid synthetases. However the so called "soluble" enzymes of the glycolytic sequence are seen to form a specific, if less tightly associated, complex. The term soluble in terms of enzyme location, may now be regarded purely as a function of the disruption procedure followed to isolate that enzyme. It is possible that many other weak associations of macromolecules may occur which have not yet been

detected, either because they have not yet been sought or because the association is so weak that dilution on isolation may be sufficient to cause disruption.

Compartmentation was defined by Lynen (1961) as the isolation of intermediate metabolites in a specific pathway and their separation from competing reactions. This can be achieved by surrounding a multienzyme system with a selective membrane, which allows the influx of some metabolites and excludes the passage of others, for example in mitochondria and other sub-cellular particles. Compartmentation may also arise by embedding the enzyme in a lipid membrane which may act as a support and as a preferential solvent for hydrophobic substrates, such as the system for the synthesis of cholesterol. Compartmentation may be achieved by covalent binding of intermediates of a reaction sequence, as in fatty acid synthesis. Enzyme sequences may be organized into multienzyme complexes. These are aggregates of different but functionally related enzymes bound together by non-covalent forces into a highly organized structure. They function to increase the catalytic efficiency by channelling metabolite flux through the complex.

Two levels of organization have been distinguished by Friedrich (1984): purely functional organization and structural-functional organization.

Functional organization describes enzymes which have evolved in structure to have functional properties, such as V_{max}, K_m, K_i, allosteric constants. These allow enzymes to exist alongside other enzymes in the same or other systems. This involves not only

kinetic complementarity of sequentially consecutive enzymes but also affects feedback inhibition or allosteric transitions.

On the other hand, structural organization denotes macromolecular associations involving enzymes, underlining the importance of precise spatial disposition of enzymes in certain cellular niches, for example, the membrane integrated respiratory chain of mitochondria. In other cellular compartments structural organization is not so evident. Although soluble enzymes are catalytically competent when isolated in aqueous medium, it is now thought that they are not just subjected to functional organization but are also intimately organized in a structural dimension.

Metabolite compartmentation occurs when two enzymes are spatially juxtaposed in such a way that product from the first enzyme binds to the second enzyme without leaving the complex or being mixed in the bulk medium. This form of compartmentation is described as the channelling of product. The transfer is achieved by diffusion in the microenvironment between the two enzymes, for example, unstirred layers. An example of this channelling effect is found in tryptophan synthetase, where the intermediate indole does not leave the complex.

Dynamic compartmentation

That multienzyme complexes exist showing classical metabolite compartmentation is irrefutable, as shown by the pyruvate dehydrogenase complex. The fact that complexes of enzymes, which function as segments of metabolic pathways, have not been isolated may be a direct result of the nature of their association.

Compartmentation may exist without rigid clustering; if the enzymes of a complex dissociated, or a membrane-bound enzyme array were in rapid equilibrium between adsorbed and free species.

Friedrich (1974) put forward a hypothesis that consecutive enzymes may achieve direct metabolite transfer even if the complex is very loose and the rate constants for both association and dissociation are high. From this he proposed several models to describe dynamic compartmentation.

Consequences of compartmentation

There are a number of theoretical advantages of enzyme organization and compartmentation. These include a decreased diffusion time, leading to catalytic efficiency between sequential enzymes; through metabolic channelling, segregation of competing pathways and restriction to a microenvironment would give an effectively high concentration at the active site. Specific reactions may be enhanced through, for example, a hydrophobic milieu and potentially unstable intermediates may be protected. Protein- protein or protein-membrane interactions may exist. Additionally, finer tuning of metabolic control may be achieved, resulting in coordinate activation or inactivation or a change in pH optima.

Stable Multienzyme Complexes

Stable multienzyme complexes are readily isolated as the functionally active whole. They consist of two or more enzymes with different catalytic activities physically clustered together either non-covalently in the form of complexes, e.g. tryptophan synthetase

or the oxoacid dehydrogenase complexes, or covalently as conjugates, e.g. the "arom" conjugate of *Neurospora* (Welch and Gaertner, 1980).

Tryptophan synthetase catalyses the final step in tryptophan biosynthesis in prokaryotes and plants and consists of two enzymes α and β. The intermediate between α and β is indole (Yanofsky and Crawford, 1972). Indole is channelled from the α enzyme to the β enzyme and does not mix with the bulk medium as a result of the juxtaposition of the α and β enzymes.

The oxoacid dehydrogenase complexes, including pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH), have been studied in considerable detail (Reed and Cox, 1966; Ginsburg and Stadtman, 1970). The three component enzymes are represented in the native complex in several copies and the covalently attached substrate undergoes consecutive transformation by each of the three enzymes, preventing its diffusion into the bulk and giving rise to channelling efficiency.

The activities of five of the seven enzymes of the polyaromatic pathway in *Neurospora* have been shown to be present on a single polypeptide chain (Gaertner and Cole, 1976). this "arom" conjugate shows channelling *in vitro*, which may serve to isolate competing metabolic pathways (Giles, 1978).

Organization in Glycolysis

A means of obtaining a functional compartmentation where loosely associated enzyme aggregates can be obtained as a result of various microenvironmental effects, may be exemplified by the

aggregation of soluble enzymes catalysing consecutive steps in a metabolic pathway. This may be achieved even in the least structured parts of the cell, such as the cytoplasm.

In the cytoplasm the major set of soluble enzymes is that of glycolysis; in yeast they constitute 65% of total soluble protein (Hess *et al.*, 1969). Considerable problems attend the interpretation of experimental approaches to studying soluble enzyme systems. Interaction *per se* does not mean that the *in vitro* phenomenon occurs *in vivo* or has any physiological significance. Conversely, many interactions existing at the high protein concentration *in vivo* may escape detection *in vitro*.

Green and coworkers (1965) reported the disruption of erythrocytes and yeast cells and the derivation of fractions which were capable of catalysing the complete sequence of glycolytic reactions with greatly increased specific activity over that of the whole homogenate. This led to the postulate that the entire glycolytic sequence was membrane bound and indeed that this may be the case with all metabolic sequences. Further support for the concept of a glycolytic complex has more recently been presented. In *Escherichia coli*, evidence has been obtained for a multienzyme complex with glycolytic activity (Mowbray and Moses, 1976; Gorringe and Moses, 1980) and the localization of nine glycolytic enzymes in a micro-body-like organelle has been established in trypanosomes (Opperdoes and Borst, 1977).

Interactions between individual enzymes in the glycolytic sequence have included the aldolase-glyceraldehyde 3-phosphate dehydrogenase associations. Ovadi and Keleti (1978) provided

kinetic evidence for these interactions and for channelling of the intermediate metabolite glyceraldehyde 3-phosphate. Batke *et al.* (1980) used active enzyme centrifugation to illustrate complex formation between aldolase and glycerol 3-phosphate dehydrogenase.

A high degree of multiplicity is observable in the individual glycolytic enzymes, possessing demonstrably different adsorption characteristics. This may lead to the presence of a number of discrete systems of glycolytic components in individual tissues and the effective partitioning of these functional systems between subcellular or tissue compartments (Masters, 1978). The binding of a variety of glycolytic enzymes to the muscle proteins F-actin and G-actin have been reported (Arnold and Pette, 1968). Binding of the enzymes to the erythrocyte cell membrane was suggested by Gourley (1952) and subsequent work has confirmed these findings. Interactions between enzymes and subcellular structures can have various functional consequences, such as changes in V_{max} and K_m through microenvironmental effects (Masters, 1977), alignment of several enzymes to form a scaffolded enzyme array leading to metabolite channelling, coordinate regulatory effects, catalytic facilitation and proximity effects. If binding forces are weak, small perturbations such as conformational changes induced by ligands can influence the degree of association leading to regulatory potential.

Organization in Other Soluble Enzyme Systems

Organization by clusters of enzymes may simply be a mechanism by which the cell reduces the "clutter" of freely diffusing

intermediate substrates in the cytoplasm. Some of these could interfere with co-existing or competing enzyme systems or tax the cytoplasmic solvent capacity. This would be especially important in pathways that produce common intermediates and use separate isoenzymes, showing the potential for substrate channelling. The reversible aggregation of enzymes could allow the direct transfer of the product of one enzyme to the active site of the next and would prevent its diffusion into the cellular space and transformation by competing reactions. This is distinct from the old view of cell metabolism in which each enzyme moves independently in the cellular milieu and consequently interacts at random with each of their corresponding substrates. The mechanism by which such compartmentation may occur is not known. Theoretically, the intermediate could be transferred by conformational changes in a protein from one site to another. Alternatively it may simply be adsorbed or contained in the microcompartment somewhere within the protein structure of the enzyme cluster.

Organization in the Citric Acid Cycle

It is becoming evident that there may be a form of organization in the enzymes of the citric acid cycle (CAC). This cycle, also known as the Krebs or tricarboxylic acid cycle, has a central role in energy metabolism, particularly in aerobic organisms. The CAC enzymes are located in the mitochondria of eukaryotes and the cytoplasm of prokaryotes.

The CAC has a dual role in the cell. Firstly the cycle is a producer of reducing equivalents for the electron transport chain,

by the degradation of sugars, fatty acids or amino acids. This provides an input of acetyl units, which are completely converted to CO_2 and reducing equivalents. The latter lead to production of ATP, by the phosphorylation of ADP as a result of the flow of electrons through the electron transport chain and vectorial transport of protons through the membrane. The terminal electron acceptor in aerobic organisms is molecular oxygen.

The second, biosynthetic, role of the CAC is provided by several cycle intermediates - oxaloacetate, oxoglutarate and succinyl-CoA. These function as precursors for biosynthetic reactions such as gluconeogenesis, lipogenesis and amino acid biosynthesis.

The CAC catalyses the overall reaction:

$$\text{Acetyl-CoA} + 3\text{NAD(P)}^{+} + \text{FAD}^{2+} + \text{NDP} + \text{Pi} + 2\text{H}_2\text{O} \rightleftharpoons$$

$$2\text{CO}_2 + \text{CoA-SH} + 3\text{NAD(P)H} + 3\text{H}^{+} + \text{FADH}_2 + \text{NTP}$$

$$G^{\ominus} \text{ overall} = -9.8 \text{ Kcal mole}^{-1},$$

where NDP/NTP represent nucleoside diphosphate and triphosphate respectively (both adenine and guanine nucleotides participate).

In aerobic organisms the reduced coenzyme NADH is reoxidized by Complex I (NADH dehydrogenase) in the electron transport chain. In total, 11 molecules of ATP are generated by this process per single cycle turn.

Cycle intermediates are replenished by anaplerotic reactions, including the enzymes pyruvate carboxylase, malic enzyme or the involvement of the glyoxylate cycle in microorganisms and plants.

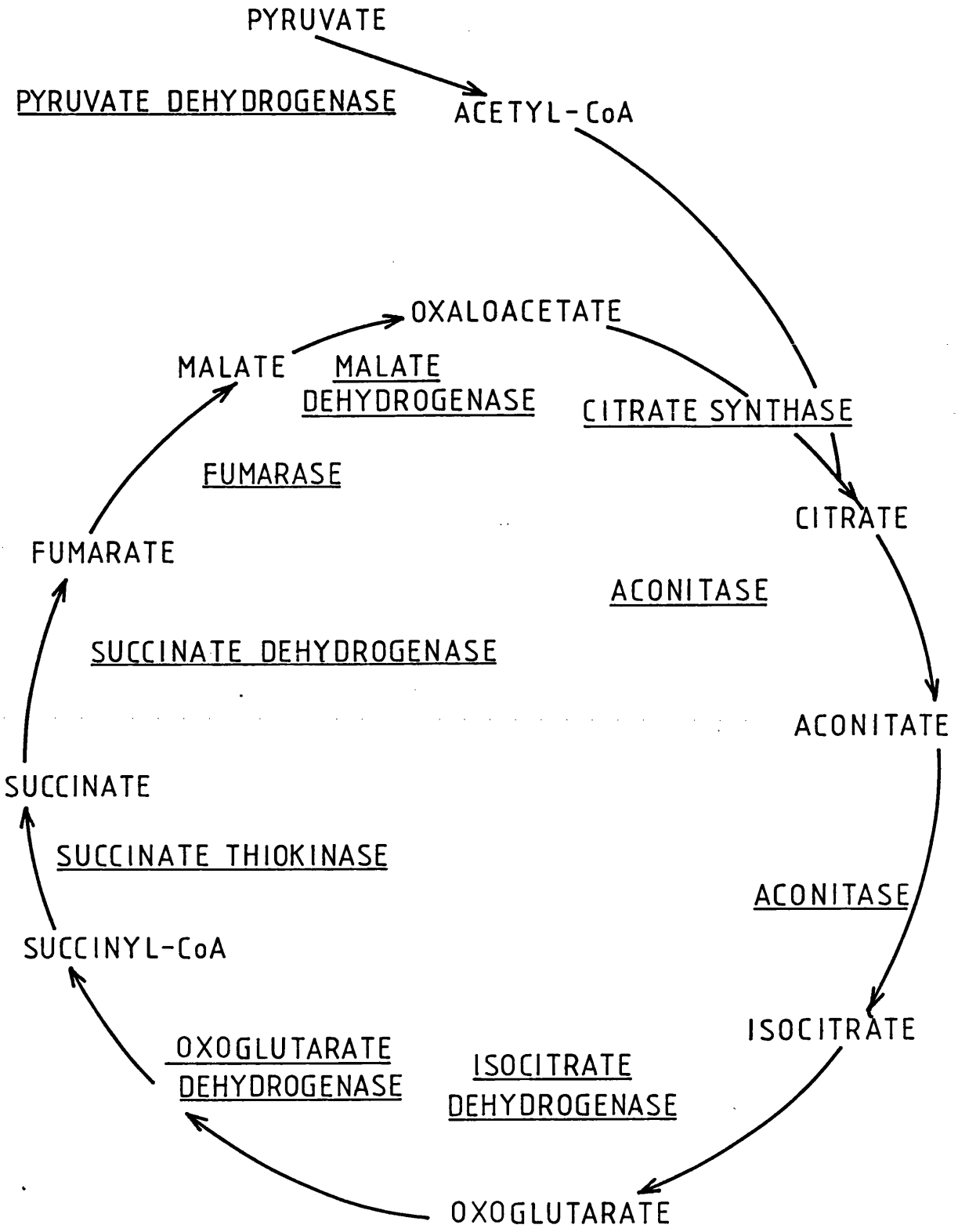


Figure 2.1

THE CITRIC ACID (KREBS) CYCLE

The Enzymes of the Citric Acid Cycle

Figure 1 indicates the enzymes of the citric acid cycle.

Citrate synthase (CS)

CS is often regarded as the "first" enzyme of the cycle, catalysing a condensation reaction between the acyl group of acetyl-CoA and the carbonyl moiety of oxaloacetate. The reaction proceeds far in the direction of citrate formation, as a result of the energy produced by the hydrolysis of the thioester bond of the citroyl-CoA intermediate.

Aconitase (AC)

AC catalyses the reversible interconversion of citrate and isocitrate, the removal of the latter ensuring the reaction proceeds in a forward direction. It is an iron-sulphur protein, the Fe-S cluster possibly having a structural role and all four Fe-S groups being required for catalytic activity.

Isocitrate dehydrogenase (IDH)

IDH catalyses an oxidative decarboxylation and occurs in two forms in mitochondria, an NAD^+ and an $NADP^+$-linked enzyme. There is some dispute as to which occurs in the CAC, the more likely being NAD linked enzyme. The NAD^+-IDH is allosteric and is regarded as one of the rate-controlling enzymes of the cycle. In bacteria, generally only NADP-IDH is found. In *Acinetobacter calcoaceticus* two isoenzymes have been isolated (Self and Weitzman, 1972), one of which is under allosteric control.

Oxoglutarate dehydrogenase (OGDH)

OGDH is a classical multienzyme complex composed of three different enzymes, in varying ratios. The complex performs three successive coordinated reactions: a decarboxylation, resulting in the formation of an aldehyde group, an oxidation, the aldehyde is converted to an acid and the formation of a thioester.

Succinate thiokinase (STK)

The substrate level phosphorylation step of STK involves the hydrolysis of succinyl-CoA, the energy from which is used to phosphorylate a nucleoside diphosphate, in an energy conserving reaction. The phosphate acceptor has recently been found to be either ADP, GDP or IDP (Weitzman *et al.*, 1986; Jenkins, personal communication).

Succinate dehydrogenase (SDH)

Succinate is oxidised to fumarate by SDH, an integral membrane flavoprotein. SDH also functions as Complex II in the electron transport chain.

Fumarase (FM)

Fumarate is hydrated to L-malate by FM in a reversible reaction.

Malate dehydrogenase (MDH)

The reaction catalysed by MDH is highly endergonic but proceeds rapidly due to the efficient removal of the products oxaloacetate and NADH.

Generally, two of the major sources of acetyl-CoA are the pyruvate dehydrogenase complex and β-ketoacyl-CoA thiolase of the fatty acid oxidising system. Additionally, the transaminases generate oxoglutarate, oxaloacetate and pyruvate from amino acid precursors for oxidation in the cycle. Succinyl-CoA can also be generated from methyl malonyl-CoA, formed from propionyl-CoA and CO_2.

In mitochondria, citric acid cycle activity is carried out by inner membrane and matrix components. It is certain that SDH is a protein of the inner membrane. The oxoacid dehydrogenase complexes are attached to the membrane but are more easily removed than is SDH. The other CAC enzymes may or may not be attached to membrane, as discussed later.

Various general observations have been made about the content of the CAC enzymes in mitochondria. There is a direct correlation between the oxidative capacity of the cell and the content of CAC enzymes (Srere, 1969). Moreover, the enzymes of the CAC seem to be a constant proportion group of enzymes, with a consistency of relative activities in a range of cell types (Pette *et al.*, 1962). The induction of one enzyme such as vitamin B_{12} deficiency in rats which leads to an increase in CS levels, causes a subsequent induction in all CAC enzymes (Matlib *et al.*, 1979). A stoichiometric relationship among the CAC has never been directly calculated but Srere (1972), using data from a number of laboratories, compared the molar ratios of active sites of the enzymes and showed that they are within one order of magnitude. These results indicate a close control over the total amount of

each enzyme with no one enzyme being rate-limiting (Srere, 1985). For many years it was assumed that citrate synthase is the rate-limiting step, but evidence provided using the method of Kacser and Burns (1973) distributes control between several positions in the cycle (Ottaway, 1976).

Interactions between Enzymes of the Citric Acid Cycle

A model of the mitochondrial matrix

Srere and coworkers have postulated that the enzymes of the CAC exist within the matrix of the mitochondrion as a multienzyme complex which he has termed a "metabolon" (Srere, 1985). A model was worked out based on the structure of the mitochondrial inner membrane-matrix compartment. Srere has stated that the integration of metabolic function may be due to structural and spatial interactions or that it is diffusion controlled. An intracellular microenvironment, in which locally high concentrations of metabolites could be maintained despite a low cellular metabolite concentration, may be created by arranging enzymes of a single pathway next to each other spatially. This would result in a high rate of enzyme activity in a region of high metabolite concentration with an overall low metabolite concentration.

Srere proposed that the inner membrane matrix compartment may have a structure on which many of the matrix proteins are adjacent or bound to the inner membrane and that this is important in control, either diffusional or structural, of the energy-producing pathway.

Calculations based on the theoretical surface area/volume ratio indicated that every protein molecule in the matrix contacted an inner membrane surface of mitochondria. The cross-section of a hypothetical CAC complex would be 2.3×10^{-4} μm^2. Therefore, if a rat heart mitochondrion has 45 μm^2 of inner membrane surface and 20,000 CAC assemblies, as much as one fifth of its inner surface membrane would be occupied by CAC assemblies (Srere, 1985).

Evidence for close links between aerobic energy production of the inner matrix and its structures include the fact that several CAC enzymes are apparently bound to the inner surface of the matrix and that there is a strong correlation between the amount of these enzymes and the area of the inner membrane surface, whilst kinetic considerations require a microenvironmental structure.

Matrix proteins are very densely packed, which may result in the formation of protein-bound pores limiting the free diffusion of large metabolites including nucleotides. This may also give rise to a possible change in local water pools in the cell, resulting in large changes in local concentrations of metabolites.

Therefore, although high concentrations of CAC enzymes exist in mitochondria (10^{-4} - 10^{-5} M) with free diffusion possible, Srere and Mosbach (1974) postulated that these enzymes exist as a multienzyme system bound to the inside of the inner mitochondrial membrane. The molecular arrangement of the enzymes is random but increasing evidence, based on structural and metabolic considerations, shows specific interactions for the CAC enzymes.

According to Beeckmans and Kanarek (1981), the prediction of the organization of enzymes as a multienzyme complex was based on

the observation that the free oxaloacetate concentration is so low that the calculated rate of CS is slower *in vivo* than *in vitro*, as measured by mitochondrial oxygen consumption. Oxaloacetate is a key metabolite in the CAC, aspartate-malate shuttle, in the regulation of SDH and is a starting metabolite for gluconeogenesis, as a substrate of PEP carboxykinase. The multiple role of this metabolite suggests that some form of channelling must exist and that it is likely that there is a physical association of previously called soluble enzymes in both the cytoplasm and mitochondria. The creation of a microenvironment would give the cell the ability to maintain a high flux of substrates through the CAC with a moderate number of intermediate molecules.

Beeckmans (1981) reported the possibility of a physical interaction between enzymes of the citric acid cycle and the aspartate-malate shuttle and also the compartmentation of different mitochondrial matrix enzymes to form a loose association with the inner membrane. The means by which the cell separates the amount of oxaloacetate as flux, from oxaloacetate in the shuttle, may be regulated by promoting or inhibiting a direct interaction of aspartate aminotransferase with malate dehydrogenase in the cycle, thereby forming a large CAC/shuttle complex.

Other workers have used different approaches to argue the need for enzyme compartmentation. Krebs and Lowenstein (1960) showed vast differences existing between cytosol and mitochondria in eukaryotic cells in the redox potential (NAD^+/NADH), phosphorylation potential (ATP/ADP.Pi) and acylation potential (acyl-CoA/CoA). They concluded that metabolic compartmentation of the CAC

would be necessary in the cell. Pollard (1963) observed that cells with dimensions of less than 1 μm must have a sub-microscopic order to secure the closeness of reacting elements which are in low concentrations, for example oxaloacetate, and also the presence of an activation energy will require that successive reactions be produced in an organized system. He used the argument that 60% of the enzymes of the rat liver cell are bound and therefore, to some extent, organized.

Interactions between Individual Citric Acid Cycle Enzymes

Organization of metabolically related enzymes may lead to a smaller number of intermediate molecules confined to a micro-environment, so giving a higher chemical potential than in a disorganized system. The advantages of such a system have been reviewed (Welch, 1977; Welch and Keleti, 1981).

Early work on the physical interactions between CAC enzymes included the work of Backman and Johansson (1976). They showed that mitochondrial-aspartate aminotransferase (mAAT) and mitochondrial MDH (mMDH) specifically interacted by using a counter-current distribution analysis of the enzymes in a biphasic system. This was demonstrated to be specific to the mitochondrial rather than cytosolic form.

Halper and Srere (1977) showed CS and mMDH co-precipitated in polyethylene glycol (PEG) as measured by an increase in optical density of the solution. Meikka and Ingham (1980) reported that PEG acts by reducing the solubility of heterocomplexes of proteins; it can therefore not induce complex formation.

Beeckmans and Kanarek (1981) have studied and confirmed the interactions between mMDH, CS and fumarase and between these and mAAT using an immobilized-enzyme gel system. They also used an immunoimmobilization system involving antibody to one of the enzymes and were able to calculate a stoichiometry of four molecules of MDH bound to each molecule of fumarase. The MDH-fumarase complex can bind either CS or AAT but not both, and it was proposed that alternate binding by substrates may constitute a switch mechanism between the CAC and the shuttle. The interactions were specific and showed sensitivity to ionic strength.

An alteration in the molecular weight of CS on gel filtration in the presence of mMDH was reported by Dulin and Harrison (1983). The shift between 87 000 and 138 000 was seen to be dependent on ionic strength, enzyme concentration and presence of anions.

Sumegi *et al.* (1980) reported an interaction between CS and PDH, demonstrated by using ultracentrifugation, whereby CS binds to the E_2 (transacetylase) portion of PDH. The co-precipitation of CS and PDH in 3.5% v/v PEG was reported. Along similar lines Porpaczy *et al.* (1983) showed an interaction between OGDH and STK.

Interactions between glutamate dehydrogenase and mMDH have been demonstrated using a variety of techniques including gel filtration, fluorescence, PEG precipitation and kinetics (Fahien *et al.* 1977, 1978, 1979). A complex was also seen between AAT and CS, enhanced by the presence of NADH and malate and palmityl-CoA.

Kinetic advantages of the association of two sequential enzymes are probably due to the fact that diffusion does not occur as in bulk solution and increased substrate concentrations in the

microenvironment of the second enzyme can occur (Koch-Schmidt *et al.*, 1977).

The kinetic advantage of immobilized enzyme over free enzyme was demonstrated with CS and MDH by Srere (1973). The rate of the coupled reaction was greater than that of the enzymes free in solution. The resulting restricted diffusion is probably the best explanation for this since the K_m for OAA did not change, discounting a change in enzyme properties on immobilization.

Koch-Schmidt *et al.*, (1977) reduced the proximity of CS and mMDH from about 2 000 nm (in free solution) to 2 nm (cross-linked) using an immobilization technique. This did not confer any kinetic advantage but did reduce the lag time such that steady state was reached almost immediately. Immobilization therefore creates a favourable condition; diffusional hindrance by the gel phase of the oxaloacetate intermediate results in its higher microenvironmental concentration and the elimination of the lag phase. Kinetic enhancement was observed when the enzymes alcohol dehydrogenase and lactate dehydrogenase were immobilized on the same bead such that their active sites were facing each other (Mansson *et al.*, 1983).

Kinetic analysis by Sumegi *et al.* (1980) demonstrated a decrease in K_m for CoA for the PDH complex from 10 µM to 1.5 µM for the PDH-CS interaction; the K_m for acetyl-CoA of CS also decreased from 12 µM to 3.1 µM. The interpretation of this observation was that not only is there a proximity effect, but some alteration in the active sites of the two enzymes occurs when the complex forms.

With the interaction between OGDH and STK (Porpaczy *et al.*, 1983) a decrease in K_m for CoA of OGDH was observed from 5.5 µM to

3.5 μM in the complex. The K_m for succinyl-CoA of STK shifted from 65 μM in the free enzyme to 1.5 μM in the OGDH-STK complex. No other kinetic constants altered.

Aims of this Study

It was proposed, in the light of previous work described above, to explore the possibility that the enzymes of the CAC are in some way organized. The description of these enzymes as 'soluble' in the cytoplasm may reflect the extraction procedure employed and does not necessarily reflect the *in vivo* state of the enzymes.

It was decided to focus on bacterial cells as a model system in which to study the associations between the enzymes. The use of bacteria offers several advantageous features, i.e. permits a homogeneous preparation of cells, manipulation of culture conditions, such as the effects of different growth media and cell age, and the exploitation of mutant strains deficient in a specific enzyme. In order to minimize damage to fine intracellular structures and loose enzyme associations, it was planned to prepare bacterial spheroplasts and lyse them in a medium of high viscosity, with minimum dilution. This would allow enzyme interactions to be studied using conventional biochemical techniques of chromatography, electrophoresis, ultracentrifugation and enzyme kinetic and inhibition studies.

The aim of the project was to study the enzymes under conditions thought to be closer to those *in vivo* than are presented by conventional sonic extracts. This was to be achieved by creating environments of high viscosity and high protein.

It was hoped to be able to extrapolate any findings from studies on bacteria by examining mitochondria from eukaryotic cells for the display of any similar associations.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1 Organisms

The following bacterial strains were used in this work: *Acinetobacter calcoaceticus* 4B and 4B5, *Pseudomonas aeruginosa* 1978, *Escherichia coli* K12 D500, K1.1 r3 and W620, *Bacillus subtilis* 168 and CU1323, from the culture collection of Prof. P.D.J. Weitzman, University of Bath.

2.2 Chemicals

Nutrient broth, Bacto yeast extract, Bacto yeast peptone and nutrient agar were from Oxoid Ltd., London, U.K.; CoA, OAA, NADPH and $NADP^+$ were from Boehringer, Mannheim, F.R.G.; acrylamide, ammonium persulphate, cysteine hydrochloride, 2-mercaptoethanol, magnesium chloride, sodium dodecyl sulphate, sodium succinate, silica gel H, N,N,N',N'-tetramethylethylene diamine and triethanolamine were from BDH Chemicals Ltd., Poole, U.K.; bromophenol blue and PEG6000 were from Fisons, Loughborough, U.K.; blue dextran, Sephadex G-200 and Sepharose 4B were from Pharmacia, Sweden; bovine serum albumin (pentax fraction V) was from Miles Scientific, Slough, U.K.; DMDBP was from Pierce Chemicals Co., Rockford, U.S.A; Penicillin G was from Glaxo U.K. Ltd., Greenford, Middlesex, U.K.; all other fine chemicals were from Sigma, Poole, U.K.

2.3 Enzymes

Pig heart citrate synthase, lactate dehydrogenase, glucose 6-phosphate dehydrogenase, egg white lysozyme, porcine NADP-isocitrate dehydrogenase and malate dehydrogenase were from Boehringer, Mannheim, F.R.G.

METHODS

2.4 Enzyme Assays

All assays were carried out at 25°C.

A unit of enzyme activity is defined as that amount that catalyzes the conversion of 1 μmole of substrate per min.

2.4.1 Aconitase (EC 4.2.1.3)

Aconitase (AC) was assayed by following the increase in absorbance at 340 nm due to the reduction of $NADP^+$ (ϵ_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 0.5 mM $NADP^+$, 5 mM trisodium citrate and 0.02 units of porcine NADP-isocitrate dehydrogenase in MET8 buffer.

2.4.2 Aspartate aminotransferase (EC 2.6.1.1)

Aspartate aminotransferase (AAT) was assayed by following the oxidation of NADH at 340 nm (ϵ_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2 mM oxoglutarate, 0.2 mM aspartate 2.0 mM NADH and excess malate dehydrogenase in 0.1 M Tris-HCl, pH 7.5.

2.4.3 Citrate synthase (EC 4.1.3.7)

Citrate synthase (CS) was assayed by the method of Srere *et al.* (1963) using Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The increase in absorbance at 412 nm due to the production of the yellow-coloured thio-nitrobenzoate anion was followed. The rate of DTNB cleavage in the absence and

presence of OAA was determined, to account for any deacetylation of acetyl-CoA not caused by CS (ϵ_{412} 13,600 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2 mM AcCoA, 0.2 mM OAA and 0.1 mM DTNB, in 20 mM Tris-HCl, pH 8.0, + 1 mM EDTA. For *E. coli* CS, 0.1 M KCl was included in the assay buffer.

2.4.4 Fumarase (EC 4.2.1.2)

Fumarase (FM) was assayed by monitoring the increase in absorbance at 250 nm on production of fumarate (ϵ_{250} 2,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 5 mM sodium malate in MET8 buffer.

2.4.5 Hexokinase (EC 2.7.1.1)

Hexokinase was assayed by following the increase in absorbance at 340 nm due to the reduction of $NADP^+$ (ϵ_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 0.5 mM $MgCl_2$, 4 mM $NADP^+$, 0.02 unit ml^{-1} glucose 6-phosphate dehydrogenase and 10 $mg.ml^{-1}$ glucose in 50 mM Tris-HCl-EDTA buffer, pH 7.6, followed by 0.2 mM ATP.

2.4.6 Isocitrate dehydrogenase (EC_{NADP} 1.1.1.42) (EC_{NAD} 1.1.1.41)

Isocitrate dehydrogenase (IDH) was assayed by following the increase in absorbance at 340 nm as a result of the reduction of either $NADP^+$ or NAD^+ (ϵ_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 4 mM isocitrate, 0.2 mM $NADP^+$ or NAD^+ in MET8 buffer. 1 mM AMP was included in the assay for the NAD-linked enzyme.

2.4.7 Isocitrate lyase (EC 4.1.3.1)

Isocitrate lyase was assayed by following the formation of phenylhydrazone on reaction with the carbonyl group of isocitrate, absorbing at 324 nm (ε_{324} 17,000 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 2 mM $MgCl_2$, 2 mM isocitrate, 5 mM phenylhydrazine in 100 mM phosphate buffer, pH 6.8.

2.4.8 Lactate dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase (LDH) was assayed by following the oxidation of NADH, as a decrease in absorbance at 340 nm (ε_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2 mM pyruvate and 0.2 mM NADH in 20 mM Tris-HCl, pH 8.0. The oxidation of NADH by non-specific NADH dehydrogenase activity was taken into account.

2.4.9 Malate dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase (MDH) was assayed by following the decrease in absorbance at 340 nm due to the oxidation of NADH (ε_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2 mM OAA and 0.2 mM NADH in 20 mM Tris-HCl, pH 8.0 NADH dehydrogenase activity was accounted for, as in 2.4.8.

2.4.10 Oxoglutarate dehydrogenase (EC 1.2.4.2);
Pyruvate dehydrogenase (EC 1.2.4.1)

Oxoglutarate dehydrogenase (OGDH) was assayed by following the increase in absorbance at 340 nm due to the reduction of NAD^+ (ε_{340} 6,220 $1.mol^{-1}.cm^{-1}$). The assay mixture contained 2 mM 2-oxoglutarate, 2.5 mM NAD^+, 0.2 mM thiamine pyrophosphate, 0.13 mM

CoA, 2.6 mM cysteine-HCl and 1 mM $MgCl_2$ in 50 mM KPO_4 buffer, pH 8.0.

Pyruvate dehydrogenase (PDH) was assayed as for OGDH, but substituting 2 mM sodium pyruvate for 2-oxoglutarate.

2.4.11 Phosphoglucose isomerase (EC 5.3.1.9)

Phosphoglucose isomerase (PGI) was assayed by monitoring the reduction of $NADP^+$ at 340 nm (ε_{340} 6,220 $l.mol^{-1}.cm^{-1}$). The assay contained 0.2 mM $NADP^+$, 0.2 mM fructose 6-phosphate and 0.2 units glucose 6-phosphate dehydrogenase in 0.1 M triethanolamine, pH 7.6, + 4.0 mM $MgCl_2$.

2.4.12 Succinate dehydrogenase (EC 1.3.99.1)

Succinate dehydrogenase (SDH) was assayed by following the increase in absorbance at 600 nm due to the reduction of the methosulphate by indophenol reagent (ε_{600} 16,100 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 0.2 mM 2,6,dichlorophenolindophenol, 1.5 mM N-methylphenazinium methosulphate, 3 mM sodium succinate and 0.75 mM sodium cyanide in 0.1 M phosphate buffer, pH 7.5.

2.4.13 Succinate thiokinase (GDP; EC 6.2.1.4, ADP; EC 6.2.1.5)

Succinate thiokinase (STK) (succinyl-CoA synthetase) was assayed spectrophotometrically, using a continuous method, employing DTNB, following the appearance of yellow-colour due to the production of the thio-nitrobenzoate anion. The assay mixture contained 0.5 mM ADP or GDP, 0.2 mM DTNB, 0.2 mM succinyl-CoA in 20 mM phosphate buffer, pH 7.5, + 1 mM $MgCl_2$.

STK was also assayed polarographically using 0.5 mM ADP or GDP, 0.2 mM succinyl-CoA in 100 mM phosphate buffer, pH 8 + 1 M $MgCl_2$. The rate of formation of CoA-SH was monitored by the dropping mercury electrode (Weitzman and Kinghorn, 1978). "Deacylase" rates were measured by omitting ADP or GDP and were subtracted from the rates measured with the complete system to give a rate for STK activity.

2.5 Estimation of Proteins

The micro-protein assay of Bio-rad (Bio-rad Laboratories Ltd., Watford, U.K.) (Bradford, 1976) was used for protein estimation of concentrations anticipated to be less than 0.1 - 0.2 $mg.ml^{-1}$. Protein concentration greater than 0.1 - 0.2 $mg.ml^{-1}$ was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

2.6 Preparation of other reagents

2.6.1 Preparation of Acetyl-coenzyme A

Acetyl-CoA was prepared by the method of Stadtman (1957). 10 mg of CoA were dissolved in 1 ml of double-distilled water, on ice. 0.2 ml of 1 M $KHCO_3$ was added to give a solution of pH 7.5. 0.2 ml of freshly diluted 0.1 M acetic anhydride was added and incubated on ice for 10 min. Complete acetylation of CoA was tested with DTNB.

2.6.2 Estimation of Acetyl-coenzyme A

The concentration of acetyl-CoA was determined using the

citrate synthase assay (2.5.3) with CS and OAA in excess of acetyl-CoA. The reaction was allowed to go to completion and the acetyl-CoA concentration calculated from the absorbance increase at 412 nm.

2.6.3 Preparation of succinyl-coenzyme A

Succinyl-CoA was prepared by dissolving 5 mg of CoA in 0.5 ml double distilled water on ice. A pH of 7.5 was achieved by the addition of 0.1 ml of 1 M $KHCO_3$. A few granules of succinic anhydride were added and the solution mixed thoroughly. Fresh succinyl-CoA was used at all times.

2.7 Bacterial Cultures

2.7.1 Maintenance and growth of organisms

Bacterial strains were maintained on nutrient agar plates at 4°C and sub-cultured every two weeks. Culture of organisms took place in liquid media at 30°C with shaking.

2.7.2 Culture media

Nutrient broth was used for the routine growth of organisms. When a specific carbon or energy source was required, growth was carried out in a medium of basal salts: 50 mM Na/K PO_4 buffer, pH 7.2 with 50 mM NH_4Cl, 0.18 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.018 mM $MnSO_4$ and 0.014 mM $FeSO_4$, with the addition of the energy/carbon source.

2.8 Preparation of Spheroplasts

2.8.1 Preparation of spheroplasts using lysozyme-EDTA

Spheroplasts were prepared according to the protocol of Gorringe and Moses (1980). A culture of *E. coli* K12 D500 in late exponential phase was harvested at 4°C by centrifugation for 15 mins at 13 000 g in a Sorval centrifuge. The pellet was washed twice in ice-cold 10 mM Tris-HCl buffer, pH 7.2, + 30 mM NaCl. The cells were resuspended at room temperature, to give 1.0 g wet weight/80 ml 0.033 M Tris-HCl, pH 7.2 buffer, containing 20% w/v sucrose. Disodium EDTA (5 ml), pH 7.0 was added, to give a final concentration of 10 mM. This was followed 15 sec later by lysozyme solution (40 mg in 5 ml 0.033 M Tris-HCl, pH 7.2, to give a final concentration of 0.5 $mg.ml^{-1}$).

The cells were incubated at room temperature with gentle stirring for 30 min or until spheroplast formation was complete. This formation was gauged either by examination under a microscope or by lysis. The spheroplasts were harvested at 9 000 g for 15 min and washed once in 20% w/v sucrose solution and re-sedimented.

2.8.2 Preparation of spheroplasts using penicillin

Penicillin was used in the preparation of spheroplasts according to the method of Mowbray and Moses (1976). Cells of *E. coli* K12 D500 were grown overnight at 37°C in basal medium supplemented with 0.9% w/v glucose. 1 ml of the overnight culture was transferred to 100 ml of basal medium for 14 hours at 37°C, with shaking. The 100 ml was transferred to 900 ml of nutrient medium containing; 0.9 $g.l^{-1}$ glucose, 3.15 $g.l^{-1}$ NaCl, 3.31 $g.l^{-1}$

K_2HPO_4, 1.19 $g.l^{-1}$ KH_2PO_4, 1.35 $g.l^{-1}$ Bacto yeast extract, 2.25 $g.l^{-1}$ Bacto peptone, 3.6 $g.l^{-1}$ Bactone nutrient broth.

When $E_{650} \simeq 1$, 500 ml of pre-warmed sucrose nutrient broth (i.e. nutrient broth + 68.5 g/100 ml sucrose) was added + 2.09 $g.l^{-1}$ $MgCl_2$ + 1.8667 units ml^{-1} penicillin. The E_{650} dropped to 0.2 - 0.3 and then increased over the next 2 - 3 hours. Samples were taken to assess completion of spheroplast formation. The spheroplasts were harvested at 4°C, 9 000 g for 15 min.

2.9 Ultrafiltration

Small sample volumes (~ 5 ml) were concentrated using a "Minicon" concentrator B15 (Amicon Corporation, U.S.A.).

Other samples were concentrated using an Amicon Ultrafiltration Cell (Amicon Corporation, U.S.A) at 60 psi with a P_{10} membrane.

2.10 Preparation of Mitochondria

2.10.1 Preparation of mitochondria from rat liver

The freshly dissected liver was washed in ice-cold extraction buffer containing 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1% w/v BSA and 2.0 mM EGTA. The liver was dried, weighed and cut into 0.5 cm^3 cubes into the extraction media at 1 g tissue/2 ml media and homogenized for three seconds on ice. The supernatant was spun at 10 000 g for 10 min at 4°C, the supernatant discarded and the mitochondrial pellet gently washed twice in medium. The pellet was kept on ice until use.

2.10.2 Preparation of mitochondria from locust flight muscle

Locusta schistocerca gregaria were used for mitochondrial isolation. 150 adult locusts were dissected and the flight muscle removed into 20 ml ice-cold media containing 50 mM Tris, pH 7.5, 0.25 M sucrose + 1 mM EDTA. This was hand-homogenized gently using a Potter/Dounce homogenizer with a teflon/glass plunger for 10 strokes. The homogenate was spun for 10 min at 1 000 g, the supernatant filtered through a nylon filter to remove fat globules and spun at 3 000 g, 30 min. The small 'halo' of membrane material was gently removed from the mitochondrial pellet and the pellet stored on ice until use.

2.10.3 Preparation of mitochondria from etiolated barley

Plant mitochondria were prepared by the method of Bonner (1967). The chilled hypocotyls from barley seedlings were cut into sections 2 - 3 cm long, suspended in grinding medium containing 0.3 M mannitol, 1.0 mM EDTA, 0.1% w/v BSA, 0.05% w/v cysteine at pH 7.2 and hand ground for 30 seconds. The pH was adjusted to 7.2 and the ground material squeezed through muslin, spun at 1 000 g for 15 min, the resulting supernatant being spun at 10 000 g for 15 min. The pellet was suspended in wash medium containing 0.3 M mannitol, 1.0 mM EDTA, 0.1% w/v BSA at pH 7.2 and spun at 250 g for 10 min. The supernatant from this was centrifuged at 6 000 g for 15 min and the resulting pellet of mitochondria suspended in an equal volume of wash medium. All procedures were carried out at 4°C.

2.10.4 Preparation of mitochondria from bovine heart

In mitochondrial preparations from fresh bovine heart, muscle tissue was cut into cubes and homogenized in a Waring blender, 10 sec low power, with 0.25 M sucrose + 50 mM Tris-HCl + 1 mM EDTA + 0.1% w/v BSA at pH 7.5. Second homogenization was for 15 sec, medium power in a Waring blender, pH adjusted to 7.5. Homogenate was spun at 1 600 g, 15 min. The supernatant was filtered through double cheesecloth and centrifuged at 14 500 g, for 30 min. The mitochondrial pellet was resuspended in sucrose-Tris buffer, as above but with no BSA present. Final centrifugation was at 30 000 g, 30 min. The pellet was retained on ice.

2.11 Methods of Cell Disruption

2.11.1 Osmotic-Lysis

The pelleted spheroplasts or mitochondria were gently resuspended in cold 10 mM Tris-acetate, pH 7.5, with 20% w/v glycerol and incubated for 4 - 12 hours at 4°C.

2.11.2 Freeze-Thaw

A pellet of spheroplasts was sealed in a microfuge tube and subjected to successive -198°C and 37°C treatments for 30 min periods, repeated three times.

2.11.3 Mechanical

Packed spheroplasts were resuspended in a small volume of 0.9% w/v NaCl and disruption achieved by passing the suspension

twice through a pre-cooled French Pressure cell at a pressure of 62 MPa (9 000 lb f/in^2).

2.11.4 Sonication

An Ultrasonics 180 Watt sonicator was used for all cell disruption with a 5 mm probe at a power of 50W for 1 min, in 15 sec bursts, with cooling.

In all cases the cell debris was removed by centrifugation. Viscosity of the lysate from the spheroplasts, due to the presence of DNA, was reduced by the addition of a crystal of DNAse and incubation at 37°C for 20 min.

2.12 Gel Filtration Chromatography

2.12.1 Preparation of gel matrices

Sephadex G-200 (particle size 40 - 120 µm, bed volume per gram dry gel: 30 - 40 ml) was suspended in distilled water (5 g.l^{-1}) and swollen overnight. The slurry was heated to 90°C for 5 hours, cooled and fixed.

Sepharose 4B (wet bead diameter 60 - 140 µm, protein fraction range = 6 x 10^4 - 20 x 10^6) is manufactured in a pre-swollen form.

For the preparation of filtration columns, both gel types were suspended in twice its volume of buffer, loaded and equilibrated.

2.12.2 Calculation of void volume

The void volume of a column was determined using 0.2% w/v blue dextran 2000 (Molecular weight ~ 2 x 10^6).

2.12.3 Calibration of column

Calibration curves for the gel-filtration columns were constructed using globular proteins of known molecular weights, see Table 2.12. The marker proteins were eluted from the columns under standardized conditions, at 4°C with TAG buffer and the protein elution profile followed at 280 nm.

A standard calibration curve was constructed by relating the peak elution volume to the logarithm of the molecular weight, enabling unknown molecular weights to be determined.

Table 2.12. Calibration of gel-filtration separation columns; molecular weight markers.

Protein	Source	Molecular weight $x10^3$	Amount loaded on gel-filtration (mg)
Oxoglutarate dehydrogenase	*E. coli*	2 000	5
Glutamate dehydrogenase	Bovine liver	1 015	5
ß-galactosidase	*E. coli*	520	5
Catalase	Bovine liver	247	5
Aldolase	Rabbit muscle	145	2
Serum albumin	Bovine	67	5
Cytochrome C	Horse heart	12.3	5

A mixture of the marker proteins was loaded onto Sepharose 4B gel filtration matrix. 1-ml fractions were collected. The column was eluted with TAG buffer.

2.13 Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation analyses were performed in a Beckman L5-50B ultracentrifuge.

Discontinous gradients of 10 - 50% w/v sucrose in 10 mM TAG, pH 7.5, were prepared by underlayering each gradient. A sample (200 µl) was overlayed onto the gradient and spun at 120 000 g (35 000 rpm) for 14 hours at 4°C.

Fractionation of the gradient was achieved using a peristaltic pump giving 400 µl samples.

2.14 Differential Centrifugation

Differential centrifugation analyses were performed in a Sorvall RC 5B superspeed centrifuge (Du Pont Instruments) and a Beckman L5-50B ultracentrifuge.

The lysate from spheroplasts of *E.coli* was centrifuged at 1 000 g (av), 15 min giving a pellet (P_1) and supernatant (S_1). S_1 was centrifuged at 9 000 g (av), 15 min giving P_9 and S_9. This supernatant after centrifugation at 40 000 g (av), 30 min yielded P_{40} and S_{40}. S_{40} was ultracentrifuged for 1 hour at 150 000 g (av) giving P_{150} and S_{150}. The samples were analysed for activities of enzymes of the citric acid cycle.

2.15 Polyacrylamide Gel Electrophoresis

2.15.1 Non-denaturing polyacrylamide gel electrophoresis

The method of Davis (1964) was used to prepare disc gels of 7.5% w/v polyacrylamide at pH 8.9 in 9 cm x 0.5 cm glass tubes. The resolving gel buffer was 0.375 M Tris-HCl, pH 8.9 and a reservoir

buffer of 5 mM Tris + 39 mM glycine, pH 8.3. A mixture of acrylamide-bisacrylamide (30% - 0.8%, w/v) was used with ammonium persulphate as initiator and N,N,N',N'-tetramethylethylenediamine (TEMED) as catalyst. Dried samples of approximately 10 µg protein per gel were suspended in 50 µl of 20 mM Tris-HCl buffer, pH 8.0 containing 1 mM EDTA, 10% w/v sucrose and 0.001% w/v bromophenol blue and applied to the gels.The sample was allowed to enter the gel at 0.5 mA per gel and then run at 2 mA per gel until the marker dye had travelled approximately 8 cm. Visualization of protein was achieved by staining according to one of the procedures stated below.

2.15.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The procedure of Weber and Osborn (1969) was used for electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). Disc gel electrophoresis at pH 6.7 with 7.5% acrylamide was carried out in 9 cm x 0.5 cm glass tubes which had been pre-soaked in SDS (1%). The gel and reservoir buffer was 0.1 M $NaPO_4$, pH 6.9 with 0.1% w/v SDS. Polymerization of the acrylamide-bisacrylamide solution was achieved using ammonium persulphate and TEMED. Samples were resuspended in 50 µl of 0.1 M $NaPO_4$, pH 6.7, 1% w/v SDS, 10% v/v glycerol, 1% v/v 2-mercapto-ethanol and 0.001% w/v bromophenol blue. The suspensions were heated at 100°C for 2 min, cooled and loaded onto the gel surface. Samples were allowed to enter the gel at 2 mA per gel and then run at 6 mA per gel until the marker dye had run approximately 8 cm. The gels were stained according to one of the procedures given below.

2.15.3 Staining of polyacrylamide gels

Coomassie Blue staining

Protein was visualized firstly by fixing for 1 hour in a mixture of methanol : acetic acid : water (5 : 1 : 5) and then by staining for 30 - 45 min with Coomassie brilliant blue dissolved in the above mixture, at 37°C. The gels were then destained in a methanol : acetic acid : water mixture (2 : 3 : 35).

Silver staining

A more sensitive stain for gels from the procedure of Morrisay (1981), capable of visualizing 1 - 5 µg protein, was used. The gels were pre-fixed in methanol : acetic acid : water (5:1:4), 30 min, 37°C, followed by a second pre-fix in methanol : acetic acid : water (5 : 7 : 88), 30 min, 37°C. The gels were incubated in 10% v/v glutaraldehyde, 30 min and washed in a large volume of distilled water. A freshly made solution of 5 $mg.l^{-1}$ dithiothreitol was then added to the gels for a 30 min incubation, followed by 0.1% w/v silver nitrate for 30 min. The gels were rinsed in double-distilled water. Development of the stain was achieved using 3% w/v $NaCO_3$ + 50 µl 37% v/v formaldehyde in 100 ml double-distilled water. The reaction was stopped by adding 0.75 ml citric acid and the gels washed before adding 0.3% $NaCO_3$ for 10 min. The gels were stored in double-distilled water.

2.16 Thin-Layer Chromatography

A modified Folch extraction method was used for the extraction of lipid from samples, from Folch *et al.* (1957). The preparation

was dialyzed to remove salts and glycerol and dried under a vacuum pump. This was resuspended and homogenized with a glass-glass homogenizer in 1 : 1 methanol : chloroform on ice. One volume chloroform was added to give a 2 : 1 v/v mixture and homogenized. The protein was spun down in a glass conical centrifuge tube 2 500 g, 10 min. The solvent was removed and the pellet washed with the 2 : 1 v/v chloroform : methanol mixture and centrifuged as before. The two supernatants were pooled into a glass stoppered tube and 0.2 volume 0.1 M KCl in water was added. The solutions were mixed carefully and stood until two layers formed. The top layer was drawn off and the interface washed with 'Folch Upper Phase', containing chloroform : methanol : water (3 : 48 : 47). The lower phase was concentrated nearly to dryness with a rotary evaporator. For storage, 1 ml benzene : methanol (4 : 1) was added, the tubes sealed and frozen at -80°C.

For analysis of the extracted lipid, the benzene : methanol was evaporated and the resulting sample was resuspended in chloroform : methanol (2 : 1).

Chromatography was carried out according to the method of Skipski *et al.* (1964), on 20 x 20 cm glass plates coated with 0.5 mm silica gel H (50 g in 115 ml 0.1% w/v $NaCO_3$), pre-run in chloroform : methanol (2 : 1). Samples were spotted onto the TLC plates, the solvent system used was chloroform : methanol : acetic acid : water (25 : 15 : 4 : 2). Plates were run until the solvent front just reached the top: they were allowed to dry in air in a fume-cupboard. Development of the spots was achieved by placing the TLC plate in an iodine tank and the brown colour denoted the separated phospholipids.

2.17 Electron Microscopy

Examination of samples by electron microscopy was carried out using negative contrast techniques.

Samples were fixed for 20 min in 0.5% v/v glutaraldehyde. 5 µl of the fixed sample was dried down onto a copper grid and stained with uranyl acetate (1% w/v) for 30 sec and blotted dry.

Grids were examined in a Jeol 100 CX Electron Microscope at an accelerating voltage of 100 KV and a range of magnifications.

2.18 Immunochemical Techniques

2.18.1 Single Radial Immunodiffusion

Single radial immunodiffusion was performed using pre-cut wells in agarose (1.5% w/v) on microscope slides.

10 µl antigen was loaded into the central well and dilutions of antibody loaded into the outer wells. Diffusion took place at 37°C overnight and the plate washed three times with PBS followed by water. The agarose on the slide was dried down, stained with Coomassie blue, 15 min and destained.

2.18.2 Preparation of immunoglobulin IgG from whole serum

A partially pure preparation of IgG was prepared by carrying out a 50% w/v ammonium sulphate precipitation cut on the whole serum obtained from rabbit. This suspension was centrifuged at 26 700 g (17 000 rpm) and the pellet resuspended and dialyzed against PBS, pH 7.2. As a further purification step, the dialyzed ammonium sulphate cut from the serum was run on a DEAE 52 chromatography column and eluted with PBS, pH 7.2. The partially pure IgG

preparation (~ 80% IgG, of which 10% will be specific) was stored in 50% v/v glycerol.

2.19 Cross-Linking

Before conducting cross-linking experiments, due to the nature of the reaction between the cross-linking reagent and amino groups, it was necessary to remove all traces of Tris buffer. Thus the samples were dialyzed extensively against 20 mM triethanolamine acid buffer, pH 8.0.

Dimethyl 3,3'-dithiobis-propionimidate-2HCl (DMDBP) was made to a concentration of 142 mM by dissolving 40 mg ml^{-1} in 20 mM triethanolamine pH 8.0 and diluted to give a range of concentrations 0 - 40 mg ml^{-1}. DMDBP was added to the dialyzed enzyme fraction in a 1 : 1 ratio and incubated for 30 min at room temperature. The reaction was stopped by the addition of an equal volume of 100 mM Tris-EDTA, pH 8.0 and incubation on ice for 30 min.

CHAPTER 3

RESULTS

Preliminary Studies on the Association between Enzymes of the Citric Acid Cycle

There is increasing evidence in the literature that there may be a specific compartmentation of enzymes of the citric acid cycle within the mitochondria of eukaryotes or within the cytomatrix of prokaryotes. Much of this research has been carried out using purified preparations of the enzymes from eukaryotic cells, investigating the various ways and permutations that individual enzymes of the cycle may appear to cluster.

In order to approach the problem of whether specific associations between these enzymes could be demonstrated in prokaryotic cells, the technique of gel filtration, i.e. the separation of molecules on the basis of their molecular size and shape, was applied. Crude extracts of bacterial cultures were prepared by sonication of an overnight growth in nutrient broth. After centrifugation to remove cell debris, a volume (1 ml) of supernatant was loaded onto a Sephadex G-200 gel filtration column and the resulting enzyme elution profiles examined. Sephadex G-200 has a fractionation range of 5 000 to 600 000.

In addition to evidence in the literature (Beeckmans and Kanarek, 1981) it has been observed in this laboratory that in a purification preparation of citrate synthase (CS) there was

frequently a high level of contamination with malate dehydrogenase (MDH). These two enzymes have quite distinct molecular weights and so on gel filtration would not be expected to demonstrate co-elution. The molecular weight of CS in Gram-negative bacteria is around 250 000 and in Gram-positive bacteria and eukaryotes is in the region of 100 000 (Weitzman, 1981). In MDH, the molecular weight in eukaryotes and a range of bacteria is 60 000 although in *Bacillus* species and some other bacteria a molecular weight of 120 000 has been recorded (Murphey *et al.*, 1967). The elution profiles of CS and MDH were studied under the above conditions.

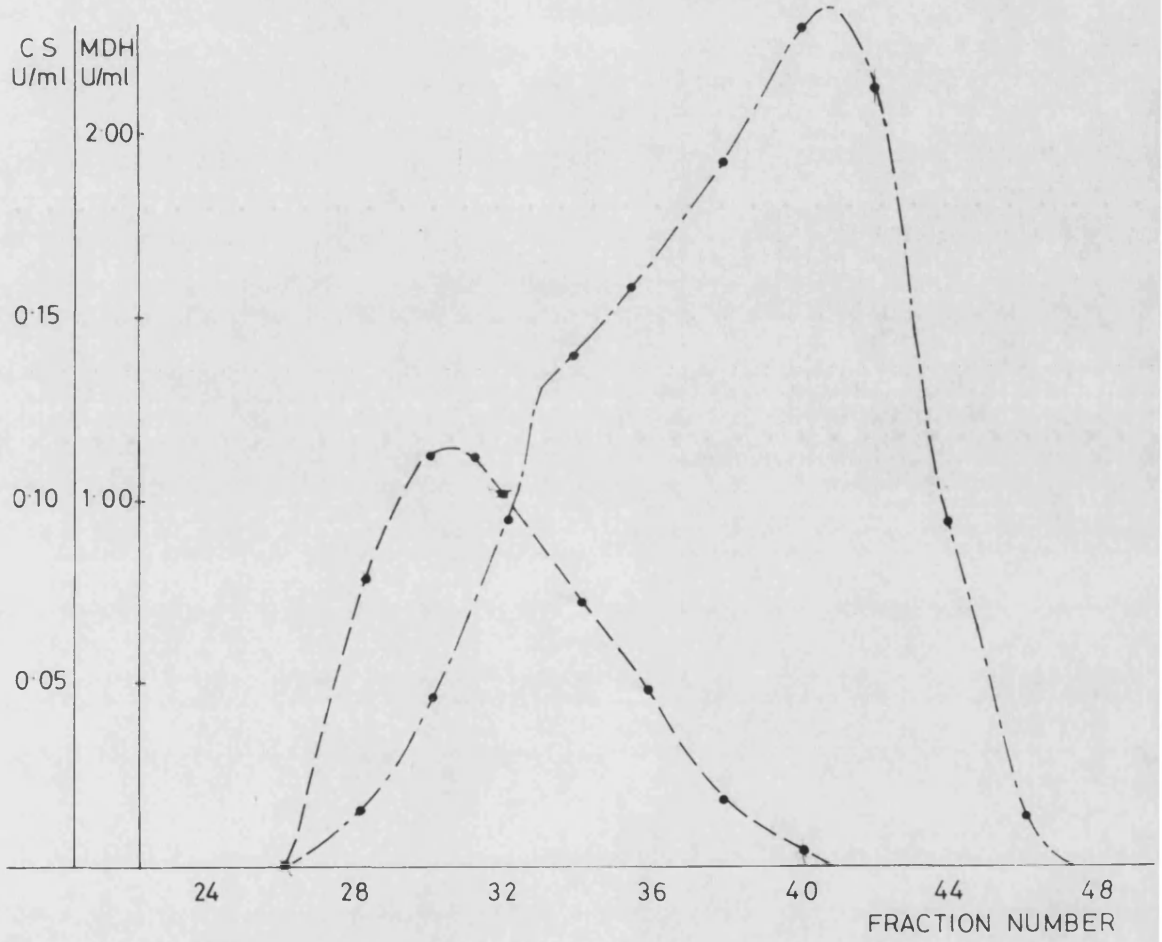
Gel filtration of the supernatant of a crude extract of *E. coli* K12 D500 showed the elution profile in Figure 3.1. All gel filtration experiments were carried out using 10 mM Tris-acetate, pH 7.5, at 4°C. A definite 'shoulder' was seen on the leading edge of MDH with considerable overlap between MDH and CS peaks, despite clearly defined peaks for marker proteins, blue dextran and lactate dehydrogenase. This observation was also noted in a preparation from *Acinetobacter calcoaceticus*.

The Effect of Increasing Salt Concentration on Gel Filtration of CS and MDH

A range of KCl concentrations (0.002 - 0.8 M) was present in the extraction, column and elution buffers to investigate whether high salt content would disturb ionic bonding between CS and MDH. Experimental conditions were as before. As the concentration of KCl increased so did the separation between the two enzymes, with the simultaneous disappearance of the shoulder in MDH. In 0.8 M KCl,

Figure 3.1.

CS and MDH activity on gel filtration of *E. coli* K12 D500 extract.



Gel filtration on Sephadex G-200, 1 ml extract eluted with 10 mM TAG, pH 7.5, at 4°C. Enzyme activity CS (— — — —) and MDH (—— - —).

the overlap of peaks was the equivalent of two fractions (2 ml) compared to 15 fractions (15 ml) when no salt was present (Figure 3.2). Therefore ionic bonding between CS and MDH may have been disturbed by the presence of KCl.

The specific effect of salt was ensured by the dialysis of a bacterial preparation which had been extracted in the presence of 0.8 M KCl. This was loaded onto Sephadex G-200 and the resulting enzyme pattern showed the appearance of 'shoulders' to the MDH and CS peaks and an overlap of mixed enzyme of twelve fractions. The interaction between the enzymes could be restored by the removal of salt.

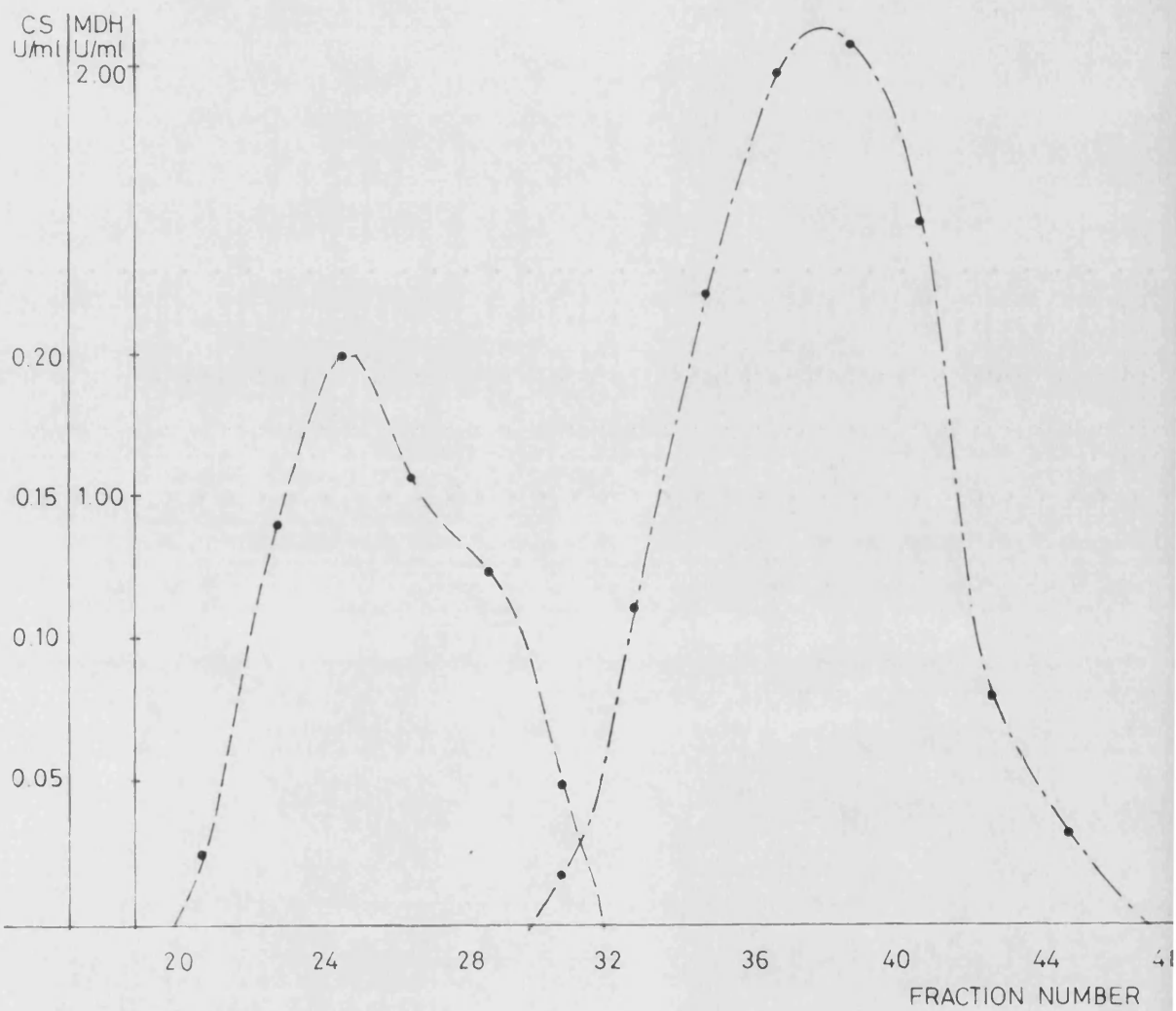
The Use of Polyethylene Glycol in Interaction Studies

Non-ionic polymer media for proteins have been used in an attempt to obtain an environment predicted to be similar to intracellular conditions; such media may be more favourable than water for protein complex formation. One such medium is polyethylene glycol 6 000 (PEG). PEG (14%, w/v) was included in the extraction and elution buffers (10 mM Tris-acetate, pH 7.5) on a bacterial extract of *E coli* cells. Figure 3.3 shows the gel filtration of the lysate in the presence of PEG, demonstrating an enhanced overlap between the two enzymes and elution at slightly higher molecular weights than would be predicted. Both these features may be indicative of increased complex formation between CS and MDH in the environment created by the non-ionic polymer media.

The specific effect of PEG was demonstrated by the separation of CS and MDH enzymes by gel filtration in the presence of 0.8 M

Figure 3.2.

The effect of increasing salt concentration on gel filtration of CS and MDH.

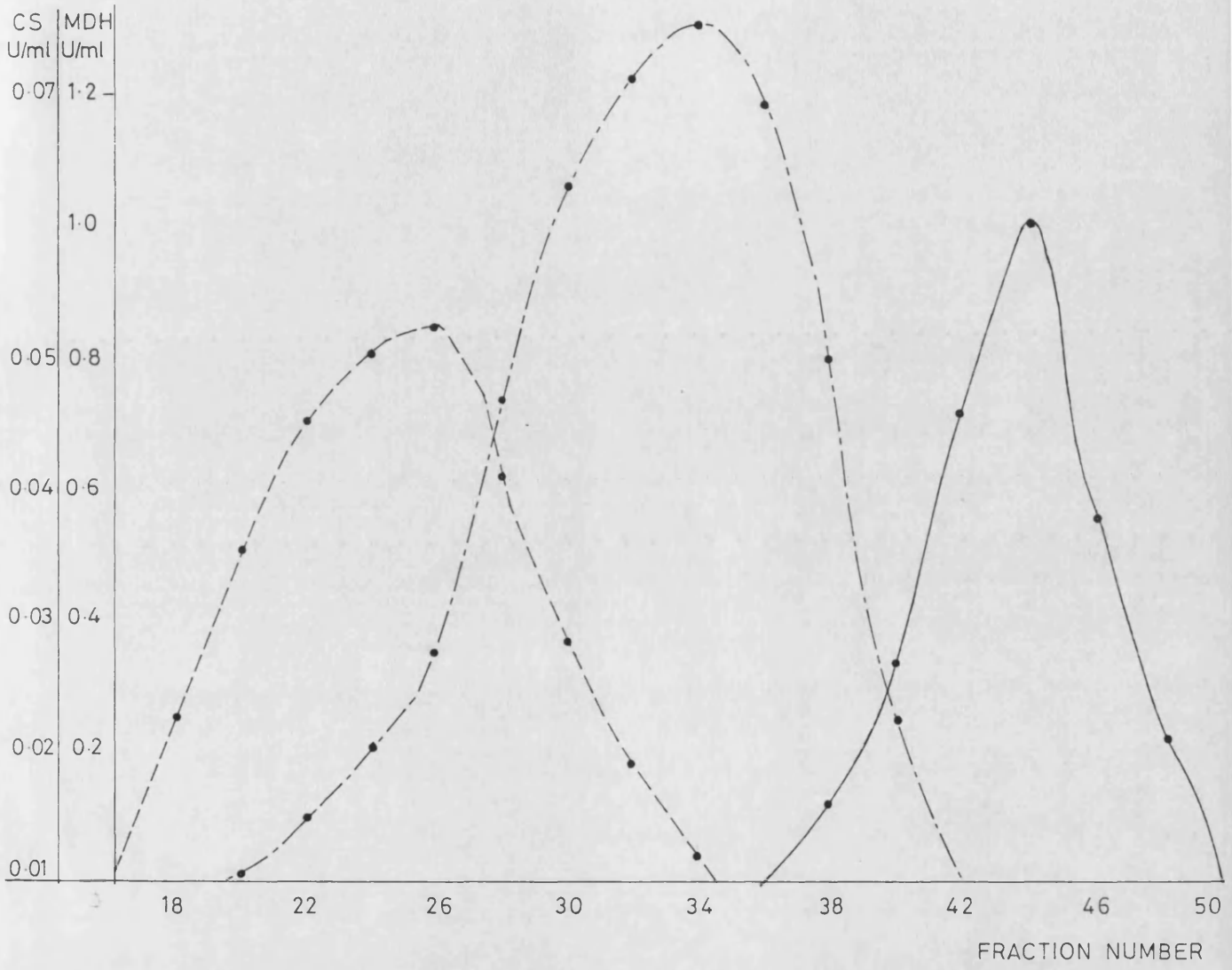


Gel filtration on Sephadex G-200. Bacterial extract (1 ml) *E. coli* K12 D500 eluted with 10 mM TAG, pH 7.5 + 0.8 M KCl, at 4°C.

Enzyme activity CS (———) and MDH (— - —).

Figure 3.3.

Gel filtration of CS and MDH from *E. coli* K12 D500 in presence of PEG (14% w/v).



Elution of Sephadex G-200 gel filtration with 10 mM TAG pH 7.5 + PEG (14% w/v) at 4°C.

KCl. The two enzymes were dialyzed to remove salt and were then incubated in equal volumes, in the presence of 14% (w/v) PEG (1 hour, 10°C) and loaded onto the gel filtration column. Analysis of the resulting fractions showed a pattern similar to that in Figure 3.1. The enzymes were then dissociated again using 0.8 M KCl fractionation, to give rise to two sharply defined peaks with little co-elution. Enzyme interaction between CS and MDH could therefore be demonstrated by manipulation of the environment into which they had been isolated.

Co-Elution of CS and MDH from Various Bacterial Strains

To study the specificity of the co-elution of bacterial CS and MDH, extracts of various bacterial strains were made, in order to separate the required enzymes. These were then mixed in equal proportions, incubated in the presence of PEG, as described previously, and run on Sephadex G-200. These results are expressed in Table 3.1.

Interactions between CS and MDH were demonstrated in media thought to reduce the water concentration in the vicinity of the proteins. The specific interaction of CS with MDH was not restricted to enzymes isolated from the same species. The significance of these results was explored further.

The Site of Interaction Between CS and MDH Enzymes

Modification of an enzyme by a chemical agent may be a means of exploring possible sites of association between two interacting enzymes. CS enzyme from *Pseudomonas* species is sensitive to 5,5'-

Table 3.1. Co-elution of CS and MDH from various bacterial strains

ENZYME COMBINATION	ELUTION PATTERN
4B CS + 4B MDH	Co-elution
Pseudomonas CS + 4B MDH	Co-elution
Pseudomonas CS + 4B MDH in 14% (w/v) PEG	Co-elution PEG shifts peaks forward
4B CS + 4B MDH in 14% (w/v) PEG	Co-elution PEG shifts peaks forward
4B CS + mitochondrial MDH	Co-elution
Pseudomonas CS + mitochondrial MDH	Co-elution

4B indicates the use of *Acinetobacter calcoaceticus*, *Pseudomonas* sp. indicates the use of *Pseudomonas aeruginosa* and mitochondrial extract was from bovine heart (10 µl mMDH from a commercial preparation, Boehringer). Gel filtration on Sephadex G-200 in 10 mM Tris-acetate buffer, pH 7.5.

dithiobis-(2-nitrobenzoate) (DTNB). DTNB reacts with sulphydryl groups of CS, rendering the enzyme insensitive to the regulatory effects of NADH and is believed to act at the NADH binding site (Weitzman and Danson, 1976). The subsequent modification to the CS enzyme, on treatment with DTNB, may result in an alteration of its ability to interact with MDH.

A semi-pure preparation of CS from *Pseudomonas aeruginosa* was incubated with 0.2 mM DTNB for 5 min, at 25°C. An aliquot of the treated CS enzyme was added to an equal volume of MDH, isolated from *Acinetobacter calcoaceticus*, before gel filtration under conditions similar to those in previous experiments.

The resulting elution profiles are shown in Figure 3.4. The co-elution and overlap of enzyme peaks were reduced significantly in the preparation of CS treated with DTNB. This decreased interaction between CS and MDH enzymes may be the result of modification of sulphydryl groups on CS bringing about some slight conformational change in the region of the NADH-binding site, an alteration to charge distribution or a physical blocking of the site caused by reaction with DTNB. Enzyme associations are likely to be over some area of the protein structure, rather than at single points. In order for two proteins to associate they must have surface areas complementary to each other. Complementarity means that the association partners share the same van der Waals' envelope over some area (Morgan *et al.*, 1979). Complementary surfaces need not be continuous and may tolerate holes (Friedrich, 1984).

It may be that an alteration to part of the complementary surface of an enzyme results in a decreased ability to form enzyme

56.

a. *Pseudomonas* sp. CS with MDH (*Acinetobacter calcoaceticus*)

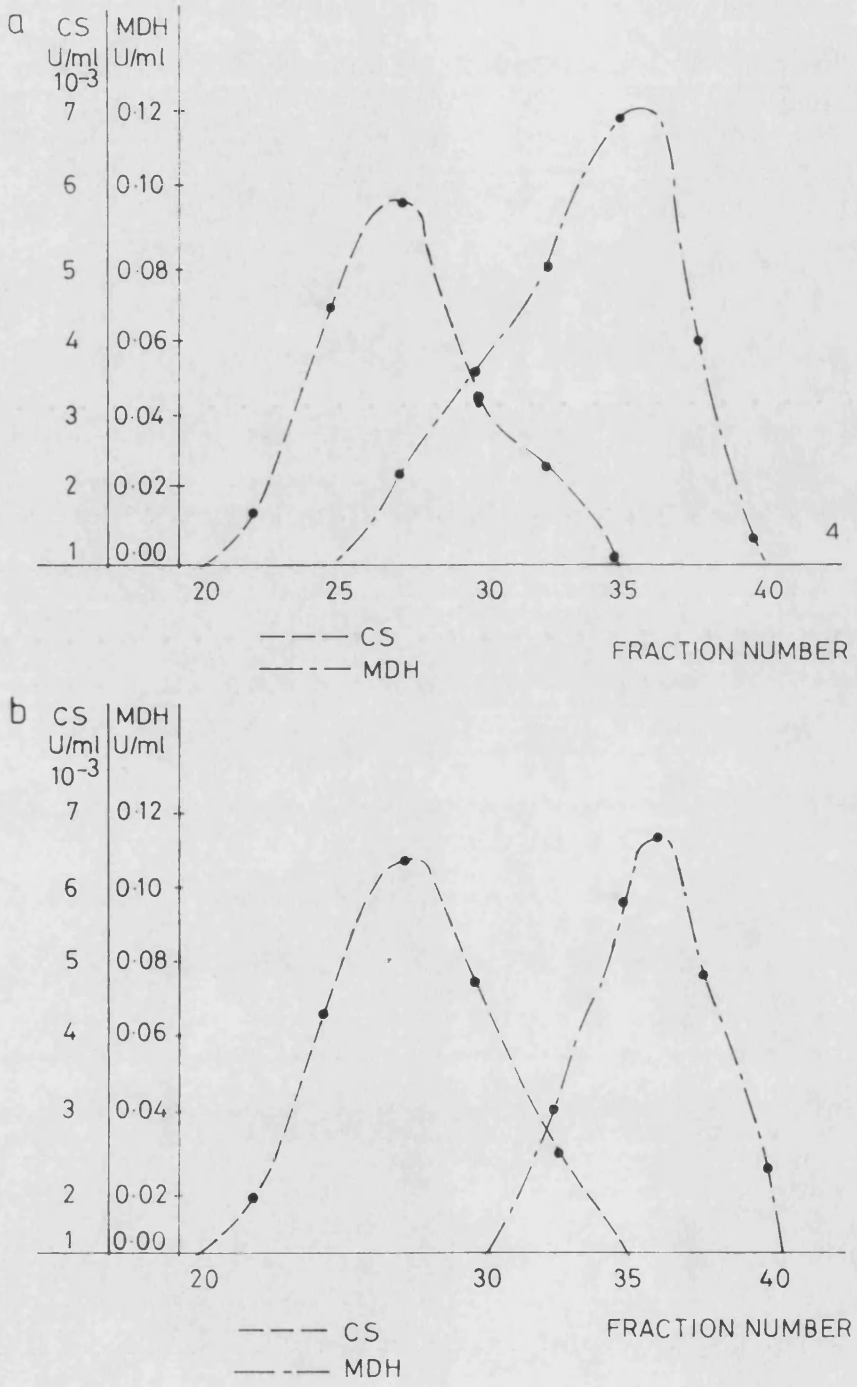
b. *Pseudomonas* sp. CS treated with DTNB (0.2 mM) with MDH (*Acinetobacter calcoaceticus*)

Gel filtration on Sephadex G-200 with 10 mM TAG, pH 7.5, 4°C.

Similar elution profiles were obtained on repeated examination of enzyme interaction in the presence and absence of DTNB.

Figure 3.4.

The site of interaction between CS and MDH, a probe of the NADH binding site of CS.



associations. The NADH-binding site may therefore be involved in the surface between the interacting CS and MDH enzymes.

Organization of Glycolytic Enzymes in *E. coli*

A tentative identification of a multi-enzyme complex with activity of the glycolytic pathway was made by Mowbray and Moses (1976). This was followed by a further investigation by Gorringe and Moses (1980). Both these studies were based on observations by other workers of specific associations between enzymes of what was regarded as a soluble enzyme system. Their studies in *E. coli* acknowledged the fact that conventional separation techniques may not be conducive to maintaining delicate associations between enzymes and so used lysed bacterial spheroplasts as source material.

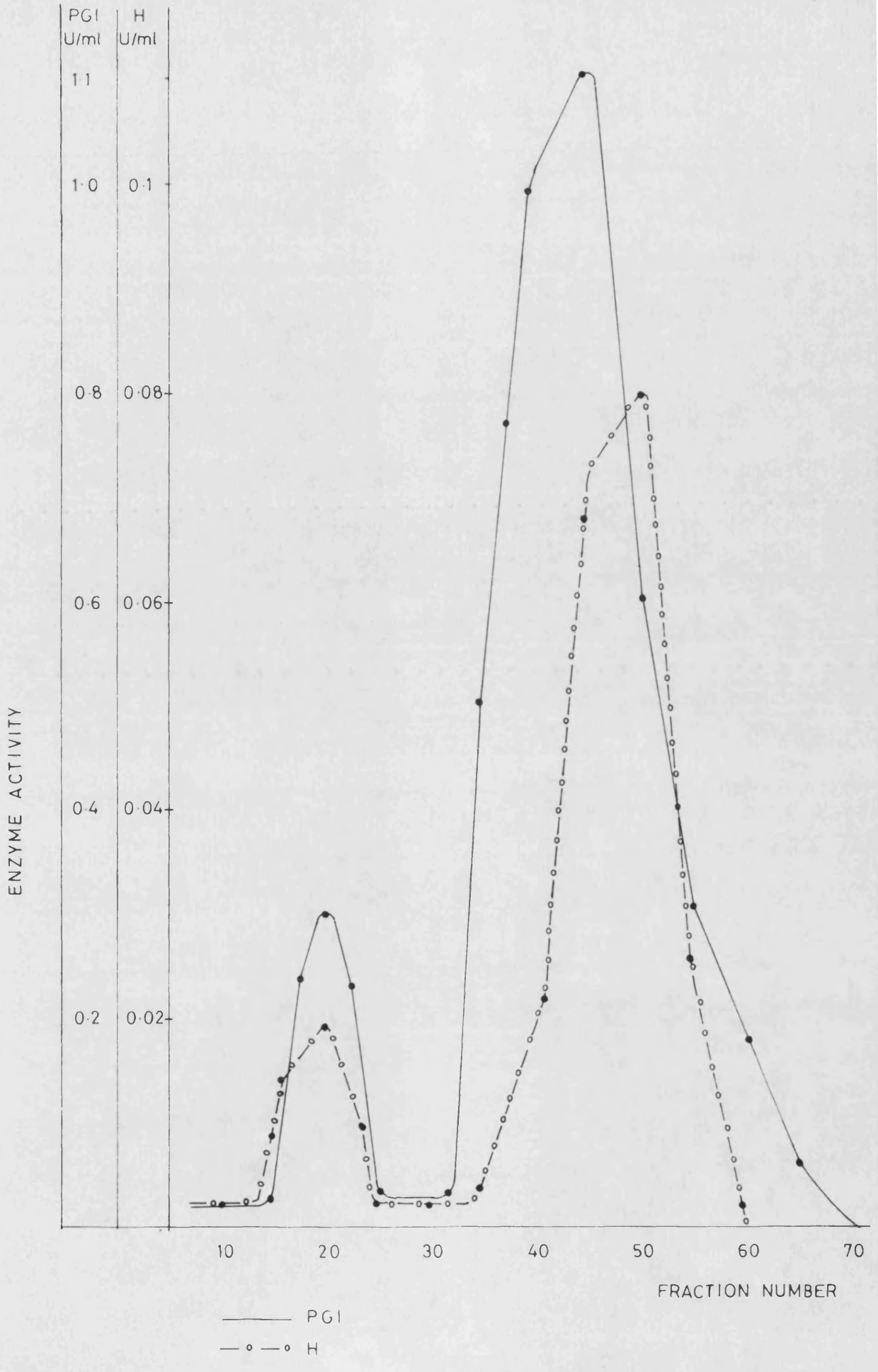
The observations of Gorringe and Moses (1980) were repeated. A preparation from lysed *E. coli* spheroplasts (prepared using the lysozyme-EDTA method) was analysed by gel filtration on a column (35 x 1.5 cm) of Sepharose 4B, with 10 mM Tris-acetate, pH 7.5, + 20% (v/v) glycerol (TAG). Fractions (1 ml) were collected and assayed for two glycolytic enzymes - hexokinase and phosphoglucose isomerase. The results are shown in Figure 3.5.

For each enzyme, two distinct peaks were detected. This agrees with the results of Gorringe (1980). Both enzymes eluted at positions consistent with their recorded molecular weights. However, the two enzymes also co-eluted at a position indicative of a much higher molecular weight (approx. 1.65×10^6; Mowbray and Moses, 1976). This peak has been termed the high molecular weight species (HMWt species).

Enzyme profile of glycolytic enzymes hexokinase (—o—) and phosphoglucose isomerase (———) on lysis of *E. coli* spheroplasts.

Gel filtration on Sepharose 4B. Elution with 10 mM TAG pH 7.5 at 4°C.

Figure 3.5.


PGI U/ml
H U/ml
1·1
1·0
0·8
0·6
0·4
0·2
0·1
0·08
0·06
0·04
0·02
ENZYME ACTIVITY
10
20
30
40
50
60
70
FRACTION NUMBER
PGI
H

Organization of the Citric Acid Cycle Enzymes in *E. coli*

An extension of the study from the glycolytic enzymes to those of the citric acid cycle employed similar techniques, of gentle lysis of *E. coli* spheroplasts prepared by removal of the outer cell wall using the lysozyme-EDTA method. After a slow-speed centrifugation to remove cell debris and whole cells, the supernatant was loaded in the presence of 0.5% $^{sucrose}_{\wedge}$ (v/v) onto a Sepharose 4B gel filtration column, equilibrated with TAG. Fractions (1 ml) were collected and assayed for each of the citric acid cycle (CAC) enzymes and the elution profiles are shown in Figure 3.6.

As with the glycolytic enzymes, two distinct peaks of enzyme activity were detected for; fumarase (FM), malate dehydrogenase (MDH), citrate synthase (CS), aconitase (AC), NADP-isocitrate dehydrogenase (IDH) and ADP- and GDP-succinate thiokinase (STK). The peak of activity in the low molecular weight form (LMWt) corresponded to the predicted molecular weight for each enzyme.

However, a small proportion of the activity of each of these enzymes was seen to co-elute as a high molecular weight (HMWt) species. This observation was reproducible, despite the low levels of enzyme activity seen in the HMWt species when compared with the LMWt species.

One of the enzymes of the CAC, succinate dehydrogenase (SDH) is regarded as an integral membrane protein, involved in directing the transfer of electrons to molecular oxygen in the electron transport chain, from the cycle. SDH activity was detected only in the pellet fraction from the lysed bacterial spheroplasts, no activity being present in the cytoplasmic component.

62

Elution profile of citric acid cycle enzyme

Gel filtration of lysed *E. coli* K12 D500 spheroplasts on Sepharose 4B with 10 mM TAG, pH 7.6, at 4°C.

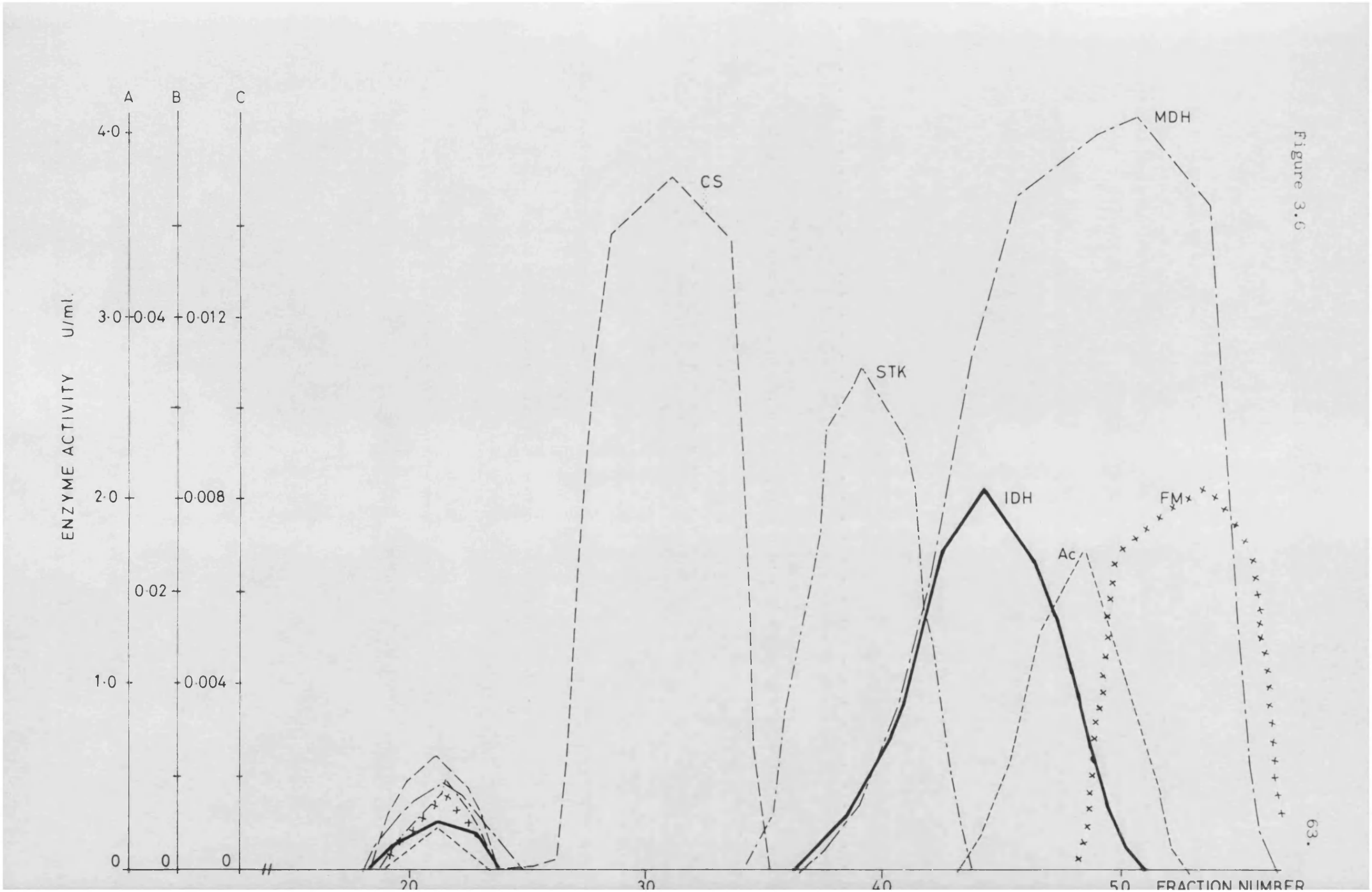

A
B
C
4·0
3·0
2·0
1·0
0
0·04
0·02
0
0·012
0·008
0·004
0
ENZYME ACTIVITY U/ml.
CS
STK
MDH
IDH
Ac
FM
20
30
40
50
FRACTION NUMBER

Figure 3.6

The final enzyme in the CAC to be described is oxoglutarate dehydrogenase (OGDH). The relationship between this multienzyme complex and the HMWt species containing six of the enzymes of the cycle was studied by examination of elution profiles of the enzymes on gel filtration, using Sepharose 4B equilibrated and eluted with TAG buffer.

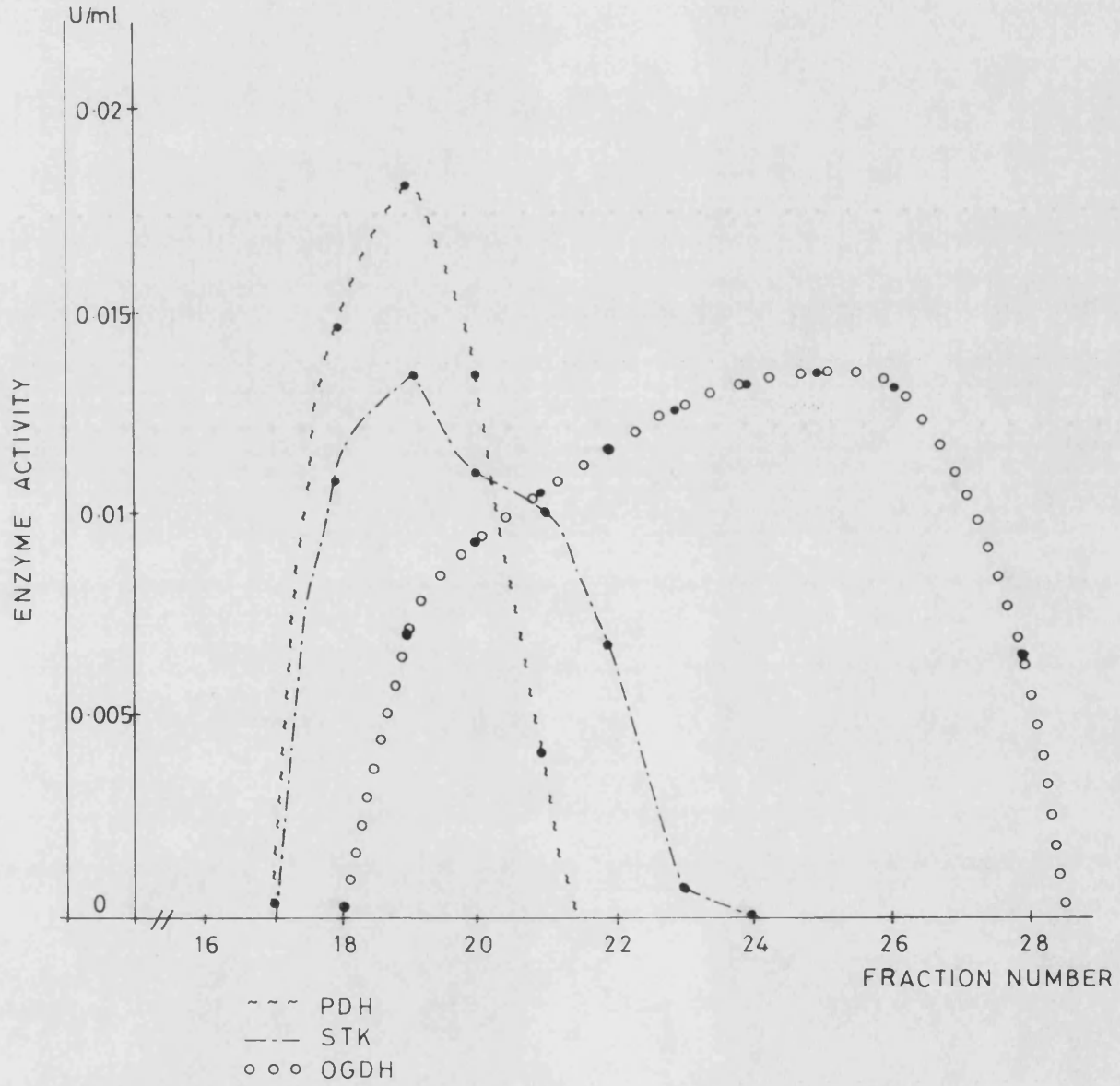
As illustrated in Figure 3.7, OGDH eluted in a position adjacent to the HMWt species although some overlap of peaks was seen, with a 'shoulder' corresponding to the HMWt species. The profile of the pyruvate dehydrogenase (PDH) multienzyme complex is also shown on this graph to elute at a similar position to the HMWt species. It would be expected that PDH and OGDH would elute within a similar fraction range, based on their similar and relatively high molecular weights. PDH may be regarded as a component enzyme of the glycolytic sequence and serves in part to direct acetyl-CoA into the citric acid cycle. The co-elution of PDH and OGDH in the region of the HMWt species may be simply indicative of their elution at a particular molecular weight or may reflect an association between one or both of these complexes with the other enzymes of the cycle in the HMWt species. This point was investigated further.

The specific activities of the enzymes present in the HMWt species were calculated and are tabulated in Table 3.2. Examination of the fractions from gel filtration for protein content showed a similar profile of protein elution to enzyme elution; a peak of protein activity corresponded to the peak of HMWt species activity.

Figure 3.7

Enzyme profiles of PDH and OGDH:

Co-elution of STK



Gel filtration of lysed *E. coli* K12 D500 spheroplasts on Sepharose 4B with 10 mM TAG, pH 7.5 at 4°C.

Table 3.2. The activities and specific activities of enzymes in the HMWt species

ENZYME	ENZYME ACTIVITY μmole $min^{-1}ml^{-1}$	SPECIFIC ACTIVITY μmole $\mu g^{-1}min^{-1}$
Fumarase	0.0140	0.0200
Malate dehydrogenase	0.2900	0.4200
Citrate synthase	0.0059	0.0084
Aconitase	0.0016	0.0023
Isocitrate dehydrogenase	0.0110	0.0160
Oxoglutarate dehydrogenase	0.0056	0.0081
Succinate thiokinase	0.0170	0.0250
Succinate dehydrogenase	-	-
Pyruvate dehydrogenase	0.0190	0.0275

Methods of Disruption

Osmotic lysis may be one of the more gentle methods of cell disruption and may be least destructive to delicate intracellular compartmentation of components of the cell. Therefore in the study of fine associations between specific enzymes, it was interesting to look at the effect of other conventional methods of cell disruption on these associations.

The Effect of Sonication

Bacterial spheroplasts prepared by the lysozyme-EDTA method were sonicated by 4 x 15 sec sonications with cooling. Cell debris was spun off using a short centrifugal spin, 3 000 g, 10 min and the supernatant loaded and run on Sepharose 4B, equilibrated and eluted with TAG.

The 1-ml fractions, when assayed for enzyme activity, showed no HMWt species; each of the CAC enzymes eluted as a single peak.

Disruption Using the French Press

The details in the Methods section describe the conditions used to disrupt *E. coli* spheroplasts by the French press. Examination of the enzyme elution profile from passage of the resulting supernatant showed single peaks for each enzyme, with no HMWt species detected.

Disruption by Freeze-Thaw Technique

Spheroplasts from *E. coli* prepared using lysozyme were disrupted using a freeze-thaw technique as described in the Methods section; the cell debris was removed by centrifugation and the supernatant was analysed using gel filtration for enzyme associations. When compared with cells disrupted by osmotic lysis, a similar elution profile was obtained, showing a high molecular weight activity peak containing MDH, CS and FM. However, the levels of enzyme present in the HMWt peak were much lower, despite a similar total enzyme activity compared with the lysed spheroplast preparation.

The Effect of Osmotic Lytic Conditions upon the Presence of a HMWt Species

The effects of varying conditions in which the bacterial spheroplasts were lysed were investigated. The aim of such a study was to identify physical conditions which may be destructive or conducive to the maintenance of the fine enzyme associations which may exist *in vivo*.

The results of this investigation are tabulated in Table 3.3. The pellet of spheroplasts were lysed in the presence of 10 mM Tris-acetate, pH 7.5 plus the agent under study. All subsequent filtration and elution was carried out using these buffer conditions. The presence and level of HMWt was recorded.

The Effect of Changing Physical Conditions on Enzyme Association

Figure 3.8 illustrates the effects of altering the physical milieu into which the spheroplasts were lysed and subsequently treated. An effect on enzyme association was seen as an increase or decrease in the proportion of enzyme present in the HMWt species, compared with the total enzyme activity loaded on the chromatography column. The results were compared to a control using TAG, pH 7.5.

The presence and the proportion of HMWt species was shown to be dependent on the method of cell disruption, the conditions of osmotic lysis and variations in the physical environment into which the spheroplasts were lysed.

Disruption techniques of sonication and the French pressure cell must be presumed to be too drastic for the maintenance of the

Table 3.3. The effect of varying conditions of lysis of bacterial spheroplasts from *E. coli* K12 D500 in 10 mM Tris-acetate, pH 7.5.

Physical agent	Presence of HMWt species	Level of HMWt species compared to lysis in TAG	Comments
Glycerol (20% v/v)	yes	standard level	Glycerol reduces the polarity of the gel in gel-filtration column. It also increases the viscosity of the medium.
PMSF (0.15 mM)	yes	high	PMSF is a protease inhibitor. Its effect may be to reduce the destructive ability of intracellular proteases on enzyme associations.
Triton X100 (0.01% v/v)	yes	high	Triton acts as a detergent and which would remove membrane fragments which serve to trap enzyme.

delicate balance of intracellular organization. A proportion of fine enzyme associations were maintained on disruption of the cells using the freeze-thaw technique, but the osmotic lysis of spheroplasts was found to be the most appropriate for the isolation of the HMWt species of CAC enzymes.

By preventing the activity of protease enzymes, enzyme associations were maintained and a higher proportion of HMWt species isolated. Organization of enzymes into multienzyme clusters may be a way of protecting delicate interactions against the action of

The effects of physical processes on associations between enzymes.

Gel filtration of lysed *E. coli* K12 D500 spheroplasts under varying conditions as stated.
Values normalised to compare with control conditions.
Values taken as proportion of total enzyme/ml present in HMWt peak.

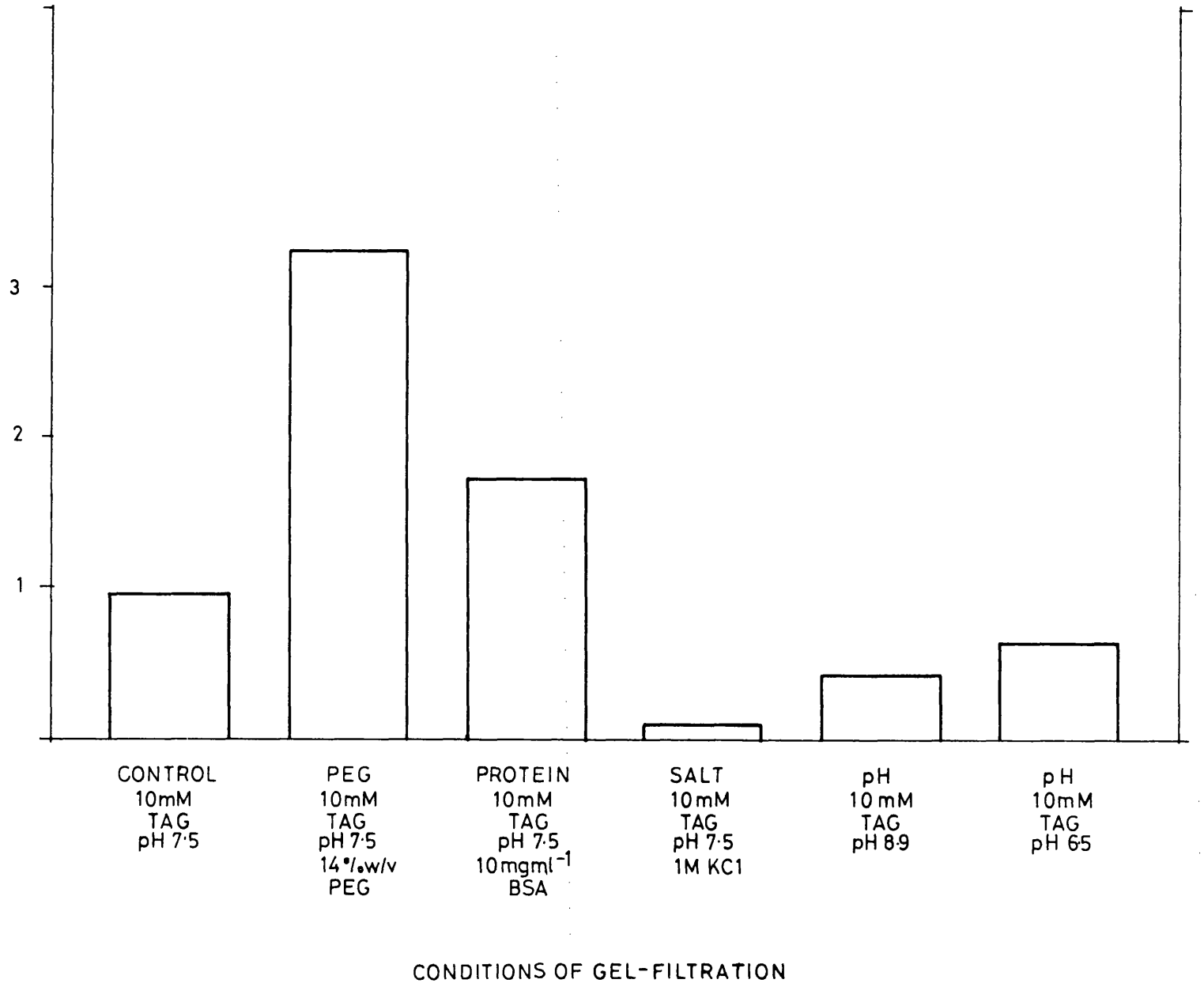

MDH ACTIVITY U/ml
3
2
1
CONTROL
10mM
TAG
pH 7·5
PEG
10mM
TAG
pH 7·5
14 %w/v
PEG
PROTEIN
10mM
TAG
pH 7·5
10mgml⁻¹
BSA
SALT
10mM
TAG
pH 7·5
1M KCl
pH
10mM
TAG
pH 8·9
pH
10mM
TAG
pH 6·5
CONDITIONS OF GEL-FILTRATION

Figure 3.8

intracellular proteases. The use of Triton X100 in the extraction medium may have been a way of releasing enzymes trapped in membrane fragments. If, as has been suggested by D'Souza and Srere (1983) and by results presented in this thesis, the location of a CAC cluster may be adjacent to the inner membrane of cells, it is conceivable that all the HMWt species present would not be released on lysis of the cell. A higher proportion of HMWt species was detected on lysis of spheroplast in the presence of Triton X100. This result indicates that the HMWt species may be located in the vicinity of the inner membrane of the cell. A further investigation is presented later.

The environment into which the CAC enzymes were released was shown to effect the enzyme association and interactions which formed the HMWt species. These results are discussed later.

Specificity of Enzyme Association

Lactate dehydrogenase (LDH) commercial preparation, 10 µl, was included in the supernatant aliquot loaded onto the gel filtration column as a marker enzyme and to see if any enzyme would spontaneously associate with the HMWt species. LDH eluted as a single tight peak and showed no association with the HMWt cluster of CAC enzymes. However LDH is metabolically unrelated to the enzymes of the cycle, so in order to test the specificity of association, an enzyme which is involved in a shuttle system with malate dehydrogenase, aspartate aminotransferase (AAT) was examined. AAT has been reported to complex with mitochondrial MDH in the presence of 14% (w/v) PEG (Fahien, 1977). A study of the

elution profile from lysed *E. coli* spheroplasts for the presence of AAT in the HMWt species showed a single peak of activity within the low molecular weight range, with no enzyme activity detected in the HMWt species.

When a pooled sample of CAC enzymes, obtained from commercial sources, was treated with the gel filtration analysis system for the detection of physical interactions between enzymes, again no HMWt species was detected. The enzymes all eluted as single activity peaks. It may therefore be concluded that either the presence of a HMWt cluster of CAC enzymes is a result of the use of a 'gentle' isolation technique or that something in the preparation of the commercially 'pure' enzymes renders them unable to associate spontaneously.

Stability of HMWt species

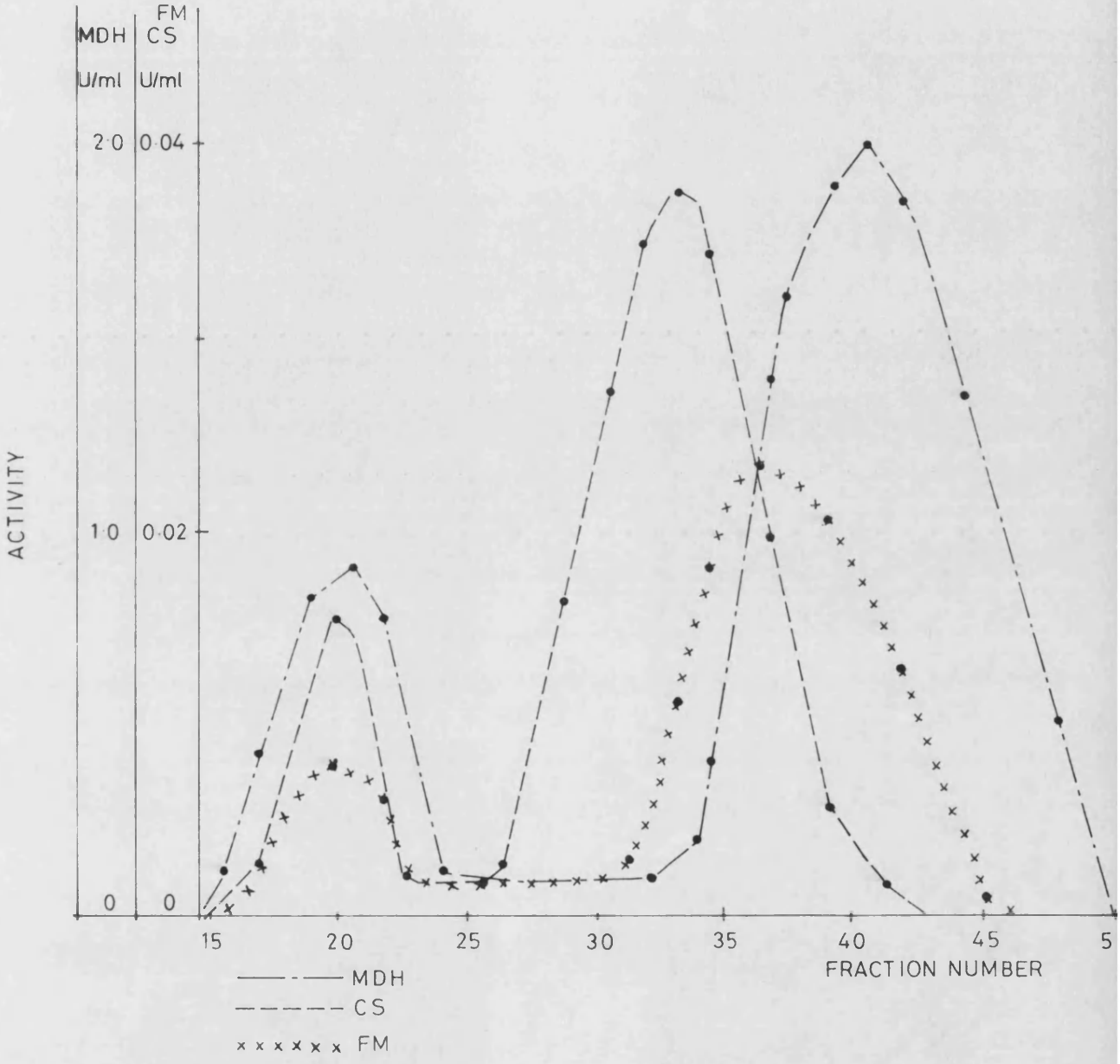
The stability of the HMWt species was examined by studying its re-passage on Sepharose 4B + 10 mM TAG. As Figure 3.9 illustrates, although there is some dissociation of the HMWt species into its constituent enzymes, a proportion of the enzymes remains associated and elutes in the high molecular weight range.

Re-Association of LMWt species Enzymes

As reported earlier, gel filtration of a lysed spheroplast preparation from *E. coli* showed the presence of two distinct peaks of activity for the CAC enzymes. The HMWt species has been described. The LMWt species of the enzymes were examined for their ability to re-associate. Pooled LMWt fractions were concentrated

Figure 3.9

Re-passage of high molecular weight peak on gel filtration.



Gel filtration of isolated HMWt species from *E. coli* K12 D500 on Sepharose 4B, 10 mM TAG, pH 7.5 at 4°C.

one hundred times using an Amicon micro-filtration cell with a P10 membrane. The concentrate was re-applied to the Sepharose 4B column and eluted with 10 mM TAG, pH 7.5. The elution profile of the resulting fractions is shown in Figure 3.10, demonstrating the presence of a HMWt species. Although only CS, FM and MDH elution profiles are shown, the presence of AC and NADP-IDH were also detected in the HMWt peak. In addition, each enzyme also eluted at points corresponding to their respective molecular weights.

The specificity of this re-association was tested by the inclusion of LDH in the concentrated enzyme sample. LDH eluted as a single peak with no activity in the HMWt species.

The effect of polyethylene glycol,which acts to mimic the intracellular milieu by exclusion of water, was examined on the re-association of LMWt enzymes. These results are illustrated in Figure 3.11. They clearly demonstrate that PEG has the effect of enhancing enzyme interaction by the exclusion of water molecules, increasing the proportion of enzyme specifically associating to form the HMWt species.

Consistently, only five of the CAC enzymes were seen to re-form as a HMWt species, as shown below:

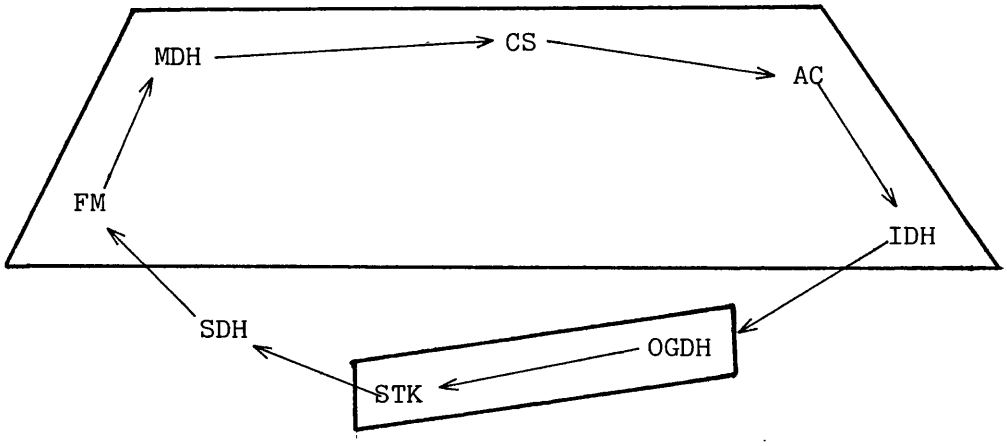
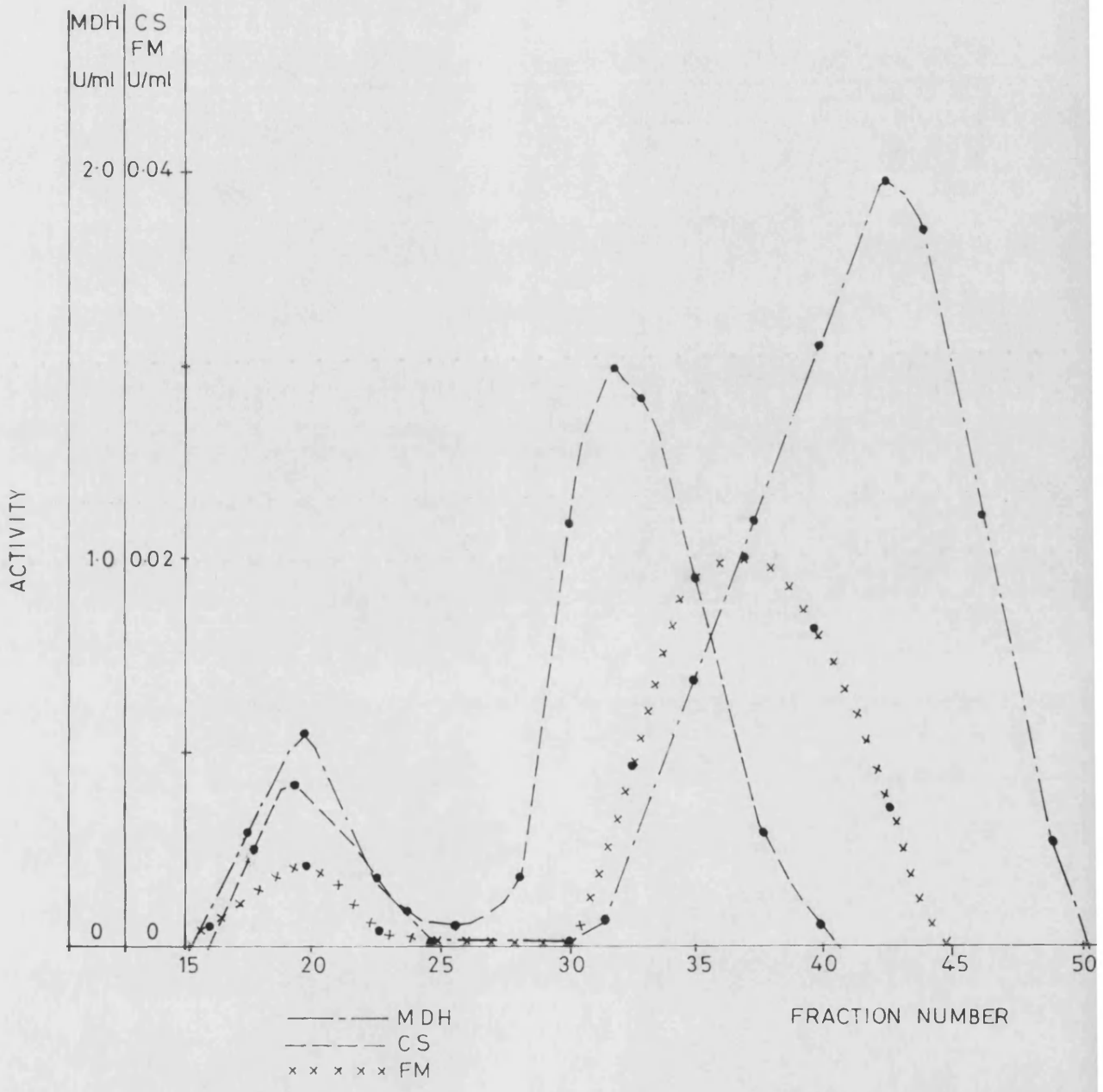


Figure 3.10

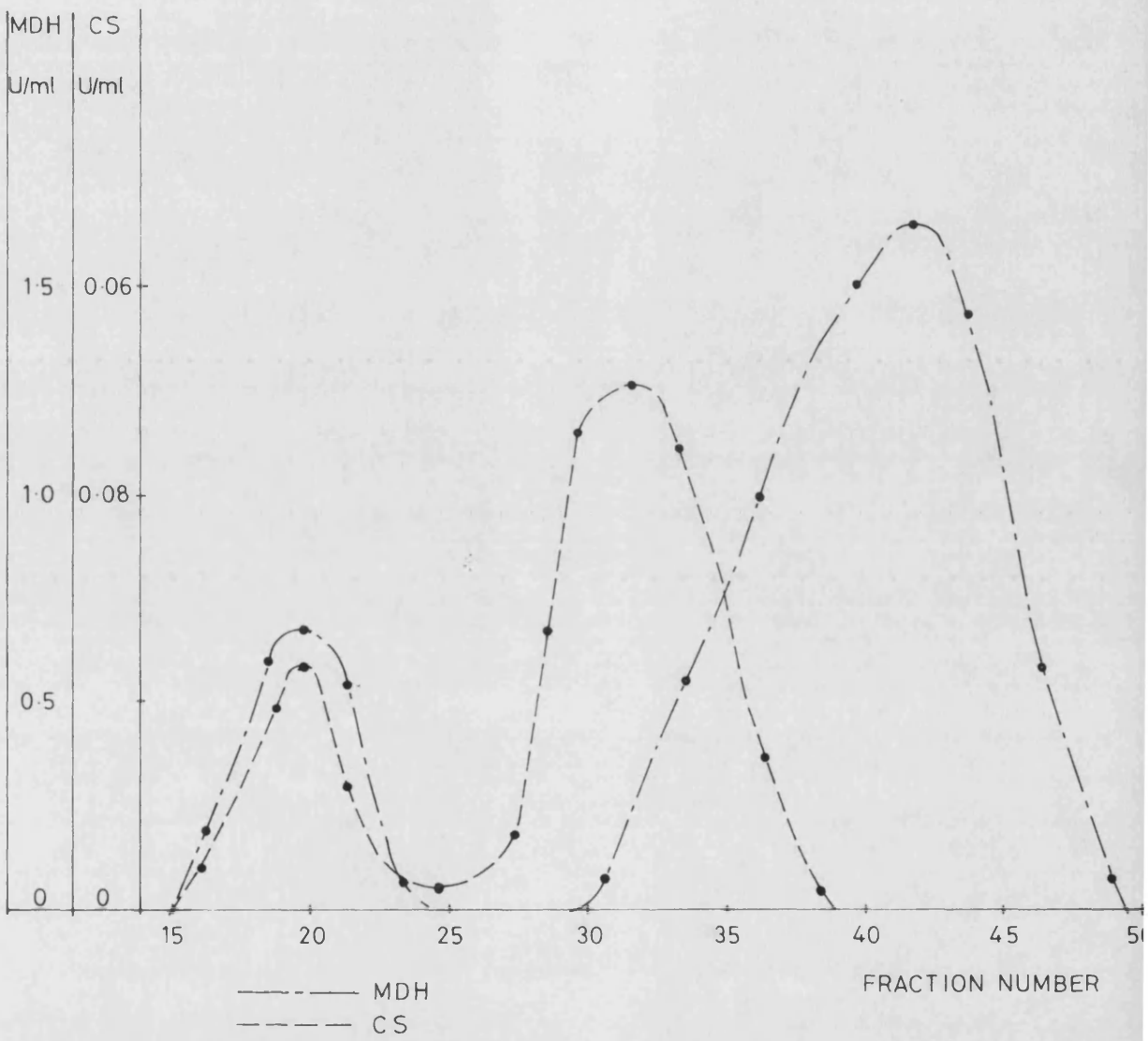
Re-association of low molecular weight fractions into high molecular weight species.



Gel filtration of pooled and concentrated LMWt fractions of *E. coli* K12 D500, run on Sepharose 4B, 10 mM TAG, pH 7.5 at 4°C.

Figure 3.11

The effect of PEG on the re-association of enzymes.



Isolated LMWt fractions pooled and concentrated by ultrafiltration in presence of PEG (14%, w/v). Gel filtration of sample on Sepharose 4B with 10 mM TAG, pH 7.5 + 14% w/v PEG at 4°C.

SDH would not be expected to re-associate with other CAC enzymes due to its location as an integral membrane protein. OGDH has a molecuilar weight of approx. 2×10^6 and so would not be included in the range of pooled LMWt fractions.

The position of STK was investigated since it did not appear to re-assemble with the other enzymes on concentration. Sumegi *et al.* (1983) reported an association between STK and OGDH using PEG precipitation and demonstrated the kinetic advantage of this coupled system.

It can be demonstrated that STK will only elute in the high molecular weight range when OGDH is present. The ability of STK to associate with other CAC enzymes was investigated in the presence and absence of OGDH. The samples were concentrated by ultrafiltration and analysed on gel filtration for the presence of a HMWt species. Figure 3.12 shows that STK would only elute at a higher molecular weight when OGDH was present. These results are discussed later.

Formation of Spheroplasts using Penicillin

The possibility that the enzyme associations seen in previous experiments were due to the method of preparation of the spheroplasts using lysozyme-EDTA, was examined. Pencillin-G was used as a means of obtaining the osmotically-sensitive spheroplasts.

The procedure of Mowbray and Moses (1976) was employed. Penicillin prevents the formation of cross-links between N-acetyl glucosamine and N-acetylmuramic acid in the cell wall of actively dividing bacteria. Growth of the bacteria in the presence of this

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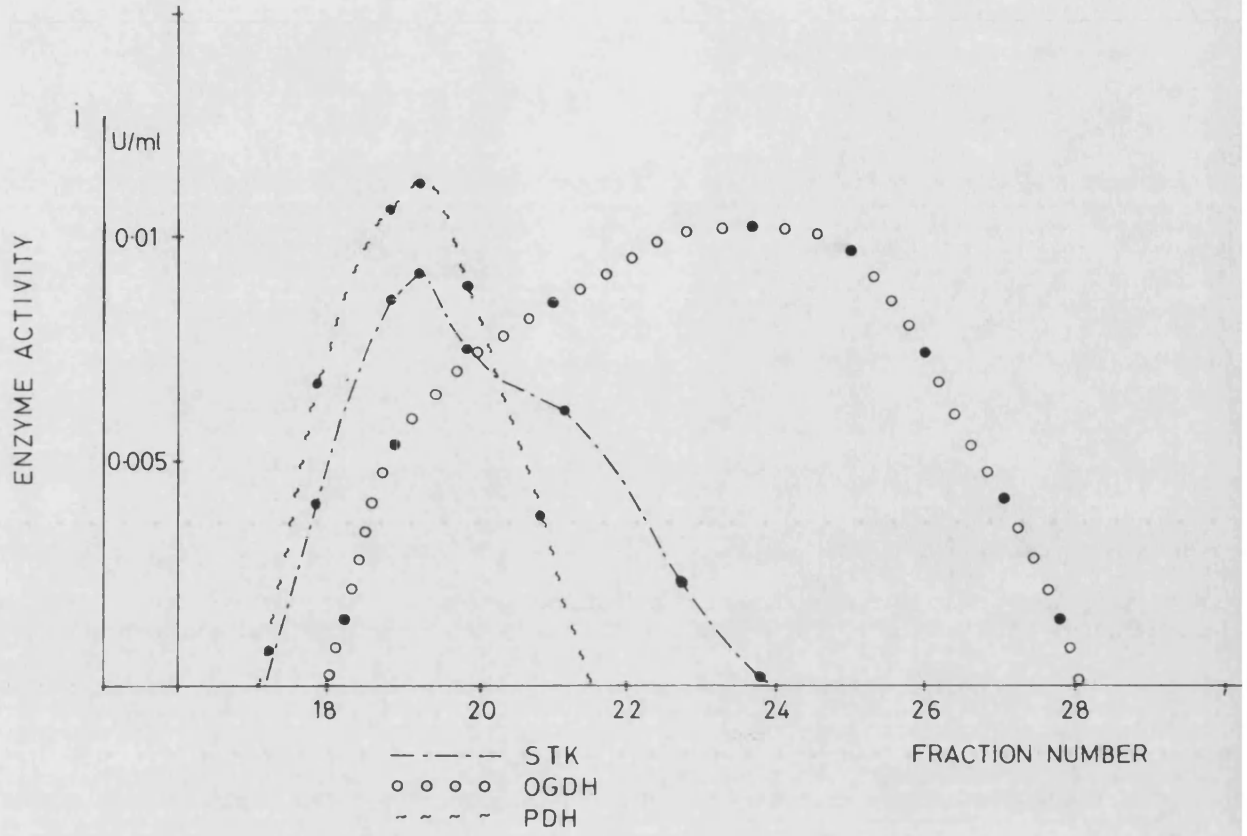
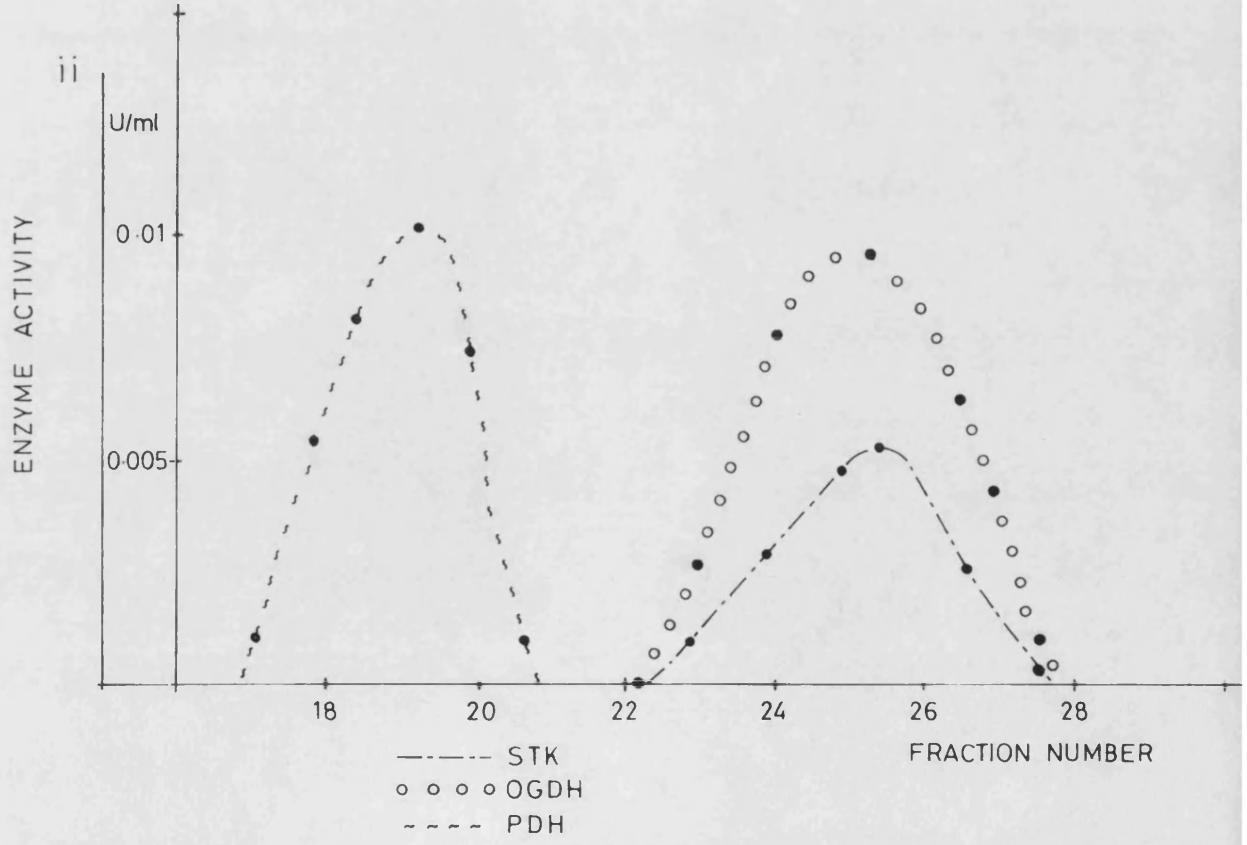
Specific Association of STK with OGDH.

i. Initial passage by gel filtration of lysed *E. coli* K12 D500 spheroplasts on Sepharose 4B, 10 mM TAG, pH 7.5 at 4°C.

ii. LMWt fractions from (i) pooled and concentrated by ultrafiltration in presence of OGDH (isolated by chromatography). Gel filtration of sample on Sepharose 4B with 10 mM TAG, pH 7.5 at 4°C.

Figure 3.12.


i
U/ml
0·01
0·005
ENZYME ACTIVITY
18
20
22
24
26
28
FRACTION NUMBER
STK
OGDH
PDH


ii
U/ml
0·01
0·005
ENZYME ACTIVITY
18
20
22
24
26
28
FRACTION NUMBER
STK
OGDH
PDH

antibiotic therefore produces a culture of osmotically-sensitive spheroplasts. The time-course of *E. coli* growth and spheroplast formation are shown in Figure 3.13.

Lysis of the penicillin spheroplasts and the subsequent analysis by gel filtration, under similar conditions to those stated for previous experiments, resulted in the elution profile of enzymes (Figure 3.14).

A cluster of CAC enzymes was demonstrated from penicillin-treated *E. coli* cells. The presence of the cluster was not merely a consequence of the experimental protocol of spheroplast formation using lysozyme-EDTA.

The Effect of Metabolic Regulators on the HMWt species

The possibility that specific metabolic regulators of key enzymes of the CAC have some effect on the proportion of enzyme in the HMWt cluster was investigated.

Since intermediates of the cycle do not normally accumulate it has been concluded that the rate-limiting step of the cycle may be that catalysed by CS (Krebs and Lowenstein, 1960). NADH is a powerful and specific inhibitor of *E. coli* CS *in vitro*, competitive with acetyl-CoA (Weitzman, 1966). A positive allosteric modifier AMP was also investigated due to its known *in vitro* effect on IDH, OGDH and PDH.

NADH (0.2 mM) and AMP (0.5 mM) were separately included in the lysis column and elution buffers (10 mM TAG, pH 7.5) of lysed *E. coli* spheroplasts. Examination of the elution profiles with regulators, compared with a control with no modifiers, showed no

Figure 3.13

Time course of *E. coli* K12 D500 growth and spheroplast formation using penicillin

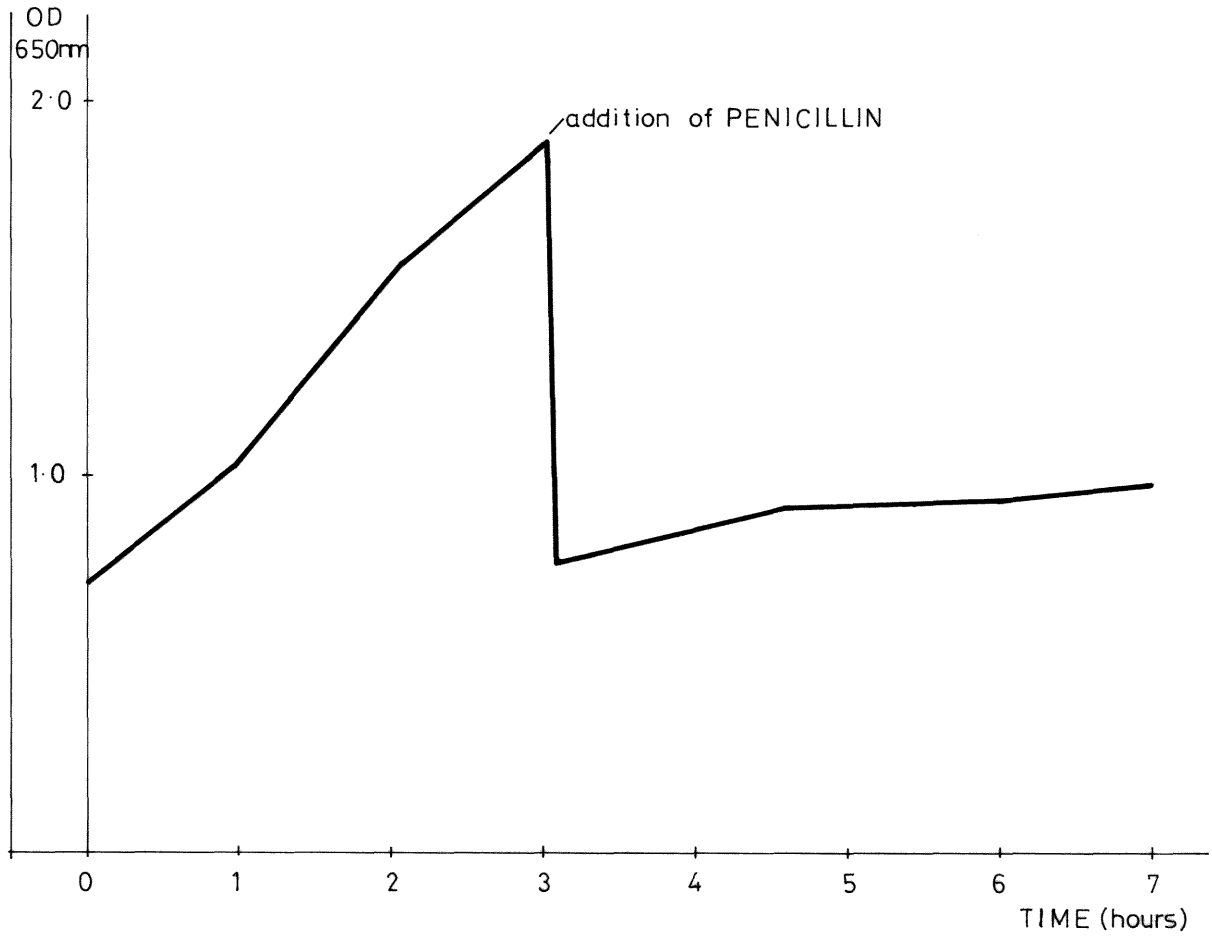
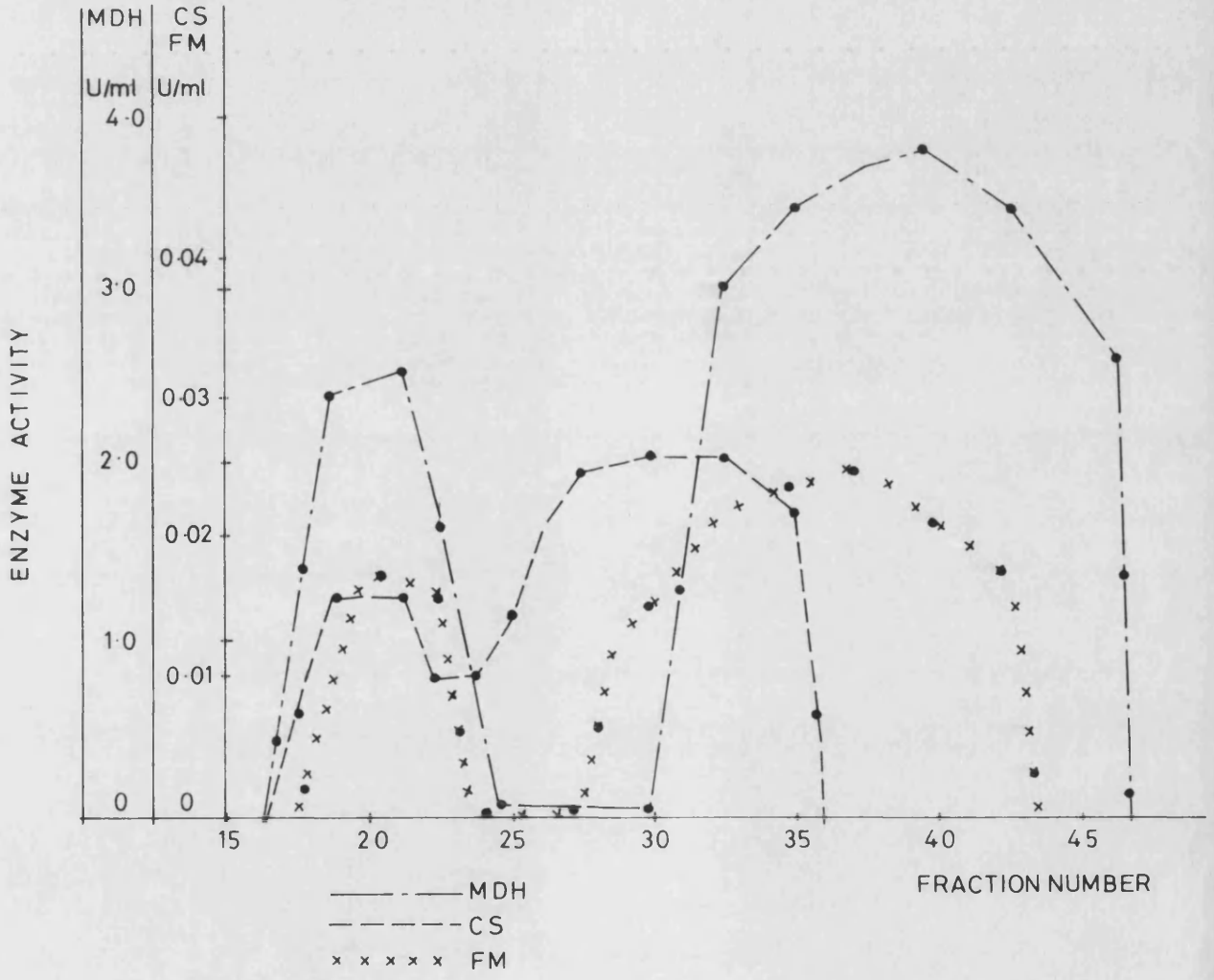


Figure 3.14

Elution profiles of citric acid cycle enzymes from lysed penicillin spheroplasts.



Gel filtration on Sepharose 4B, 10 mM TAG, pH 7.5 at 4°C.

difference in the proportion of enzyme in the HMWt species. These metabolic regulators appeared therefore to have no effect on enzyme association.

The Effect of Sonication on Enzyme Associations in the CAC

As reported earlier, the effect of sonication on bacterial spheroplasts was to disrupt the cell in such a way as to destroy interactions between the CAC enzymes, which could be detected using a more gentle disruption technique. Since sonication is frequently used in the isolation of enzymes, it was interesting to study its effect on less defined features of intracellular compartmentation.

The HMWt species was prepared as described previously and subjected to a standard sonication of 4 x 20 sec bursts, with cooling. The sample was re-applied to a Sepharose 4B column, with TAG buffer, pH 7.5 and the resulting fractions assayed for CAC enzyme activity. No HMWt peak was detected but the enzymes were eluted as individual activities.

Similarly, the reassociation of LMWt fractions isolated from lysed spheroplasts, was examined. Pooled fractions containing the activities of the CAC enzymes were sonicated on ice for 4 x 20 sec bursts with cooling. After ultra- filtration to concentrate the sample, it was reapplied to the Sepharose 4B column and fractions assayed. Again, no HMWt species was detected, the enzymes eluting as single LMWt peaks.

The controls for these experiments were non-sonicated preparations, isolated in a similar way, the results of which have been presented earlier.

In sonication, material is ruptured by sonic vibrations, although the physical basis of disruption is not understood. It may possibly be the result of the production of free radicals, oxygen or a mechanical disruption. The forces, however, are sufficient to cause dissociation of weakly-bound clusters.

An interesting observation was made on examination of the levels of enzyme measured before and after sonication, as tabulated in Table 3.4.

Comparison of the enzyme levels measured in the HMWt species, of sonicated and non-sonicated preparations, revealed that enzyme activity appeared to be greater in disrupted HMWt species. The dissociation resulting from the disruption of the HMWt species by sonication, gave rise to an apparently higher level of enzyme activity. For each of the five enzymes in the cluster, the apparent level of activity rose by between 2 to 3.3 times the level recorded in the non-disrupted form.

The HMWt species which was formed by re-association by the concentration of LMWt fractions, as reported ealrier, was also examined. Comparison of the sonicated and non-sonicated forms of this second type of HMWt species showed no difference between the levels of enzyme recorded.

The significance of these results will be examined later.

The Time-Dependent Stability of the HMWt Cluster

The cluster of CAC enzymes, isolated by the techniques previously described was regarded as relatively stable. It was possible to subject the HMWt species to gel filtration and for the

Table 3.4. Enzyme levels in sonicated and non-sonicated HMWt species.

ENZYME	SONICATION Enzyme activity $\mu mole.min.^{-1}ml^{-1}$	RATIO	NON-SONICATION Enzyme activity $\mu mole.min.^{-1}ml^{-1}$
FM	0.0227	2 : 1	0.0136
MDH	0.4440	3 : 1	0.1550
CS	0.0280	3 : 1	0.0088
AC	0.0032	2 : 1	0.0016
NADP-IDH	0.0160	3.3 : 1	0.0047

Enzyme levels in sonicated and non-sonicated HMWt species obtained by re-association of LMWt fractions, by concentration.

ENZYME	SONICATION Enzyme activity $\mu mole.min.^{-1}ml^{-1}$	RATIO	NON-SONICATION Enzyme activity $\mu mole.min.^{-1}ml^{-1}$
MDH	9.4×10^{-3}	1 : 1	9.4×10^{-3}
CS	0.1×10^{-3}	1 : 1	0.1×10^{-3}
AC	0.56×10^{-3}	1.1 : 1	0.53×10^{-3}
NADP-IDH	0.03×10^{-3}	1.2 : 1	0.025×10^{-3}

enzymes to remain associated, although some dissociation was evident presumably due to dilution on the gel filtration column. Over several days the isolated HMWt species remained enzymically active. From spectroscopic measurements only it was not possible however to deduce whether or not the enzymes had remained associated with each other.

Based on the observations of sonication, of the 'cryptic' enzyme activity on dissociation of the cluster, a study of the time- dependent stability of the cluster was designed. If sonication results in dissociation of the cluster and a concomitant increase in apparent enzyme activity, the dissociation of the cluster over a period of time may produce a similar apparent increase in activity.

The activity of one of the CAC enzymes, CS, was examined in a preparation of isolated HMWt species, over a period of seven days. Control for the experiment was a similar preparation which had been subjected to sonication (4 x 15 sec bursts, with cooling), to dissociate the HMWt species. Levels of CS activity were recorded at 24 hour intervals from lysis of the spheroplast.

Examination of the levels of CS enzyme activity revealed that activity remained constant for two days. After this point, the level of activity apparently increased very slightly over the next three days before falling, six days after the lysis of spheroplast. Since the level of control CS remained constant until day 6, it was concluded that the apparent increase in activity was due to a dissociation of the enzyme cluster, or at least, of the CS enzyme from the cluster. A fall in both HMWt and control samples from day 6 would probably be the result of loss of enzyme activity due to the age of the preparation.

Table 3.5. Time-dependent stability of the CAC cluster

TIME (days)	Citrate synthase activity (μmole.min^{-1}.ml^{-1})	
		CONTROL
	HMWt	HMWt (sonicated)
1	0.0114	0.055
2	0.0114	0.055
3	0.0158	0.055
4	0.0162	0.055
5	0.0184	0.055
6	0.0154	0.0515
7	0.0120	0.048

From these findings it would appear that the optimum time in which to examine the CAC cluster was in the first 48 hours after lysis of the spheroplasts.

Determination of Molecular Weight of HMWt Species

Globular proteins of known molecular weights covering the fractionation range of the gel were used as molecular weight markers for the construction of a calibration curve. The gel matrix of Sepharose 4B has a fractionation range of 6 x 10^4 to 20 x 10^6 molecular weights for proteins. Molecular weight markers of a range

of 1.2×10^4 to 2×10^6 were used to prepare the calibration curve of the gel filtration column, according to the procedure of Andrews (1964).

The relationship between the approximate molecular weight of proteins and their elution volumes from gel filtration columns, as discussed by Andrews (1964), is described by the equation:

$$V_e = V_o + K_d V_i$$

where V_e is the elution volume, V_o is the void volume (the elution volume of a solute completely excluded from the internal cavities of the gel), V_i is the volume of solvent imbibed by the gel and K_d is the volume fraction of solvent imbibed by the gel which is accessible to a solute.

Gel filtration measures the Stokes' radius of a protein; the radius of a perfect unhydrated sphere having the same rate of passage through the gel as an unknown protein. If unknown and marker proteins are regarded as spherical globular proteins, then in most cases a reasonable estimate of their molecular weight can be made. The molecular weight of an unknown protein can be estimated by plotting $\log_{10}$ molecular weight of marker proteins against their elution volumes. The relationship is linear over a large molecular weight range.

The following proteins of known molecular weight were used as markers: glutamate dehydrogenase (1 015 000), β-galactosidase (520 000), catalase (240 000), aldolase (145 000), serum albumin (67 000) and cytochrome c (12 300). 2 mg of each of the proteins

was applied in 1 ml of elution buffer, to a 1.5 x 35 cm column of Sepharose 4B. Proteins were detected in the eluant by spectrophotometric measurement at 280 nm. Blue dextran (molecular weight approximately 2×10^6) was also used in the calibration.

The column elution profile was used to construct the calibration curve; Figure 3.15, indicating a good linear relationship between $\log_{10}$ molecular weight and elution volume.

By extrapolating the calibration line beyond blue dextran marker, an estimation of the molecular weight of the HMWt species was obtained. The peak of activity for the HMWt species corresponded to a molecular weight of 2.6×10^6. This was consistent with a protein which eluted between PDH and OGDH on gel filtration. The molecular weight of a complex containing one of each of the CAC enzymes detected would be estimated to be approximately 700 000. However it was reported earlier that sonication of the HMWt species revealed a degree of crypticity, such that three times the level of each enzyme was assayed when the complex was broken apart. If three copies of each enzyme are present in the HMWt species, the estimated molecular weight would be approximately 2×10^6. This compares quite well with calculated molecular weight from the calibration curve.

However, the high molecular weight species derived from concentration of low molecular weight fractions was shown to contain one of each of the enzymes of the cycle. This difference should be reflected in the molecular weight, as determined by its elution peak when compared to standards. The molecular weight of this reconstituted HMWt species was determined

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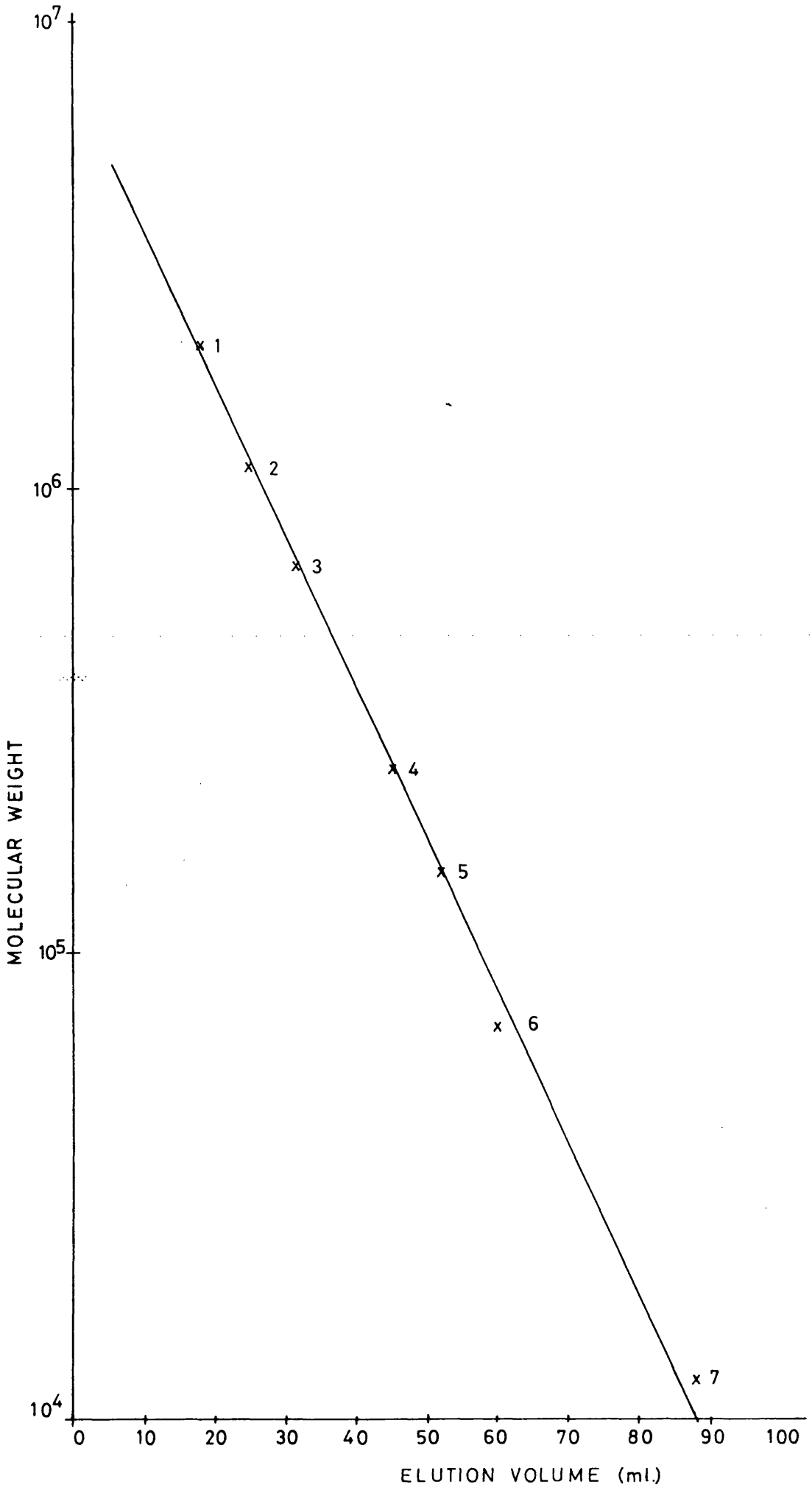
Calibration curve of a Sepharose 4B column

dimensions 1.5 cm x 35 cm marker proteins (2 - 4 mg) loaded

1. Blue dextran
2. Glutamate dehydrogenase
3. Thyroblobulin
4. Catalase
5. Aldolase
6. Bovine serum albumin
7. Cytochrome C

Figure 3.15


10^7
10^6
10^5
10^4
MOLECULAR WEIGHT
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
100
ELUTION VOLUME (ml.)

as approximately 900 000. This figure approaches that estimated. The functional significance of this apparent crypticity will be discussed later.

Inhibition and Inactivation Studies on the CAC Cluster

Inhibition and inactivation studies are used routinely in enzymological investigations. They allow specific parameters of an enzyme to be defined, differences between enzymes, especially isoenzymes to be determined, and comparisons between similar enzymes from different sources to be made.

Inhibition and inactivation studies were made on the isolated CAC cluster as a means of probing the structure and organization of the enzymes. Any differences between enzyme in the HMWt and LMWt species would highlight differences in the associated and dissociated forms of the enzyme.

Thermal Inactivation

The thermal stability of the associated and dissociated forms of CAC enzymes was investigated in a number of species. Samples were pre-incubated at each temperature and further incubated for 5 min. Aliquots (500 µl) were removed and assayed for enzyme activity. A temperature range of 30 - 60°C was used and the results reported in Figures 3.16 and 3.17.

A distinct difference was seen between the thermal stability of the HMWt and LMWt forms of the enzymes. The presence of other enzymes perhaps not surprisingly conferred a degree of stability on the enzyme examined, protecting it against thermal inactivation. At

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Thermal inactivation of CS and MDH comparison of HMWt and LMWt species isolated from lysed *E. coli* spheroplast preparation.

Figure 3.16

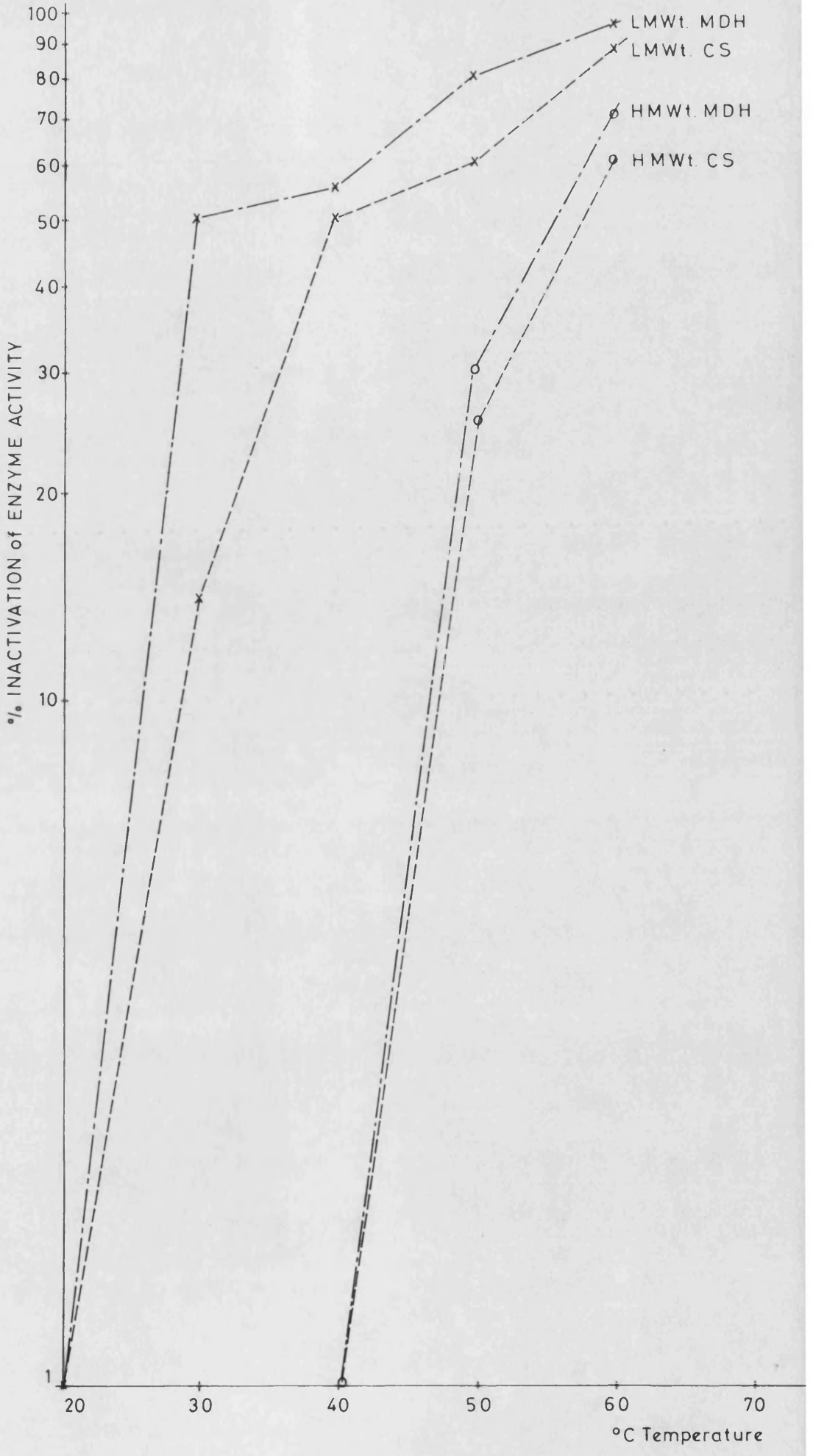
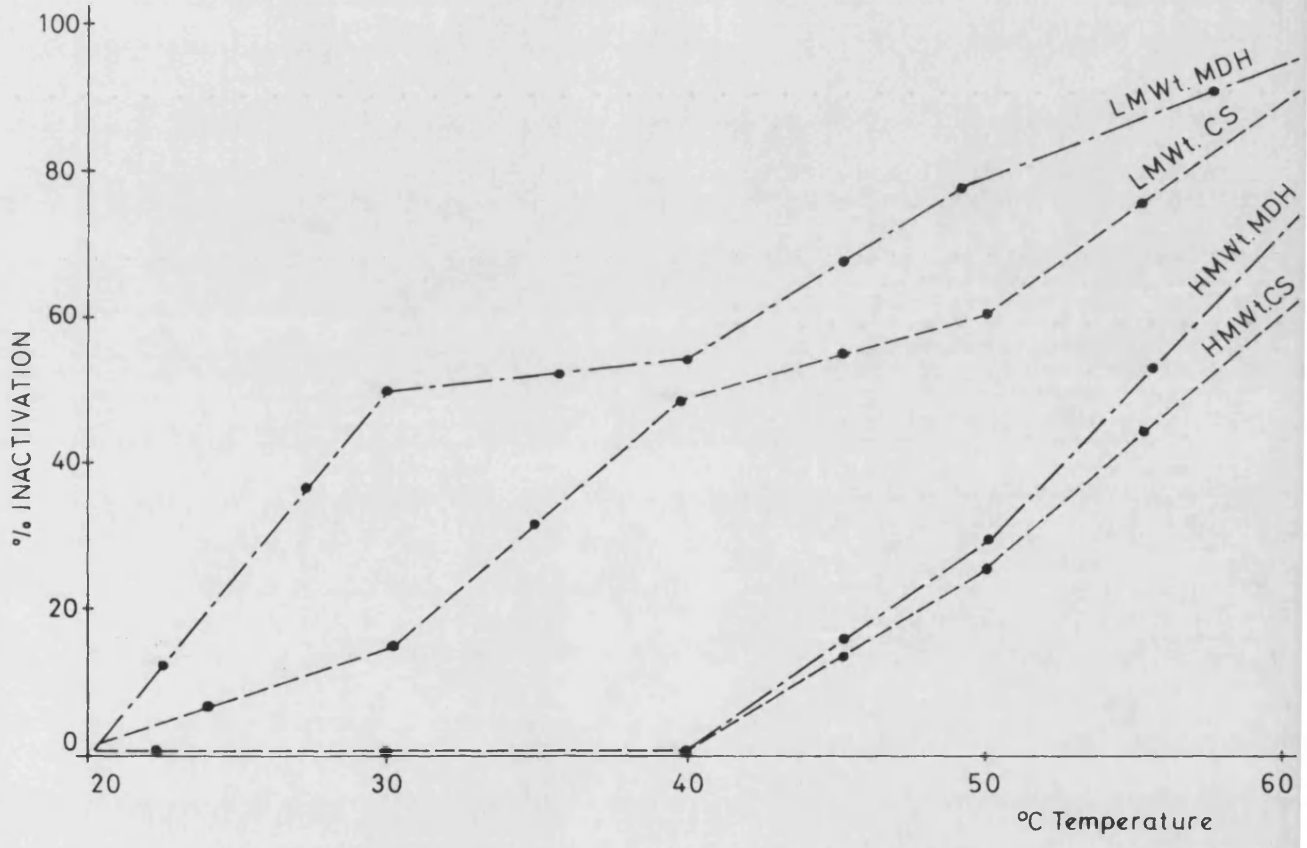

100
90
80
70
60
50
40
30
20
10
1
% INACTIVATION of ENZYME ACTIVITY
LMWt. MDH
LMWt. CS
HMWt. MDH
HMWt. CS
20
30
40
50
60
70
°C Temperature

Figure 3.17

Thermal inactivation of CS and MDH - comparison of HMWt and LMWt from lysed *B. subtilis* spheroplast preparation.



60°C, the free form of *E. coli* CS was inactivated by 90%, whereas the clustered CS was only inactivated by 50%. In *B. subtilis* the associated forms of CS and MDH remained fully active until 40°C, when they were inactivated in a similar pattern. Conversely, the dissociated CS and MDH enzymes were inactivated in a linear fashion from 20°C.

This form of protection against thermal inactivation of the enzyme when it is associated with other enzymes, is facilitated by maintenance of the enzymes' native state by forces of interaction with surrounding proteins which are absent in enzymes in free solution.

Inactivation of CS by DTNB

Modification of sulphydryl groups on CS by the use of DTNB has been used as a means of identifying functional groups in the reaction mechanism of the enzyme. In *E. coli*, DTNB has been found to cause inactivation of CS (Weitzman, 1966).

A series of experiments was carried out to study the effect of DTNB on CS in associated and dissociated forms. Table 3.6 indicates the results of incubating varying concentrations of DTNB with HMWt and LMWt species from a lysed spheroplast preparation of *E. coli*.

CS from the HMWt species was insensitive to DTNB at 0.5 mM concentration whereas the single enzyme (LMWt form) was inactivated to the extent of 60%. The presence of $MgCl_2$ (5 mM) in addition to DTNB (0.5 mM) resulted in inactivation of both forms of the CS enzyme to approximately the same extent. This finding is consistent with the idea that low concentrations of magnesium ions greatly

enhance the rate of inactivation of *E. coli* CS by DTNB, suggesting the possibility that Mg^{2+} ions alter the conformation of the enzyme (Weitzman and Danson, 1976). As expressed in the table, the effect of divalent salt $MgCl_2$ itself appeared to differ between the two forms of CS. The HMWt CS was inactivated by 85% in the presence of $MgCl_2$ (50 mM) and the LMWt CS by 60%. This inhibition has been suggested to be due to chelate formation between the divalent cation and the polyphosphate chain of acetyl-CoA (Faloona *et al.*, 1969).

The difference in inactivation of CS by DTNB suggests that there may be a difference in the accessibility of the enzyme. The orientation of CS in association with other CACenzymes may be such that the part of the enzyme structure which reacts with DTNB is inaccessible to the externally added DTNB. As stated earlier, the complementary surfaces between CS and MDH may be in the region of the NADH-binding site, in Gram negative bacterial CS. It is possible that in the CAC cluster DTNB has restricted access to this site.

The presence of a divalent cation caused an alteration in the conformation of the CS enzyme such that the HMWt species became susceptible to the inactivating effects of DTNB. These results will be discussed later, in the context of the physical association of CAC enzymes.

Table 3.6. DTNB inactivation of *E. coli* citrate synthase

TREATMENT	% INACTIVATION	
	HMWt	LMWt
0.1 mM DTNB	0	0
0.5 mM DTNB	0	60
0.1 mM DTNB + 5.0 mM $MgCl_2$	0	0
0.5 mM DTNB + 5.0 mM $MgCl_2$	30	30
1.0 mM DTNB + 5.0 mM $MgCl_2$	100	100
0.1 mM DTNB + 50 mM $MgCl_2$	85	60

These results express the average readings of three separate experiments.

In each case the protein concentration of the HMWt and LWMt forms were comparable.

Sucrose Density Gradient Centrifugation

Sucrose density gradient (SDG) centrifugation can be used in either a preparative or an analytical way to separate macromolecules by exploiting differences in their sedimentation coefficients.

Sucrose density gradients were prepared by underlaying increasing concentrations of sucrose in 5 ml nitrocellulose tubes, giving a gradient of 10 - 50% (w/v) sucrose in TAG buffer. Samples (200 µl) were overlayed and run as described in Methods Section 3.9.

This technique allowed a comparison between the sedimentation profiles of associated (HMWt) and dissociated (LMWt) forms of the CAC enzymes. The results of SDG centrifugation are given, with experimental conditions in Figures 3.18 and 3.19.

It has been reported later in this study, that the chemical crosslinking agent DMDBP inactivated CS to different extents, depending on whether the enzyme was in the associated or dissociated form. This means of differentiation between the two forms was applied to samples which had been subjected to SDG centrifugation. After the centrifugal spin the gradients were fractionated and assayed for enzyme activity. For each sample, fractions containing CS activity were pooled, dialysed extensively to remove Tris buffer and tested for DMDBP inactivation. At a concentration of 20 $mg.ml^{-1}$ DMDBP, the LMWt sample showed complete inactivation of CS activity. However, the HMWt CS remained active under these conditions with an 8% loss of activity.

Thus ultracentrifugation of the complex of CAC enzymes on sucrose density gradients showed co-sedimentation within a defined

Figure 3.18

Sucrose density gradient centrifugation of HMWt species on 10 - 30% w/v sucrose gradient.

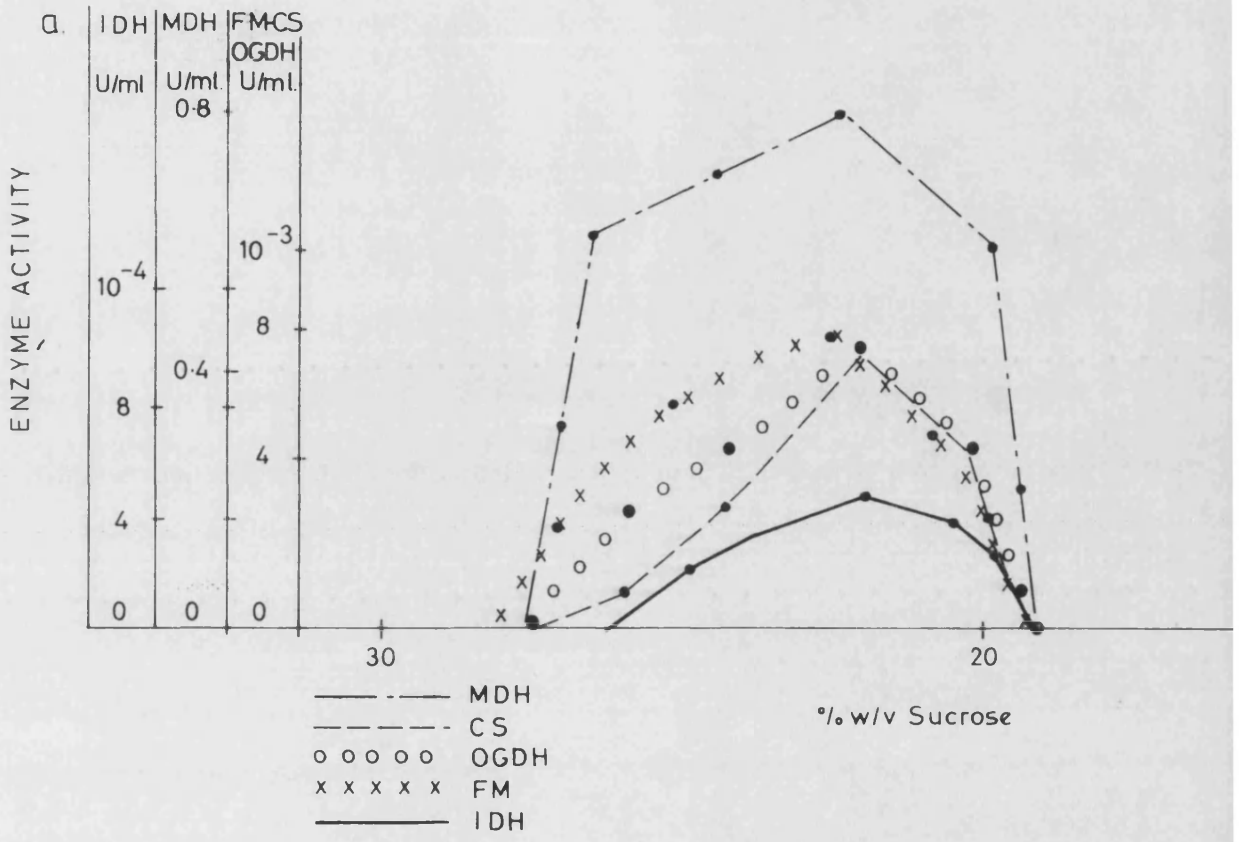
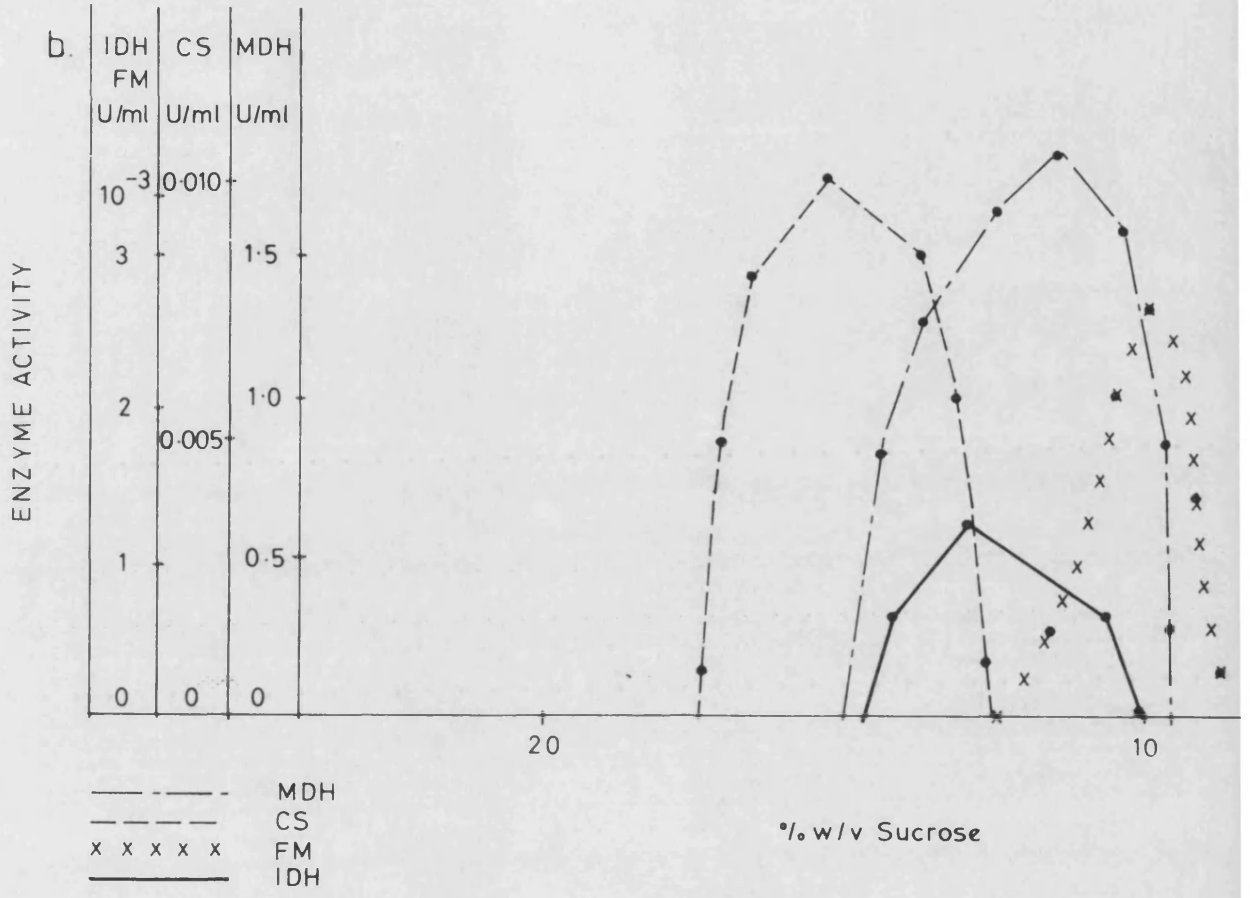


Figure 3.19

Sucrose density gradient centrifugation of pooled sample of LMWt fractions on 10 - 30% w/v sucrose gradient.



band of activity. Conversely, with a sample containing enzymes eluted from gel filtration in the low molecular weight form, analysis showed that each enzyme sedimented to a different extent. The CAC cluster therefore remained as a physical entity on ultracentrifugation and did not dissociate. The high density medium created by the sucrose-buffer solution would have probably prevented dissociation of the cluster by dilution effects. The use of DMDBP as an indicator of the associated state of enzymes confirmed that the inter-enzyme forces were sufficient to withstand dilution on SDG centrifugation.

Enzyme Localization

Examination of fractions from the lysis of spheroplasts

By studying the presence and the level of enzyme activity from the lysis of bacterial spheroplasts, it may be possible to gain some idea of the position of the different enzymes in the cell. The location of the HMWt species *in vivo*, if it exists as such, may also be implied.

Those enzymes released immediately on spheroplast lysis may be regarded as purely cytoplasmic. By washing the pellet resulting from the lysis, a proportion of enzymes were released, which may not only be cytoplasmic components entrapped by membrane, but also may be loosely membrane-bound proteins. Enzymes remaining in the pellet represented integral membrane proteins and those more tightly associated with the membrane surface.

The results expressed in Table 3.7 are from osmotically lysed spheroplasts. The pellet from the lysate, spun at 1 000 g, 10 min,

was washed with 0.01% (v/v) triton and re-sedimented. The activities of enzymes of the CAC were recorded.

Table 3.7. Intracellular location of CAC enzymes

Enzyme activity in supernatant from lysed *E. coli* preparation, spun 1 000 g, 10 min.(S_1); supernatant from washed pellet from S_1, spun 1 000 g, 10 min.(S_2); pellet from S_2 (P_2). Activity in mole.min^{-1}.ml^{-1}.

ENZYME	S_1	S_2	P_2
Fumarase	0.091	0.250	0.205
Malate dehydrogenase	25.44	23.44	16.40
Citrate synthase	2.570	2.200	1.980
Aconitase	0.056	0.059	0.047
NADP-Isocitrate dehydrogenase	1.125	1.500	0.840
Oxoglutarate dehydrogenase	0.063	-	0.280
Succinate thiokinase	0.121	0.088	0.048
Succinate dehydrogenase	-	0.044	0.113

The activity of the CAC enzymes was generally found to be present in all of the fractions. Each of the enzymes, apart from SDH which is known to be an integral membrane protein, were present in S_1. S_1 corresponds to the cytoplasmic component of the cell. Examination of the supernatant (S_2) from the washed pellet from S_1 however, revealed that a substantial level of each of the enzymes

was present. This result was surprising since the CAC enzymes have been regarded for many years as being soluble in the cytoplasm of the cell. The levels of enzyme in S_1 and S_2 were generally comparable, in the case of FM and IDH, the levels were higher in S_2. The pellet P_2 also showed a measurable amount of each of the CAC enzymes.

For so-called soluble enzymes, their presence would not be expected in association with a pellet fraction, which in bacteria would be composed of membrane fragments and higher molecular weight proteins as well as cell debris. The fact that CAC enzyme activity was detected in the pellet fraction may be indicative of enzyme entrapment in membrane vesicles, which are permeable to substrate. Alternatively, it may reflect the intracellular location of these enzymes, near the inner membrane.

Fractions S_1, S_2 and P_2 were analysed by gel filtration for the presence of a HMWt species. Both supernatants S_1 and S_2 showed the presence of a HMWt peak, with the activity of those enzymes identified in the CAC cluster. Comparison of the levels of CS enzyme in S_1 and S_2, measured in the HMWt species as a proportion of the total enzyme activity, showed that twice as much CS was present in the HMWt species of S_2 than in S_1. A greater proportion of the enzyme was therefore found in the loose membrane-bound fraction. It would be unlikely that this activity was due to entrainment of the CAC in selectively permeable vesicles since the fraction had been treated with the detergent Triton X100.

This result may indicate that CS, when it associated with other enzymes as a CAC cluster, may be preferentially located near the inner membrane of the bacterial cell.

Gel filtration of the pellet fraction revealed no HMWt species. This experiment had inherent problems due to the presence of membrane fragments in the sample which provided obvious difficulties on gel filtration.

Based on the findings shown above, the investigation into the intracellular location of the CAC enzymes was continued by examination of wash-out patterns.

Wash-out patterns

An alternative approach to studying intracellular enzyme location was used by Gorringe and Moses (1980) by examination of wash-out patterns. These patterns are drawn up by examination of enzyme levels resulting from successive washing and differential centrifugation of lysed bacterial spheroplasts. The protocol was as described in the legend of Figure 3.20. The histogram shows wash-out patterns from lysed spheroplast membranes of CAC enzymes.

Although a proportion of CAC enzyme activity was released on lysis of the spheroplast, successive washings of the pellet yielded a further release of enzyme activity. If this enzyme, which was present in the initial pellet P_1, was the result of entrainment in semipermeable membrane vesicles, washing of the pellet would give rise to enzyme which was readily released and would leave the proportion of enzyme remaining in the pellet fraction relatively constant. On the other hand, if the proportion of enzyme released into the supernatant, on washing the membrane fragments, varies from enzyme to enzyme, this would reflect a difference in attachment to the membrane of the CAC enzymes. The enzymes would

therefore show different degrees of binding to the inner cytoplasmic membrane of the bacterium.

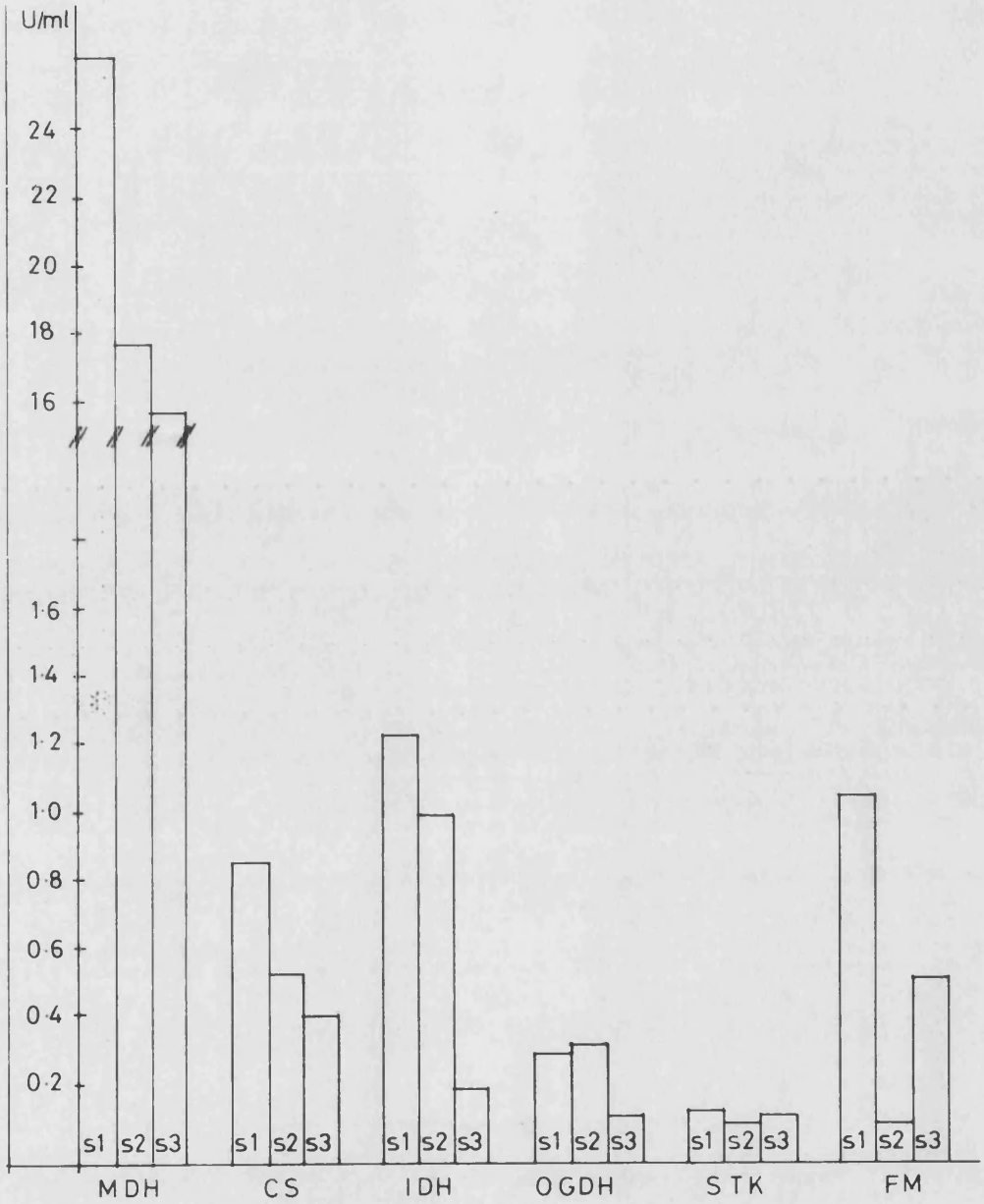
Examination of the histogram in Figure 3.20 reveals that successive washing of the pellet released the CAC enzymes in varying proportions. With each enzyme, the greatest activity was that in the initial supernatant (S_1) reflecting that readily released on lysis. The exception was OGDH where an almost equal amount was washed out in S_1 and S_2. MDH and CS expressed similar wash-out patterns, possibly reflecting a closer association of these two enzymes. IDH activity was more easily washed out of the membrane fragments, the majority being released in S_1 and S_2. Conversely, FM exhibited a tighter degree of binding to the membrane, with approximately one third only of the activity released after two washings of the membrane. OGDH and STK showed fairly similar patterns of enzyme release. This may be a further indication of some form of specific interaction between these two enzymes. The fact that OGDH and STK exhibited a tendency to be released from the washed pellet in similar proportions and ratios, may reflect the fact that they are located at similar positions in the cell, or that they are associated to some extent *in vivo*.

Differential Centrifugation Analysis

Differential centrifugation analysis has been widely used in the study of subcellular localization of enzymes (Hogeboom, 1955). The technique was employed as a means of locating the glycolytic enzymes within the bacterial cell (Mowbray and Moses, 1976; Gorringe and Moses, 1980).

Figure 3.20

Washout patterns of citric acid cycle enzymes from lysed *E. coli* spheroplasts.



Enzyme activities in supernatants from :
lysed spheroplast spun at 1000g (S_1),
resuspended S_1 pellet spun at 1000g (S_2),
resuspended S_2 pellet spun at 9000g (S_3)

Pellets washed with 10mM TAG, pH 7.5

A similar approach was adopted for the investigation of the intracellular organization of the CAC. If no *in vivo* associations between the enzymes existed, one may expect a random differential centrifugation pattern. If such associations were taking place in the cell, however, a pattern of sedimentation may exist. A comparison between the ratio of enzymes sedimented at each stage of the differential centrifugation may reflect co-sedimentation between certain enzymes.

The results of one such experiment are shown in Table 3.8. Repeated examination of the differential centrifugation showed similar patterns.

Examination of the distribution of the cycle enzymes on differential centrifugation showed that no fraction was particularly rich in enzyme activity, instead enzymes appeared to be sedimented in each of the fractions. This perhaps may be viewed as unexpected for enzymes which are commonly referred to as soluble in the cytoplasm of the cell.

Whilst not showing clear cut co-sedimentation, a trend can be detected in the ratios of enzymes sedimented, as a percentage of the total enzyme present. Each of the enzymes of the cycle were seen to be pelleted to varying degrees at each stage of the differential centrifugation. For each enzyme examined, the percentage of enzyme present decreased from the P_2 spin through the P_9 spin to the P_{40} spin. In the P_{150} pellet all the enzyme levels rose simultaneously. The exception to this pattern was FM, where a different distribution was detected. Almost one quarter of the total FM enzyme present was pelleted in the final P_{150} spin,

Table 3.8. Differential centrifugation of lysed spheroplasts from *E. coli* K12 D500. Distribution of citric acid cycle enzymes. Each enzyme is expressed as enzyme activity per ml for each fraction and as % of total activity.

Enzyme	Total Recovered	Pellet Fractions				Supernatant Fractions	
		P_2	P_9	P_{40}	P_{150}	S_1	S_{150}
	mole.min.$^{-1}$ml^{-1}	2000g	9000g	40000g	150000g	1000g	150000g
OGDH	0.95	0.28	0.13	0.09	0.16	0.06	0.07
% total activity		29%	13.5%	9%	16%	6.5%	7%
ADP-STK	1.44	0.26	0.18	0.11	0.16	0.22	0.20
% total activity		18%	13%	8%	11%	15%	14%
FM	4.10	0.79	0.18	0.51	0.97	0.11	0.51
% total activity		19%	4%	12%	24%	3%	12%
MDH	513.00	84.40	30.90	17.80	23.40	75.00	93.75
% total activity		16%	6%	3.5%	5%	15%	18%
CS	16.77	2.87	1.76	1.10	2.44	2.43	2.87
% total activity		17%	10%	3%	15%	14%	17%
NADP-IDH	3.85	0.29	0.23	0.04	0.58	0.336	1.64
% total activity		7%	6%	1%	15%	9%	43%

perhaps tentatively reflecting some association with cellular membrane components.

43% of NADP-IDH activity remained in the supernatant after repeated centrifugation, with low levels sedimenting along with the other CAC enzymes. This may be an indication of two pools of IDH enzyme, one existing in association with other enzymes of the cycle and the other reflecting a soluble or 'free' pool of IDH. Alternatively, and perhaps more realistically, this shows the nature of the IDH enzyme as soluble in the cytoplasm with no membrane-association, with a proportion of the enzyme not freely dispersed but involved with other cellular processes, be they membranous, proteinaceous or enzymological.

Conversely, OGDH shows a greater sedimentation in the P_2 fraction of the centrifugation with low levels of activity remaining in the final S_{150} supernatant. This may be a reflection of the high molecular weight of OGDH (2×10^6 kdal), being sedimented at low speeds or alternatively may be as the result of a tentative association with membrane components.

The data are interpreted with relation to intracellular location of the enzymes; in reiteration, there appears to be a trend in the sedimentation ratios between the enzymes, which may reflect some degree of association between them. These points will be discussed and expanded later.

Use of Mutants in Studies of Enzyme Association

Examination of several bacterial species

The studies up to this point have largely been carried out on

E. coli K12 D500 (wild-type). However, the presence of a HMWt cluster has been investigated in a number of species as shown in Table 3.9.

Table 3.9. Bacterial species in which a cluster of citric acid cycle enzymes was identified.

GRAM POSITIVE	GRAM NEGATIVE
Bacillus subtilis 168	*E. coli* K12 D500
CU1323	K.1.1.r3
	W620
	Acinetobacter calcoaceticus 4B
	4B5
	Pseudomonas aeruginosa 1978

The use of bacterial mutants to define the associations between CAC enzymes

A series of mutants of wild-type *E. coli* are available which may give an indication of the nature of the association between the CAC enzymes seen in the previous work. If, for example, CS were vital to the organization of a weakly bonded complex, then one may expect that a lysed protoplast preparation of a CS negative mutant may not yield a HMWt species, if the mutation was as the result of a deletion of that protein from the cell repertoire.

A strain of *E. coli* which is deficient in CS is unable to grow on glucose as a sole source of carbon and has a requirement for glutamate. Glutamate feeds into the CAC at oxoglutarate ensuring effective functioning of electron transport and that essential amino acids continue to be formed.

Growth profiles (Figure 3.21) of wild-type *E. coli* K12 D500 and CS negative *E. coli* W620 were established and the cultures harvested at exactly the same phase in their growth curve.

On lysis and subsequent analysis of spheroplasts prepared from these species, a HMWt cluster was detected in the K12 control and also in W620 (CS negative). Activities corresponding to MDH, NADP-IDH and FM were measured in the HMWt cluster. The proportion of enzyme activity of FM and MDH in the HMWt form varied in the two mutants, as illustrated in Figure 3.22. The implications drawn from this experiment are discussed later.

Specificity of association between mutants

To show the specificity of re-association of CS with the HMWt peak from a CS -ve mutant, the LMWt peak of CS activity from wild-type K12 D500 was concentrated and combined with the CS -ve HMWt species. Figure 3.23 shows that CS re-associated into a complex with other enzymes of the cycle. At the peak of HMWt activity, 3% of total CS activity became associated whilst the remainder of the enzyme eluted later.

The procedure was repeated using eukaryotic CS (from a commercial preparation from pigeon breast) and CS -ve HMWt. Figure 3.24 shows the apparent lack of species specificity of association between CS and

Figure 3.21

Growth profiles of *E. coli* K12 D500 wild type and *E. coli* W620, CS negative.

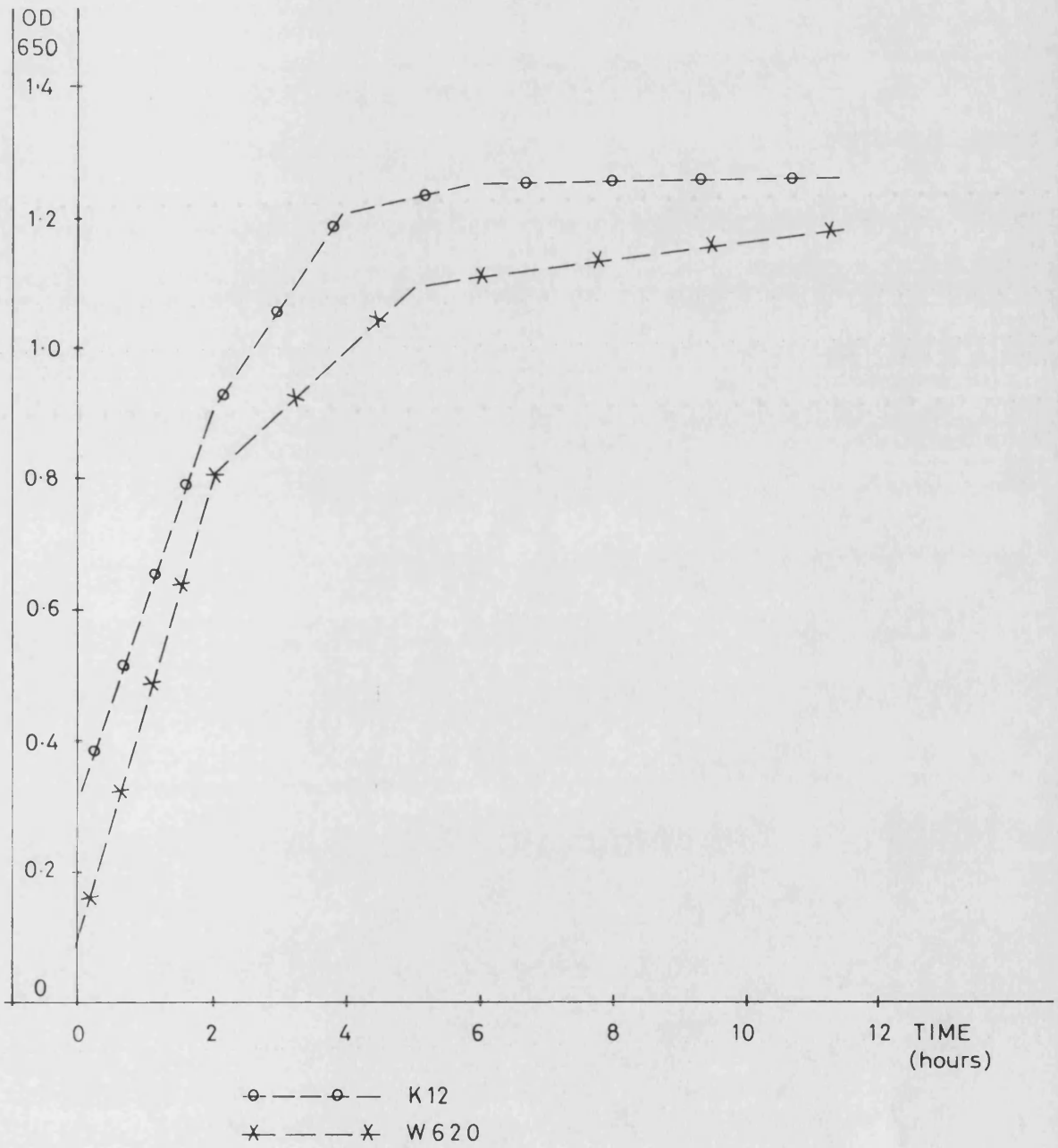
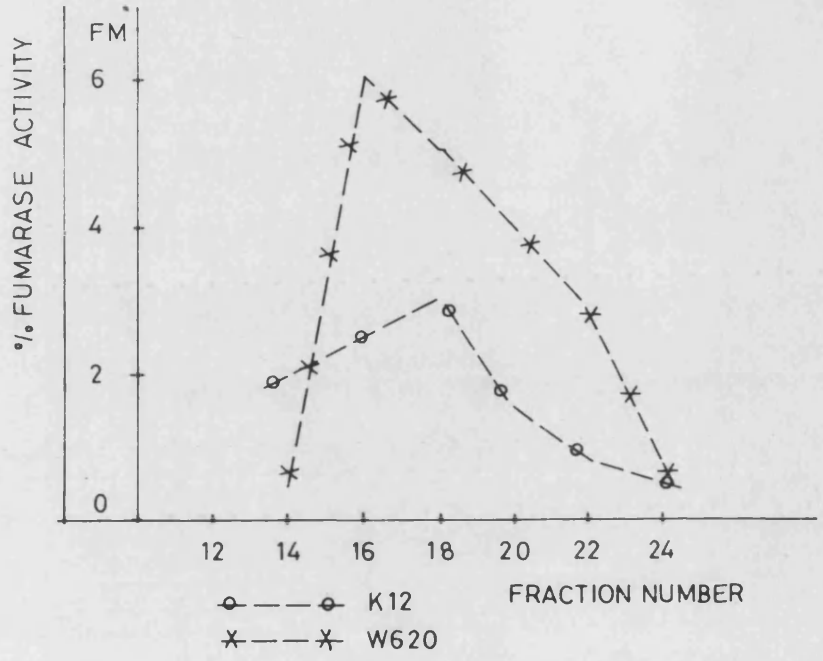
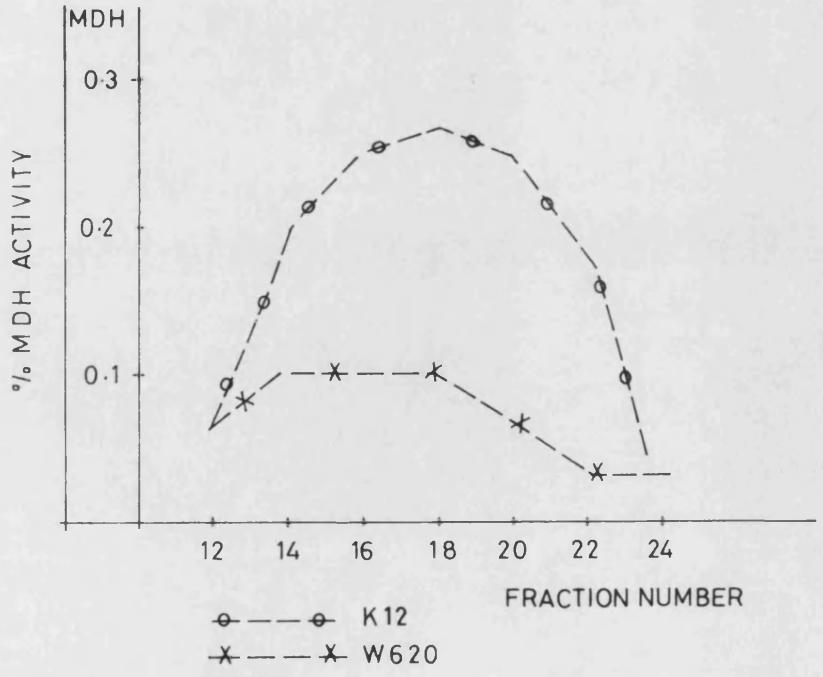


Figure 3.22

Levels of enzyme in HMWt species of wild type *E. coli* K12 D500 and CS negative *E. coli* W620.

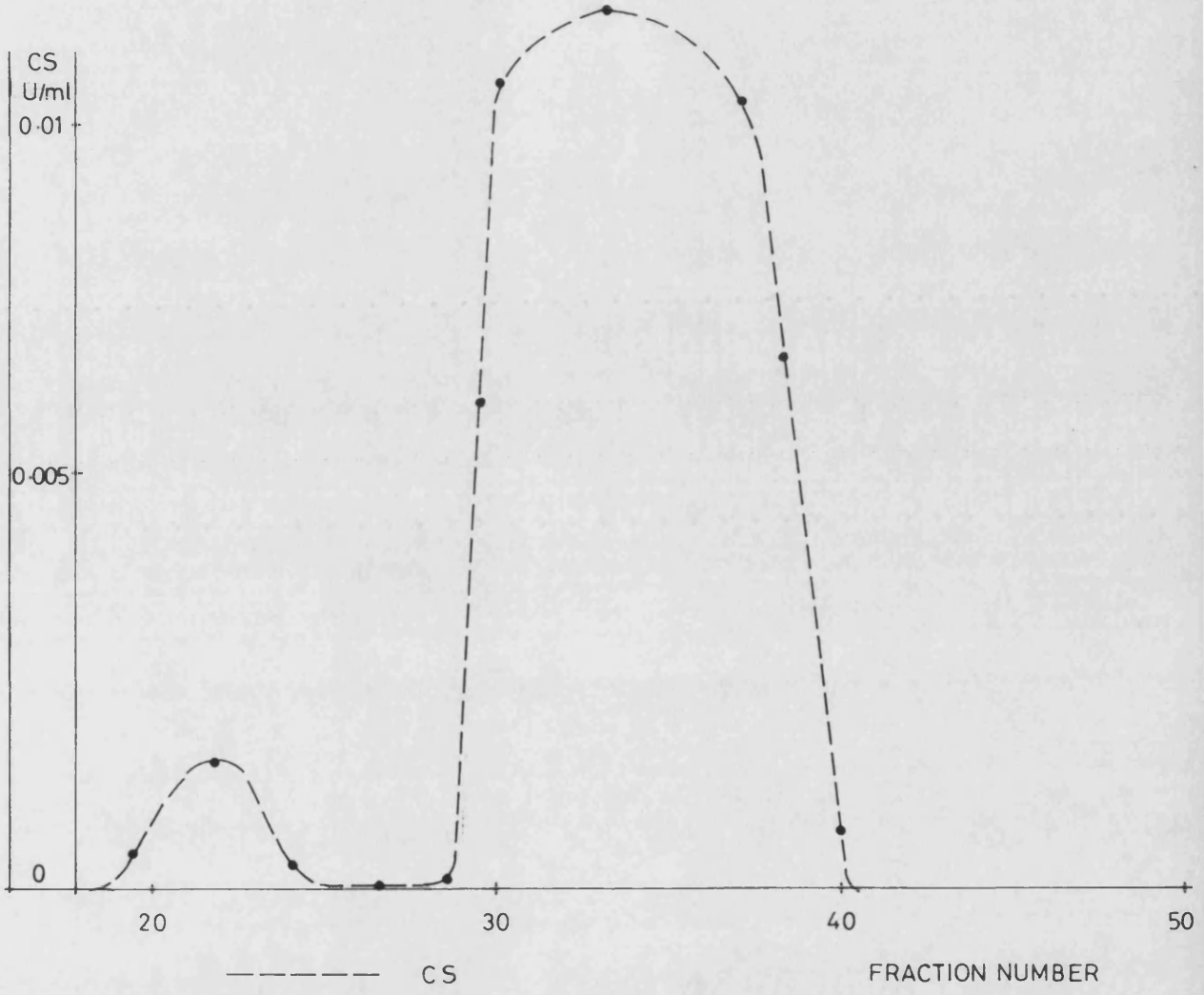




Gel filtration on Sepharose 4B, 10 mM TAG, pH 7.5 at 4° C.

Figure 3.23

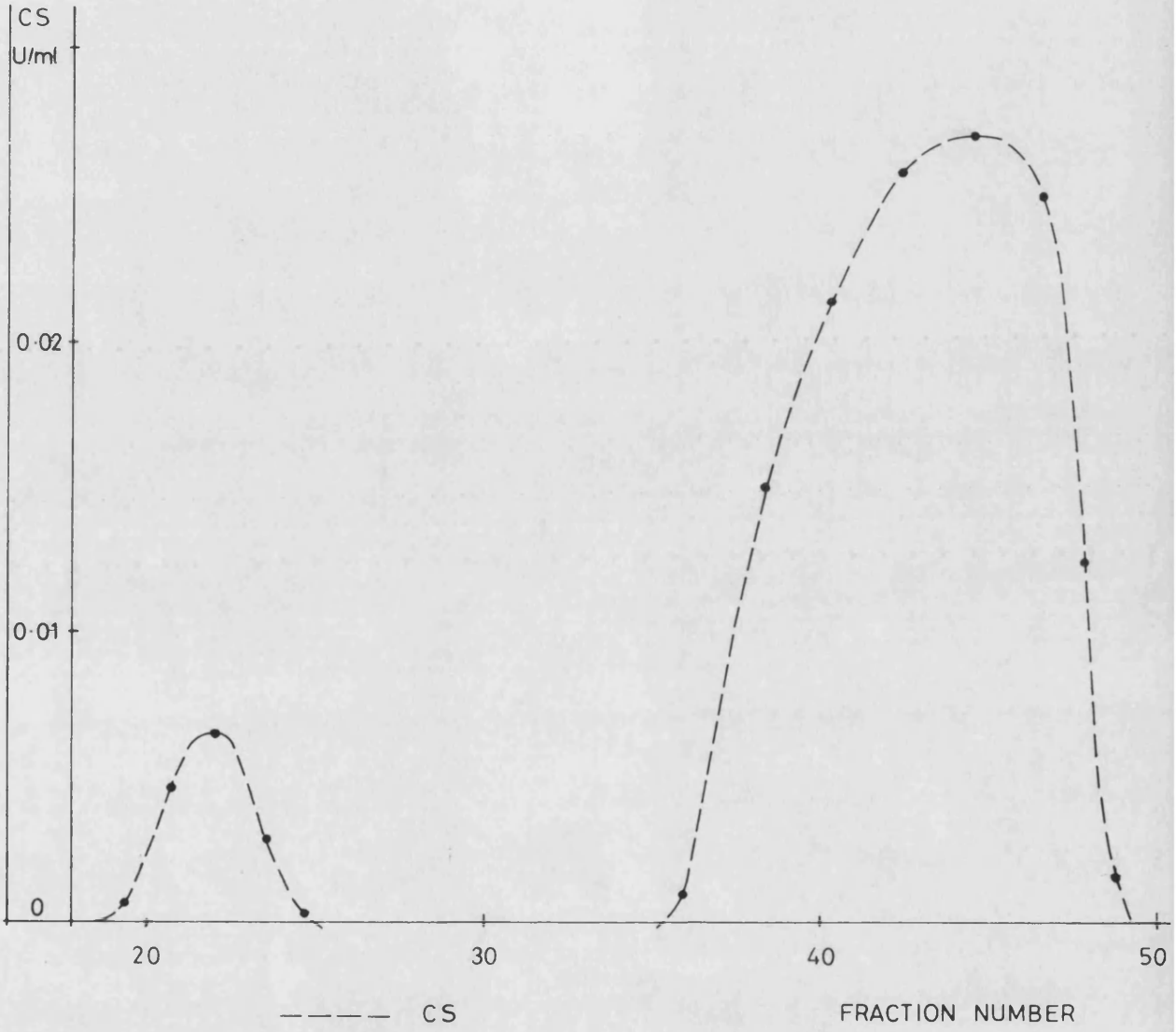
Re-association of CS from wild type *E. coli* with HMWt species from CS negative *E. coli* W620 mutant



Gel filtration on Sepharose 4B with 10 mM TAG, pH 7,5 at 4°C.

Figure 3.24

Re-association of 'small' CS from eukaryotic source with HMWt species from CS negative *E. coli* W620 mutant.



Gel filtration on Sepharose 4B with 10 mM TAG, pH 7.5 at 4°C.

the CS -ve HMWt peak, with 5% of total CS activity at the peak HMWt fraction. This lack of species specificity will be expanded later.

The revertant mutant *E. coli* K 1.1r3 possesses a 'small' (Gram positive type) CS with a molecular weight of around 100 000. A preparation of this mutant was used to see whether an association is possible between this type of CS and other enzymes of the cycle derived from a wild-type *E. coli* (possessing the large Gram negative type CS, with a molecular weight of approximately 250 000). Figure 3.25 illustrates that the size of CS does not appear to be important to any association, the regions of bonding to the other enzymes remains constant despite differences in molecular structure, size and shape. It may be concluded that the complementary surfaces in 'large' and 'small' type CS are essentially similar.

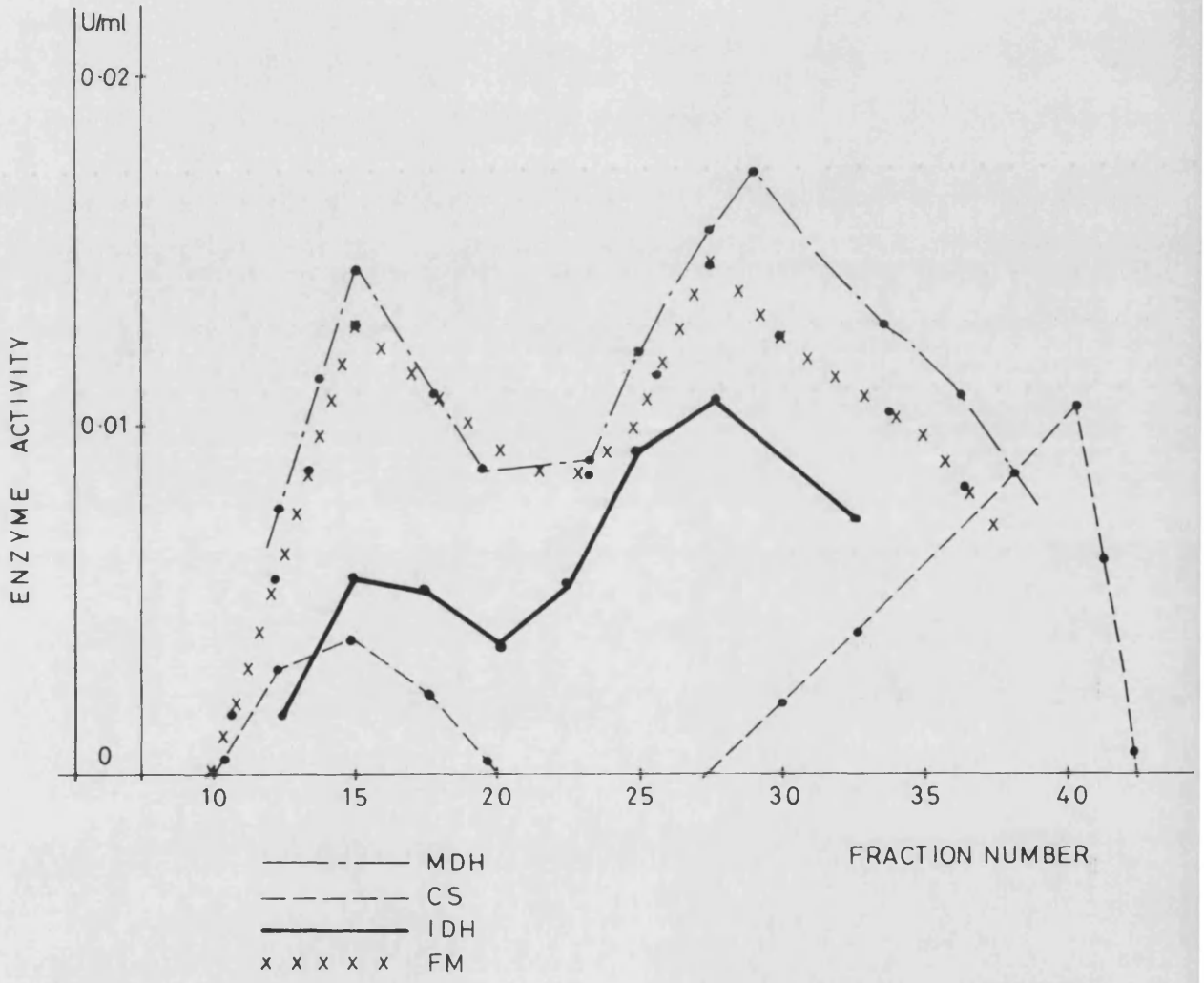
Immunochemical Study of *E. coli* Mutant W620

The bacterial mutant *E. coli* W620 has been shown to be CS-negative. In order to determine whether the lack of CS activity was due to a deletion of the protein from the enzyme repertoire of the cell or due to an enzymically inactive protein, a bacterial extract of W620 was studied immunochemically.

The single radial immunodiffusion technique was set up using wells cut into an agarose support. Anti *E. coli* CS IgG was used as antibody. Cross-reactivity was viewed as an arc formed mid-way between the well containing antibody and that containing the W620 extract (antigen). The arc is formed as the result of precipitation on complex formation between the antibody and antigen, visualized using Coomassie blue staining.

Figure 3.25

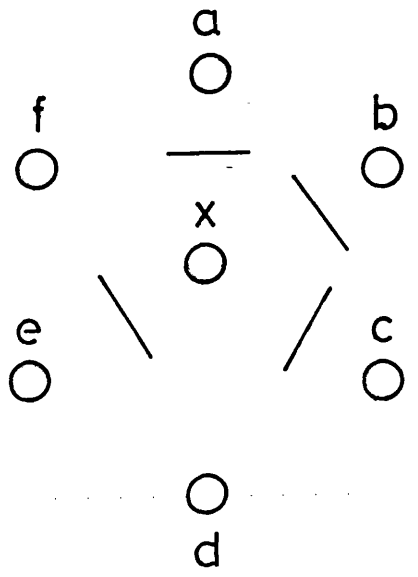
Chromatography of lysed spheroplast preparation of *E. coli* K1.1-3 mutant, possessing 'small' type CS.



Gel filtration on Sepharose 4B with 10 mM TAG, pH 7.5 at 4°C.

The diagram below shows cross-reactivity between anti *E. coli* CS antibody and antigen.

Single radial immunodiffusion of CS and anti-CS antibody.



Key: a 10 µl *E. coli* W620 extract

b 10 µl 1/10 dilution *E. coli* W620 extract

c 10 µl 1/100 " " "

d 10 µl 1/1000 " " "

e 10 µl 1/100 *E. coli* wild-type

f 10 µl normal rabbit serum (control)

x anti *E. coli* CS IgG antibody (raised in rabbit)

The results show that the antibody recognised specific epitopes on the surface of a protein in the W620 extract. It may be presumed that the immunochemical cross-reactivity was a result of the presence of an enzymically inactive, but structurally intact, CS protein interacting with the anti-CS antibody.

Enzyme association and cell ageing

The use of bacteria as a model system in which to study the organization of the CAC means that the environment in which the cells are grown may be manipulated and any subsequent alteration to the metabolic state of the enzymes observed.

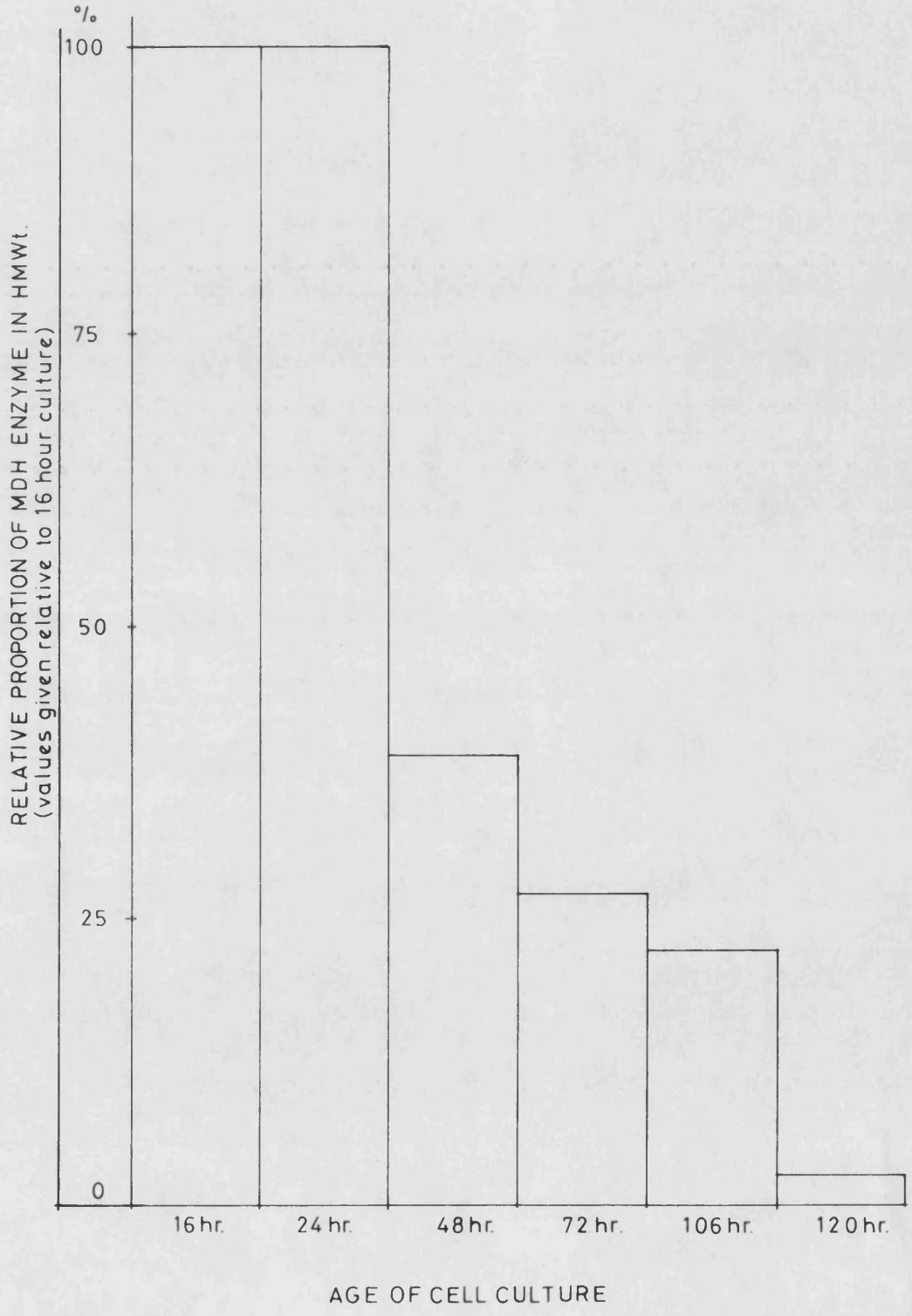
The effect of the age of the cell in culture on enzyme organization was examined over a time scale of 120 hours. A series of culture flasks was inoculated simultaneously with *E. coli* and cultured under standard conditions. At given time intervals the cultures were taken and spheroplasts prepared, using the lysozyme-EDTA method, lysed and run on gel filtration. The presence of a HMWt species was noted and the level of enzyme present related to the total enzyme level. Figure 3.26 illustrates the proportion of enzyme association as the cell ages. The values were compared to that for a bacterial culture of 16 hours, used in the standard spheroplast preparations. The proportion of enzyme association decreased with the period of culture of the cells.

In batch culture, bacterial growth follows a defined growth path. The growth phase of the bacterial culture can therefore be related to the degree of association of certain CAC enzymes.

As the bacterial culture progressed from late stationary phase into exponential death phase, the proportion of enzyme present in an organized CAC cluster decreased. This may be an indication that the association of CAC enzymes only exists in actively growing bacterial cells; this requirement being unnecessary when active metabolism is not taking place. Thus enzyme organization of such a metabolic pathway may only be manifested in certain growth phases

Figure 3.26

Relationship between enzyme association and cell age.



of the cell. Enzyme organization and cell ageing will be discussed later.

Enzyme associations in mitochondria

As discussed in the Introduction, a considerable amount of research has been carried out in the area of metabolic compartmentation in the mitochondria of eukaryotic cells. Associations between specific CAC enzymes have been reported and the existence of the cycle as a multienzyme complex has been proposed by Srere (1976).

The use of gentle isolation techniques was applied to mitochondria in order to detect whether the observations made in bacterial cells were also seen in the eukaryotic cell.

Mitochondria were prepared from rat liver and gently lysed over 30 min in TAG 10 mM, pH 7.5. The supernatant containing both matrix and periplasmic enzymes was eluted from a Sepharose 4B gel filtration column. This gel filtration revealed the presence of several CAC enzymes which co-eluted with OGDH and PDH. These enzymes (FM, MDH, CS, AC, and NAD-IDH) show a further elution peak at their predicted fraction number (Figure 3.27). The presence of these enzymes in a HMWt form may have been due to their attachment to fragments of inner mitochondrial membrane or may be the result of an association between these enzymes similar to that seen in bacteria.

NAD-IDH is thought to be specific to the matrix of mitochondria but was found with its isoenzyme NADP-IDH in the HMWt peak. However, in the HMWt peak, the percentage of NAD form was far

greater (8%) than the percentage of NADP form (2%), when compared to their total enzyme levels. The NAD-IDH was stimulated by ADP.

The low molecular weight peak fractions were pooled, concentrated by ultrafiltration and analysed by gel filtration in order to see if enzymes prepared in this way are capable of re-association and to see whether the presence of these enzymes in the HMWt peak was due to their attachment to a high molecular weight entity or a membrane fragment. The elution profile of CS and MDH showed that a proportion of the LMWt form of the enzymes had converted to the HMWt species and eluted some 20 - 40 fractions ahead of the single enzymes. Only NAD-IDH was detected in the HMWt species from concentration of LMWt fractions.

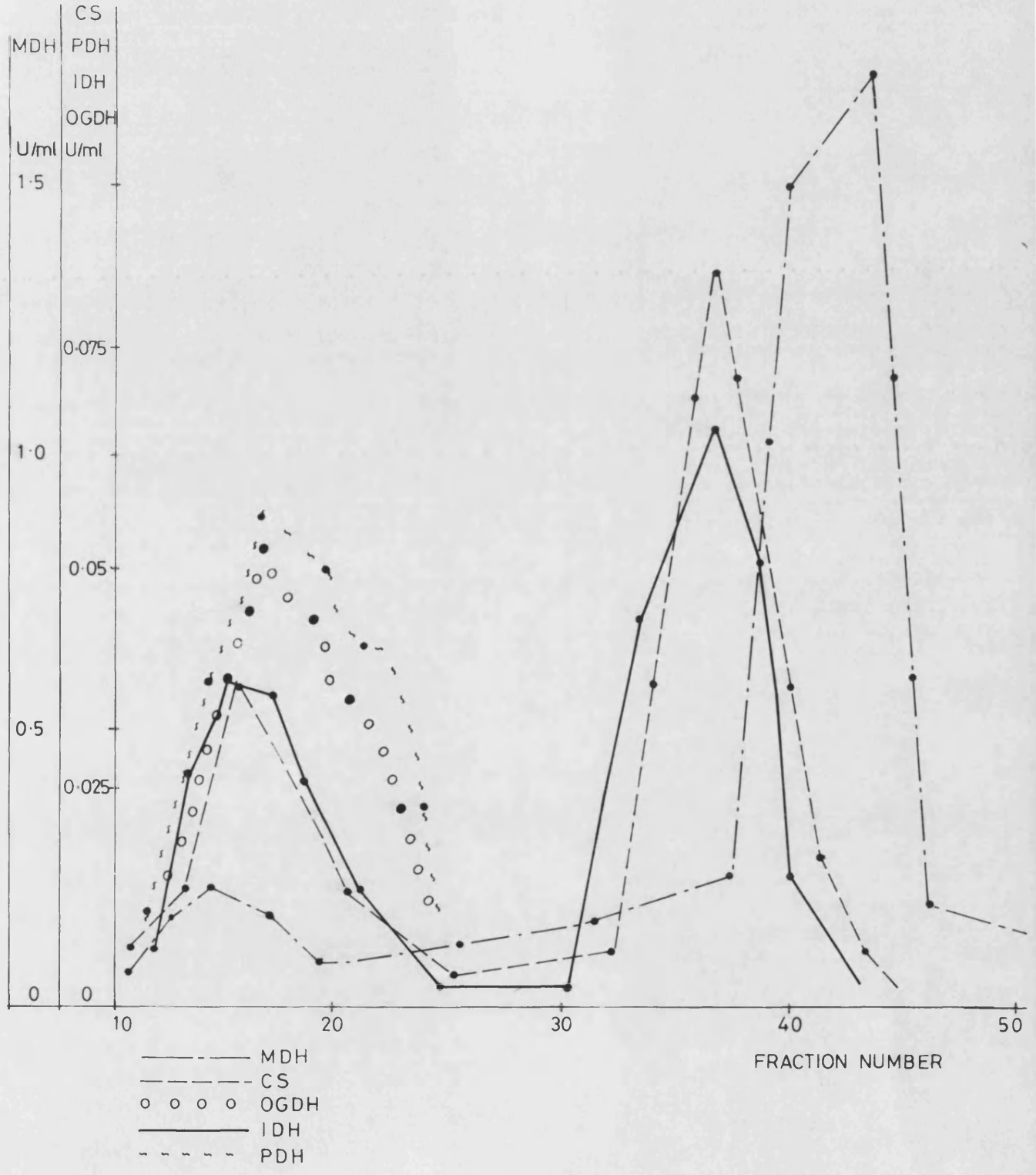
An alternative method to examine the role of membrane in the presence of CAC enzymes in the HMWt fractions was to lyse the mitochondria in the presence of 10 mM TAG, pH 7.5 + 0.01% (v/v) Triton X100. This detergent would remove membrane fragments in the supernatant. Gel filtration of this preparation again showed a defined HMWt species, containing activities of the enzymes as detected in the preparation without Triton X100.

The dependence of these observations on the use of freshly prepared mitochondria was highlighted when a sample of mitochondria, which had been stored frozen, was examined. It appears that freezing the tissue causes irreparable damage to the fine infrastructure of the cell resulting in the destruction of the weak associations between enzymes which have been observed.

In addition to rat liver, mitochondria were also prepared from bovine heart, locust flight muscle and etiolated barley. With all

Figure 3.27

Gel filtration of lysed mitochondria (rat liver) on Sepharose 4B 10 mM TAG, pH 7.5 at 4°C.



these preparations the enzymes of the CAC were seen to co-elute as a HMWt peak. A CAC cluster was therefore identified in four, quite distinct, mitochondrial sources.

Inhibition of Enzyme Activity by Antibody

Polyclonal antibodies have been used in topographical studies on proteins. Antibody raised against a specific enzyme will interact and bind to that enzyme at defined epitopes on the surface of the enzyme. The predominant type of antibody will be raised against hydrophilic regions on the enzyme. In a polyclonal antibody preparation there may be a variety of different antibodies raised against different epitopes on the protein antigen.

Having detected cross-reactivity between antibody and enzyme by a simple method such as single radial immunodiffusion, the influence of the antibody on enzyme activity was investigated.

Pooled HMWt fractions containing the activities of the CAC enzymes, and LMWt fractions of the corresponding second elution peaks were examined. Antibody raised against both *E. coli* CS and *Acinetobacter calcoaceticus* CS (4B), raised in rabbit, was used in this study. Antibody was isolated by ammonium sulphate precipitation, as detailed in Methods 2.18.2. CS levels in HMWt and LMWt samples were assayed and diluted in TAG such that enzyme levels were within a similar range.

50 µl of enzyme sample were incubated with 10 µl of antibody diluted to the appropriate concentration in PBS for 5 min, at room temperature and then placed on ice. The resulting enzyme activity

was plotted against the antibody/antigen ratio, as shown in Figure 3.28.

With both species, the CS enzymes from HMWt and LMWt were inhibited to different extents. The single CS enzyme was inactivated at lower concentrations and to complete inactivation. When CS was organized with other enzymes of the CAC, inhibition occurred at higher Ab/Ag ratios and the enzyme was not completely inhibited, even at relatively high Ab/Ag ratios. Normal serum, containing a range of non-specific antibodies, exerted no influence over CS activity.

It appears that antibody raised against CS from either *E. coli* or *Acinetobacter calcoaceticus* bound to the enzyme in such a way as to influence the activity of the enzyme. This influence may have been manifested by holding the enzyme in such a way that conformational changes essential to the effective functioning of the enzyme were prevented. Alternatively, the binding of antibody close to the active site may have meant that steric considerations prevailed.

When CS exists in association with other enzymes, as distinct from when it is free in solution, two observations can be made. These will be expanded on in the Discussion section.

First, the results indicate that the HMWt species isolated from the gel filtration of a gently lysed preparation of spheroplasts was not contained within a type of membrane-bound vesicle. If this were the case then the antibody would be unable to enter and exert any inhibitory effects. The HMWt species therefore existed as an entity itself, held together by the forces between the enzymes or

Inhibition of CS enzyme activity with

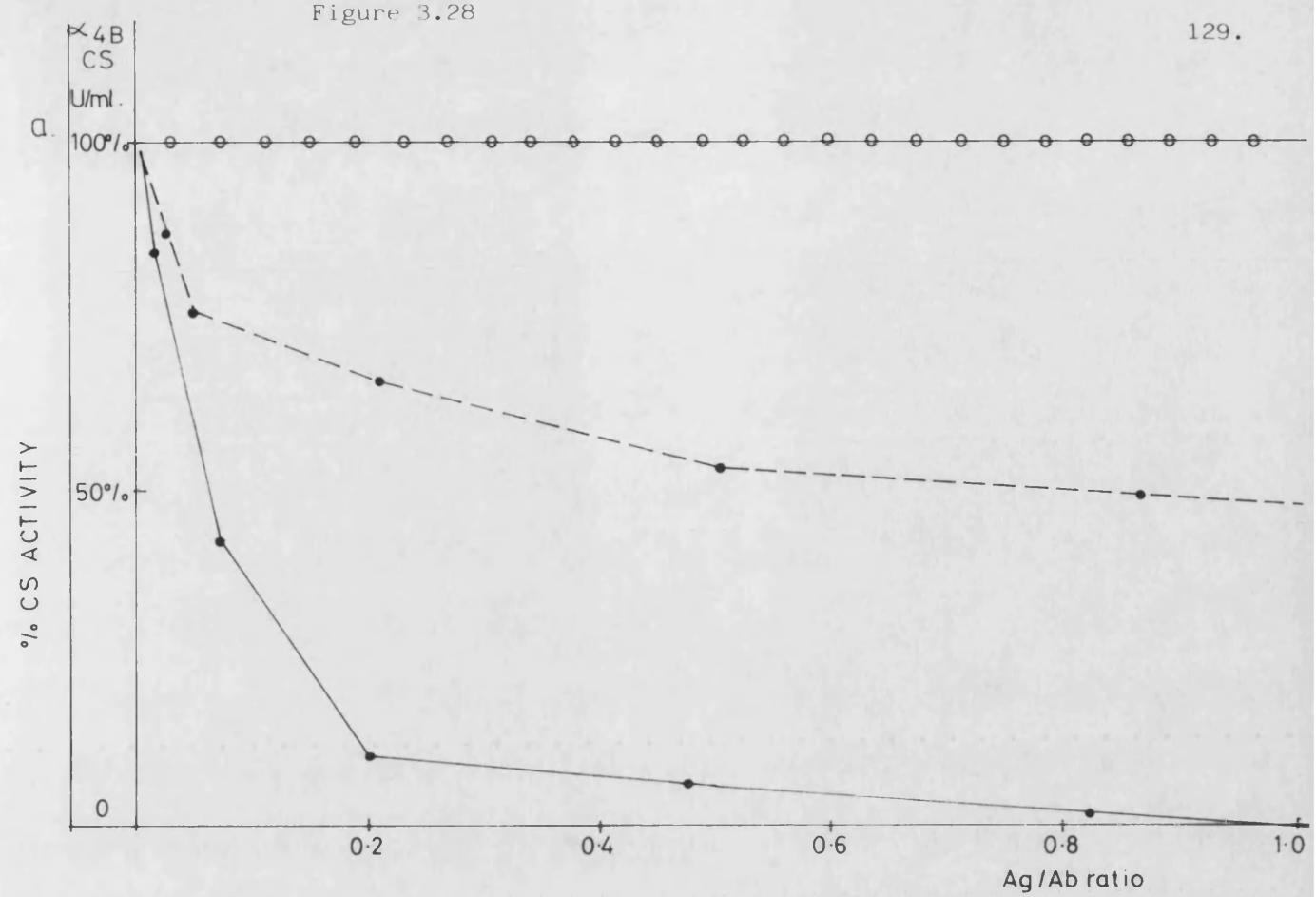
polyclonal antibody

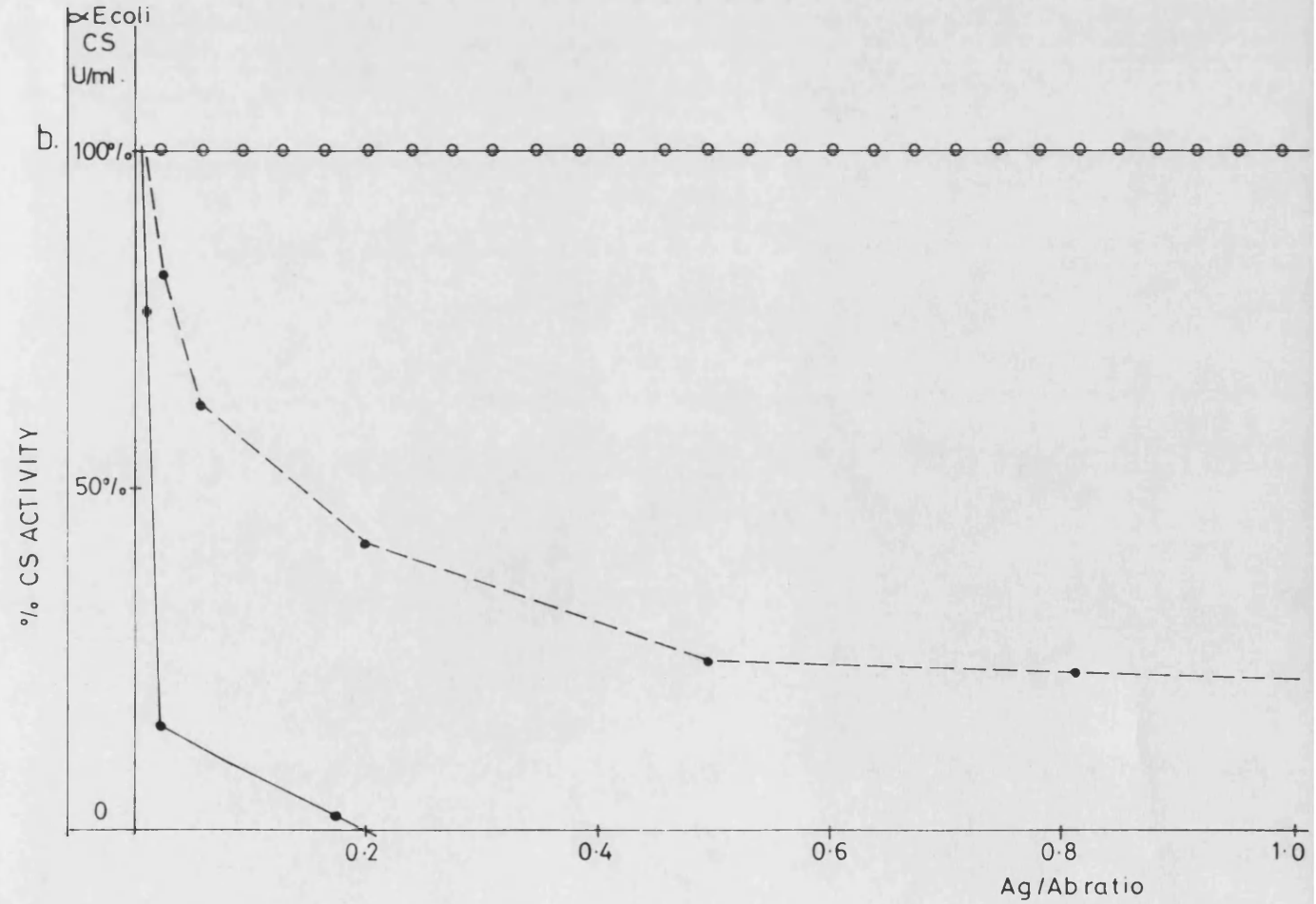
a. anti-4B CS antibody

b. anti-*E. coli* CS antibody

HMWt (— — •); LMWt (—•—); normal serum (—o—o—).

Figure 3.28


a.
∝4B
CS
U/ml.
100%
50%
0
% CS ACTIVITY
0·2
0·4
0·6
0·8
1·0
Ag/Ab ratio


b.
∝Ecoli
CS
U/ml.
100%
50%
0
% CS ACTIVITY
0·2
0·4
0·6
0·8
1·0
Ag/Ab ratio

another moiety rather than existing as a unit due to physical restraint.

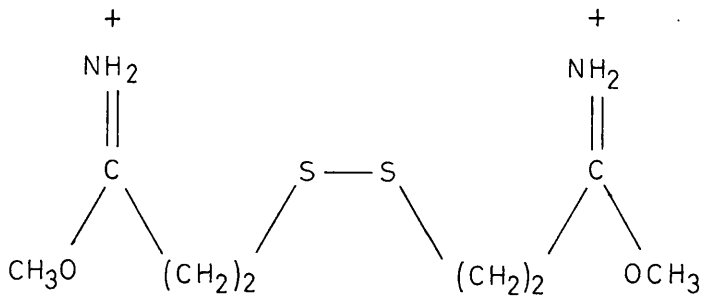
Second, the fact that CS activity was inhibited to a lesser extent in the HMWt than in the single enzyme suggests that there is some access restriction to the antibody binding site. This may be because the enzyme was oriented in such a way that some sites were shielded from the effects of antibody, possibly by the presence of other enzymes existing in association with CS. This is borne out by the observation that the CS enzyme was never completely inactivated by antibody, even at relatively high Ab/Ag ratios.

CROSS-LINKING STUDIES

Covalent modification of proteins using bifunctional reagents for chemical cross-linking

Cross-linking reagents have proved to be useful tools in the study of structure and organization of proteins and biological membranes (Peters and Richards, 1977; Ji, 1979; Freedman, 1979). Cross-linking reagents are composed of two linked reactive groups which are open to attack from nucleophilic groups on proteins, such as thiols and amines (Freedman, 1979). The development of commercially available cleavable cross-linkers such as dimethyl 3,3'-dithiobis-propionimidate allows a cross-linked product to be reconverted into its original, if modified, form. This may allow the analysis of complex mixtures of individual components as well as determining restrictive changes induced by a cross-linker.

The structure of the cross-linker used in this study: dimethyl 3,3'-dithiobis-propionimidate-2HCl (DMDBP) is as follows:



The ability of cross-linker to "freeze" a protein in a given conformation was exploited. The effect of DMDBP on preparations from *E. coli*, *B. subtilis* and *Acinetobacter calcoaceticus* were examined. It has been reported that the bisimidoester DMDBP inactivated CS from *Acinetobacter calcoaceticus*, such that enzyme

activity was 50% inhibited by concentrations of DMDBP of 16.2 mM (Lloyd, 1985).

Through an attempt to study the effect of cross-linkers on the HMWt complex of enzymes from the CAC, a clear distinction was made between the associated and dissociated forms of CS. It was noted that whereas 20 mg ml^{-1} DMDBP caused over 60% inactivation of the "free" CS enzyme from *E. coli*, when this enzyme was associated with the other enzymes in the HMWt form, there was a markedly reduced level of inactivation. These results are expressed graphically in Figure 3.29.

The effect of sonication on weak associations between enzymes in the complex was highlighted by the use of DMDBP. As Figure 3.29 shows, when the HMWt species was sonicated it became susceptible to inactivation by DMDBP, illustrating that sonication must have altered some conformational or structural aspects of the association, allowing for access of the inactivator to its site of action.

Variations in pH of an isolation medium have been noted to influence enzyme associations, as reported earlier and shown in Figure 3.30 . These effects have been shown again using DMDBP inactivation of CS, following the protocol given on page 133.

SDS-Polyacrylamide gels were used as a means of detecting the effectiveness of cross-linker. Figure 3.31 shows that DMDBP was effective as a cross-linker of the HMWt species despite the fact that it had not inactivated the CS enzyme. The untreated enzyme preparations were used in comparison for the detection of an altered band profile on treatment with cross- linker. The disappearance or decrease in low molecular weight bands

Experimental Protocol

The use of cross-linking agent DMDBP to show the effect of a change in pH on the associations between the citric acid cycle enzymes.

I. HMWt peak isolated by running lysed *E. coli* speroplasts on Sepharose 4B in 10 mM triethanolamine, pH 7.5

↓ +DMDBP 20mg ml^{-1}

HMWt peak run on G25 Sephadex gel filtration equilibrated with 10 mM triethanolamine, pH 8.9

↓

II. Eluant pooled and concentrated. Aliquot applied to G25 equilibrated with 10 mM triethanolamine, pH 7.5

↓

III. Eluant pooled and concentrated.

SAMPLES	I	:	HMWt, pH 7.5
	II	:	HMWt, pH 8.9
	III	:	HMWt, pH 7.5
	IV	:	LMWt, pH 7.5

Figure 3.29

Inactivation of CS activity by DMDBP.

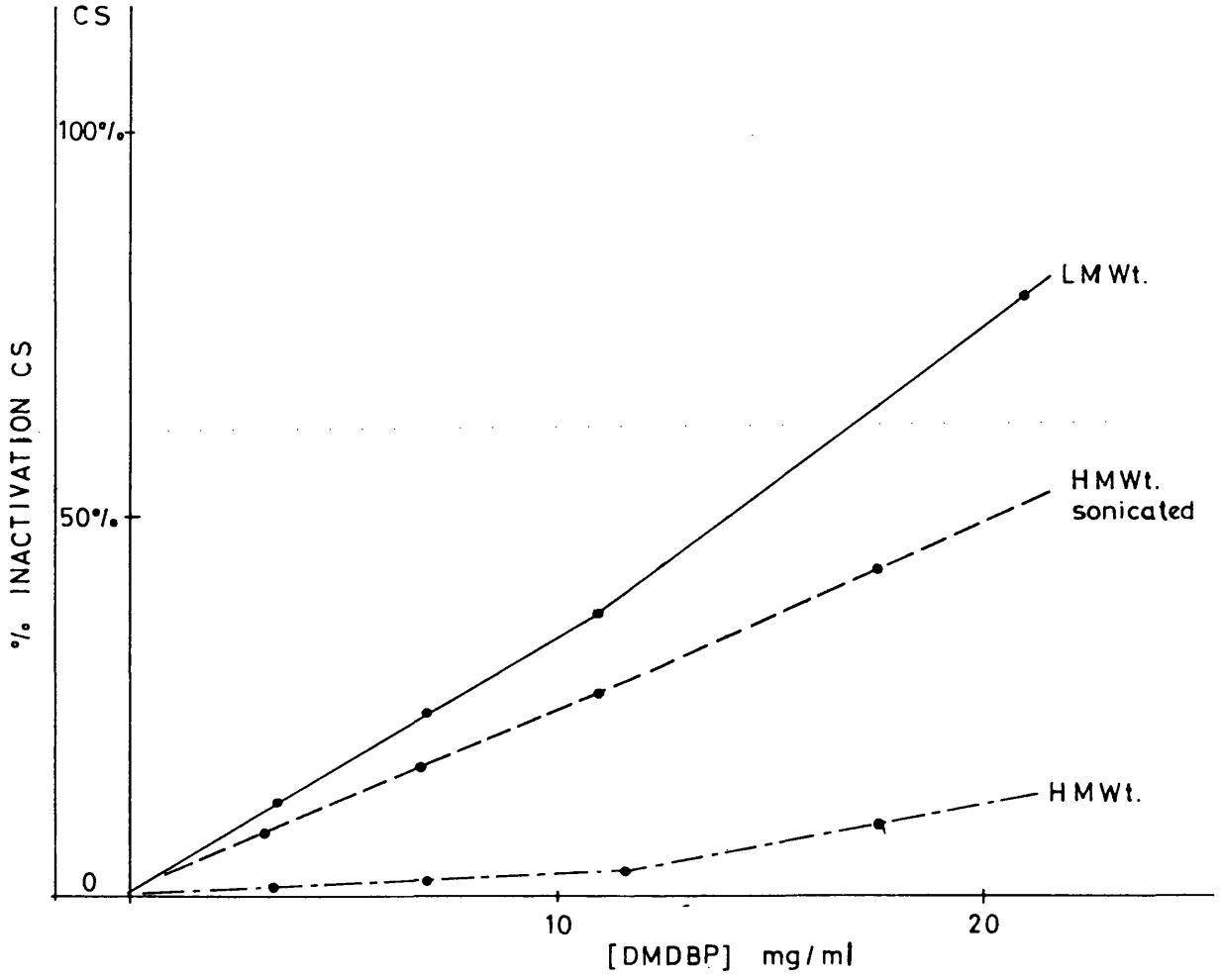


Figure 3.30

Inactivation of CS activity by DMDBP:

effect of pH on enzyme associations.

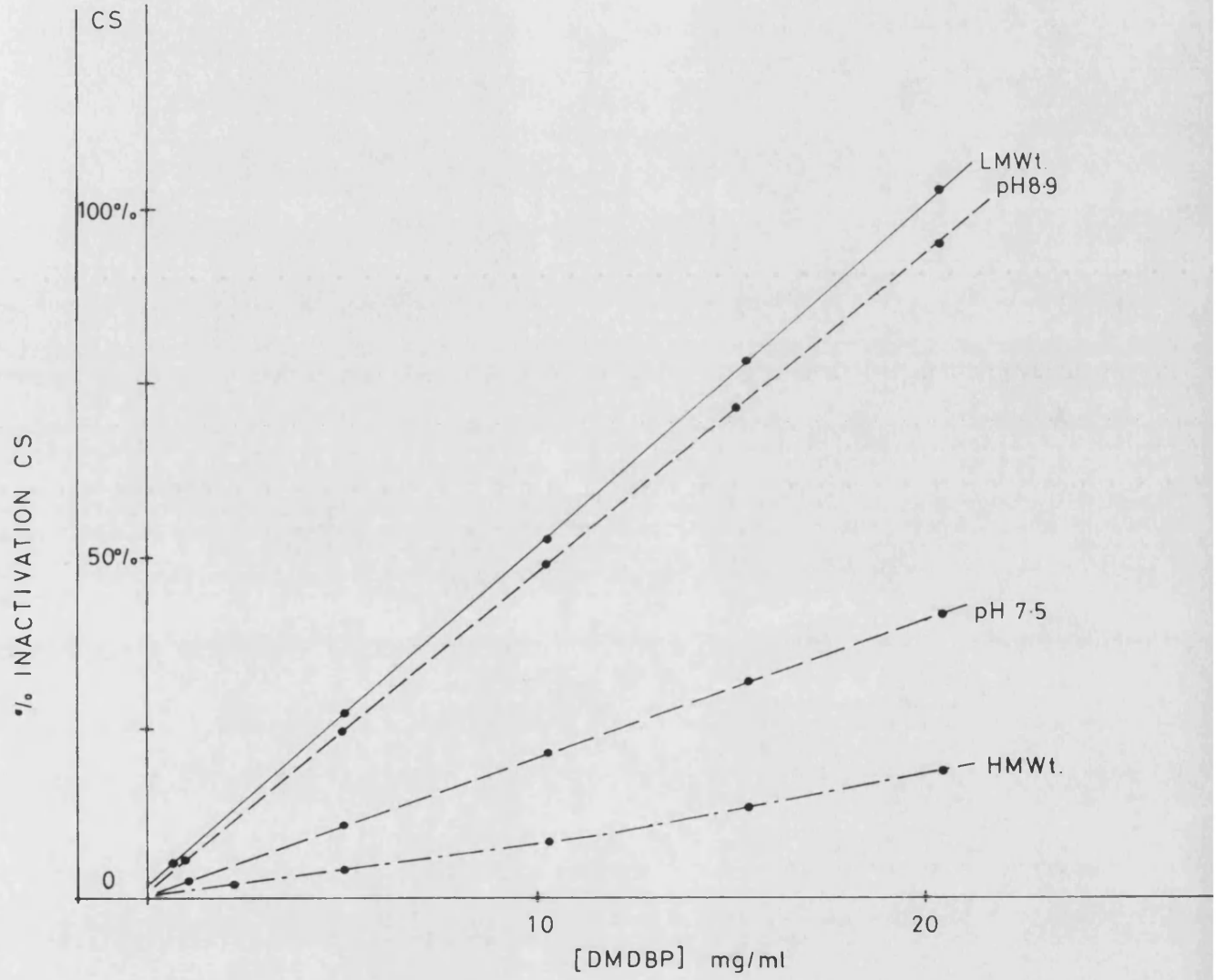
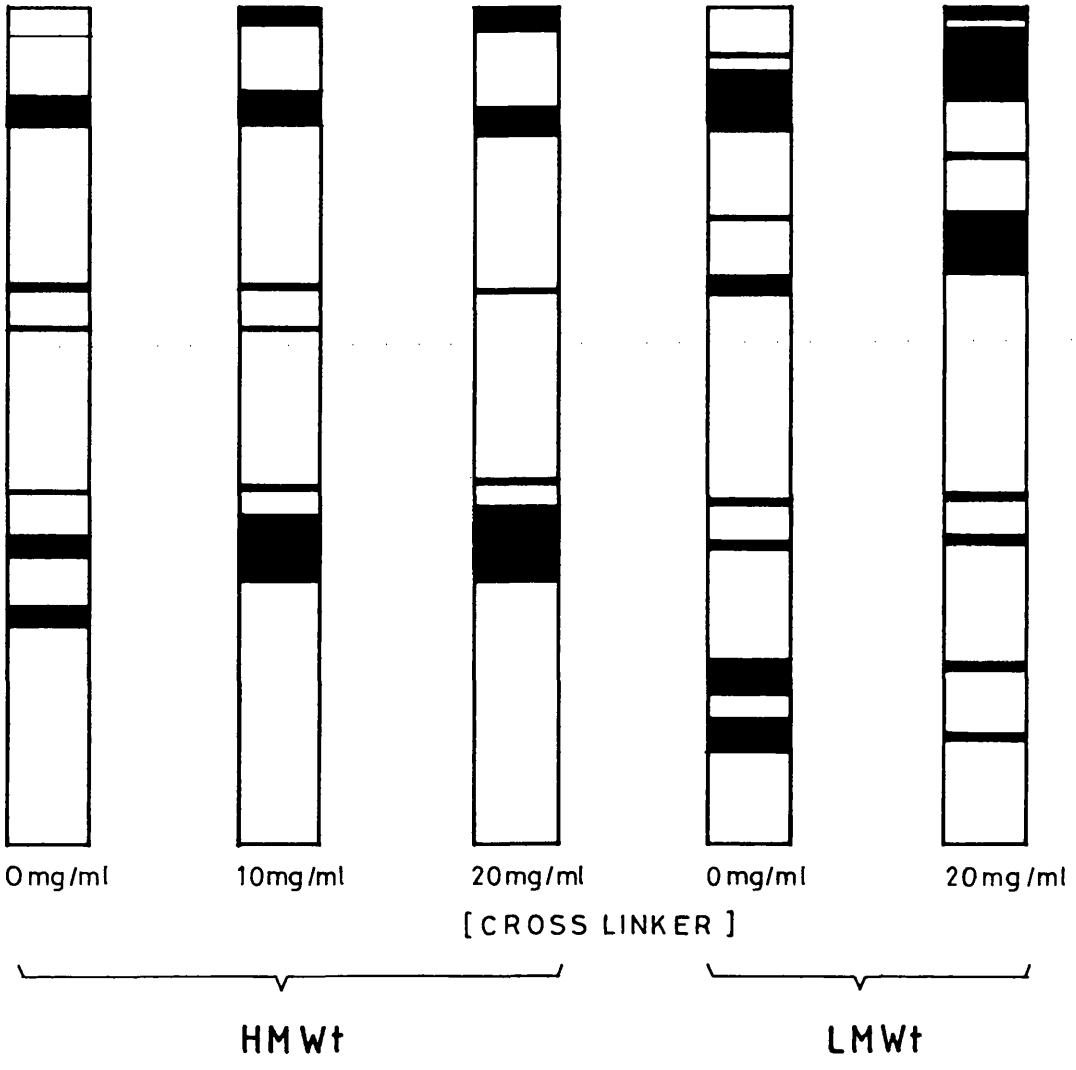


Figure 3.31

SDS-polyacrylamide gels used to detect the effect of cross-linker DMDBP on HMWt and LMWt samples.



and the concomitant appearance in bands corresponding to a higher molecular weight were indicative that the enzymes were indeed cross-linked.

Chromatography of Cross-Linked Species

It was of interest to examine the chromatographic pattern of enzymes which had been cross-linked. Two features of the chemical cross-linker were exploited; first the DMDBP preparation had a given "arm length", i.e. it could cross-link molecules which exist within its 1.1 nm arm length span, thus giving some indication of distances involved between the enzymes. Secondly, the use of a cleavable cross-linker such as DMDBP allowed a cross-linked complex to be cleaved enabling the reversibility of the chemically imposed association to be examined.

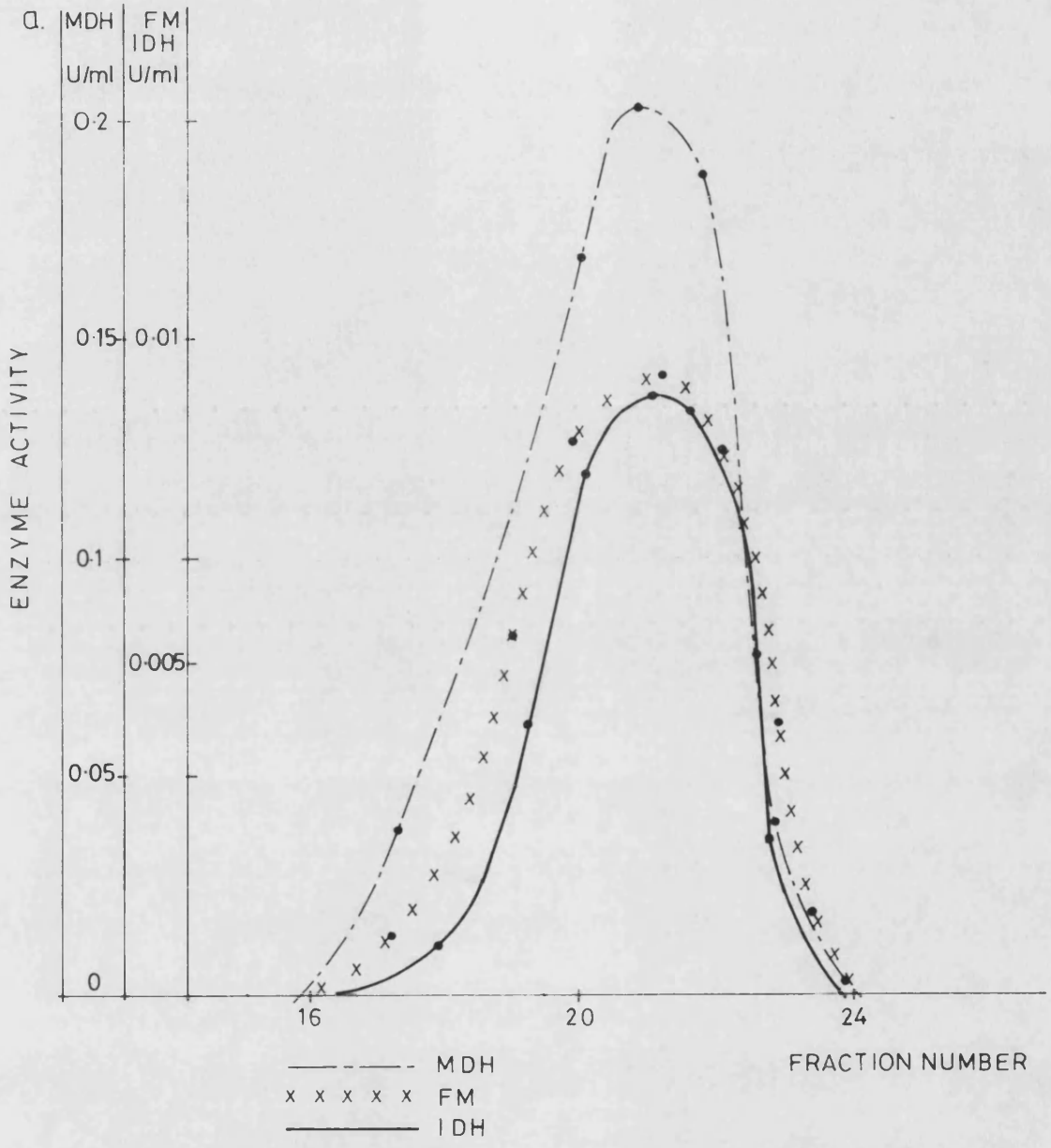
A HMWt species was isolated from *E. coli* and treated with DMDBP (10 mg ml^{-1}) as described. Half of this preparation was loaded onto Sepharose 4B gel filtration and the resulting fractions assayed for enzyme activity. The remaining half was cleaved by treatment with DTT, dialyzed extensively and run on Sepharose 4B. Enzyme profiles were compared with the non-cleaved sample.

Chromatography of the cross-linked HMWt species on Sepharose 4B resulted in a complex of enzymes containing the activities of CS, MDH, IDH and FM (Figure 3.32). Cross-linking may have existed between individual CAC clusters since a slightly higher molecular weight of approximately 3×10^6 was recorded.

On cleavage of the cross-linked species, with DTT (Figure 3.33), gel filtration elution profiles of the HMWt and cross-linked

Figure 3.32

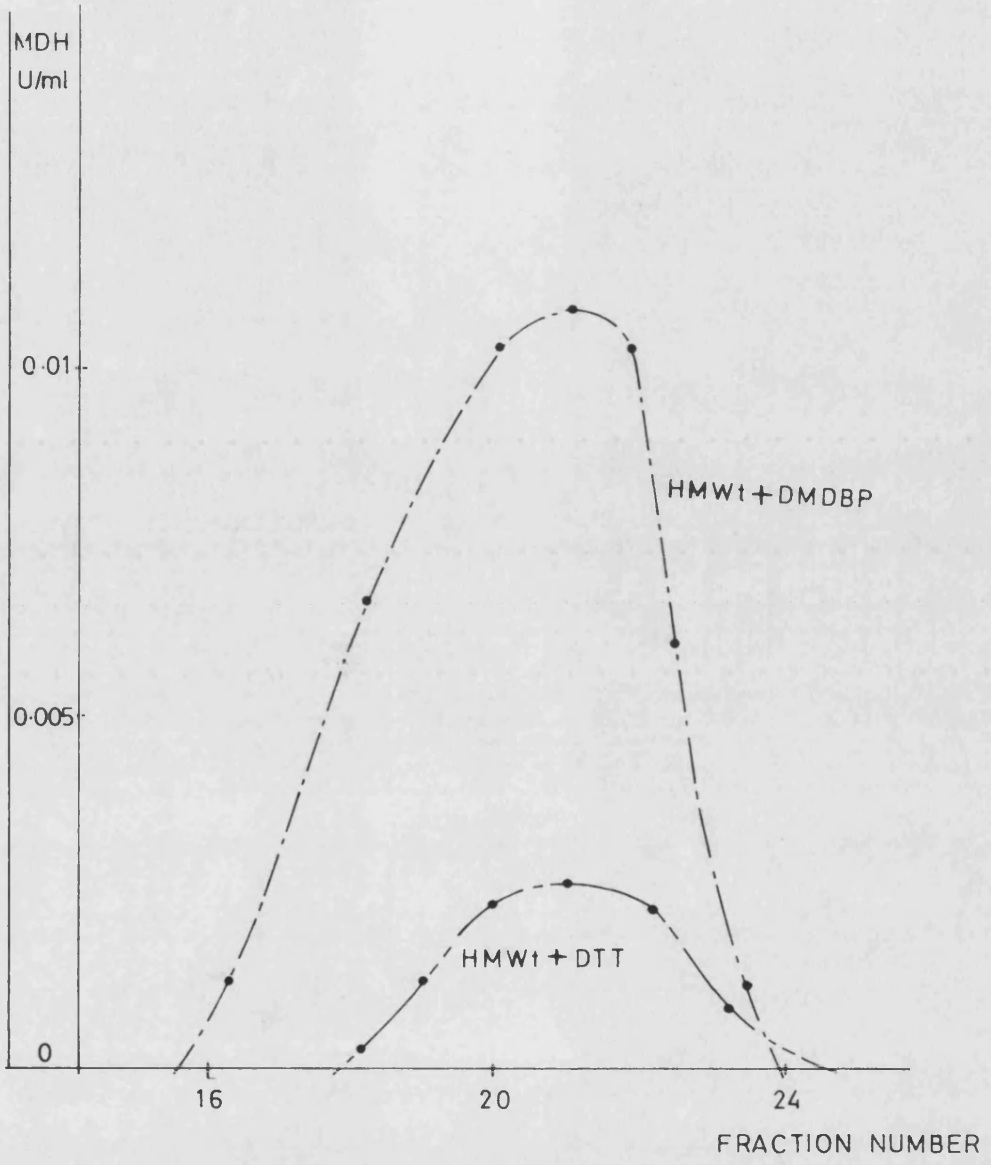
Chromatography of cross-linked species.



Gel filtration of HMWt species cross-linked with DMDBP ($10\ mg/ml^{-1}$) on Sepharose 4B, 10 mM TAG, pH 7.5 at 4°C.

Figure 3.33

'Chromatography of cross-linked species with DMDBP (- — -) and cleaved cross-linked species with DDT (-- —--).



Gel filtration on Sepharose 4B with 10 mM TAG, pH 7.5 at 4°C.

HMWt species overlapped. This showed that the integrity of the HMWt species remained, despite the relatively harsh treatment of the cross-linking reaction.

Determination of the Presence of Lipid

It has been established that the interaction between the CAC enzymes was a result of the physical association of the enzymes and not merely entrainment in a membrane vesicle. Experiments have shown, however, that a functional CAC cluster may exist in the cell in a location adjacent to the inner membrane.

Thin layer chromatography was used to investigate whether any lipid was present in fractions containing the CAC cluster. Although a fairly crude technique, TLC can be used qualitatively to detect the presence of phospholipid and give an indication of the presence of low density lipids.

Lipid was extracted from a dried sample of HMWt species as indicated in the Methods section. The extract was run in the presence of standards - 10 µl of each of phosphatidylcholine, phosphatidylserine, phosphatidylinositol and phosphatidyl-ethanolamine distearoyl.

After chromatography, the TLC peak was developed in an iodine tank and the presence of phospholipid marked by a brown colouration.

Table 3.10 lists the RF values for the standards used. Examination of the sample extract of HMWt species showed trace amounts of a lipid of RF value of 0.70. This corresponds to that of phosphatidylinositol.

Table 3.10.

LIPID	RF VALUE
Phosphatidylcholine	0.43
Phosphatidylethanolamine distearoyl	0.78
Phosphatidylinositol	0.70
Phosphatidylserine	0.88

If the enzymes were surrounded by a membrane vesicle, it may be logical to deduce that any such lipid extract would contain a mixture of lipid types. However, just one lipid type was evident.

TLC of the HMWt species revealed the presence of low density lipid, which ran with the solvent front in the separation and stained as lipid. The low density lipid was not identified but may give a vital indication of a core component around which the CAC enzymes associate or which acts as an "anchor" inside the cell. These observations are discussed later.

ELECTRON MICROSCOPY

To visualize the observations made through enzymological techniques that the enzymes of the CAC appear to associate with each other to form a loosely-bound multi-enzyme complex, a series of experiments were devised with the use of electron microscopy.

The series of electron micrographs shows the examination of the "multienzyme complex" by the technique of negative-staining

with uranyl acetate (1%, w/v). The protocol followed is explained fully in the Methods Section.

Plates 1 - 3 are electron micrographs from lysed *E. coli* spheroplasts. Plate 1 is that of material from the HMWt peak, showing defined particles, the majority of which were between 20 and 30 nm in diameter. The electron micrograph shown in Plate 2 is that of sonicated HMWt material. There was an absence of the large particles seen in Plate 1, with little uniformity in appearance of the material present. Plate 3 is representative of pooled LMWt fractions; range different sized materials was evident, with none of the defined particles, seen in Plate 1, visible. The 2 cm scale bar in Plate 3 represents 400 nm.

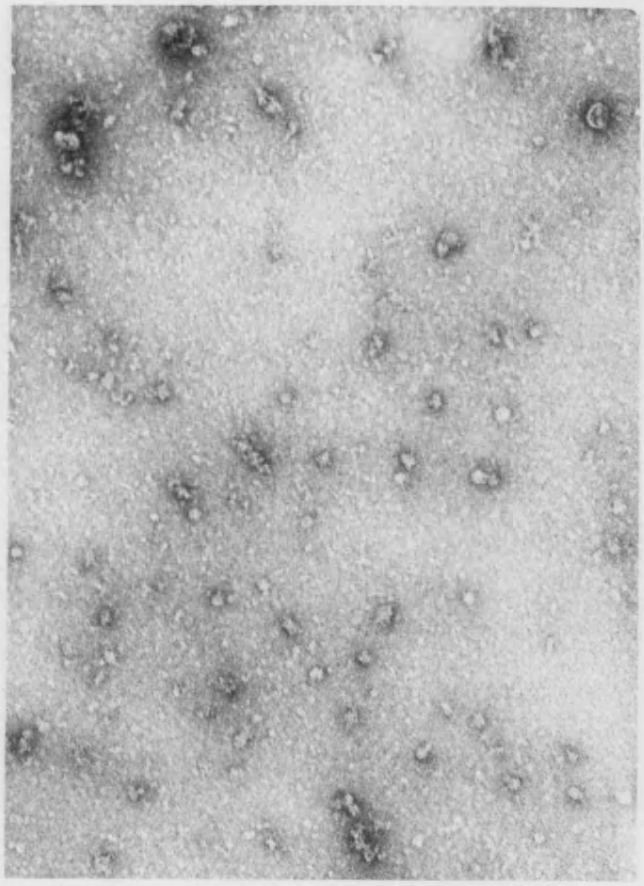


Plate 1

Electron micrograph of material negatively stained with 1% (w/v) uranyl acetate from the HMWt peak of lysed *E. coli* spheroplasts. Magnification of 70,000x.

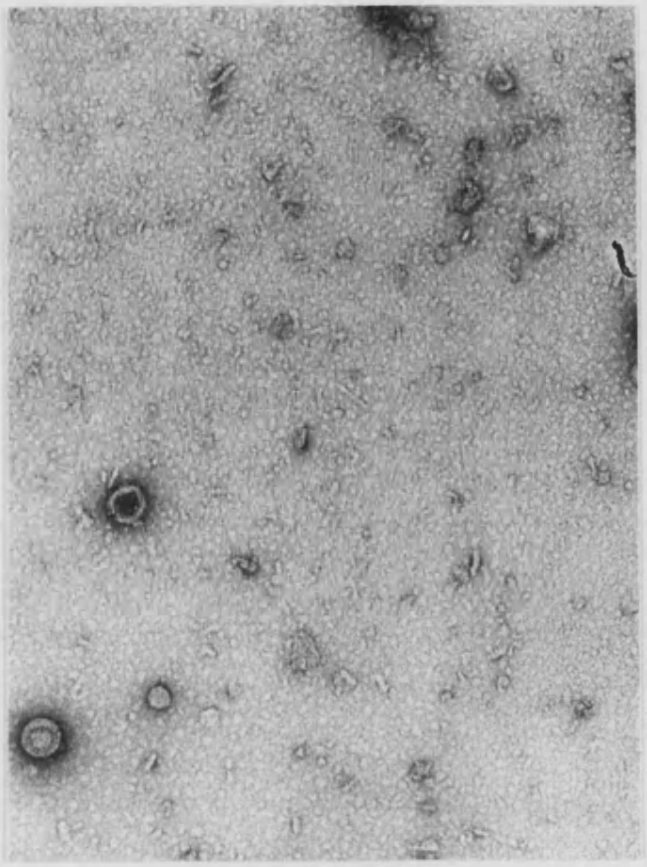


Plate 2

Electron micrograph of material from HMWt peak sonicated (4 x 15 sec) of lysed *E. coli* spheroplasts. Magnification of 50,000x, stained as in plate 1.

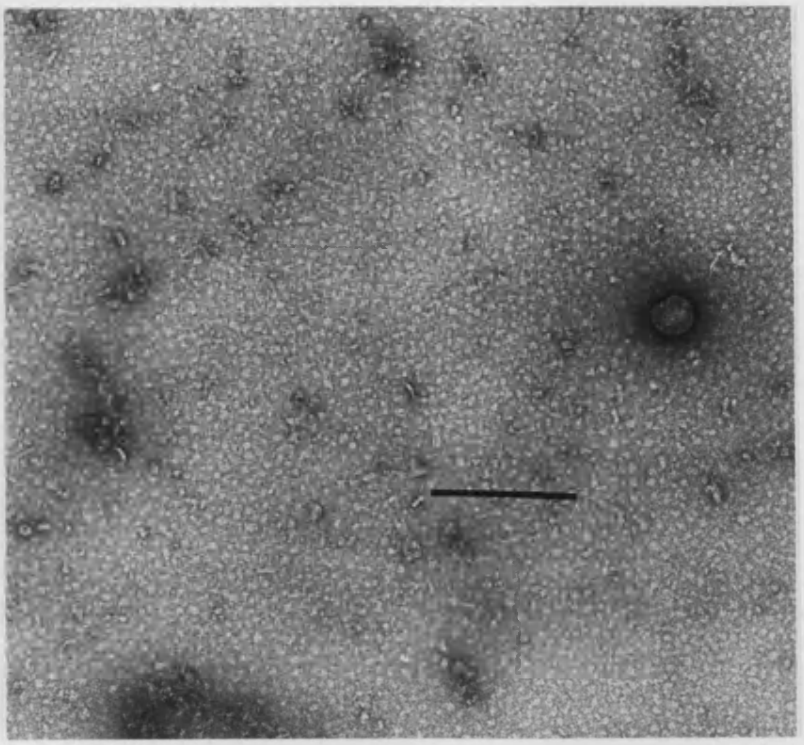


Plate 3

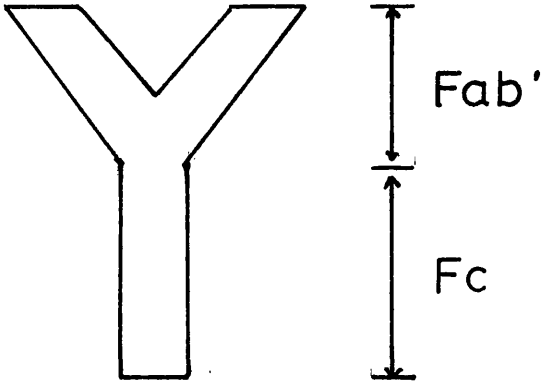
Electron micrograph of material from LMWt fractions of lysed E. coli spheroplasts. Magnification of 70,000x, stained as in plate 1.

Immunoelectron Microscopy

Although a structure was located and visualized by electron microscopy, it was necessary to show that this structure contained an enzyme or enzymes of the cycle and was not just a protein with a relatively high molecular weight which coincidentally was disrupted by sonication.

The technique of immunoelectron microscopy was applied. IgG raised against *E. coli* citrate synthase was used in an attempt to demonstrate the presence of that enzyme in the complex.

IgG is described as having a "Y-shaped" structure, with the two "arms" corresponding to the Fab' portions of the immunoglobulin. Each of these Fab' fragments forms an interaction site between the antibody and the antigenic site on the corresponding protein. Therefore theoretically two antigen molecules can be bound per IgG molecule, as shown diagrammatically below:



In the case under study, the Ag (antigen) molecules correspond to the HMWt cluster. On interaction of the HMWt species with IgG raised against *E. coli* citrate synthase it may be possible to see,

using negative contrast electron microscopy, pairs or groups of complexes. These would reflect the interaction between citrate synthase in the complex and one Fab' arm of an anti-CS IgG molecule. Interaction of the other Fab' arm of the IgG molecule would be with citrate synthase existing as part of another HMWt cluster.

Enzymological Evidence in Support of Immunoelectron Microscopy

Following work by Lunsdorf *et al.* (1984) on the use of antibody in immunoelectron microscopy and in support of the observations of the interaction between the anti *E. coli* CS IgG and CS in the HMWt species, the putative antibody-antigen complexes were chromatographed. A Sepharose 4B gel filtration system was used in the analysis.

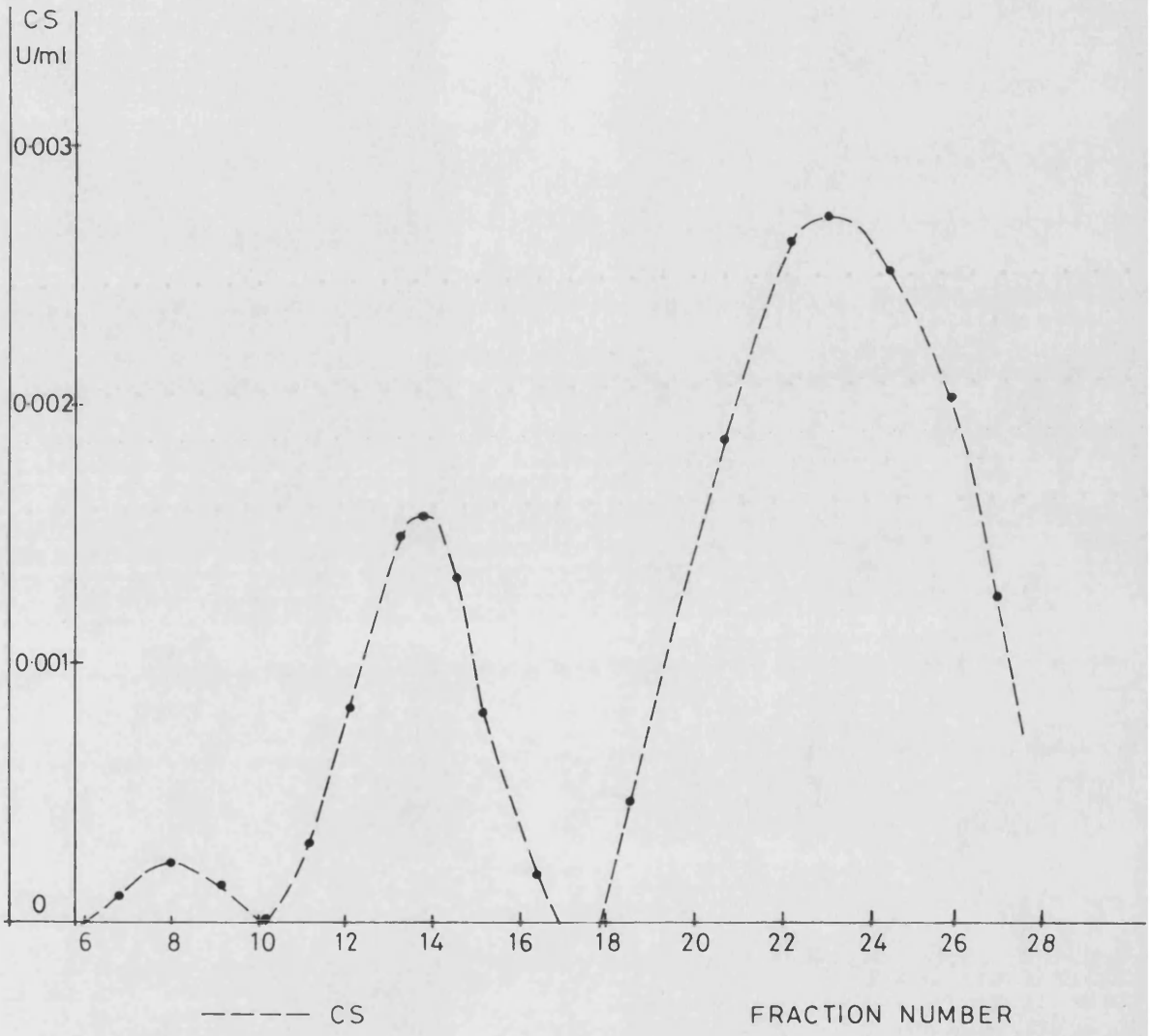
Isolated HMWt species was incubated with 1/100 dilution of anti-CS-antibody, 30 min, 37°C. A sample (0.5 ml) of the IgG-HMWt complex was loaded onto a Sepharose 4B column (10 x 0.5 cm) and eluted with TAG buffer. Fractions (250 µl) were collected and assayed for MDH activity, as an indicator of the presence of HMWt species. MDH was chosen due to its relatively high activity in these samples and also because CS, as stated earlier, is partially inactivated on interaction with its antibody.

The results of this experiment are expressed diagrammatically in Figure 3.34.

Previous experiments have shown the presence of two peaks of enzyme activity on re-passage of the HMWt species (Figure 3.9) and with normal serum as a control (Figure 3.35). However, gel

Figure 3.34

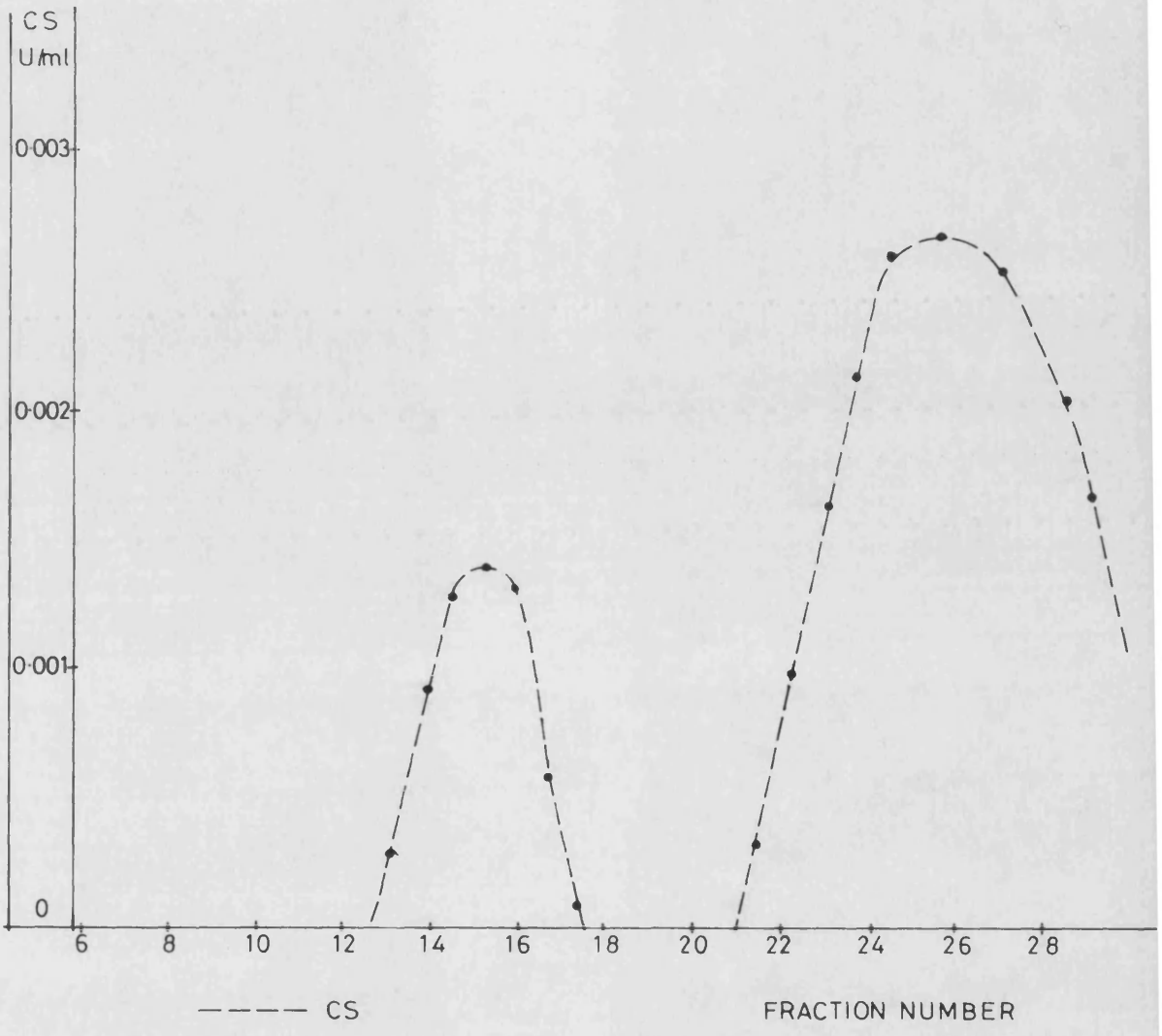
Chromatography of immuno-enzyme complex.



Gel filtration of HMWt - anti CS antibody complex on Sepharose 4B 10 mM TAG, pH 7.5 at 4°C.

Figure 3.35

Chromatography of HMWt species in presence of normal serum.



Gel filtration on Sepharose 4B, 10 mM TAG, pH 7.5 at 4°C.

filtration of the anti *E. coli* CS IgG-HMWt complex showed three peaks of enzyme activity for MDH. It may be surmised that the first of these peaks corresponded to the antigen-antibody complexes formed between CS in the CAC cluster and the anti *E. coli* CS IgG. The second peak corresponded to the HMWt species and the final peak was that of the single enzyme.

Plates 4 and 5 show electron micrographs of peaks 1 and 2 respectively. The arrows indicate the clusters of immuno-enzyme complexes isolated on gel filtration. The samples were negatively-stained with uranyl-acetate 1% (w/v) and examined and photographed under comparable conditions.

From both the enzymological evidence and from that shown in the electron micrographs, it may be deduced that CS appears to exist in association with particles of high molecular weight. It appears probable that these particles, therefore, correspond to clusters of CAC enzymes.

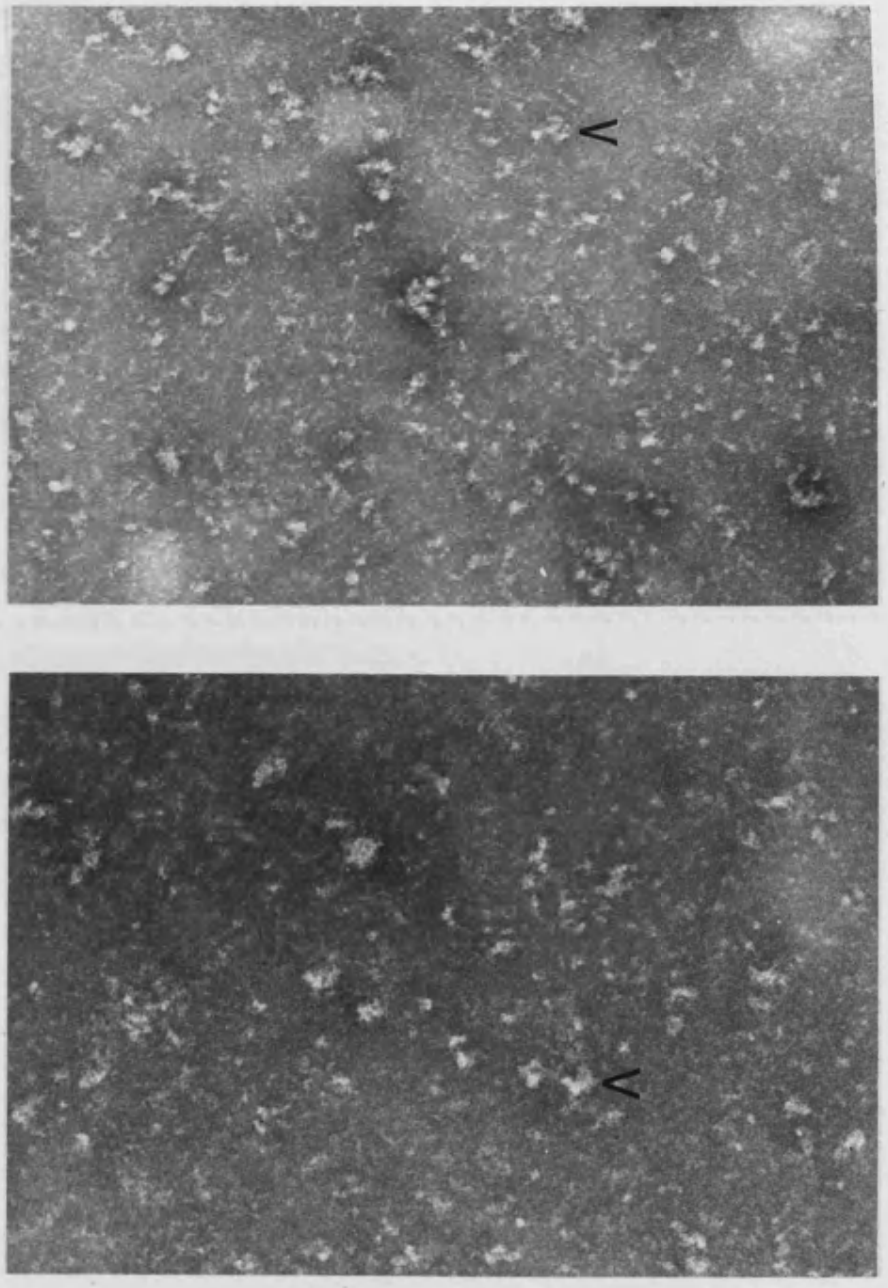


Plate 4

Electron micrograph of immuno-enzyme complexes from lysed E. coli spheroplasts. HMWt peak incubated in presence of anti-CS antibody; peak 1 from gel filtration. Complexes indicated by arrows. Magnification of 70,000x, stained as in plate 1.

Plate 5

Electron micrograph of immuno-enzyme complexes from lysed E. coli spheroplasts. HMWt peak incubated in presence of anti-CS antibody; peak 2 from gel filtration. Photograph taken and developed under comparable condition as in plate 4. Magnification of 70,000x, stained as in plate 1.

Functional aspects of the CAC cluster

Kinetic and regulatory properties of enzymes have been shown to vary according to the conditions under which the enzymes are assayed, an example of which was the study by Weitzman and Hewson (1973) on the ATP inhibition of CS activity in permeabilized yeast cells. It was shown that the inhibitory effect of the modulator, evident *in vitro*, was not present *in vivo*. This led to the conclusion that ATP may not be an *in vivo* regulatory mechanism.

Having established that a multienzyme cluster of CAC could be isolated from bacterial cells and from mitochondria, it was of interest to examine the functional aspects of this multienzyme system. It should be emphasised at this point that the data presented in this section represent a preliminary examination of functional aspects of the CAC cluster.

Kinetic parameters of particular enzymes in the CAC cluster were examined. For each enzyme-substrate pair, the results obtained were examined using linear transformations to see whether valid Michaelis-Menten kinetics applied. If at any substrate concentration the line resulting from a plot of v/s against v, deviated from a straight line, Michaelis-Menten kinetics could not be applied.

With kinetic data which obeyed Michaelis-Menten kinetics, the direct linear plot was employed to give the best estimate of kinetic constants (Eisenthal and Cornish-Bowden, 1974). A computer program was employed, based on the direct linear plot, to calculate kinetic constants from the data given.

Kinetic analyses of CS from *B. subtilis* and *Acinetobacter calcoaceticus* were carried out for the substrates acetyl-CoA and oxaloacetate (Table 3.11). Subsequent analyses demonstrated a similar trend to that shown in Table 3.11, for the kinetic constants of CS in HMWt and LMWt species. A direct comparison between different preparations may be unwise due to the unstable nature of the CAC cluster, as shown earlier.

The results of kinetic analysis of data on two of the enzymes present in the CAC cluster are shown in Table 3.12.The results are displayed as half-reciprocal plots and included in the Appendix section of the thesis.

The action of regulatory effectors on CS in the CAC cluster

Due to its position in the CAC, CS has been the subject of intensive experimental work, including the examination of possible *in vivo* regulatory mechanisms. Reviews of studies on this enzyme include those of Weitzman and Danson (1976) and Weitzman (1981), which emphasise the diversity and patterns of regulation of CS in a wide range of prokaryotic and eukaryotic organisms.

In the study of the organization of the CAC enzymes, an investigation into the effect of a metabolic regulator of one of the components of the cycle may reveal some useful information of the role of regulators on the multienzyme system.

The regulation of enzyme activity by cellular metabolites in prokaryotes is often exerted by feedback control on the initial enzyme of a pathway. The dehydrogenation reaction of later steps of the CAC led to the formation of NADH, the subsequent oxidation of

Table 3.11. Comparison of Km values for CS from different bacterial species.

Bacterial species	Form	Substrate	Km (μM)
Acinetobacter calcoaceticus	H	OAA	2 ± 1
	L	OAA	11 ± 4
	H	AcCoA	232 ± 30
	L	AcCoA	500 ± 50
Bacillus subtilis	H	OAA	7 ± 1
	L	OAA	14 ± 4
	H	AcCoA	135 ± 50
	L	AcCoA	485 ± 35

Samples prepared from lysed spheroplasts, isolated on gel filtration on Sepharose 4B, eluted with TAB buffer. Readings taken at 25°C with constant conditions. In each of the two substrate analyses, the concentration of the second substrate was under fixed, saturating conditions.

Table 3.12. Kinetic analysis of citric acid cycle enzymes from *E. coli*. Samples prepared from lysed *E. coli* spheroplasts, isolated on gel filtration on Sepharose 4B eluted with TAG buffer. Readings taken at 25°C with constant conditions.

Enzyme	Form	Substrate	Km (μM)
Malate dehydrogenase	H	OAA	3.1 ± 2
	L	OAA	46.3 ± 20
	H	Malate	4.5 ± 1
	L	Malate	7.8 ± 1
	H	NAD^+	400.0 ± 20
	L	NAD^+	334.0 ± 12
	H	NADH	67.0 ± 2
	L	NADH	51.0 ± 4
Isocitrate dehydrogenase	H	Isocitrate	3.6 ± 1
	L	Isocitrate	9.1 ± 2
	H	$NADP^+$	7.3 ± 1
	L	$NADP^+$	16.1 ± 3

which is coupled to the production of ATP. Thus both NADH and ATP have been considered to be putative regulators of CS (Weitzman, 1981). A third possible regulator of CS is oxoglutarate (OG), thought to be a feedback inhibitor of certain CS enzymes (Weitzmann and Dunsmore, 1969b). The regulatory behaviour of ATP and OG were examined on CS present as a free enzyme and in association with the CAC cluster.

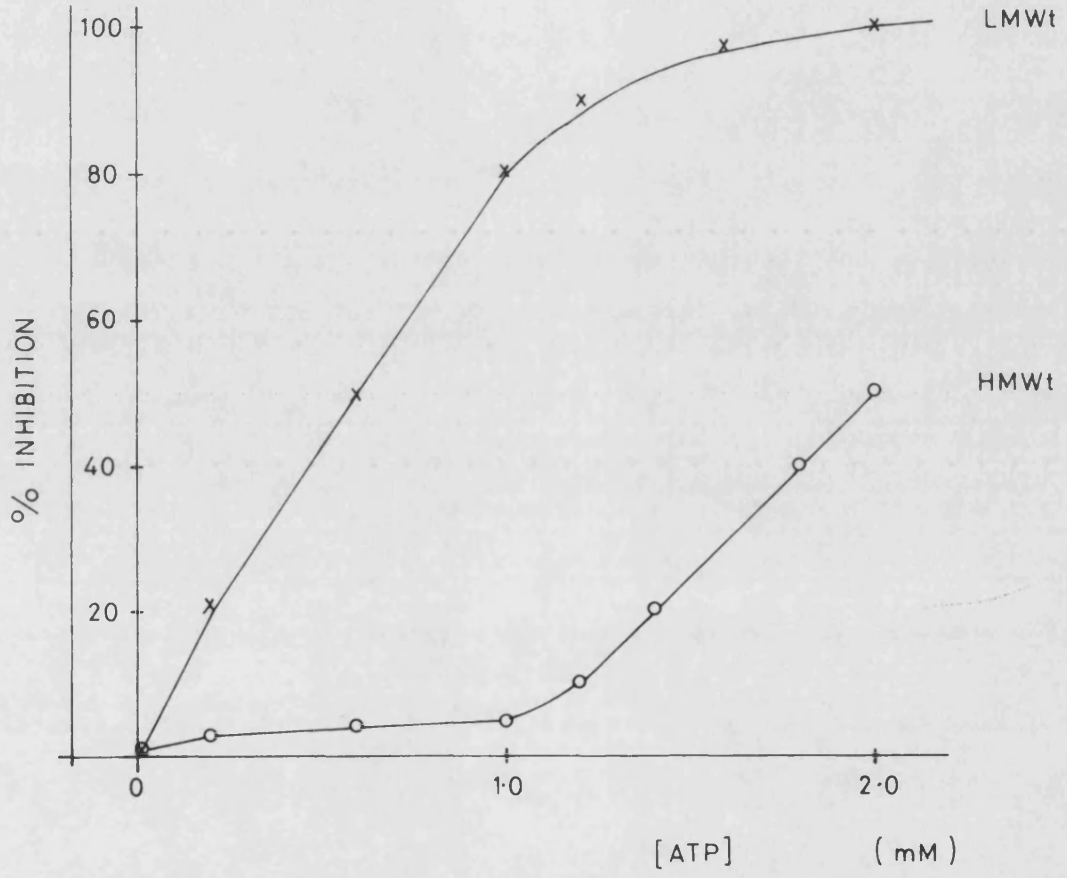
The regulation of CS by ATP

A competitive inhibition against acetyl-CoA by adenine nucleotides in CS was first reported by Hathaway and Atkinson (1965) using yeast CS, with ATP producing the greatest effect; it was subsequently also observed in a wide range of other eukaryotic and prokaryotic organisms.

Inhibition of CS by ATP occurred to different extents in the HMWt and LMWt. In *E. coli*, the free CS enzyme expressed a hyperbolic dependence of the degree of inhibition on inhibitor concentration, resulting in almost 100% inhibition of 2 mM ATP. The CS in the CAC cluster also expressed a sigmoidal dependence of the degree of inhibition on ATP concentration but showed a greater lag and did not show significant inhibition until 1.2 mM ATP, reaching a level of 50% inhibition at 2 mM ATP (Figure 3.36).

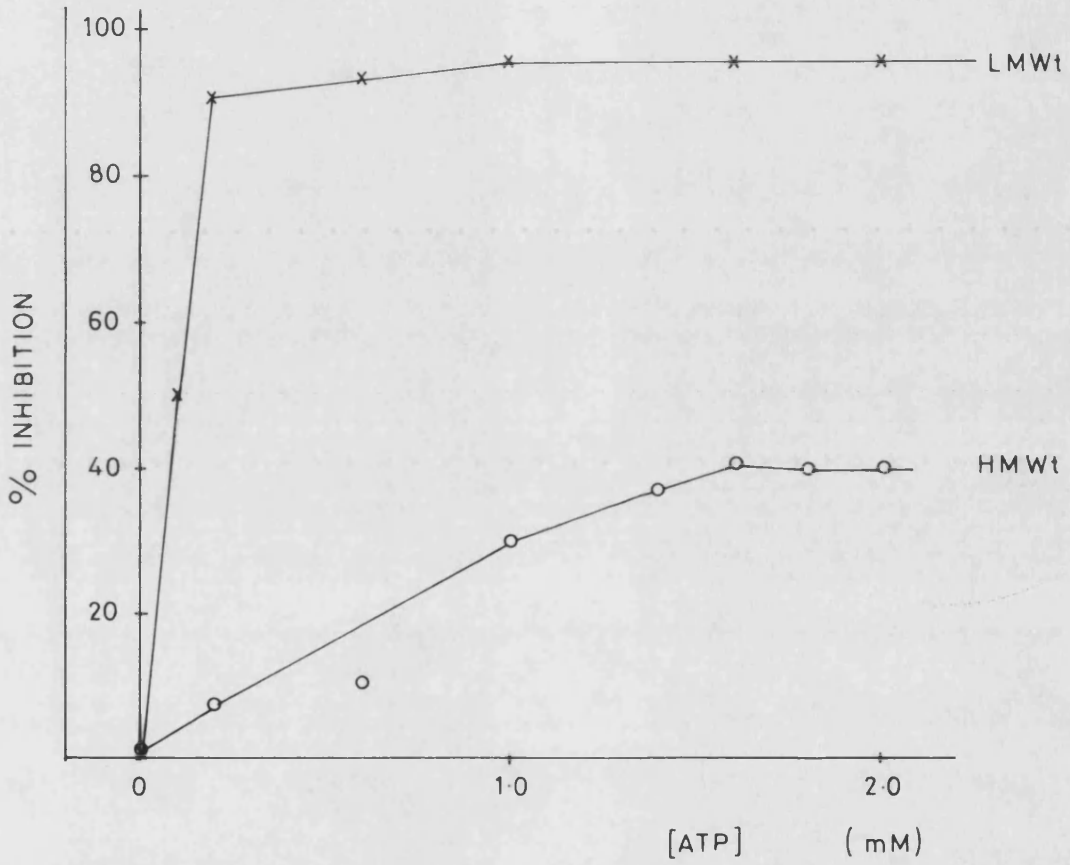
In CS from *B. subtilis*, both HMWt and LMWt species expressed a hyperbolic dependence of inhibition on inhibitor concentration (Figure 3.37). The data were analysed using a Dixon plot to give an estimate of the dissociation constant (K_i) for the inhibition of *B. subtilis* CS by ATP (Figure 3.38). The plot of 1/v against [I]

Figure 3.36



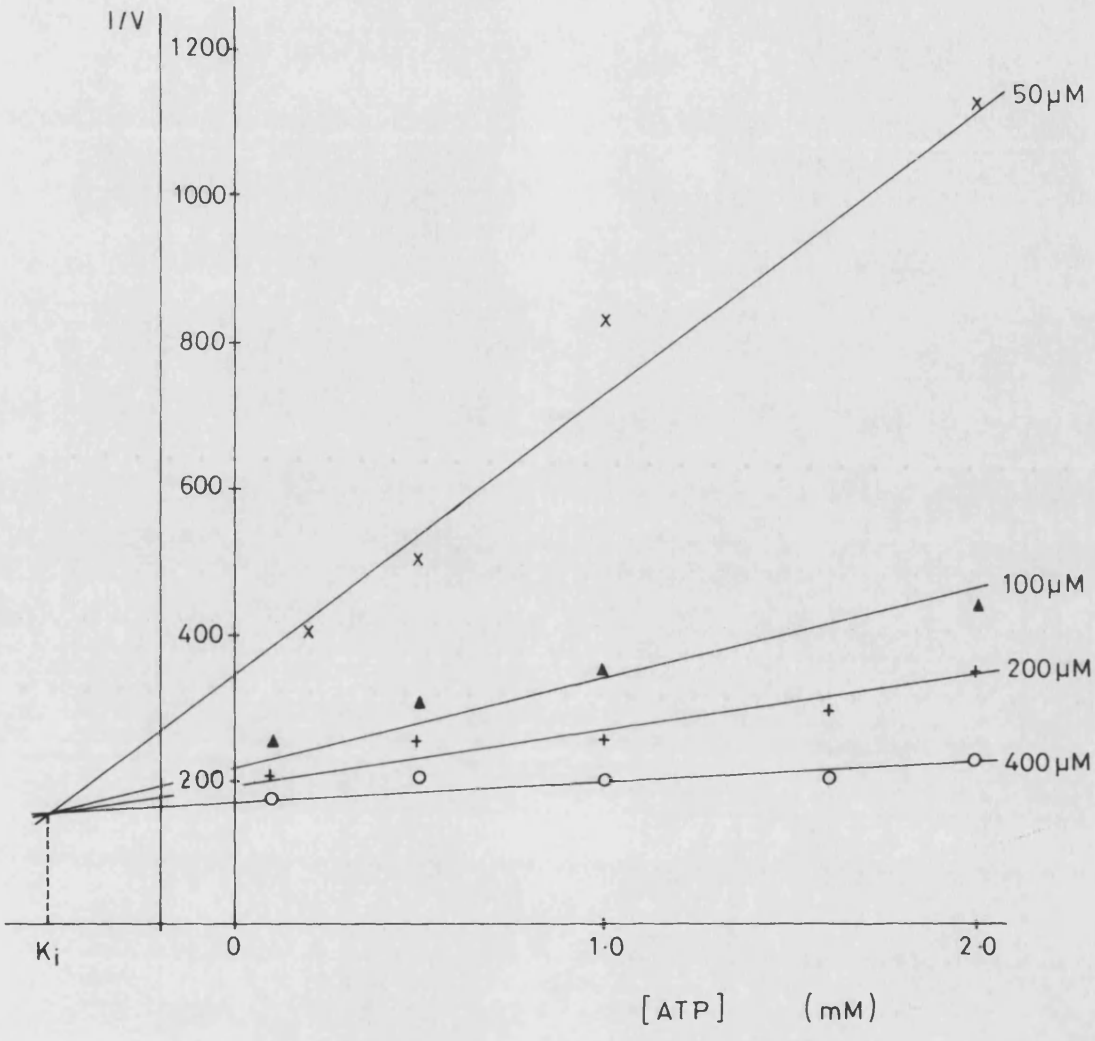
ATP inhibition of CS from *E. coli*.

Figure 3.37



ATP inhibition of CS from *Bacillus subtilis*

Figure 3.38



Dixon plot for the estimation of K_i of CS from *Bacillus subtilis*. Inhibition by ATP at varying concentrations of acetyl-CoA.

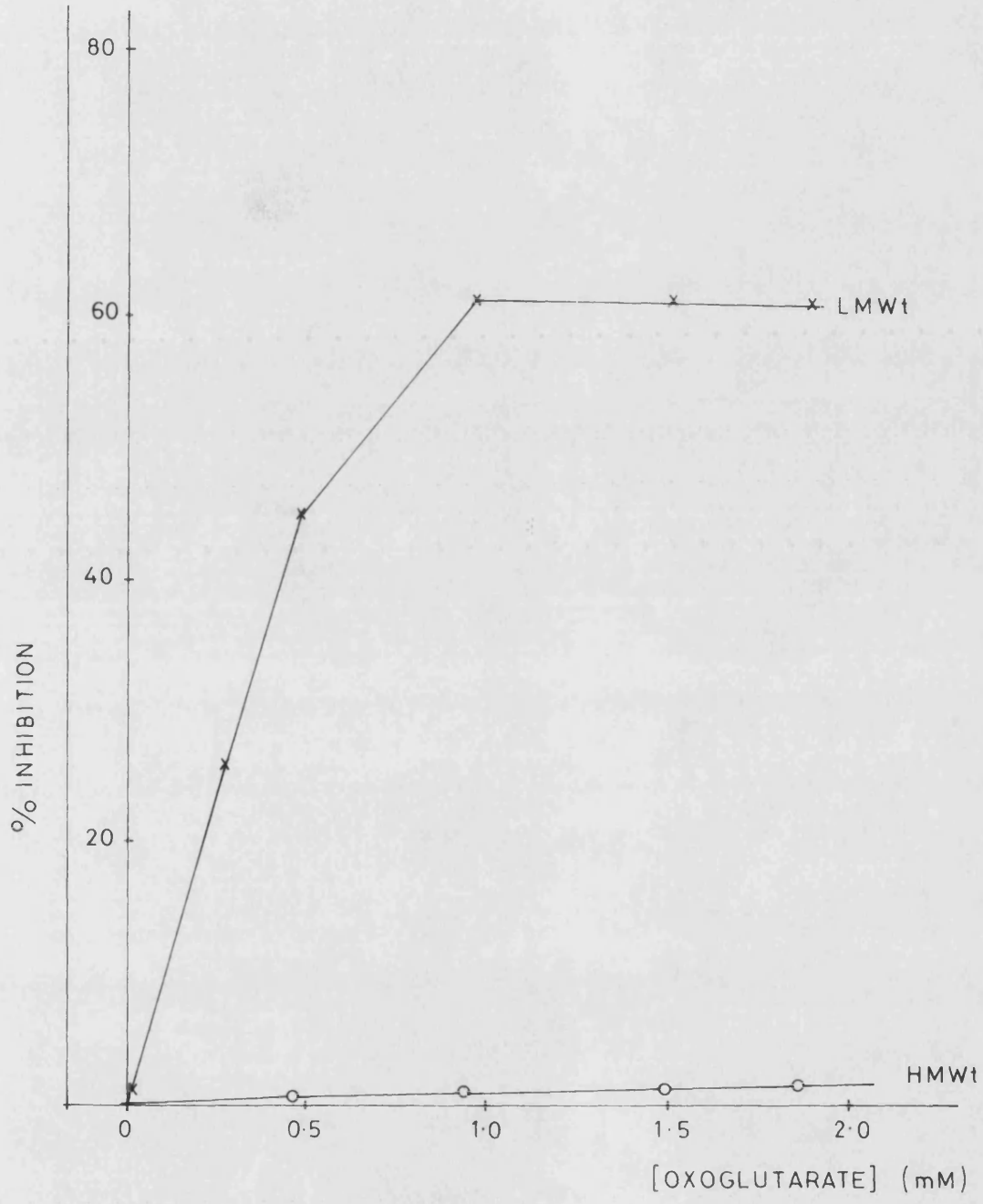
at different fixed substrate concentrations gave an intersection in the second quadrant, providing the estimate of K_i. The Dixon plot for inhibition of CS by ATP suggested that mixed or competitive inhibition occurred. The value of K_i obtained from the plot was approximately 550 μM. This value compares with that obtained from pure CS from eukaryotic sources (100 - 500 μM) (Srere *et al.*, 1973; Shepherd and Garland, 1969).

Regulation of CS by oxoglutarate (OG)

It is regarded that some inhibition by OG is an inherent feature of all CS enzymes, perhaps because of the structural analogy to oxaloacetate.Some organisms however have developed and amplified this response in order to make it a physiologically sensitive regulatory mechanism. This indeed is the case with the Gram-negative facultative anaerobic bacteria, such as *E. coli*, which under anaerobic conditions derives its energy from fermentation and lacks the enzyme OGDH. The effect of OG under such conditions suggests a type of feedback inhibition of CS (Weitzman and Dunmore, 1969b).

The inhibitory effects of OG on HMWt and LMWt species of *E. coli* CS are shown in Figure 3.39. The free CS showed a hyperbolic dependence of the degree of inhibition on oxoglutarate concentration. On the other hand, CS in the enzyme cluster was not inhibited to any extent over the concentration range of inhibitor used.

Figure 3.39



Oxoglutarate inhibition of CS in *E. coli*.

Throughput activity in the CAC cluster

If sequential enzymes of a metabolic pathway are located in close proximity, as has been suggested in the CAC cluster, it may be expected that intermediates would be passed directly from one catalytic site to the next in the sequence. If all enzymes in a sequence are present, product formation would indicate that these enzymes existed in a catalytically competent state. The examination of product formation was used by workers on the glycolytic sequence to identify a functionally organized multienzyme complex (Mowbray and Moses, 1976; Gorringe and Moses, 1980). In this work the term 'throughput' was used to describe the synthesis of pyruvate from hexose.

Throughput activity in the CAC cluster was examined by providing the substrate for what may be regarded as the first enzyme of the sequence, so that product formation from an enzyme later in the pathway might be detected.

Throughput activity in the HMWt cluster of CAC enzymes, isolated from lysed *E. coli* spheroplasts, was examined by providing fumarate, as initial substrate, in the presence of cofactors. Fumarate (10 mM) was incubated in the presence of HMWt species, NAD^+ (4 mM), acetyl-CoA (2 mM) and DTNB (1 mM). A similar incubation was set up using the LMWt species, diluted in buffer to give similar apparent activities. The production of citrate was monitored spectrophotometrically at 412 nm.

i.e. throughput was followed:

$$\text{fumarate} \xrightarrow{FM} \text{malate} \xrightarrow{MDH} \text{oxaloacetate} \xrightarrow{CS} \text{citrate}$$

Recycling of $NAD^+ \rightarrow$ NADH was achieved by the addition of lactate dehydrogenase (10 µl, commercial preparation) and 10 mM pyruvate. Figure 3.40 shows that citrate formation was observed, indicating throughput between the three-enzyme sequence FM → MDH → CS.

A difference was observed in the steady-state rate in the associated and dissociated forms of the enzymes (Figure 3.40). Product formation would be expected in both HMWt and LMWt species, but a difference in steady state rates would not be predicted unless the interaction of the enzymes in some way affected the enzyme of lowest velocity, in that sequence. This is because the steady state rate is dependent on the enzyme of lowest velocity.

The investigation was extended to all five enzymes of the multienzyme cluster, by the inclusion of cofactors for the enzymes AC and IDH in the assay system. The reaction was followed by recording the rate of reduction of $NADP^+$ to NADPH, for the final IDH step.

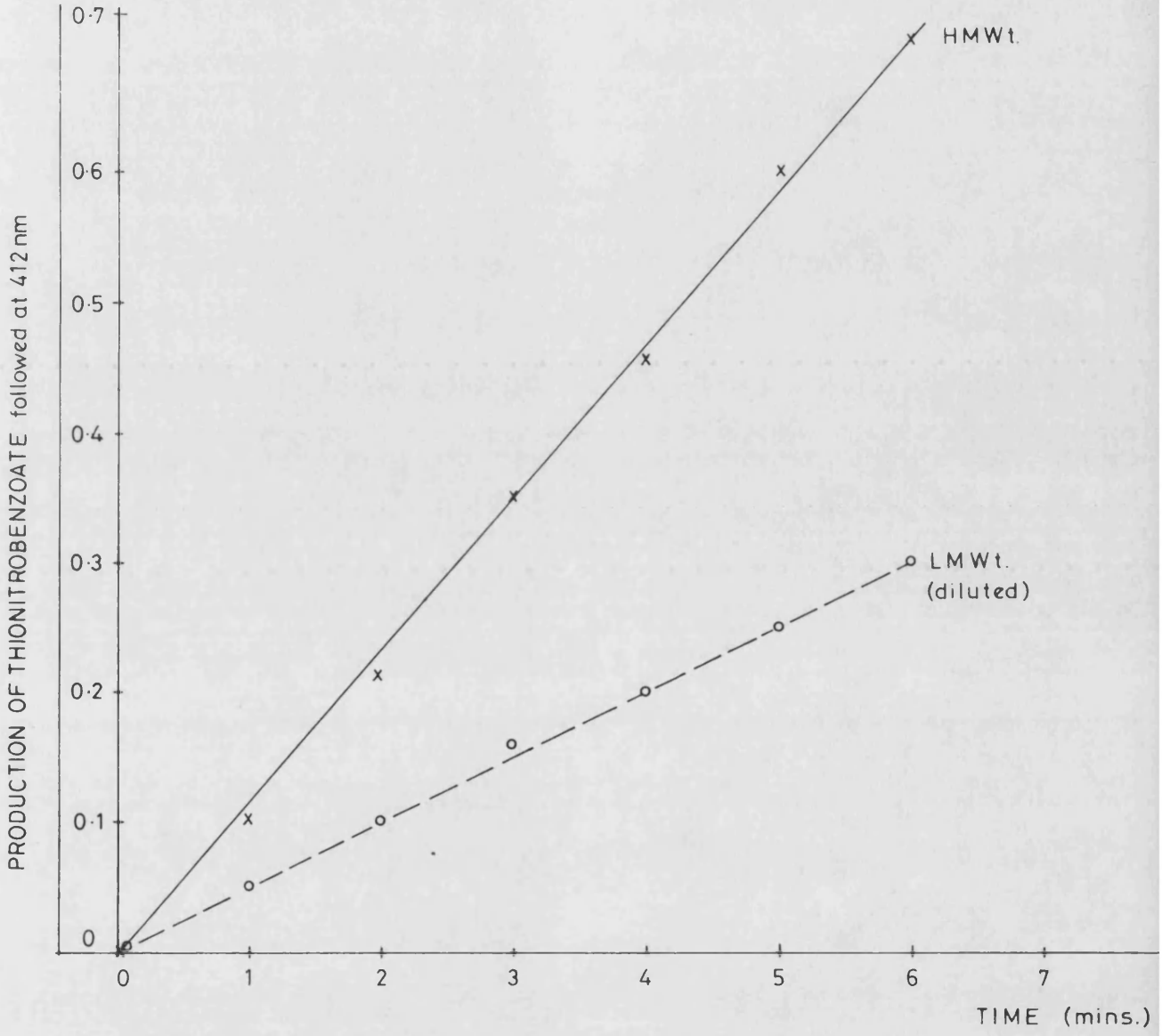
i.e. throughput was followed of:

fumarate → malate → oxaloacetate → citrate → aconitate → isocitrate → oxoglutarate

Throughput activity was examined in the reassociated HMWt cluster (Figures 3.41 and 3.42). This CAC cluster was shown earlier to be formed by the reassociation of enzymes, on a 1 : 1 ratio, from pooled and concentrated LMWt fractions. Examination of the data revealed that throughput had proceded from fumarate, in both the three-enzyme and five-enzyme sequences. Sonicatio n of the

Figure 3.40

Throughput in HMWt species.

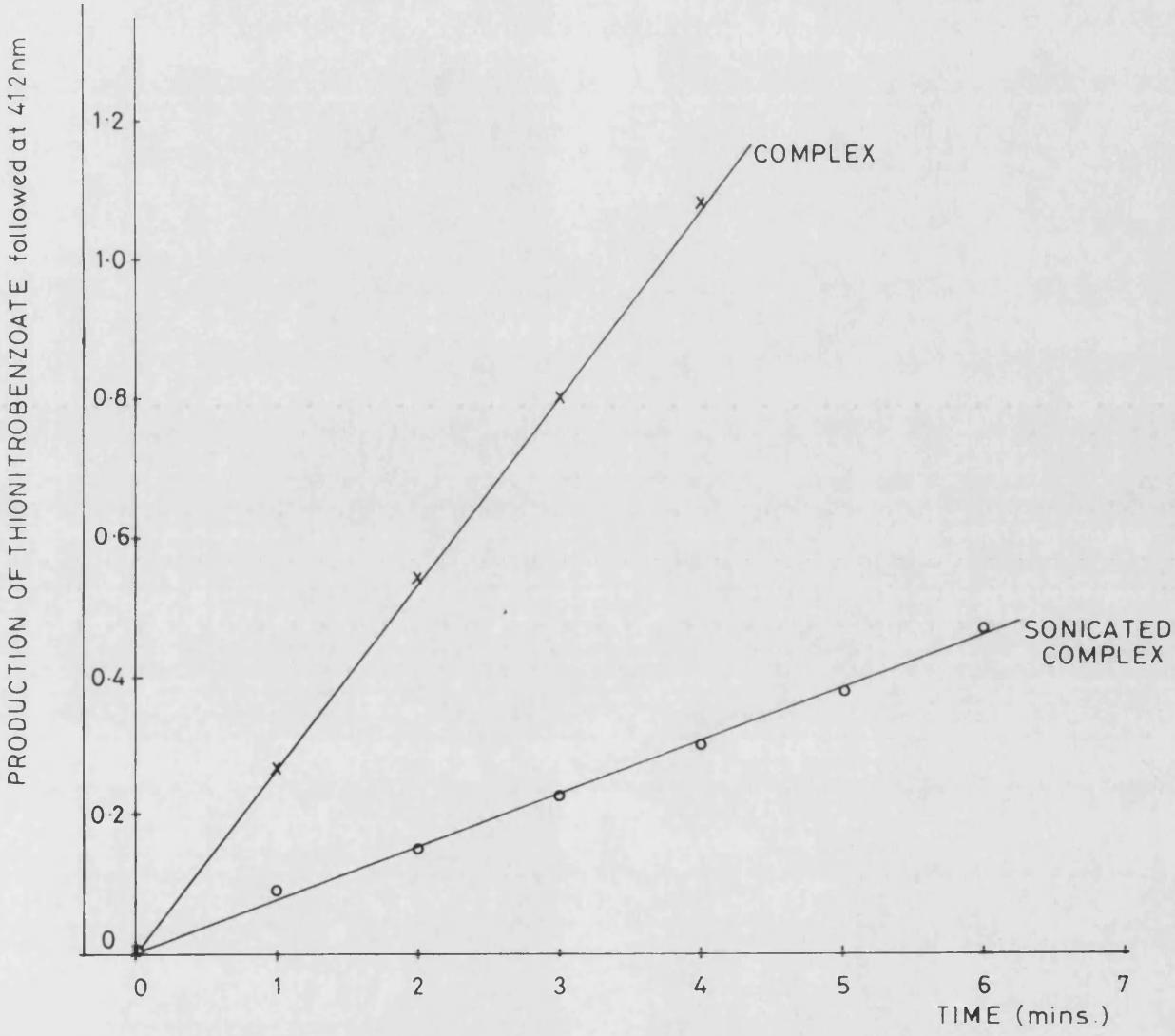


Production of thionitrobenzoate as indicator of throughput from fumarate → citrate. Comparison of HMWt species from *E. coli*, and LMWt pooled fractions diluted to give similar activity.

Method as in text.

Figure 3.41

Throughput in HMWt species from concentrated LMWt fractions.

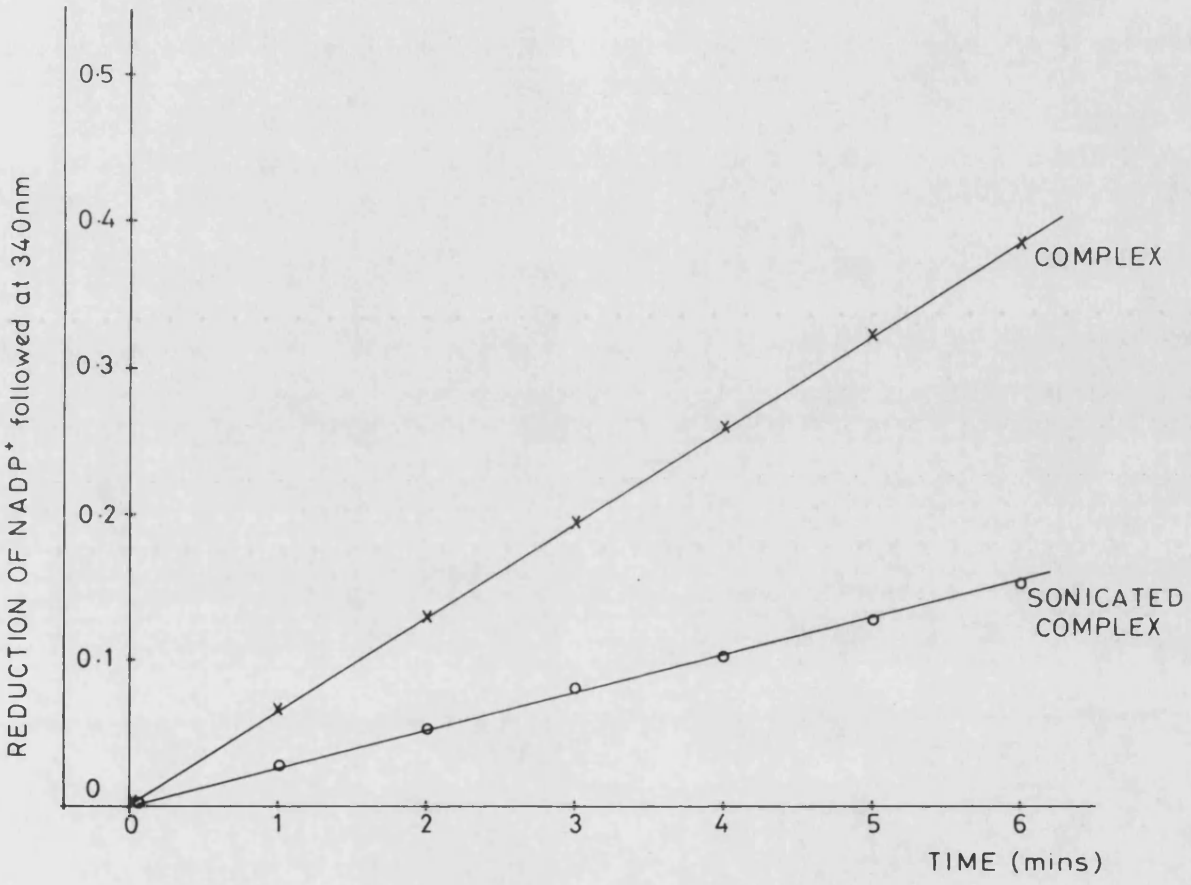


Production of thionitrobenzoate as indicator of throughput from fumarate ⟶ citrate. Comparison of complex and sonicated complex from *E. coli*.

Method as in text.

Figure 3.42

Throughput in HMWt from concentrated LMWt fractions.



Reduction of $NADP^+$ as indicator of throughput from fumarate $\longrightarrow$ oxoglutarate.

Comparison of complex and sonicated complex from *E. coli*

Method as in text.

reassociated cluster, which destroys the loose enzyme interactions, was shown to give rise to a reduction in steady-state rate, when compared to the HMWt cluster. This result, again, would not be predicted unless by the sonication or the disruption of the cluster, affects the enzyme of lowest velocity in the sequence.

Dissociation from the HMWt cluster was shown earlier to be avoided by the use of bifunctional imidate cross-linker (DMDBP), which effectively froze the cluster by cross-linking between the enzymes. Throughput activity was determined in the cross-linked HMWt and compared with the non-cross-linked form. The rate at which citrate was formed from fumarate was detected as above.

	cross-link	non cross-link
Throughput activity (moles.$min^{-1}ml^{-1}$)	0.020	0.001

Therefore, for the first three enzymes of the sequence at least, throughput activity was greater when dissociation of constituent enzymes was avoided.

The implications from these observations are discussed later.

CHAPTER 4

DISCUSSION

'No man is an Island, entire of itself;
everyman is a piece of the continent,
a part of the main'.

John Donne (Devotions)

This project set out to investigate whether the citric acid cycle (CAC) is composed of soluble enzymes, existing in free and random diffusion in the cell, or whether there is specific order and organization within the metabolic pathway. The CAC enzymes have been described as soluble; however, this term only arises as a result of the extraction procedure employed and does not logically reflect the *in vivo* situation. Evidence presented by other workers and in this thesis supports a case for the metabolic compartmentation of the CAC enzymes in the cell; no enzyme acts as a chemical catalyst in isolation, but exists as a functional part of a complex network of interactions with other proteins, membranes and cytoskeletal elements.

Interactions between isolated enzymes of the CAC have been reported and have been discussed at length in the Introduction. Perhaps the most extensively studied interaction has been that between CS and MDH (Beeckmans and Kanarek, 1981; D'Souza and Srere, 1983; Fahien and Kmiotek, 1983; Halper and Srere, 1977; Srere *et al.*, 1973; Srere *et al.*, 1978).

Gel filtration was used as a means of studying enzyme

interactions. A distortion of the elution profile of an enzyme, producing overlapping peaks or a shift of peak elution position, may be indicative of an enzyme interaction. Similarly, consecutive enzyme elution, from a crude preparation, of enzymes with dissimilar molecular weights indicated the possibility of specific enzyme interaction (Beeckmans and Kanarek, 1981; McEvily *et al.*, 1985).

Examination of CS and MDH from gently sonicated bacterial extracts of wild type *E. coli* on gel filtration chromatography, showed that these two CAC enzymes had overlapping elution profiles, despite having quite different molecular weights. The presence of 0.8 M KCl in the elution buffer eliminated this interaction and the two enzymes were eluted as single enzymes. The use of polyethylene glycol in a similar experiment highlighted the effects of the non-ionic polymer on the creation of a favourable enviroment for protein interaction, as CS and MDH elution profiles overlapped to a greater extent than in its absence. The overlap may be interpreted in terms of aggregation between CS and MDH, more apparent in the presence of polyethylene glycol, due to its exclusion effects.

The use of salt and polyethylene glycol emphasized the role of the physical environment in which the enzymes exist. Attempts to simulate intracellular conditions may lead to a clearer understanding of protein-protein interactions.

CS and MDH isolated from different bacterial strains confirmed work by Halper and Srere (1977) and other workers on species specificity. The specific interaction of CS and MDH was not

restricted to enzymes originating from the same species. The site of interaction must be a common feature of CS and MDH enzymes irrespective of their origin. One may conclude that the determinants on the surface of these enzymes have been very resistant to mutation throughout the course of evolution. Fahien and Kmiotek (1979) observed that interaction may be detected between glutamate dehydrogenase and MDH even with ~~the~~ enzymes ~~were~~ extracted from different eukaryotic species. It was observed that *E. coli* CS could replace the enzyme from pig heart in the interaction with pig heart mitochondrial MDH (Halper and Srere, 1977). Beeckmans and Kanarek (1981) made similar observations in their studies of the interaction of CS, FM and MDH. It would appear that the outer surfaces of some of the CAC enzymes are as conservative as the inner contact surfaces of the subunits of oligomeric enzymes, which can very often be exchanged between species (Levinthal *et al.*, 1962; Zablin and Villarejo, 1975; Feldmann *et al.*, 1976; Heib and Lebherz, 1978).

It has been shown that enzyme interactions can be manipulated by alteration of their environment. It is easy to see how subtle associations may have been overlooked in the past as the result of use of conditions which were unfavourable for the maintenance of delicate *in vivo* organization.

CS and MDH enzymes may interact at or near the NADH-binding site on CS, since the action of DTNB at this site on CS resulted in a reduction in enzyme association, as shown by elution profiles on gel filtration. However, this would only apply to Gram negative

bacterial CS, which possess an NADH site, since Gram positive bacteria and eukaryotes have NADH-insensitive CS enzymes.

Refinement of existing methods of enzyme isolation led to the discovery of a multienzyme complex in the so called 'soluble' glycolytic enzymes (Mowbray and Moses, 1976). By employing similar 'gentle' treatments of cell fractionation a cluster of CAC enzymes was observed using gel filtration chromatography. A multienzyme cluster is the collective name for physically associated enzymes (Welch and Gaertner, 1980).

The presence of a high molecular weight species of CAC enzymes was dependent on the protocol adopted for cell disruption. Harsh extraction procedures such as sonication and the French press were too drastic for the maintenance of fine associations. Similarly, the environment into which the enzymes were released affected the proportion of enzymes existing as the high molecular weight species. Attempts to simulate intracellular conditions of high viscosity, high protein concentration and restricted diffusion using glycerol, protein and polyethylene glycol illustrated the promotion of specific inter-enzyme interactions.

A parallel set of experiments indicated that enzymes showed less tendency to associate, when subjected to variations in pH and ionic strength, from the arbitrary experimental conditions used. It is difficult to obtain precise values of the pH in the interior of the cell. Although an overall pH value may be calculated at one particular point in the cell, the pH may differ from that at another. A similar situation arises on consideration of the ionic

strength of the cell; an overall value of the disrupted cell may be obtained but this does not necessarily reflect a homogeneous ionic strength throughout the cell. A decrease in the proportion of associated enzymes was detected in an environment of increased ionic strength. This does not preclude the fact that, *in vivo*, a CAC cluster may exist in a restricted microenvironment where the ionic strength may differ from the overall value obtained for the cell.

It was observed by Duchon and Collier (1971) that the pH of the medium had little effect on the binding of 'firmly' bound enzymes to isolated membranes, but more so on loosely 'bound' enzymes. However, the possibility of these associations resulting from artificial electrostatic adsorption due to the method preparation, was highlighted by Gorringe (1977).

Beeckmans and Kanarek (1981) noted that interactions between CS, MDH and FM were mostly of an electrostatic nature since they were all broken by higher ionic strenth. The involvement of electrostatic interaction in the loose binding of some mitochondrial matrix enzymes to the inner membrane has been proposed by Addink *et al.*, (1972). It may not be relevant that the ionic strength at which interactions between enzymes are seen is not physiological. With a high enzyme concentration, most metabolites will be bound by different enzymes, water molecules will be ordered in layers and free metabolites will be divided into compartments throughout the matrix (Beeckmans and Kanarek, 1981). As a consequence of the high cellular protein concentration even small associative forces between protein molecules would be favoured so that weak

interactions not apparent in dilute solution may be important.

The specificity of those enzymes which eluted as a multienzyme cluster from gel filtration was precise. Neither externally added enzyme nor enzyme not involved in the CAC was detected in association with the cluster. Also, a preparation of commercially prepared CAC enzymes did not spontaneously associate when pooled and run on gel filtration. The fact that these enzyme sources were diverse was not important since it has been shown that there has been conservation of complementary surfaces of associating enzymes through the course of evolution (Srere *et al.*, 1978; Friedrich, 1985).

The high molecular weight species was stable to passage on gel filtration and could be formed by the ultraconcentration of low molecular weight fractions. Consistently, five of the enzymes re-associated on concentration of low molecular weight fractions. STK only interacted with the other enzymes of the cycle when OGDH was present. This observation is consistent with the work of Porpaczy *et al.* (1983) who showed a specific association between OGDH and STK. It is therefore concluded that a multienzyme cluster containing at least five of the enzymes of the CAC may be isolated. A further two enzymes, OGDH and STK, co-eluted with the five, possibly reflecting additional interactions. The association of STK with the cluster is, however, dependent on the presence of OGDH.

A dynamic system between associated and dissociated forms of the enzymes may exist in the CAC, as has been identified with the glycolytic sequence. It would be unlikely that all the enzymes of

the CAC would exist within a rigid supramolecular structure, seen in the multienzyme complexes PDH and OGDH, with their defined metabolic functions. Since the CAC has both anabolic and catabolic roles in the cell and intermediates from the cycle are involved in other metabolic processes, the necessity for flexibility is apparent. The cell does not require that substrate is fed into the cycle and product is released as the end result of a metabolic sequence; rather, that product is released at specific positions in the cycle as required. It might however be advantageous to the cell to have a variable proportion of the enzymes involved in a pathway in close physical association with each other, thus being able to achieve high local concentrations of metabolites with an overall low metabolite cellular concentration. The maintenance of low metabolite concentration avoids the problems of taxing the solvent capacity of the cell.

Essentially, flexibility in a metabolic sequence may be a means of achieving regulatory function on pathway flux by the association and dissociation of its constituent enzymes. Such enzymes have been termed 'ambiquitous' by Wilson (1978) who described such behaviour in hexokinase. An ambiquitous enzyme is one whose distribution between soluble and particulate forms may vary with the metabolic status of the cell, as reflected in levels of certain metabolites capable of influencing that distribution. Ambiquity thus imparts a dynamic character to intracellular distribution.

Demonstration of a Physical Association between CAC Enzymes

1. Sonication

The use of sonication techniques on cell extracts or isolated enzymes was shown to be drastic and its effects irreversible. Whilst not affecting the activity of the enzyme, it was seen to give rise to changes in the enzyme such that after treatment it would be unable to form specific interactions with other enzymes.

Ultrasound or the production of sonic waves is a type of liquid shear method of disruption. Sonication causes cavities to form in the cell suspension, which lead to acoustic or micro-streaming of liquid around each bubble. It is thought that the acceleration due to streaming generates sufficient force to disrupt the cells in suspension. The technique has been widely used, although little attention has been given to the effects that sonication may have on fine cellular structures. Clearly, apart from releasing enzymes and other components from the cell, some damage must arise. In this study the use of sonic disruption altered some feature of enzymic structure or the microenvironment around the enzyme, such that enzyme associations, which are evident when more gentle disruption techniques are used, were no longer apparent.

Again the premise holds that conventional methods of examining cellular components are not readily applicable to the more subtle study of intracellular organization of enzymes.

Sonication of the cluster of CAC enzymes revealed that three times the level of enzyme was actually present in the fraction than

could be measured by assaying the undisrupted cluster. The CAC cluster isolated by gel filtration therefore, shows a degree of "crypticity". Sonication of the high molecular weight species released the five enzymes in equal ratios, such that each level of enzyme was approximately three times greater. Conversely, sonication of the high molecular weight species, which was obtained by ultraconcentration of low molecular weight fractions, revealed that these enzymes associated on a 1 : 1 basis. This difference between the initial CAC cluster and that obtained by re-association was also reflected in their slightly different elution position on gel filtration.

The fact that three times the apparent activity of enzyme was present, was consistent with the molecular weight value obtained on gel filtration for the HMWt species. A cluster containing one of each of the enzyme (MDH, CS, IDH, AC and FM) would not be predicted to elute between PDH and OGDH (molecular weights of approx. 4×10^6 and 2×10^6 respectively), unless it was itself associated with either multienzyme complex. However, three copies of each enzyme would give a molecular weight of 2.1×10^6 compared to an estimated molecular weight of the HMWt species on gel filtration of 2.6×10^6.

The observation of an increase in enzyme activity may be interpreted in several ways. The HMWt species may be composed of three copies of each of the five enzymes which are now regarded as being physically associated as a loose CAC cluster. Alternatively, one copy of each enzyme may be present, but the apparent activity of

those enzymes was less when they were in association with each other than if they were dissociated. This would indicate that external substrate was prevented from entering the cluster; enzyme activity would only give a measure of activity of enzyme active sites facing away from the internal environment of the cluster.

A combination of both views may be a more realistic interpretation of the difference in enzyme activity in associated and dissociated forms of the enzymes. Sonication and consideration of the estimated molecular weight of the HMWt species indicated that the cluster may be composed of more than one copy of each of the five enzymes. The structural organization of these enzymes may be arranged in such a way that externally added substrate, as in an enzymic assay, measures approximately one third of the potential activity of the enzymes. Steric and structural conditions may be such that free passage of substrate into and out of the cluster is prevented. A microenvironment is thus formed within the metabolic compartment of the associated enzymes. A potential advantage of compartmentation of the enzymes of a metabolic pathway, such as the CAC, would be to create a restricted microenvironment for substrate. Substrate may be 'channelled' directly from one active site to another, preventing diffusion into the bulk phase of the cell. A high local substrate level may be achieved with an overall low substrate level, for example, with oxaloacetate. This point will be expanded and discussed later.

The observation of an increase in enzyme activity on disruption of the multienzyme system may be similar to the phenomenon termed 'crypticity' reported by Duchon and Collier (1971) where an in-

crease in activity of enzymes released to the supernatant after washing a membrane fragment was recorded, despite a sixfold dilution of the pellet. Similar observations were made by Gorringe (1977) and in both cases the interpretation of their findings was based on restricted access to substrates and co-factors if the enzymes were specifically associated, whether it be with the inner surface of the membrane or with each other.

2. Differences in inactivation profiles of associated and dissociated enzymes

Examination of inactivation and inhibition plays a useful part in enzyme studies. They reflect the individual enzyme's characteristic and unique chemical and catalytic behaviour, the interpretation of data leading to a clearer understanding of the function of the enzyme.

The CAC enzyme CS has been studied extensively and metabolic regulators, chemical inhibitors and inactivators of the enzyme are well documented (Weitzman and Danson, 1976). The study of the inactivation of an enzyme demonstrated as existing in specific association with other enzymes in its metabolic sequence was undertaken. A distinct difference between CS in the HMWt species and that which eluted as a LMWt species on gel filtration may be interpreted as a reflection of the actual physical association of CS with the CAC cluster. A distinction could be thus made between associated and dissociated enzyme.

The association conferred thermal stability on the enzymes from *E. coli* and *B. subtilis* species. The protection against thermal inactivation of the enzyme when it is associated with other enzymes is facilitated by maintenance of the enzyme's native state, by forces of interaction with surrounding proteins which are absent in enzyme in free solution.

In *E. coli*, DTNB has been found to cause inactivation of CS (Weitzman, 1966). The site of inactivation may be at the the NADH-binding site on the enzyme. The inability of the chemical modifier DTNB to inactivate CS when it was in the HMWt species demonstrated that there was restricted access to this enzyme when it existed in association with other CAC enzymes. As reported in Results, the inaccessibility of DTNB illustrated that CS was either surrounded by other enzymes or proteins or that it was embedded within another structure.

The presence of a divalent cation (Mg^{2+}) resulted in a conformational change of the enzyme and allowed access of the inhibitor to its site of interaction, resulting in inactivation of CS. The fact that a change in conformation in one of the enzymes of the cluster resulted in a modification in access to that enzyme, may indicate that slight conformational changes have a role in the regulation and control of the association of enzymes. A possibility exists for the role of divalent cations in enzyme interactions, as discussed by Sols and Marco (1970).

3. Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was used to demonstrate that the HMWt species isolated on gel filtration of lysed bacterial spheroplasts existed as a physically associated entity. Ultra-centrifugation of the HMWt species on sucrose gradients resulted in co-sedimentation of the enzyme activities. A control experiment using dissociated enzymes showed that they sedimented to varying degrees. Despite the possibility of dissociation of the cluster by dilution through the gradient, the enzyme cluster remained intact, the viscosity of the sucrose medium may have been sufficient to prevent dilution effects.

By employing three, quite distinct, biochemical techniques, it was shown that the CAC cluster, isolated on gel filtration existed as a physical entity. The multienzyme cluster possessed characteristic behaviour distinct from that displayed by 'free' enzyme.

The intracellular location of the CAC cluster was investigated using centrifugation techniques in an attempt to confirm findings by other workers in the field. Association between the CAC cluster and structural components in the cell may lead to an indication of a possible *in vivo* location. Association of CAC enzymes with membrane was first suggested by Mitchell (1963).

Intracellular Location of the CAC Complex

Techniques of differential centrifugation and the examination of enzyme released on washing the lysed pellet were used in an attempt to identify the location of the CAC enzymes in bacterial

cells. Data on the centrifugation patterns of the enzymes may be interpreted in terms of the differential location of the CAC enzymes within the cell.

In separate experiments, the supernatant and the pellet of lysed bacterial spheroplasts were examined. The supernatant was subjected to a series of differential centrifugations from 1 000 g to 150 000 g and the resultant pellets examined. The pellet of lysed spheroplasts was washed with buffer and enzyme activity released from each was assayed. Both these procedures indicated similar findings.

The CAC enzymes were not all immediately released from the cell, as would be predicted if they were wholly soluble. The enzymes appeared to be freed from the cell in varying ratios. CS and MDH showed similar washout and differential centrifugation patterns. FM appeared to be more associated with the membrane fragments. FM is the enzyme which follows the integral membrane protein SDH, in the metabolic pathway of the CAC. If organization of the CAC exists in association with the inner surface of the inner bacterial cell membrane, one can envisage that SDH may be the core component on which, through FM, the other CAC enzymes might be organized.

IDH, on the other hand, showed less of a tendency to be sedimented with the membrane fragments, a possible indication that IDH itself is not organized on the membrane but exists with the CAC cluster through association with AC, the preceding enzyme in the cycle.

The observation made on the proximity of FM to the inner membrane reflects similar findings by Matlib and O'Brien (1975), where concentration of digitonin necessary to unmask FM activity in treated mitochondria were lower than those for MDH and CS. D'Souza and Srere (1983b) reported findings in studies of cross-linked mitochondria, indicating a preferential membrane location of CAC enzymes from liver and a close proximity of all proteins in the matrix. Wit-Peeters *et al.* (1971) concluded that for a number of mitochondrial matrix enzymes, 'no clear distinction can be made between membrane-bound and soluble matrix enzymes' but the enzymes only differed in the tightness of their binding to the inner membrane.

D'Souza and Srere (1983a) have shown that CS, MDH and FM bind to the inner mitochondrial matrix in a saturable process. The binding was fairly tight in 2 mM Hepes, pH 7.0 but was quite sensitive to ionic strength. Rendon and Waksman (1973) observed that MDH and aspartate aminotransferase could be bound by inner mitochondrial membranes and various metabolites could trigger the specific release of these enzymes.

The data presented on the putative intracellular location of CAC enzymes is consistent with the scheme drawn up by Srere (1985) on the binding of these enzymes to the inner mitochondrial matrix.

Specific binding components for CAC enzymes have been proposed on the inner mitochondrial matrix membrane. NADH : ubiquinone reductase (complex 1) of the mitochondrial inner membrane

respiratory chain was shown to bind a number of mitochondrial matrix NAD-linked dehydrogenases including MDH and OGDH (Sumegi and Srere, 1984). The binding of CS to the citrate receptor on the surface of the membrane was suggested by Srere (1985). Porpaczy *et al.* (1986) presented good evidence for the existence of a ternary complex; NADH : ubiquinone reductase-OGDH-NAD^+-IDH suggesting that a part of the CAC is located in the vicinity of the respiratory complexes. Each of the proposed binding components for the CAC enzymes are sites which have other functions in the cell. The coordinated organization of mitochondrial membrane and matrix enzymes would facilitate the channelling of intermediates of the CAC and on the other hand, the channelling of NADH to the respiratory chain.

The metabolic advantages of enzyme binding to membranes may be based on the microenvironment existing in the proximity of membranes. Water is now regarded as taking on a different structure from bulk water when it is adsorbed to subcellular cytoskeletal structures and membranes, being more ordered, with reduced rotational and translational mobility and altered solvent properties. The reduced dielectric constant in non-bulk water and the subsequent alteration in ionic interactions would influence molecular interactions. The result of these differences could give rise to an uneven distribution of solutes and to partition effects.

Enzymes existing in this environment may have significantly altered classical control parameters, such as Michaelis constants, and if so,further regulatory controls are introduced (Masters, 1981).

Clegg (1984) visualized that in evolutionary terms early cells 'devised' a means of escaping the chaos of solution chemistry by attaching their enzymes to a framework that itself could be controlled. The existence of an intimate connection between cellular architecture and metabolic processes could lead to a dynamic relationship under tight control, the disruption of which would lead to malfunction and loss of regulation.

If the CAC enzymes exist *in vivo* in association with each other, possibly on or adjacent to the inner surface of the bacterial membrane, a metabolic compartment would be formed. A metabolic compartment is regarded as a subcellular region of biochemical reactions metabolically isolated from other cellular processes. Reactions occurring within a compartment depend on the metabolism of the rest of the cell, both for the supply of initial reactants and removal of final products. Gain or loss of intermediates from individual steps of the sequence of enzymes is contrary to the concept of compartmentation and such reaction systems are described as 'leaky'. This form of compartmentation may parallel that seen in the CAC, with its apparent tendency for dynamic associations.

The use of Bacterial Mutants in the Study of Enzyme Associations

Bacterial mutants deficient in one or more of the enzymes of the CAC provided an alternative approach to studying enzyme organization. A change or absence of enzyme as the result of genetic manipulation may be manifested as a disturbance to the

delicate balance of enzyme organization in the cell. As such it provides a true *in vivo* method of studying metabolic processes.

The approach used in previous experiments, of examining the lysate from wild-type *E.coli* spheroplasts for the presence of a HMWt species, was applied to a series of bacterial mutants.

E. coli W620 is a mutant which possesses no CS activity. It is unable to grow on glucose as sole carbon source and has a requirement for glutamate, which feeds into the cycle at oxoglutarate. Examination of W620 lysate on gel filtration revealed the existence of enzymes co-eluting as a HMWt species. The proportion of enzymes MDH and FM varied from that obtained in the wild-type *E. coli*. The levels of MDH, normally the most active enzyme in the enzyme complex of wild-type *E. coli*, were reduced in the CS-negative mutant. Already considerable evidence exists for close association between CS and MDH enzymes. It may be possible that in mutants deficient in CS, the MDH enzyme shows less of a tendency to associate with other enzymes of the complex. With a lower proportion of MDH present in the complex, sites on FM which had previously been shielded may become exposed.

The presence of a HMWt species from a mutant deficient in one the enzymes of the cycle,may indicate that although a genetic change has taken place at the active site or binding site of co-factor,such that the enzyme is essentially inactive, the CS protein itself remains intact. Interactions with other enzymes through complementary surfaces on the protein continue to take place.

The molecular structure of CS and the taxonomic status of the source organism show a close correlation (Weitzman and Jones, 1968; Weitzman and Danson, 1976; Weitzman, 1981). CS's fall into two major groups, those of the Gram-negative bacteria which have 'large' hexameric enzyme allosterically inhibited by NADH and those of the Gram-positive bacteria, archaebacteria and eukaryotes which have 'small'dimeric enzymes isosterically inhibited by ATP.

Experiments on the recombination of low molecular weight fractions into a HMWt species illustrate that either 'large' or 'small' CS type would associate with the other enzymes of the cycle. There apppeared to be no species specificity; CS from a eukaryotic source associated with enzymes from *E. coli* to yield a functional complex.

This lack of species specificity has been shown by Halper and Srere (1977) using PEG turbidity studies as an indicator of enzyme association; pig heart CS or *E. coli* CS interacted with pig heart mMDH. Beeckmans and Kanarek (1981) demonstrated specific interactions of CS with MDH and FM, these reactions not being restricted to enzymes originating from the same species; for example, *E. coli* CS replaced enzyme from pig heart in an interaction with pig mMDH. One current theory on this aspect is that, during evolution, the surface of at least these CAC enzyme molecules must have been subjected to severe selectional pressure.

The revertant mutant of *E. coli* K.1.1r3, possessing a 'small' (Gram-positive type) CS in place of the wild-type 'large' CS, was used to ascertain the dependence on enzyme size for specific association. Organization of CAC enzymes was still apparent despite a change in size of one of the component enzymes. The regions of bonding or association with other enzymes obviously remain constant despite changes in CS from a hexamer to dimer and the resulting differences in molecular structure, size and shape.

At this point a note of caution may be pertinent. Although the use of bacterial mutants would provide an excellent means of investigating specific enzyme interactions and cellular organization, unless the mutation is well defined, a false picture may emerge. If the mutation is such that a complete protein is deficient, the use of the bacterial mutant may give useful indications of enzymes with which it has specific intracellular interactions. If the mutation, however, has resulted in an inactive enzyme protein being formed, inter-enzyme associations may still exist through unchanged contact surfaces.

Immunochemical examination using single radial immunodiffusion of the mutant CS-negative *E. coli* extract with anti- *E. coli* CS antibody indicated cross-reactivity between the proteins. It therefore appears likely that the mutant W620 possesses an enzymically inactive CS protein. The use of bacterial mutants in the study of enzyme associations therefore has some shortcomings but may be potentially useful in mutants where the bacterial mutation is well defined.

Enzyme Association and Cell Ageing

Enzyme association was seen to decrease as the age of bacterial cells in culture increased. The decrease in association was manifested in a lower proportion of enzyme being present in the HMWt species, compared to that of a standard 16 hour cell culture. Although the overall enzyme activity decreased with age, there was a drastic reduction in the relative proportion of enzyme associated with other CAC enzymes.

The apparent decrease in enzyme association coincided with the exponential death phase of the bacterial culture. Within the culture during this phase, a proportion of bacterial cells will be dying whilst other cells will be actively growing. Therefore, although statistically the bacterial culture is undergoing exponential death, the system remains complex. Examination of the early log phase of bacterial growth, may give further indication of the interaction of enzyme organization and cell age.

The abundance of theories on the question of cellular ageing suggets that it cannot be traced back to a single causative factor. It appears rather to be a systemic process, the consequence of a disturbance in the balance of a number of interactions including alterations in the various macromolecular and enzyme organizational patterns.

Cell ageing and disease processing may not only be due to genetic and post-translational events affecting catalytic activity but also the occurrence of enzymes with unchanged catalytic activity *in vitro* but impaired ability to assemble *in vivo*. These enzymes therefore fail to build up the correct physiological superstructure possibly due to amino acid substitution in a heterologous contact

surface region (Friedrich, 1984). Weak macromolecular interactions such as those of the CAC cluster may be more vulnerable to degradative processes which possibly occur during cell death.

The fact that the incidence of CAC cluster was found to be higher in actively dividing bacteria than in cells in the stationary or death phase of growth may be indicative of some form of *in vivo* role for organized enzymes. It might be speculated that it is advantageous to the cell that, during active growth and division, the enzymes of key metabolic pathways are in close proximity. The advantage of sequential steps of a metabolic pathway being organized, such that product is channelled from one enzyme to the next, may be important during phases of active growth, from a view of metabolic efficiency or for the maintenance of a high level of flux through the enzyme sequence.

Enzyme Associations in Mitochondria

One of the earliest comments on compartmentation of the CAC was made over forty years ago in a communication by Green and co-workers on 'cyclophorase'. In the paper Green *et al.* (1948) documented the properties of a cluster of enzymes which catalysed the complete oxidation of pyruvic acid by way of the Krebs CAC and which was termed the cyclophorase system. It was suggested that the enzymes constituting the cyclophorase complex was either just a loose association of chemically and physically discrete enzymes or that they were all inextricably associated wih one another as in a mosaic.

Although the observations of Green *et al.*(1948) were later shown to be consistent with the compartmentation of CAC enzymes in the mitochondria of eukaryotic cells, further work on the organization of these enzymes has revealed another level of compartmentation within the mitochondrion. Evidence for specific organization of these enzymes in mitochondria was presented in the Introduction. Despite a considerable amount of work in the area a multienzyme cluster containing the activities of the CAC has not, as yet, been isolated.

The knowledge that conventional biochemical techniques are not conducive to the maintenance of fine intracellular associations between enzymes, has been established. By extending the technique developed for the prokaryotic cell, the lysate of freshly prepared rat liver mitochondria was examined by gel filtration on Sepharose 4B. Two elution peaks were noted for at least five of the CAC

enzymes. These enzymes co-eluted as a HMWt species with a molecular weight of approximately 2×10^6..The HMWt species was similar to that obtained from *E. coli* and other bacteria in the ability to be formed by ultrafiltration of low molecular weight fractions. The specific associations which formed the CAC cluster were demonstrated in plant, insect and mammalian tissue preparations.

The HMWt species detected by this approach may be similar to the sedimentable five-enzyme cluster reported by Robinson and Srere (1985). This complex of enzymes was described as: containing the activities of MDH, CS, IDH, AC and FM, sedimentable at 32 000 g, 30 min, from a gentle sonic or osmotically disrupted mitochondrial preparation, and stable at conditions presumed to exist *in situ*. More disruptive isolation techniques, e.g. longer sonication, or freeze-thaw, did not yield the complex of enzymes.

It has now been demonstrated in mitochondria that interactions between CAC enzymes exist, as well as interactions between the enzymes and the matrix surface of the inner membrane (Srere, 1985). The theoretical existence of functional organization of the CAC on the supramolecular level was proposed several years ago (Srere, 1976). Physical evidence for the existence of specific CAC compartmentation may now be emerging. It is possible that the five-enzyme HMWt species isolated on gel filtration, the five-enzyme sedimentable cluster described by Robinson and Srere (1985) and the putative CAC 'metabolon' (Srere, 1985) are one and the same entity.

The view of the enzymes of the CAC existing within the mitochondrion in an ordered array, bound to be matrix side of the inner membrane, is emerging from that which is theoretically plausible to that which is an indication of the functional *in vivo* state. The altered kinetic behaviour of organized enzymes as reported by Mosbach and Mattiasson (1970) might achieve higher throughput or flux at low substrate concentrations than would a random array of enzymes at the same substrate concentration.

Topology of the Enzyme Cluster

Immunochemical techniques were used to study the topology of one of the enzymes of the CAC multienzyme cluster. Antibodies are used as probes to locate specific targets, i.e. epitopes on antigen surfaces, interaction with which results in cross-reactivity and complex formation. When antibody is raised to an enzyme, interaction between the two may give rise to an inactivation of the activity of that enzyme. This property was exploited in a study on the CAC cluster isolated by gel filtration.

The specific inactivation of CS in the HMWt species indicated that the enzyme cluster could not be interpreted as enzyme entrapment in a substrate-permeable, but enzyme-impermeable, vesicle. Antibodies (Mr = 160 000) would not be expected to penetrate a vesicle that retained CS (Mr = 250 000). Since the exposed CS in these clusters could be inactivated by antibody, the enzymes were not merely entrapped in substrate-permeable vesicles.

Incomplete inhibition was observed for CS in the cluster when compared with the free enzyme, it may therefore be concluded that

specific epitopes on the surface of the CS molecule were not exposed. Thus a proportion of the enzyme may have been buried within another structure. These structures may be other enzymes to which CS was bound, membrane or other unidentified components. Although the associations between the CAC enzymes are described as being weak, the spatial proximity of the enzymes may result in a microenvironment which affords only limited access.

A similar explanation accounted for other inactivation profiles given earlier. The orientation of the enzyme in the cluster was such that specific sites were shielded from the effects of antibody, or other inactivator and limited access results from the microcompartmentation of enzyme.

The Presence of Lipid in Association with the Multienzyme Cluster

The possibility that a lipid component may be involved in the CAC cluster was examined using thin layer chromatography. The developed chromatogram showed the presence of a single phospholipid corresponding to that of phosphatidylinositol. This lipid is a minor component of the plasma membrane of bacteria (Rose, 1976). Co-chromatography of phosphatidylinositol with the extracted sample from the cluster may be coincidental and is not firm evidence for its existence in association with the cluster. It is highly unlikely that if the cluster were enclosed in some form of membrane vesicle, only one form of lipid would be present. The fact that multiple forms of lipid were not detected is evidence in itself that the cluster was not surrounded by a vesicle.

An interesting observation emerged on examination of the lipid content of the cluster. It was evident that a low density lipid fraction may be present. This fraction ran with the solvent front and was present in all HMWt species samples examined. The identification of this lipid would provide support for the theory that some form of lipid core component may exist in these dynamic soluble multienzyme systems.

As pure conjecture, it may be possible that the low density lipid corresponds to evidence presented by Else (1986) on the physiological role of palmitoyl- CoA. Palmitoyl-CoA, a fatty acyl-CoA, has been suggested to be involved with anchoring CS to a membrane and/or to other metabolically related proteins (Webster *et al.*, 1980; Fahien and Kmiotek, 1983). The inhibitory effect of palmitoyl-CoA on CS *in vitro* may be explained; bound palmitoyl-CoA affects the entry of substrate molecules to the active site *in vitro*, whilst *in vivo* the substrate is provided by channelling through a loosely-associated multienzyme complex of CAC and related enzymes (Else, 1986). Similar effects are seen with glutamate dehydrogenase, which is also thought to be involved in multienzyme complexes (Taketa and Pogell, 1966).

Experimental evidence is obviously necessary to ascertain whether the low density lipid bears any relation to palmitoyl-CoA. Only when this is shown can further conclusions be drawn.

It may be noted at this point that in preliminary studies on the binding of isolated CAC cluster to membranes from *E. coli*, the

binding of cluster was enhanced in the presence of palmitoyl-CoA (the results of the study are not presented in this thesis). Palmitoyl-CoA also markedly increased the binding of glutamate dehydrogenase to MDH (Fahien and Kmiotek, 1979).

The Use of a Chemical Cross-Linker to Demonstrate Enzyme Association

Previous studies using a bifunctional chemical cross-linker to examine enzyme associations include those of D'Souza and Srere (1983) on mitochondrial matrix proteins, Fahien *et al.* (1978) on glutamate dehydrogenase-aspartate aminotransferase complexes and Mattiasson *et al.*, (1984) on MDH and CS.

Cross-linking of the HMWt species with DMDBP produced a complex of CAC enzymes. Although cross-linking between the enzymes had taken place, as shown by SDS-polyacrylamide gel electrophoresis, the inactivation of CS usually produced by DMDBP was not evident probably due to restricted access to CS. Dissociation of the CAC cluster has been shown to occur on gel filtration of the HMWt species, probably due to the dilution effects of buffer. Dissociation of the cluster was prevented by cross-linking with DMDBP, by the formation of intra-molecular links between enzyme proteins. The covalent constraints imposed by the cross-linker were reversed by cleavage of the cross-linked species with dithio-threitol, to the dissociable form. This was probably due to the removal of covalent constraints which provided a physical barrier to dissociation.

It has been shown, for example by Peters and Richards (1977), that the conditions of cross-linking do not favour intermolecular cross-links between enzymes free in solution. It may therefore be assumed that cross-linking between different proteins of the low molecular weight form probably did not take place. The distances, however, between individual enzyme proteins in the cluster, may have been sufficiently small to facilitate intermolecular cross-linking between enzyme proteins. It may be deduced that the distances between the CAC enzymes in the cluster must be in the order of 1.1 nm since this figure corresponds to the 'arm' length of the cross-linking agent DMDBP.

The differential inhibition of CS by DMDBP of the associated and dissociated forms of the enzymes, was exploited in other studies such as sucrose density gradient centrifugation.

Kinetic analysis of enzymes in the CAC cluster

Kinetic analysis of enzymes present in the CAC cluster has been presented. In three bacterial species, examination of the kinetic properties displayed by CS for each of its substrates oxaloacetate and acetyl-CoA revealed a trend in the Km values obtained. In each case, the Km for both substrates was lower in the HMWt than in the LWMt species. It therefore appears that the incorporation of CS into a multienzyme cluster results in some change in the apparent kinetic parameters of that enzyme.

An investigation of the kinetic parameters displayed by other enzymes of the multienzyme cluster showed a similar trend. Generally, the Km for each substrate was found to be lower in the associated enzyme than in the free enzyme. The relative differences in Km values varied for each enzyme-substrate pair. A difference in V_{max} was also noted in the associated and free forms of each enzyme; generally the values for V_{max} were lower in the associated form.

Extreme caution is required in the interpretation of the kinetic data. It should be recognised that the results presented represent only a preliminary examination of the kinetic parameters of the multienzyme cluster; analysis of the data was limited and the system is still relatively undefined.

A difference in kinetic parameters has been observed by other workers. Sumegi *et al.* (1980) reported that the Km for CoA in the PDH complex is decreased from 10 µM to 1.5 µM when the enzyme interacts with CS to form a PDH-CS cluster. No changes in the Km for pyruvate or NAD^+ or in the V_{max} were noted. The Km of CS for acetyl-CoA was 3.1 µM for the PDH-CS cluster compared to 12 µM for

CS alone. These results indicate not only a proximity effect in the cluster but also a possible alteration in the active sites of the two enzymes, when the cluster is formed (Welch, 1977; Srere, 1985).

In the analogous interaction of OGDH and STK, Porpaczy *et al.* (1983) have shown that the Km of OGDH for CoA is 5.5 μM for the enzyme alone and 3.5 μM for the OGDH-STK complex. The other kinetic constants did not change. For STK alone, the Km for succinyl-CoA was 65 μM and for the OGDH-STK cluster the Km was 1.5 μM. As in the previous case, the complex of sequential metabolic enzymes demonstrated an apparent kinetic difference from the individual enzymes.

Backman and Johansson (1976) showed that complex formation occurred between mitochondrial MDH and aspartate aminotransferase and Bryce *et al.* (1976) showed that a kinetic advantage existed for the coupled system.

The kinetics of CS *in situ* were studied by Matlib *et al.* (1977, 1978) in permeabilized toluene-treated mitochondria. In this system, there was no change in the V_{max} of CS or its Km for oxaloacetate. In the permeable mitochondria the enzyme concentration was unchanged from its *in vivo* concentration, compared to the enzyme in dilute solution. The Km for acetyl-CoA was increased but this appeared not to be due to a diffusion barrier against acetyl-CoA. Thus the hypothesis that the environment *in situ* would change the kinetic characteristics of CS, so that faster rates could be achieved at low substrate, but high enzyme concentrations, was not upheld by these results.

Thus, previous work in this area has led to no specific conclusions for the kinetic differences between associated and

dissociated enzyme sequences. However, the differences observed in the Km values for a number of CAC enzymes must reflect a modification of the kinetic parameters of the enzymes as a result of their interaction. It remains possible that some form of alteration (conformational change?) of the active sites of the enzymes may have arisen from the specific association of the enzymes into a cluster, this change being manifested as a lowering of Km values for substrates.

The role of metabolic regulators in the CAC cluster

Evaluating the *in vivo* regulation of enzymes is obviously a much more difficult task than determining the inhibition patterns for a purified enzyme *in vitro*. Studies of substrate uptake and accumulation of cycle intermediates under various conditions with perfused hearts and with isolated mitochondria in different metabolic states have shed some light on the possible *in vivo* control of the CAC, as reviewed by La Noue and Williamson (1971), Newsholme and Start (1973), Williamson (1976) and Lane and Mooney (1981). It is generally accepted that essentially CS, together with IDH (the $NAD^{\pm}$ and $NADP^{\pm}$dependent enzymes) and the OGDH complex, are the subject of regulatory control and influence the flux of substrates through the CAC (Randle *et al.*, 1970; Tischler *et al.*, 1977; Williamson and Cooper, 1980; Greksak *et al.*, 1982).

In eukaryotes, CS activity can be regulated by the availability of either of its two substrates, oxaloacetate and acetyl-CoA. The regulation of the eukaryotic CS does not appear to be due to the existence in the enzyme of any special regulatory binding sites or

to covalent modification, but seems to be achieved by subtle changes in the cellular compartments of the availability of the two substrates, particularly oxaloacetate, in response to fluctuating needs of the cell.

A clear relationship has been demonstrated and extensively documented by Weitzman and coworkers between the regulation of CS activity and the taxonomic status of the source organism (Weitzman and Jones, 1968; Weitzman and Dunmore, 1969; Weitzman and Danson, 1976; Weitzman, 1981). Two metabolites which have been shown to regulate the activity of bacterial CS, ATP and OG, were examined for their inhibitory action on CS activity when it exists in association with other CAC enzymes in a multienzyme cluster.

Regulation of CS by ATP

ATP demonstrated differential inhibition of the free CS enzyme and the associated enzyme. In *B. subtilis*, almost 100% inhibition was reached in the LMWt species at 0.2 mM ATP, whereas inhibition in the HMWt species was less than 10%. The level of inhibition in the HMWt species appeared to plateau at about 40% (at 1.5 mM ATP).

The difference observed between the associated and dissociated forms of the same enzyme may be explained by the orientation and accessibility within the microenvironment of the enzymes. In the restricted microenvironment, as would probably exist in a multienzyme cluster of enzymes, the enzyme active site may be less accessible to the freely diffusing substrate. Alternatively, the differential inhibitory effects of ATP may be a reflection of the altered kinetic parameters of the enzyme in the associated state.

Thus the apparent lower level of ATP inhibition observed in the multienzyme HMWt species may reflect the observed lower Km value for CS, when compared to the free enzyme. Although this latter interpretation may apply to CS from *B. subtilis*, it may not be applicable to *E. coli* CS, where the addition of KCl was shown to lower the Km of the enzyme for acetyl-CoA and also to lower the K_i for ATP (Weitzman, 1981). The nucleotide therefore appeared to be a better inhibitor in the presence of KCl. The situation is obviously far from clear and further conclusions may be unwise due to the ill-defined nature of the multienzyme cluster.

Hathaway and Atkinson (1965) first reported a direct inhibitory action of adenine nucleotides on CS of yeast, this inhibition being exerted competitively against acetyl-CoA. Weitzman and Danson (1976) noted a wide range of organisms which have been reported to be sensitive to inhibition by ATP. Multiple-inhibition studies were used by Harford and Weitzman (1975) to show that ATP is an isosteric inhibitor binding at the acetyl-CoA site. ATP inhibition has also been observed, however, with CS enzymes from organisms in which which the CAC is believed to fulfil a purely biosynthetic role, rather than in energy production, for example, in cyano-bacteria (Lucas and Weitzman, 1975, 1977).

Doubt has been cast on the *in vivo* role of ATP in the regulation of CS. Weitzman and Hewson (1973), using a system of permeabilized yeast cells in which to study *in situ* regulation of CS, found that the enzyme was insensitive to ATP inhibition. Moreover, although ATP in the cell is probably largely chelated

with magnesium, ATP inhibition of CS was reduced or abolished in the presence of magnesium ions.

Regulation of CS by oxoglutarate

The inhibition of CS by OG was first reported by Wright *et al.* (1967). Weitzman and Dunmore (1969) showed OG to be an allosteric regulator of CS in several facultatively anaerobic Gram-negative bacteria. No such regulation of CS activity by OG was observed in strictly aerobic Gram-negative bacteria or in other types of organisms.

Regulation of CS from *E. coli* in the associated and dissociated states was examined with increasing concentrations of oxoglutarate. Inhibition of the free CS enzyme showed a hyperbolic dependence on inhibitor concentration. No inhibition of CS activity was noted, however, in the associated form of the enzyme, even up to 2 mM OG.

Steric considerations prevail as an explanation of the non-inhibitory action of OG on associated CS activity. The allosteric sites on CS, to which the OG modulator binds, may presumably be inaccessible. It is possible that the site on the enzyme to which OG binds is buried in the CAC cluster and therefore remains 'hidden' from the externally-added inhibitor. An alternative interpretation of the results may be that the OG binds to CS in the CAC cluster but, because of the juxtaposition of the enzyme next to other enzymes, the CS cannot undergo the conformational alteration normally produced by OG in the free enzyme.

Throughput activity of the CAC enzymes

The ability to measure overall pathway activity was a reflection of the presence and the catalytic competence of the component enzymes of that pathway. A system was established by which throughput activity could be measured in the CAC cluster, dissociated enzymes, re-associated HMWt cluster and cross-linked HMWt cluster. In each case, synthesis of product was observed. However, the steady-state rate of throughput was greater when the CAC enzymes were in an associated state. Dilution and sonication, both techniques which appear to destroy the delicate inter-enzyme interactions, resulted in a reduction of the steady-state rate of throughput activity.

As stated earlier, this result would not be predicted, since it is understood that steady-state rates are dependent on the enzyme of lowest velocity in a pathway. However, if the association of enzymes were to bring about an alteration in that enzyme, it is possible that a difference in steady-state rate may be due to the associated/dissociated state of that enzyme. Interpretation of such data is tentative, however, due to the undefined nature of the system under study. The observations, although preliminary, are interesting and warrant further investigation.

In the words of Oscar Wilde, "the truth is rarely pure and never simple". This may well be applied to the examination of the functional consequences of the organization of enzymes into multienzyme clusters.

GENERAL DISCUSSION

Compartmentation is one of the fundamental principles in the organization of living matter. The very existence of cells is a manifestation of biological compartmentation. The division of space does not stop at the boundaries of cells and is highly developed within eukaryotic cells. Within the least structured parts of the cell, spatial organization of metabolic processes may exist. Compartmentation at such a level is easy to propose, but hard to prove. If one accepts that metabolic processes *in vivo* are spatially ordered, the source of such order must lie in the organization of component enzymes, either among themselves or together with a supporting matrix.

> 'A cell is nothing but the population of component entities that constitute it. But these entities are not just of molecular rank, nor can their ordered behaviour in the group be fully appreciated and understood solely by studying them in isolation, out of context'.
>
> Weiss, 1963

The traditional 'grind and find' (Wilson, 1980) techniques of enzymology preclude the possibility of demonstrating 'cyto-sociological' influences on enzyme activity, unless physical interactions are strong enough to withstand harsh extraction techniques.

These 'cyto-sociological' aspects of enzyme function *in vivo* have been used by Welch and Keleti (1981) to describe the intricate and finely coordinated forms of spatio-temporal interactions among cellular components. It is therefore possible that many loose enzyme interactions have been overlooked in the past, as a result of the techniques employed to examine those enzymes.

The existence of multienzyme clusters of so-called soluble enzymes have now been demonstrated *in vitro* in the glycolytic system (Mowbray and Moses, 1976; Gorringe and Moses, 1980) and, as presented in this thesis, in the CAC enzymes of prokaryotes and eukaryotes. The true physiological significance of the aggregated state however can be realized only if it relates to the structural-functional integration of the metabolic framework of the cell as a whole.

Several specific advantages of the integration of cell metabolism have been postulated for enzyme clusters (Srere and Mosbach, 1974). The clustering of the component moieties may produce entities that have intrinsic catalytic properties unlike those of separate proteins, and the physical association may stabilize or enhance the overall activity of the enzyme sequence. The assembly into a cluster may increase the efficiency of the overall process, even if the intrinsic catalytic activities of the components are not altered on association: advantages may result from the proximal juxtaposition of constituent active sites within the enzyme system.

An important advantage of the aggregated state relates to metabolic compartmentation or 'channelling' at the molecular level.

The physiological advantage of channelling, as described by Davis (1967) lies in the potential ability of enzyme clusters with active sites in close proximity to effect an intracellular compartmentation of intermediate substances, common to one or more pathways (for example, oxaloacetate). Compartmentation therefore serves to maintain a high **local** concentration of intermediate substrate, with an **overall** low concentration of that metabolite in the cell. If each enzyme in a reaction sequence were separate and operated wholly individually, the concentrations of intermediates in the cell might be excessive and detrimental to the vitality of the cell. If, however, each sequence were composed of closely-linked enzymes, the optimal concentration could be kept localized. Where substrates are present at low concentrations, spatial organization at the enzyme level becomes significant with respect to diffusion considerations, for example with coenzyme A, and oxaloacetate. Completely free diffusion of metabolites and the absence of pathway segregation could lead to chaotic and inefficient systems *in vivo*.

It now seems highly probable that dynamic associations of enzyme systems, such as those of the CAC, may be the rule rather than the exception in cellular metabolism.

Directions for future work

As with many scientific investigations, the questions that have arisen as the result of the work presented in this thesis far outweighs the answers it has provided. Nevertheless the study may have gone some way towards identifying the existence of a form of structural organization within one of the so-called soluble metabolic systems.

Further work in the area could include many different and diverse directions of investigation. The intracellular location of the CAC cluster should be defined, possibly using binding studies to inner membrane preparations. Binding to and association with succinate dehydrogenase, the CAC enzyme which exists as an integral membrane protein, should be investigated. The relationship between succinate thiokinase and oxoglutarate dehydrogenase and the possible role of pyruvate dehydrogenase could be studied. This latter enzyme may provide some indication of the involvement of the multienzyme glycolytic sequence in directing acetyl-CoA into the cycle. This may be particularly pertinent in bacterial cells, devoid of classical intracellular compartmentation.

The demonstration of a functional role of enzymes existing as a multienzyme cluster, rather than in a free state and the subsequent kinetic advantage to the cell, are of prime importance. Detailed kinetic analysis using techniques such as stop-flow spectroscopy would allow transit and transient times to be calculated for the complete enzyme sequence. Radioactive channelling experiments may identify the form of metabolic compartmentation of the CAC enzymes. Calculation of the flux control coefficient would demonstrate

whether the cluster exists in bulk or non-bulk phase. The potential advantages conferred by multienzyme association into complexes include the segregation of competing pathways (metabolic channelling), catalytic enhancement (decreased transit and transient times) and the provision of a finer system of metabolic control (coordinate regulation, creation of new allosteric sites, etc.).

Exploitation of bacterial mutants with defined enzyme mutations may provide valuable information as to the central role of specific enzymes in cluster formation. Manipulation of culture conditions, anaerobic growth, use of defined media such as acetate or glucose may present an *in vivo* modification of the association of the enzymes. Examination of a variety of nutritional variables may be of particular value because of the influences that various growth conditions will have on the balance between metabolic pathways; such as between glycolysis and gluconeogenesis or between the CAC and glyoxylate cycle.

Electron microscopic investigations might include the use of gold or radiolabelled antibody directed to one component of the cluster, which may be used to identify the intracellular location of that enzyme.

Wilson (1980) debated whether the transient dynamic structuralization of the cytosolic enzymes of glycolysis in higher eukaryotes may in fact be the evolutionary vestige of the glycolytic "pathway particle" found in prokaryotes by Mowbray and Moses (1976) and Gorringe and Moses (1980). In this case, the rigid organization of the complex may have given way to a more flexible form of interaction, for regulatory purposes, in mammalian systems.

It may indeed emerge that a similar picture is reflected in the organization of the CAC enzymes.

It is now evident that 'soluble' enzyme systems, such as those of the CAC, can no longer be viewed solely in one dimension, as a metabolic pathway but must be incorporated into the three-dimensional map of metabolism with which they are intrinsically and inextricably combined.

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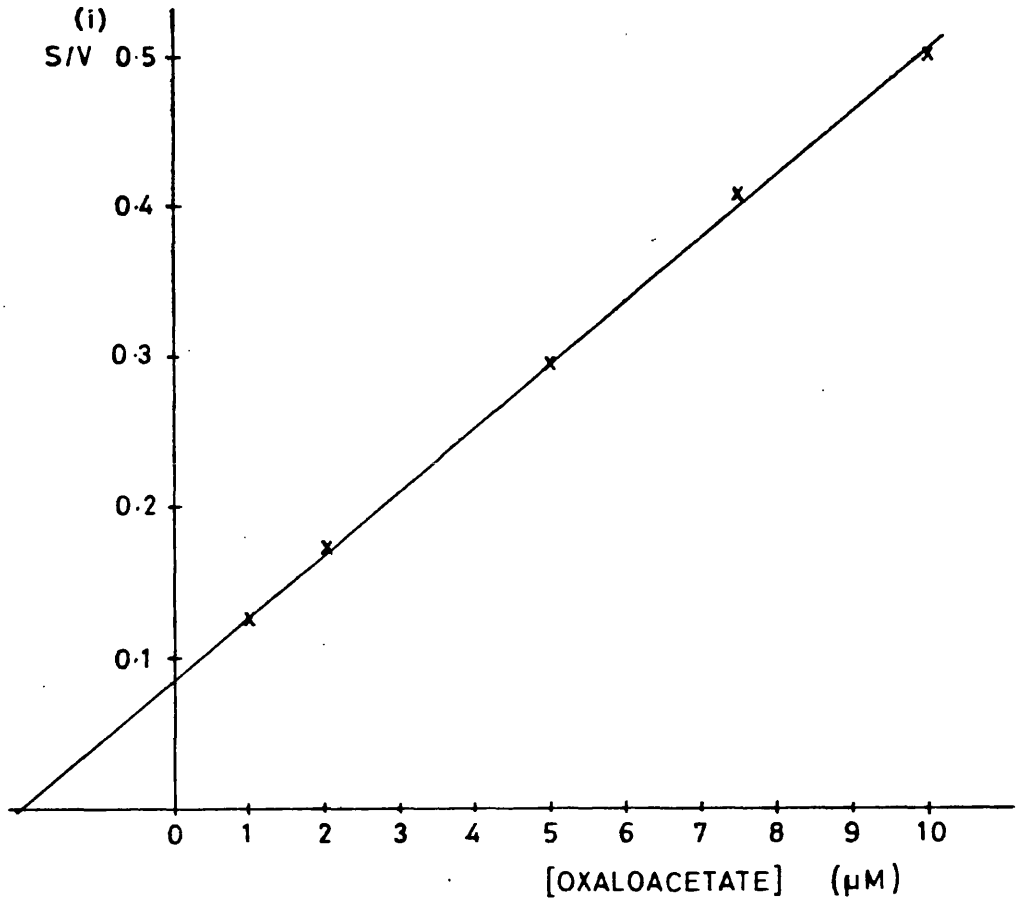
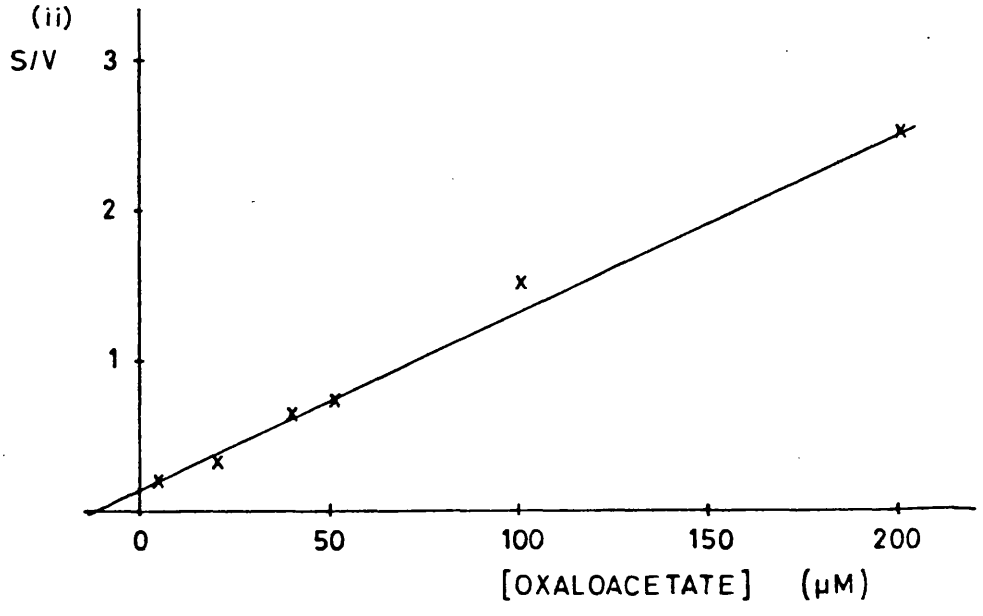
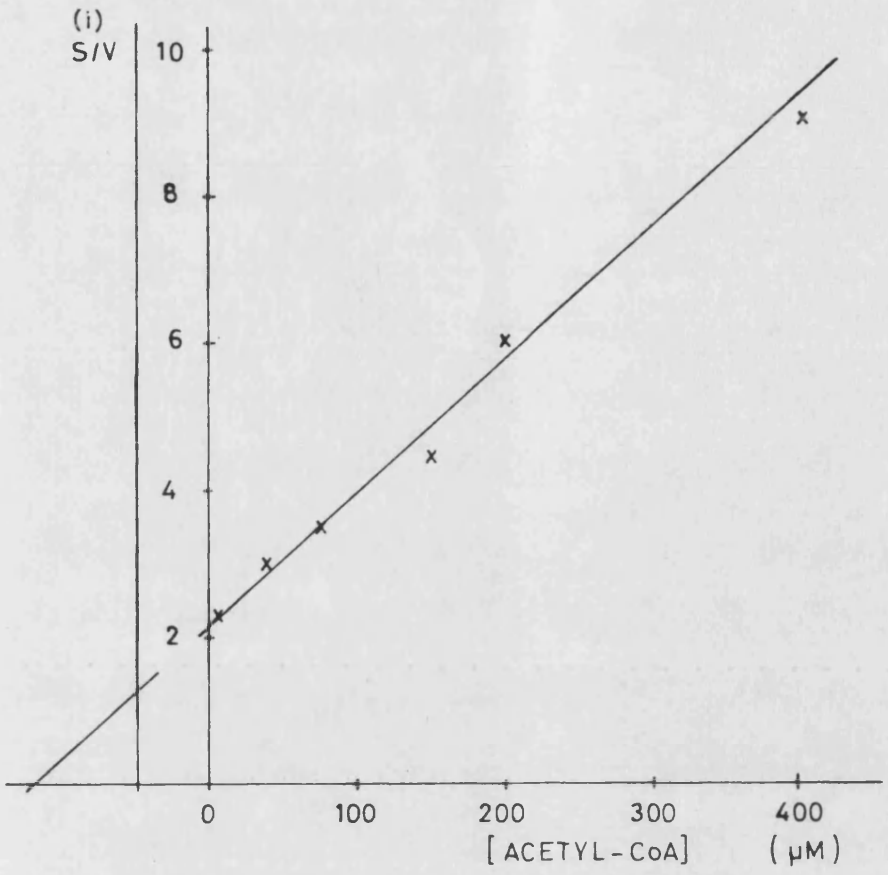
APPENDIX

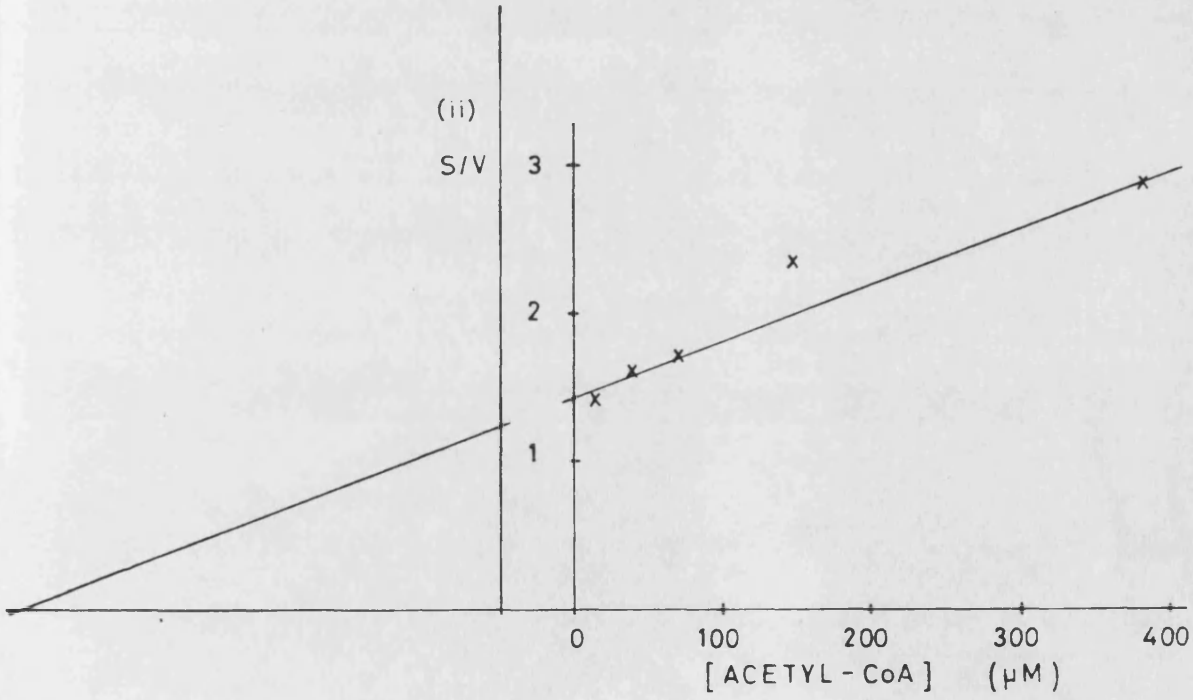




Half-reciprocal plots for the determination of the K_m of CS for oxaloacetate in Acinetobacter calcoaceticus

(i) HMWt (ii) LMWt





Half-reciprocal plots for the determination of K_m of CS for acetyl - CoA in Acinetobacter calcoaceticus

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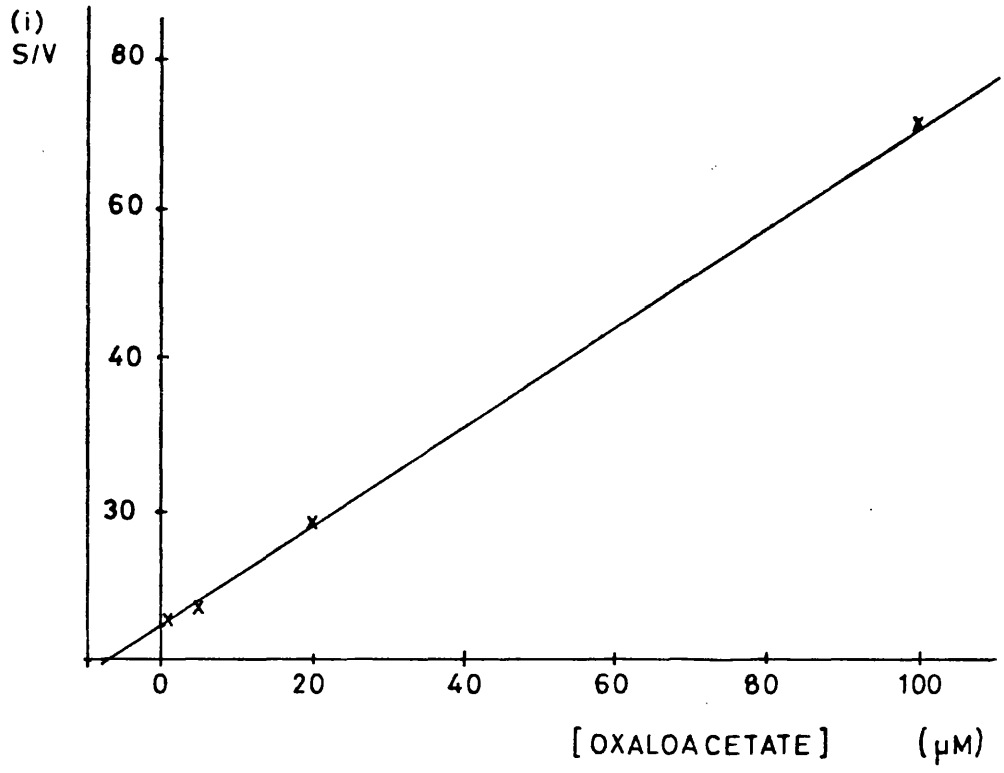

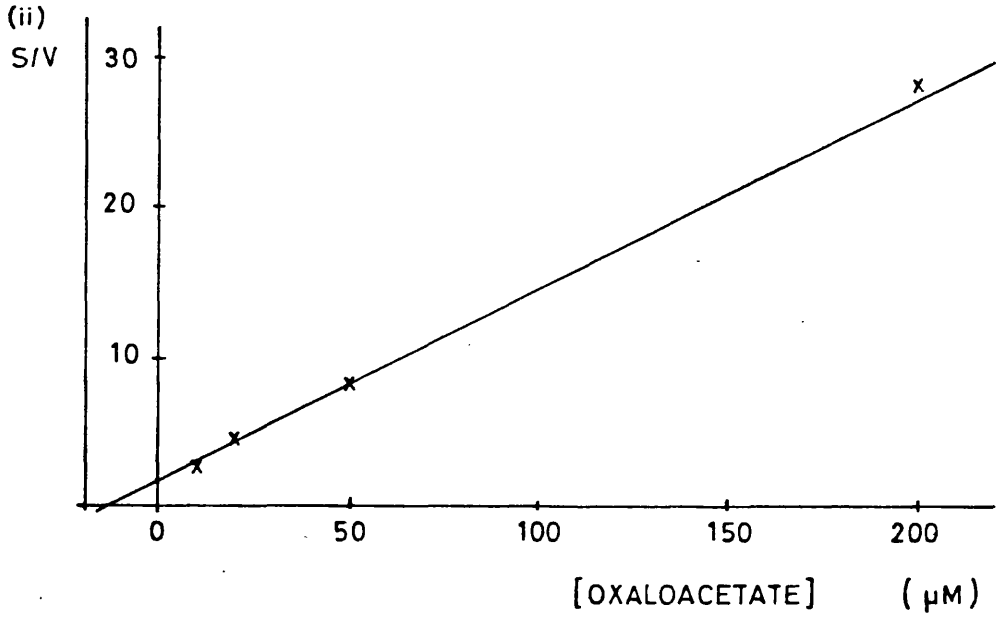

Half-reciprocal plots for the determination of Km of CS for oxaloacetate in Bacillus subtilis

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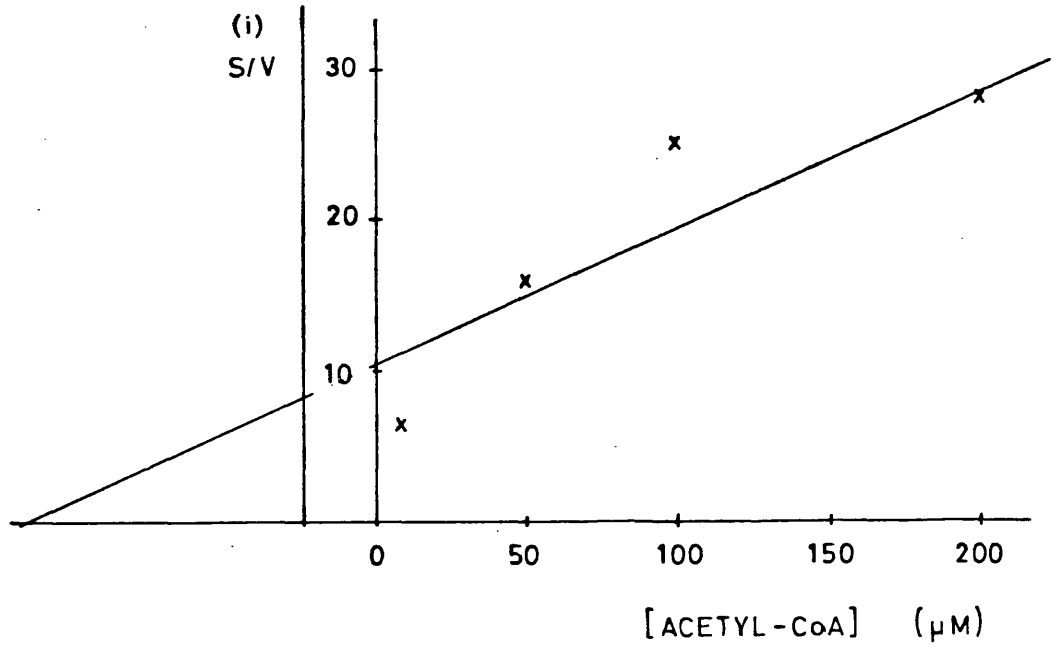

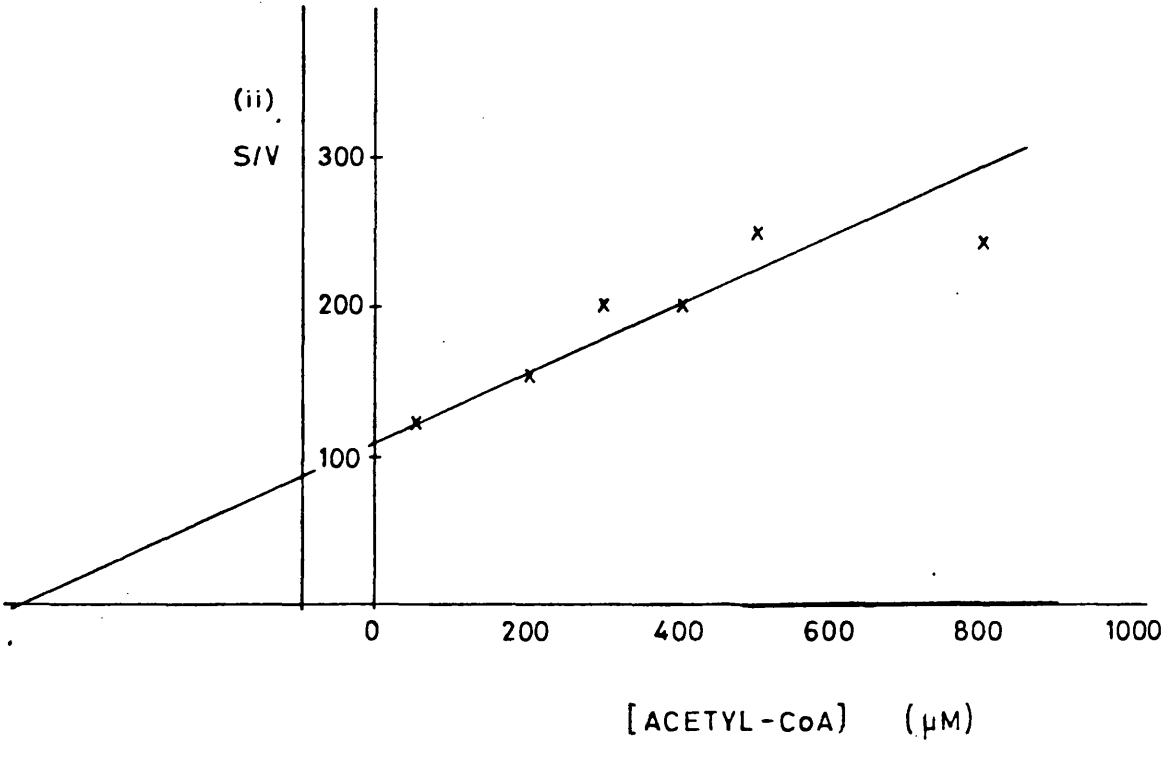

Half-reciprocal plots for the determination of Km of CS for acetyl - CoA in Bacillus subtilis

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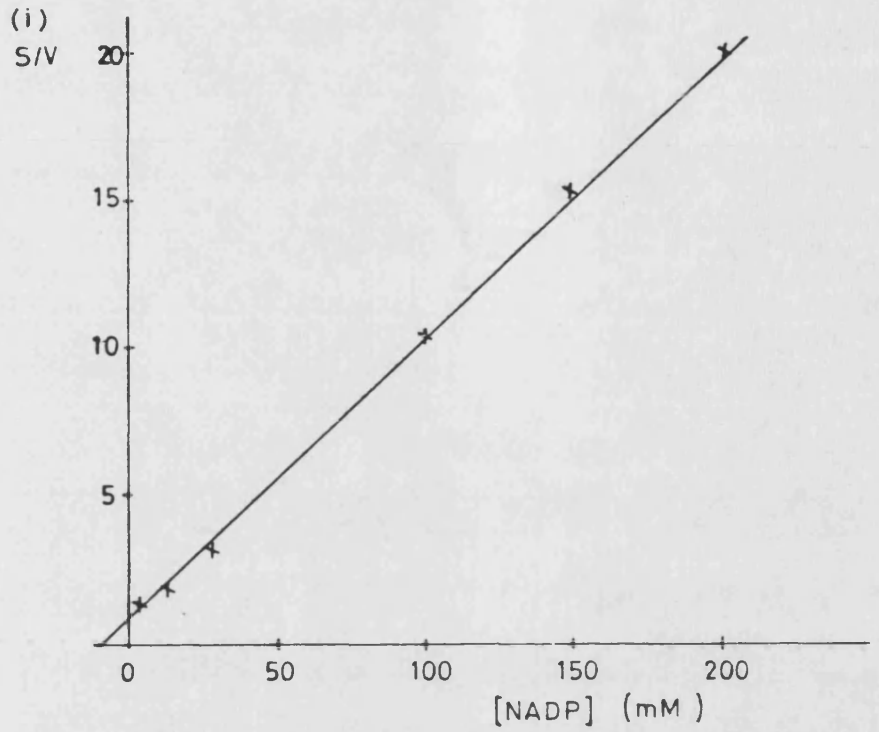
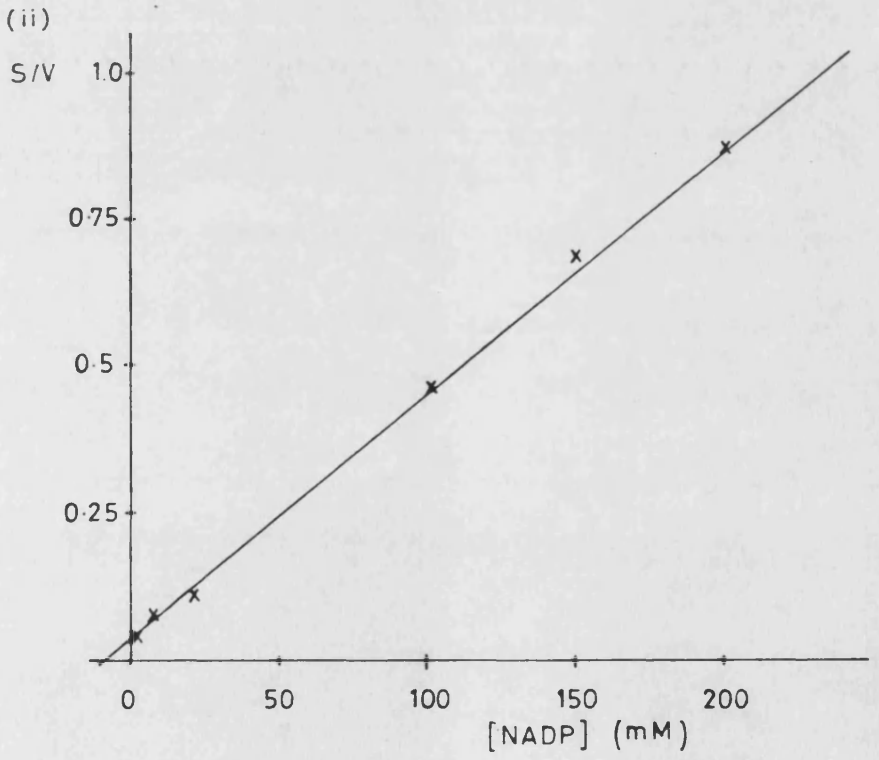
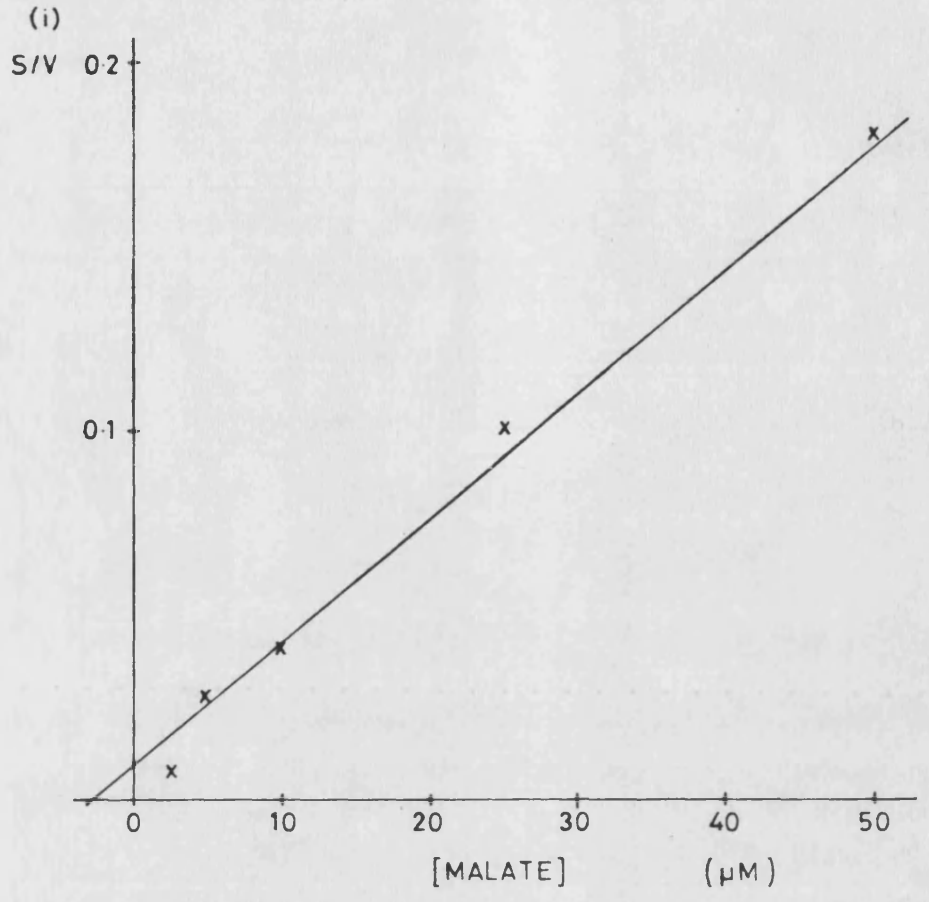


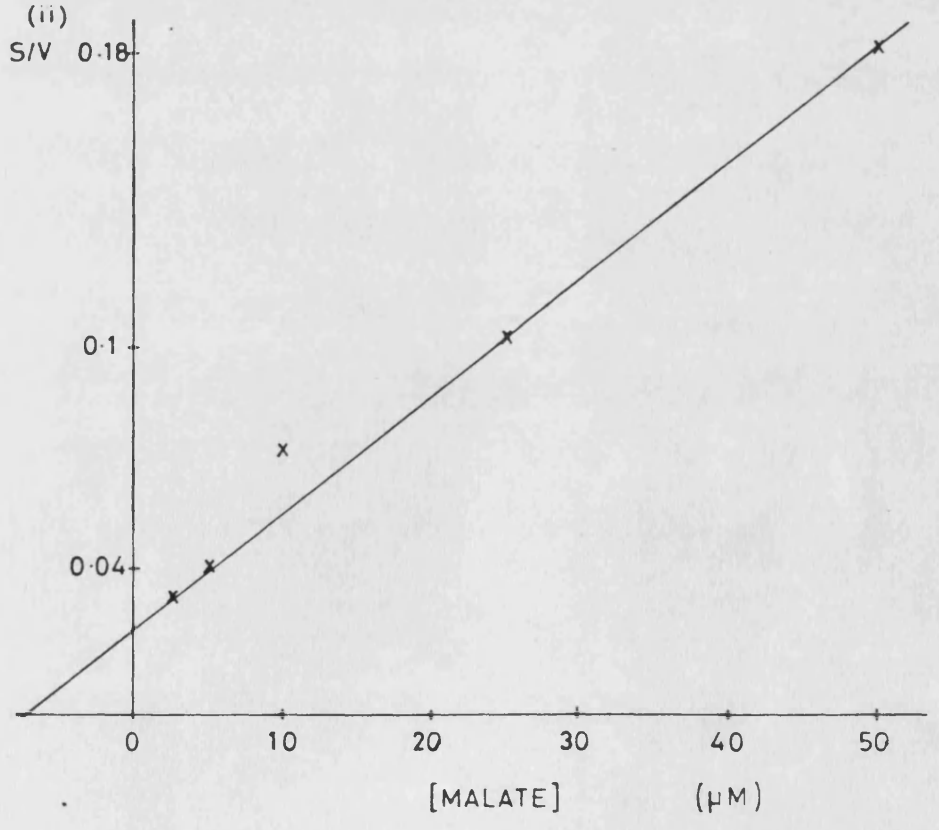


Half-reciprocal plots for the determination of the Km of IDH for NADP

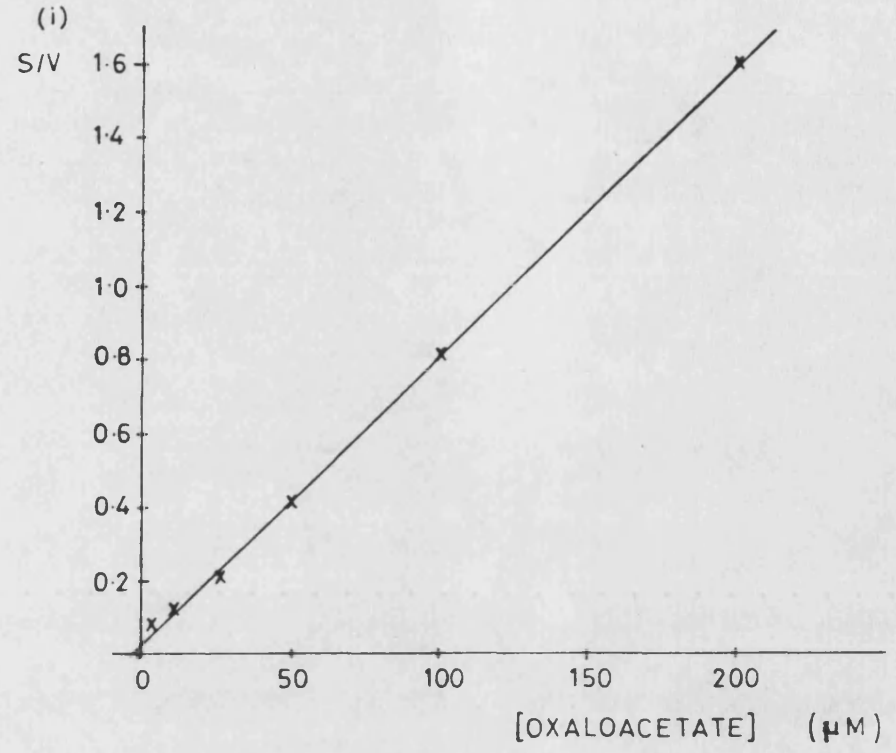
(i) HMWt

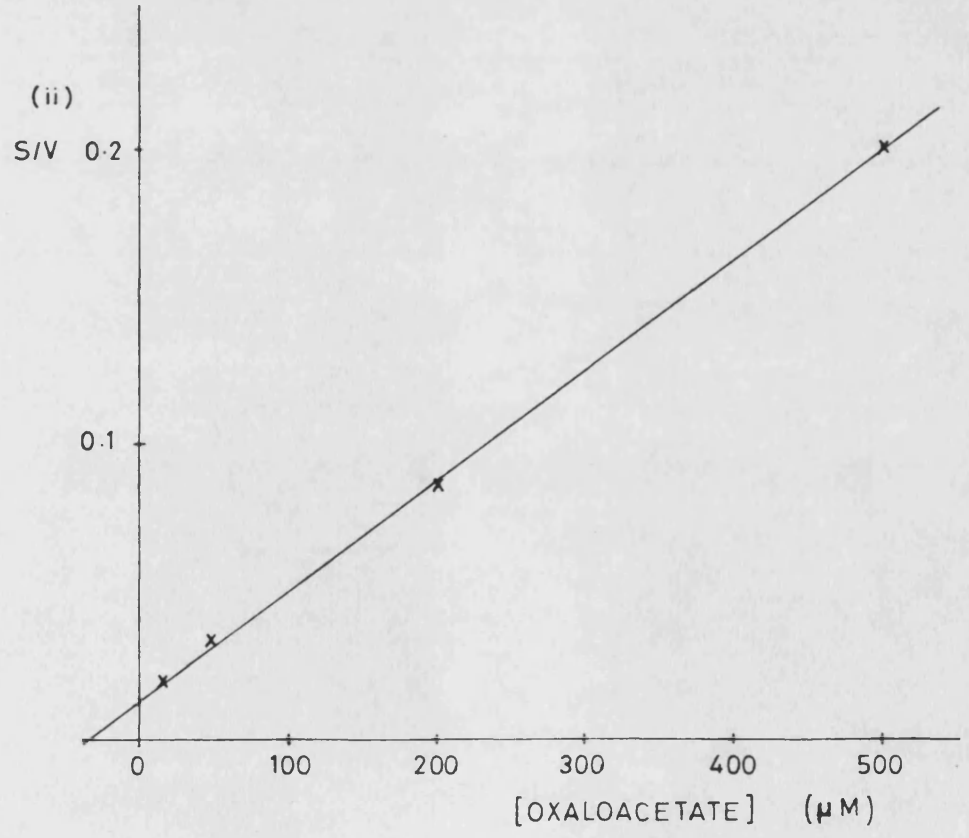
(ii) LMWt





Half-reciprocal plots for the determination of Km of MDH for malate. (i) HMWt (ii) LMWt

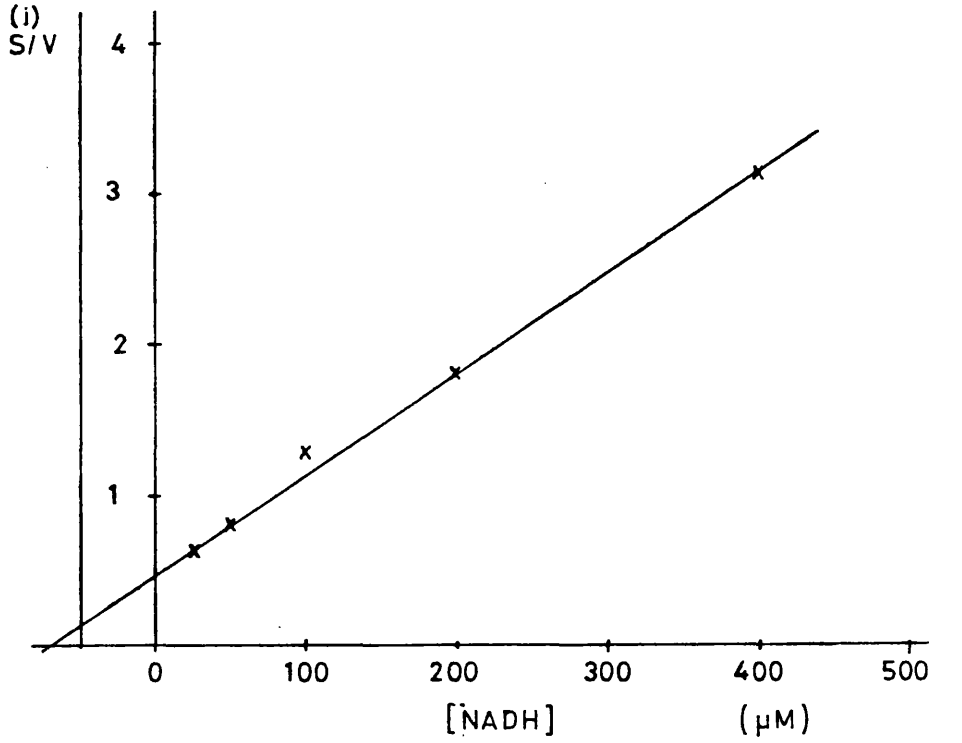


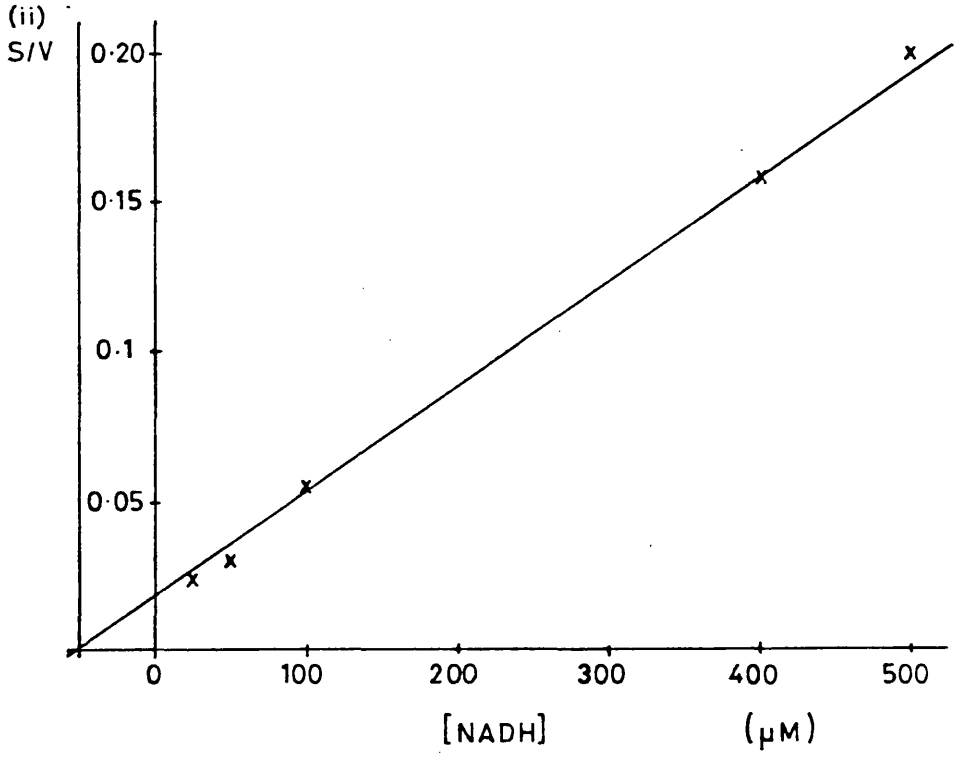


Half-reciprocal plots for the determination of the Km of MDH for oxaloacetate

(i) HMWt

(ii) LMWt





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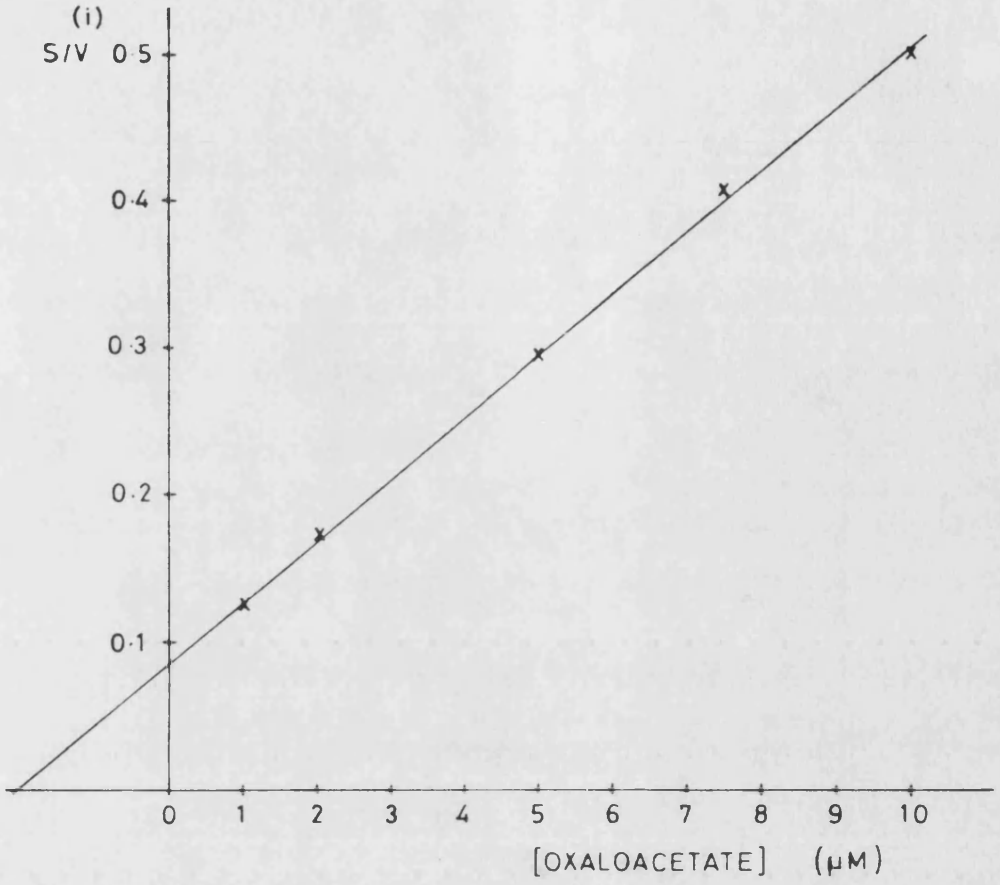
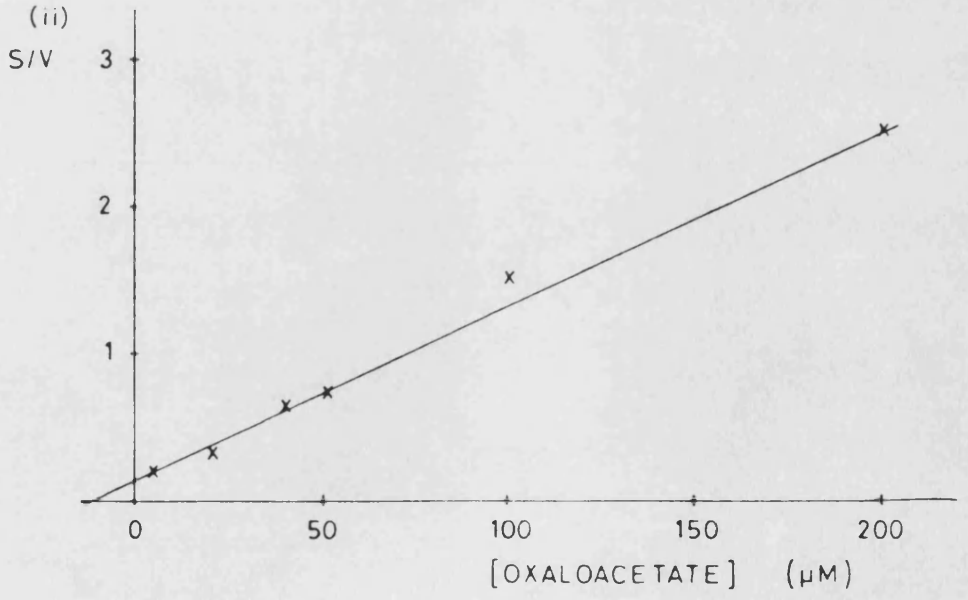
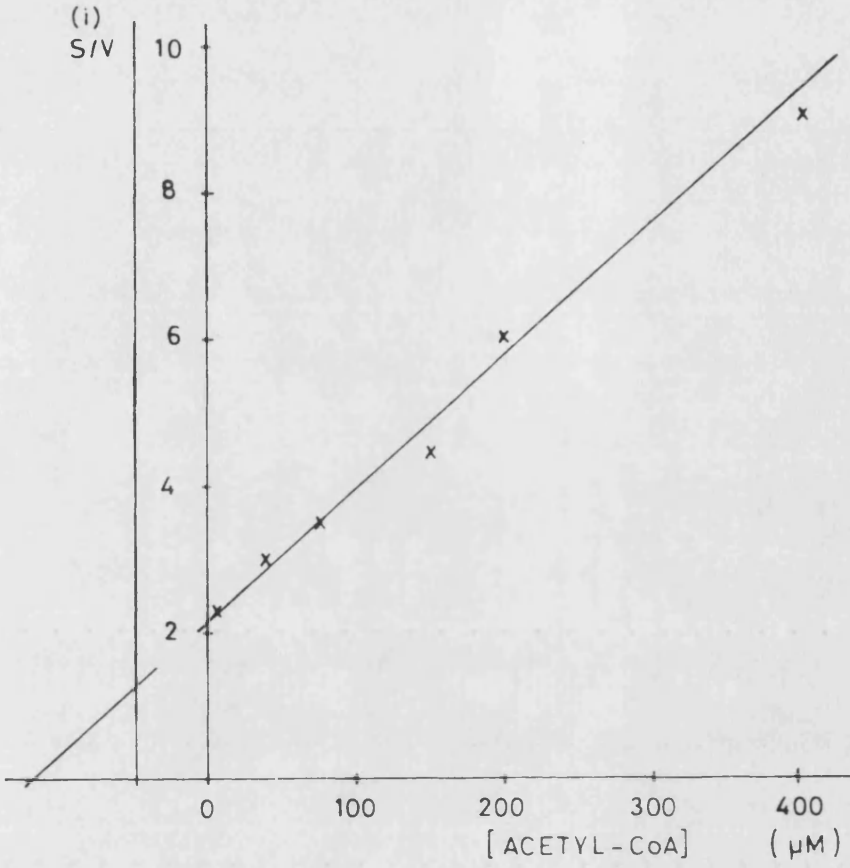
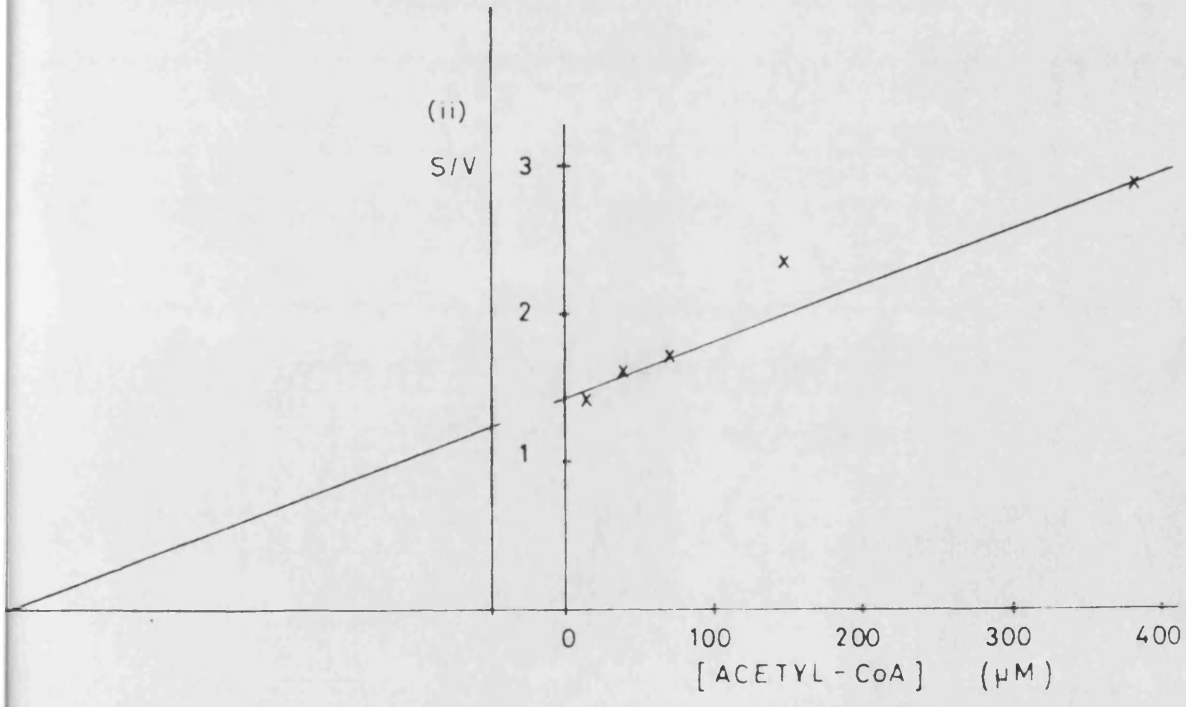




Half-reciprocal plots for the determination of the K_m of CS for oxaloacetate in Acinetobacter calcoaceti..

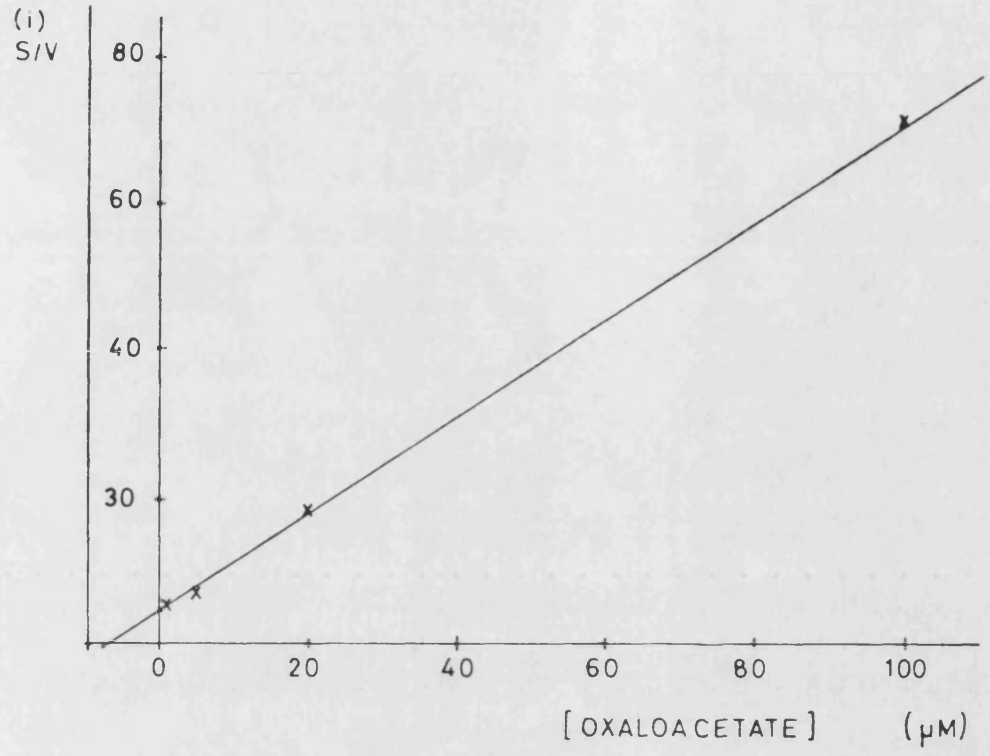
(i) HMM (ii) LMM

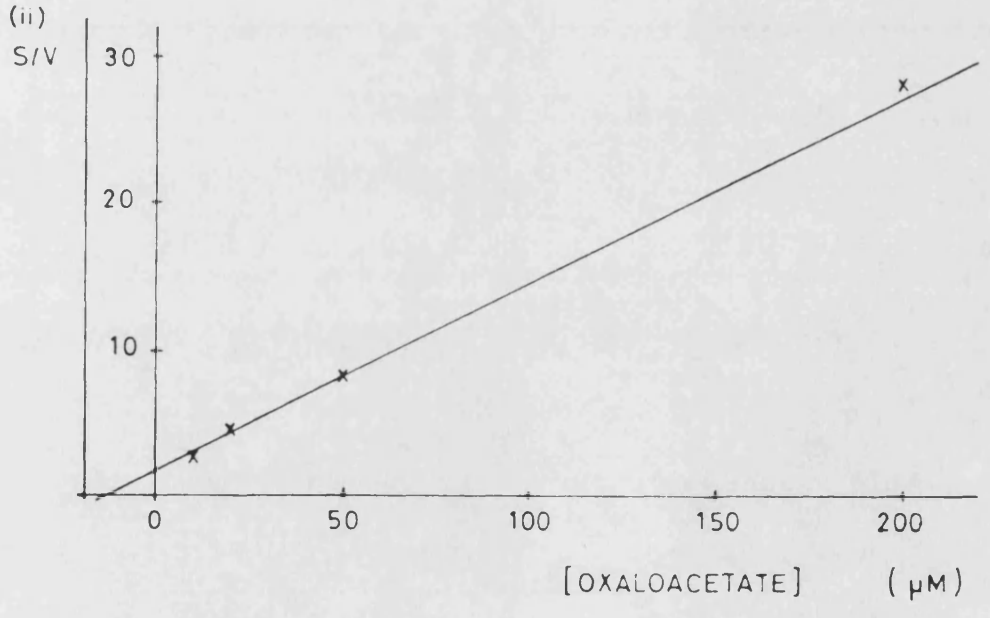




Half-reciprocal plots for the determination of K$_m$ of CS for acetyl - CoA in Acinetobacter calcoaceticus

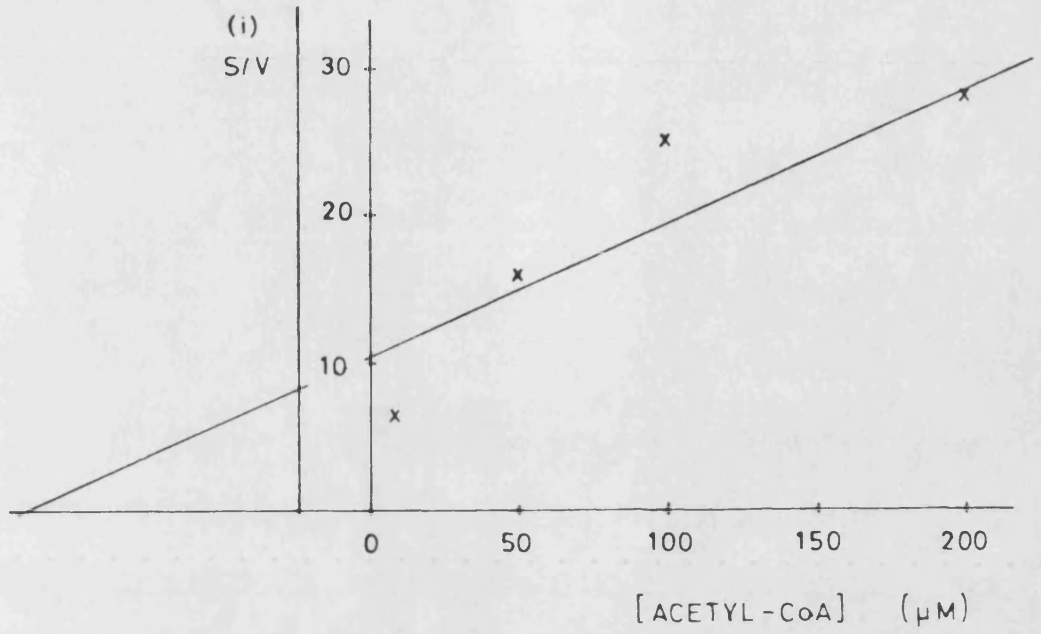
(i) HMWt (ii) LMWt

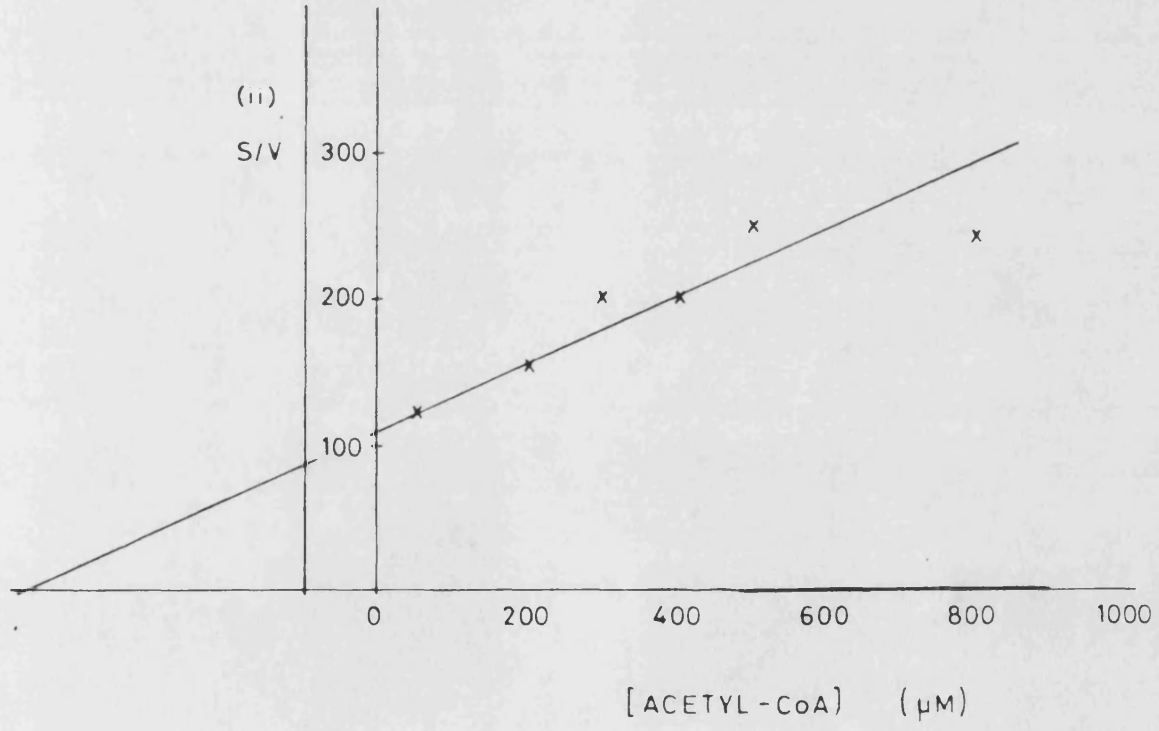




Half-reciprocal plots for the determination of Km of CS for oxaloacetate in Bacillus subtilis

(i) HMWt (ii) LMWt





Half-reciprocal plots for the determination of Km of CS for acetyl - CoA in Bacillus subtilis

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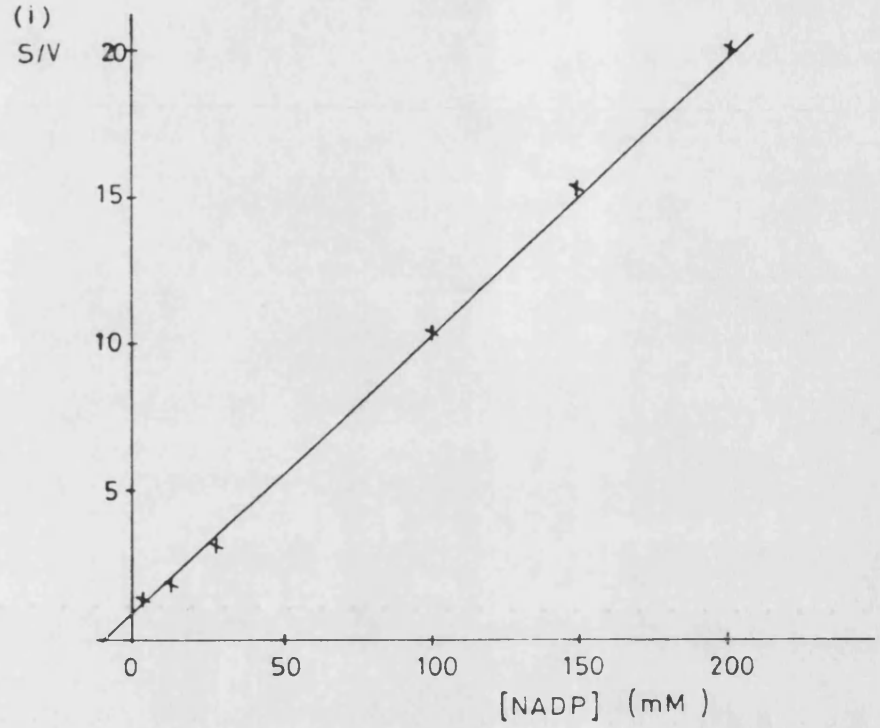

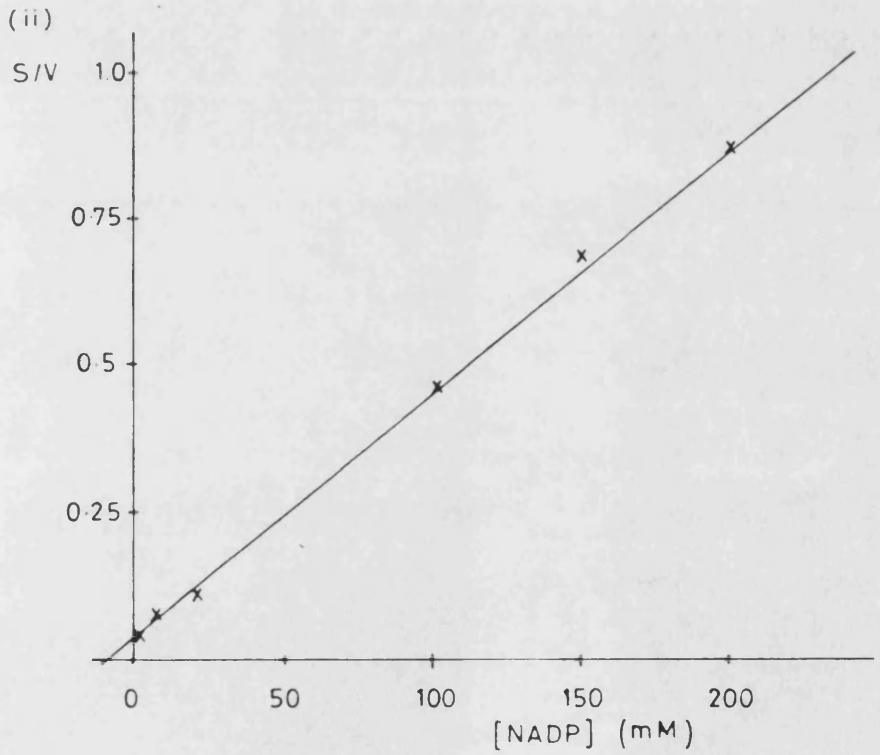

Half-reciprocal plots for the determination of the Km of IDH for NADP

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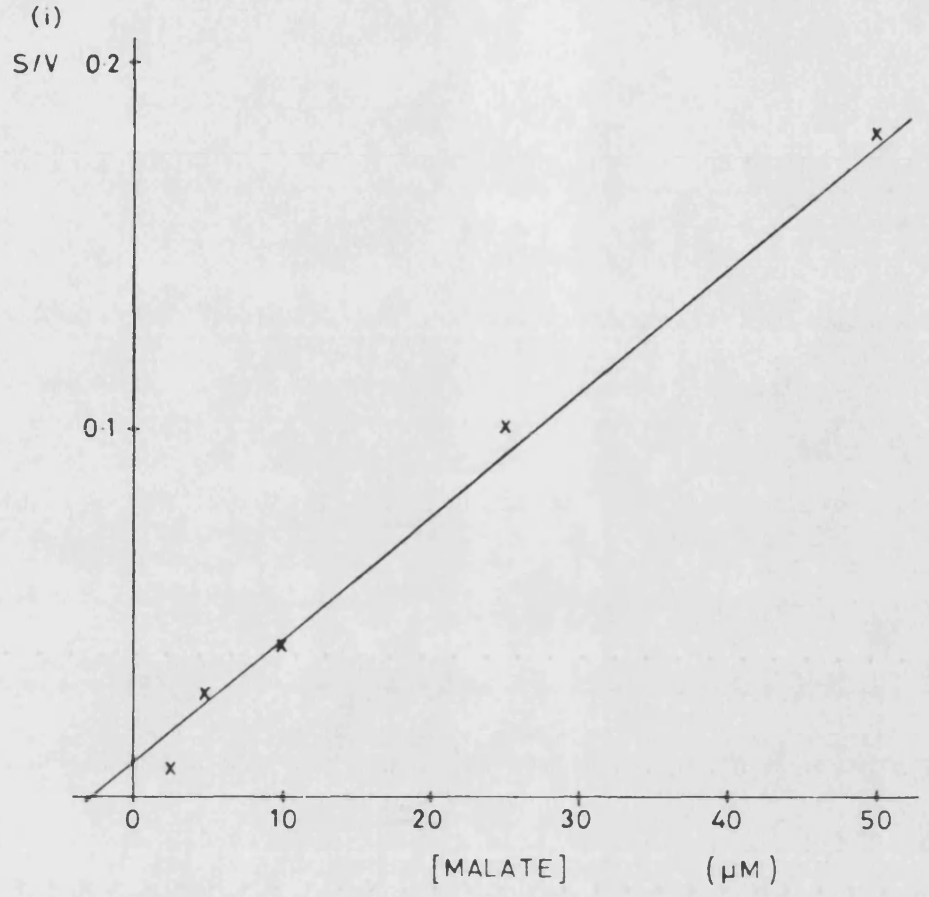
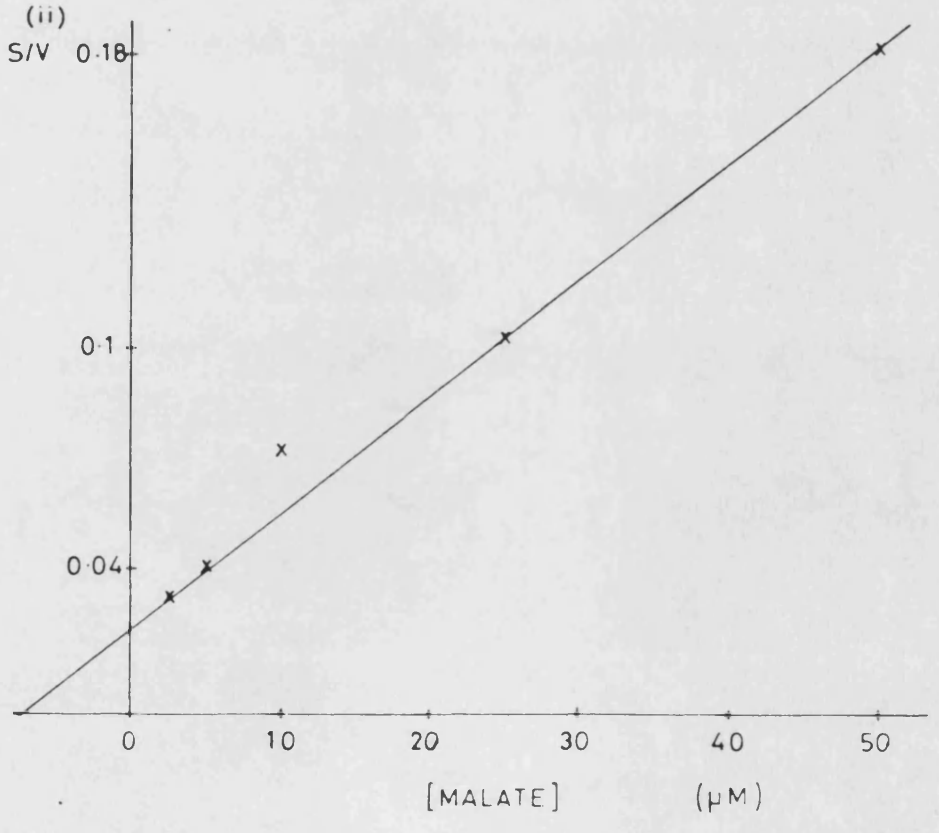
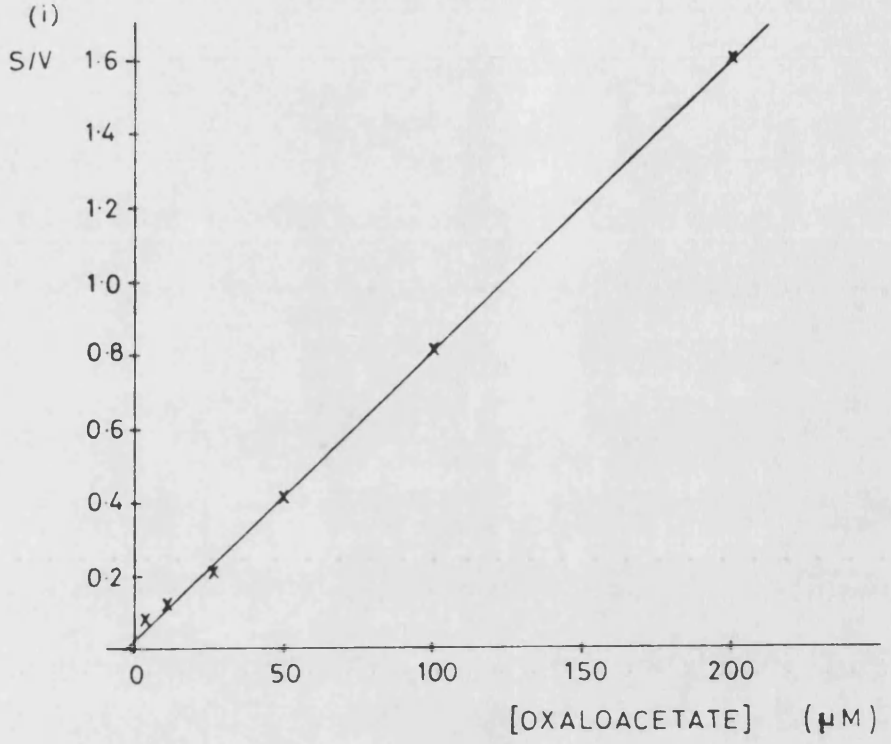
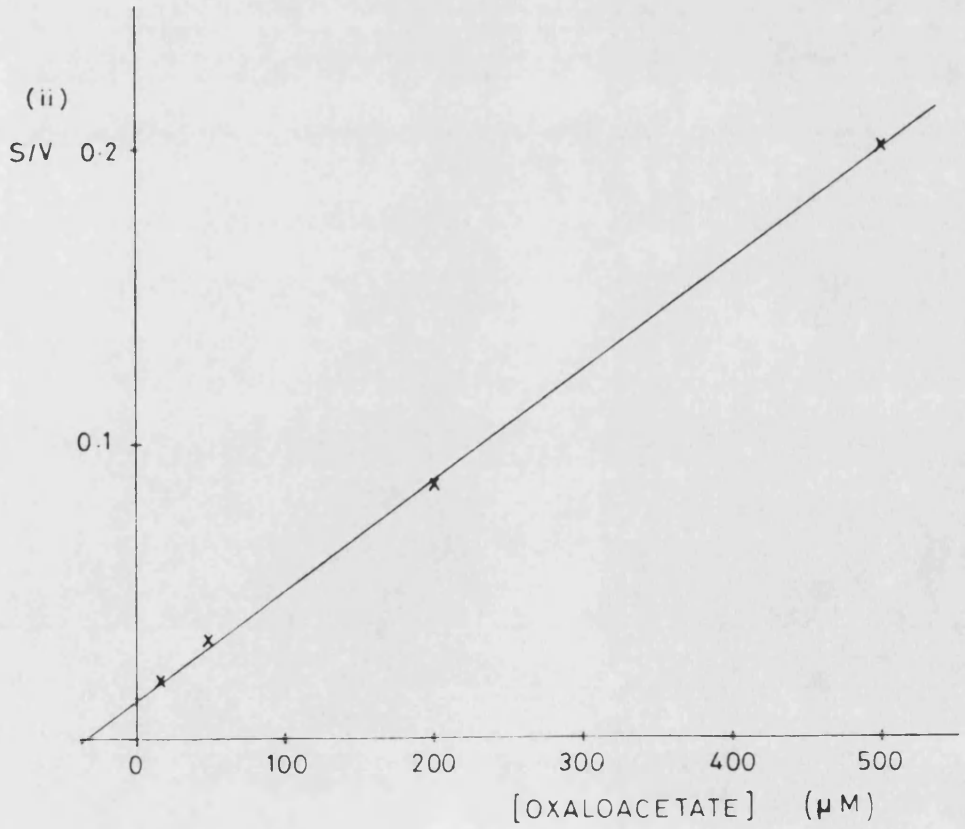
(ii) LMAt





Half-reciprocal plots for the determination of K_m of MDH for malate. (i) HMWt (ii) LMWt





Half-reciprocal plots for the determination
of the Km of MDH for oxaloacetate
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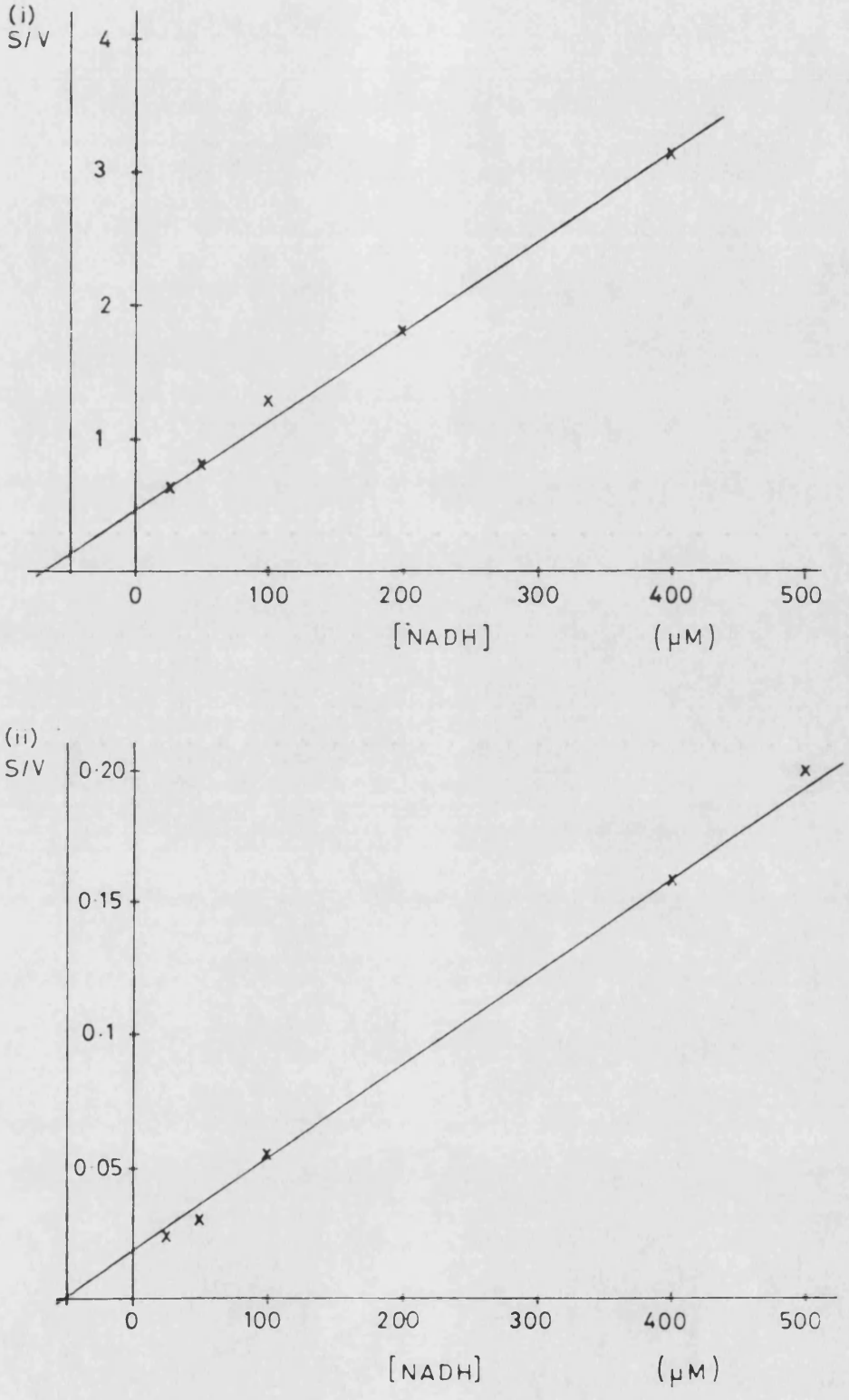
(ii) EMH



Half-reciprocal plots for the determination of Km of MDH for NADH. (i) HMWt (ii) LMWt