THE L-SERINE DEHYDRATASE FROM *ESCHERICHIA COLI* B

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ABSTRACT

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The discovery that the L-serine dehydratase (E.C. 4.2.1.13) from *Escherichia coli* B could be stabilised by iron and dithiothreitol has allowed this enzyme to be purified to a level approaching homogeneity. The purification scheme involved chemical treatments with streptomycin sulphate and ammonium sulphate, and elution from DEAE cellulose, pentyl agarose, Mono Q, Reactive Green and Phenyl superose chromatography columns. The purified dehydratase had a specific activity of 1653 μmoles of pyruvate min^{-1} mg^{-1} enzyme and was, as judged by SDS PAGE, about 90 % pure. Overall recoveries were in the region of 4 % of the starting activity.

A series of spectroscopic investigations and inhibitor studies showed that L-serine dehydratase did not utilise pyridoxal phosphate as cofactor. However, the purified enzyme did show an absolute requirement for iron and dithiothreitol for activity. The activation produced by these reagents was characterised and found to be slow, markedly influenced by both temperature and pH, and could be prevented, or reversed, by metal chelators, such as EDTA and o-phenanthroline. The activation process was also oxygen-dependent, and appeared to involve the production of an oxygen radical, since it was subject to inhibition by catalase and stimulation by hydrogen peroxide. Activation of L-serine dehydratase by iron and DTT also appeared to involve iron binding, at a ratio of 2 - 3 μmoles of Fe per μmole enzyme. However, UV/visible and EPR investigations were unable to identify the structural form in which this bound iron existed.

L-Serine dehydratase was found to be specific for L-serine; D-serine, L-threonine and L-cysteine were not deaminated. The timecourse of pyruvate formation was found to be non-linear, and the substrate saturation curve for L-serine sigmoidal, with an $S_{[0.5]}$ value of 2.6 mM, and a Hill coefficient of 2.13. The dehydratase could be activated by its substrate, L-serine, or substrate analogue, D-serine, which resulted in the production of a linear timecourse and hyperbolic substrate saturation profile ($S_{[0.5]}$ 2.8 mM, Hill coefficient 1.13). The molecular basis of this substrate activation process was investigated, and appeared to have its origins in a slow, serine-dependent rearrangement of the tertiary structure of the enzyme rather, than had previously been suggested from studies of the dehdyratase in crude extracts, a dimerisation reaction.

In common with other microbial L-serine dehydratases, the purified *E. coli* B enzyme showed a broad pH optimum for pyruvate production, with maximal activity occurring between pH 7.8 and 8.2. It was inhibited competitively by L-cysteine and D-serine, with K_i values of 1.6 and 4.2 mM, respectively, and irreversibly by sulphydryl-active agents such as DTNB, N-ethylmaleimide and $HgCl_2$. In addition, the N-terminal amino acid sequence of the *E. coli* B L-serine dehydratase was analysed, and was found to show a high level of similarity with the predicted N-terminal sequence of the L-serine dehydratase from *E. coli* K12.

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Abbreviations and Symbols.

A	Absorbance unit.
ADP	Adenosine diphosphate.
AMP	Adenosine monophosphate.
cAMP	Cyclic adenosine monophosphate
Bicine	N,N-bis[2-hydroxyethyl]-glycine
Bis-tris	N-bis[2-hydroxyethyl] iminotris [hydroxymethyl]-methane
BSA	Bovine serum albumin.
CoA	Coenzyme A.
Da	Dalton unit of molecular weight.
DEAE	Diethyl amino ethyl.
DNA	Deoxyribose nucleic acid.
DNase	Deoxyribonuclease
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5'-Dithio bis nitro benzoate.
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EPPS	N-[-2-Hydroxyethyl]-piperazine-N-3-propanesulphonic acid
EPR	Electron paramagnetic resonance
FPLC	Fast protein liquid chromatography (Pharmacia)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IEF	Isoelectric Focussing
K_i	Inhibition constant.
K_m	Apparent Michaelis constant.

LDH	Lactate dehydrogenase.
L-SD	L-Serine dehydratase
MMTS	Methylmethanethiosulphonate
Mono Q	Mono quaternary amine.
NADH	Nicotinamide adenine dinucleotide (reduced).
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced).
NTCB	2-Nitro-5-thio cyanobenzoic acid
PLP	Pyridoxal 5'-phosphate.
PAGE	Polyacrylamide gel electrophoresis.
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute.
SDS	Sodium dodecyl sulphate.
$SmSO_4$	Streptomycin sulphate
$S_{[0.5]}$	Substrate concentration producing half maximal velocity
TCA	Trichloroacetic Acid
TEMED	N,N',N',N'-tetramethylethylenediamine
TAPS	N-tris[Hydroxymethyl]methylpropanesulphonic acid
TES	N-tris[Hydroxymethyl]-methyl-2-aminoethane sulphonic acid
Tricine	N-tris [Hydroxy-methyl]methylglycine
Tris	Tris-hydroxymethylaminomethane.
UV	Ultraviolet
V_{max}	Apparent maximal velocity.
X	Unspecified amino acid

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CHAPTER 1 - INTRODUCTION

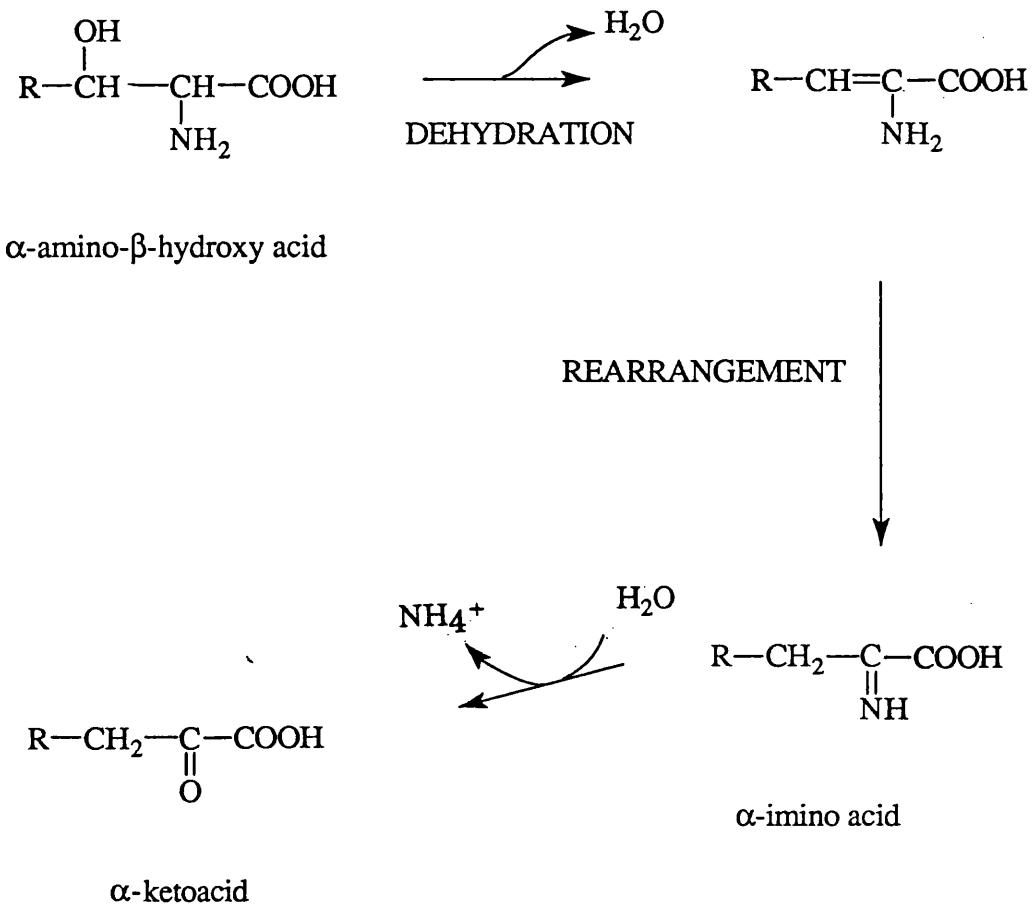
Chapter 1

Introduction

1.1 The Discovery of L-Serine Deamination in *Escherichia coli*

An enzyme activity capable of the deamination of L-serine has been known since 1938 when Gale and Stephenson made measurements of serine deamination in non-proliferating cultures of *Escherichia coli*. Later work by Chargaff and Sprinson (1943), using extracts of liver and permeabilized cells of *E. coli* and *Clostridium welchii*, showed that the enzymatic deamination of serine and threonine yielded pyruvate and α-ketobutyrate, respectively, and that the deamination reaction occurred by a non-oxidative mechanism. They also predicted, on the basis of the observation that several O-substituted serine derivatives were not substrates for these enzyme systems, that the deamination of serine and threonine would involve an initial dehydration step leading to the formation of an α-imino acid intermediate. This α-imino acid would then be converted in water to the corresponding α-keto acid (Figure 1.1). Later studies have verified this prediction (Wood, 1969).

A certain amount of confusion is evident in the early work on L-serine and L-threonine degradation in bacteria concerning the question of whether the deamination of these amino acids was catalyzed by the same or different enzymes. This confusion was in part due to the presence, in cells and crude extracts, of a multiplicity of enzymes, formed under different growth conditions and of different specificities, which have the ability to convert L-serine to pyruvate and ammonia. Wood and Gunsalus (1949), working with extracts of *E.coli*, were unable to separate L-serine and L-threonine deaminating activities after extensive purification, and so concluded that a single enzyme was responsible for the deamination of both amino acids. For similar reasons, a single enzyme with dual specificity was also suggested by Yanofsky and Reissig (1953) for *Neurospora crassa*. However, later work by Pardee and Prestidge (1955) and by Boyd and Lichstein (1955) found that growth of *E. coli* under different culture conditions affected the relative rates of deamination of L-serine and L-threonine to different extents, suggesting that there was a separate enzyme acting on each amino acid. Evidence to support this suggestion was provided by Artman and Markenson (1956), who showed that a strain of *E. coli* B/r which lacked L-threonine dehydratase (biosynthetic) had relatively unchanged levels of L-serine dehydratase.



R = H L-Serine and Pyruvate

R = CH_3 L-Threonine and α-ketobutyrate

Figure 1.1

The Deamination of L-Serine and L-Threonine.

The uncertainty concerning the nature of the enzyme(s) responsible for the deamination of L-serine and L-threonine was partially resolved by the work of Umbarger and Brown (1957), who showed that under different growth conditions, *E. coli* formed two L-threonine dehydratases, each of which was also able to act on L-serine. The existence of a separate L-serine-specific dehydratase, however, did not become clear until a number of years later, when Alfoldi *et al* (1968) described such an activity in *E. coli* K12. Since then, L-serine dehydratases have been identified in a number of other microbial species including *Clostridium acidiurici* (Carter and Sagers, 1972), and *Arthrobacter globiformis* (Gannon, 1973, Gannon *et al*, 1977).

The L-serine-specific dehydratases are proteins of considerable instability, and as a consequence, only a few members of this class have purified to a level which has allowed their properties to be studied in detail (Carter and Sagers, 1972, Gannon, 1973, Gannon *et al*, 1977). In contrast, the L-threonine dehydratases and the D-serine dehydratase, are much more stable, and this has allowed them to be the subject of detailed investigations. The information gained from these enzymes has been used as a starting point for the study of the activity of the L-serine dehydratases.

1.2 The L-Threonine Dehydratases (E.C. 4.2.1.16) and D-Serine Dehydratase (E.C. 4.2.1.14) of *E. coli*

1.2.1 The L-Threonine Dehydratases of *E. coli*

The L-threonine-deaminating enzymes of bacterial origin are of two functionally distinct types, termed by Umbarger and Brown (1957) the degradative and biosynthetic L-threonine dehydratases. The degradative enzyme is produced when *E. coli* is grown on rich media under anaerobic conditions and is involved in the degradation of L-threonine to produce energy. The biosynthetic L-threonine dehydratase is formed during growth on simple media lacking L-isoleucine, and serves to supply the α-ketobutyrate required for L-isoleucine biosynthesis.

1.2.1.1 Regulation of Biosynthesis

The synthesis of the degradative and biosynthetic L-threonine dehydratases is subject to regulatory patterns that are a reflection of the catabolic and anabolic roles of these two classes of enzymes.

The degradative dehydratase was first obtained in significant quantities during the anaerobic growth of *E. coli* on a sugar-free medium containing an enzymatic digest of tryptone (2 %) and yeast extract (1 %), and buffered with phosphate (Wood and Gunsalus, 1949). Later work by Shizuta and Hayaishi (1970) showed that a polypeptone medium supplemented by 0.2 % each of L-serine and L-threonine gave improved levels of the dehydratase. Toshikuge (1970) demonstrated that a phosphate buffered-medium containing L-serine and L-threonine could only induce the degradative enzyme if yeast extract, or a mixture of all the other 18 L-amino acids was included in the growth medium. Later studies of the regulation of synthesis of the degradative L-threonine dehydratase give a clearer picture of the requirements for induction of this enzyme. Egan and Philips (1977) presented evidence which suggested that only L-threonine, L-valine and L-leucine were necessary for the induction of the degradative enzyme. However, Yui *et al* (1977) showed that, L-serine, L-aspartate, L-arginine and L-methionine, as well as L-threonine, L-valine and L-leucine were also able to stimulate the production of the degradative L-threonine dehydratase. These workers called the induction produced by these amino acids multivalent induction. The differences in the induction requirements obtained by these two groups of workers may have arisen from the different growth conditions under the which the induction was studied. Egan and Phillips (1977) studied the induction in anaerobically growing cultures, while Yui *et al* (1977) used non-growing cultures of *E. coli*.

The synthesis of the degradative L-threonine dehydratase is also subject to catabolite repression. Umbarger and Brown (1957) were the first to note that the presence of glucose led to a repression of the synthesis of this enzyme. Shizuta and Hayaishi (1970) later showed that 3',5'-cAMP participated in the induction of the *E. coli* degradative dehydratase. These workers observed that 1 mM cyclic AMP was able to reverse the repression caused by 10 mM glucose and that the nucleotide was also able to stimulate the production of the dehydratase in the absence of a fermentable sugar.

By contrast, the biosynthetic L-threonine dehydratase is formed by *E. coli* grown anaerobically on minimal salts medium supplemented with a carbon source (Davis and Mingioli 1950). The biosynthetic dehydratase is coded for by the *ilv A* gene of the *ilv ADE* operon which codes for the enzymes of L-isoleucine, L-leucine and L-valine biosynthesis (Umbarger, 1973). The formation of the biosynthetic L-threonine dehydratase (and also the other enzymes of the isoleucine and valine biosynthetic pathway) is controlled by a multivalent system of repression, with repression of synthesis only occurring when L-isoleucine, L-leucine and L-valine

are all present in excess. When one of these amino acids is present in limiting amounts in the presence of the other two, a greater than five-fold stimulation of the production of the biosynthetic dehydratase is observed (Freundlich *et al*, 1962).

1.2.1.2 Regulation of Enzyme Activity

The degradative L-threonine dehydratase is a tetrameric enzyme of molecular weight 200 000, composed of identical subunits. The activator of the dehydratase is AMP, a feature first demonstrated by Wood and Gunsalus (1949) and explored in more detail by Phillips and Wood (1964). They showed that AMP promoted the association of the inactive monomeric form of the enzyme to the activated tetrameric form. Although Phillips and Wood (1964) were able to show that tetramer formation could occur when the enzyme concentration was high, the tetramer formed in the presence of AMP binding had a greater affinity for its substrate than that formed by increasing the enzyme concentration. Whanger *et al* (1968) showed that activation by AMP decreased the K_m for L-threonine by nearly 25-fold, without appreciably affecting the V_{max} for the deamination reaction. Each subunit is thought to contain a binding site for a molecule of AMP (Shizuta and Hayaishi, 1970).

The biosynthetic L-threonine dehydratase is also a tetrameric protein, of molecular weight 160 000, consisting of identical subunits. Unlike the response of the degradative L-threonine dehydratase to its regulatory effector AMP, the regulation of the activity of the biosynthetic enzyme does not appear to involve any changes in the overall number of enzyme subunits. Instead, this enzyme, which catalyzes the first committed step in the pathway of L-isoleucine biosynthesis, is allosterically inhibited by the end product of the pathway, L-isoleucine. Umbarger (1956) showed that the dehydratase was also subject to allosteric regulation by another naturally occurring effector, L-valine, which stimulates the activity of the enzyme by antagonising the feedback inhibition produced by L-isoleucine. Burns and Zarlengo (1968) demonstrated that the L-isoleucine inhibition of the biosynthetic L-threonine dehydratase resulted in an increase in both the $S_{[0.5]}$ and cooperativity of L-threonine binding, without substantially affecting the maximal velocity of the reaction. These workers also showed that L-valine was able to act as an activator of the dehydratase only when there was cooperativity in L-threonine binding.

1.2.1.3 The Deamination of L-Serine by the Biosynthetic and Degradative L-Threonine Dehydratases

Both the biosynthetic and degradative L-threonine dehydratases are able to act upon L-serine, although more slowly than the rate at which they act upon L-threonine. Umbarger and Brown (1957) showed that the deamination of L-serine by the *E. coli* enzymes occurred at approximately one-quarter of the rate of L-threonine deamination.

The deamination of L-serine by the L-threonine dehydratases is subject to the same regulatory effectors as L-threonine deamination. Thus, Wood and Gunsalus (1949) were able to demonstrate activation by AMP of L-serine degradation by the degradative enzyme, and Umbarger and Brown (1957) observed inhibition by L-isoleucine of L-serine deamination by the biosynthetic enzyme.

Although the L-threonine dehydratases are able to deaminate L-serine, both types of enzyme are subject to an essentially irreversible form of inactivation when acting on L-serine as substrate. This phenomenon, termed the serine-suicide reaction, was first observed by Wood and Gunsalus (1949) using the degradative dehydratase. These investigators found that the the rate of L-serine deamination decreased rapidly, approaching zero within 15 minutes after the initiation of the enzymatic reaction. Later work has shown that the inhibition produced by L-serine could be relieved by pyridoxal phosphate (Nishimura and Greenberg, 1961) or pH alteration (Mclemore and Metzler, 1968). A number of suggestions have been made to account for the mechanism of this inactivation, including cofactor resolution (Nishimura and Greenberg, 1961) and the modification of a catalytically important reactive group by a derivative of L-serine. For example, Phillips and Wood (1965) have suggested that the α-aminoacrylate formed by the dehydration of L-serine (Figure 1.1), forms a covalent bond with an essential sulphydryl group in the active site, while Shizuta and Hayaishi (1970) argued that the covalent attachment of a reaction intermediate to pyridoxal phosphate was involved in the process of enzyme inactivation by L-serine.

1.2.1.4 Pyridoxal Phosphate

Pyridoxal phosphate (PLP) is utilised by both of the *E. coli* L-threonine dehydratases. The reaction mechanism has been extensively characterised for the degradative enzyme (Wood, 1969, Flavin and Slaughter, 1969) and is an α,β-elimination reaction (Figure 1.2). Each of the subunits of the dehydratase

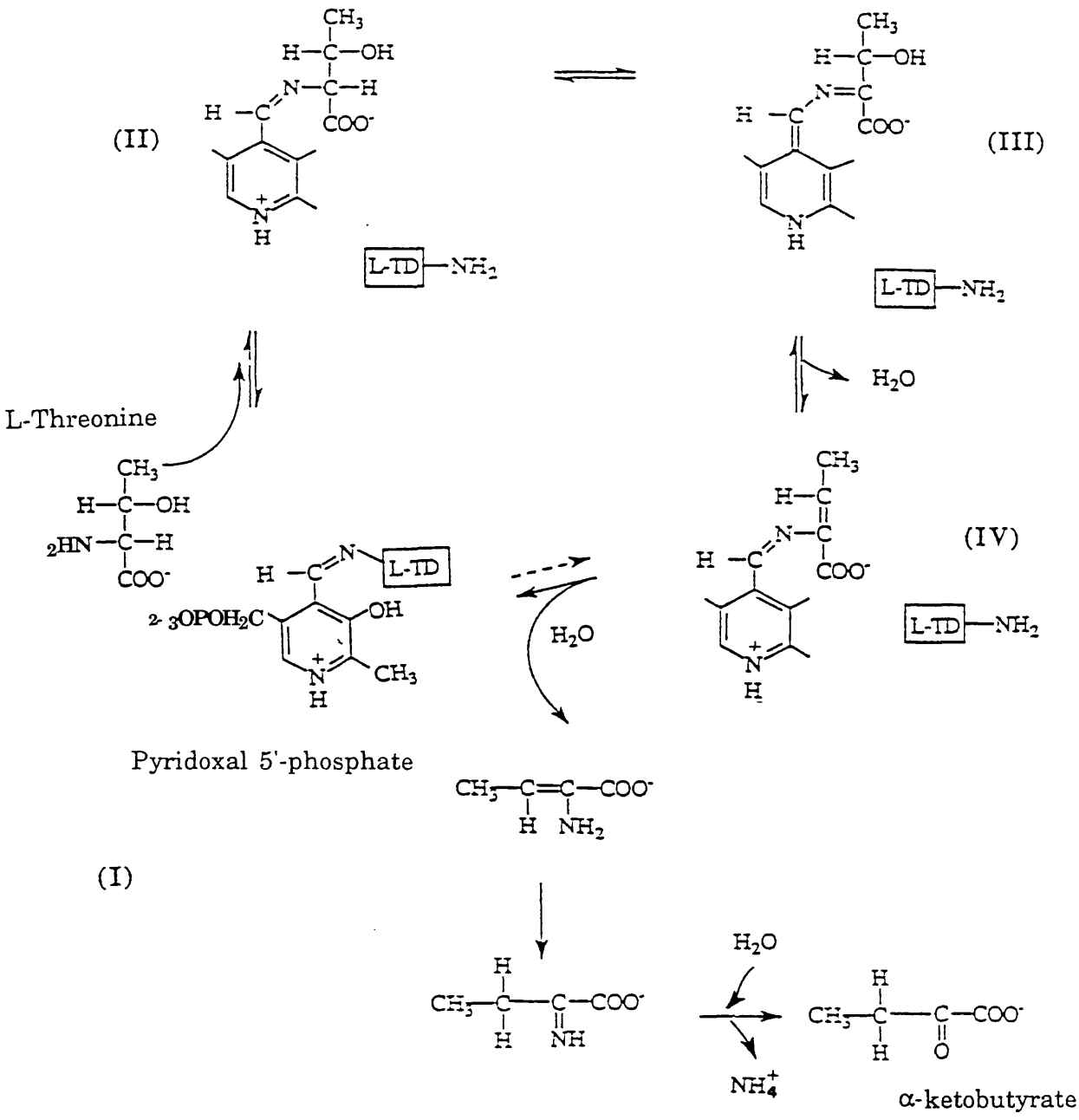


Figure 1.2

The Role of Pyridoxal 5'-Phosphate in the Reaction Mechanism of the Degradative L-Threonine Dehydratase

The reaction mechanism shown proceeds from the initial transaldimination (I to II) through α-elimination (II to III), β-elimination (II to IV) and transaldimination again (IV to I). In the interests of clarity, the phosphate, methyl and hydroxyl group have been omitted from pyridoxal phosphate in stages II to IV of the reaction mechanism.

tetramers has been shown to contain one molecule of PLP attached to the ε-amino group of specific lysyl residues (Whanger *et al*, 1968). Both enzymes possess spectra which show the absorption maximum at 412 nm characteristic of bound pyridoxal phosphate (Snell and Dimari, 1970). The PLP cofactor may be resolved from the L-threonine dehydratases by dialysis against L-cysteine, which results in the loss of both enzymatic activity and the 412 nm absorption maximum. Preincubation of the resolved enzymes with pyridoxal phosphate restored both the catalytic activity and the spectrum of the dehydratases (Umbarger, 1973). The L-threonine dehydratases are also inactivated by reagents which can interact with the carbonyl group of pyridoxal phosphate, such as hydroxylamine (Umbarger and Brown, 1957).

1.2.1.5 Keto Acid-containing Threonine Dehydratases

Most microbial and mammalian threonine dehydratases require pyridoxal phosphate. There exist, however, several L-threonine deaminating enzymes which do not contain pyridoxal phosphate, but instead employ covalently bound keto acids as cofactor. The L-threonine dehydratase from *Pseudomonas putida* and the serine-threonine dehydratase of sheep liver (Kapke and Davis, 1975) are two such examples, both of which contain an α-ketobutyrate cofactor. Dehydratases which contain keto acid prosthetic groups do not show the absorption maximum at 410 to 425 nm indicative of bound PLP, and do not fluoresce when excited at the wavelengths normally associated with enzyme-bound pyridoxal phosphate (340 or 420 nm). They do, however, show sensitivity to certain carbonyl reagents, and are inactivated by reduction with sodium borohydride. Protocols for the isolation and identification of these cofactors usually involve reduction with ^{3}H-containing borohydride, followed by acid hydrolysis and chromatographic separation and identification of the tritiated acid (Riley and Snell, 1968).

1.2.2 The D-Serine Dehydratase of *E. coli*

1.2.2.1 Regulation of Biosynthesis

The D-serine dehydratase of *E. coli* is an inducible enzyme, the primary function of which, is detoxification. D-Serine is toxic to *E. coli* in minimal medium because it blocks L-serine and pantothenate biosynthesis (Cosloy and McFall, 1973). The enzyme can therefore be obtained by adding an inhibitory concentration of D-serine (150 $\mu g\ ml^{-1}$) to cultures of *E. coli* grown on minimal medium (McFall, 1964). Growth ceases for one to two hours after the addition of the D-

serine, and is resumed at its former rate when sufficient enzyme has been induced to degrade the inhibitor.

The D-serine dehydratase operon has been mapped and sequenced and has been found to consist of three specific genetic loci: the enzyme's structural gene, *dsdA*, the operator-promoter region, *dsdO* and a control system consisting of two positively acting elements, a specific activator protein, *dsdC*, that is functional only in the presence of its inducer D-serine, or its analogue D-threonine, and the cyclic AMP binding protein complex (cAMP-CAP) (McFall, 1975). The *dsdA* and *dsdC* genes are adjacent, and located at 50 minutes on the *E. coli* K-12 linkage map (McFall, 1967). The *dsdC* activator protein has been partially purified and, based on sucrose gradient analysis, is a dimer of molecular weight 66 000. It is absolutely required for *dsdA* gene gene expression, whose transcription it is able to enhance several-thousand fold (Heincz *et al*, 1984).

D-Serine is also a good nitrogen source and a moderately good carbon source (pyruvate and NH_3), and so the dehydratase also has a secondary catabolic role in *E. coli* (Bloom and McFall. 1975). The addition of glucose to a culture of *E. coli* K12 growing with D-serine resulted in a significant repression of enzyme synthesis. This catabolite repression was relieved by the addition of cAMP (McFall, 1975).

1.2.2.2 Enzyme Activity

Most pyridoxal phosphate-dependent proteins exist as multimeric complexes (Umbarger, 1973). The *E. coli* D-serine dehydratase is unusual in that it exists as a monomer, incorporating one mole of pyridoxal phosphate per mole of protein (Dowhan and Snell, 1970). The dehydratase is a fairly small protein of molecular weight 47 000, consisting of 442 amino acids, whose sequence has been determined (Schiltz and Schmitt, 1981). The active site is centred at amino acid 118, a lysine residue, whose δ-amino group forms a Schiff base with pyridoxal phosphate. The dehydratase is very stable, easily purified and shows a high level of specificity for D-serine, for which it has a K_m of 0.32 mM (Federink and Shafer, 1983). The D-serine dehydratase reaction scheme is well understood, (Federink and Shafer, 1983, Marceau *et al*, 1988) and has been used as a model for other reactions which involve pyridoxal phosphate as cofactor. This has led to the widely held, if somewhat premature view, that all serine-deaminating activities, including those belonging to the L-serine-specific class of enzymes, contain pyridoxal phosphate.

Parsot (1986) has called attention to the extensive homologies between the amino acid sequence of the *E. coli* D-serine dehydratase and the predicted amino acid sequences of the *Bacillus subtilis* and *E. coli* threonine synthases and the threonine dehydratase from *Saccharomyces cerevisiae*. He argues that this sequence homology, which correlates with similarities in the PLP-dependent mechanisms of these enzymes, indicates that these enzymes may have evolved from a common, multi-specific ancestral protein.

1.3 The Properties of L-Serine-specific Dehydratases (E.C. 4.2.1.13) from *E. coli* and Other Microbial Sources

The study of the properties of the L-serine dehydratase from *E. coli* B is the primary concern of the work presented in this thesis. However, since the enzyme from *E. coli* shares many similarities with the L-serine dehydratases from other microbial species, a description of the properties of these enzymes is also included for the purposes of comparison.

1.3.1 Functions of the Microbial L-Serine Dehydratases

Most microbial L-serine dehydratases are involved in degradative pathways, the pyruvate formed from the deamination of L-serine being utilised for growth. In *A. globiformis* (Bridgeland, 1968) and *Diplococcus glycinophilus* (Klein and Sagers, 1961), L-serine dehydratase is involved in growth on glycine as the sole carbon source; the glycine is converted into L-serine, which is then deaminated. In *Cl. acidiurici*, the dehydratase is one of the enzymes of the uric acid fermentation pathway. L-Serine is formed as an intermediate of this pathway and is converted to pyruvate by means of this enzyme (Benziman *et al*, 1961).

The function of the *E. coli* L-serine dehydratase is uncertain, although a considerable amount of information has been obtained about the conditions under which it is formed. Pardee and Prestidge (1955) were the first to show that the dehydratase is inducible and that glycine and leucine were able to stimulate its production. These observations were confirmed by Isenberg and Newman (1974) and studied further by Roberts (1983), who has made a detailed investigation of the nutritional factors which influence the biosynthesis of the *E. coli* L-serine dehydratase. She was able to show that the synthesis of this enzyme could be induced by the amino acids glycine, L-glutamine, L-leucine, L-alanine, L-tryptophan, L-valine and L-methionine. The induction of the dehydratase by these inducers was generally found to be additive, although the induction produced by glycine and L-methionine appeared to be synergistic, with this

synergism being reduced by L-tryptophan. In contrast to the D-serine dehydratase, the L-serine-specific dehydratase was not induced by its substrate. Under certain circumstances, L-serine was found to actually repress the synthesis of this enzyme. Roberts also presented data which showed that the production of L-serine dehydratase by *E. coli* was markedly influenced by nitrogen availability. The presence of an inorganic nitrogen source, such as ammonium sulphate, in the culture medium was found to reduce the induction of the dehydratase in a concentration-dependent manner. For example, in the absence of ammonium sulphate the induced activity of the dehydratase was 0.27 μmoles of pyruvate min^{-1} (mg dry weight cells)$^{-1}$; in the presence of 75 mM ammonium sulphate the induced activity of the enzyme fell to 0.03 μmoles of pyruvate min^{-1} (mg dry weight cells)$^{-1}$. This prompted Roberts to suggest that L-serine dehydratase may play an important role in the nitrogen metabolism of the cell.

A number of other environmental factors have been found to influence the induction of the *E. coli* L-serine dehydratase. Newman *et al* (1982) showed that the activity of this enzyme could also be induced by growth at 42 °C, by ultraviolet irradiation and by exposure to DNA-damaging agents, such as mitomycin and nalidixic acid as well as by mutation (Newman *et al*, 1981). Although the work of Ramotar and Newman (1986) showed that L-serine dehydratase can deaminate L-serine *in vivo*, there is as yet no evidence to indicate what might be the physiological role of this activity.

1.3.2 Stability

Microbial L-serine dehydratases tend, in general, to be rather unstable, a feature which has proved to be a barrier to their purification and study. The L-serine dehydratases from *Salmonella typhimurium* and *Bacillus cereus* are particularly unstable. For example, the *S. typhimurium* dehydratase lost 95 % of its initial activity when stored for 120 minutes at 30 °C and 0.41 mg of protein ml^{-1}, while the *B. cereus* enzyme lost 77 % of its activity over the same time interval at 0.15 mg of protein ml^{-1} and 30 °C (Rasko *et al*, 1969). The addition of L-serine to crude extracts containing these enzymes provided some protection against inactivation, although not enough to allow their purification to be undertaken.

The *E. coli* L-serine dehydratase is also unstable, and until the work of Roberts (1983), studies of this enzyme had been limited to whole cells or crude extracts (Alfoldi *et al*, 1968, Newman and Kapoor, 1980). Alfoldi *et al* (1968) undertook the

first study of the instability this dehydratase and found that it was particularly unstable to dilution in cell-free extracts. They showed that the rate of loss of activity was dependent on the protein concentration: 94 % of the activity of the dehydratase was lost in 120 minutes at 0.6 mg ml^{-1} and 30 °C, while only 27 % was lost under the same conditions at 27 mg ml^{-1} of protein. Alfoldi *et al* also found that the activity of the cell-free dehydratase could be stabilised by the addition of L-serine (80 mM) or D-serine (250 mM), although this protection was of limited duration (60 minutes), after which the activity of the dehydratase decayed to the same extent as that of enzyme incubated in the absence of these amino acids. Newman and Kapoor (1980) also demonstrated stabilisation of L-serine dehydratase by D- and L-serine. In addition, they found that the activity of the dehydratase slowly increased when incubated with these amino acids. For instance, when L-serine (20 mM) was added to a crude extract containing the dehydratase and incubated at 37 °C, the activity of the enzyme was found to increase 2.6-fold over a five minute period. The addition of 1 mM D-serine to a sample of the extract resulted in a 1.3-fold increase in the initial activity of the dehydratase over a 6 minute interval.

Roberts (1983) also investigated the factors influencing the stability of the *E. coli* L-serine dehydratase. She found that its stability could be improved by maintaining the dehydratase at high enzyme concentrations and by preparing the enzyme in buffers containing D-serine (40 mM) and glycerol (15 %). By using the stabilisation that these conditions provided, Roberts was able to develop a partial isolation scheme for this enzyme. This involved an initial digest with ribonuclease and deoxyribonuclease to remove nucleic acids, a salt precipitation with ammonium sulphate followed by anion exchange chromatography on DEAE cellulose. Although the instability of the dehydratase limited it to only a partial purification (a 17-fold increase in specific activity to a figure of 5.0 μmoles pyruvate min^{-1} mg^{-1} of protein, at a recovery rate of 20 %), a preparation was obtained which was free from the two L-threonine deaminating activities.

Not all L-serine dehydratases are as labile as those just discussed. The enzyme from *A. globiformis* is unstable in dilute solution, losing 50 % of its activity in 4 hours at 30 °C and 0.075 mg of protein ml^{-1}. However, it is considerably more stable at higher protein concentrations (1 mg of protein ml^{-1} or higher) so that it has been possible to purify it to a level approaching homogeneity (Gannon, 1973, Gannon *et al*, 1977). The L-serine dehydratase from *Clostridium acidiurici* is stable in the presence of reducing agents (10 mM DTT), which has also allowed it to be extensively purified (Carter and Sagers, 1972).

1.3.3 Timecourses and Substrate Saturation Curves

All bacterial L-serine dehydratases, with the exception of the *E. coli* and *A. globiformis* enzymes (which are discussed in more detail in the next section), have been reported to show linear timecourses of activity.

The microbial L-serine dehydratases are highly specific for L-serine. This is in contrast to their mammalian counterparts which are also able to act on L-threonine (Tokushige, 1972). Hyperbolic substrate saturation curves have been reported for the L-serine dehydratase from *Cl. acidiurici* (Carter and Sagers, 1972), *B. circulans* (Nabe, 1971) and *Streptomyces rimosus* (Szentirmai and Horvath, 1962). However, sigmoidal substrate saturation profiles have been demonstrated for the dehydratases from *B. cereus* and *S. typhimurium* (Rasko *et al*, 1969), *E. coli* (Alfoldi *et al*, 1968, Roberts, 1983), and *A. globiformis* (Gannon, 1973, Gannon *et al*, 1977).

With the exception of the enzyme from *Str. rimosus*, which has been reported to possess a K_m for L-serine of 48 mM (Szentirmai and Horvath, 1962), the K_m for L-serine for most of the microbial L-serine dehydratases is approximately 6 mM L-serine. For example, Roberts (1983) found that the partially purified enzyme from *E. coli* B showed an $S_{[0.5]}$ value of 6.5 mM L-serine. However, Newman and Kapoor (1980), working with the enzyme from a crude extract of *E. coli* K12, have obtained a K_m of 420 mM L-serine. This value is very high, and appears to have been misquoted, since the calculation of the K_m from their Lineweaver-Burke plot gives a value of 42 mM L-serine. Even so, this value is still high, almost an order of magnitude higher than the K_m of 6.6 mM L-serine determined by Alfoldi *et al* (1968) for the same enzyme, and may consequently be somewhat questionable.

1.3.3.1 The Substrate-Activation of the L-Serine Dehydratase from *E. coli*

Alfoldi *et al* (1968) were the first workers to show that the L-serine dehydratase from cell free preparations of *E. coli* possessed a sigmoidal substrate saturation curve. They also showed that an extract prepared in the presence of L-serine (80 mM) displayed a hyperbolic profile. A similar situation was later found with the L-serine dehydratase from *A. globiformis*, where it was found to be due to the slow activation of this enzyme by its substrate, L-serine (Gannon,1973). This suggested that the cooperativity which had been shown by Alfoldi *et al* to exist in the substrate saturation kinetics of the *E. coli* L-serine dehydratase may have had

a similar underlying cause, and prompted Roberts (1983) to investigate whether the enzyme from this microorganism was also activated by its substrate.

The results that Roberts (1983) obtained showed that the *E. coli* L-serine dehydratase was activated by its substrate in a manner that was remarkably similar to that shown earlier (Gannon 1973, Gannon *et al*, 1977) for the corresponding enzyme from *A. globiformis*. The timecourse of pyruvate formation by the cell-free *E. coli* L-serine dehydratase was found to be non-linear, with a pronounced lag phase of several minutes duration during which the rate of pyruvate formation was found to increase progressively until it reached a maximal, linear rate. The presence of this lag phase is a consequence of the activation of the enzyme by its substrate, and allows L-serine dehydratase to be classified as a 'hysteretic' enzyme, of the type described by Frieden (1971). Hysteretic enzymes possess more than one state each having different kinetic properties, with the transition between the two states being slow with respect to the rate of catalysis. The addition of a ligand (substrate, inhibitor, or activator) shifts the interconversion equilibrium from one state to another, the slowness of the response giving rise to the non-linear phase of the timecourse. In the case of the *E. coli* L-serine dehydratase, the substrate activated form has a higher catalytic activity than the non-activated from, the slowness of the interconversion between the two forms giving rise to the lag phase evident in the timecourse of activity of this enzyme.

Marked differences exist in the properties of the activated and non-activated forms of L-serine dehydratase. Roberts (1983) found that the timecourse of enzyme that had been activated by preincubation with with a saturating concentration of L-serine (100 mM) or D-serine (150 mM) possessed no lag phase. Significant cooperativity was evident in the substrate saturation profile of the non-activated enzyme, which was sigmoidal with a Hill coefficient of 2.36. However, activation of the enzyme with either L-serine, or the competitive inhibitor D-serine resulted in the production of a substrate saturation curve with a lower level of cooperativity, as judged by the reduction in the value of the Hill coefficient (1.55 for enzyme activated by L-serine, 1.1 for dehydratase preincubated with D-serine).

The slow transition periods evident in the timecourses of hysteretic enzymes may be due to one of several possible mechanisms: a conformational change, a dissociation-association reaction, or the displacement of a bound ligand by a second ligand. In the case of the L-serine dehydratase from *E. coli* an association (dimerisation) reaction appears to underly the slow response of the enzyme to its

substrate. Roberts (1983) showed that the molecular weight of L-serine dehydratase which had been activated by preincubation with L-serine was double that of the non-activated form of the dehydratase (97 000 and 42 000, respectively). Enzyme which had been activated by D-serine was also found to undergo a doubling of its molecular weight. A series of kinetic analyses of the activation process was also performed. However, these gave somewhat inconclusive results, and were unable to define the nature of the activation process.

The evidence that Roberts (1983) presented suggested that the molecular basis of the slow activation of L-serine dehydratase by its substrate, L-serine, involved the dimerisation of inactive enzyme monomers. However, to show unequivocally that activation and dimerisation are facets of the same process, a direct physical measurement of the molecular weight of the enzyme during activation would be required. Roberts was unable to undertake these experiments because of the instability and heterogeneity of the L-serine dehydratase preparations with which she worked.

1.3.4 Cofactor Requirements

Although it has often been assumed that the microbial L-serine dehydratases contain pyridoxal phosphate, unequivocal spectrophotometric and fluorimetric evidence for the presence of this cofactor has only been obtained for the enzyme from *Cl. acidiurici* (Carter and Sagers, 1972). The UV/visible spectrum of this L-serine dehydratase was found to possess absorption maxima at 420 and 340 nm, typical for enzymes containing pyridoxal phosphate. This enzyme was also found to fluoresce when excited at 340 or 420 nm, with emission occurring at 390 and 520 nm, respectively. Although the dehydratase was not activated by PLP, and could not be resolved by prolonged dialysis against L-cysteine, the enzyme was inactivated when reduced by sodium borohydride, which resulted in stimulation of the 390 nm fluorescence emission maximum of the protein and the elimination of the 520 nm fluorescence emission. The presence of pyridoxal phosphate was also inferred in the L-serine dehydratase from *Str. rimosus* when the enzyme was found to be inactivated by the inhibitors isonicotinic acid hydrazide, semicarbazide and hydroxylamine, even though this enzyme showed no stimulation of activity when incubated with pyridoxal phosphate (Szentirmai and Horvath, 1962). Studies to discover if this cofactor was utilised by the L-serine dehydratase from *E. coli* (Alfoldi *et al*, 1968, Newman and Kapoor, 1980 and Roberts, 1983) were inconclusive. The non-homogeneity of the enzyme preparations used by these workers did not permit the undertaking of spectroscopic investigations which would have unequivocally shown if this

cofactor was present. However, a number of experiments which examined the effects of pyridoxal phosphate and carbonyl inhibitors on the activity of this dehydratase failed to produce any results which would appear to indicate its presence. In addition, the enzyme was not stimulated by PLP, nor significantly inhibited by hydroxylamine. Attempts to determine the nature of the cofactor utilised by the *A. globiformis* L-serine dehydratase also did not meet with success. The enzyme from this microorganism was not activated by pyridoxal phosphate, could not be resolved by dialysis against L-cysteine or hydroxylamine and was resistant to a variety of carbonyl reagents. Although a purification scheme was devised for the *A. globiformis* L-serine dehydratase, the quantities of purified enzyme obtained were not enough to allow definitive spectroscopic investigations of this enzyme to be undertaken (Gannon, 1973, Gannon *et al*, 1977)

The microbial L-serine dehydratases are not affected by compounds such as AMP or ADP, which have been shown to be important effectors in the regulation of the activity of the degradative L-threonine dehydratase (Umbarger, 1973). However, a requirement for cations has been demonstrated for most of the L-serine-specific dehydratases. The activation of the L-serine dehydratase from *A. globiformis* was found to have an absolute, but non-specific, requirement for mono- or divalent cations (Gannon, 1973, Gannon *et al*, 1977). They affected both the rate and extent of the activation process. However, once the enzyme had been activated in the presence of cations, it could be assayed in their absence, indicating that cations were not essential for the catalytic process. Divalent cations were found by Gannon to be more effective at lower concentrations that monovalent cations, for example, 1 mM $MgCl_2$ produced the same degree of activation of the dehydratase as 22.5 mM KCl.

Until the work of Newman *et al* (1985), the importance of cations to the *E. coli* L-serine dehydratase was uncertain. Roberts (1983) had found that this dehydratase possessed no absolute or specific cation requirement, although cations such as Ca^{2+} or K^{+} were stimulatory to the activity of the enzyme, producing an average increase in the catalytic activity of the enzyme of between 1.3- and 1.6- fold. Cations were not found to be necessary for the activation of the dehydratase by its substrate, L-serine.

The work of Newman *et al* (1985) showed that the activity of the *E. coli* L-serine dehydratase could be substantially increased if the enzyme was supplied with ferrous iron and dithiothreitol. An extract assayed in the presence of 1 mM $FeSO_4$ and 10 mM DTT showed a six-fold increase in the rate at which it produced

pyruvate compared to enzyme assayed in buffer only. Preincubation of the dehydratase with 1 mM ferrous sulphate and 10 mM dithiothreitol (plus 5 mM L-serine) also resulted in a greater than 300 % increase in the activity of the enzyme, compared with only 60 % when the dehydratase was preincubated with the L-serine alone. Newman *et al* (1985) also showed that activation of the dehydratase was very specific in terms of its metal requirement: Fe^{2+} could not be replaced by Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Ni^{2+}, and Na^{+}. Similarly, mercaptoethanol, glutathione and ascorbate were unable to substitute for the dithiothreitol. The activation was also shown to require the presence of both the iron and DTT.

An earlier example of a similar requirement of an L-serine-specific dehydratase for iron was provided by Carter and Sagers (1972) for the highly purified dehydratase from *Cl. acidiurici*. This enzyme was found to have an absolute and highly specific requirement for ferrous iron, and a reducing agent, most effectively met by dithiothreitol. Carter and Sagers also presented indirect evidence which would appear to indicate that iron was directly incorporated into the protein during the process of activation by these reagents. This suggestion is of particular interest in view of the fact that this L-serine dehydratase has also been conclusively shown to contain pyridoxal phosphate. Carter and Sagers suggested that iron may play a direct role in the catalytic reaction, possibly by chelating to the 3-hydroxyl group of pyridoxal phosphate and forming a ring system with the PLP cofactor and the Schiff base-linked L-serine molecule, in a manner similar to the model suggested by Metzler and Snell (1952) for the non-enzymatic deamination of serine. The iron would then act by promoting the formation of unstable Schiff base intermediates, and ultimately the products of the deamination reaction.

The work of Newman *et al* (1985) is also important with respect to the investigations made by these workers into the effects of buffer types on the stability of the *E. coli* K12 L-serine dehydratase. They showed that the activity of L-serine dehydratase preparations made in glycylglycine buffer was substantially more stable than enzyme prepared in other buffers, such as potassium phosphate.

The observations made by Newman *et al* (1985) suggested that it might now be possible to stabilise the *E. coli* L-serine dehydratase and so a re-examination of the question of the basis of the instability of th *E. coli* dehydratase was undertaken. This ultimately led to the development of the purification scheme described in Chapter 3.

1.3.4 Inhibitor Studies

Microbial L-serine dehydratases show considerable sensitivity to mercurial compounds and other reagents which modify sulphydryl groups (Gannon, 1973, Roberts, 1983). This suggests that, as is the case with many enzymes, a sulphydryl group plays an essential role in the L-serine dehydratase reaction.

D-Serine and L-cysteine have been found to be inhibitors of the most of the microbial L-serine dehydratases investigated. In the cases where the inhibition has been analysed, these amino acids have been found to act competitively, with K_i values of approximately 3 mM and 1 mM, respectively. The inhibitor constants for the *E. coli* L-serine dehydratase are 3.5 mM D-serine and 1.04 mM L-cysteine (Roberts, 1983).

The L-serine dehydratases are generally resistant to reagents which interact with carbonyl groups, and frequently show little inhibition at inhibitor concentrations which would normally completely inactivate pyridoxal phosphate containing enzymes such as the L-threonine dehydratases (section 1.3). For instance, Umbarger and Brown (1957), showed that the L-threonine dehydratases from *E. coli* were completely inactivated when assayed with 0.1 mM hydroxylamine. In a similar experiment by Roberts (1983), the L-serine dehydratase from *E. coli* B still retained 34 % of its initial activity when assayed with a thousand-fold greater concentration of this inhibitor.

1.3.5 pH Optimum

The pH activity curves of L-serine-specific dehydratases tend to be very broad, with the pH optimum often spanning up to one pH unit, usually around pH 8. The pH optimum of the *E. coli* enzyme in 100 mM Tris-phosphate buffer was found to lie between pH 7.5 and 8.2 (Roberts, 1983).

1.3.6 Molecular Weight Studies

The molecular weights of the L-serine dehydratases have been determined in only a few instances, and have generally been found to be fairly low. For example, the dehydratase from *Cl. acidiurici* was found to have a molecular weight of 72 000 (Carter and Sagers, 1972), while the molecular weight of the enzyme from *B. circulans* was less than 100 000 (Nabe, 1971). The activated form of the L-serine dehydratase from *E. coli* was shown by Roberts (1983) to exist as a dimer of molecular weight 97 000.

1.4 Iron Sulphur-containing (De)Hydratases

The work of Newman *et al* (1985) has shown that the L-serine dehydratase from *E. coli* can be substantially activated by ferrous iron and dithiothreitol (section 1.3.4). The characteristics of this metal and reducing agent requirement bear striking similarities to those shown by a number of other enzymes catalysing dehydration-hydration reactions, including the citric acid cycle enzyme aconitase, from beef heart mitochondria (Kennedy *et al* 1983a), maleate hydratase from rabbit kidney (Dreyer, 1985), tartrate dehydratase from *Pseudomonas putida* (Kelly and Scopes, 1986) and 6-phosphogluconate dehydratase from *Zymomonas mobilis* (Scopes and Griffiths-Smith, 1984). All of these enzymes have been shown to contain iron-sulphur (FeS) centres, which raised the possiblity that the *E. coli* L-serine dehydratase may also contain such a centre. For this reason a discussion of the properties of the better characterised of these (de)hydratases is included in this section.

1.4.1 FeS Centre Stability and Metal and Thiol Requirements

Iron-sulphur containing dehydratases differ considerably in terms of the type of their FeS clusters, the stability of these centres, reducing agent requirements and reactivation procedures (Table 1.1). While the iron within [2Fe-2S] centre in the dihydroxy acid dehydratase is quite stable and the enzyme does not require a reducing agent or iron for activity (Flint and Emptage, 1988), the [4Fe-4S] cluster in maleate hydratase is considerably more labile. Loss of iron from this centre results in a loss of catalytic activity. Dreyer (1985) has shown that this enzyme can be reactivated by anaerobic preincubation with 5 mM sodium sulphide and 1 mM Fe^{2+}. The *P. putida* L-tartrate dehydratase rapidly loses activity unless supplied with 0.1 mM Fe^{2+}, 5 mM cysteine, 5 mM glutathione and 10 mM ascorbate (Kelly and Scopes, 1986). Aconitase, which contains an unstable [4Fe-4S] centre, is also non-specific in its requirement for a reducing agent and is activated by a variety of compounds, including NAD(P)H, ascorbate, dithiothreitol, sodium sulphide and sodium dithionite, although the enzyme is highly specific in respect of its metal requirement (Fe^{2+}) (Kennedy *et al*, 1983).

Aconitase is the best characterised of the FeS class of (de)hydrolyases. A series of detailed studies using Mossbauer (Telser *et al*, 1986) and EPR spectroscopy (Emptage *et al*, 1983), as well as radiochemical and analytical approaches (Kennedy *et al*, 1983) have shown that the loss of iron and

Table 1.1

Iron Sulphur Dehydratases

(De)hydratase	Molecular Weight	Reactivation Requirements	FeS Centre	Reference
Aconitase (beef heart mitochondria)	81 000	Fe^{2+}, DTT or Anaerobic Reduction	[4Fe-4S]	Kennedy *et al* (1983)
Maleate Hydratase (rabbit kidney)	68 000	Fe^{2+}, Na_2S, Mercapto-ethanol	[4Fe-4S]	Dreyer (1985)
Dihydroxyacid Dehydratase (spinach)	105 000	None (FeS centre is stable)	[2Fe-2S]	Flint and Emptage (1988)
Lactyl CoA Dehydratase	1 000 000 (multimer)	None (FeS centre is stable)	[3Fe-4S]	Kuchta *et al* (1986)
Tartrate Dehydratase (*P, putida*)	100 000 (dimer)	Fe^{2+}, Cysteine, Glutathione Ascorbate	[4Fe-4S] or two [2Fe-2S]	Kelly *et al* (1986)
6-Phospho-gluconate Dehydratase (*Z. mobilis*)	105 000 (dimer)	Fe2+, DTT, Ascorbate	Not yet known	Scopes *et al* (1984)

re-activation of aconitase involves an interconversion between [3Fe-4S] and [4Fe-4S] iron-sulphur centres:

$$\underset{\text{(inactive)}}{\text{[3Fe-4S]}} \underset{-\,Fe^{2+}\,/\,\text{Oxidation}}{\overset{+\,Fe^{2+}\,/\,\text{Reduction}}{\rightleftharpoons}} \underset{\text{(active)}}{\text{[4Fe-4S]}}$$

Loss of iron from the [4Fe-4S] centre results in the loss of activity, a process which may be reversed, as already mentioned, by preincubation of the enzyme with ferrous iron and a reducing agent. Kennedy and co-workers have shown that inactive, Fe-deficient aconitase can also be partially reactivated by anaerobic preincubation with reducing agents in the absence of added iron (Kennedy *et al*, 1983). Electron paramagnetic resonance studies have shown that this partial activation (up to 75 % of the activity attainable in the presence of iron) is due to the breakdown and redistribution of iron between the [3Fe-4S] centres: active [4Fe-4S] centres are built up from inactive, iron-deficient [3Fe-4S] centres.

1.4.2 UV/Visible and EPR Spectra

Iron-sulphur containing proteins, including the (de)hydratases aconitase (Kennedy *et al*, 1983), maleate hydratase (Dreyer, 1985) and dihydroxy acid dehydratase (Flint and Emptage, 1988), are characteristically brown in colour. However, there is no common identifying feature to the absorption spectra of these enzymes, although most possess one or more maxima in the 400 to 500 nm region of the spectrum. Their EPR spectra are more clearly defined, however, and typically show several component EPR signals whose *g*-average value is close to 2.00. For example, [4Fe-4S] aconitase has a g-average value of 2.01 (Emptage *et al*, 1983).

1.4.3 The Role of FeS Centres in Catalysis

The addition of substrate to either maleate hydratase, dihydroxyacid dehydratase or lactyl CoA dehydratase results in significant changes in the visible and EPR spectra of these enzymes, suggesting that the iron-sulphur centres within these proteins may play a role in the catalytic process (Dreyer, 1985, Flint and Emptage, 1988, Kuchta et al, 1986). A more clearly defined role, however, has been assigned to the FeS centre in aconitase. Mossbauer and ^{17}O ENDOR spectroscopy have shown that citrate is bound via its hydroxyl group to a single Fe site in the [4Fe-4S] centre, Fe_a, the iron which is added on re-activation of the inactive

[3Fe-4S] form of the enzyme. Telser et al (1986) have presented evidence which shows that the Fe_a is directly involved in the conversion of citrate to isocitrate, probably by acting as a Lewis acid during the dehydration-rehydration reaction.

1.5 Binuclear Iron-oxo Proteins

The functions of iron in biology are often associated with haem, or with iron-sulphur proteins, such as electron carriers (ferredoxins) or the (de)hydratases described in the previous section. There exists, however, a third group of iron-containing proteins in which the iron is not coordinated to a porphyrin ligand or to sulphur clusters, but is instead linked to another iron atom via one or more oxygen atoms. These oxo bridged iron centres are involved in a range of functions, including dioxygen transport, oxidation of methane to methanol, acid phosphatase activity, iron storage and the reduction of ribonucleotides to deoxyribonucleotides (Lippard, 1988). Certain aspects of the iron requirement of L-serine dehydratase, most notably the involvement of oxygen in the iron-activation process (Chapter 6, section 6.7) bear similarity to the reactivation procedures for one of these iron oxo proteins, ribonucleotide reductase, and so a description of some of the prominent features of this enzyme is included.

1.5.1 Ribonucleotide Reductase

Ribonucleotide reductase catalyses the first committed step in the biosynthesis of DNA. The *E. coli* ribonucleotide reductase consists of two different subunits, proteins B1 and B2, which are required in equimolar amounts for enzyme activity. The B1 subunit is a dimer of molecular weight 160 000. It contains 4 binding sites for effector molecules as well as 2 substrate binding sites with redox-active sulphydryl groups. The B2 subunit, a dimer of molecular weight 78 000, can either contain a Cobalt cofactor derived from vitamin B12, or an oxo bridged binuclear iron centre. Associated with the iron-containing group is an unusual tyrosyl free radical (Reichard, 1980). The presence of these two groups confers distinctive features upon the UV/visible and EPR spectra of the enzyme, with absorption maxima at 390, 410 and 600 nm, (Petersson *et al*, 1980) and *g*-average values in the 1.7 to 1.8 range (Que and Scarrow, 1988).

The production of the tyrosyl free radical is dependent on the presence of the binuclear centre. Destruction of the iron centre by dialysis against a reductant (hydroxylamine) and an iron (II) chelator results in the loss of the both the free radical and enzymatic activity. Full activity can be restored by preincubating the

reductase with ferrous iron, and sulphydryl reducing agents in the presence of oxygen, which is essential for the reactivation process (Petersson *et al*, 1980).

1.6 Aims and Objectives

Until the present study, investigations into the properties of the L-serine-specific dehydratase from *E. coli* had been limited, because of its instability, to material that was contained in either crude cell extracts (Alfoldi *et al*, 1968, Newman and Kapoor, 1980), or that was at best only slightly purified (Roberts, 1983). The lack of purified enzyme has meant that, hitherto, studies of L-serine dehydratase have largely been restricted to kinetic analyses of enzyme activity. While these studies have revealed much about the properties of this enzyme, a number of interesting questions remain which can only be answered by using purified protein.

The first of these is the question of pyridoxal phosphate. It has often been assumed that the microbial L-serine dehydratases, like their mammalian counterparts, contain pyridoxal phosphate, even though conclusive evidence for the presence of this cofactor has been obtained for only one member of this group of enzymes (Carter and Sagers, 1972). The availability of purified L-serine dehydratase from *E. coli* would allow a series of spectrophotometric and fluorimetric studies to be undertaken, which would unequivocally show if the enzyme from this source contained pyridoxal phosphate.

The characteristics of the activation of *E. coli* L-serine dehydratase by ferrous iron and DTT, described by Newman *et al* (1985), were seen to be very similar to those shown by a number of other (de)hydratase-type enzymes, all of which had been shown to contain FeS centres (section 1.4). This raised the possibility that such a centre might also be present within the *E. coli* dehydratase. With a preparation of purified enzyme the presence of such a centre, and its possible involvement in the deamination reaction, could be investigated using techniques such as EPR spectroscopy.

Another unresolved question is that concerning the molecular mechanism of dimerisation and its relationship to activation by serine (section 1.3.3.1). Roberts (1983) presented evidence which suggested that the dimerisation of enzyme monomers accompanied the activation of L-serine dehydratase by its substrate. However, the low purity of the material with which she worked prevented her from undertaking the biophysical analyses, such as laser light scattering, which could have directly shown if dimerisation and activation by L-serine were

causally related. With enzyme of high purity, such a series of experiments could be carried out, and an answer to this question obtained.

During the course of this work a sequence for the *E. coli* L-serine dehydratase gene, *sda*A, was published by Newman and co-workers (Su *et al*, 1989). Although it is likely that this sequence does represent the structural gene for the dehydratase, a possibility remains that the sequence obtained by these workers is that of an activator of the enzyme (Chapter 4, section 4.7) This uncertainty arises because of the somewhat indirect method used to isolate the gene sequence (an *in vivo* complementation technique), and the lack of a primary sequence obtained directly from the dehydratase protein with which to compare it. If purified enzyme was available, an analysis could be made of its amino acid terminal sequence, which could then be used to determine if the sequence proposed by Su *et al* (1989) was that of the gene for L-serine dehydratase.

All of these objectives require purified enzyme, and so the first aim of this project was to purify L-serine dehydratase to a level as near to homogeneity as possible. This in turn required the activity of the dehydratase to be stabilised, and so a detailed investigation into the underlying cause of the apparent instability of this enzyme was a necessary preliminary to the attempt to purify it. The work of Newman *et al* (1985), supplemented by work undertaken by the present author as part of an undergraduate project, showed that the stability of the *E. coli* L-serine dehydratase could be improved by appropriate treatment. These findings, together with the application of recently developed purification techniques, particularly FPLC, held out the prospect that the purification of the enzyme might now be successfully accomplished.

CHAPTER 2 - MATERIALS AND METHODS

2.9.3 Native Isoelectric Focussing Gel Electrophoresis.

2.10 Materials.

2.10.1 Instruments.

2.10.2 Enzymes and Proteins.

Chapter 2

Materials and Methods

2.1 Organisms Used

Wild-type strains of *Escherichia coli* (strains B, W, C, K12 and Crookes) were obtained from laboratory stocks. Strain B/S53 was a variant of *E. coli* B containing elevated levels of L-serine dehydratase; it was isolated from L-serine selection plates as described in Chapter 3, section 3.2.2 All strains were maintained by subculturing at 7 to 10 day intervals onto nutrient agar slopes. These were incubated overnight at 37 ^{o}C and then stored at room temperature.

2.2 Growth Media

Two growth media were used for the culture of *E. coli*. These were the medium of Alfoldi *et al* (1968), and that of Davis and Mingioli (1950).

2.2.1 Alfoldi's Medium

The basal medium of Alfoldi *et al* (1968) had the following composition (per litre):

	g
NH_4Cl	2.0
$NaH_2PO_4.2H_2O$	7.8
$K_2HPO_4.3H_2O$	18.4
NaCl	8.0
$MgSO_4.7H_2O$	0.25
Tryptone	10.0
Yeast Extract	1.0
pH	7.5

This mixture was sterilised by autoclaving at 15 lbs in^{-2} for 15 minutes. The final component of the medium, glucose, was autoclaved separately and added aseptically prior to inoculation, to a final concentration of 0.2 %.
Growth of *E. coli* on this medium took place aerobically at 37 ^{o}C in Erlenmeyer

flasks, baffled to aid aeration, and shaken at 150 to 200 r.p.m.

2.2.2 Davis and Mingioli Minimal Medium

The basal medium of Davis and Mingioli (1950) had the following composition (per litre):

	g
K_2HPO_4	7.0
KH_2PO_4	3.0
$(NH_4)_2SO_4$	1.0
Na_3-Citrate	0.5
$MgSO_4.7H_2O$	0.1
pH	7.0

This solution was sterilised by autoclaving at 15 lbs in^{-2} for 15 minutes. Just prior to inoculation of the medium a sterile carbon source, usually glucose, and filter-sterilised thiamine were added aseptically to final concentrations of 0.2 % (w/v) and 1 μg ml^{-1}, respectively. Small-scale cultures (up to 800 ml) of medium were inoculated either from a slope culture or from cell suspensions aseptically harvested, washed and resuspended in sterile basal medium. These cultures were grown aerobically at 37 °C in baffled 2 litre Erlenmeyer flasks shaken at 150 to 200 r.p.m.

For large scale growth of *E. coli*, 12 litres of this basal medium were sterilised *in situ* at 121 °C in a LH 13 litre capacity fermenter (LH Fermentation Ltd., Slough, U.K.), according to the manufacturers instructions. Just prior to inoculation, glucose and thiamine were aseptically added at the concentrations stated earlier. The medium was inoculated with a 750 ml culture of *E. coli* grown on the medium of Davis and Mingioli (1950) to a cell density of between 1.0 to 1.5 mg dry wt. ml^{-1}. Growth in the fermenter took place at 37 °C. The culture was stirred at 500 r.p.m., and aerated by sparging with compressed air (pressure setting 15 lbs in^{-2}) which had been sterilised by passage through a 0.2 μm sterile filter.

2.2.2.1 Cell Growth on the Medium of Davis and Mingioli and the Production of L-Serine Dehydratase Activity

The growth of *E. coli* on the medium of Davis and Mingioli took place in two stages. During the first stage, the bacteria were allowed to grow to late logarithmic/early stationary phase (1.2 to 1.5 mg ml^{-1}) on the basal medium specified above, supplemented with glucose (0.2 %) and thiamine (1 μg ml^{-1}). In the second stage, further cell growth (an approximate doubling of the cell density) and the induction of L-serine dehydratase activity were achieved by the aseptic addition of 0.1 % glucose, 0.1 % glycine, 0.2 % yeast extract and 1 % Tryptone. At this stage, fermenter grown cultures required the extra addition of an anti-foaming agent (200 to 500 μl of sterile polyethylene glycol 4000). The cells were harvested towards the end of the second phase of exponential growth, when the induction of the dehydratase was maximal and the cell density was between 2.0 and 2.3 mg ml^{-1}.

A more detailed discussion of the growth conditions under which L-serine dehydratase is formed is provided in Chapter 3, section 3.2.2 and 3.2.3

Cell material required for purification work was usually obtained from fermenter-grown cultures. On occasion, however, it was found necessary to grow *E. coli* B intended for this purpose by flask culture. When this occurred, 12 to 15 flasks of Davis and Mingioli minimal medium (800 ml Medium per 2 l baffled Erlenmeyer flask) were prepared, and cell growth and L-serine dehydratase production achieved as described above. Although the specific activity of extracts prepared from cells grown by flask culture was usually higher than that of cells grown in the fermenter (by some 15 to 25 %), the extra time required to prepare the flasks offset the advantage gained by higher specific activity. More L-serine dehydratase activity, although of a somewhat lower specific activity, could be produced by fermenter culture in a given period of time than could be produced by growth in flasks.

2.2.3 Solid Growth Media

When the medium of Davis and Mingioli was required in solid form it was solidified by the addition of 1.5 % (w/v) Bacteriological agar (Oxoid, England) prior to autoclaving. Carbon sources, amino acids, thiamine and other additions were made aseptically after autoclaving, and the medium poured

before use.

Nutrient agar slopes were made from nutrient agar tablets as specified by the manufacturer's instructions (Oxoid, England).

2.3 Estimation of Bacterial Growth

Bacterial cell densities were estimated by measuring the optical density (OD) of a sample of the culture, diluted if necessary, at 680 nm. The value obtained was converted into mg dry weight of cells ml^{-1} by reference to a previously constructed standard curve. This curve shows a linear relationship between the optical density at 680 nm and dry weight of cells ml^{-1} up to an absorbance of 1.0, and has a gradient of 1.43 OD units per mg dry weight of cells ml^{-1}.

2.4 Harvesting of Cells and Preparation of Cell-free Extracts

2.4.1 Harvesting of Cells

When bacterial growth was complete, the cell cultures were quickly cooled on ice and centrifuged for 10 minutes at 4 ^{o}C and 6000 *g* in a Beckman J2-21 centrifuge. The harvested cell pellet was then washed by resuspension in chilled buffer A (2 mM DTT in 50 mM glycylglycine, pH 7.8) followed by centrifugation as already described. The cell pellet was washed and centrifuged once more before re-suspension in buffer A and extraction by one of the methods described in sections 2.4.2, and 2.4.3.

A number of experiments have shown that L-serine dehydratase activity in whole cells is stable for up to 2 days when stored at 4^{o} C, and for up to 4 days when frozen at -20 ^{o}C. Usually, however, the cells were extracted immediately after harvesting.

2.4.2 Extrusion Under High Pressure ; French Press

Large quantities of cell material, intended for purification work, were extracted by several passes through a French Pressure cell (American Instrument Co., Silver Springs, Md., U.S.A.). Portions (40 ml) of the chilled cell suspension (60 to 80 mg dry wt. ml^{-1}) were passed through the pre-cooled French Press at a pressure of between 9000 to 12 000 lbs in^{-2}. The treated cell preparation was collected in a flask in an ice-water bath, cooled and recycled at

French Press at a pressure of between 9000 to 12 000 lbs in^{-2}. The treated cell preparation was collected in a flask in an ice-water bath, cooled and recycled at least once. After breakage, the bacterial suspension was diluted with buffer A to reduce viscosity, and centrifuged as described below.

2.4.3 Sonication

Ultrasonication was used for volumes of cell suspensions of less than 15 ml. The washed cell suspension (approximate cell density 30 to 40 mg dry wt. ml^{-1}) was placed in a beaker surrounded by an ice-water mixture and treated with ultrasound for a total period of 3 to 5 minutes, depending on the volume of the suspension, in 30 second treatments with 1 minute intervals for cooling. The sonication was performed on a MSE 60W ultrasonic oscillator set at 9 microns. Cell debris was removed as described in section 2.5.3.

2.4.4 Centrifugation of Crude Extracts

After cell breakage, cell debris was removed by centrifugation for 60 minutes at 4 ^{o}C, 20 000 *g* in a Sorvall RC5B Centrifuge (Dupont Ltd., Stevenage, Herts, U.K.). All of the L-serine dehydratase activity remained in the supernatant.

2.5 Permeabilisation of Cells

Bacterial cell membranes were made permeable to the passage of small molecules by the addition of toluene. Toluene (0.025 ml per ml of bacterial suspension) was added to whole cell suspensions of known cell density (2 to 4 mg ml^{-1}). The cell suspension and toluene were vigorously mixed on a vortex mixer and incubated at 37 ^{o}C for 15 minutes. During this time the vortexing was repeated every 3 to 4 minutes to ensure complete mixing. Cells treated in this way were either used immediately, or stored on ice for no more than 20 minutes. Storage for longer periods resulted in a significant loss of L-serine dehydratase activity.

2.6 Measurement of L-Serine Dehydratase Activity

L-Serine dehydratase activity was assayed by measurement of the pyruvate formed during the enzymatic reaction with L-serine. Two methods were used to achieve this: one using a discontinuous (2,4-dintrophenylhydrazine) assay,

the other a continuous (lactate dehydrogenase coupled) method.

2.6.1 Discontinuous Assay

In the discontinuous assay, pyruvate was measured as its 2,4-dinitrophenylhydrazine (DNPH) derivative. The procedure used was based on the assay system developed by Roberts (1983), which was a modification of the method of Friedemann and Haugen (1943). The basal mixture for this assay had the following composition:

100 μmoles of glycylglycine-NaOH buffer, pH 8.0
50 μmoles of L-serine, pH 8.0
10 μmoles of DTT
1 μmole of $FeSO_4$
appropriate volume of toluene-treated cell suspension or cell-free enzyme preparation
and distilled water, to a final volume of 1 ml

Isoleucine (10 μmoles) and neutralised hydroxylamine (1 μmole) were included in assays of L-serine dehydratase activity in crude extracts, in toluene-treated cells and in partially purified enzyme preparations up to, and including, the DEAE cellulose stage of the purification (step 4) to inhibit biosynthetic and degradative L-threonine dehydratase activities. These inhibitors were omitted from assays of L-serine dehydratase after the DEAE cellulose step, when all traces of these enzymes had been removed.

Discontinuous assays were also performed, where appropriate, without additions of iron or dithiothreitol. L-Serine dehydratase used in such assays was either Fe-independent enzyme from a crude extract which did not require ferrous iron and dithiothreitol for activity, or purified protein that had been iron-activated by preincubation with Fe^{2+} and DTT (as described in section 2.6.4). A definition of the terms 'Fe-independent' and 'iron-activated' are provided in Chapter 4. section 4.1. Except where stated in individual experiments, the assay procedure followed was as described below.

The discontinuous assay was initiated by the addition of enzyme to basal assay mixtures that had been preincubated at 37 ^{o}C for at least ten minutes. The complete reaction mixture was then incubated at this temperature for

precisely 10 minutes. After the incubation period, the enzyme-catalysed reaction was terminated by the addition of 0.1 ml of the incubation mixture to a tube containing 0.9 ml of water and 0.33 ml of 0.1 % dinitrophenylhydrazine in 2 M HCl (the addition of only a proportion of the reaction mixture to the DNPH reduced the carryover of DTT, and where appropriate, of hydroxylamine, to a level which did not interfere with the 2,4-dinitrophenylhydrazine assay). The DNPH mixture was allowed to stand for 10 minutes, when 2 ml of 2 M NaOH were added. After a further 10 minutes, the absorbance was measured at 445 nm against a suitable blank (usually an assay mixture lacking substrate). If the absorbance was greater than about 1.5, the mixtures were diluted with 1 M NaOH. The amount of pyruvate formed was calculated by reference to a standard curve prepared with sodium pyruvate. This showed a linear relationship between pyruvate concentration and absorbance, with a gradient of 5.5 absorbance units per μmole of pyruvate.

In experiments where the timecourse of a reaction was followed, the reaction was initiated either by the addition of enzyme, or by the addition of substrate to 2 or 3 ml assay mixtures. At the times indicated in individual experiments, 0.1 ml samples were withdrawn, and the amount of pyruvate formed determined as already described.

Dithiothreitol and hydroxylamine caused considerable interference with the 2,4-dinitrophenylhydrazine method of pyruvate estimation. This was investigated by examining the absorbance of range of pyruvate concentrations measured by the DNPH method in the presence of various components of the discontinuous assay mixture (Figure 2.1). It can be seen that the concentrations of DTT and NH_2OH present in the complete 1.0 ml of the assay medium (10 mM and 1 mM respectively) caused a substantial reduction of the colour intensity of the developed pyruvate-dinitrophenylhydrazone mixtures. This effect was particularly pronounced when both components were present in the assay media. However, the concentrations of DTT and hydroxylamine which were present in 0.1 ml of the basal assay mixture (1 mM and 0.1 mM) did not significantly interfere with pyruvate estimation.

2.6.2 Continuous Assay

In the continuous mode of assay, pyruvate formation was coupled to NADH oxidation by the action of lactate dehydrogenase (E.C. 1.1.1.27). The

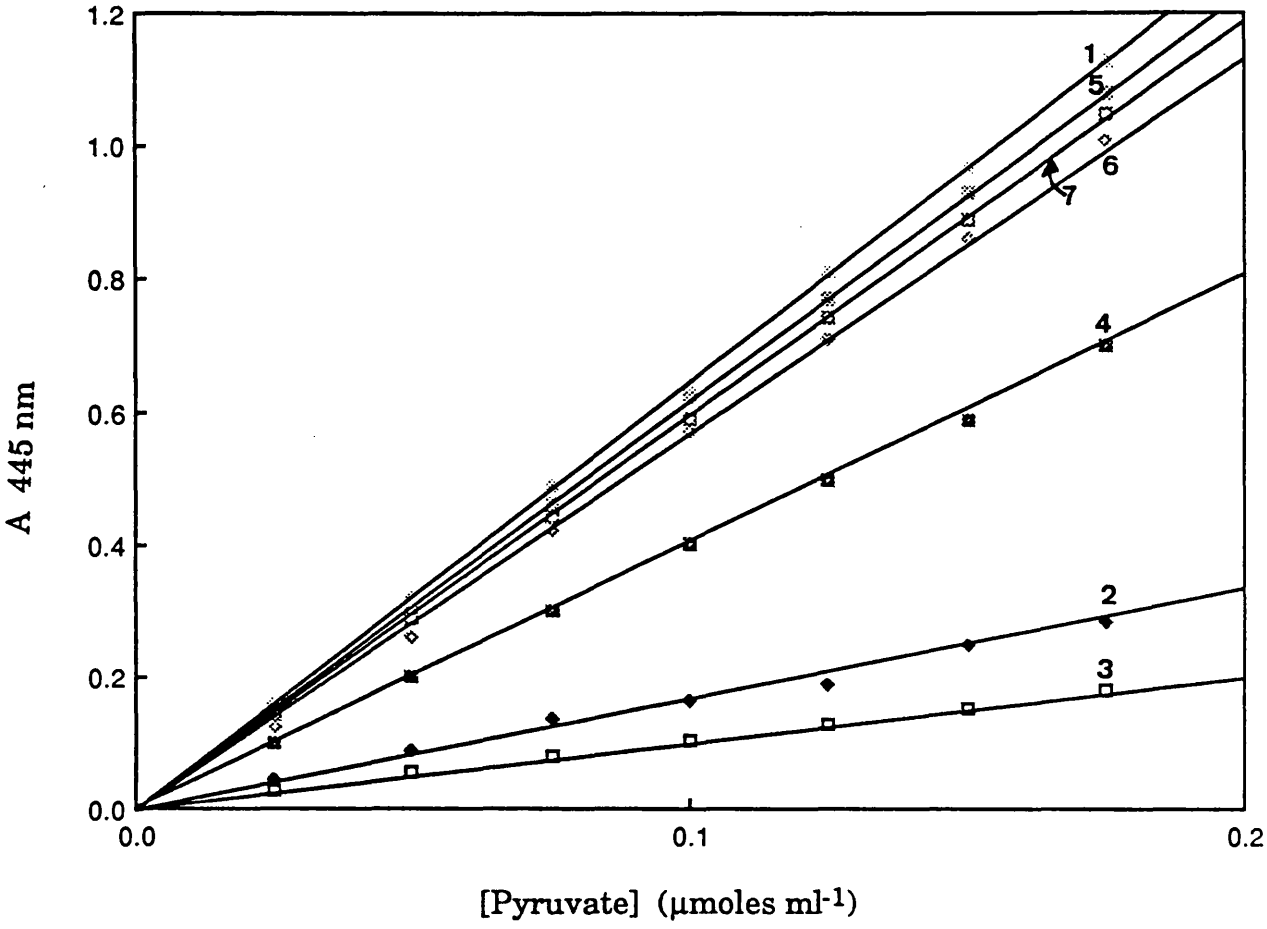


Figure. 2.1

The Interference Effects of Ferrous Iron, Dithiothreitol and Hydroxylamine on the Measurement of Pyruvate Concentration by the 2,4-Dinitrophenylhydrazine Assay.

The absorbance of a range of 1ml reaction mixtures containing pyruvate concentrations from 0.025 to 0.175 μmole ml^{-1} were measured at 445 nm after the addition of 0.33 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl. This procedure was repeated several times, each time with a different additions of ferrous iron, DTT and hydroxylamine, as indicated below:

1 100 mM Glycylglycine, pH 8.0 (control).
2 100 mM Glycylglycine, pH 8.0 + 1mM $FeSO_4$ + 10mM DTT
3 100 mM Glycylglycine, pH 8.0 + 1mM $FeSO_4$ + 10mM DTT + 1 mM hydroxylamine.
4 100 mM Glycylglycine, pH 8.0 + 1mM hydroxylamine.
5 10 mM Glycylglycine, pH 8.0 + 1mM DTT + 0.1mM $FeSO_4$
6 10 mM Glycylglycine, pH 8.0 + 1mM DTT + 0.1mM $FeSO_4$ + 0.1mM hydroxylamine.
7 10 mM Glycylglycine, pH 8.0 + 0.1mM hydroxylamine.

disappearance of NADH was followed spectrophotometrically at 340 nm, at which wavelength NADH has a molar absorbance of 6220 M^{-1} cm^{-1} (Dawson *et al*, 1969).

The basal assay for this method of pyruvate estimation had the following composition:

100 μmoles of glycylglycine-NaOH buffer, pH 8.0
50 μmoles of L-serine, pH 8.0
0.25 μmoles of NADH
15 units of lactate dehydrogenase
appropriate volume of cell-free enzyme preparation
and distilled water, to a final volume of 1 ml

The dehydratase reaction was usually initiated by the addition of enzyme to assay mixtures that had been preincubated at 37 °C in the thermostatically controlled cell-block of a spectrophotometer. When extracts of *E. coli* were likely to contain L-threonine dehydratase activity, 10 μmoles of isoleucine and 1 μmole of hydroxylamine were added to the above assay mixture. In addition, with crude enzyme preparations, which contain NADH oxidase activity, the blank rate of NADH oxidation was determined and its value subtracted from the apparent rate of L-serine dehydratase activity.

2.6.3. Choice of Assay

The choice of assay method was determined by the nature of the enzyme preparation to be assayed, and by the experiment to be performed.

The lactate dehydrogenase coupled assay is sensitive and continuous, and was used for most of the kinetic analyses of L-serine dehydratase activity, particularly those experiments that required the presence of high concentrations of hydroxylamine (10 mM or more), which does not inhibit lactate dehydrogenase. It could not be used, however, when ferrous iron and dithiothreitol were components of the assay medium. At the concentrations normally used, Fe^{2+} plus DTT absorb very strongly over the 300 to 400 nm range, thereby preventing an accurate measurement of NADH consumption. For experiments which required the presence of these substances as components of the assay medium, the 2,4-dintrophenylhydrazine method of

pyruvate estimation was always employed. Purified L-serine dehydratase is catalytically inactive in the absence of iron and DTT (see section 3.1 for a fuller discussion) and so continuous assays could only be performed with unpurified enzyme that was either Fe-independent, or with Fe-dependent enzyme that had first been activated by preincubation with iron and DTT (section 2.6.4.). Experience has shown that there is a limit to the volume of this iron-activated enzyme that can be added before the concentration of the $FeSO_4$ and DTT carried over into the lactate dehydrogenase-coupled assay causes significant interference. This has been empirically determined to be approximately 150 µl per ml of assay mixture.

The 2,4-dinitrophenylhydrazine assay was used when there were large numbers of enzyme samples to be assayed, for example with column fractions obtained during the purification. It was always chosen for measurement of enzyme levels in toluene-treated cells. The discontinuous method was also used when possible inhibitors of lactate dehydrogenase were present, such as mercurial compounds and other sulphydryl modifying reagents.

For best results, the discontinuous assay system requires 0.1 to 0.2 units of L-serine dehydratase. The continuous method of pyruvate estimation functioned most effectively with 0.05 to 0.1 units of enzyme activity. Good correlation was observed between the two methods of assay provided that the appropriate number of enzyme units were used.

2.6.4 The Activation of Purified L-Serine Dehydratase by Ferrous and Dithiothreitol

When assays of purified L-serine dehydratase were performed in the absence of added iron or dithiothreitol, the enzyme was first activated by preincubation at 37 °C with 100mM glycylglycine-NaOH pH 8.0, 1 mM $FeSO_4$ and 10 mM DTT. The time taken for maximal activation varied slightly between preparations, but typically required between 15 and 20 minutes. Once activation had occurred, the dehydratase remained fully activated for only a limited length of time: at 37 °C the activity of the enzyme was stable for about 40 minutes, increasing to 45 - 50 minutes at room temperature (20 to 25 °C) but decreasing to about 20 minutes when stored at 0 °C. After these time intervals had elapsed, the activity of the dehydratase slowly declined. As a consequence, considerable care was taken during the course of an experiment to monitor the

activity of the iron-activated enzyme and to compensate for any losses of activity.

2.6.5. Definition of a Unit of L-Serine Dehydratase Activity

One unit of L-serine dehydratase activity is defined as the quantity of enzyme which catalyses the formation of 1 μmole of pyruvate, at a linear rate, in 1 minute at 37 ^{o}C.

2.7 Protein Estimation

Protein concentration was routinely measured by one of two methods. The A_{280}/A_{260} procedure of Warburg and Christian (1942) was used for protein concentrations of less than 5 mg ml^{-1}. At higher concentrations, and when compounds were present which absorb in the 260 to 280 nm region - such as nucleic acids or pyruvate - the protein concentration was measured by the Biuret method of Gornall *et al* (1949), using bovine serum albumin as the standard protein. Glycylglycine is a dipeptide, and so gives a positive result with the Biuret reagent. Consequently, all assays of protein preparations prepared in glycylglycine buffers were corrected for the absorbance due to this buffer.

2.8 Molecular Weight Determinations

2.8.1 Gel Filtration

The molecular weight of L-serine dehydratase was determined by the method of Andrews (1964). This method involves comparison of the elution volume of the dehydratase from a gel filtration column with those of standard proteins of known molecular weight.

2.8.1.1 Preparation and Calibration of The Gel Filtration Column

Columns of Sephadex G-100 (2.5 cm diameter x 45.0 cm length; bed volume 220 ml) were prepared as recommended by the manufacturer (Pharmacia Fine Chemicals A.B., Milton Keynes, England). The column was equilibrated by passing a minimum of 3 column volumes of the appropriate eluting buffer through the gel bed, at a flow rate of 20 ml hr^{-1}. All gel filtration work was

carried out at 4 ^{o}C.

The Sephadex G-100 column was calibrated by the application of a mixture of the following standard proteins dissolved in 0.5 ml of eluting buffer:

lactate dehydrogenase, M_r132 000, (15 units), detected by the oxidation of NADH at 340 nm in the presence of 15 mM sodium pyruvate;
bovine serum albumin, M_r 66 000, (5 mg), detected by A_{280};
chymotrypsinogen, M_r24 500, (3 mg), detected by A_{280};
cytochrome c, M_r 12 400, (1 mg), detected by A_{412}.

Eluting buffer was passed through the column at a flow rate of 20 ml hr^{-1}; 2 ml fractions were collected and the standard proteins located in them by the methods shown above. The elution volume of each protein was then calculated and plotted against the $\log_{10}$ of the corresponding molecular weight. A typical calibration curve is shown in figure 2.2. This standardisation procedure was repeated for each eluting buffer used. Very little variation was seen between different calibration curves obtained in this way.

2.8.1.2 Molecular Weight Determination

Lactate dehydrogenase, bovine serum albumin and cytochrome c, each at the amounts listed above, were included as internal standards in each molecular weight estimation. A mixture consisting of 0.2 ml of L-serine dehydratase preparation (20 to 30 units) and 0.3 ml of eluting buffer containing the internal standards was applied to the Sephadex G-100 column and eluted with buffer at a flow rate of 20 ml hr^{-1}, using the procedures described above.

L-Serine dehydratase activity was detected by the 2,4-dinitrophenylhydrazine assay. The elution volume of the enzyme was calculated and the molecular weight determined by comparison of this value with the calibration curve obtained using the standard proteins. Corrections were made for any small variation in the position of elution of the internal standard proteins

2.8.2 Sucrose Gradient Centrifugation

The method followed was based upon the procedures described by Martin and Ames (1961).

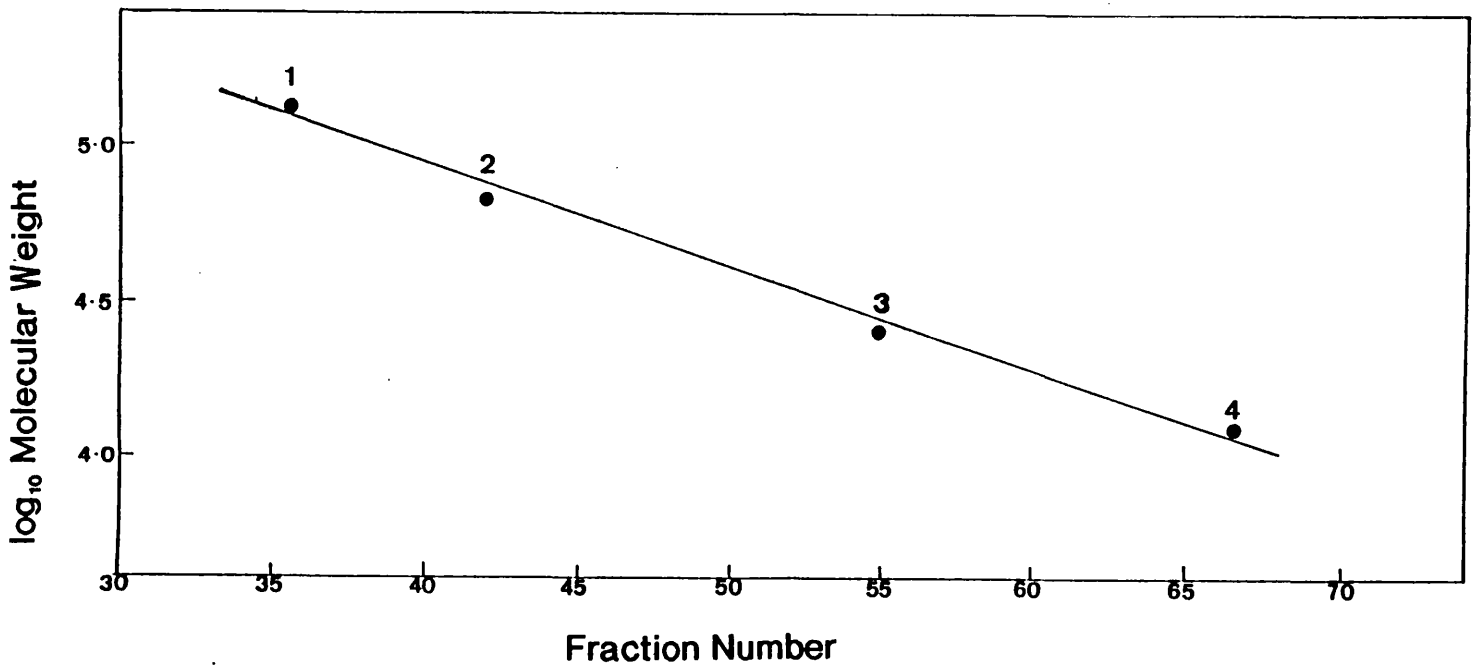


Figure 2.2

The Calibration Curve of a Sephadex G-100 Gel Filtration Column in the Presence of 100 mM D-Serine

A column of Sephadex G-100 (2.5 cm diameter X 45 cm length, bed volume 220 ml) was calibrated using the proteins shown. The column was eluted with 50 mM Tris-HCl, pH 7.8, 100 mM KCl and 100 mM D-serine. The elution volumes of these proteins were calculated and plotted against the corresponding $\log_{10}$ (molecular weight).

1 Lactate Dehydrogenase (132 000)
2 Bovine Serum Albumin (66 000)
3 Chymotrypsinogen (25 000)
4 Cytochrome c (12 400)

2.8.2.1 Preparation of the Gradients

Sucrose gradients were prepared using the freeze-thaw method of Baxter-Gibbard (1972). The 5 to 20 % linear gradients (of 4.2 ml volume) were prepared from a 20 % (w/v) sucrose solution containing 50 mM buffer and 100 mM KCl, and other additions as appropriate. These were frozen at - 70 °C for a minimum of 3 hours. The tubes were thawed at 4 °C to minimize disturbance of the gradients by convection currents and stored for up to one hour before use.

2.8.2.2 Sucrose Gradient Centrifugation

A 0.15 ml sample containing up to 10 units of L-serine dehydratase, 1 mg of lysozyme, and 15 units of lactate dehydrogenase was carefully layered onto the surface of the gradient. The gradients were then centrifuged for 17 hours, at 4 °C in a Beckman SW 50.1 swing-out bucket rotor at 36 000 r.p.m. (78 000*g*), in a Beckman model L5-65 ultracentrifuge. After the centrifugation, fractions (150 µl) were manually removed from the top of the tubes using a P200 Gilson pipette. L-Serine dehydratase activity was assayed by the 2,4-dinitrophenylhydrazine method. Lactate dehydrogenase was assayed by the reduction of NADH at 340 nm using 10 mM sodium pyruvate as substrate, and Lysozyme assayed as protein by the method of Lowry *et al* (1951).

Tangents were drawn to the elution profile of each protein to obtain its elution position. The S_{20W} of the L-serine dehydratase was obtained by comparing its position in the gradient with that of the standard proteins. Lactate dehydrogrenase has a S_{20W} of 7.43 and lysozyme a S_{20W} of 1.95 (Rawitch, 1972).

2.9 Analytical SDS Polyacrylamide Gel Electrophoresis

2.9.1 SDS Polyacrylamide Gel Electrophoresis

Routine sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed using a discontinuous pH 8.9 system, with Tris-glycine buffer, and 0.1 %, SDS (Hames, 1988). A Biorad Protean II vertical minigel system was used for all routine SDS PAGE. The 12.5 % resolving gel (8 x 7-cm x 1 mm) was cast from the following mixture:

4.0 ml of 30 % acrylamide : bis-acrylamide (30 g : 0.8 g)
3.7 ml of 1 M Tris-Cl, pH 8.9
100 µl of 10 % (w/v) SDS
50 µl of 10 % (w/v) ammonium persulphate
10 µl of TEMED
2.1 ml of distilled water

The resolving gel was allowed to polymerize at room temperature for at least 45 minutes, before the addition of a 5 % stacking gel. This had the following composition:

0.65 ml of 30 % acrylamide : bis-acrylamide (30 g : 0.8 g)
0.625 ml of 0.5 M Tris-Cl, pH 6.8
50 µl of 10 % (w/v) SDS
25 µl of 10 % (w/v) ammonium persulphate
5 µl of TEMED
3.65 ml of distilled water

Protein samples were mixed with an equal volume of SDS sample buffer (5.0 ml of 0.5 M Tris-Cl, pH 6.8; 2.0 ml of 10 % (w/v) SDS; 4.0 ml of glycerol; 10 mg of bromophenol blue and 9.0 ml of distilled water) containing a small quantity of DTT, and heated in a boiling water bath for at least 5 minutes. Bio-rad low molecular weight SDS PAGE marker proteins were used as standards (5 µg of the standard mixture per gel track). The electrophoresis was performed at room temperature in Tris-glycine running buffer (0.25 M Tris, 1.92 M glycine and 0.1 % SDS, pH 8.9) using a constant voltage of 150 V, for an average of 1.5 to 2 hours. After electrophoresis, the gels were fixed and stained in 0.05 % (w/v) coomassie blue in an isopropanol/acetic acid/water mixture (25 % : 10 % : 65 %) for a minimum of 3 hours. Destaining was performed in 25 % isopropanol,10 % acetic acid (v/v). Gels were stored in 10 % acetic acid (v/v).

2.9.2 Native Polyacrylamide Gel Electrophoresis

Non-denaturing polyacrylamide electrophoresis was performed using a modification of the pH 8.9 system of Ornstein and Davis (1961).
An 8 cm x 7 cm x 1 mm native gel containing 7.5 % (w/v) polyacrylamide was cast from the following mixture:

3.2 ml of 30 % Acrylamide : bis-acrylamide (30 g : 0.8 g)
1.55 ml of 3 M Tris-Cl, pH 8.2
50 µl of 10 % (w/v) ammonium persulphate
15 µl of TEMED
7.7 ml of distilled water

The gels were cast with a sample well comb in place, and allowed to polymerise at room temperature, before cooling to 4 ^{o}C. The addition of a stacking gel (5 %) afforded no extra resolution, and so was not used.

The protein samples (up to 50 µl, total volume) were mixed with native sample buffer (0.3 ml of glycerol, 0.3 ml of 1 M Tris-Cl, pH 6.8 and 0.4 ml of distilled water), at a ratio of 1 part sample buffer to 3 parts protein solution. Bromophenol Blue (10 µl of a 1 mg ml^{-1} solution mixed with 20µl of distilled water and 10 µl of sample buffer) was used as a tracking dye. Electrophoresis was performed in native Tris-glycine running buffer (0.25 Tris, 1.92 M Glycine, pH 8.2), at 200 V constant voltage, 4 ^{o}C for 2 to 2.5 hours. After electrophoresis, the gels were assayed for L-serine dehydratase activity as described in section 3.4. Protein bands in the gel were detected using the coomassie blue staining procedure described in section 2.9.1.

Attempts were made to locate the position of L-serine dehydratase in native polyacrylamide gels using the activity stain devised by Gannon and Jones (1977). However, although the DNPH assay was able to detect the activity of the dehydratase in gel slices, it did not prove possible to directly visualise the presence of the L-serine dehydratase activity in the intact polyacrylamide gel using this method.

2.9.3 Native Isoelectric Focusing Gel Electrophoresis

Native IEF (isoelectric focusing) gels were run by the method of Robertson *et al* (1987). A vertical minigel system (8 x 7-cm cell format) was used. The gels were cast 1.5 mm thick from the following mixture:

2 ml of 30 % acrylamide : bis-acrylamide (30 g : 0.8 g)
2.4 ml of 50 % (v/v) glycerol
0.6 ml of ampholyte (pH range 3 - 10)
50 µl of 10 % (w/v) ammonium persulphate

20 µl of TEMED;
7.0 ml of distilled water

The gels were cast with a 10 well comb in place and allowed to polymerise at room temperature for 1 hour.

The cathode solution was 25 mM NaOH and the anode solution 20 mM acetic acid. These solutions were cooled to 4 °C prior to electrophoresis. After polymerization was complete, the comb was removed and the wells rinsed and then filled with the cathode solution. Protein samples were mixed with an equal volume of 60 % (v/v) glycerol and 4 % (v/v) ampholyte (pH range 3 - 10). Electrophoresis was performed at room temperature for 1.5 hours at 200 V constant voltage, then increased to 400 V constant for an additional 1.5 hours. After electrophoresis, the gels were rinsed briefly in 10 % (w/v) TCA (trichloroacetic acid), and then more extensively in 1 % (w/v) TCA, to remove ampholytes. The IEF gels were fixed in 1 % (w/v) TCA for at least 2 hours, before rinsing with distilled water and staining with coomassie as described in section 2.10.1. Protein standards used included trypsin inhibitor, pI 4.55; bovine carbonic anhydrase, pI 5.85; myoglobin, pI 6.76, 7.16; and lactate dehydrogenase, pI 8.3, 8.4, 8.55.

2.10 Materials

2.10.1 Instruments

Continuous Spectrophotometric assays, and readings of absorption were performed on a Unicam SP 1800 Ultraviolet spectrophotometer (Pye, Unicam Ltd.,Cambridge, U.K.) fitted with a Unicam AT 25 linear chart recorder. Where appropriate, the temperature was controlled by circulating water at 37 °C through the cell block. Ultraviolet and visible spectra were recorded in 1 cm path length 0.10 ml quartz microcuvettes on a Pye SP8-100 UV/Visible Spectrometer Pye, Unicam Ltd., Cambridege, U.K.)
Fluorescence spectra were determined using a Baird Atomic Ratio Recording Spectrofluorimeter, and EPR spectra on a Bruker ER 200-D Electron Spin Resonance spectrometer (Bruker Ltd., FRG).

2.10.2 Enzymes

Lactate dehydrogenase (E.C. 1.1.1.27) (Rabbit Muscle, type I), ribonuclease

(E.C. 3.1.27.5) (bovine pancreas), deoxyribonuclease (E.C. 3.1.21.1) (bovine pancreas), catalase (bovine liver), superoxide dismutase (bovine erythrocytes), cytochrome c (horse heart, type IV), lysozyme (chicken egg), bovine serum albumin and IEF protein standards (myoglobin, lactate dehydrogenase, carbonic anhydrase and trypsin inhibitor), from Sigma Chemical Co., Ltd; Low molecular weight SDS PAGE standards from Bio-rad; chymotrypsinogen A from BDH Chemicals Ltd., Poole, Dorset, U.K.

2.10.3 Chemicals

Glycine and ferrous sulphate from Fisons plc, Loughborough, Leicestershire; glycylglycine, L-amino acids, dithiothreitol, NADH (disodium salt, yeast), thiamine HCl, streptomycin sulphate, pyridoxal 5'-phosphate, isonicotinic acid hydrazide, hydroxylamine hydrochloride, phenylhydrazine hydrochloride, semicarbazide hydrochloride, TEMED, Pentyl Agarose, and Reactive Green Agarose from Sigma; Sephadex, Mono Q and Phenyl Superose from Pharmacia Fine Chemicals AB, Uppsala, Sweden; DEAE cellulose (type 52, microgranular) from Whatman Chemical Separation Ltd., Maidstone, Kent, England; sodium dodecyl sulphate, acrylamide and bis-acrylamide from Serva Fine Biochemicals ; and $^{59}FeSO_4$ from NEN, Du Pont (U.K.) Ltd., NEN Research Products, Stevenage, Hertfordshire.

Other chemicals were obtained from the usual sources, principally BDH, Sigma and Fisons and were of AnalaR quality where available.

CHAPTER 3 - THE PURIFICATION OF L-SERINE DEHYDRATASE

Chapter 3

The Purification of L-Serine Dehydratase

One of the most notable properties of many of the microbial L-serine-specific dehydratases is the instability of their activity in cell-free extracts, a feature which has proved to be a serious barrier to the purification and study of these enzymes. Until the present study only two L-serine-specific dehydratases had been purified to homogeneity or near-homogeneity. These were the L-serine dehydratases from *Arthrobacter globiformis* (Gannon *et al*, 1977) and *Clostridium acidiurici* (Carter and Sagers, 1972). The L-serine dehydratase from *E. coli* is particularly unstable, both to time and dilution. This instability prevented Alfoldi *et al* (1968), and Newman and Kapoor (1980) from undertaking the purification of the dehydratase from *E. coli* K12, and limited Roberts (1983) to only a slight purification of the enzyme from *E. coli* B.

Later work by Newman *et al*, (1985) showed that the stability of the L-serine dehydratase from *E. coli* K12 was greater when prepared in glycylglycine buffer, compared with enzyme prepared in either tris or potassium phosphate buffers. They also showed that the dehydratase underwent a substantial increase in activity when incubated with ferrous iron and dithiothreitol . These observations prompted a re-examination of the stability of the *E. coli* B L-serine dehydratase, the results of which are presented in section 3.1. It became clear that the dehydratase was not as unstable as previously found (Roberts, 1983), and that much of the apparently lost activity of this enzyme could be restored by its preincubation with ferrous iron and dithiothreitol. This discovery was used in the development of a protocol for the reactivation of the dehydratase which, along with the introduction of new, faster methods of protein isolation, most notably FPLC, allowed the purification of the *E. coli* L-serine dehydratase to be successfully undertaken.

3.1 The Stabilisation of L-Serine Dehydratase in Crude Extracts

3.1.1 The Stability of L-Serine Dehydratase in Cell-free Extracts of *E. coli* B

Before work began on developing an isolation scheme, experiments were undertaken to re-examine the stability of L-serine dehydratase and to discover conditions which would protect the activity of the cell-free enzyme. Figure 3.1 shows that when a crude extract of *E. coli* was diluted into either 50 mM Tris-HCl, pH 8.0, or 50 mM glycylglycine-NaOH, pH 8.0, incubated at 37 °C and then assayed in the absence of iron and DTT, the activity of the dehydratase decayed at

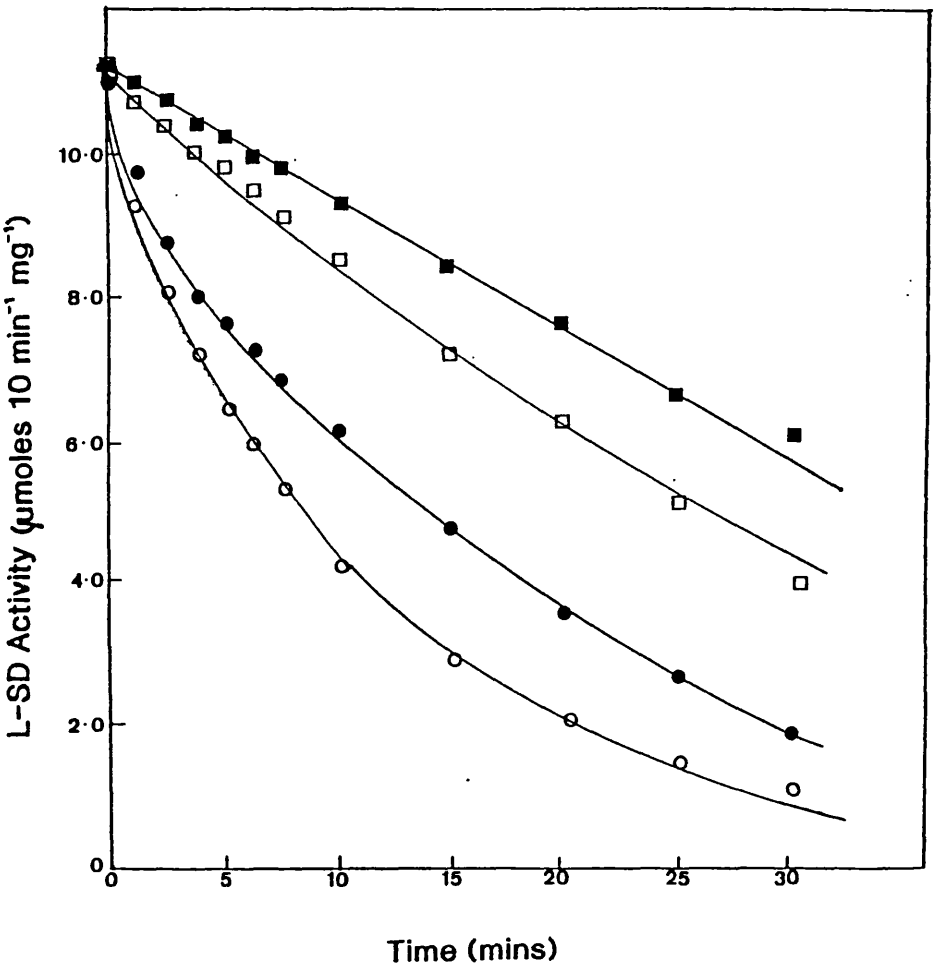


Figure 3.1

The Decay of L-Serine Dehydratase Activity in Diluted Cell-free Extracts

An extract of *E. coli* B made in 50 mM glycylglycine-NaOH buffer, pH 8.0 was diluted into 50 mM glycylglycine-NaOH, pH 8.0 to the protein concentrations indicated below, and incubated at 37 °C. At the times shown, samples were removed and assayed in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM L-isoleucine and 1 mM hydroxylamine. Pyruvate formed during the asssays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1. The resulting timecourses were then used to calculate the half-life of activity ($t_{1/2}$) at each of the protein concentrations shown.

		$t_{1/2}$ (minutes)
■ ■ ■ ■	20 mg of protein ml^{-1}	31.7
□ □ □ □	10 mg of protein ml^{-1}	22.5
● ● ● ●	3.0 mg of protein ml^{-1}	10.45
○ ○ ○ ○	1.0 mg of protein ml^{-1}	6.5

a rate, and to a final level, that was dependent upon the protein concentration. At a protein concentration of 1 mg ml^{-1}, the dehydratase lost over 85 % of its starting activity during the course of the 30 minute incubation ($t_{1/2}$ = 6.5 mins). As the protein concentration of the preincubation mixtures was increased, correspondingly less L-serine dehydratase activity was lost, and enzyme incubated in an extract containing protein at a concentration of 20 mg ml^{-1} lost only 40 % of its initial activity over the same time interval ($t_{1/2}$ = 31.7 minutes). These observations are consistent with the results obtained by Roberts (1983) during an earlier study of the *E. coli* B L-serine dehydratase. They show that, although the dehydratase was more stable at higher protein concentrations, further stabilisation was necessary before this enzyme could be purified.

3.1.2 The Stabilisation of L-Serine Dehydratase Activity

The ability of various compounds to stabilise the activity of L-serine dehydratase was assessed by their inclusion in dilutions of cell-free extracts. Their effect on the decay of activity was measured by taking samples for assay in media lacking iron and DTT over a 60 minute period, and comparing the rate of decay with that of a control containing diluted cell-free enzyme only (Figure 3.2). It can be seen that incubation of the dehydratase with ferrous iron and dithiothreitol resulted in both the stabilisation and activation of the enzyme (a phenomenon that is discussed in detail in Chapter 6). The inclusion of additional agents into preincubation media containing iron and dithiothreitol, such as glycine or D-serine and glycerol, did not substantially increase the stability of the dehydratase. Ferrous iron or dithiothreitol alone were unable to afford any significant stabilisation, although 50 mM D-serine and 15 % glycerol were able to provide some protection against inactivation, a finding made earlier by Roberts (1983).

Although $FeSO_4$ and dithiothreitol were good agents for the protection of the activity of the cell-free dehydratase, under the slightly alkaline conditions in which they were used (pH 8.0, the optimum pH of L-serine dehydratase for both activity and stability) a tendency was shown to form a precipitate.
This presented serious obstacles to the performance of column chromatography, particularly FPLC work, and so a search was made for a buffer which would maintain the iron in a soluble form, without adversely affecting the stability of the dehydratase. A number of buffers were examined (Table 3.1). It was found that, although tricine and bicine buffers were able to prevent the formation of an iron precipitate, the activity of L-serine dehydratase was considerably reduced in the presence of these buffers. When the diluted enzyme was preincubated with

Figure 3.2

The Stabilisation of L-Serine Dehydratase Activity in Diluted Cell-free Extracts of *E. coli* B

The experimental conditions are the same as those in the legend to Figure 3.1, except that the dilution of the extract was to 1 mg of protein ml^{-1}. All incubation media contained 50 mM glycylglycine-NaOH buffer, pH 8.0 in addition to the reagents shown below.

● ● ● ●	10 mM DTT, 1 mM $FeSO_4$, 50 mM D-serine and 15 % glycerol
■ ■ ■ ■	10 mM DTT and 1 mM $FeSO_4$
▲ ▲ ▲ ▲	10 mM DTT, 1 mM $FeSO_4$ and 150 mM glycine
o o o o	50 mM D-Serine and 15 % glycerol
□ □ □ □	10 mM DTT
△ △ △ △	Extract only
◇◇◇◇	1 mM $FeSO_4$

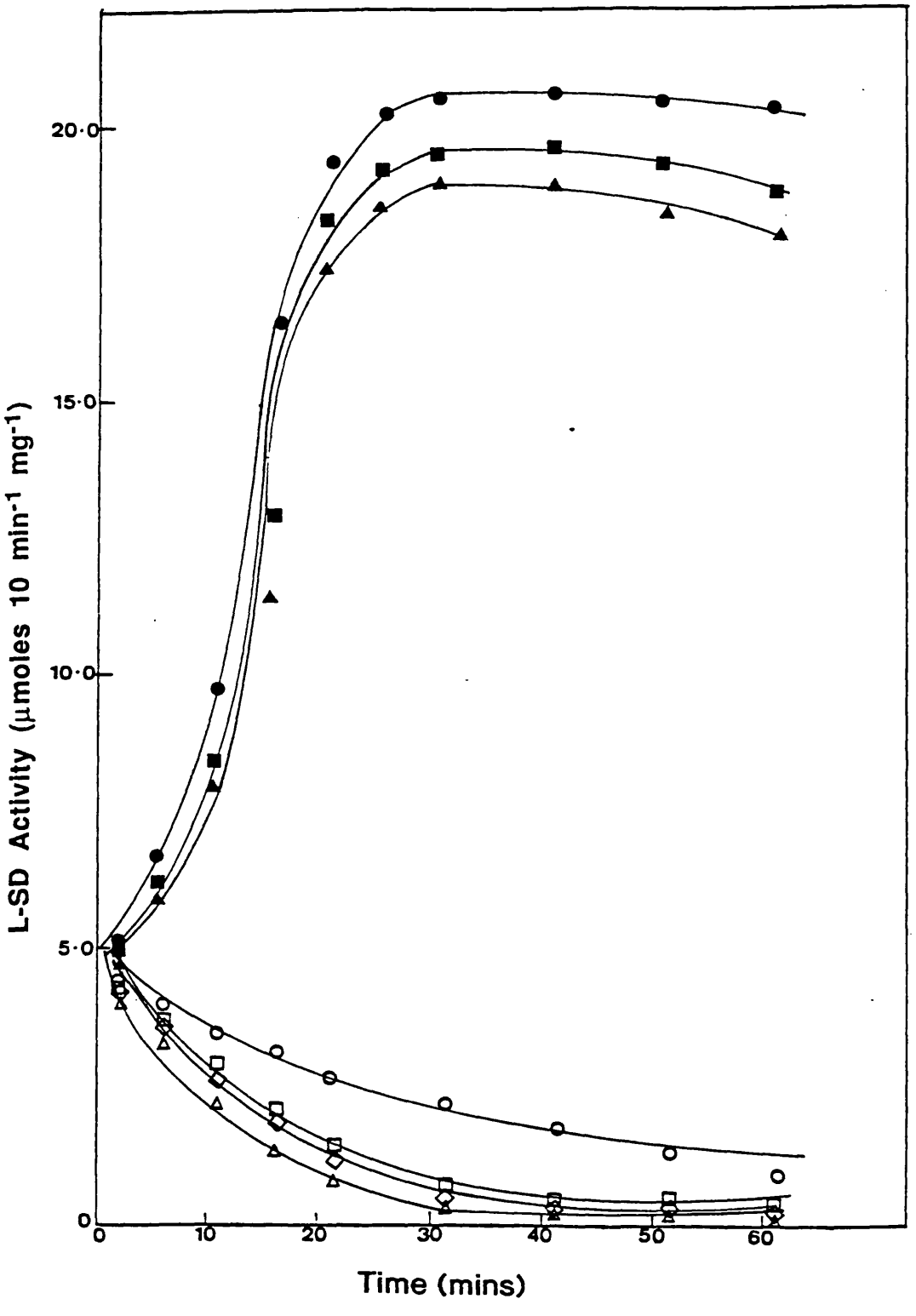

20·0
15·0
10·0
5·0
0
0
10
20
30
40
50
60
L-SD Activity (μmoles 10 min⁻¹ mg⁻¹)
Time (mins)

Table 3.1

The Effect of Different Buffers on the Levels of Activity and Iron-activation of L-Serine Dehydratase

An extract of *E. coli* B was prepared in 50 mM glycylglycine-NaOH buffer, pH 8.0 containing 2 mM DTT, as described in Chapter 2, section 2.4. Samples of the extract (0.1 units) were then assayed in 1 ml mixtures containing 50 mM L-serine, pH 8.0 10 mM L-isoleucine, 1 mM hydroxylamine and the buffers shown in the table below (all at 50 mM final concentration and pH 8.0). Assays in the buffers shown below were also performed in the presence of 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during both sets of assays was measured by the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

Buffer	**L-Serine Dehydratase Activity (μmoles pyruvate $10\ min^{-1}\ mg^{-1}$) X 10 in:**		**Activation by Fe^{+2} plus DTT**
	Buffer only	**Buffer + Fe^{2+} + DTT**	
Glycylglycine	0.205	0.75	3.7
Bicine	0.20	0.50	2.5
Tricine	0.20	0.40	2.0
EPPS	0.15	0.45	3.0
HEPES	0.20	0.60	3.0
TES	0.155	0.37	2.4
TAPS	0.17	0.46	3.2
Bis-Tris-propane	0.20	0.64	3.2
Tris-HCl	0.20	0.68	3.4

50 mM bicine pH 8.0, or 50 mM tricine, pH 8.0, in the manner shown in Figure 3.1, it was found to be less stable than dehydratase similarly incubated with tris or glycylglycine buffer. A number of other experiments also showed that inclusion of 150 mM glycine, pH 8.0 in glycylglycine buffer containing 10 mM DTT and 1 mM $FeSO_4$ could also prevent the formation of an insoluble precipitate. Table 3.1 also shows that activation by Fe^{2+} and DTT was not restricted to bicine, tricine and glycylglycine buffers: to varying degrees it was also seen with tris, bis-tris, bis-tris propane, HEPES, EPPS, TAPS and TES buffers.

Although conditions were found which allowed the iron to be maintained in a soluble form, preliminary work on the early stages of the purification scheme subsequently revealed that it was not necessary to include $FeSO_4$ and dithiothreitol in the extraction and elution buffers. Although the cell-free enzyme appeared to be very unstable when prepared in buffers lacking these factors, a large proportion of the enzyme activity 'lost' during storage, DEAE cellulose purification or other procedures, could be recovered when the dehydratase was re-activated by assay, or preincubation, with ferrous iron and DTT. For example, in the experiment described in Table 3.2, L-serine dehydratase was stored at -20 °C as an ammonium sulphate fraction for up to thirteen days. Over this period, the activity of the dehydratase assayed in media lacking iron and DTT appeared to undergo a progressive decay such that, by the end of the thirteenth day of storage, only 1 % of the initial activity still remained. However, if the dehydratase was assayed or preincubated with ferrous iron or DTT, nearly 90 % of this 'lost' activity could be recovered.

The instability of the *E. coli* L-serine dehydratase is a consequence of two distinquishable, and possibly inter-related, processes. The first involves the loss of the native, or Fe-independent, activity of the dehydratase (defined, along with other related terms in Chapter 4, section 4.1). This form of inactivation is most clearly demonstrated by the progressive loss of catalytic activity which occurs during the early stages of the purification of the dehydratase, or by treatment of Fe-independent enzyme with metal chelators such as EDTA or *o*-phenanthroline (Chapter 6, section 6.5). Inactivation from either of these causes is reversible by incubation with Fe^{2+} and DTT. The dehydratase is also subject to a second form of inactivation that cannot be reversed by treatment with iron and dithiothreitol. This may represent a loss of tertiary structure, which suggests that the dehydratase is also structurally unstable. Conditions encountered during the purification which cause an irreversible inactivation include extended exposure times to temperatures above 20 °C, and prolonged periods in a dilute state or on a chromatography column. It is this second form of irreversible inactivation,

Table 3.2

The Stability of L-Serine Dehydratase as an Ammonium Sulphate Fraction

L-Serine dehydratase (step 3) was stored at - 20 °C for 13 days. At the intervals indicated in the table, samples were thawed and assayed in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM L-isoleucine, 1 mM hydroxylamine with, and without, additions of 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method of estimation (Chapter 2, section 2.6.1).

* The enzyme sample from day 13 was also preincubated with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$, as described in section 2.6.4, prior to being assayed for L-serine dehydratase activity as already shown.

Length of Storage (days)	**% Activity Remaining When Assayed in:**	
	Buffer only	**Buffer + Fe^{2+} + DTT**
0	100	100
1	51	96
6	15	89
13	1	70
* 13	85	89

which is a result of the structural instability of the enzyme, rather than the reversible inactivation caused by the loss of Fe-independent activity, that has proved to be the factor presenting the most serious obstacle to the development of a purification scheme for the dehydratase during the present study.

In contrast to the findings of Newman *et al*, (1985), it was found that glycylglycine did not significantly preserve the initial, Fe-independent activity of L-serine dehydratase. It was found that the half-life of the Fe-independent activity of dehydratase prepared in glycylglycine was shorter than that of enzyme extracted in tris (24 hours as opposed to 32 hours at 4 ^{o}C). A similar phenomenon was observed when iron-activated purified dehydratase was extensively diluted into glycylglycine buffer in the absence of a stabilising agent, such as L-serine. Glycylglycine is able to chelate metals, and it may be that the instability of the Fe-independent enzyme in this buffer is due to the chelation and extraction of an essential cofactor from the dehydratase, possibly iron. For this reason many of the metal chelator studies described in Chapter 6, section 6.3 were performed in tris buffer. Despite this, the L-serine dehydratase protein was intrinsically more stable when prepared in glycylglycine buffer, particularly to long term storage and column separations. Recoveries of purified enzyme were always considerably higher when glycylglycine was the choice of purification buffer.

The isolation scheme described below was developed using protein purified in glycylglycine only. Dithiothreitol was included into the extraction and purification buffer after the completion of a series of experiments which showed that DTT could protect against inhibition by certain sulphydryl modifying reagents (Chapter 4, sections 4.3 and 4.4). Its inclusion produced an average increase in the final yield of purified enzyme of up to 50 %. The optimal DTT concentration for protection of the dehydratase was determined empirically to be 2 mM. Higher concentrations of DTT afforded no extra stabilisation.

3.2 Optimisation of the Production of L-Serine Dehydratase Activity

In order to obtain the largest amounts of purified L-serine dehydratase possible, a number of experiments were performed to increase the starting levels of this enzyme. These involved a search for a strain of *E. coli* which produced L-serine dehydratase in high quantities, and an examination of the growth conditions under which the enzyme was formed.

3.2.1 Choice of Organism

The first efforts to obtain a richer source of the dehydratase involved screening some of the many strains of *E. coli* for enhanced L-serine dehydratase synthesis. This was achieved by monitoring the levels of dehydratase formed during growth on Alfoldi's medium, using the protocol described in the legend to Figure 3.3. Of the strains of *E. coli* examined (W, C, Crookes, K12 and B) extracts made from *E. coli* B contained the largest amounts of L-serine dehydratase and also had the highest ratio of L-serine to L-threonine-deaminating activities. For instance, at maximal induction the L-serine dehydratase from *E. coli* K12 had a specific activity of less than one-quarter of the maximal value attained by the B enzyme (0.47 μmole of pyruvate min^{-1} mg^{-1} protein as opposed to 2.07 μmole of pyruvate min^{-1} mg^{-1} protein). Most of the early work on the *E. coli* L-serine dehydratase was performed on the enzyme from *E. coli* K12, and so an additional set of studies, similar to those described in section 3.1, was undertaken to compare the stability of this dehydratase with the corresponding enzyme from *E. coli* B. These experiments showed that the K12 dehydratase was unable to retain its Fe-independent activity for as long as the corresponding *E. coli* B enzyme and was also much less stable to storage. Accordingly, *E. coli* B was chosen as the source of starting material for the purification of L-serine dehydratase.

The purification described in section 3.3 was initially developed using L-serine dehydratase obtained from cultures of wild-type *E. coli* B. Experiments were later undertaken to obtain variants of this strain of *E. coli* which produced L-serine dehydratase in higher quantities. This involved selecting for cells of *E. coli* B which were able to grow on solid minimal medium lacking ammonium sulphate, in which L-serine replaced ammonium sulphate as the nitrogen source. It was hoped that by starving the cells of nitrogen in this way, and by supplying L-serine as an alternative source, the bacteria would be forced to induce L-serine dehydratase production. Colonies growing quickly on such plates might contain elevated levels of the dehydratase. A considerable number of these variants were isolated and assessed for L-serine dehydratase activity by following the timecourse of induction of the enzyme during growth on the medium of Davis and Mingioli, using the method described in the legend to Figure 3.4. The *E. coli* variants were found to contain only modestly increased amounts of dehydratase, on average 1.5 - 2 times the maximal activity obtainable from the parental strain. The best of these (variant S53) was used as the source of material for the work described in this thesis. A series of studies showed that the L-serine dehydratase from variant *E. coli* B/S53 displayed no noticeable

differences in its catalytic properties and cofactor requirements from those shown by the enzyme from the parental strain.

3.2.2 Optimisation of Growth Conditions

A series of experiments was performed to discover the optimal growth conditions for the production of L-serine dehydratase. These studies were performed before the purification scheme had been developed to completion and used wild-type *E. coli* B as the source of L-serine dehydratase. However, the more important of the experiments described below were also performed on variant S53 and the results obtained showed no obvious changes in either the pattern of induction of L-serine dehydratase or the growth conditions required for its production. Roberts (1983) had shown that the component of Alfoldi's medium responsible for the induction of dehydratase was tryptone. Roberts further demonstrated that the active constituents of this casein hydrolysate were glycine, L-leucine, L-valine, L-tryptophan, L-alanine, L-glutamine and L-methionine. The effectiveness with which this cocktail of amino acids induced L-serine dehydratase was assessed by the addition of this mixture (plus 0.2 % glucose and 1 $\mu g.ml^{-1}$ of thiamine) to a late logarithmic/early stationary phase culture of *E. coli* B grown in Davis and Mingioli medium (Table 3.3). Although this cocktail induced L-serine dehydratase of high specific activity, cells grew slowly on culture media containing these amino acids, which resulted in a low total activity (per ml of culture). An examination was made of the efficiency of enzyme induction in cells grown with tryptone only and with tryptone with various combinations of components previously shown to have been inducers of the enzyme, such as yeast extract or glycine. Roberts (1983) had shown that the induction of L-serine dehydratase was subject to catabolite repression, and so the effects of substituting the glucose with other carbon growth supplements was also investigated.

The results (Table 3.3) show that tryptone alone was insufficient to produce high yields of enzyme. Considerably more dehydratase was produced, and of a greater specific activity, when 0.2 % glucose was added to the tryptone. The addition of yeast extract further increased the levels of enzyme induced by the tryptone and glucose. The substitution of glycerol for glucose as growth supplement did not lead to an elevation in the level of enzyme produced, and actually resulted in a reduction of both specific and total activity. The substitution of 2 % casein hydrolysate for the 1 % tryptone resulted in the production of enzyme low in both quantity and quality. The largest production of dehydratase activity was obtained using a combination of tryptone, yeast extract, glycine and glucose

Table 3.3

The Induction of L-Serine Dehydratase Activity

Flasks (700 ml) of the medium of Davis and Mingioli were supplemented with 0.2 % glucose and thiamine (1 μg ml^{-1}) and inoculated with an aseptically harvested and washed suspension of *E. coli* B to an initial cell density of 0.2 mg dry wt. of cells ml^{-1}. The cultures were then shaken at 37 °C until bacterial growth was decreasing. At this point the additions of the inducer and carbon source shown in the table were made, and the cultures allowed to continue to grow. Samples were periodically withdrawn for measurement of cell density, and then harvested and extracted as described in Chapter 2, section 2.4. L-Serine dehydratase activity was measured in assay mixtures containing 100 mM glycylglycine-NaOH buffer, pH 8.0, 50 mM L-serine, 10 mM L-isoleucine, 1 mM hydroxylamine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method of estimation, as described in Chapter 2, section 2.6.1.

Key:

YE	Yeast Extract
Trypt	Tryptone

Inducer	Carbon Source	Maximal Specific Activity (μmoles pyruvate min^{-1} mg protein)	Maximal Total Activity (μmoles pyruvate min^{-1} ml $culture^{-1}$)
Alfoldi's Medium	0.2 % Glucose	2.06	2.98
1 % Trypt	None	1.09	1.27
2 % Trypt	None	0.76	1.09
1 % Trypt	0.2 % Glucose	1.24	2.16
1 % Trypt 0.1 % YE	0.2 % Glucose	1.45	2.98
1 % Trypt 0.2 % YE	0.2 % Glucose	2.06	4.05
1 % Trypt 0.2 % YE	0.2 % Glycerol	1.90	3.60
1 % Trypt 0.2 % YE	0.2 % Glycine	1.72	2.86
1 % Trypt 0.2 % YE 0.1 % Gly	0.2 % Glucose	1.98	4.14
1 % Trypt 0.2 % YE 0.1 % Gly	0.2 % Glycerol	1.80	3.26
2 % Casein Hydrolysate 0.2 % YE	0.2 % Glucose	0.96	1.26
0.1 % L-Leu, Gly, L-Ala, L- Met, L-Trp, L-Gln	0.2 % Glucose	1.76	1.49

(1 % : 0.2 % : 0.1 %: 0.2 %). Accordingly, this combination was used for the routine induction of L-serine dehydratase in cells of *E. coli* grown on the medium of Davis and Mingioli.

3.2.3 Cell Growth and the Conditions under which L-Serine Dehydratase is Formed

Roberts (1983) showed that the production of L-serine dehydratase activity by cells of *E. coli* B grown on Alfoldi's medium occurred at a specific stage of the growth cycle. Maximal induction of the enzyme occurred towards the end of the exponential phase of growth. Roberts demonstrated that L-serine dehydratase activity was not stable once induced, and that the enzyme in cells maintained in the stationary phase underwent an apparently irreversible decline in activity. She suggested that this fall in activity was due to the dehydratase being 'destroyed' in some manner. To investigate these observations further, and to ensure that the starting levels of L-serine dehydratase activity were as high as possible, a number of experiments were performed to determine during which phase of the growth cycle this enzyme was induced.
The timecourse of the induction of L-serine dehydratase from cells of *E. coli* B grown on Alfoldi's medium is shown in Figure 3.3. This shows that the specific activity of the dehydratase increased during exponential growth, with the peak of enzyme induction occurring towards the end of the logarithmic growth phase. This period of maximal enzyme production was maintained for only a limited length of time. Maintenance of the cells in the stationary phase resulted in a rapid loss of activity that was only partially reversible by Fe^{2+} plus DTT. This decline in specific activity was mirrored in the total activity of the culture, indicating that the fall in activity was not due simply to a diluting-out effect. Figure 3.3 also shows that the extent of the iron-DTT activation of the dehydratase was dependent upon the age of cells from which the enzyme was extracted. Very little activation was seen during logarithmic growth, but at least part of the loss of activity occurring during the stationary phase was due to the conversion of the dehydratase into an inactive form, which required iron and dithiothreitol for activity.

A similar pattern was observed in the timecourse of induction of L-serine dehydratase activity when *E. coli* was grown on the suitably supplemented medium of Davis and Mingioli (1950) (section 2.2.2). In these experiments, growth was allowed to occur in two stages. In the first stage, *E. coli* was grown to stationary phase on minimal medium supplemented with glucose and thiamine; no L-serine dehydratase was induced during this stage. In the second stage

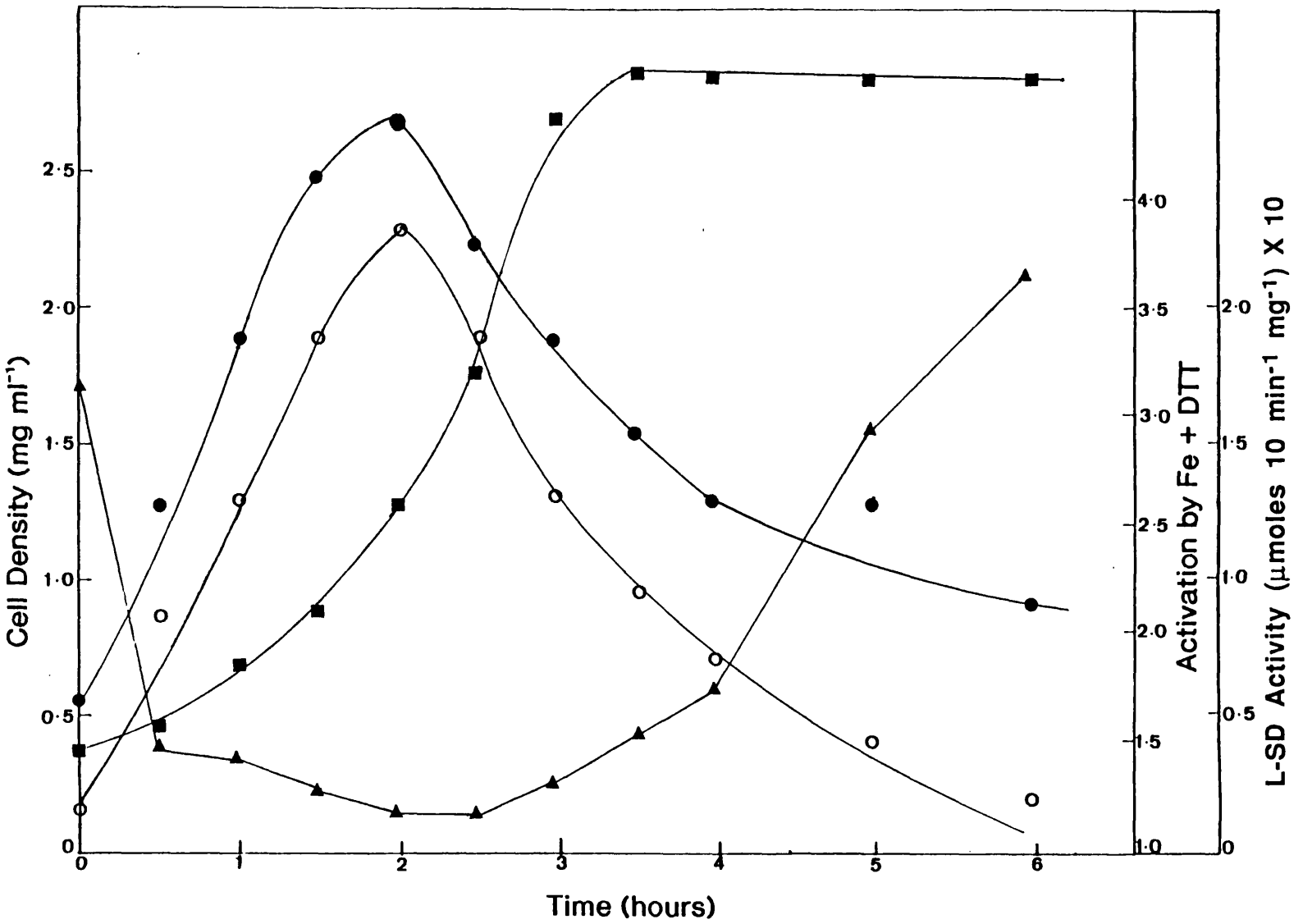
Figure 3.3

The Timecourse of Induction of L-Serine Dehydratase Activity on Alfoldi's Medium + 0.2 % Glucose

A 750 ml flask of Alfoldi's medium supplemented with 0.2 % glucose was inoculated with an aseptically harvested and washed suspension of *E. coli* B to and initial cell density of 0.4 mg dry wt. of cells ml^{-1}. The culture was then shaken at 37 °C for six hours. At the times shown in the figure, samples of the culture were removed, measured for cell density and then harvested and extracted as described in Chapter 2, section 2.4. The extracts were then assayed for L-serine dehydratase in assay mixtures containing 100 mM glycylglycine-NaOH buffer, pH 8.0, 50 mM L-serine, 10 mM L-isoleucine, 1 mM hydroxylamine with, and without, additions of 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

■ ■ ■ ■	A	Cell density (mg dry wt. of cell ml^{-1})
● ● ● ●	B	L-Serine dehydratase activity assayed with $FeSO_4$ and DTT (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10
o o o o	C	L-Serine dehydratase activity assayed without $FeSO_4$ and DTT (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10
▲ ▲ ▲ ▲	D	Degree of activation by $FeSO_4$ and DTT (B/C)

Note: The initial degree of iron-activation of L-serine dehydratase is dependent upon the physical age of the culture used as innoculum, and therefore varied somewhat from experiment to experiment.


Cell Density (mg ml⁻¹)
Activation by Fe + DTT
L-SD Activity (μmoles 10 min⁻¹ mg⁻¹) X 10
Time (hours)
0
0·5
1·0
1·5
2·0
2·5
3·0
3·5
4·0
1
2
3
4
5
6

further cell growth and the induction of L-serine dehydratase activity were achieved by the addition of glucose, glycine, yeast extract and tryptone at concentrations of 0.2 %, 0.1 %, 0.2 %, and 1.0 %, respectively. Figures 3.4 and 3.5 show the timecourse of induction of L-serine dehydratase under these conditions in cells of *E. coli* B grown by flask culture and by fermenter culture. Maximal production of the enzyme occurred only after the addition of the inducers, when the second phase of exponential growth was beginning to decline, at which point the cells were harvested.

These results suggest that the fall in L-serine dehydratase levels of stationary phase cultures is the result of at least two processes, one of which is reversible, and may involve the loss of an essential cofactor (possibly iron) and another which is irreversible, and may involve denaturation or degradation of the enzyme.

3.2.4 Further Optimisation of L-Serine Dehydratase Production

A number of further experiments were undertaken to increase the levels of the deydratase. The first of these included several 'Fed-batch' experiments, in which wild-type *E. coli* B and variant S53 were cultured under conditions of nitrogen limitation, with L-serine supplied as N source. These attempts did not meet with success; the levels of enzyme induced were lower than those achievable using the standard growth conditions for the production of this enzyme.

The second involved production of the dehydratase under conditions of heat stress. Newman *et al*, (1984) have stated that the *E. coli* L-serine dehydratase is induced by growth at 42 °C. This possibility was examined by growing cells of wild type *E. coli* B and variant S53 at 42 °C, on Davis and Mingioli media, as described in the legend to Figure 3.4. In contrast to the findings of Newman and co-workers, no production of L-serine dehydratase was observed during the first phase of growth at 42 °C. Enzyme was formed during the second growth phase, however, although the amount produced at maximal induction was about 25 % lower than that obtained at the usual growth temperature of 37 °C.

3.3 Purification Scheme for L-Serine Dehydratase

During the purification of L-serine dehydratase considerable care was taken to ensure that conditions were optimal for the stability of the enzyme. Separation methods were designed to be relatively rapid in nature, and steps were taken to ensure that the dehydratase was kept in a dilute state for the minimal length of

Figure 3.4

The Timecourse of Induction of L-Serine Dehydratase Activity on the Medium of Davis and Mingioli.

(I) Flask Culture

A 750 ml flask of the medium of Davis and Mingioli supplemented with 0.2 % glucose and thiamine (1 μg ml^{-1}) was inoculated with an aseptically harvested and washed suspension of *E. coli* B to an initial cell density of 0.2 mg dry wt. of cells ml^{-1}. The culture was then shaken at 37 °C until bacterial growth was decreasing. At this point (shown in the Figure) further cell growth and the production of L-serine dehydratase activity were induced by the addition of sterile solutions of 1 % tryptone, 0.2 % yeast extract, 0.1 % glycine, and glucose and thiamine at the concentrations stated above. Further growth was allowed to continue, and samples of the culture were removed, measured for cell density and then harvested, extracted and assayed for L-serine dehydratase activity as described in the legend to Figure 3.3.

■ ■ ■ ■	A	Cell density (mg dry wt. of cell ml^{-1})
● ● ● ●	B	L-Serine dehydratase activity assayed with $FeSO_4$ and DTT (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10
o o o o	C	L-Serine dehydratase activity assayed without $FeSO_4$ and DTT (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10
▲ ▲ ▲ ▲	D	Degree of activation by $FeSO_4$ and DTT (B/C)

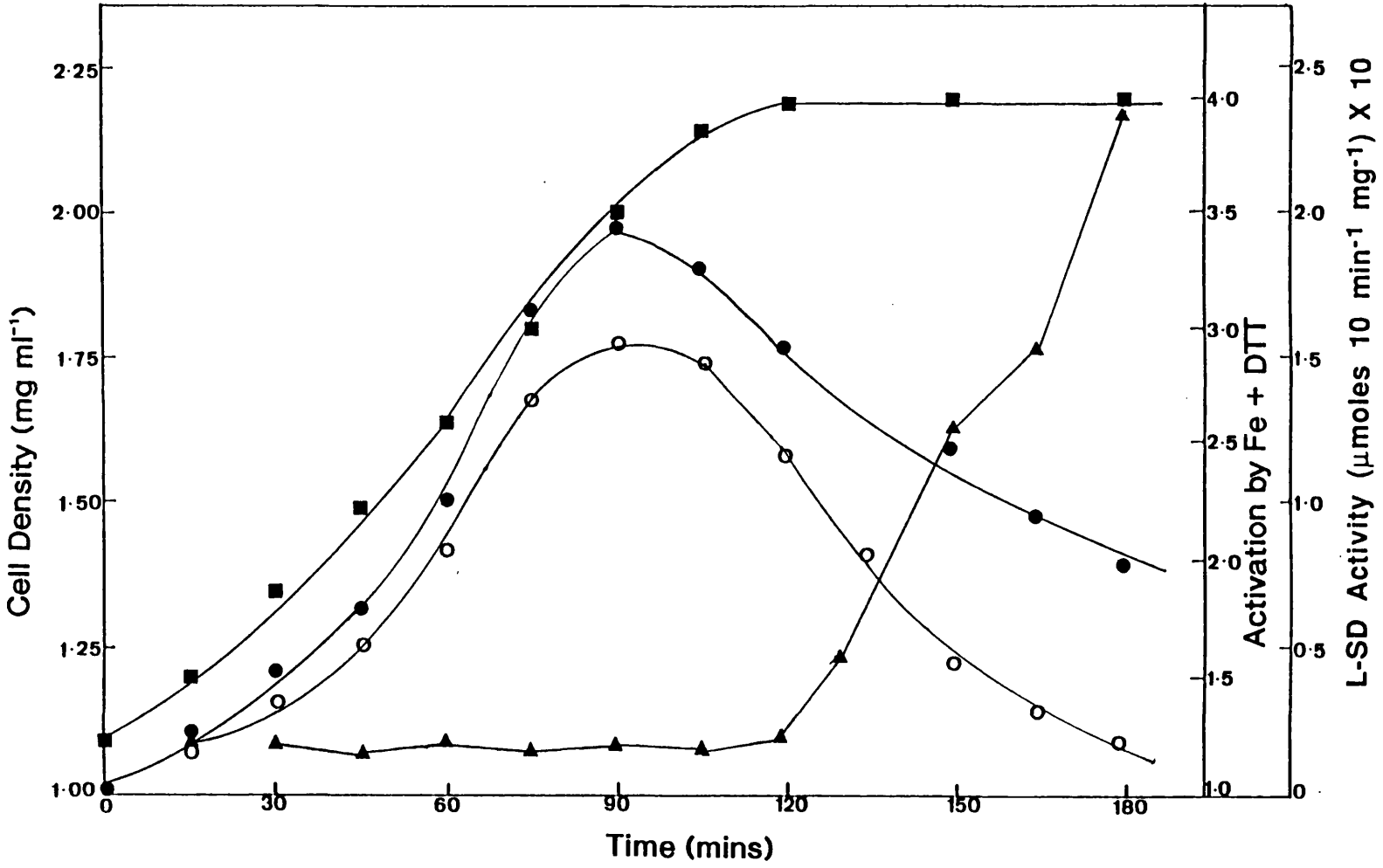

Cell Density (mg ml⁻¹)
2·25
2·00
1·75
1·50
1·25
1·00
Activation by Fe + DTT
4·0
3·5
3·0
2·5
2·0
1·5
1·0
L-SD Activity (μmoles 10 min⁻¹ mg⁻¹) X 10
2·5
2·0
1·5
1·0
0·5
0
0
30
60
90
120
150
180
Time (mins)

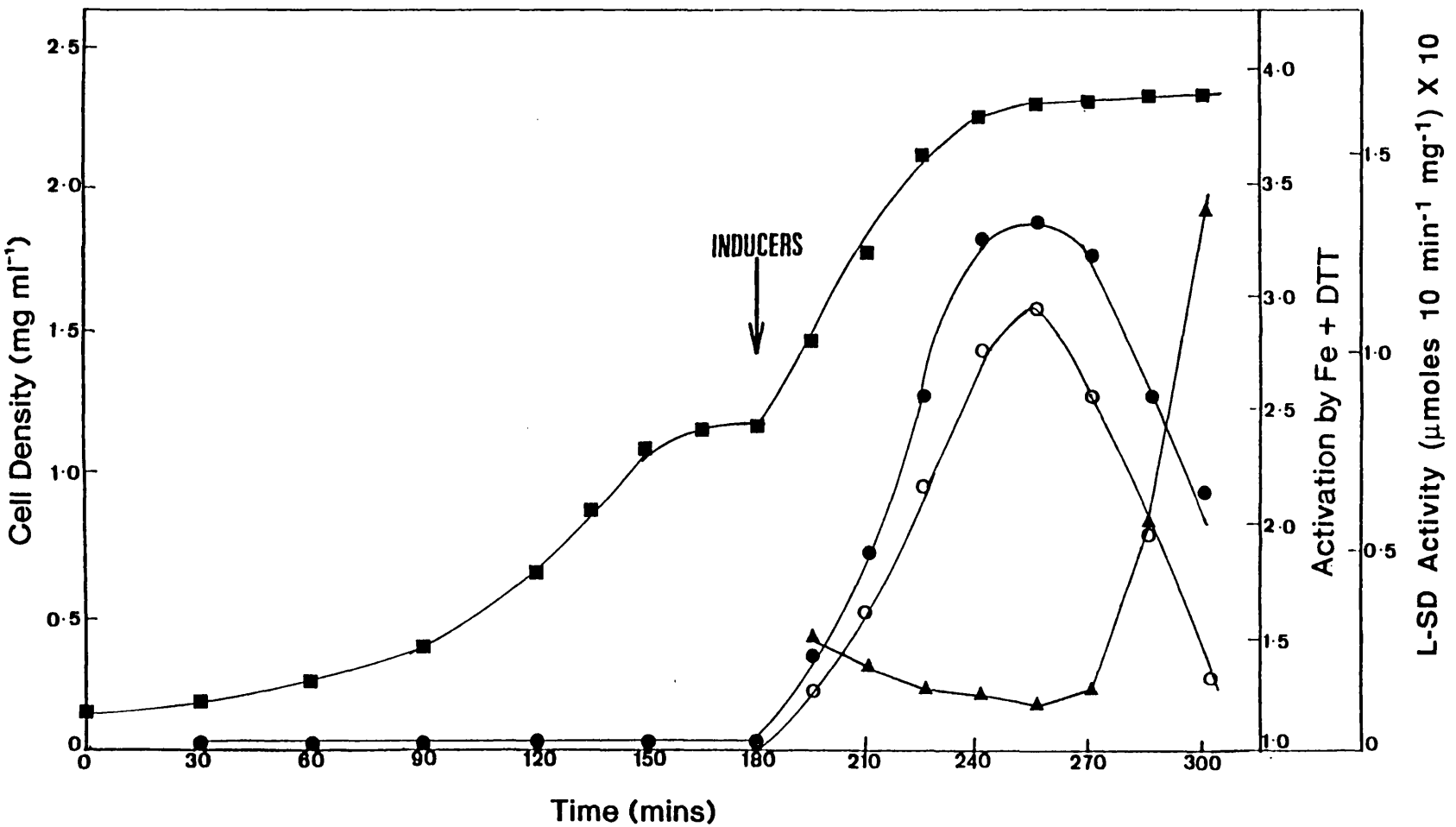
Figure 3.5

The Timecourse of Induction of L-Serine Dehydratase Activity on the Medium of Davis and Mingioli.

(II) Fermenter Culture

Twelve litres of Davis and Mingioli minimal medium supplemented with 0.2 % glucose and thiamine (1 μg ml^{-1}) was inoculated with an aseptically harvested and washed suspension of *E. coli* B to and initial cell density of 0.15 mg dry wt. of cells ml^{-1}. The culture was then allowed to grow as described in Chapter 2, section 2.2.2 until bacterial growth was decreasing. At this point further cell growth and the production of L-serine dehydratase activity were induced by the addition of sterile solutions of 1 % tryptone, 0.2 % yeast extract, 0.1 % glycine, and glucose and thiamine at the concentrations stated above. Further growth was allowed to continue, and samples of the culture were removed, measured for cell density and then harvested, extracted and assayed for L-serine dehydratase activity as described in the legend to Figure 3.3.

■ ■ ■ ■	A	Cell density (mg dry wt. of cell ml^{-1})
● ● ● ●	B	L-Serine dehydratase activity assayed with $FeSO_4$ and DTT (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10
o o o o	C	L-Serine dehydratase activity assayed without $FeSO_4$ and DTT (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10
▲ ▲ ▲ ▲	D	Degree of activation by $FeSO_4$ and DTT (B/C)


Cell Density (mg ml⁻¹)
2·5
2·0
1·5
1·0
0·5
0
INDUCERS
0
30
60
90
120
150
180
210
240
270
300
Time (mins)
4·0
3·5
3·0
2·5
2·0
1·5
1·0
Activation by Fe + DTT
1·5
1·0
0·5
0
L-SD Activity (μmoles 10 min⁻¹ mg⁻¹) X 10

time. Steps 1 to 5, and Step 7 were all performed at 0-4 °C. The separations requiring a FPLC system (steps 6 and 8) had to be performed at room temperature, although efforts were made to minimise heat denaturation of the dehydratase by using chilled buffers, by pumping ice-cold water around cooling jackets specially constructed for each column, and by placing enzyme fractions on ice immediately after elution.

3.3.1 Step 1
Growth and Extraction

E. coli B strain S53 was fermenter-grown in 13 l batches on the minimal medium of Davis and Mingioli (1950) or, occasionally, on the medium of Alfoldi *et al* (1968), as described Chapter 2, section 2.2 2. The bacteria (20 to 25 g of cells dry weight per 13 l of culture, approximately 100 g wet weight) were harvested, washed and a cell-free extract prepared as described in Chapter 2, section 2.4. Although the cells were usually extracted and processed to step 3 on the day of harvesting, it was possible to store the washed cell suspensions at 0-4 °C for up to two days without incurring an irreversible loss of L-serine dehydratase activity.

3.3.2 Step 2
Removal of Nucleic Acids

Immediately after centrifugation, the crude extract from Step 1 was treated with a freshly made 20 % (w/v) solution of neutralised streptomycin sulphate, to give a final concentration of 1.5 % (w/v), and stirred at 4 °C for 15 minutes. This was then followed by centrifugation at 20 000 *g*, as described in section 2.4.4. Although no purification or significant loss of activity occurred at this stage, the removal of RNA and DNA molecules considerably improved the fractionations obtained in the later stages of the purification.

The quantity of streptomycin sulphate used was determined empirically, and represents the amount giving the greatest increase in the A_{280}/A_{260} ratio of a crude extract without causing a precipitation of L-serine dehydratase activity.

3.3.3 Step 3
Ammonium Sulphate Fractionation

Solid ammonium sulphate (331 g l^{-1}) was added slowly with stirring to the supernatant from Step 2, to give a final saturation of 55 %. The pH of the extract

was maintained at 8.0 by the addition of 1 M glyclyglycine-NaOH, pH 9.0. The mixture was then stirred for a further 15 minutes, and then centrifuged at 20 000 *g*, as described in section 2.4.4.

The supernatant contained less than 5 % of the L-serine dehydratase activity and was discarded. The precipitate was dissolved in about 50 ml of buffer A. This contained 90 to 95 % of the activity present after Step 2, and showed a 2 to 3-fold increase in specific activity.

3.3.4 Step 4

DEAE Cellulose Column Chromatography

A column (3.5 cm diameter x 43 cm, bed volume 430 ml) of DEAE cellulose was prepared according to the manufacturer's recommendations, and equilibrated with buffer A (50 mM glycylglycine-NaOH, pH 7.8 containing 2 mM DTT). The re-dissolved pellet from Step 3 was diluted with 600 ml of this buffer in order to lower the concentration of ammonium sulphate carried over from the previous step to below the point at which it would interfere with the binding of the dehydratase to the DEAE cellulose (approximately 100 mM $(NH_4)_2SO_4$). The protein solution was then applied to the column at a flow rate of 250 ml hr^{-1}. The DEAE cellulose column was eluted, first with 150 ml of 150 mM KCl in buffer A, and then with a 1500 ml gradient of 150-700 mM KCl in A, at a flow rate of 120 ml hr^{-1}. A typical elution profile is shown in Figure 3.6.

L-Serine dehydratase activity was eluted over the 300-400 mM KCl range. Fractions (10 ml) containing enzyme purified by a factor of 4 or more, were pooled for further purification. These contained 50 to 60 % of the activity applied to the column, and showed a 6 to 8-fold increase in specific activity.

3.3.5 Step 5

Pentyl Agarose Column Chromatography

Sufficient ammonium sulphate was added to the pooled fractions from Step 4 to give a final concentration of approximately 800 mM. This solution was then loaded at a flow rate of 150 ml hr^{-1} on to a column of pentyl agarose (2.50 cm diameter x 20.5 cm, 100 ml bed volume) equilibrated in 1 M $(NH_4)_2SO_4$ in buffer A. The extract was washed onto the column with 50 ml of the equilibration buffer, followed by elution, first with 50 ml of 700 mM $(NH_4)_2SO_4$ in buffer A, and then by a 500 ml gradient of decreasing ammonium sulphate (700 to 100 mM) in buffer A.

Figure 3.6

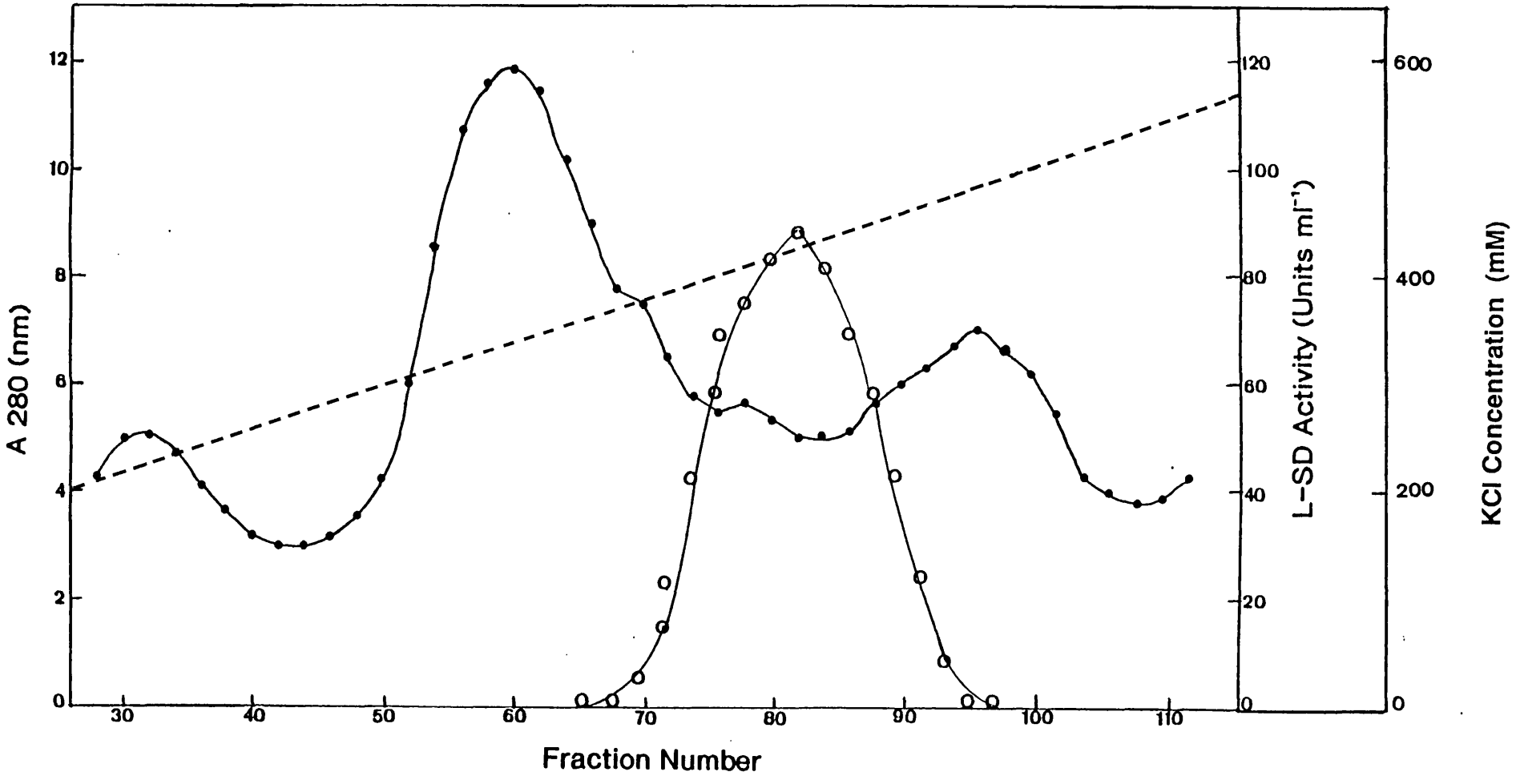
Elution Profile of L-Serine Dehydratase from a Column of DEAE Cellulose (Step 4)

L-Serine dehydratase activity was eluted from a column of DEAE cellulose (3.50 cm diameter, by 43 cm long - bed volume 430 ml) as described in Chapter 3, section 3.2.4. The fractions (10 ml) were eluted at a flow rate of 120 ml hr^{-1}, and assayed for L-serine dehydratase activity in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM DTT, 1 mM hydroxylamine, 10 mM L-isoleucine, and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2, 4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

● ● ● ● A_{280} nm

\- - - - - - - KCl Concentration (mM)

o o o o L-Serine Dehydratase Activity (units ml^{-1})


A 280 (nm)
L-SD Activity (Units ml⁻¹)
KCl Concentration (mM)
Fraction Number
0
2
4
6
8
10
12
0
20
40
60
80
100
120
0
200
400
600
30
40
50
60
70
80
90
100
110

The flow rate was 90 ml hr^{-1}. A typical elution profile is shown in Figure 3.7. L-Serine dehydratase activity was eluted over a 400 to 200 mM $(NH_4)_2SO_4$ range.

Fractions (4 ml) containing enzyme purified by a factor of 3 or more were pooled for concentration (to about 15 ml) by ultrafiltration in a 200 ml Amicon ultrafiltration cell (Amicon Corp., Woking, Surrey, U.K.) using a U.M.5, 5 K cut-off ultrafiltration membrane. This procedure usually resulted in a 10-15 % loss of activity. Overall recoveries from this step, including losses suffered during the ultrafiltration, were usually between 40 and 65 %, with purifications of between 3- and 5-fold.

Despite the losses incurred during the ultrafiltration step, the concentration was necessary for two reasons. Firstly, L-serine dehydratase was unstable in dilute solution: the protein concentration of the pentyl agarose fraction, usually 1.5 mg ml^{-1} or less, was too low to allow even short-term storage of the enzyme without considerable losses of activity (up to 50 % when stored overnight at 4 °C, and 20 to 30 % when stored for the same period at -20 °C). Secondly, the next step in the purification, anion exchange on a FPLC Mono Q column, required a preparation of low ionic strength. Dialysis of the dilute pentyl agarose preparation resulted in an extensive and irreversible inactivation of the enzyme, with losses of 50 % or more of the initial activity. Concentration by ultrafiltration was rapid, it minimized the time spent by the dehydratase in a dilute state and produced a concentrated extract of much reduced volume that could readily be de-salted on a Sephadex G-25 column, or diluted with a minimum of low salt buffer.

3.3.6 Step 6
Mono Q 10/10 Chromatography

The concentrated extract from step 5 was filtered through a 0.2 μm filter, and divided into two or three batches, according to the volume of the concentrated protein solution. Each batch of concentrate (about 6 ml) was diluted with chilled buffer A to give a final volume of 50 ml to reduce the $(NH_4)_2SO4$ carryover to a point which did not interfere with binding of the dehydratase to the column (approximately 75 mM). The solution was then loaded via a 50 ml Pharmacia superloop at a flow rate of 4 ml min^{-1} onto a Mono Q 10/10 column equilibrated in buffer A. The column was eluted, first with 10 ml of 100 mM KCl in buffer A, and then with a 150 ml gradient of 100 to 500 mM KCl, at a flow rate of 3 ml min^{-1}. The elution pattern of protein and L-serine dehydratase activity is shown in Figure 3.8.

Figure 3.7

Elution Profile of L-Serine Dehydratase from a Column of Pentyl Agarose (Step 5)

L-Serine dehydratase activity was eluted from a column of Pentyl agarose (2.5 cm diameter, by 20.5 cm long - bed volume 100 ml) as described in Chapter 3, section 3.2.5. The fractions (4 ml) were eluted at a flow rate of 90 ml hr^{-1}, and assayed for L-serine dehydratase activity in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2, 4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

● ● ● ● A_{280} nm

- - - - - - $(NH_4)_2SO_4$ Concentration (mM)

o o o o L-Serine Dehydratase Activity (units ml^{-1})

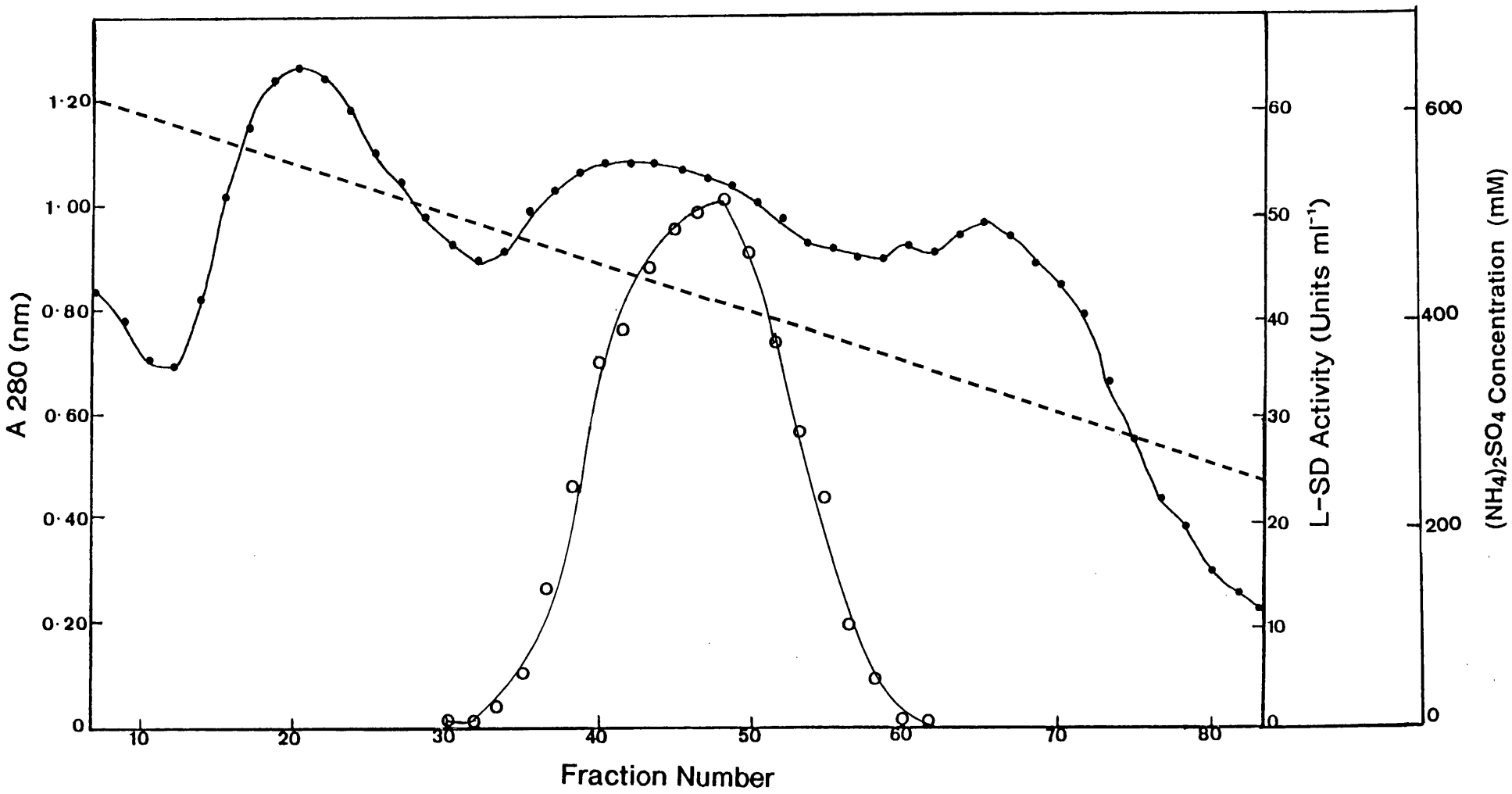

A 280 (nm)
1·20
1·00
0·80
0·60
0·40
0·20
0
10
20
30
40
50
60
70
80
Fraction Number
L-SD Activity (Units ml⁻¹)
60
50
40
30
20
10
0
(NH4)2SO4 Concentration (mM)
600
400
200
0

Figure 3.8

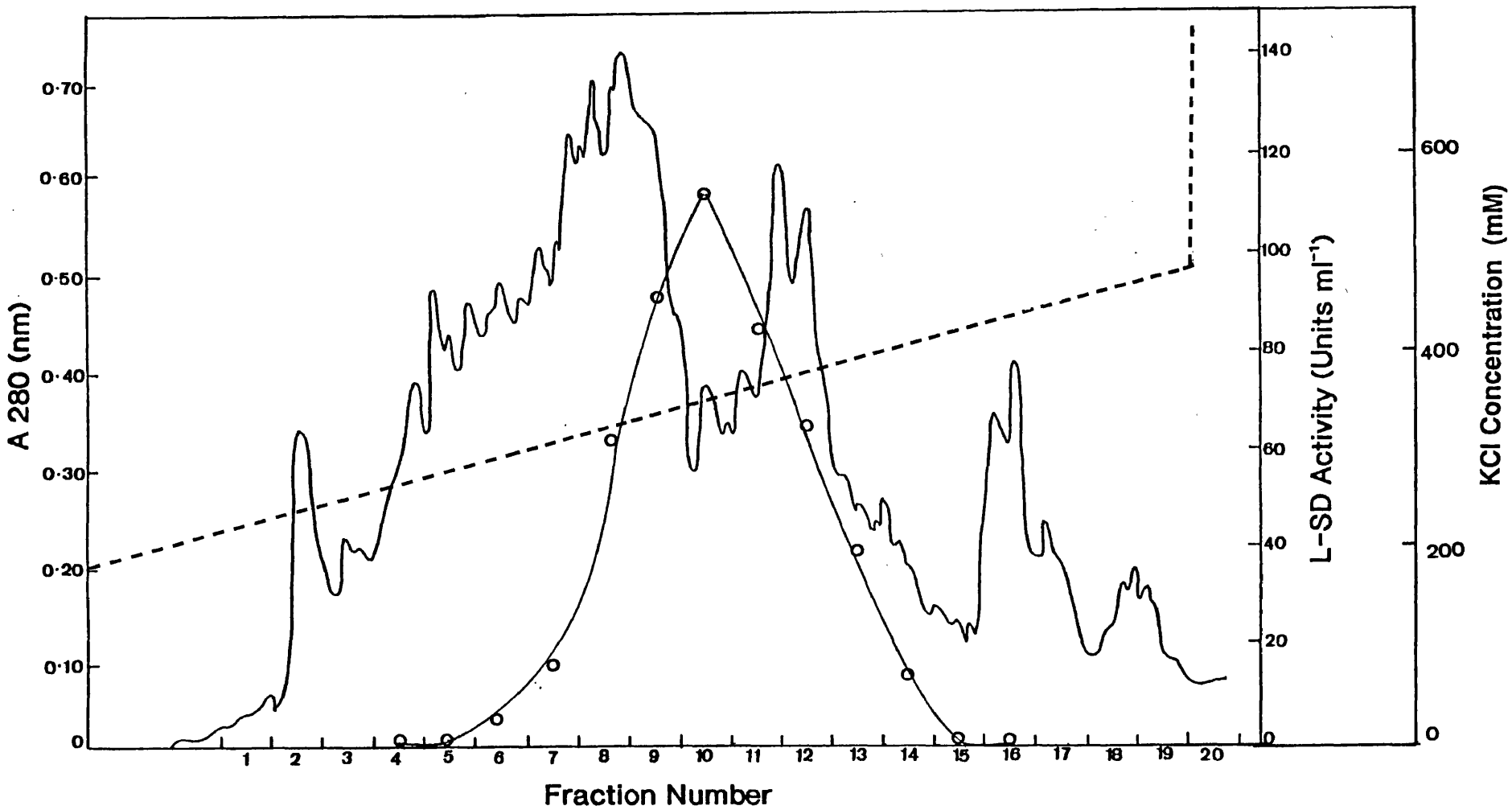
Elution Profile of L-Serine Dehydratase from a Mono Q 10/10 Column (Step 6)

L-Serine dehydratase activity was eluted from a Pharmacia Mono Q 10/10 column as described in Chapter 3, section 3.2.6. The fractions (3 ml) were eluted at a flow rate of 180 ml hr^{-1}, and assayed for L-serine dehydratase activity in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2, 4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

———— A_{280} nm

- - - - - - - KCl Concentration (mM)

o o o o L-Serine Dehydratase Activity (units ml^{-1})


A 280 (nm)
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0
L-SD Activity (Units ml⁻¹)
140
120
100
80
60
40
20
0
KCl Concentration (mM)
600
400
200
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Fraction Number

Fractions (3 ml) containing enzyme purified by a factor of greater than 3 were pooled. Overall recoveries of enzyme activity were of the order of 50 to 65 %, with purifications of between 4 and 7-fold.

3.3.7 Step 7

Reactive Green Agarose Chromatography

The pooled protein solution from step 6 was diluted approximately 1 : 3 to lower the KCl concentration, prior to loading onto a Reactive green agarose column (1.6 cm diameter x 12.5 cm, 25 ml bed volume) equilibrated in buffer A, at a flow rate of 120 ml hr^{-1}. The enzyme was washed onto the column with 20 ml of 150 mM KCl in buffer A, and then eluted with a 200 ml gradient of 150 to 700 mM KCl in buffer at a flow rate of 90 ml hr^{-1}. Fractions (3 ml) were collected and assayed for protein and enzyme activity (Figure 3.9). Fractions containing dehydratase with a purification factor of greater than 3 were pooled. Recoveries were of the order of 40 to 60 %, with overall purifications of between 3 and 5-fold.

3.3.8 Step 8

Phenyl Superose 5/5 Chromatography

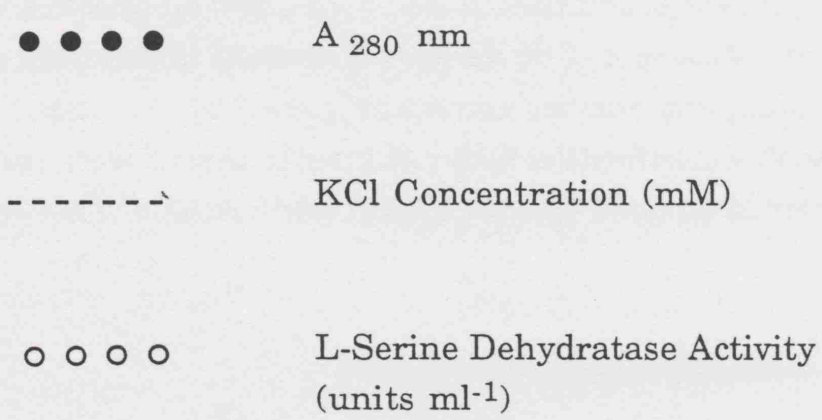
Enough solid ammonium sulphate was added to the pooled fractions from the Reactive Green agarose column to give a final concentration of 700 mM. The protein solution was then filtered through a 0.2 μm filter and divided into two batches. Each batch of protein was then loaded via a Pharmacia 50 ml Superloop at a flow rate of 1 ml min^{-1}, onto a phenyl superose 5/5 column, equilibrated with 1 M $(NH4)_2SO_4$ in buffer A, and washed in with 2 ml of the same buffer. L-Serine dehydratase was eluted with a 20 ml gradient of decreasing $(NH_4)_2SO_4$ (700 to 0 mM) in buffer A, at a flow rate of 0.50 ml min^{-1}. The enzyme was typically eluted between 300 and 200 mM ammonium sulphate, as a distinct peak (Figure 3.10). The most active L-serine dehydratase Fractions (0.5 ml) were collected and assayed for protein and dehydratase activity. Those containing enzyme with specific activities of greater than 1000 μmole min^{-1} mg^{-1} were pooled, to give a total volume of 3 to 4 ml and a combined specific activity of between 1300 and 1500 μmole min^{-1} mg^{-1}. Recoveries were usually between 60 and 75 % of the activity applied to the column.

Although the loading capacity of the Phenyl superose column was more than sufficient to bind all of the protein produced in the preceding step of the purification, a number of trials showed that L-serine dehydratase in step 7

Figure 3.9

Elution Profile of L-Serine Dehydratase from a Column of Reactive Green Agarose (Step 7)

L-Serine dehydratase activity was eluted from a column of Reactive Green agarose (1.6 cm diameter, by 12.5 cm long - bed volume 25 ml) as described in Chapter 3 ,section 3.2.7. The fractions (3 ml) were eluted at a flow rate of 90 ml hr^{-1}, and assayed for L-serine dehydratase activity in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2, 4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.



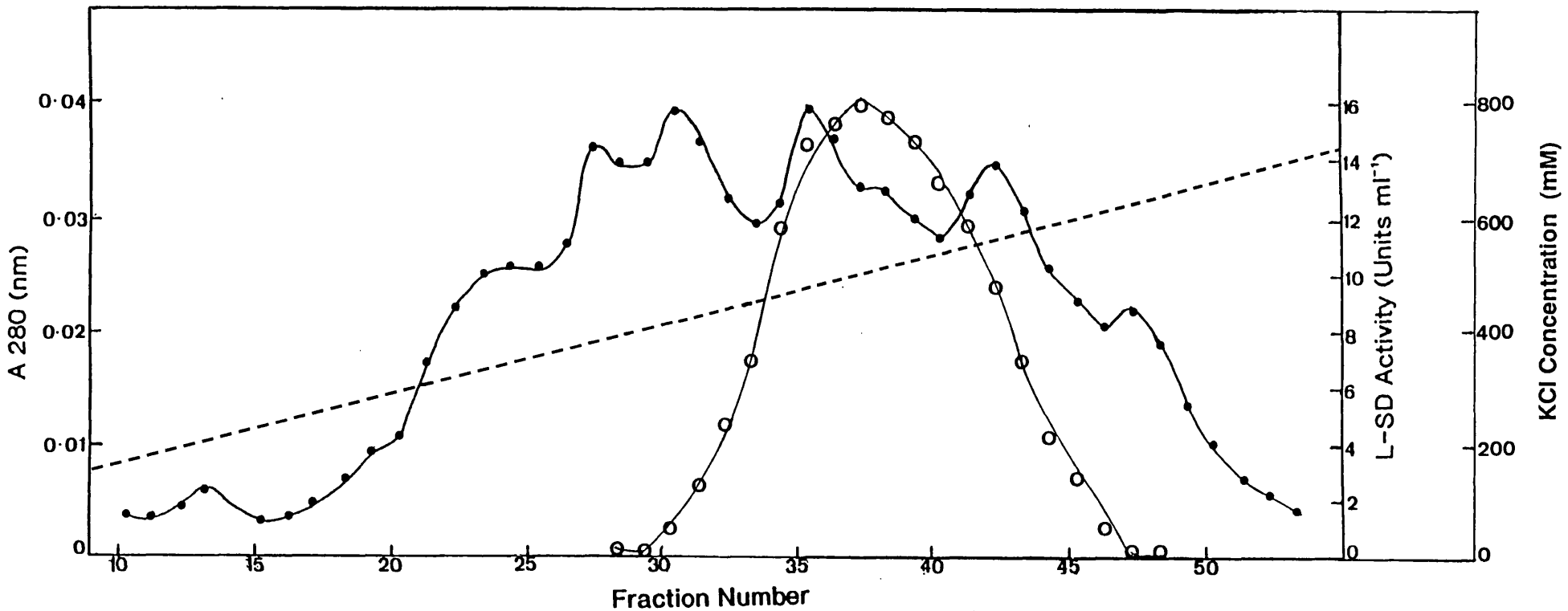
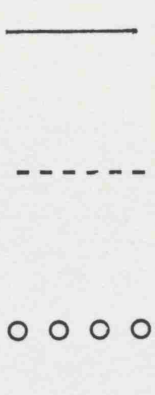
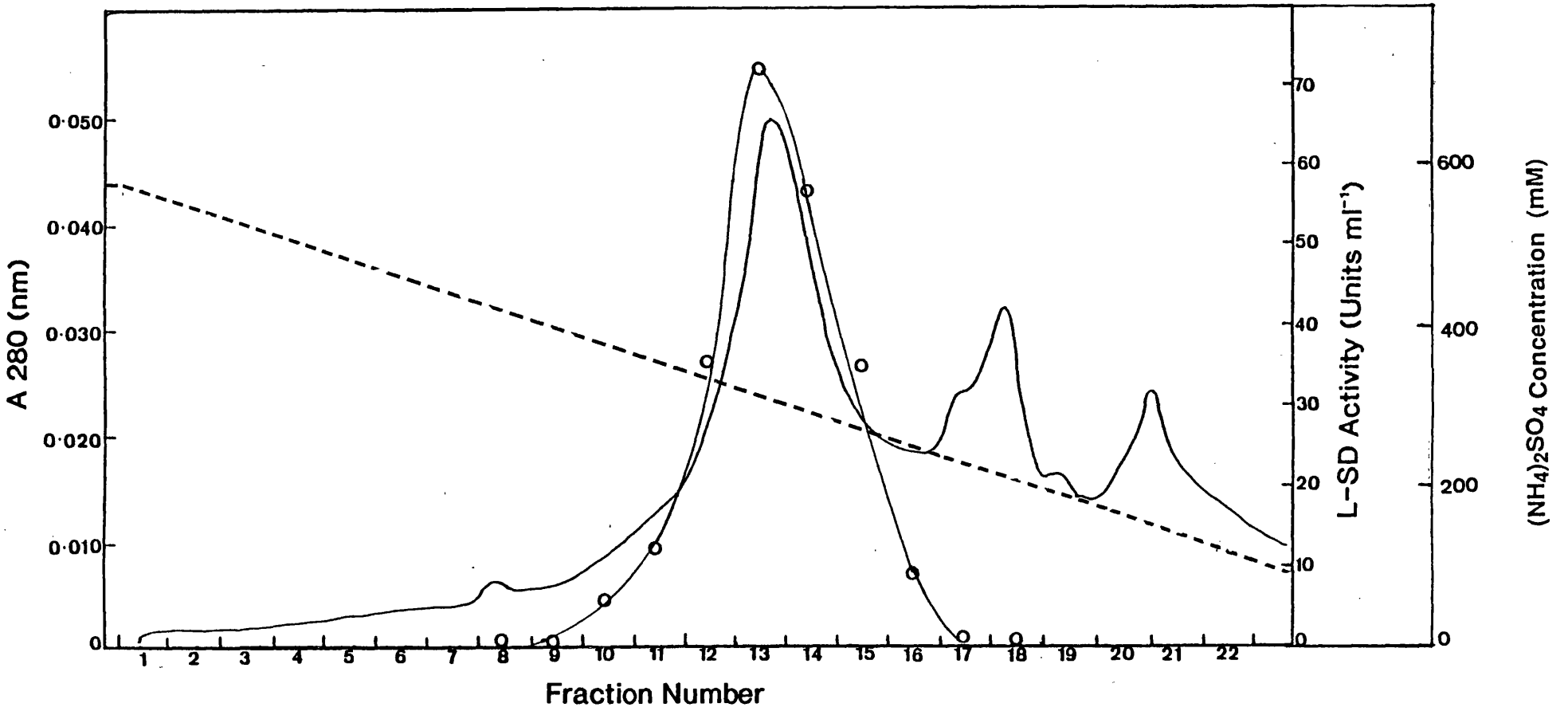

A 280 (nm)
0·04
0·03
0·02
0·01
0
10
15
20
25
30
35
40
45
50
Fraction Number
L-SD Activity (Units ml⁻¹)
16
14
12
10
8
6
4
2
0
KCl Concentration (mM)
800
600
400
200
0

Figure 3.10

Elution Profile of L-Serine Dehydratase from a Phenyl Superose 5/5 Column (Step 8)

L-Serine dehydratase activity was eluted from a Pharmacia phenyl superose 5/5 column as described in Chapter 3 section 3.2.8. The fractions (1 ml) were eluted at a flow rate of 30 ml hr^{-1}, and assayed for L-serine dehydratase activity in 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine, 10 mM DTT, and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2, 4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.




A 280 (nm)
0·050
0·040
0·030
0·020
0·010
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Fraction Number
70
60
50
40
30
20
10
0
L-SD Activity (Units ml⁻¹)
600
400
200
0
(NH4)2SO4 Concentration (mM)

preparations was particularly unstable to handling and underwent an extensive and irreversible loss of activity when bound to this column for long time intervals (in excess of one hour). This was in large part a consequence of the low pressure limits of the phenyl superose column, which prevented the attainment of flow rates of more than about 1 ml min^{-1}. This resulted in long periods of loading onto and of adsorption to the column. Purifying the Reactive green extract in two separate batches substantially reduced the overall time spent by the dehydratase bound to the phenyl superose column, and produced much higher recoveries of enzyme activity than were achieved when the step 7 material was purified in a single column run.

When enzyme was required for spectroscopy it was usually necessary to concentrate the phenyl superose-purified dehydratase. This initially proved to be difficult, because of the small volume to be reduced (3 to 6 ml) and the instability of the protein. A number of techniques were examined including concentration in Amicon minicon-A25 concentrators, precipitation with high concentrations of ammonium sulphate, and dessication with dry Sephadex G25. None of these methods proved to be successful, and a satisfactory concentration of the dehydratase was eventually achieved by reapplying the enzyme to the phenyl superose column, and eluting it with a small gradient. The step 8 material was mixed with an equal volume of 2 M $(NH_4)_2SO_4$ in buffer A, and loaded onto the Phenyl superose column at a flow rate of 1 ml min^{-1}. The enzyme was eluted with a 3 ml gradient of 1 M - 0 M $(NH_4)_2SO_4$, at a flow rate of 0.25 ml min^{-1}; 0.2 ml fractions were collected. This procedure produced a 5 - 7-fold reduction in volume, and resulted in a 10 to 25 % loss of activity.

3.3.9 Summary of the Purification

The procedure described above results in a 1300 to 1700-fold purification of L-serine dehydratase (depending on the specific activity of the enzyme in the crude starting material) to a specific activity of between 1450 and 1700 μmoles of pyruvate formed min^{-1} mg^{-1}, at a final yield of between 2 and 4 % of the initial activity. On average, a single fermenter preparation (20-25 g dry weight of cells) produced about 0.20 to 0.25 mg of highly purified protein. The results of a typical preparation are shown in Table 3.4

Table 3.4

The Purification of the L-Serine Dehydratase from *E. coli* B

The table describes the purification of L-serine dehydratase from the material produced from 1.5 fermenter preparations.

Step	Total Protein (mg)	Total Activity (units)	Specific Activity (μmoles pyruvate min^{-1} mg^{-1})	Purification (-fold)	Recovery (%)
1 Crude Extract	12 000	14 400	1.2	1	100
2 $SmSO_4$ Treatment	11 700	14 110	1.2	1	98
3 $(NH_4)_2SO_4$ Extraction	5 200	12 960	2.4	2	90
4 DEAE Cellulose	435	6 480	14.9	12	45
5 Pentyl Agarose	73	3 499	47.9	40	24
6 Mono Q	7.6	1 820	239.6	199	12.6
7 Reactive Green	1.2	909	766.7	639	6.3
8 Phenyl Superose	0.3	545	1653	1377	3.7

3.3.10 Analytical Polyacrylamide Gel Electrophoresis of Purified L-Serine Dehydratase

Samples from key stages of the purification were analysed by SDS polyacrylamide electrophoresis as described in Chapter 2, section 2.9.1 (Figure 3.12). The purity of the dehydratase, as judged by gel densitometry, was usually between 70 and 90 %. A gel densitometry scan of the dehydratase preparation shown in Figure 3.12 is illustrated in Figure 3.13. This particular preparation is about 80 % pure.

The final dehydratase preparation, although of high purity, is not completely homogeneous, and a small number of contaminating proteins are still present at the end of the purification (Figure 3.12). Several lines of evidence, for example the high specific activity of the purified protein, the close agreement of values obtained for the molecular weight of the enzyme by gel filtration and SDS PAGE, and the observation that the sequencing of the amino terminus produced only one set of data which agreed with the predicted N-terminal sequence for the *E. coli* K12 L-serine dehydratase (Su *et al*, 1989) strongly suggested that the principal component of the final preparation in the purification was L-serine dehydratase. However, a number of experiments were performed to confirm that the dehydratase was the major band seen on SDS gels of step 8 enzyme.

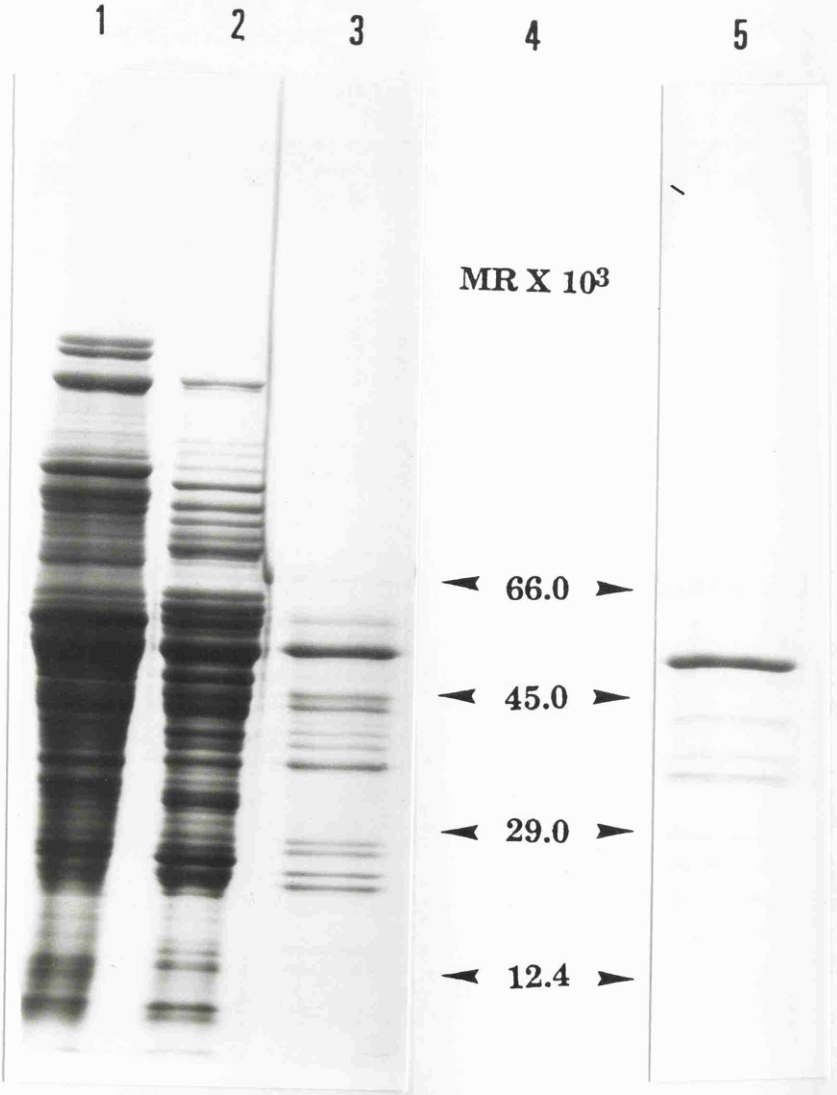
In one, a 10 % native polyacrylamide gel (prepared as described in Chapter 2, section 2.9.2) was run, containing 3 tracks of purified dehydratase (15 units, about 10 μg of protein). One enzyme track was stained for protein, while the other two were sliced into sections corresponding to individual protein bands and tested for activity by the 2,4-dinitrophenylhydrazine assay in media containing ferrous iron and DTT (Chapter 2, section 2.6.1). Pyruvate formation was only ever seen with the major protein band. Protein was electroeluted from the gel sections giving positive results for enzyme activity, and run out on a SDS 12.5 % polyacrylamide minigel, in addition to a control sample of purified L-serine dehydratase, and molecular weight standards. The electroeluted protein ran as a single band to the same position as the major band in the control sample of dehydratase. On isoelectric focussing gels, the major band of protein shown on native gels to possess L-serine dehydratase activity always ran as a single band, suggesting that it consisted of a single protein. All of these approaches would seem to confirm that L-serine dehydratase was the major component of the final protein preparation.

Figure 3.12

SDS-PAGE Showing the Purification of the *E.coli* B L-Serine Dehydratase.

Proteins were separated using SDS-PAGE and stained with Coomassie blue, as described in Chapter 2, section 2.9.1.

Track 1 Pooled Pentyl agarose fractions (approx.100 μg).
Track 2 Pooled Mono Q 10/10 fractions (approx. 70 μg).
Track 3 Pooled Reactive green agarose fractions (approx. 25 μg).
Track 4 Molecular weight standards (cytochrome c (M_R12 400); carbonic anhydrase (29 000); egg albumin (45 000); bovine serum albumin (66 000).
Track 5 Pooled Phenyl superose fractions (approx.10 μg).



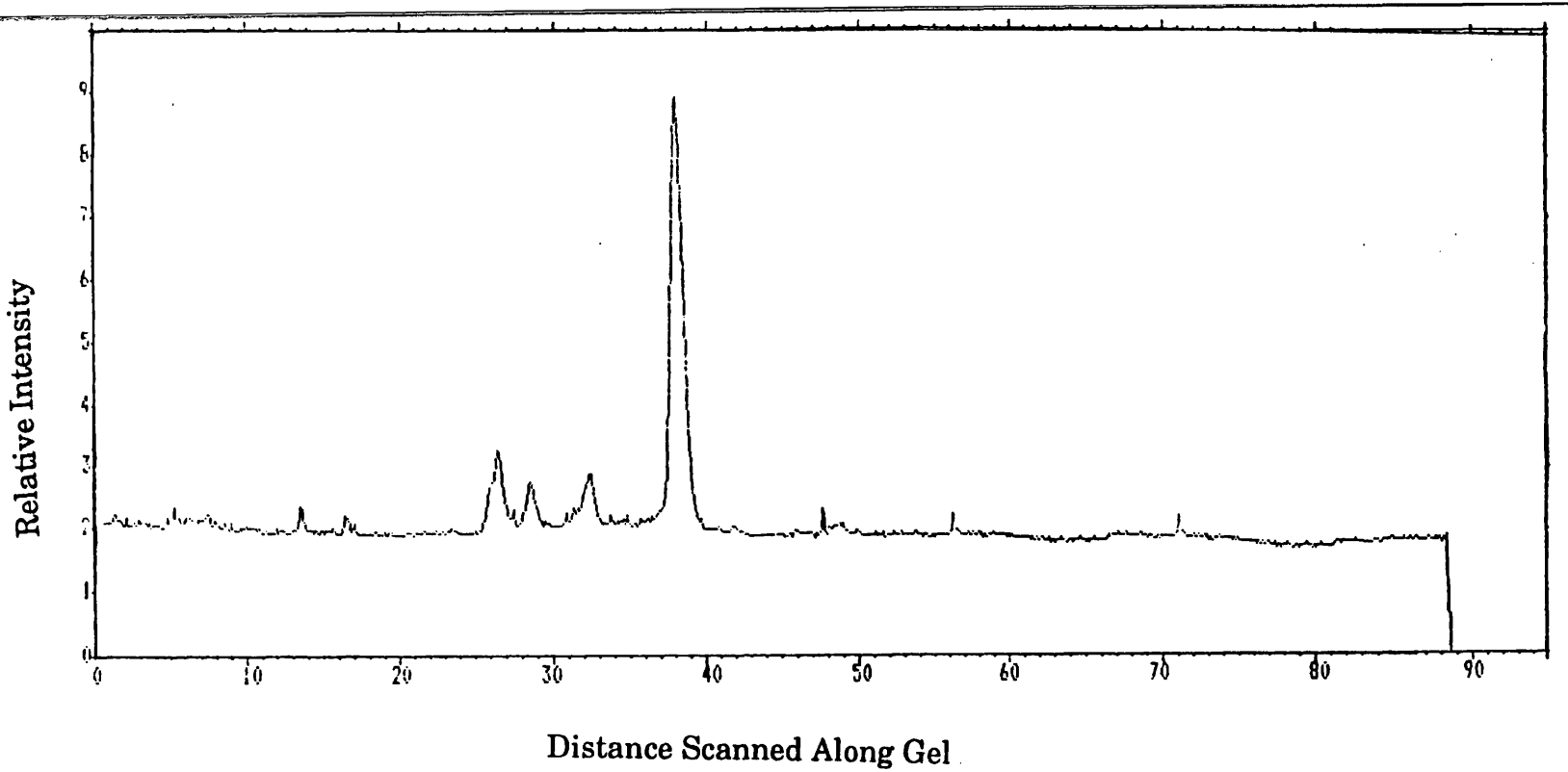


Figure 3.13

Scanning Laser Densitometry of Phenyl Superose Purified L-Serine Dehydratase.

Samples of Phenyl superose purified L-serine dehydratase were electrophoresed in a dissociating SDS polyacrylamide gel and stained with Coomassie blue, as described in Chapter 2, section 2.9.1. The purity of the dehydratase (M_R 52 000) was determined by an LKB scanning laser densitometer linked to a recording integrator.

3.3.11 Timespan of the Purification

Steps 1, 2 and 3 were usually performed on the same day, steps 4, 5 and 6 on successive days. Because of the extreme lability of L-serine dehydratase in relatively dilute solutions, the final two column separations (steps 7 and 8) had to be performed on the same day. Consequently, a small scale purification, dealing with material from 1 to 1.5 fermenter preparations would take some 5 working days for completion. In practice, depending upon the requirements of individual experiments, considerably more material than this was usually processed, typically 3 fermenter batches. The cells from these fermentations were extracted and processed to step 3 on the day of harvesting, and stored at -20 °C until required (at this stage the dehydratase is most stable to storage). The ammonium sulphate extracts were then pooled, halved and further purified as two separate batches. Each batch of pooled ammonium sulphate extract required one DEAE cellulose column purification. The pooled enzyme from this step could be further processed by a single pentyl agarose fractionation. The relatively small size of the Mono Q 10/10 column (8 ml) imposed a limit upon the amount of concentrated material from step 5 that could be purified in a single column separation. Depending on its concentration (and volume) the protein solution from the pentyl agarose stage of the purification required up to three Mono Q column runs. The pooled enzyme from the Mono Q step could be dealt with by a single Reactive green agarose separation. The enzyme solution from Reactive green agarose stage of the purification in turn required two phenyl superose column purifications. With careful scheduling of the use of fermenter and chromatography columns, the amount of protein produced from three fermenter preparations could be purified within 9 or 10 working days.

3.3.12 Storage and Stability

The extract at the end of Step 3 was stable to storage at - 20 °C for many weeks, that from Step 4 for up to three weeks, the concentrated pentyl agarose extract for 10 to 14 days and the Mono Q preparation for up to 1 week. Because of the instability of L-serine dehydratase in very dilute solution, the Reactive green preparation was not stable to storage, losing up to 30 % of its activity when stored overnight at - 20 °C, and had to be further purified, and thereby concentrated, within a few hours of elution. Thus, with the exception of the very dilute step 7 material, it was possible to store the dehydratase after most of the intermediate stages of the purification programme.

Unconcentrated L-serine dehydratase from step 8 of the purification scheme, (approximately 40 to 60 μg of enzyme ml^{-1}), was most stable to storage at - 20 °C. Enzyme which was stored at 0-4 °C for 24 hours underwent a 50 % loss of activity that could not be restored by ferrous iron or DTT. This half-life of activity increased to about 14 days when the enzyme was stored at - 20 °C. Step 8 enzyme that had been concentrated to 0.3 mg ml^{-1} or higher was considerably more stable to storage, with a half-life at -20 °C of approximately four weeks.

There was no noticeable shift in the pH for optimal stability as the purification of the dehydratase became more advanced. This is in contrast to the situation described for the L-serine dehydratase from *Arthrobacter globiformis*. Gannon (1973) found that the optimum pH for storage of the enzyme from this microorganism shifted from 7.0 - 8.0 in the early stages of the purification, to 6.4 - 7.2 in the latter part of the isolation. No such trend was observed with the *E. coli* L-serine dehydratase, which was most stable to storage in the 7.8 to 8.2 pH range.

3.4 Comments on the Purification Scheme

3.4.1 Problems Encountered

The purification of L-serine dehydratase was a difficult and timeconsuming process. The reasons for this were several-fold. The first is that L-serine dehydratase is produced by *E. coli* in only very small amounts - even when maximally induced it appears to constitute only some 0.05 - 0.1 % of the total cellular protein, a feature shared by the *A. globiformis* enzyme (Gannon, 1973). This meant that large amounts of starting material were required to produce even a modest quantity of purified enzyme. As a consequence, the most important influence on the choice of chromatography column during the early stages of the purification was the capacity to bind large amounts of protein, rather than resolving power. For this reason DEAE cellulose was chosen in preference to Mono Q for the first anion exchange step, even though the selectivity and resolution of the latter is considerably greater.

A second factor is the somewhat limited purifications and significant losses obtained at each step in the protocol, which has resulted in a rather long, and as far as expenditure of enzyme is concerned, expensive purification programme. A number of trials have shown that all of the stages in the scheme are necessary to achieve a high degree of purity. Attempts to increase the resolution at each step have been made, for example by increasing the length of a gradient. However,

any gains in resolution that a longer gradient might have produced were counterbalanced by the instability of the dehydratase to longer periods on a column, resulting in reduced yields. Efforts were also made to increase the overall recovery of the purification by, for instance, removing a step in which there were particularly heavy losses of enzyme activity, or by including more of the less purified dehydratase in the pool of enzyme carried over from each column. All of these modifications resulted in the production of a much poorer quality enzyme preparation. All of the steps in the scheme are needed to produce L-serine dehydratase of high purity. The individual column programmes described in the protocol have been designed to achieve an optimal balance between separation and recovery.

3.4.2 Alternative Purification Techniques

During the development of the programme described above, a number of alternative purification techniques and schemes were investigated. The more notable of these are described below.

3.4.2.1 Removal of Nucleic Acids

Previous work on the purification of the *E. coli* B L-serine dehydratase (Roberts, 1983) had involved an initial digestion of nucleic acids using DNase and RNase, and the precipitation of the dehydratase with ammonium sulphate, the digested nucleotides remaining behind in the supernatant. This was also the method of choice for the *Arthrobacter* enzyme (Gannon 1973,Gannon *et al*, 1977). Several small-scale trial purifications were performed using L-serine dehydratase prepared in this way, and the results compared with enzyme which had been treated with streptomycin sulphate before purification. These experiments showed that purity of L-serine dehydratase obtained from streptomycin sulphate-treated crude extracts was consistently greater than dehydratase purified from enzyme preparations that had undergone nuclease digestion, even though the final yields of enzyme activity were very similar.

A number of other methods of removing nucleic acids were examined. Treatment of the enzyme with the positively charged synthetic polymer Polymin P resulted in co-precipitation of dehydratase with nucleic acids. Salt extraction of precipitated protein produced only a slight overall purification and recovered less than 45 % of the starting activity. Protamine sulphate was almost as effective as streptomycin sulphate at removing nucleic acids, but was not used because of a tendency to occasionally co-precipitate L-serine dehydratase with RNA and DNA.

3.4.2.2 Ammonium Sulphate Precipitation

A number of trial fractionations were performed using ammonium sulphate concentrations lower than the 0-55 % cut used in the purification, e.g. 0-25 %, 0-35 %, followed by an appropriate increase to 55 % saturation. However, the quantities of protein brought down by the former fractionations were so small that no advantage was gained by including them. At saturation of greater than 30 %, L-serine dehydratase begins to be precipitated. A number of attempts were made to improve the purification obtained from this step by using reverse $(NH_4)_2SO_4$ fractionation, or by precipitation by high concentrations of KCl. No significant increase in the purity of the dehydratase fractions was achieved with the former, and the latter resulted in considerably lower recoveries of enzyme activity (an average yield of approximately 40 %).

3.4.2.3 Hydrophobic Interaction Chromatography

Initial attempts were made to purify L-serine dehydratase using phenyl sepharose CL 4B. These were unsuccessful, largely because, although the dehydratase readily bound to the column, it did not prove possible to elute the activity, even with 50 % ethylene glycol. It became clear that what was required was a hydrophobic material that would interact with L-serine dehydratase less tightly, while binding as little extraneous protein as possible. Such a medium was found by screening a series of hydrophobic agaroses of increasing alkyl chain length (Table 3.5). It can be seen that the affinity of L-serine dehydratase for butyl agarose was too weak to allow a productive interaction (only 30 % of the activity applied to the column actually adsorbed). Conversely, the strength of the interaction of the dehydratase with hexyl agarose was too great: the column required more stringent elution conditions, and the recovery of activity was low, although the purification was good. Pentyl agarose achieved a satisfactory balance between these two extremes. The dehydratase adsorbed to, and was readily eluted from the column and the purification and the recovery of activity were both good. Pentyl agarose was therefore chosen for the first hydrophobic interaction step in the purification scheme.

A number of trial separations of DEAE cellulose-purified enzyme were made using a phenyl superose 5/5 column. Although the purifications obtained with this hydrophobic medium were better than those achieved using pentyl agarose, the small size of the phenyl superose column (1.0 ml) and, as a consequence, its low binding capacity for protein (10 to 15 mg of protein per column) meant that in

Table 3.5

The Binding of L-Serine Dehydratase to Hydrophobic Interaction Media of Increasing Carbon Chain Length

Minicolumns (1 ml) of the hydrophobic agaroses shown in the table were prepared and equilibrated in 50 mM glycylglycine-NaOH buffer, pH 7.8 containing 1 M $(NH_4)_2SO_4$ and 2 mM DTT. L-Serine dehydratase (3 units of a step 4 extract prepared in the same buffer) was applied to each column. The columns were then sequentially eluted at a flow rate of 0.6 ml min^{-1} with two volumes (2 ml) of 50 mM glycylglycine-NaOH, pH 7.8 containing 2 mM DTT and the following additions:

1.0 M $(NH_4)_2SO_4$

0.8 M $(NH_4)_2SO_4$

0.6 M $(NH_4)_2SO_4$

0.4 M $(NH_4)_2SO_4$

0.2 M $(NH_4)_2SO_4$

0 M $(NH_4)_2SO_4$

10 % Ethylene glycol

50 % Ethylene glycol

Fractions (0.5 ml) were collected and assayed for protein by the A_{280}/A_{260} method of Warburg and Christian (1942) and L-serine dehydratase activity in assay media containing 100 mM glycylgycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM L-isoleucine, 1 mM hydroxylamine, 10 mM DTT and 1 mM FeSO4, as described in Chapter 2, section 2.6.1.

Key: **L-SDA** - L-Serine Dehydratase Activity

Specific Activity - (μmoles pyruvate 10 min^{-1} mg^{-1}) X 10

Carbon Chain Length (C_n)	% L-SDA adsorbed to column	Conditions required for elution	Specific Activity in Peak Fraction	% Activity Recovered in : Peak Fraction	Total Recovery
Ethyl (C_2)	0 %	Did not adsorb	2.58	-	100 %
Propyl (C_3)	0 %	Did not adsorb	2.50	-	98 %
Butyl (C_4)	30 %	0.8 - 0.6 M $(NH_4)_2SO_4$	3.69	18 %	79 %
Pentyl (C_5)	100 %	0.4 - 0.2 M $(NH_4)_2SO_4$	10.40	52 %	75 %
Hexyl (C_6)	100 %	Buffer A only	9.60	15 %	30 %
Octyl (C_8)	100 %	10 % Ethylene Glycol	0.79	6 %	10 %
Decyl (C_{10})	100 %	Did not elute with 50 % Ethylene glycol	0	0 %	0 %

the intermediate stages of the purification, when the amounts of protein to be processed still constituted many hundreds of mg, it could not really be used. The considerable selectivity and resolving powers of the Phenyl superose column found a more useful application towards the end of the purification scheme, when the quantities of protein to be handled had been reduced to only a few mg.

3.4.2.4 Affinity Chromatography

The Reactive green affinity agarose used in Step 7 of the purification scheme was selected from a series of triazine dye-coupled agaroses screened for their ability to selectively bind the dehydratase from a Mono Q extract. It is of interest that L-serine dehydratase in cruder preparations does not bind to this affinity medium. In addition, it did not prove possible to elute the enzyme using D- or L-serine.

A number of attempts were also made to purify L-serine dehydratase in a more selective manner using a commercially prepared L-serine-linked agarose. This was unsuccessful in that the dehydratase failed to adsorb to this material, possibly because the nature of the bonding of the serine to the agarose (via the $-NH_2$ terminus) presented the functional group in the wrong orientation for productive interaction.

Several attempts were also made to fractionate the dehydratase using hydroxylapatite chromatography. However, L-serine dehydratase is particularly unstable in phosphate buffer, and the very low recoveries of activity achieved with this method severely limited its usefulness.

3.4.2.5 Gel Filtration Chromatography

A number of trials were also made of various types of gel permeation chromatography media, including Sephacryl S-300, Sephadex G-100 and Superose 6 and 12. Because this separation method is only suitable for use with rather limited amounts of material, it did not find an application until after Step 6 (Mono Q) of the protocol. Although analysis by SDS PAGE showed that some purification was usually achieved using this method of purification (a 2 - 3-fold increase in specific activity), the recoveries tended to be too low for this type of separation procedure to be of any real value.

3.4.3 Alternative Purification Schemes

Several attempts were made to purify the *E. coli* L-serine dehydratase using the purification scheme developed by Gannon (1973) for the corresponding enzyme from *Arthrobacter globiformis*. This took the following form:

$(NH_4)_2SO_4$ -----> DEAE Cellulose -----> Gel Filtration -----> Gel Filtration		
Extraction		plus D-Serine
Approx. Molecular Weight	50 000	100 000

The success of this method relies heavily upon the separations obtained at two different molecular weights (50 000 and 100 000), made possible by the form of substrate activation undergone by the *Arthrobacter* dehydratase (a dimerisation). Unfortunately, this scheme was unable to substantially purify the *E. coli* B dehydratase, largely because, unlike the L-serine dehydratase from *A. globiformis*, the enzyme from *E. coli* does not appear to undergo dimerisation in the presence of either D-, or L-serine once it is purified beyond the first anion exchange step of its purification (Chapter 7, section 7.3.2)

3.4.4 The Nature of the Enzyme Used in the Experiments

Except where stated in individual experiments, all of the work described in this thesis was performed using enzyme that had been purified to Step 8.

3.4.5 Future Prospects

The losses that occur during the purification of L-serine dehydratase are heavy, and because each of the separation steps is an integral and essential part of the overall scheme, it was realised that there was little scope for the improvement in the yields obtained by manipulation of the purification programme without compromising the purity of the final enzyme preparation (section 3.4.1). The only recourse left was to attempt to increase the amount of L-serine dehydratase produced at its source, as described in section 3.2. Although these experiments were able to bring about some improvement in the starting levels of the dehydratase, they were not as successful as had been hoped. Possibly the most effective way to achieve an increase in the starting levels of L-serine dehydratase this would have been by the isolation of the gene for this enzyme, and its cloning and insertion into a high expression vector. At the beginning of this work such

an undertaking was contemplated. One possible way to achieve this might have involved an *in vivo* complementation method, similar to that used by Su *et al* (1989) (Chapter 4, section 4.7). However, the non-availability of a rapid screening method for the identification of those clones which which might have contained high levels of the dehydratase, made the following of such a course uncertain. It was decided, instead, to adopt a different approach which involved sequencing the amino terminus of the dehydratase and using this information to construct an oligonucleotide probe. This probe would have been used to screen an *E. coli* DNA library in order to isolate the gene for L-serine dehydratase, which would then have been inserted onto an appropriate high copy number plasmid and transfected back into *E. coli*. This approach first required that purified enzyme be obtained. Unfortunately, the purification of L-serine dehydratase was less straight forward than anticipated, and by the time an amino terminal sequence was available it was judged that insufficient time remained to attempt the cloning of the L-serine dehydratase gene.

In order to supply the amounts of L-serine dehydratase for those studies requiring large (mg) amounts of enzyme, a strategy of multiple fermenter preparations and large scale purifications was, *per force*, adopted. Although this does not represent an ideal approach, in that it is costly in both time and effort, it did result in the production of enough L-serine dehydratase to allow an extensive study of the kinetic properties of the dehydratase and a partial characterisation of the physical qualities of the enzyme. The availability of sufficient quantities of purified enzyme has been something of a limiting factor throughout the course of this work, and it is clear that in order to fully investigate the nature of the chemical and physical changes that occur following activation by iron, and by serine, a much richer source of L-serine dehydratase activity must be obtained. The most likely means of achieving this would be by the cloning and expression of the L-serine dehydratase gene, or by a substantial improvement in the percentage yields obtained in the purification procedure.

CHAPTER 4 - PROPERTIES OF L-SERINE DEHYDRATASE

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4.2 Substrate Specificity.

4.3 The Influence of Substrate Concentration.
4.3.1 Substrate Saturation Curve.
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4.8 The Properties of L-Serine Dehydratase in Permeabilised Cells.
4.8.1 Time-course of Activity.
4.8.2 Substrate Saturation Profile.

Chapter 4

Properties of L-Serine Dehydratase

The instability of the *E. coli* L-serine dehydratase has prevented the purification of this enzyme, which has meant that previous studies of the dehydratase have been confined to either crude or only slightly purified extracts (Alfoldi *et al*, 1968, Newman and Kapoor, 1980 and Roberts, 1983). With the preparations of L-serine dehydratase of high purity that are now available, many investigations can be undertaken that were previously not possible. The most important of these is the discovery of the nature of the cofactor utilised by the dehydratase. It has long been assumed that the microbial L-serine dehydratases, like their mammalian counterparts, utilise pyridoxal phosphate as cofactor, even though firm evidence for this assumption has only been provided for the enzyme from *Clostridium acidiurici* (Carter and Sagers, 1972). An unequivocal answer to the question of whether or not the *E. coli* L-serine dehydratase contains pyridoxal phosphate is provided in Chapter 5. Chapter 6 continues the investigations into the nature of the cofactor utilised by L-serine dehydratase and studies the characteristics, and molecular basis, of the iron requirement of the enzyme mentioned earlier (section 3.1). The nature of the slow-activation of L-serine dehydratase by its substrate is considered in Chapter 7. The present Chapter examines some of the kinetic and other properties of the dehydratase, and includes the determination of the amino terminal sequence of the dehydratase.

4.1 Definitions

The terms described below will be used in the description of the changes in L-serine dehydratase activity which result from the preincubation of this enzyme with ferrous iron and dithiothreitol, and with L-serine.

(i) Fe-independence Purified L-serine dehydratase is inactive unless assayed in the presence of, or preincubated with, ferrous iron and dithiothreitol. L-Serine dehydratase which requires these reagents for activity has been termed Fe-dependent. However, enzyme in whole cells or in crude extracts does not have an absolute requirement for iron and DTT, and shows a significant amount of activity in the absence of these reagents. This initial *in vivo* activity of L-serine dehydratase has been termed the Fe-independent activity of the enzyme. The Fe-independent activity of L-serine dehydratase is at its highest in whole cells and crude extracts (section 3.2). It is unstable, and is progressively lost during storage (Table 3.1), during protein purification procedures such as ammonium

sulphate precipitation and ion exchange chromatography (Table 7.2), and by treatment with metal chelators, such as EDTA (section 6.3). In all of these instances, this apparently lost activity can be restored by preincubation with Fe^{2+} and DTT. The Fe-independent activity of L-serine dehydratase is, therefore, a measure of the ability of the enzyme to deaminate L-serine in the absence of ferrous iron and DTT.

(ii) Iron-activation of L-Serine Dehydratase This term describes the process which occurs when the activity of Fe-dependent L-serine dehydratase is restored by preincubation with ferrous iron and dithiothreitol, under the conditions described in Chapter 2, section 2.6.4. The activation produced by iron and DTT is distinct from that produced by D- or L-serine in that L-serine dehydratase which has been iron-activated is still subject to a further form of activation by these amino acids.

(iii) Lag Period (or Lag Phase) This is the period between the start of the enzyme-catalysed reaction and the attainment of the steady state rate, during which enzyme activity increases with time.

(iv) Serine-Activation of L-Serine Dehydratase The term serine-activation is used to describe the transition which occurs when L-serine dehydratase is converted from the non-serine activated form of the enzyme, which is characterised by a non-linear timecourse of activity and a sigmoidal substrate saturation curve, to the serine-activated form, which shows a linear timecourse of pyruvate formation and a hyperbolic substrate saturation profile. Reagents which can cause this change are termed activators, or activating ligands; they do not necessarily increase the linear catalytic rate, but are responsible for converting non-serine-activated dehydratase to the serine-activated form. In this sense of the use of the term 'activator', substances which inhibit the linear catalytic rate, but which are able to induce the transition to the serine-activated form of the dehydratase, such as D-serine, are also referred to as activators.

4.2 The Timecourse of L-Serine Dehydratase Activity

Roberts (1983) showed that, when assayed continuously, the timecourse of pyruvate formation by the L-serine dehydratase from *E. coli* B was non-linear, this non-linearity taking the form of an initial lag phase of several minutes duration during which the rate of product formation increased until pyruvate was formed at a constant rate. Roberts was able to demonstrate that the initial lag phase was a consequence of the slow activation of L-serine dehydratase by its

substrate. Gannon (1973) described similar properties for the corresponding enzyme from *A. globiformis*. Both workers used enzyme preparations that were Fe-independent, that is active in the absence of added ferrous iron and dithiothreitol. The purified dehydratase used in the present study loses this Fe-independent activity during the course of its purification (section 3.1), and is inactive unless preincubated with, or assayed in the presence of, Fe^{2+} and DTT. In view of this difference, it was important to determine if purified L-serine dehydratase which had been restored to activity by preincubation with iron and DTT was still subject to activation by L-serine.

This was investigated by examining the continuous timecourse of activity of iron-activated L-serine dehydratase which had been activated by preincubation at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$, as described in Chapter 2, section 2.6.4. The timecourse of dehydratase activated in this way was found to be non-linear, and very similar in form to the timecourses obtained earlier by Roberts (1983). The initial rate of enzyme-catalysed reaction was low, but progressively increased over a period of several minutes until pyruvate was being produced at a constant rate, which continued until the NADH in the assay became limiting (Figure 4.1). The presence of this lag phase shows that L-serine dehydratase which has been activated by ferrous iron and DTT is still subject to further activation by its substrate, L-serine. Other features of L-serine dehydratase activity which arise from the slow activation of the enzyme by its substrate, and the possible underlying molecular basis of this activation process, are considered in more detail in Chapter 7.

The activation of L-serine dehydratase by ferrous iron and DTT is also, like the activation produced by L-serine, characterised by an initial slow, or lag phase (Chapter 6, section 6.2). The process of iron-activation which restores the activity of inactive dehydratase needed up to 20 minutes for completion. Although the time required for maximal activation by iron and DTT is reduced if L-serine was present in the incubation medium, the reaction profile of enzyme incubated with all three reagents was still characterised by a distinct initial lag phase, during which the activity of the enzyme was found to increase with time (Figure 6.3). The forms of activation produced by iron and DTT and by L-serine, and their possible inter-relationships, are considered further in Chapter 6, section 6.2 and Chapter 7, section 7.4.

4.2. Substrate Specificity

L-Serine dehydratase at all stages of its purification was highly specific for

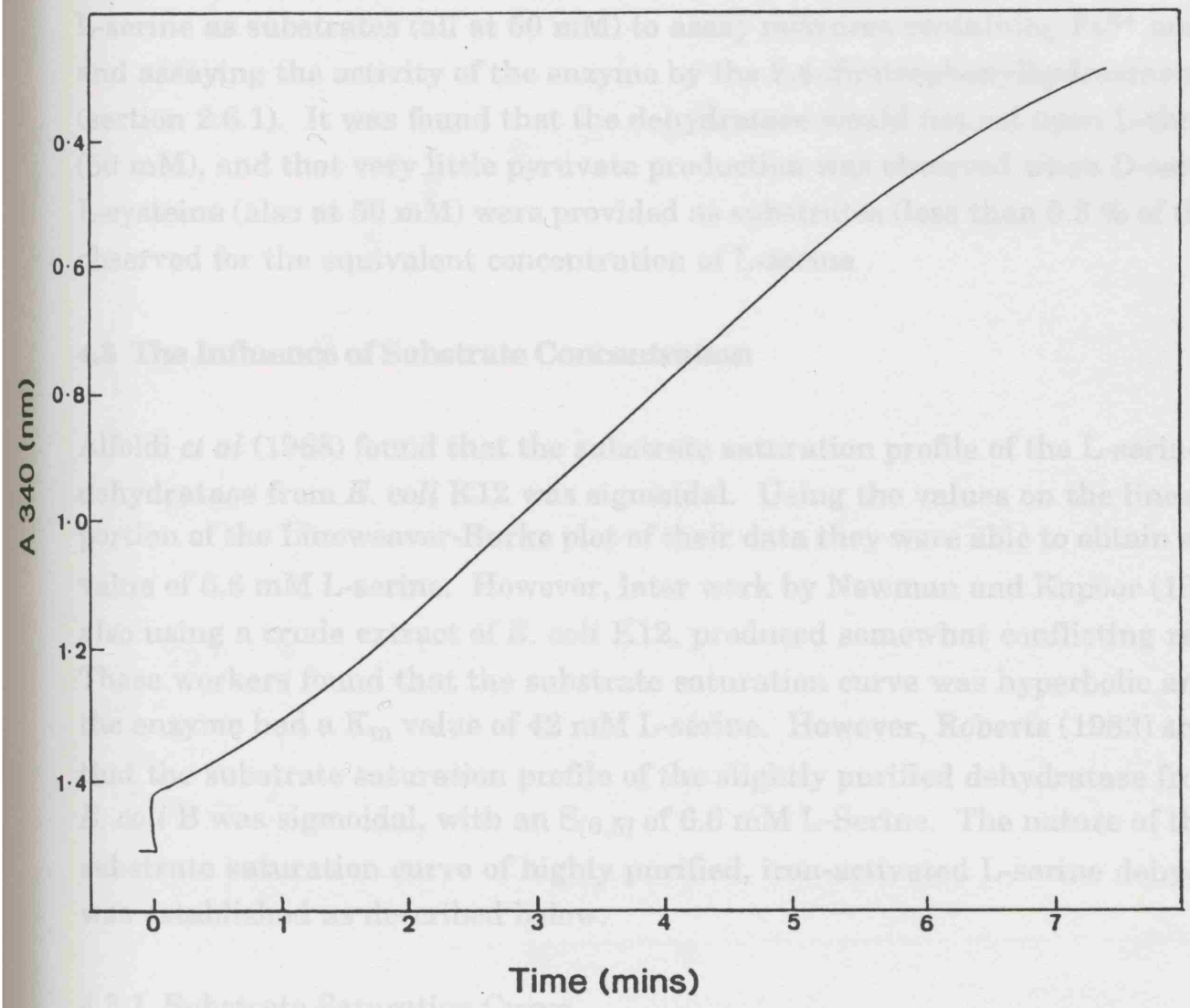


Figure 4.1

The Timecourse of L-Serine Dehydratase Activity

L-Serine dehydratase (step 8, enzyme concentration 0.10 mg ml^{-1}) was iron-activated as described in Chapter 2, section 2.6.4. Samples (0.025 units) of the iron-activated enzyme were then added to a lactate dehydrogenase-coupled assay mixture containing 50 mM L-serine. The assay was performed at 37 °C

L-serine. This specificity was demonstrated by adding amino acids other than L-serine as substrates (all at 50 mM) to assay mixtures containing Fe^{2+} and DTT and assaying the activity of the enzyme by the 2,4-dinitrophenylhydrazine method (section 2.6.1). It was found that the dehydratase would not act upon L-threonine (50 mM), and that very little pyruvate production was observed when D-serine and L-cysteine (also at 50 mM) were provided as substrates (less than 0.5 % of that observed for the equivalent concentration of L-serine .

4.3 The Influence of Substrate Concentration

Alfoldi *et al* (1968) found that the substrate saturation profile of the L-serine dehydratase from *E. coli* K12 was sigmoidal. Using the values on the linear portion of the Lineweaver-Burke plot of their data they were able to obtain a K_m value of 6.6 mM L-serine. However, later work by Newman and Kapoor (1980), also using a crude extract of *E. coli* K12, produced somewhat conflicting results. These workers found that the substrate saturation curve was hyperbolic and that the enzyme had a K_m value of 42 mM L-serine. However, Roberts (1983) showed that the substrate saturation profile of the slightly purified dehydratase from *E. coli* B was sigmoidal, with an $S_{[0.5]}$ of 6.6 mM L-Serine. The nature of the substrate saturation curve of highly purified, iron-activated L-serine dehydratase was established as described below.

4.3.1 Substrate Saturation Curve

The relationship between substrate concentration and linear rate was determined by assaying iron-activated L-serine dehydratase (Step 8, 0.05 units) by the lactate dehydrogenase method, with different concentrations of L-serine (Figure 4.2). The substrate saturation curve obtained was sigmoidal.

4.3.2 Analysis of Substrate Saturation Data

The concentration of L-serine required to give half maximal velocity ($S_{[0.5]}$) and the value of the Hill coefficient, were calculated from the Hill plot of the substrate saturation data (Figure 4.3). The $S_{[0.5]}$ value calculated from the x-axis intercept of the plot, has a value of 2.6 +/- 0.10 mM L-serine. This is somewhat lower than the value obtained by Roberts (1983) for the *E. coli* B enzyme (6.6 mM), and Gannon for the dehydratase from *A. globiformis* (6.5 mM), but is within the range of values observed for other microbial L-serine dehydratases.

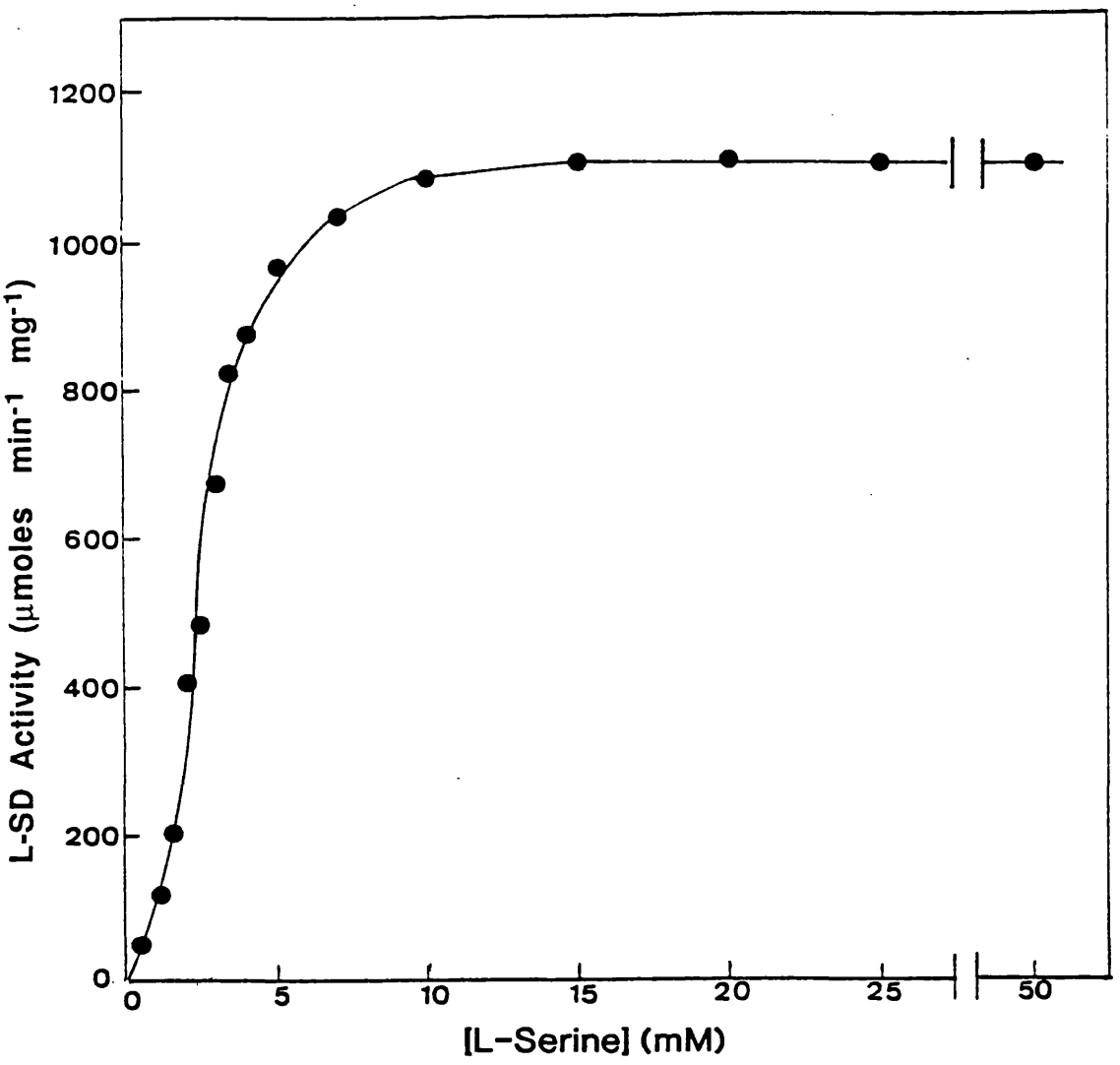


Figure 4.2

The Substrate Saturation Curve of L-Serine Dehydratase

L-Serine dehydratase (step 8, enzyme concentration 0.10 mg ml^{-1}) was iron-activated, as described in Chapter 2, section 2.6.4. Samples of the activated enzyme (10 μl) were then transferred to lactate dehydrogenase-coupled assay mixtures equilibrated at 37 °C containing 100 mM glycylglycine-NaOH, pH 8.0 and the concentrations of L-serine shown in the Figure. The linear rates of pyruvate formation for each reaction trace were then plotted against the substrate concentration at which they were obtained.

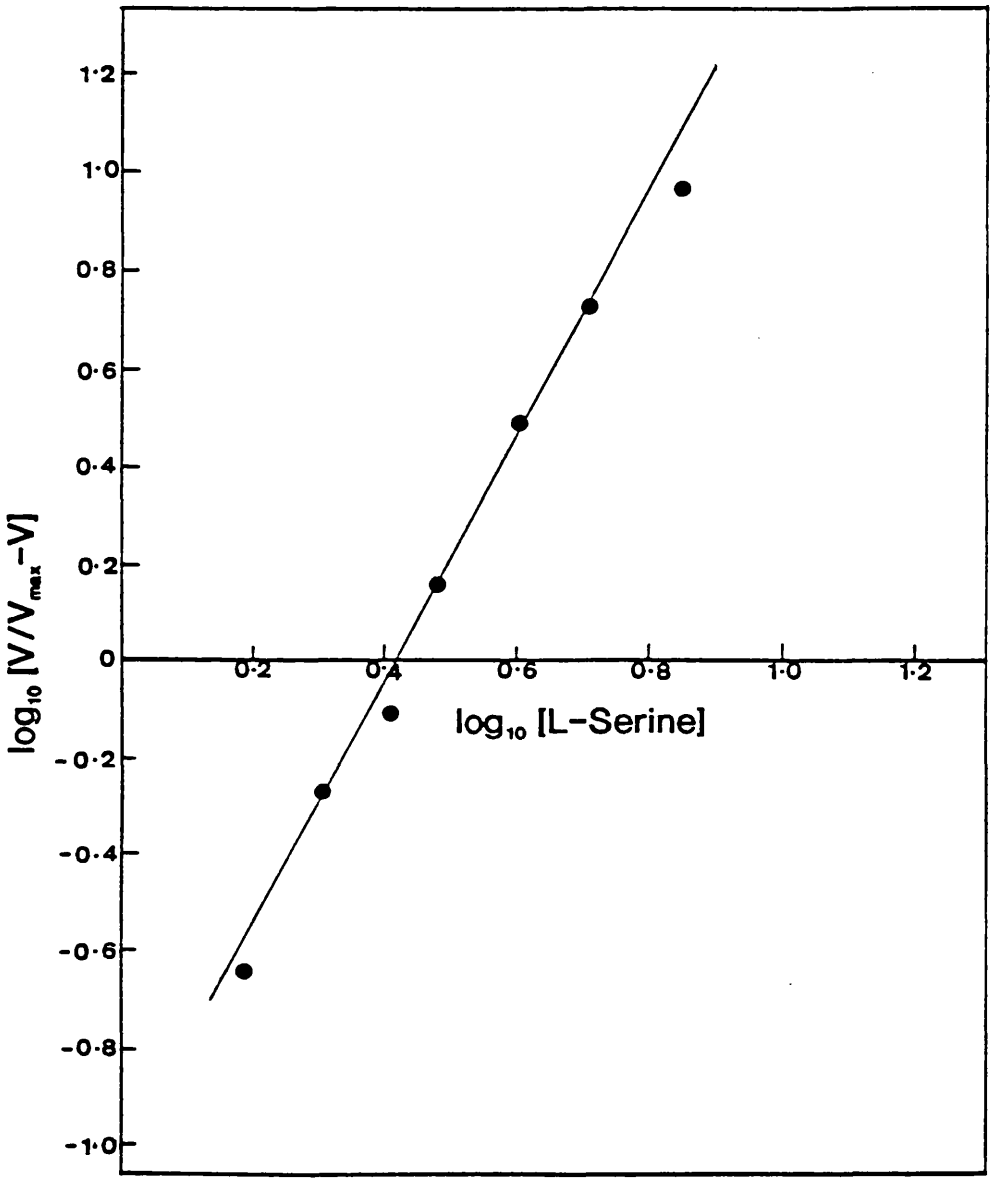


Figure 4.3

Hill Plot of the Substrate Saturation Data

The substrate saturation data for L-serine concentrations below 10 mM from Figure 4.2 were plotted on a Hill plot:

$$(\log_{10}[S] \text{ vs } \log_{10} (V/(V_{max} - V)$$

The Hill coefficient is the slope of this plot, and the $[S]_{0.5}$ value is the x-intercept value where

$$\log_{10} (V/V_{max} - V) = 0$$

The Hill coefficient,*h*, which is a measure of the cooperativity of the interaction between substrate binding sites, was determined from the slope of the Hill plot and has a value of 2.53. This agrees well with the earlier result of 2.36 obtained by Roberts for the partially purified *E. coli* B L-serine dehydratase (Roberts, 1983). However, since it is shown later in this thesis that dimerisation is not the underlying molecular basis of the cooperativity of L-serine-binding by L-serine deydratase, (Chapter 7),*h* cannot be considered to be a quantitative representation of the number of binding site(s) possessed by this enzyme

A computer fit of the substrate saturation data in Figure 4.2 was also obtained, producing an $S_{[0.5]}$ value of 2.52 mM L-serine (5 % standard error) and a Hill Coefficient of 2.72 (13 % standard error).

Sigmoidal substrate saturation profiles were also obtained for Fe-independent L-serine dehydratase in crude and partially purified extracts. The $S_{[0.5]}$ and Hill coefficient values obtained did not significantly differ from those achieved with purified enzyme.

4.3.4 The Effect of Protein Concentration on the Substrate Saturation Curve

Roberts (1983), working with the partially purified enzyme from *E. coli* B, was able to show that the $S_{[0.5]}$ value for L-serine varied with the protein concentration used in the assays. At 50 μg of protein ml^{-1} the $S_{[0.5]}$ was 2.43 mM L-serine, compared with 6.5 mM L-serine for 2.5 μg protein ml^{-1}. The Hill coefficient remained relatively constant at 2.31. A similar trend was also demonstrated for the L-serine dehydratase from *A. globiformis* (Gannon *et al*, 1977). Both of these studies were performed with Fe-competent enzyme.

A similar set of experiments was performed using iron-activated preparations of purified *E. coli* L-serine dehydratase. Substrate saturation curves were obtained at enzyme concentrations of 0.1 and 1.3 μg of protein ml^{-1}, respectively. The graphs obtained were both sigmoidal. Although a decrease in the $S_{[0.5]}$ was seen with the higher protein concentration, the trends observed were not as pronounced as those reported by Roberts or Gannon. At 0.1 μg ml^{-1} of protein , the $S_{[0.5]}$ value was 2.8 +/- 0.16 mM L-serine, compared with 2.2 +/- 0.10 mM L-serine for 1.3 mg ml^{-1} of protein. The values for the Hill coefficient were 2.02 +/- 0.10 and 2.12 +/- 0.11, respectively.

4.4 Inhibitor Studies

4.4.1 Method of Analysis

The nature of the inhibition of L-serine dehydratase by L-cysteine and D-serine was determined using the procedures for the statistical analysis of kinetic data developed by Dixon (1953) The non-linearity of the timecourse and the lability of iron-activated enzyme necessitated the adoption of a number of precautionary procedures. Firstly, the dehydratase has a non-linear timecourse, and so the reaction velocities described in the analysis below refer to the linear rates of pyruvate production, and not the initial, non-linear rate. Secondly, purified L-serine dehydratase which has been activated by preincubation with ferrous iron and DTT remains in a maximally activated state for only a limited length of time (Chapter 2, section 2.6.4). Consequently, care was taken to monitor the activity of the dehydratase during the course of each experiment and to compensate for any small losses of activity. In addition, although the K_i determinations for each inhibitor were performed with L-serine dehydratase obtained from a single purification, a separate batch of iron-activated enzyme was used for each set of substrate concentrations. The data from each batch of iron-activated dehydratase were normalised by adjusting the actual amount of enzyme preparation used, such that the same reaction velocity in the presence of a control concentration of L-serine in the absence of inhibitor (25 mM) was obtained.

Preliminary experiments were performed over a wide range of inhibitor and substrate concentrations to give an indication of the K_i value of each inhibitor. More refined ranges of inhibitor concentrations were then used in to determine the K_i values accurately. The concentrations of inhibitor and L-serine are described in the text for individual experiments. All assays were performed in duplicate.

The reciprocal velocities at constant L-serine concentration (1/v) were plotted against the inhibitor concentrations (i) according to the equation for competitive inhibition:

$$1/v = K_m/V_s + 1/V + K_m/V_s \,.\, i/K_i$$

The K_i value of each inhibitor was directly determined from the point of intersection of the 1/v and [inhibitor] plots.

4.4.2 L-Cysteine

4.4.2.1 Reversible Inhibition

When increasing concentrations of L-cysteine were included in assay mixtures, L-serine dehydratase was inhibited in the manner shown in Figure 4.4 Almost complete inhibition of enzyme activity occurred at approximately 70 mM L-cysteine.

An analysis of this inhibition was undertaken, using the method described in the previous section. Figure 4.5 shows the plots obtained by assaying the enzyme over a range of concentrations of L-serine (2.5, 3.33 and 5.0 mM) and L-cysteine (1.5, 3.0 and 5.0 mM), and in the absence of L-cysteine; the form of these plots indicates that the inhibition of L-serine dehydratase by L-cysteine is competitive.

The value of the K_i for L-cysteine was calculated as described in section 4.4.1, and was found to have a value of 1.6 +/- 0.10 mM L-cysteine. This figure is close to the K_i values of 1.2 mM L-cysteine for the L-serine dehydratase from *A. globiformis* (Gannon, 1973, Gannon *et al*, 1977), and 1.05 mM for the partially purified enzyme from *E. coli* B (Roberts, 1983)

4.4.2.2 Longer-term Inhibition

Gannon (1973) showed that the L-serine dehydratase from *Arthrobacter globiformis* underwent a slow, irreversible inhibition when incubated with L-cysteine over a period of several hours. Roberts (1983) reported similar findings for the enzyme from *E. coli*. Neither worker was able to discover the underlying mechanism of this inactivation.

To test for the occurrence of this long-term inhibition, L-serine dehydratase (Step 8, protein concentration 0.11 mg^{-1} ml^{-1}) was preincubated at 0 °C with 150 mM L-cysteine for time intervals of up to 5 hours, after which samples (10 µl) were removed for a 10 minute assay in the presence of Fe^{2+} and DTT. (Chapter 2, section 2.6.1). Controls were run in which the dehydratase was preincubated with an equal volume of water to allow for corrections for losses of activity resulting from enzyme instability.

The results obtained with the L-cysteine-treated enzyme showed no significant reduction in L-serine dehydratase activity other than that resulting from competitive inhibition caused by the carryover of 1.5 mM L-cysteine into the assay

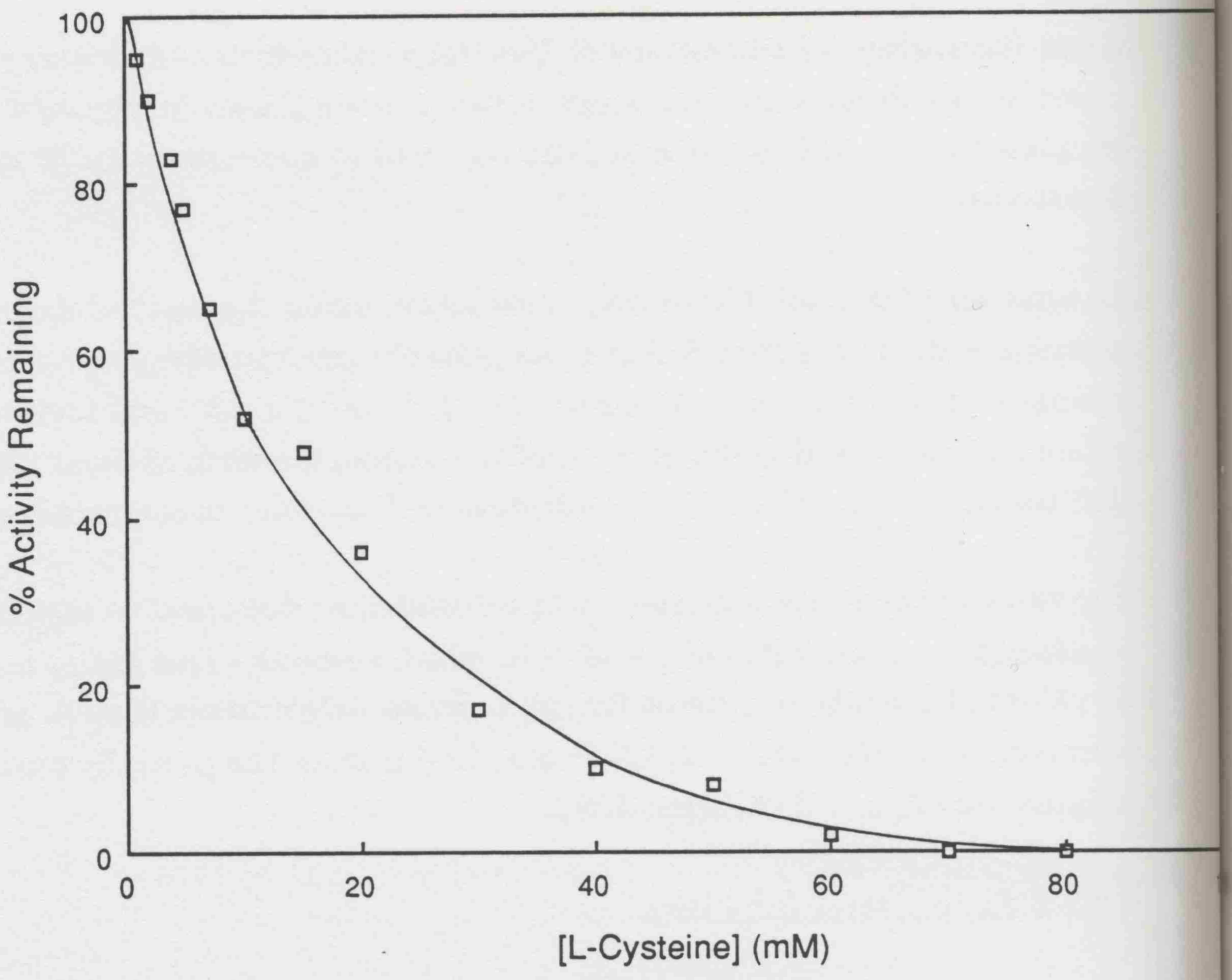


Figure 4.4

The Inhibition of L-Serine Dehydratase by L-Cysteine Present in the Assay Mixture

Iron-activated L-serine dehydratase (step 8, 0.05 units) was assayed at 37 °C lactate dehydrogenase-coupled assay mixtures (Chapter 2, section 2.6.2), containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine plus the concentrations of L-cysteine shown in the Figure. The linear rate of pyruva production from each reaction trace was then compared with those obtaine when no L-cysteine was present, to give the percentage activity remaining.

Figure 4.5

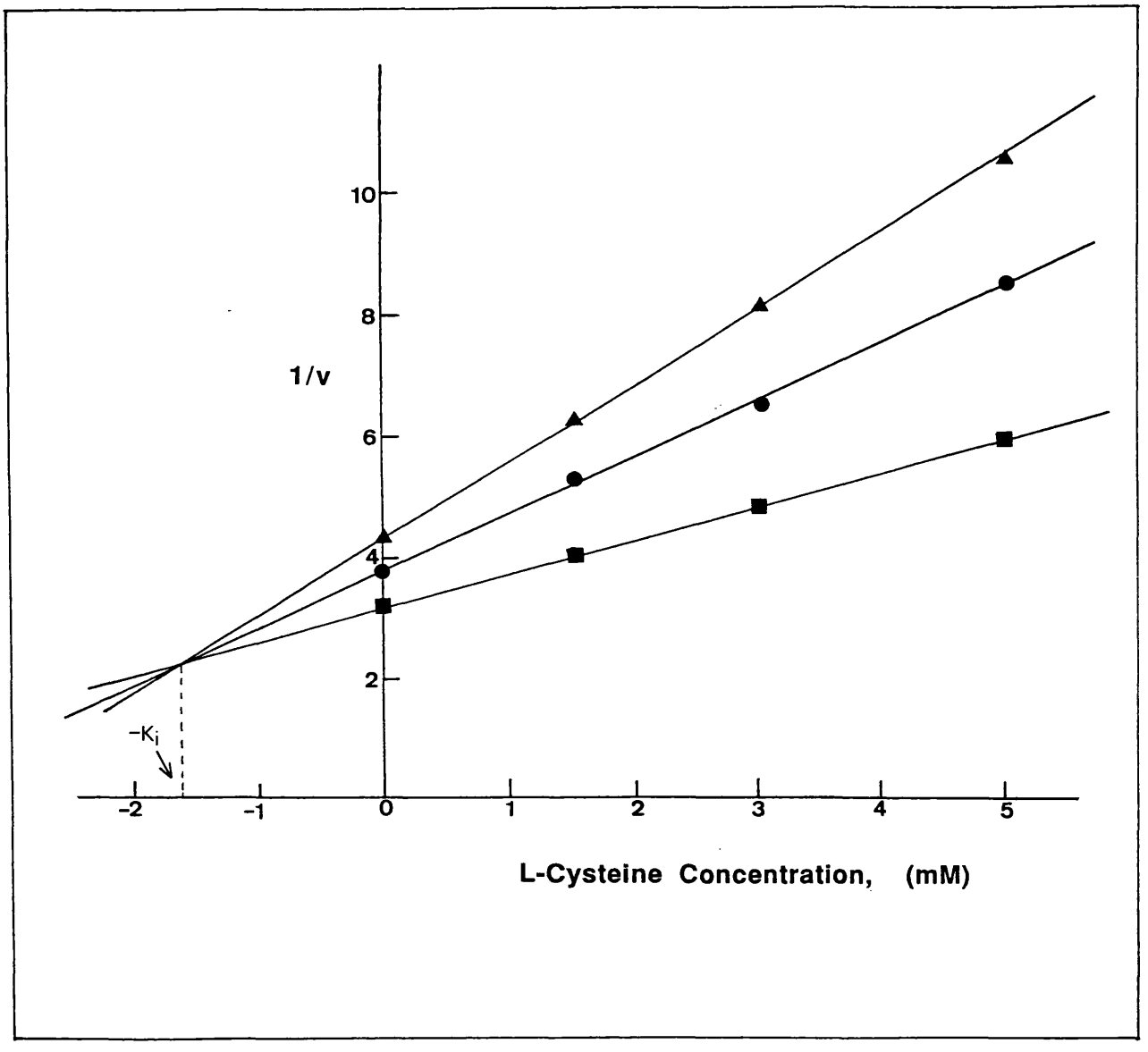
Analysis of the Data from the Inhibition of L-Serine Dehydratase by L-Cysteine

The inhibition of L-serine dehydratase by L-cysteine was investigated by measuring the activity of enzyme (0.10 units of a Step 8 preparation which had been iron-activated as described in Chapter 2, section 2.6.4) over a range of concentrations of L-serine (2.5, 3.33 and 5 mM) and L-cysteine (0, 1.5, 3.0 and 5.0mM). The continuous assay method was used (Chapter 2, section 2.6.2). The reciprocals of the linear reaction velocities obtained were then plotted as described in Chapter 4, section 4.4.1.

▲ ▲ ▲ ▲ 2.5 mM L-Serine

● ● ● ● 3.33 mM L-Serine

■ ■ ■ ■ 5.0 mM L-Serine


10
8
1/v
6
4
2
-Ki
-2
-1
0
1
2
3
4
5
L-Cysteine Concentration, (mM)

mixture. Several more such trials with highly purified enzyme also failed to show a time-dependent irreversible inactivation by L-cysteine. These results were not consistent with the earlier work of Roberts (1983), and Gannon (Gannon, 1973, Gannon *et al*, 1977). It was felt that a possible reason for this might lay in the nature of the enzyme used in these studies. Roberts and Gannon used enzyme preparations that were Fe-independent, that is active in the absence of iron and DTT, and measured the residual activity of the L-cysteine-treated dehydratase in lactate dehydrogenase-coupled assays which do not contain Fe^{2+} or dithiothreitol (Chapter 2, section 2.6.2). L-Cysteine is able to form complexes with certain transition metals, including iron. If the inactivation observed by Gannon and Roberts was the result of the chelation and abstraction of some catalytically essential cofactor from the dehydratase, possibly iron, the inhibition seen would have appeared irreversible, since no iron would have been available in the assay medium to replace that chelated by the cysteine. The purified L-serine dehydratase from *E. coli* used in this study may have lost this iron cofactor, since it is inactive in the absence of added iron and DTT. It would therefore be unaffected by the chelating effects of the cysteine.

To test this hypothesis, Fe-independent L-serine dehydratase from a crude extract of *E. coli* was preincubated with 150 mM L-cysteine as described above, and assayed discontinuously in the presence and absence of 1 mM Fe^{2+} and 10 mM dithiothreitol (Figure 4.6). At the end of the 5 hour incubation period, enzyme assayed in the absence of iron and DTT appeared to have lost almost all of its initial Fe-independent activity. However, when the dehydratase was assayed in the presence of iron and DTT, very little activity had, in fact, been lost. The results of this experiment are similar to those described in Chapter 6, section 6.5, which showed that purified L-serine dehydratase which has not been activated by iron and DTT was not inhibited by metal chelators, whereas similar treatment of iron-activated or Fe-independent dehydratase resulted in the complete inactivation of the enzyme. This apparent inactivation was almost fully reversible by ferrous iron and DTT.

The similarities between the two sets of experiments would appear to indicate that, for the *E. coli* B L-serine dehydratase, the long-term inhibition produced by L-cysteine was probably due to the chelation and extraction of a cofactor which could be replaced by ferrous iron and DTT. Since the L-serine dehydratase from *Arthrobacter globiformis* is also subject to activation by iron and DTT (personal observation), it is possible that the long term inhibition of this enzyme by L-cysteine is the result of a similar process.

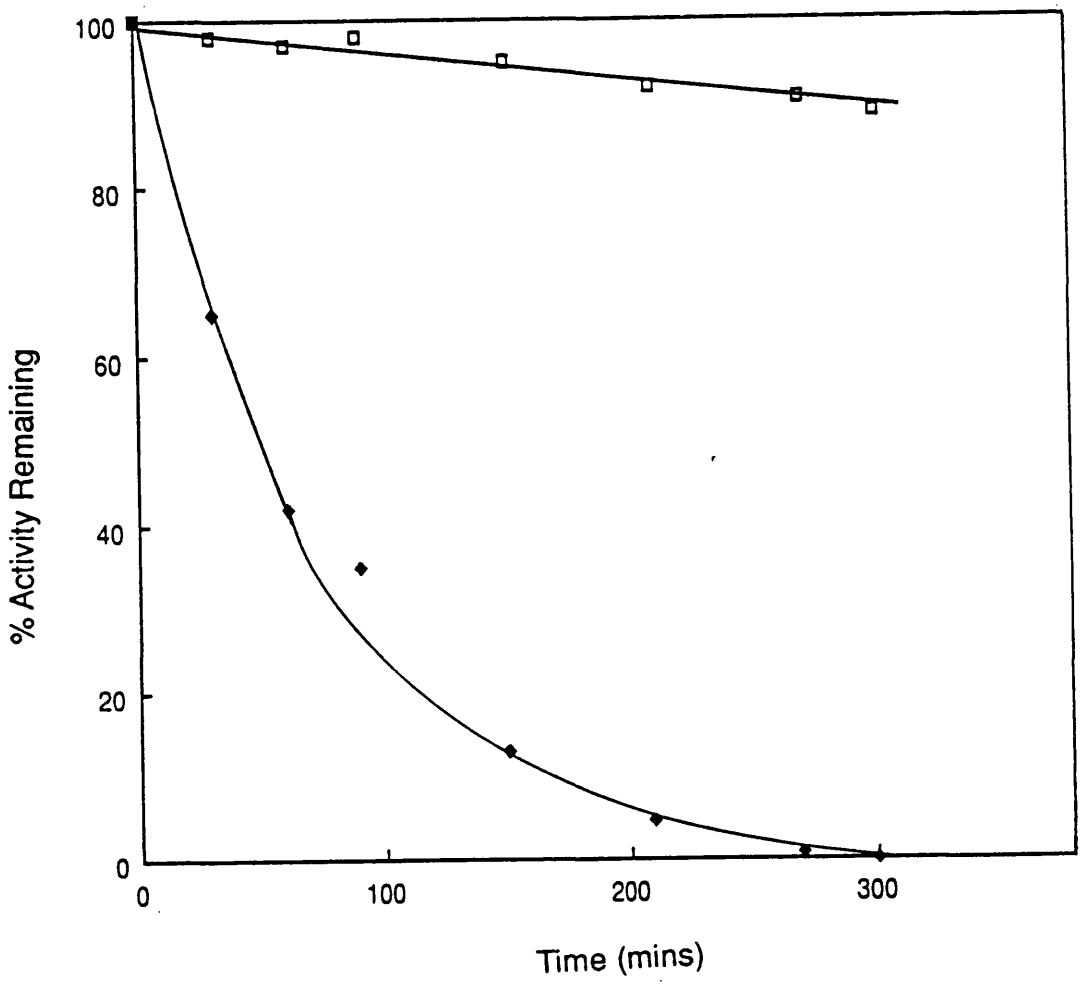


Figure 4.6

The Inhibition of L-Serine Dehydratase Activity On Prolonged Incubation With L-Cysteine

L-Serine dehydratase (step 1, 15 units) was incubated at 0 °C with 50 mM Tris-HCL, pH 8.0 and 150 mM L-cysteine. At the times shown in the Figure, samples of the preincubation mixture (10 µl) were removed and assayed at 37 °C in mixtures composed of 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine,10 mM L-isoleucine, 1 mM hydroxylamine with, and without, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1. Corrections were made for losses of activity resulting from enzyme instability by monitoring the activity of a sample of step 1 extract preincubated with tris buffer only.

□ □ □ □ L-SD Activity in the Presence of Fe and DTT

• • • • L-SD Activity in the Absence of Fe and DTT

The experiments described in this section have shown that L-cysteine inhibits L-serine dehydratase in two ways. When present in an assay mixture L-cysteine inhibits by virtue of its structural similarity to L-serine, as shown by the competitive nature of this inhibition. It also appears to inhibit by acting as a chelating agent, causing the abstraction of an essential cofactor from the dehydratase (possibly iron).

4.4.3 D-Serine

4.4.3.1 Reversible Inhibition

When L-serine dehydratase was assayed in the presence of different concentrations of D-serine, the enzyme was inhibited in the manner shown in Figure 4.7. D-Serine was unable to inhibit the enzyme more than 80 % even at the highest concentration tested of 150 mM.

An analysis of kinetic data obtained when D-serine was an inhibitor was performed as described previously for L-cysteine. The concentrations of L-serine used were 2.5, 5.0 and 15 mM, and of D-serine, 0, 6, 12 and 24 mM. The plot of 1/v vs [D-serine] is shown in Figure 4.8. The pattern observed indicates that the inhibition of L-serine dehydratase by D-serine is competitive. The K_i obtained was found to have a value of 4.25 +/- 0.05 mM D-serine, which agrees well with the value of 4.9 mM D-serine obtained by Gannon for the *A. globiformis* enzyme (Gannon, 1973, Gannon *et al*, 1977) and 3.54 mM D-serine for the partially purified enzyme from *E. coli* B (Roberts, 1983).

4.4.3.2 Long-term Inhibition

D-Serine does not inhibit L-serine dehydratase in an irreversible manner. When samples of Fe-independent enzyme from a crude extract, and inactive purified enzyme were incubated at 0 °C with 250 mM D-serine for time intervals of up to 6 hours, and assayed in mixtures containing iron and DTT, no losses of activity were observed above those accountable to enzyme instability, and competitive inhibition resulting from carry over of the inhibitor into the assay medium. The inhibition produced by D-serine is therefore of a competitive nature only.

4.4.4 Mercurials

For the study of the effects of mercurial compounds and other sulphydryl-modifying reagents on L-serine dehydratase activity, the discontinuous method

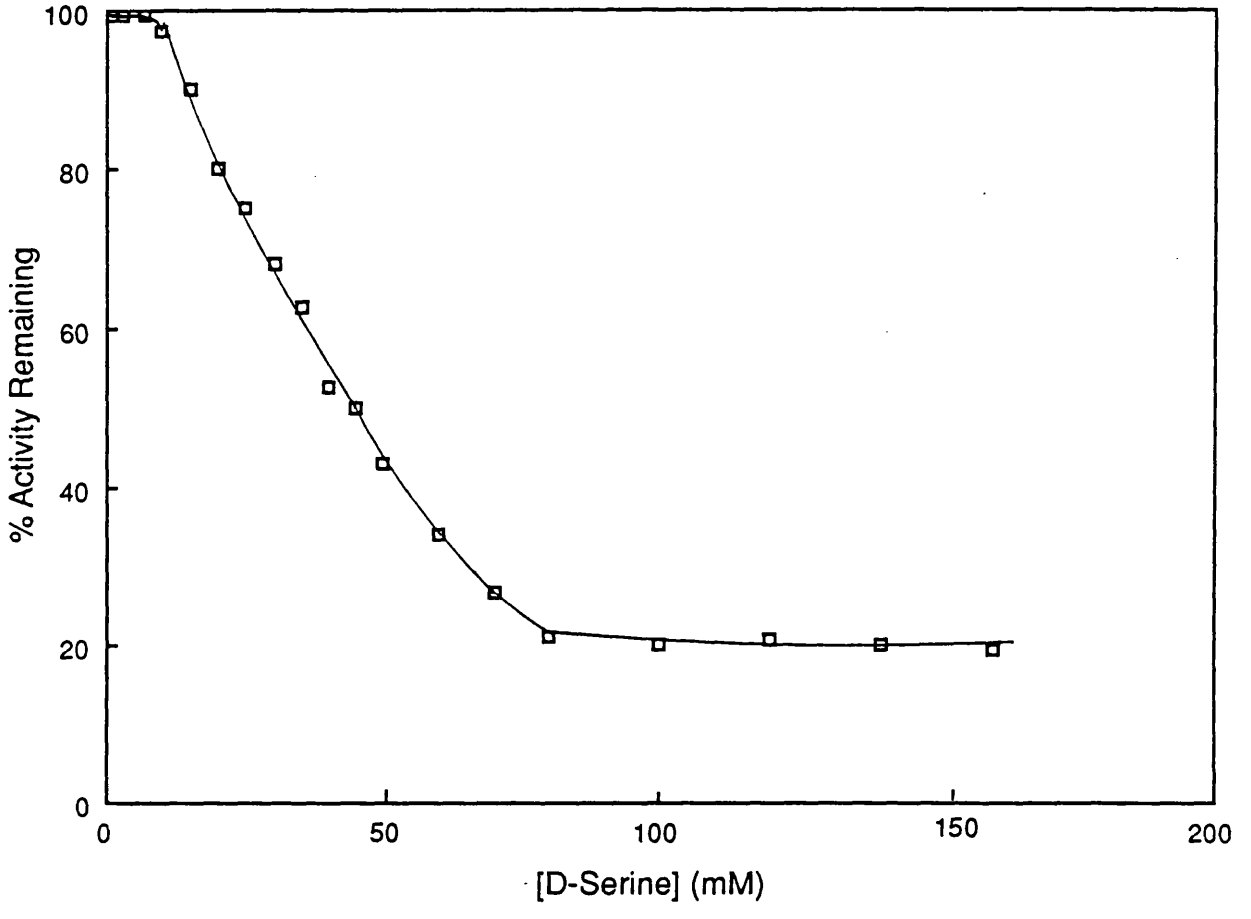


Figure 4.7

The Inhibition of L-Serine Dehydratase by D-Serine Present in the Assay Mixture

Iron-activated L-serine dehydratase (step 8, 0.05 units) was assayed at 37 °C in lactate dehydrogenase-coupled assay mixtures (Chapter 2, section 2.6.2), containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine plus the concentrations of D-serine shown in the Figure. The linear rate of pyruvate production from each reaction trace was then compared with those obtained when no D-serine was present, to give the percentage activity remaining.

Figure 4.8

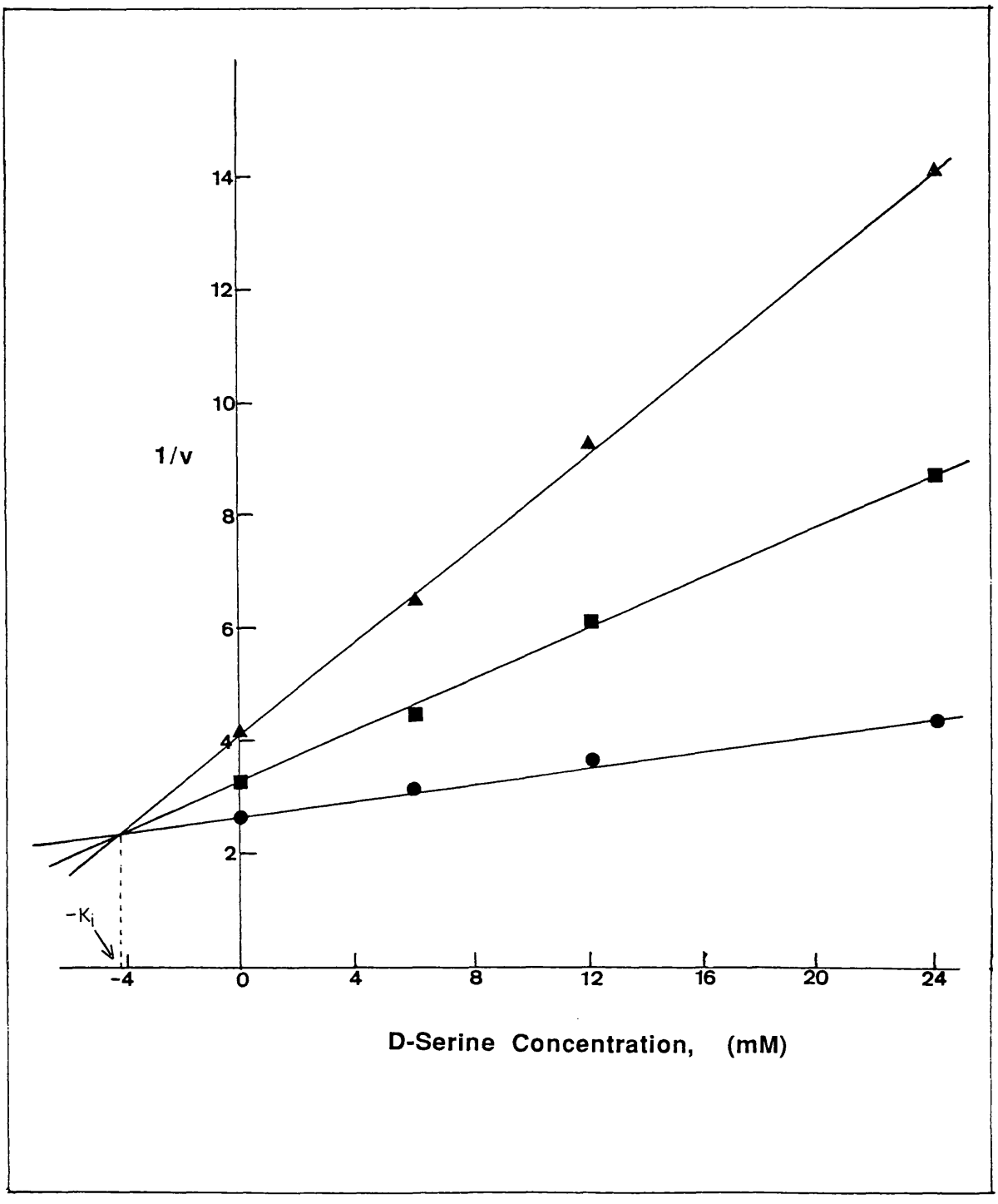
Analysis of the Data from the Inhibition of L-Serine Dehydratase by D-Serine

The inhibition of L-serine dehydratase by L-cysteine was investigated by measuring the activity of the enzyme (0.10 units of a Step 8 preparation which had been iron-activated as described in Chapter 2, section 2.6.4), over a range of concentrations of L-serine (2.5, 5.0 and 15.0 mM) and D-serine (0, 6.0, 12.0 and 24.0 mM). The continuous assay method was used (Chapter 2, section 2.6.2). The reciprocals of the linear reaction velocities obtained were then plotted as described in Chapter 4, section 4.4.1.

▲ ▲ ▲ ▲ 2.5 mM L-Serine

■ ■ ■ ■ 5.0 mM L-Serine

● ● ● ● 15.0 mM L-Serine


14
12
10
1/v
8
6
4
2
-Ki
-4
0
4
8
12
16
20
24
D-Serine Concentration, (mM)

of assay was used (Chapter 2, section 2.6.1). The continuous assay system was not used because lactate dehydrogenase has been shown to be sensitive to a variety of thiol blocking agents (Bloxham and Wilton, 1976). In addition, since L-serine dehydratase is prepared in buffer containing DTT which is able to protect against inhibition by mercurials, the following experiments were performed with enzyme that had first been chromatographed through a Sephadex G100 spun column to remove this reducing agent.

L-Serine dehydratase showed great sensitivity to mercurial agents. When the enzyme (Step 8, protein concentration 0.15 mg enzyme ml^{-1}) was preincubated with 1 mM mercuric chloride for varying lengths of time, a very rapid inhibition was observed, resulting in the loss of all enzyme activity within 10 minutes (column A Table 4.1). The inclusion of 100 mM L-serine into the incubation medium afforded only a limited amount of protection. It slowed, but did not ultimately prevent, the onset of total inhibition (column B, Table 4.1)

Sulphydryl-protective agents such as L-cysteine (5 mM) or dithiothreitol (10 mM) were able to partially reverse the mercurial inhibition of the dehydratase. To examine the characteristics of this reversal, L-serine dehydratase was preincubated with 1 mM $HgCl_2$ for time intervals of up to 10 minutes. At the end of the desired preincubation period samples were withdrawn and added to tubes containing either reducing agent or an equal volume of distilled water. Thirty seconds later, 10 µl aliquots of the incubation mixture were assayed for enzyme activity as described above.

The experiment described in Table 4.2 examines the ability of dithiothreitol (10 mM) to reverse the Hg^{2+} inhibition of L-serine dehydratase. The results show that the ability of 10 mM dithiothreitol to reverse the Hg^{2+} inhibition of L-serine dehydratase was time dependent. As the period of incubation with mercuric chloride was increased, less of the original activity could be recovered by adding DTT, and for incubation periods of greater than 5 minutes, the mercurial inhibition was no longer reversible by dithiothreitol. Even prolonged incubation (4 hours) of mercuric chloride-treated enzyme with DTT did not result in the reversal of this inhibition. In addition, enzyme which had been activated by Fe^{2+} and DTT (as described in Chapter 2, section 2.6.4), and then centrifuged through a Sephadex G100 spun column to remove excess iron and reducing agent, was still susceptible to mercurial poisoning. Iron-activation, like the protection afforded by L-serine, was able to slow the rate of inhibition, but could not prevent eventual, irreversible inactivation by the mercury (Table 4.1, column C). Very

Table 4.1

The Inhibition of L-Serine Dehydratase by Mercury (II) Chloride

L-Serine dehydratase (step 8, 0.15 mg enzyme ml^{-1}) was preincubated at 37 °C with 1 mM $HgCl_2$ with, and without, 100 mM L-serine, pH 8.0. At the times indicated in the table, samples (10 µl) of the enzyme-inhibitor mixture were assayed at 37 °C in mixtures containing 100 mM glycylglycine-NaOH buffer, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

In the experiment shown in column C, L-serine dehydratase was activated with $FeSO_4$ and DTT, as described in Chapter 2, section 2.6.4, chromatographed through a Sephadex G-100 spun column before being treated with 1 mM $HgCl_2$, and assayed as described above.

Length of Preincubation (minutes)	Percentage Activity Remaining		
	A	B	C
	No Additions	L-Serine	Iron-activated
0	100	100	100
1	93	96	98
2	69	84	90
3	52	72	86
4	43	53	70
5	30	35	60
7	20	27	40
15	0	0	3
20	0	0	0

Table 4.2

The Inhibition of L-Serine Dehydratase by Mercury (II) Chloride and its Reversal by Dithiothreitol

L-Serine dehydratase (step 8, 0.15 mg enzyme ml^{-1}) was preincubated at 37 °C with 1 mM $HgCl_2$ for the time intervals shown in the table. At the end of the appropriate preincubation period, samples were withdrawn and added to tubes containing either DTT (final concentration 10 mM) or an equal volume of distilled water; 30 seconds later, 10 µl aliquots were withdrawn and assayed at 37 °C in 100 mM glycylglycine-NaOH buffer, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

Length of Preincubation (minutes)	Percentage Activity Remaining:	
	A	B
	Water	DTT
0	100	100
1	93	99
2	69	94
3	52	86
4	43	74
5	30	52
7	20	24
10	0	0
15	0	0

similar results to those shown in Table 4.2 were obtained when the DTT was replaced by 5 mM L-cysteine.

A pattern of almost identical results was also obtained when L-serine dehydratase was preincubated with the monofunctional mercurial inhibitor, p-hydroxymercuribenzoate (data not shown).
These results show that inhibition of L-serine dehydratase by mercurial is a two-step process. The inhibition which results from the initial binding of mercurial reagents, presumably to sulphydryl groups within the enzyme, is reversible by the addition of sulphydryl reagents, such as DTT or L-cysteine. This initial process is then followed by a slower, secondary binding which appears to be irreversible.

These results confirm the earlier study made by Roberts of the mercurial inhibition of the *E. coli* L-serine dehydratase (Roberts, 1983), and show similarities to a parallel set of studies performed with the L-serine dehydratase from *A. globiformis* (Gannon, 1973).

4.4.5 Sulphydryl Modifiers

The effects of sulphydryl modifying reagents on L-serine dehydratase activity was examined by incubating dehydratase (step 8, enzyme concentration 0.10 mg ml^{-1}), which had been chromatographed through Sephadex G-100 spun columns to remove DTT, with a fixed concentration of the inhibitor (1 mM) for time intervals of up to 30 minutes. Samples of the preincubation mixtures (20 µl) were then diluted into assay containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$, as described in Chapter 2, section 2.6.1. The effects of six sulphydryl modifiers were examined: DTNB (5,5'-dithio bis nitrobenzoate), NTCB (2-nitro-5-thio cyanobenzoate), methyl methane thiosulphonate (MMTS), N-ethylmaleimide, iodacetate and iodoacetamide (Figure 4.9). The results obtained show that L-serine dehydratase was inhibited by all of these reagents. However, the extent of this inhibition, as a function of time, varied according to the inhibitor used. For example, a 30 minute incubation with iodoacetamide produced a loss of activity of less than 40 %, while a similar period of exposure to N-ethylmaleimide, NTCB or DTNB resulted in complete inactivation. Methyl methanethiosulphonate reacted most rapidly of all the modifiers tested, causing a total inactivation of the dehydratase after only 5 minutes incubation.

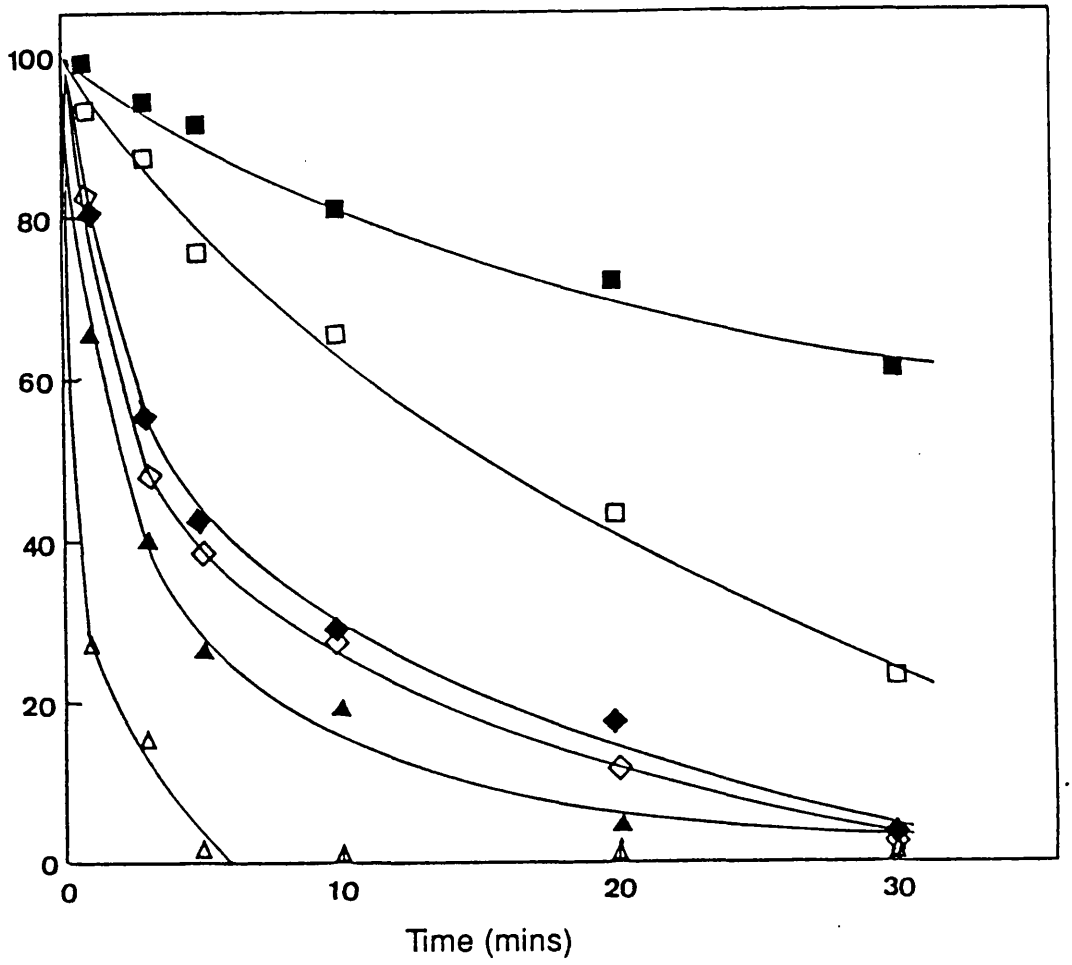


Figure 4.9

The Inhibition of L-Serine Dehydratase by Sulphydryl Modifying Reagents

L-Serine dehydratase (step 8, protein concentration 0.10 mg ml^{-1}) was incubated at 20 °C with the inhibitors shown below (all at 1 mM, final concentration). At the times shown in the Figure, samples (20 µl) of the preincubation mixtures were assayed at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM FeSO4. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

■ ■ ■ ■ Iodoacetamide
□ □ □ □ DTNB
◆ ◆ ◆ ◆ NTCB
◇ ◇ ◇ ◇ N-ethylmaleimide
▲ ▲ ▲ ▲ Iodoacetate
△ △ △ △ MMTS

The inclusion of L-serine (100 mM) into the enzyme-modifier preincubation mixtures, or pre-activation of the dehydratase by iron and DTT provided only a limited amount of protection, with inhibition being delayed, rather than prevented (results not shown).

4.5 pH and pI

For the study of the effects of changes in pH on L-serine dehydratase activity the discontinuous assay method was used (Chapter 2, section 2.6.1). The lactate dehydrogenase assay was not employed, to ensure that the pH effects seen were a product of the activity of L-serine dehydratase only. However, since the activation of the dehydratase by iron and dithiothreitol is markedly dependent on pH (Chapter 6, section 6. 4), a modification of the discontinuous assay was required. This involved activating the enzyme by preincubation with Fe^{2+} and DTT, as described in section 2.6.4, which removes the need for the presence of iron and DTT in the assay medium, followed by a 10 minute assay of the iron-activated enzyme in mixtures buffered at different pH values. At the end of the incubation, 0.1 ml samples of the reaction mixture were added to 0.33 ml of 2,4-dinitrophenylhydrazine and 0.9 ml of distilled water, and the pyruvate formed measured as its 2,4-dinitrophenylhydrazone derivative.

The pH activity curve obtained in this way shows a broad pH optimum, with maximal activity occurring between pH 7.75 and pH 8.5 (Figure 4.10). The broadness of this pH optimum is a typical feature of the microbial L-serine-specific dehydratases and is consistent with similar pH determinations performed by Roberts (1983) and Gannon (1973, Gannon *et al*, 1977) for the L-serine dehydratase from *A. globiformis*.

The isoelectric point of L-serine dehydratase under non-denaturing conditions was determined as described in Chapter 2, section 2.8.2. The results of three such determinations gave an average pI value of 5.3.

4.6 Molecular Mass Estimation

The subunit molecular weight of purified L-serine dehydratase has been measured by a variety of methods, including SDS PAGE and gel filtration on Sephadex G100 and Superose 12 media, all of which produced a value of between 52 000 and 57 000.

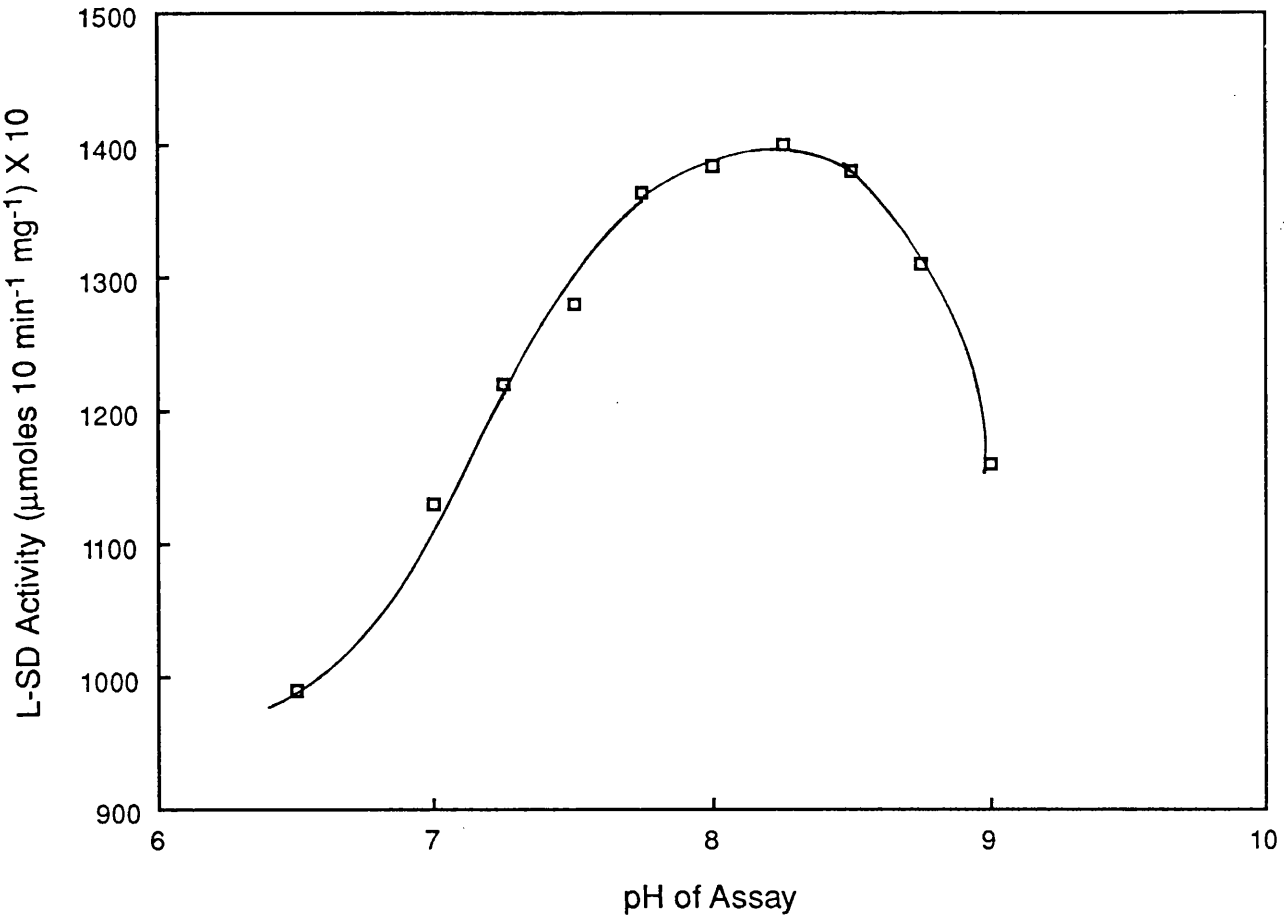


Figure 4.10

The pH Activity Curve of L-Serine Dehydratase

L-Serine dehydratase (step 8, protein concentration 0.10 mg ml^{-1}) was iron-activated as described in Chapter 2, section 2.6.4, Samples of the iron-activated enzyme (10 ml) were then assayed at 37 °C in mixtures containing 50 mM L-serine, buffered to the pH values shown in the Figure with 100 mM glycylglycine-bis-tris-propane (50 mM, each). Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

4.7 N-Terminal Sequence

4.7.1 Method of Amino Terminal Sequence Analysis

Purified L-serine dehydratase, 3 ml containing aproximately 150 μg of protein, was dialysed overnight at 4 °C, against 50 mM HCl. This dialysis treatment facilitated the freeze drying of the dehydratase, since HCl is more volatile than glycylglycine, and reduced the carryover of buffer. The dialysed enzyme was then freeze-dried, and re-suspended in 200 μl of SDS sample buffer. This preparation was run over 3 tracks on a 12.5 % SDS-polyacrylamide gel, as described in Chapter 2, section 2.8.1. The enzyme was then electroblotted by Dr K.S. Lilley onto Fluorotrans transfer membrane [PALL, Biosupport Division, Portsmouth] according to the procedure developed by Matsudaira (1987), and stained with Coomassie blue R-250. The stained protein band was excised and loaded into an Applied Biosystems 470A gas-phase sequencer. The N-terminal amino acids were removed by sequential Edman degradation, analysed by high performance liquid chromatography and the N-terminal sequence deduced from the resulting chromatograms. The sequence of the first 20 residues from the amino terminus of the *E. coli* B L-serine dehydratase is shown in Figure 4.11. The sequence determination was also repeated using a different preparation of the purified dehydratase. The results of this second analysis were identical to the first.

4.7.2 N-terminal Amino Acid Sequence of the *E. coli* B L-Serine Dehydratase

A possible primary sequence for the *E. coli* L-serine dehydratase has been suggested by Su *et al* (1989). This sequence was deduced from the nucleotide sequence of the *E. coli* K12 sdaA gene, isolated by Su *et al* (1989) and believed by these workers to be the structural gene for L-serine dehydratase. The method of isolation of the *sdaA* gene, an *in vivo* complementation technique, and the lack of a primary sequence obtained directly from the dehydratase protein with which to compare the predicted sequence, initially cast some doubt upon this suggestion, even though the predicted molecular weight of the *E. coli* K12 L-serine dehydratase (approximately 48 000) agreed well with the measured molecular weight of the *E. coli* B strain enzyme (52 000). However, a comparison of the measured N-terminal sequence of *E. coli* B L-serine dehydratase (Figure 4.12) with the predicted amino terminus of the *sdaA* gene product shows, with the exception of one unassigned residue (at position 17), a very high level of homology over the first 20 residues of the protein. The similarities between the two sequences suggests that the *sdaA* sequence isolated by Su *et al* (1989) is indeed the structural gene for L-serine dehydratase.

Figure 4.11

The N-Terminal Amino Acid Sequence of the *E. coli* B L-Serine Dehydratase

L-Serine dehydratase (step 8, 150 μg) was prepared and its amino terminus sequenced using the automated Edman Degradation procedure described in Chapter 4, section 4.7.1.

1									10
Met	Ile	Ser	Leu	Phe	Asp	Met	Phe	Lys	Val

									20
Gly	Ile	Gly	Pro	Ser	Ser	?	His	Thr	Val

The elucidation of the amino terminal sequence of the *E. coli* B L-serine dehydratase is also of interest in view of the apparent difference in the position of its initiation codon when compared with that of the *E. coli* K12 enzyme. Figure 4.12 shows the alignment of the N-terminal sequence from purified L-serine dehydratase with the DNA sequence and proposed amino acid sequence of Su *et al* (1989). The deduced sequence for the *E. coli* K12 enzyme appears to begin at position 7 of the *E. coli* B protein sequence, with an ATG start codon. The N-terminal sequence of the *E. coli* B dehydratase protein begins six residues earlier, with a much rarer GTG codon It may be that this apparent anomaly represents a genuine difference in the translational start sites of the two dehydratases, and that the *E. coli* B L-serine dehydratase is infact six amino acids longer than the *E. coli* K12 enzyme . Alternatively, it is also possible, considering the rarity with which GTG start codons occur in *E. coli* (approximately 3 % of the frequency of the ATG sequence, Watson *et al*, 1988), that Su *et al* did not recognise the GTG codon as the initiating methionine for the K12 enzyme and that the start site of the *sdaA* gene is actually some 18 bases earlier than they predicted. If this is so, the N-terminal sequences of the two dehydratases would be identical, at least over the first 20 residues of the protein.

The high, possibly complete, level of homology between the amino terminal sequences of the two dehydratases, coupled with other similarities in their properties, such as a dependence upon for iron and DTT for activity (Newman *et al*, 1985), and a non-requirement for pyridoxal phosphate (Newman and Kapoor, 1980), would suggest that the *E. coli* B L-serine dehydratase shares a quite considerable level of structural homology with the *E. coli* K12 enzyme Thus any notable structural motifs within the primary sequence of the *sdaA* gene product, such as homologies with the sequences of Fe-S-containing proteins, or pyridoxal phosphate-dependent enzymes, would also probably be possessed by the *E. coli* B enzyme. Thus, the sequence of the *E. coli* K12 L-serine dehydratase could be used to scan a protein database for homologies with other proteins, particularly those which contain FeS or pyridoxal phosphate coenzymes. The results of such a search are described in Chapter 8, section 8.1.2.

4.8 The Properties of L-Serine Dehydratase in Permeabilised Cells

It has been shown by a number of workers, that the properties of the L-serine dehydratases in permeabilised whole cells of *E. coli* (Roberts, 1983) and of *Arthrobacter globiformis* (Bridgeland, 1968 and Gannon, 1973) differ considerably from those of the enzyme in cell-free extracts of the same organism.

Figure 4.12

Comparison of the Predicted Amino Acid Sequence of the N-terminus of the *E. coli* K12 *SdaA* Gene Product with the Measured Primary Sequence of the *E. coli* B L-Serine Dehydratase

The N-terminal amino acid sequence of the *E. coli* B L-serine dehydratase was determined as described in Chapter 4, section 4.7.1. The predicted primary sequence of the *E. coli* K12 dehydratase was determined as described by Su *et al* (1989).

Notes

1. Part of the s*daA* amino terminal gene sequence determined by Su *et al*, (1989). Their predicted ribosome binding site, GTGA, and initiation codon, ATG, are shown underlined.

2. The first 20 amino acid of the L-serine dehydratase amino terminus, as predicted by Su *et al* (1989) from the DNA sequence of the s*daA* gene.

3. Alignment of the measured N-terminal sequence from purified L-serine dehydratase with the DNA sequence. An alternative ribosome binding site and initiation codon are shown in bold italics.

```
1    CCTTGTC***AGGA***GTATTATC ***GTG*** ATT AGT CTA TTC GAC ATG TTT AAG
2                                                  1  MET PHE LYS
3                       1  MET ILE SER LEU PHE ASP MET PHE LYS

1     GTG GGG AAT GGT CCC TCA TCT TCC CAT ACC GTA GGG CCT ATG
2     VAL GLY ILE GLY PRO SER SER SER HIS THR VAL GLY PRO MET
3     VAL GLY ILE GLY PRO SER SER  X  HIS THR VAL 20

1     AAG GCA GGT
2     LYS ALA GLY 20
3
```

The most important of these differences included a linear timecourse of activity and a hyperbolic substrate saturation profile, characteristics of serine-activated L-serine dehydratase. It was therefore considered worthwhile to confirm and extend these earlier investigations in the present study.

4.8.1 Time-course of Activity

The timecourse of L-serine dehydratase activity in toluene-treated cells of *E. coli* B was linear, with no lag phase. The order of addition of enzyme or substrate did not affect this linearity (results not shown). These findings are consistent with the work of both Roberts (1983) and Gannon (1973).

L-Serine dehydratase in permeabilised cells was also subject to a substantial activation by ferrous iron and DTT. The timecourse of activity in the presence of these reagents was non-linear, the initial rate of product formation being low and increasing progressively over a period of several minutes until a maximal, linear rate of pyruvate was attained (Figure 4.13). Also shown, for comparison purposes, is the equivalent timecourse produced by the same cell preparation in the absence of Fe^{2+} and DTT. These results reveal that L-serine dehydratase, in whole cells, and therefore fully activated in the serine-sense, is also subject to a further activation by ferrous iron and dithiothreitol.

4.8.2 Substrate Saturation Profile

Substrate saturation curves for L-serine dehydratase in permeabilised cells were determined both in the presence and absence of iron and DTT using the 2,4-dinitrophenylhydrazine assay. The dehydratase showed a hyperbolic substrate saturation curve under both sets of conditions, with $S_{[0.5]}$ values of 3.0 +/- 0.2 mM and 3.2 +/- 0.10 mM L-serine, and Hill coefficients of 1.13 +/- 0.05 and 1.18 +/- 0.10, respectively. The low values of the Hill coefficients, similar to those obtained with serine-activated cell-free enzyme (Chapter 7, section 7.2) are consistent with the suggestion made by Gannon and Roberts that L-serine dehydratase exists in whole cells in the serine-activated state.

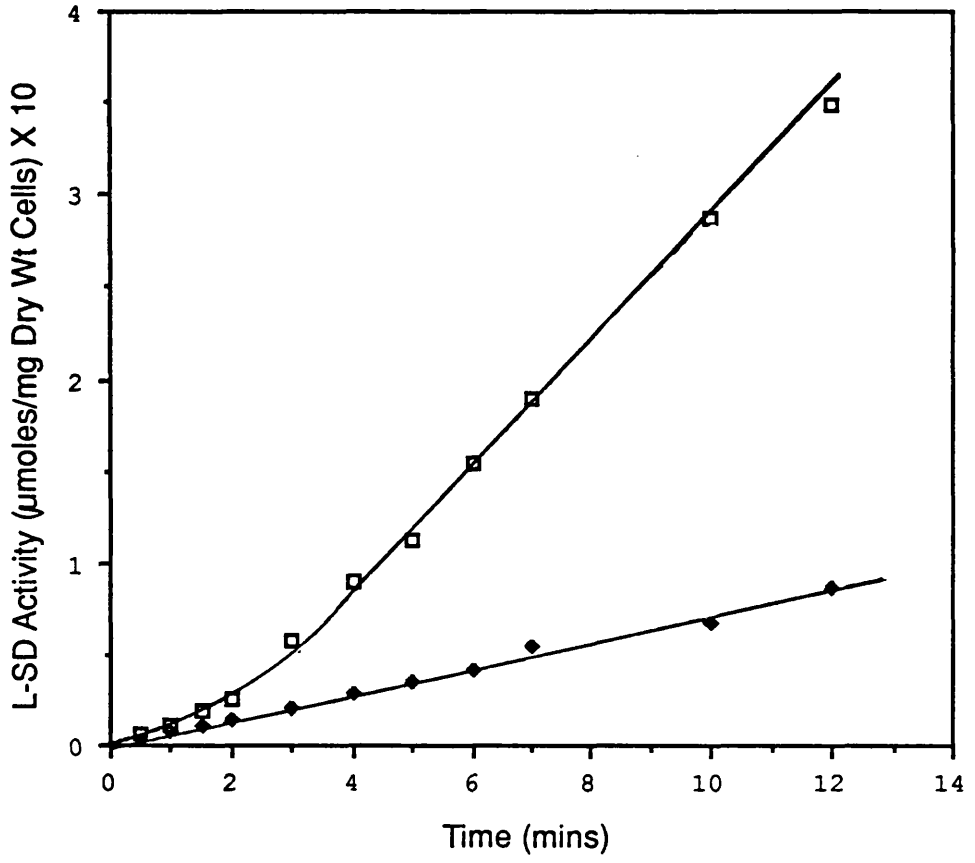


Figure 4.13

The Timecourse of Pyruvate Formation of Toluene-treated Cells in the Presence and Absence of Ferrous Iron and Dithiothreitol

E. coli B was grown overnight on Alfoldi's medium plus 0.2 % glucose. The cell were harvested, washed and toluene-treated as described in Chapter 2, sections 2.3 and 2.5. Assay mixtures (5 ml) were prepared containing 100 mM glycylglycine-NaOH buffer, pH 8.0, 50 mM L-serine, 10 mM L-isoleucine, 1 mM hydroxylamine with, and without 10 mM DTT and 1 mM $FeSO_4$, and equilibrated at 37°C. Toluene-treated cells (2.0 mg dry wt ml^{-1}) were added to the mixtures and samples (0.10 ml) removed at the times shown in the Figure, and assayed for pyruvate using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

□ □ □ □ A Timecourse of pyruvate formation in the presence of Fe and DTT

◆ ◆ ◆ ◆ B Timecourse of pyruvate formation in the absence of Fe and DTT

CHAPTER 5 - PYRIDOXAL PHOSPHATE

5.1 Cofactor Requirements.

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5.5 Spectroscopic Studies of L-Serine Dehydratase.

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Chapter 5

Pyridoxal Phosphate

The L-threonine and D-serine dehydratases of microorganisms, and the L-serine dehydratase of rat liver have been conclusively shown to contain pyridoxal phosphate (PLP) as a prosthetic group (Umbarger, 1973). By analogy, it has been widely assumed that the L-serine-specific dehydratases also contain such a cofactor, although the evidence for this assumption is by no means conclusive. For example, the presence of pyridoxal phosphate was inferred in a study of the L-serine dehydratase from *Streptomyces rimosus* in which the enzyme was found to be inhibited by carbonyl reagents, such as phenylhydrazine and hydroxylamine, even though no actual requirement for PLP was demonstrated (Szentirmai and Horvath, 1961). Only in the case of the L-serine dehydratase from *Clostridium acidiurici*, has direct spectroscopic and fluorimetric evidence been obtained to confirm the presence of pyridoxal phosphate in an L-serine-specific dehydratase (Carter and Sagers, 1972).

Investigations into the nature of the coenzyme in the L-serine dehydratase from *E. coli* have been inconclusive. This dehydratase is not activated by pyridoxal phosphate (Alfoldi *et al*, 1968, Newman and Kapoor 1980) and has a high level of resistance to inhibition by the carbonyl reagent hydroxylamine (Roberts, 1983). These observations would tend to suggest that the enzyme did not contain PLP. However, spectrophotometric and fluorimetric evidence for the presence, or otherwise, of PLP is required for any degree of certainty. Investigations of this kind require homogeneous preparations of protein, which have not been available until the present study. Now that highly purified enzyme is available, it should prove possible to unequivocally determine if the *E. coli* L-serine dehydratase contains pyridoxal phosphate.

5.1 Cofactor Requirements

Many compounds have been reported to stimulate the activity of L-serine dehydratases from microbial sources other than *E. coli*. However, none of the following coenzymes either stimulated or inhibited the activity of either Fe-independent or purified L-serine dehydratase from this organism: ATP, ADP, AMP (all at 1 mM), cyclic AMP (0.1mM), and biotin (0.01 mM). L-Serine dehydratase was not activated by pyridoxal phosphate (0.01 to 1.0 mM), either by preincubation with this cofactor before, during or after activation by Fe^{2+} and DTT, or by its inclusion into continuous or discontinuous assay mixtures. The

addition of pyridoxal phosphate to preparations of Fe-independent L-serine dehydratase from crude extracts also failed to produce any significant increase in the activity of the enzyme. The only cofactor dependence that has been observed during the course of this study is a requirement for ferrous iron and DTT. The characteristics of this requirement are discussed in more detail in Chapter 6.

5.2 The Attempted Resolution of Pyridoxal Phosphate

Attempts were made to resolve pyridoxal phosphate from purified L-serine dehydratase using dialysis against inhibitors of the enzyme, (100mM D-serine, or 100 mM L-cysteine), and a carbonyl reagent (50 mM hydroxylamine), (Table 5.1). The inhibitors were removed from the samples by dialysis against 50 mM glycylglycine-NaOH, pH 7.8, and reactivation was attempted by preincubation with 1 mM pyridoxal phosphate. The results shown in Table 5.1 reveal that dialysis against the inhibitors did not produce a loss of activity markedly below that of the control, and in the case of D-serine, resulted in a noticeable stabilisation. In addition, in none of the samples of dialysed enzyme was pyridoxal phosphate able to restore the activity of the dehydratase. Only treatment with ferrous iron and dithiothreitol was able to achieve this.

These results would appear to indicate that PLP had not been removed from the *E. coli* L-serine dehydratase, a finding that is in marked contrast to the corresponding situation described by Suda and Nakagawa (1965) for the L-serine dehydratase from rat liver. Rapid and complete resolution of this enzyme was achieved by incubation with only 1 mM L-cysteine, followed by a short period of dialysis against water. Full activity was restored to the dehydratase by preincubation with pyridoxal phosphate. In a similar experiment, a 4 hour dialysis against 50 mM L-cysteine was sufficient to cause a 90 % inhibition of the glycine decarboxylase from *Peptococcus glycinophilus* (Klein and Sagers, 1966). Preincubation of the apoenzyme with 0.1 mM pyridoxal phosphate restored all of this lost activity.

5.2. The Effect of Carbonyl Reagents on L-Serine Dehydratase Activity

5.2.1 Hydroxylamine

The action of hydroxylamine on the activity of L-serine dehydratase was measured over a wide range of inhibitor concentrations. The lactate dehydrogenase assay was used since concentrations of hydroxylamine above

Table 5.1

The Attempted Resolution of Pyridoxal Phosphate

Duplicate samples of L-serine dehydratase (step 8, enzyme concentration 0.18 mg ml^{-1}) were dialysed for 2.5 hours at 2 °C against 50 mM glycylglycine-NaOH, pH 7.8 , either alone or containing 100 mM L-cysteine, or 100 mM D-serine, or 50 mM hydroxylamine. After this treatment, the inhibitors were removed from the enzyme samples by a 2 hour period of dialysis at 2 °C, against 50 mM glycylglycine-NaOH, pH 7.8. followed by a 45 minute preincubation at 20 °C with 1 mM pyridoxal phosphate (PLP). Measurements of the activity of the dehydratase before and after treatment with the pyridoxal phosphate were performed in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

Treatment: **Dialysis against:**	**% Activity Remaining:**	
	Before Preincubation with PLP	**After Preincubation with PLP**
50 mM glycylglycine	75	73
100 mM D-serine	86	83
100 mM L-cysteine	68	65
50 mM Hydroxylamine	65	62

0.1 mM caused interference with the 2,4-dinitrophenylhydrazine method of pyruvate estimation (Chapter 2, section 2.6.3). L-Serine dehydratase appears to be very resistant to inactivation by hydroxylamine. Very high levels of this inhibitor were required to bring about significant reduction in the activity of the dehydratase. At a concentration of 40 mM, it showed an inhibition of less than 50 % (Table 5.2). Prolonged preincubation with 15 mM hydroxylamine also failed to produce a significant inactivation of the *E. coli* L-serine dehydratase. After 4 hours incubation with 15 mM hydroxylamine, the enzyme still retained nearly 90 % of its initial activity (Table 5.3).

The considerable tolerance of L-serine dehydratase to hydroxylamine, both to high concentrations of this reagent and to prolonged periods of exposure to it, is in marked contrast to the behaviour of the biosynthetic and degradative L-threonine dehydratases of *E. coli* in the presence of this inhibitor. These enzymes were completely inhibited by a 15 minute incubation incubation with only 0.1 mM hydroxylamine (Umbarger and Brown, 1957). In a similar experiment, the PLP-dependent L-serine dehydratase from rat liver underwent a 94 % loss of activity when assayed in the presence of 6.7 mM hydroxylamine (Selim and Greenberg, 1960).

5.2.2 The Effects of Other Carbonyl Reagents on L-Serine Dehydratase

An examination was made of the sensitivity of L-serine dehydratase to a variety of other carbonyl reagents frequently used in the study of pyridoxal phosphate-containing enzymes. The series of experiments described below examines the effects of a range of semicarbazide hydrochloride and isonicotinic acid hydrazide concentrations when included in assays of L-serine dehydratase activity and investigates the ability of these inhibitors to inactivate the dehydratase following more prolonged exposure. An estimation was also made of the effectiveness with which phenylhydrazine hydrochloride is able to inhibit L-serine dehydratase. Phenylhydrazine absorbs strongly over the 300 to 450 nm range of the spectrum and at concentrations above about 3 mM, caused severe interference with the methods used to measure the activity of the enzyme. As a consequence it was not possible to investigate the effects of higher levels of phenylhydrazine on L-serine dehydratase, and studies of this carbonyl inhibitor were limited to an examination of the effects of long-term preincubation.

Table 5.2

The Inhibition of L-Serine Dehydratase by Hydroxylamine

L-Serine dehydratase (step 8) was iron-activated, as described in Chapter 2, section 2.6.4. Samples of the activated enzyme (0.05 units) were assayed by the lactate dehydrogenase-coupled assay (section 2.6.2) at 37 °C with 50 mM L-serine and the concentrations of hydroxylamine shown in the table.

Concentration of Hydroxylamine (mM)	% Activity Remaining
0	100
3	100
5	99
8	90
10	91
20	79
30	63
40	58
50	49
60	43

Table 5.3

The Inhibition of L-Serine Dehydratase by Long-term Preincubation with Hydroxylamine

L-Serine dehydratase (step 8, enzyme concentration 0.1 mg ml^{-1}) was preincubated at 0 °C with 15 mM hydroxylamine, pH 7.0. At the times shown in the Table, 10 µl aliquots of the preincubation mixture were withdrawn and added to assay mixtures containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method (Chapter 2, section 2.6.1). The results shown in the Table have been corrected for losses due to enzyme instability determined from a control sample of dehydratase preincubated in buffer only.

Length of Preincubation (minutes)	**% Activity Remaining**
0	100
30	98
60	95
90	93
120	92
150	92
180	90
240	89

5.2.2.1 Isonicotinic Acid Hydrazide

The effects of isonicotinic acid hydrazide (IAH) on L-serine dehydratase activity were measured over a wide range of IAH concentrations. The dehydratase was found to be very resistant to this inhibitor. When assayed with 50 mM isonicotinic acid hydrazide, L-serine dehydratase showed an inhibition of less than 15 % (Table 5.4, column A). In contrast, when the L-serine dehydratase from rat liver was incubated with 6.7 mM isonicotinic acid hydrazide a 42 % inhibition of activity was observed (Selim and Greenberg, 1960). At this concentration the *E. coli* B L-serine dehydratase still retained more 95 % of its original activity.

5.2.2.2 Semicarbazide Hydrochloride

Treatment of purified L-serine dehydratase with semicarbazide hydrochloride resulted in very little inhibition of L-serine dehydratase. When increasing concentrations of semicarbazide were added to assays of the dehydratase, a significant reduction in the activity of the enzyme was only seen at high inhibitor concentrations. A semicarbazide concentration of 40 mM caused only a 17 % reduction in the activity of the dehydratase (Table 5.4, column B)

5.2.2.3 The Effects of Long-term Preincubation with Semicarbazide PhenylHydrazine Hydrochloride, and Isonicotinic Acid Hydrazide on L-Serine Dehydratase Activity

An examination was also made of the effects of long-term exposure of L-serine dehydratase to semicarbazide hydrochloride (25 mM), isonicotinic acid hydrazide (25 mM) and phenylhydrazine hydrochloride (15 mM). The dehydratase was preincubated with each inhibitor for time intervals of up to 4 hours. The results, shown in Table 5.5, show that little significant inhibition of the dehydratase has occurred after even prolonged incubation with these carbonyl reagents. Four hours treatment with isonicotinic acid hydrazide or semicarbazide, failed to produce an inhibition of more than 10 % or 14 %, respectively. L-Serine dehydratase treated with 15 mM phenylhydrazine lost rather more activity over the course of the preincubation, but at the end of the experiment was still in possession of nearly 75 % of its initial activity.

Table 5.4

The Inhibition of L-Serine Dehydratase Activity by Semicarbazide and Isonicotinic Acid Hydrazide

L-Serine dehydratase (step 8, 0.10 units) was assayed at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine, 10 mM DTT, 1 mM $FeSO_4$ and the concentrations of semicarbazide hydrochloride or isonicotinic acid hydrazide shown in the Table. Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

Concentration of Inhibitor (mM)	% Uninhibited Activity Remaining:	
	A	B
	Semicarbazide	Isonicotinic Acid Hydrazide
1	99	100
3	100	99
5	98	98
7	98	96
10	97	95
15	96	96
20	95	93
25	93	91
30	91	90
40	83	86
50	74	83

Table 5.5

The Inhibition of L-Serine Dehydratase After Long-term Preincubation with Semicarbazide, Isonicotinic Acid Hydrazide and Phenylhydrazine

Duplicate samples of L-serine dehydratase (step 8, enzyme concentration 0.12 mg ml^{-1}) were incubated at 0 °C with either 25 mM semicarbazide hydrochloride, 25 mM isonicotinic acid hydrazide (IAH), or 15 mM phenylhydrazine hydrochloride. At the times indicated in the Table, samples of the preincubation mixtures (10 µl) were withdrawn and assayed at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1. The results have been corrected for losses due to enzyme instability and, in the case of phenylhydrazine, carryover of inhibitor.

Length of Preincubation (minutes)	**% Activity Remaining:**		
	Semicarbazide	**IAH**	**Phenylhydrazine**
0	100	100	100
30	100	100	98
60	96	96	92
90	95	98	85
120	92	97	82
150	91	95	78
180	90	96	76
240	86	92	73

5.3 The Effects of Sodium Borohydride Reduction on the Activity of L-Serine Dehydratase

Sodium borohydride reduces Schiff bases formed between pyridoxal phosphate, or other carbonyl-containing cofactors, and the ε-amino group of a lysyl residue of the apoenzyme to a stable secondary amine. This interferes with the transimination process of the PLP-catalysed reaction, essential for the activity of the enzyme. Consequently, an examination was made of the effects of $NaBH_4$ on L-serine dehydratase activity.

Samples of L-Serine dehydratase, pre-treated with Fe^{2+} and DTT or D-serine or both, were preincubated with 10 mM sodium borohydride for time intervals of up to 30 minutes, as described in Table 5.6. At the times shown, aliquots of the incubation medium (10 µl) were withdrawn for assay in 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$ (carryover of borohydride in the assay mixtures 0.1 mM). The results shown in Table 5.6 reveal that L-serine dehydratase was subject to a time-dependent inactivation by sodium borohydride. The final degree of this inactivation was dependent on the nature of the pre-treatment of the dehydratase: enzyme that had been activated by preincubation with iron and DTT, or incubated with D-serine retained more of its initial activity than dehydratase which had undergone neither of these treatments. Optimal protection against inhibition by the borohydride was provided by pre-treatment with iron and dithiothreitol, followed by activation by D-serine. Enzyme treated in such a manner showed the greatest stability and least loss of activity.

Although L-serine dehydratase was subject to inactivation by sodium borohydride, the rate of this inhibition is slow when compared with the speed with which PLP-containing enzymes are inactivated by this reagent . For example, the homoserine deaminase-cystathionase from rat liver was completely inactivated by a 5 minute incubation with 5 mM $NaBH_4$ (Matsuo and Greenberg, 1958). Over this same time interval, L-serine dehydratase, exposed to 10 mM borohydride, lost only 18 % of its initial activity.

5.3 Spectroscopic Studies of L-Serine Dehydratase

Pyridoxal phosphate-containing proteins possess very distinctive UV/visible spectra. An absorption maximum in the range 410 to 425 nm is usually indicative of the presence of this cofactor (Snell and Dimari, 1970). Consequently, a search was made for pyridoxal phosphate in the purified L-serine dehydratase using UV/visible spectroscopy.

Table 5.6

The Effect of Sodium Borohydride Reduction on L-Serine Dehydratase Activity

Samples of L-serine dehydratase (step 8, enzyme concentration 0.12 mg ml^{-1}) were subjected to the pre-treatments described in the Table (see below). Sodium borohydride was then added, to a final concentration of 15 mM. At the times shown in the Table, 10 µl aliquots of the preincubation mixtures were removed for assay at 37 °C in mixtures containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$, according to the method described in Chapter 2, section 2.6.1.

Enzyme Pre-treatment	**Experimental Conditions**
None	No pre-treatments
Fe^{2+} and DTT	Dehydratase iron-activated by preincubation with 1 mM $FeSO_4$ and 10 mM DTT, as described in section 2.6.4.
D-Serine	20 minute preincubation of unactivated dehydratase with 100 mM D-serine.
Fe^{2+} and DTT, D-Serine	20 minute preincubation of iron-activated dehydratase, with 100 mM D-serine.

Preincubation (minutes)	% Activity Remaining:			
	None	Fe^{2+} and DTT	D-Serine	Fe^{2+} and DTT, D-Serine
0	100	100	100	100
5	74	87	86	91
10	64	74	74	81
20	56	68	67	75
30	50	65	62	69

5.3.1 UV/Visible Spectroscopy

A sample of unactivated L-serine dehydratase (Step 8, specific activity 1190 μmoles pyruvate min^{-1} mg^{-1} enzyme, protein concentration 0.65 mg ml^{-1}) was scanned over the range 240 to 600 nm (Figure 5.1a). Examination of the spectrum did not reveal the presence of absorption maxima within the 410 to 425 nm range; the only distinct peak seen was at 280 nm, which was probably due to aromatic amino acid residues within the protein. Incubation of the enzyme with 1 mM pyridoxal phosphate, followed by centrifugation of the protein through a sephadex G-100 spun column to remove excess cofactor, did not produce any change in either the activity or spectral properties of the dehydratase (results not shown). When the dehydratase was preincubated with ferrous iron and DTT (Chapter 2, section 2.6.4) a small, but consistently observed increase in absorbance over the 400 to 500 nm range of the spectrum was produced (Figure 5.1b). However, the change in spectral properties observed after iron-activation did not include the appearance of the 410 to 425 nm peak typical of PLP-containing enzymes.

The UV/visible spectrum of L-serine dehydratase did not contain any features which would indicate the presence of pyridoxal phosphate. This observation is in marked contrast to the D-serine dehydratase of *E. coli*, which has a pronounced absorbance peak at 415 nm representing the aldimine linkage between the formyl group of PLP and the ε-NH_2 group of lysine 118 of the enzyme (Marceau *et al*, 1988). In many respects, D-serine dehydratase behaves as a model PLP enzyme. Pyridoxal phosphate may be removed from the holodehydratase by dialysis against D- or L-cysteine, resulting in both a loss of enzymic activity and the 415 nm peak. Full restoration of the spectrum and catalytic activity can be readily achieved by preincubation with pyridoxal phosphate (Dowhan and Snell, 1970). The spectra of the PLP-dependent L-serine dehydratase from rat liver (Nakagawa and Kimura, 1969) and the biosynthetic L-threonine dehydratase from *Salmonella typhimurium* (Burns and Zarlengo, 1968) both contain prominent absorption maxima between 410 and 420 nm indicative of the presence of pyridoxal phosphate.

5.3.2 Fluorescence Spectroscopy

When present in proteins, the fluorescence of pyridoxal phosphate is characteristically activated at either 340 or 420 nm, with resultant emission at 390 and 520 nm, respectively.

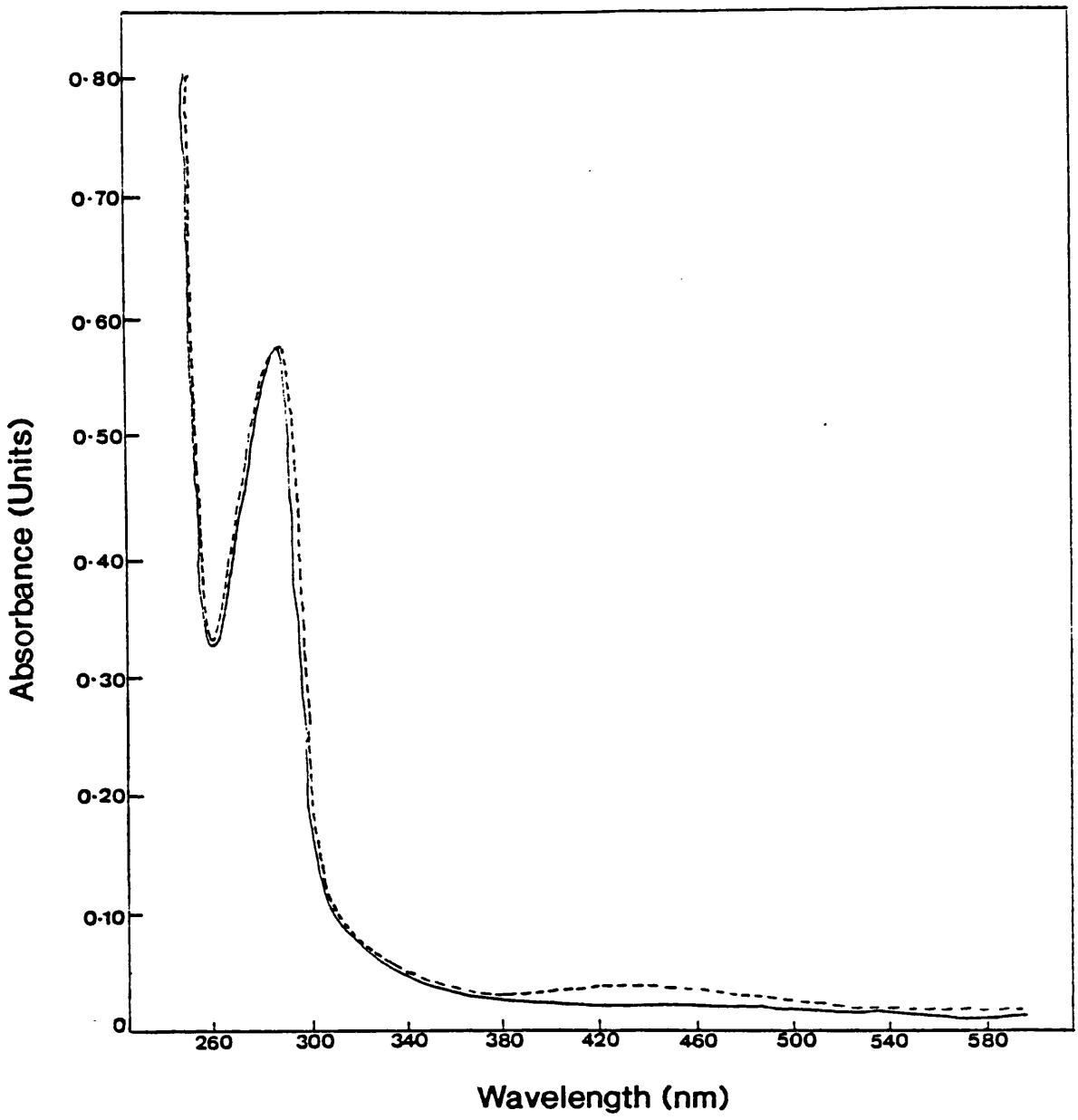


Figure 5.1

The UV/Visisble Spectrum of L-Serine Dehydratase

The UV/visible spectrum of L-Serine dehydratase (step 8, protein concentration 0.68 mg ml^{-1}) was scanned from 260 to 600 nm, before and after iron-activation (Chapter 2, section 2.6.4). Spectra were obtained using 0.10 ml 1 cm pathlength quartz cuvettes.

———— **Figure 5.1a** Spectrum of unactivated L-Serine Dehydratase.

- - - - - - - - - **Figure 5.1b** Spectrum of Iron-activated L-Serine Dehydratase.

The fluorescence spectrum of L-serine dehydratase (Step 8 protein, 0.25 mg ml^{-1}, specific activity 1100 μmoles pyruvate min^{-1} mg^{-1}) was investigated under a variety of conditions, with enzyme that was unactivated, with enzyme that had been activated by iron and DTT, and in the presence of substrate (100 mM L-serine) or substrate analogue (150 mM D-serine). Excitation at either 340 or 420 nm of enzyme treated in these ways, did not produce the emission at 390 or 520 nm typical of protein-bound pyridoxal phosphate. It was possible that the *E. coli* L-serine dehydratase does not absorb, or emit, at these wavelengths, and so an examination of the fluorescence properties of the enzyme was made over a wider range of excitation and emisson wavelengths (λ_{ex} extending from 300 to 450 nm, in 15 nm increments and λ_{em} from 320 to 550 nm, also in 15 nm increases). The only significant fluorescence that it was possible to detect, was that probably due to intrinsic tryptophan and tyrosine residues.

5.4 Discussion

No evidence has been obtained during this study to suggest that the L-serine dehydratase from *E. coli* utilises pyridoxal phosphate as cofactor. The enzyme showed none of the expected responses to a range of investigative procedures used to test for the presence of this cofactor. The dehydratase was not activated by pyridoxal phosphate, and was not resolved by dialysis against inhibitors of the enzyme. The only cofactor requirement that it was possible to demonstrate was for ferrous iron and DTT.

L-Serine dehydratase exhibited a high level of tolerance to a variety of carbonyl agents commonly used in the study of PLP-dependent proteins. A concentration of hydroxylamine which produced a 50 % inhibition of the *E. coli* L-serine dehydratase, was a thousand-fold greater than that required to cause a total inhibition of the pyridoxal phosphate-dependent L-threonine dehydratases (Umbarger and Brown, 1957).

The inhibition observed with sodium borohydride is insufficient evidence to allow the suggestion that L-serine dehydratase contains pyridoxal phosphate. It is possible that the inactivation caused by the borohydride represents the effect of non-specific reduction of essential structural elements within the dehydratase protein, such as disulphide linkages.

The most convincing evidence for the absence of pyridoxal phosphate comes from the study of the UV/visible and fluorescence spectra of the enzyme. The dehydratase did not show an absorption maximum in the 410 to 425 nm range in

which all pyridoxal phosphate-containing serine or threonine dehydratases have been shown to absorb. If pyridoxal phosphate had been present, a preparation of L-serine dehydratase of the protein concentration in used in Figure 5.1a would have had an absorbance at 415 - 435 nm of at least 0.060 absorbance units in magnitude. Further, the dehydratase did not show any of the fluorescence properties characteristic of enzymes containing this cofactor. The inability of these spectroscopic methods to detect pyridoxal phosphate in L-serine dehydratase is not likely to be a consequence of the concentration of protein available (0.65 mg ml^{-1}). The concentrations of L-serine dehydratase used in the spectroscopic studies, particularly for the fluorescence experiments, were comparable with equivalent experiments performed with well characterised PLP-enzymes, such as the D-serine dehydratase of *E. coli* (Marceau *et al*, 1988).

On the basis of the results described above it appears probable that L-serine dehydratase does not contain pyridoxal phosphate. It is also unlikely, considering the relative insensitivity of L-serine dehydratase to carbonyl reagents, and its slow inhibition by sodium borohydride, that the dehydratase contains a pyridoxal phosphate-like keto acid cofactor (Chapter 1, section 1.2.1.5). For example, the L-histidine deaminase (ammonia lyase) of higher plants, which contains a dehydroalanine prosthetic group, was fully inactivated by a 15 minute preincubation with 3 mM sodium borohydride (Givot *et al,* 1969); the histidine decarboxylase from *Lactobacillus* 30a, which has a pyruvate keto acid cofactor, was completely inhibited by a 6 minute incubation with 1.5 mM phenylhydrazine and by a 2 minute treatment with 20 mM borohydride (Riley and Snell, 1968).

The characteristics of the iron requirement of the *E. coli* L-serine dehydratase suggest that this enzyme may employ a coenzyme quite unlike that found in the serine or threonine dehydratases. The possible nature of this cofactor is the subject of the following chapter.

CHAPTER 6 - STUDIES OF THE IRON-ACTIVATION OF L-SERINE DEHYDRATASE.

6.8 Spectroscopic Studies of L-Serine Dehydratase

6.8.1 UV/Visible Spectrum of L-Serine Dehydratase

6.8 2 The EPR Spectrum of L-Serine Dehydratase.

6.9 Discussion.

Chapter 6

Studies of the Iron-activation of L-Serine Dehydratase

A requirement for cations has been demonstrated for most of the microbial L-serine-specific dehydratases. The L-serine dehydratase from *A. globiformis* required mono- or divalent cations such as Mg^{2+} or K^{+} for activity (Gannon *et al*, 1977), while the enzyme from *Cl. acidiurici* was shown to have an absolute and specific requirement for ferrous iron and dithiothreitol (Carter and Sagers, 1973). Until the work of Newman *et al* (1985) and the present study, the importance of cations for the activity of the *E. coli* L-serine dehydratase was uncertain. Roberts (1983) had shown that the effect of cations on the activity and stability of the *E. coli* B enzyme was small. However, later work by Newman *et al* (1985) demonstrated that the activity of the L-serine dehydratase in a crude extract of *E. coli* K12 could be protected and increased by incubation with $FeSO_4$ and DTT. This observation prompted a re-examination of the stability of the *E. coli* B L-serine dehydratase, which led to the realisation that a large proportion of the apparent instability of this enzyme was a consequence of the loss of an essential cofactor replaceable, *in vitro*, by ferrous iron and dithiothreitol (the experiments which led to this discovery are described in more detail in chapter 3, section 3.1).

The very high degree of specificity of the *E.coli* L-serine dehydratase for iron and a reducing agent was seen to be similar to that shown by a number of other (de)hydratases, such as aconitase (Kennedy *et al*, 1983), maleic acid hydratase (Dreyer, 1985) and 6-phosphogluconate dehydratase (Scopes and Griffiths-Smith, 1984), all of which had been shown to contain one or more FeS centres. This suggested that L-serine dehydratase may also contain an iron sulphur centre, and so a series of investigations were undertaken to determine the role of iron in the activation of L-serine dehydratase, concentrating initially on the possibility that an FeS centre may be present..

6.1. Iron and Reducing Agent Requirement

6.1.1 Iron Requirement

The specificity of the requirement of L-serine dehydratase for Fe was demonstrated by measuring the activity of enzyme assayed in 1 ml mixtures containing 100 mM glycylglycine-NaOH buffer, pH 8.0, 50 mM L-serine, 10 mM DTT and the metals shown below (all at 1 mM, final concentration).

Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method of estimation (Chapter 2, section 2.6.1).

Mn^{2+} , Ca^{2+} , Mg^{2+} , Co^{2+} , Ni^{2+} , Cr^{2+} , Cu^{2+} , Zn^{2+} , Na^{+} , K^{+}
Li^{+} , Rb^{+} , Cs^{+} , Pb^{2+} , Cd^{2+}

None of the cations shown were able to act as substitutes for Fe. Further experiments were performed to determine the effects of these metals on L-serine dehydratase activity when included in assay mixtures containing 1 mM $FeSO_4$, and glycylglycine-NaOH and DTT at the concentrations stated above. Several metals were found to inhibit the dehydratase: Zn, Pb, and Cu caused a complete and irreversible inactivation of the enzyme, while Cd was moderately inhibitory, causing, on average, a 25 to 50 % reduction in activity. The remainder either very slightly stimulated L-serine dehydratase, or had no effect on the enzyme.

Provided that dithiothreitol (10 mM) was present in the activation or assay medium, the activation of L-serine dehydratase was also possible with ferric iron, supplied in the form of $FeCl_3$. However, the requirement of dehydratase for Fe was more routinely met using $FeSO_4$.

6.1.2 Reducing Agent Requirement

The requirement of L-serine dehydratase for a reducing agent was also quite specific. None of the following reducing agents (examined at concentrations ranging from 0.1 to 20 mM) could act as a substitute for DTT (10 mM) when included in assay media containing 100 mM glycylglycine-NaOH, 50 mM L-serine and 1 mM $FeSO_4$:

2-Mercaptoethanol , Sodium Dithionite , Glutathione , L-Cysteine,
2,3-Dimercaptopropanol , Na_2S

Sodium ascorbate (20 mM) was able to produce a limited degree of activation of the dehydratase when included in assay mixtures containg 1 mM FeSO4 (approximately 15 % of the level of activity attainable in the presence of 10 mM DTT). However, the isomer of dithiothreitol, dithioerythritol; (DTE), appeared to be as effective as DTT in its ability to activate the dehydratase. Enzyme preincubated with 10 mM DTE, 100 mM glycylglycine-NaOH, pH 8.0 and 1 mM

$FeSO_4$, as described in Chapter 2, section 2.6.4, achieved the same specific activity at maximal activation as enzyme activated in the presence of an equal concentration of DTT

A number of physiologically occurring reducing agents were also tested for their ability to replace DTT. NADH or NADPH (0.10 to 10 mg ml^{-1}) alone, or in conjunction with thioredoxin (0.5 mg ml^{-1}), were unable to activate L-serine dehydratase. This is consistent with the experiments described later in section 6.1.4, which show that DTT alone is unable to activate the dehydratase. However, in the presence of added iron (1 mM $FeSO_4$), NADPH and NADH (10 mg ml^{-1}) were able to achieve a partial activation of the enzyme of up to 15 % of the activity attained in the presence of 10 mM DTT. The addition of thioredoxin (0.5 mg ml^{-1}) to assay mixtures containing 10 mg ml^{-1} NAD(P)H and 1 mM Fe^{2+} did not result in any further activation of the dehydratase.

6.1.3 Sodium Dithionite

Sodium dithionite, frequently used to reduce FeS-containing centres, appeared to have a dual effect on L-serine dehydratase activity. When included in assay mixtures containing 1 mM ferrous sulphate and 10 mM DTT, sodium dithionite concentrations of between 0.1 and 1.0 mM caused a 20 to 25 % stimulation of the activity of the dehydratase. However, higher concentrations of the dithionite caused a concentration-dependent inhibition of enzyme activity, with a 20 % inhibition occurring at 10 mM dithionite.

A similar phenomenon of both activation and inhibition was observed when an investigation was made of the effects of a more prolonged exposure of L-serine dehydratase to sodium dithionite. Non iron-activated, purified L-serine dehydratase was preincubated with sodium dithionite for time intervals of up to 45 minutes, and then assayed in media containing iron and dithiothreitol, as described in Chapter 2, section 2.6.1. The results of this experiment (Figure 6.1) show that the reduction of L-serine dehydratase by sodium dithionite appeared to occur in two distinct phases. During the first phase a slow increase in the activity of the dehydratase was observed, becoming maximal after about 5 or 6 minutes. This peak of activation (equivalent to a 30 % stimulation of the initial activity of the enzyme) was maintained for only a short interval of 2 to 3 minutes duration, and was followed by a second phase during which the activity of the dehydratase decayed with time. By the end of the 45 minute preincubation, L-serine dehydratase had lost nearly 90 % of its initial activity. This lost activity was not restored by Fe^{2+} and dithiothreitol.

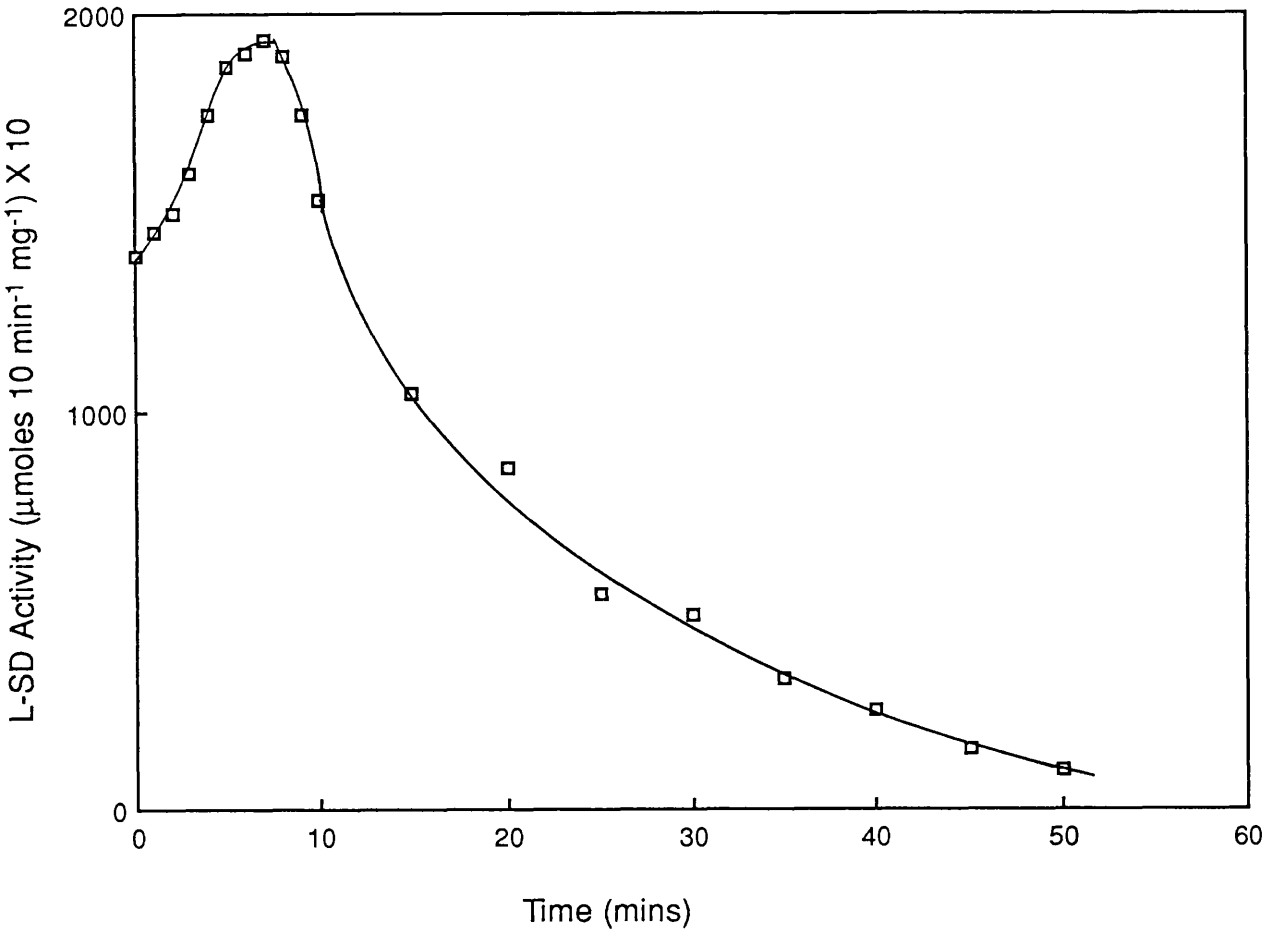


Figure 6.1

The Effect of Preincubation with Sodium Dithionite on the Activity of L-Serine Dehydratase

L-Serine dehydratase (step 8, protein concentration 0.13 mg ml^{-1}) was incubated at 0 °C with 10 mM sodium dithionite. At the times shown in the Figure, samples (10 µl) of the preincubation mixture were assayed at 37 °C in mixtures containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$ Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1

The reaction of L-serine dehydratase towards sodium dithionite is in marked contrast to the behaviour shown by this enzyme when undergoing reduction by sodium borohydride, when the only trend observed was that of a time-dependent loss of activity (Chapter 5, section 5.3). The dual pattern of activation followed by inhibition produced by preincubation of the dehydratase with sodium dithionite was not seen when the enzyme was incubated with DTT alone, which was only able to activate L-serine dehydratase in the presence of iron.

6.1.4 Both iron and DTT are required for activation

Unlike the aconitase from beef heart which may be partially activated by anaerobic incubation with reducing agents alone (Chapter 1, section 1.4.1), the L-serine dehydratase from *E. coli* required the presence of both iron and DTT for activity (Table 6.1). However, once activation by iron and dithiothreitol was complete, the dehydratase could be transferred from the activation mixture into an assay medium lacking either factor, or both, and still remain active. Table 6.1 demonstrates that activation of L-serine dehydratase could be also be achieved when Fe^{2+} and DTT were included in the assay mixtures. This is in contrast to the results obtained by Carter and Sagers (1972) when the analogous experiment was performed with the L-serine dehydratase from *Cl. acidiurici*. These workers found that Fe^{2+} and DTT were not able to activate the dehydratase when present in the assay medium; enzyme activity was only observed with enzyme that had been first been activated by preincubation with these reagents.

The concentrations of Fe^{2+} and dithiothreitol which were optimal for the activation process were determined by examining the effects of different concentrations of iron and DTT on the activity of L-serine dehydratase. This was achieved by measuring the activity of enzyme assayed in mixtures in which the concentration of ferrous iron was held constant, and the concentration of DTT varied, and *vice-versa* (Figure 6.2). It can be seen that when the concentration of the DTT was held constant (at 10 mM), maximal activation of the dehydratase was possible with $FeSO_4$ concentrations as low as 0.4 mM. Increasing the concentration of the iron beyond 1 mM did not result in any further activation of the dehydratase. Still higher concentrations of iron, 2 mM and 3 mM, resulted in an inhibition of the activity of the enzyme. When the concentration of the iron was held constant (at 1 mM), maximal activation was produced by 8 mM DTT. Further increases in the concentration of the reducing agent did not produce an increase in the level of activation of the dehydratase, and actually proved to be inhibitory.

Table 6.1

The Correlation of Enzyme Activity with the Presence or Absence of Iron and Dithiothreitol in the Activation and Reaction Mixtures

L-Serine dehydratase (Step 8, enzyme concentration 0.10 mg ml^{-1}) was incubated for 25 minutes at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 and the additions shown in column (a). Samples of each incubation mixture (10 µl, 0.2 units) were then assayed in 100 mM glycylglycine, 50 mM L-serine, pH 8.0 and the additions shown in column (b). Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method described in Chapter 2, section 2.6.1 The concentrations of $FeSO_4$ ('Fe') and dithiothreitol ('DTT') used in the activation and reaction mixtures were 1 and 10 mM, respectively.

'+' : presence of 1 mM $FeSO_4$ or 10 mM DTT
'-' : absence of 1 mM $FeSO_4$ or 10 mM DTT

(a) Activation Mixture		(b) Assay Mixture		L-Serine Dehydratase Activity (µmoles Pyruvate 10 min^{-1} mg enzyme) X 10
Fe	**DTT**	**Fe**	**DTT**	
+	+	+	+	922.1
+	+	+	-	868.8
+	+	-	+	869.5
+	+	-	-	868.1
+	-	+	+	849.2
+	-	+	-	0
+	-	-	+	0
+	-	-	-	0
-	+	+	+	861.8
-	+	+	-	0
-	+	-	+	0
-	+	-	-	0
-	-	+	+	869.6
-	-	-	-	0
-	-	-	+	0
-	-	-	-	0

A similar experiment was performed to examine the effects of variations in iron and DTT concentration on the level of activation attained by dehydratase preincubated with these reagents in the absence of L-serine, as described in Chapter 2, section 2.6.4. The results obtained showed a similar trend, although the minimum iron concentration required for maximal activation was somewhat higher, at 0.5 - 0.7 mM. For this reason, concentrations of $FeSO_4$ and DTT of 1 and 10 mM were chosen for the routine activation and assay of L-serine dehydratase activity.

6.2 Activation by Iron and DTT is Slow

6.2.1 The Timecourse of L-Serine Dehydratase Activity in the Presence of Ferrous Iron and Dithiothreitol

The timecourse of pyruvate formation of L-serine dehydratase assayed in the presence of ferrous iron and DTT was non linear, this non-linearity taking the form of an initial lag phase, during which the activity of the enzyme increased progressively over a period of several minutes to reach a final constant rate (Figure 6.3 curve A). L-Serine dehydratase which was assayed in the absence of iron and DTT produced no pyruvate (curve B).

This initial non-linear phase was seen in the timecourses of L-serine dehydratase assayed in iron-containing media in both whole, permeabilised cells (Chapter 4, section 4.8.1) and cell-free enzyme preparations from all stages of the purification, whether inactive or Fe-independent. However, when L-serine dehydratase was first activated by preincubation with 1 mM Fe^{2+} and 10 mM dithiothreitol, as described in Chapter 2, section 2.6.4, and then added to assay media containing Fe^{2+} plus DTT, there was only a small degree of further activation of the dehydratase, and the lag phase for this timecourse of activity was of a shorter duration (Figure 6.4, curve A). The equivalent timecourse of iron-activated enzyme in assay media which did not contain iron or DTT appeared to be linear (Figure 6.4, curve B). This is in apparent disagreement with the timecourse of activity of iron-activated L-serine dehydratase determined by the lactate dehydrogenase-coupled method, which was non-linear, a consequence of the activation of the dehydratase by its substrate (Chapter 4, section 4.2 and Chapter 7, section 7.2). This apparent conflict may be a consequence of the time delays in the recording process for each type of assay. The activation of L-serine dehydratase by L-serine is markedly influenced by temperature (Roberts, 1983). At the temperature at which the enzyme is assayed

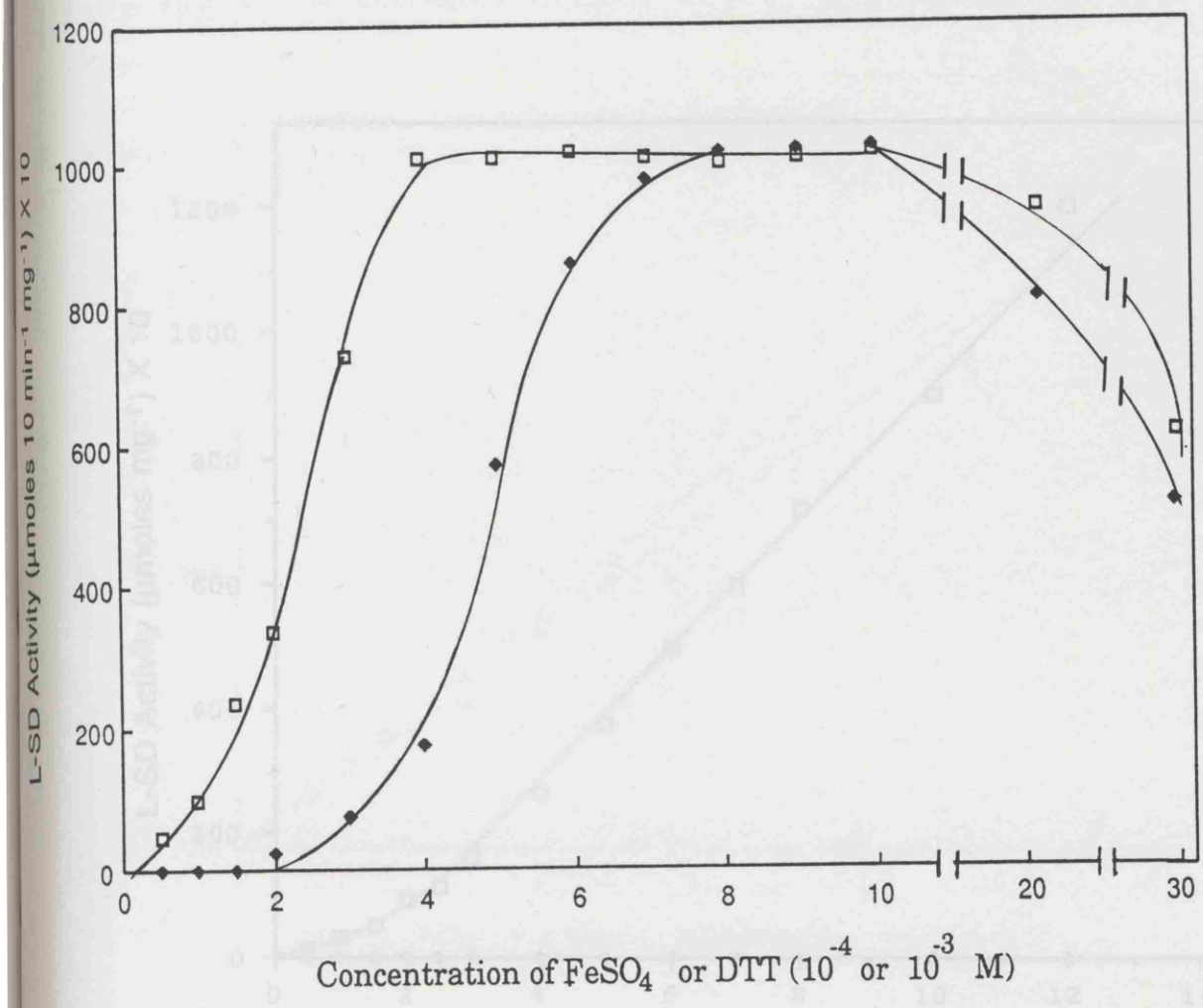


Figure 6.2

The Effects of Variation in the Concentrations of Dithiothreitol and Ferrous Sulphate on the Activity of L-Serine Dehydratase

L-Serine dehydratase (step 8, 0.2 units) was assayed either in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine, 10 mM DTT and the concentrations of $FeSO_4$ shown in the Figure or 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 1 mM $FeSO_4$ and the concentrations of dithiothreitol shown in the Figure. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

• • • • A DTT held constant (10 mM), $FeSO_4$ varied.

▫ ▫ ▫ ▫ B $FeSO_4$ held constant (1mM), DTT varied.

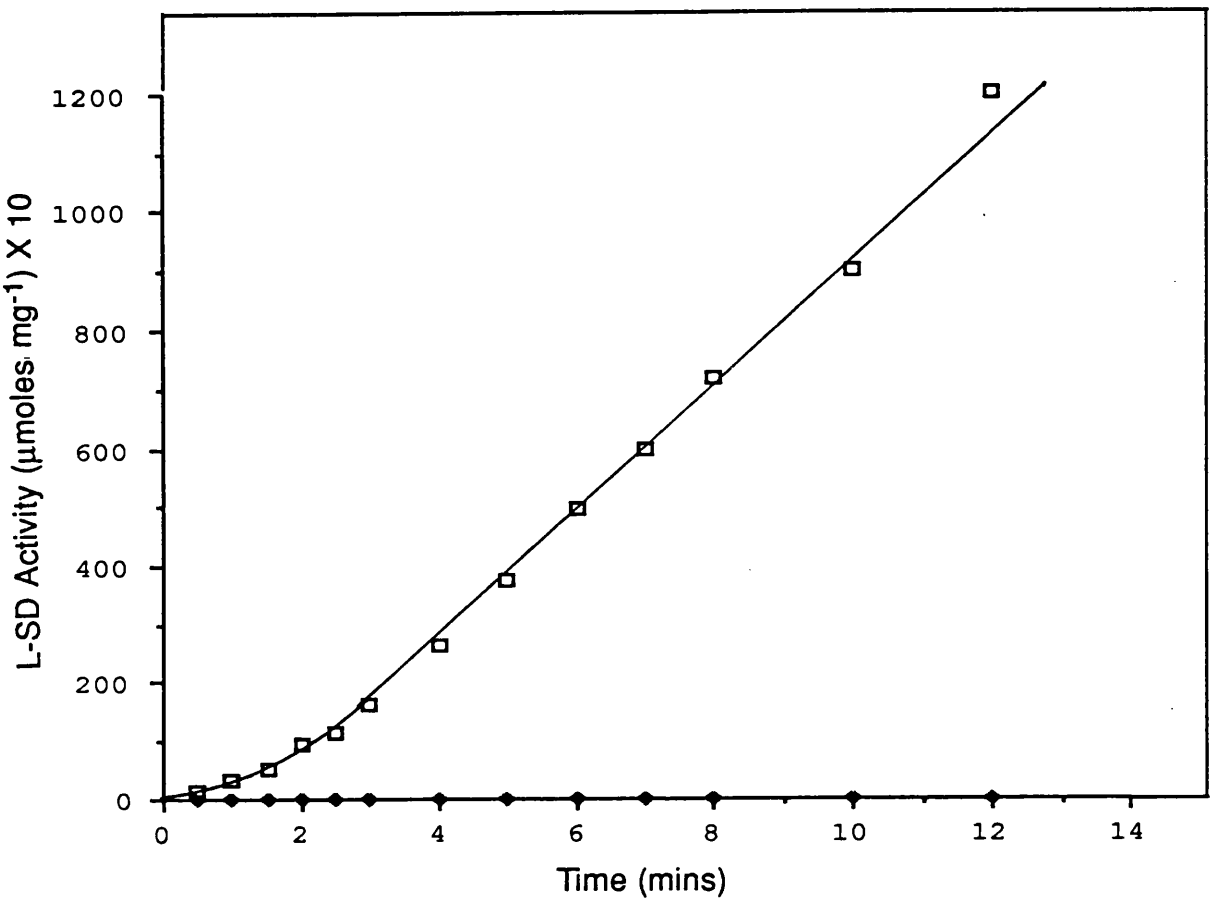


Figure 6.3

The Timecourse of Pyruvate Formation of L-Serine Dehydratase Assayed in the Presence and Absence of Ferrous Iron and Dithiothreitol

L-Serine dehydratase (step 8, 0.6 units) was added to assay mixtures (3 ml) equilibrated at 37 °C containing 100 mM glycylglycine-NaOH buffer, pH 8.0 containing 50 mM L-serine with, and without, additions of 10 mM DTT and 1 mM $FeSO_4$. At the times shown in the figure, 0.1 ml samples of the assay mixtures were removed and added to 0.9 ml of water and 0.33 ml of 2,4-dinitrophenylhydrazine and measured for pyruvate content, as described in Chapter 2, section 2.6.1.

□ □ □ □ A Timecourse of pyruvate formation in the presence of $FeSO_4$ and DTT
(µmoles pyruvate mg^{-1}) X 10

◆ ◆ ◆ ◆ B Timecourse of pyruvate formation in the absence of $FeSO_4$ and DTT
(µmoles pyruvate mg^{-1}) X 10

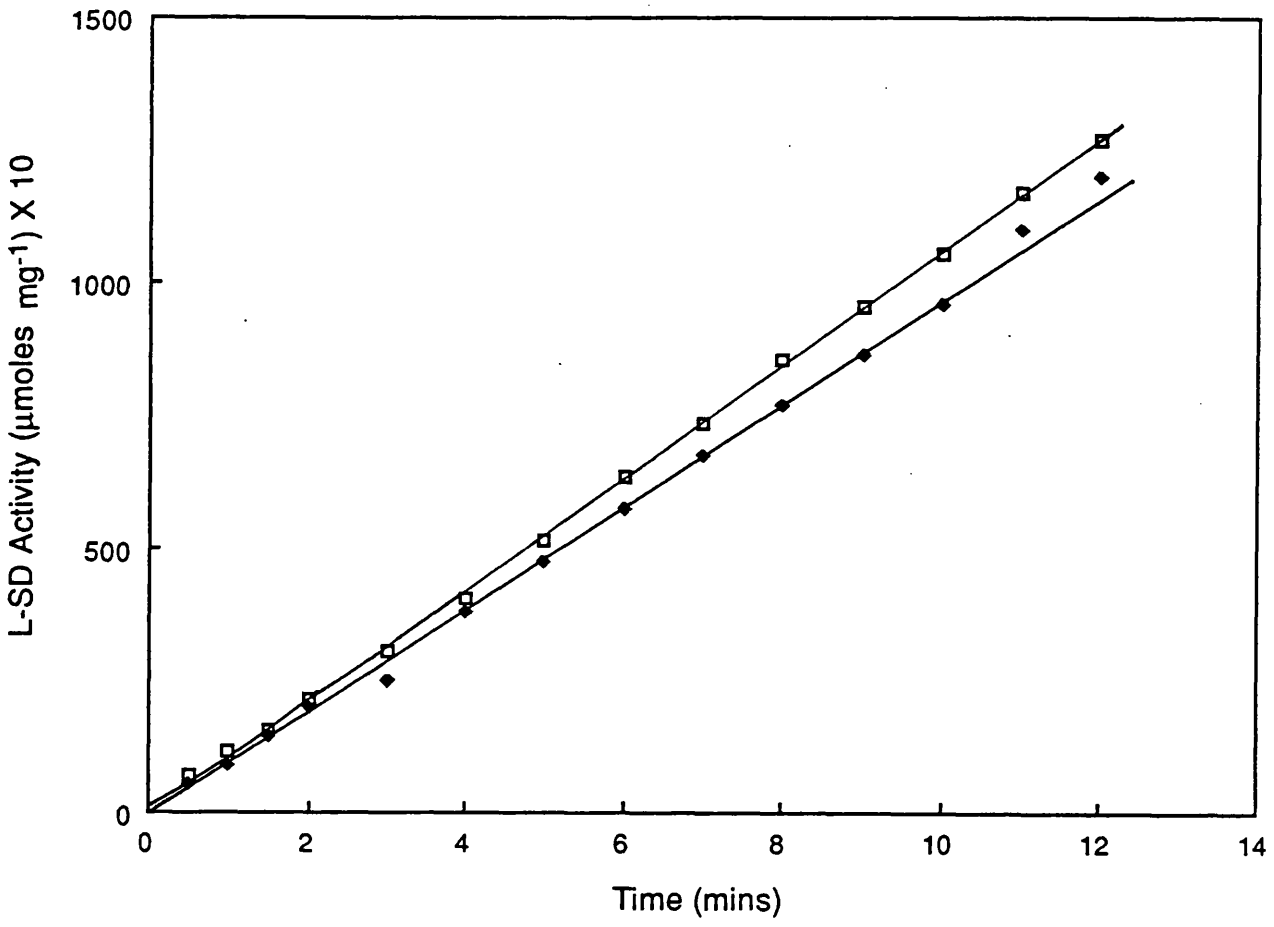


Figure 6.4

The Timecourse of Pyruvate Formation of Iron-activated L-Serine Dehydratase in the Presence and Absence of Ferrous Iron and Dithiothreitol

L-Serine dehydratase (step 8, protein concentration 0.10 mg ml^{-1}) was activated by preincubation with 100 mM glycylglycine-NaOH buffer, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$ as described in Chapter 2, section 2.6.4. Samples of the iron-activated enzyme (0.60 units) were then added to assay mixtures (3 ml) equilibrated at 37 °C containing 100 mM glycylglycine-NaOH , pH 8.0 containing 50 mM L-serine with, and without, additions of 10 mM DTT and 1 mM $FeSO_4$. At the times shown in the figure, 0.1 ml samples of the assay mixtures were removed and added to 0.9 ml of water and 0.33 ml of 2,4-dinitrophenylhydrazine and measured for pyruvate content, as described in Chapter 2, section 2.6.1.

□ □ □ □ A Timecourse of pyruvate formation in the presence of $FeSO_4$ and DTT
(µmoles pyruvate mg^{-1} of enzyme) X 10

◆ ◆ ◆ ◆ B Timecourse of pyruvate formation in the absence of $FeSO_4$ and DTT
(µmoles pyruvate mg^{-1} of enzyme) X 10

(37 °C) the activation of the dehydratase may be very rapid, and may be complete within 90 seconds or less. This results in the production of a timecourse with an initial lag phase of very short duration, which is only clearly seen when the course of the reaction is followed continuously. When timecourses of enzyme activity are obtained by the discontinuous DNPH assay system, sampling does not usually begin until 30 seconds after the start of the enzyme-catalysed reaction (prior to this time the pyruvate content of the 0.1 ml samples taken for assay is too low to measure accurately). By the time this period has elapsed the serine-activation process may be nearing completion, with the result that the differences between initial and final rates of pyruvate formation may be quite small, which may mask the initial lag phase and produce an apparently linear timecourse.

6.2.2 Iron-activation of L-Serine Dehydratase in the Absence of L-Serine

The activation of L-serine dehydratase by iron and DTT was also found to occur in the absence of L-serine. When the enzyme (step 8, protein concentration 0.1 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM dithiothreitol, and 1 mM $FeSO_4$ and assayed at regular time intervals in assay mixtures containing buffer and L-serine only, the activation profile shown in Figure 6.5 was obtained. This shows that the activity of the dehydratase slowly increased during the course of the incubation with iron and DTT in a non-linear fashion. During the first 7 minutes of the incubation, the enzyme was activated to about 20 % of the level attained at maximal activation. The major part of the activation process occurred during the next 7 to 8 minutes, when the level of enzyme activity achieved approached 85 % of the maximal. Full activation of the dehydratase was observed after a total preincubation time of about 20 minutes.

Once the dehydratase had become activated by the iron and DTT, it was able to remain in a state of maximal activity for periods of up to 40 minutes at 37 °C and 45 to 50 minutes at 20 - 25 °C. For time intervals of longer duration there was, at both temperatures, a slow decay of activity, only partially reversible by further incubation, or assay, with iron and dithiothreitol.

The time required for maximal activation by Fe^{2+} and DTT in the absence of L-serine was also found to increase as the dehydratase became more purified, and less Fe-independent (Table 7.2 shows the level of Fe-independent activity of L-serine dehydratase from the successive steps of a purification, and the degree to which it is iron-activated at each of these stages). It was found that L-serine dehydratase in crude, or streptomycin sulphate-treated extracts, which have the highest level of Fe-independent activity of all cell-free preparations, was fully

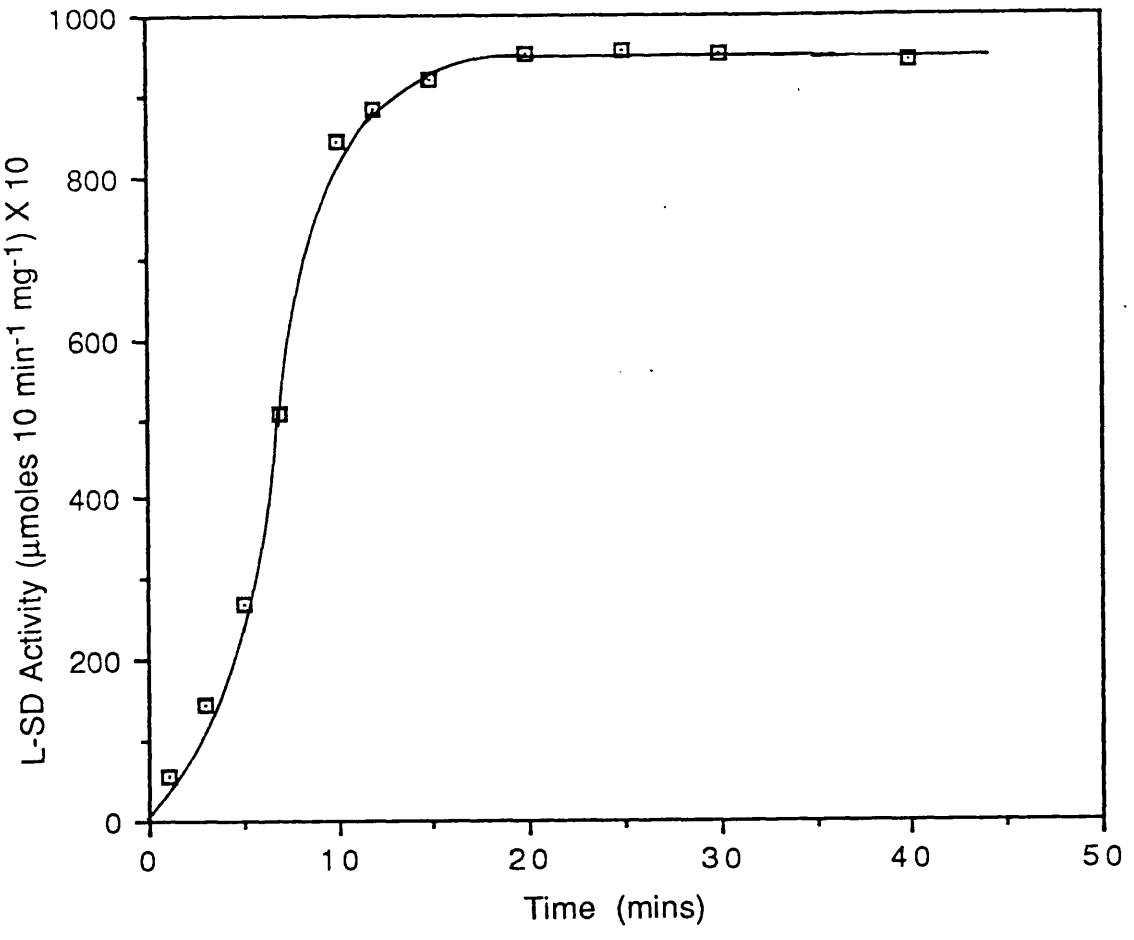


Figure 6.5

The Timecourse of the Iron-activation of L-Serine Dehydratase in the Absence of L-Serine

L-Serine dehydratase (step 8, enzyme concentration 0.12 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH buffer, pH 8.0, 10 mM DTT, and 1 mM $FeSO_4$. At the times shown in the Figure, samples of the activation mixture (10 µl) were withdrawn for assay 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine. Pyruvate formed during the assays was measured as described in Chapter 2, section 2.6.1.

activated by iron and DTT after an incubation period of only 7 to 10 minutes. This increased to 10 to 12 minutes for ammonium sulphate-precipitated enzyme, which had about a third of the residual Fe-independent activity of dehydratase from a crude extract. L-Serine dehydratase purified to step 4 of the purification scheme (the DEAE cellulose stage) had little Fe-independent activity, and required longer incubation with iron and dithiothreitol to achieve maximal activation (on average, 13 to 17 minutes). Enzyme obtained from the remaining steps of the purification (step 5 onwards) had no Fe-independent activity and had an absolute requirement for iron and DTT for activity. These L-serine dehydratase preparations usually required an incubation period of longer duration for complete iron-activation to occur (an average of 20 minutes). The levels of maximal activity attained by the iron-activated dehydratase samples shown in the timecourses in Figure 6.3 and 6.5 are comparable, even though the former iron-activation reaction took place in the presence of L-serine, and the latter in its absence. However, enzyme incubated with iron, DTT and L-serine reached maximal activation in less than a quarter of the time required by dehydratase incubated solely with Fe^{2+} and dithiothreitol, which suggests that L-serine is able to increase the rate of the iron-activation process. The mechanism by which it achieves this is uncertain. It has already been mentioned (Chapter 4, section 4.2), and is discussed more fully in Chapter 7, that iron-activated L-serine dehydatase undergoes a further form of activation when preincubated with either L- or D-serine. Enzyme incubated with iron, DTT and L-serine under the conditions described in Figure 6.3 would be therefore be simultaneously subject to two activation processes. The results obtained would seem to suggest that the two processes may be inter-related, and that activation by L-serine may facilitate the activation of L-serine dehydratase by iron and dithiothreitol.

Sections 6.1 and 6.2 have shown that purified *E. coli* L-serine dehydratase has an absolute and highly specific requirement for iron and dithiothreitol, and that activation by these reagents is slow. These characteristics are similar to the conditions needed for the re-activation of maleate hydratase from rabbit kidney, which contains an unstable [4Fe-4S] centre (Dreyer, 1985). Once this enzyme had lost its initial activity, re-activation of maleate hydratase required a 30 minute preincubation with 1 mM Fe^{2+}, 5 mM Na_2S and 5 mM mercaptoethanol. Although highly specific in its requirement for ferrous iron, beef heart aconitase was less specific in its reducing agent requirment than either maleate hydratase or L-serine dehydratase. When supplied with 0.1 mM Fe^{2+}, aconitase showed a significant degree of activation (up to 40 %) when incubated with either NAD(P)H (1 mg ml^{-1}), sodium dithionite (0.01 mM) or ascorbate (30 mM). In addition,

Kennedy and co-workers found that it was possible to partially activate aconitase with reducing agents alone (Chapter 1, section 1.4.1). However, maximal activation only be achieved if the enzyme was incubated with both iron and dithiothreitol (5 mM). Under these conditions, activation required about 10 minutes for completion (Kennedy *et al*, 1983a).

6.3 The Effect of Metal Chelators on the Activity and Iron-activation of L-Serine Dehydratase

The requirement of L-serine dehydratase for iron was further demonstrated by examining the effects of metal chelating agents on the activity and activation of the enzyme. Since glycylglycine is also able to bind iron, although very weakly compared to chelators such as EDTA and o-phenanthroline, the following experiments were performed in Tris buffer.

Metal chelating agents were found to inhibit the activation of L-serine dehydratase. When the dehydratase was assayed in media containing 10 mM DTT, and 1 mM Fe^{2+}, and either EDTA, nitrilotriacetate (NTA) or o-phenanthroline the enzyme was inhibited in a manner which depended on the concentration of the chelator (Figure 6.6). o-Phenanthroline and EDTA (both at 1 mM) were able to prevent activation of the dehydratase completely. At this same concentration NTA caused only a partial inhibition of the activation process. A similar pattern of inhibition was observed when these chelators were present in the activation mixtures (results not shown). Further experiments showed that it was possible to overcome the inhibition produced by the chelators by increasing the concentration of the iron in the assay or activation medium until it was equimolar to the chelator present, plus the concentration of iron normally required for activation (2 mM).

Purified L-serine dehydratase, which has no Fe-independent activity, and which had not been activated by iron and DTT, appeared to be very resistant to inhibition by either EDTA, or o-phenanthroline. Duplicate samples of Fe-dependent L-serine dehydratase (step 8, protein concentration 0.12 mg ml^{-1}) were incubated at 0 ^{o}C with either 5 mM EDTA or 5 mM o-phenanthroline for periods of up to 3 hours. Over the course of the incubation samples of the enzyme-chelator mixtures (10 µl) were removed for assay in iron-containing assay media as described in Chapter 2, section 2.6.1. Enzyme incubated with either chelator was found to lose no more activity than a control preparation of enzyme diluted with an equal volume of water.

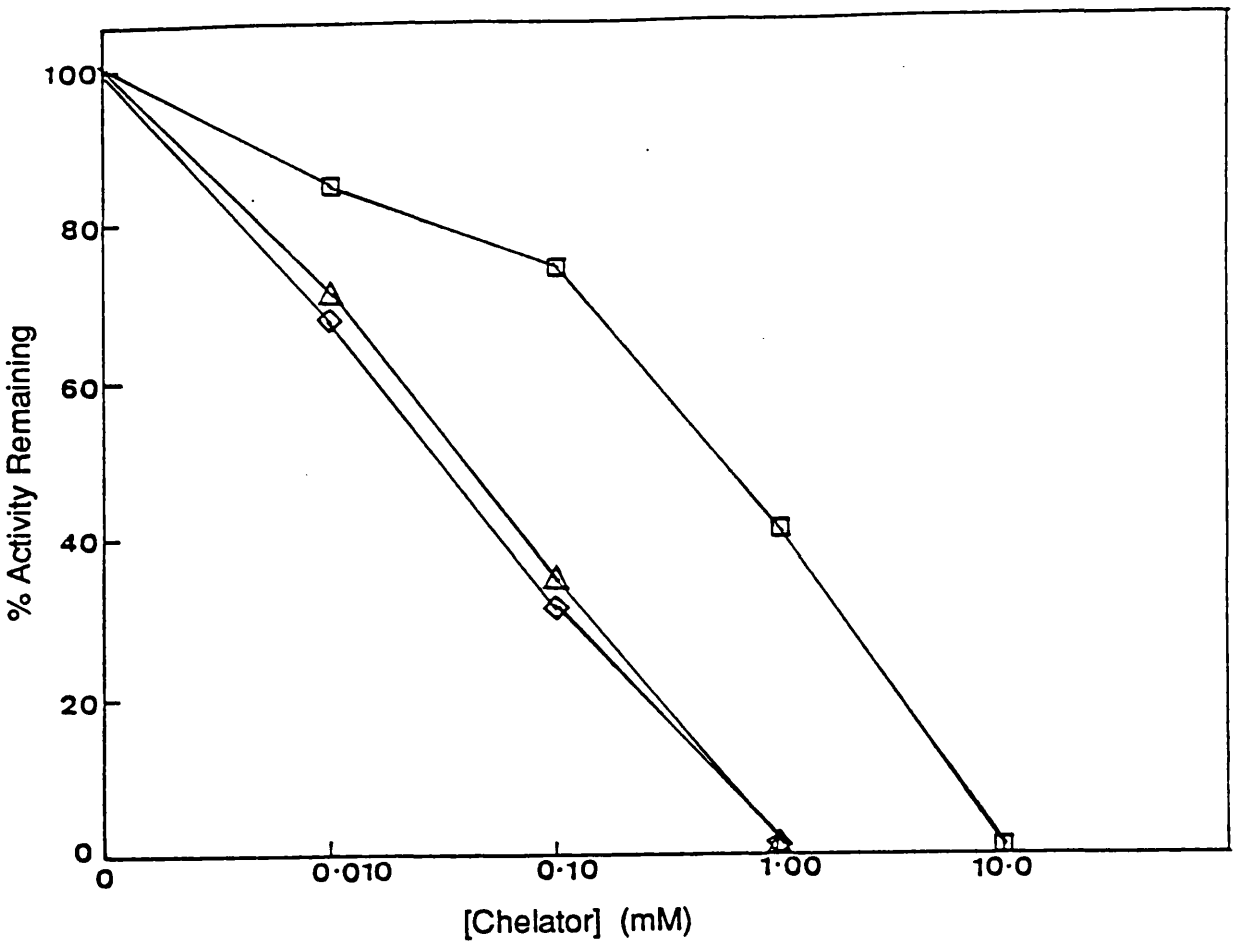


Figure 6.6

The Inhibition of the Iron-activation of L-Serine Dehydratase by Metal Chelators

L-Serine dehydatase (step 8, 0.20 units) was assayed at 37 °C in 100 mM Tris-HCl, pH 8.0 containing 10 mM DTT, 1 mM $FeSO_4$ and the concentrations of metal chelators shown in the Figure. Pyruvate formed during the incubations was measured as described in Chapter 2, section 2.6.1.

△ △ △ △ EDTA

◇ ◇ ◇ ◇ o-Phenanthroline

□ □ □ □ Nitrilotriacetate

In contrast, Fe-independent L-serine dehydratase from crude extracts was inactivated when incubated with metal chelators. When cells of *E. coli* B were extracted in the presence of either EDTA, NTA or o-phenanthroline (each at 1 mM), the results shown in Table 6.2 were obtained. Enzyme prepared in buffer only, or in buffer plus ferrous iron and DTT possessed a high level of Fe-independent activity, and showed only a small degree of activation when assayed with iron and dithiothreitol. In contrast, L-serine dehydratase from the same preparation of cells extracted in the presence of EDTA or o-phenanthroline appeared to possess very little Fe-independent activity. However, when assayed with iron and DTT, the enzyme activities of the chelator-treated extracts were very similar to those of the control preparations of dehydratase.

The inactivation of Fe-independent L-serine dehydratase by metal chelating agents was also found to be time-dependent. Figure 6.7 (graph A), shows the gradual decrease in enzyme activity that occured when L-serine dehydratase (step 1) was preincubated with 1 mM EDTA. When assayed in media containing buffer and L-serine only, nearly all of the initial activity of the dehydratase appeared to be lost by the end of the 60 minute incubation. A control sample of L-serine dehydratase lost less than 10 % of its initial activity over this same time interval. However, when the same EDTA-treated enzyme was assayed in the presence of iron and DTT, virtually all of the apparently lost activity was recovered (Figure 6.7, graph B).

It also proved possible to use EDTA (1 mM) to reversibly inactivate purified L-serine dehydratase which had been activated by preincubation with 10 mM DTT and 1 mM $FeSO_4$, as described in Chapter 2, section 2.6.4. The pattern of inactivation of iron-activated L-serine dehydratase by the EDTA (Figure 6.8, graph A) was similar to that obtained with unpurified, Fe-independent enzyme, except that considerably less time was required to bring about full inactivation of the dehydratase (30 minutes). As before, most of the original activity was recovered when the EDTA-treated dehydratase was assayed with ferrous iron and dithiothreitol (Figure 6.8, graph B).

A very similar pattern of inhibition was obtained when iron-activated L-serine dehydratase was incubated with a similar concentration of o-phenanthroline (results not shown). The inactivation of the dehydratase was found to be more rapid with this chelator, with complete inactivation occurring after only 15 to 20 minutes preincubation with 0-phenanthroline. As before, most of this apparently lost activity could be restored by assaying the enzyme with $FeSO_4$ and DTT.

Table 6.2

The Effect of Metal Chelators on the Fe-Independent Activity of L-Serine Dehydratase When Included in the Extraction Medium

Cells of *E. coli* B were grown on the medium of Alfoldi *et al* (1968), and harvested as described in Chapter 2, sections 2.2.1. and 2.3. Cell suspensions (5 ml, 30 mg dry weight cells ml^{-1}) containing 100 mM glycylglycine-NaOH, pH 8.0, 2 mM DTT and the additions shown in the Table, were extracted by sonication and centrifuged to remove cell debris as described in Chapter 2, sections 2.4.3 and 2.4.4. Samples (10 µl) of each of the extracts were then assayed in both 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM L-isoleucine and 1 mM hydroxylamine and 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM L-isoleucine, 1 mM hydroxylamine plus 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was estimated as its 2,4-dinitrophenylhydrazone derivative (Chapter 2, section 2.6.1).

The 'degree of iron-activation' of the enzyme preparations is the ratio of the activity of the dehydratase when assayed in glycylglycine, iron and DTT (b) : the activity of the dehydratase when assayed in glycylglycine buffer only (a).

Extraction Medium	**L-Serine Dehydratase Activity (Units ml^{-1})**		**Degree of Iron-activation**
	(a)	**(b)**	**(b/a)**
	Glycylglycine only	**Glycylglycine + Fe + DTT**	
No Additions	21.3	51.3	2.4
10 mM DTT + 1 mM Fe^{2+}	41.6	53.8	1.4
1 mM EDTA	0.50	49.8	93.9
1 mM NTA	3.2	52.0	16.3
1 mM o-Phenanthroline	0.07	42.5	582.2

Figure 6.7

The Inactivation of Fe-Independent L-Serine Dehydratase by EDTA

L-Serine dehydratase (step 1, protein concentration 10 mg ml^{-1}, unactivated specific activity 0.85 μmole pyruvate min^{-1} mg^{-1}) was incubated at 37 °C in the presence of 2 mM EDTA (pH 7.0). At the times shown in the figure, samples of the incubation mixture were withdrawn for assay in 100 mM tris-HCl, pH 8.0 and 50 mM L-serine and 100 mM tris-HCl, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. A control experiment, in which the EDTA was replaced by an equal volume of water, is also shown.

Figure 6.7a The activity of L-serine dehydratase treated with EDTA (■ ■ ■ ■) or an equal volume of water (● ● ● ●) when assayed in 100 mM tris-HCl, pH 8.0 and 50 mM L-serine.

Figure 6.7b The activity of L-serine dehydratase treated with EDTA when assayed in 100 mM tris-HCl, pH 8.0 and 50 mM L-serine (■ ■ ■ ■) and 100 mM tris, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$ (□ □ □ □)

Figure 6.7 a

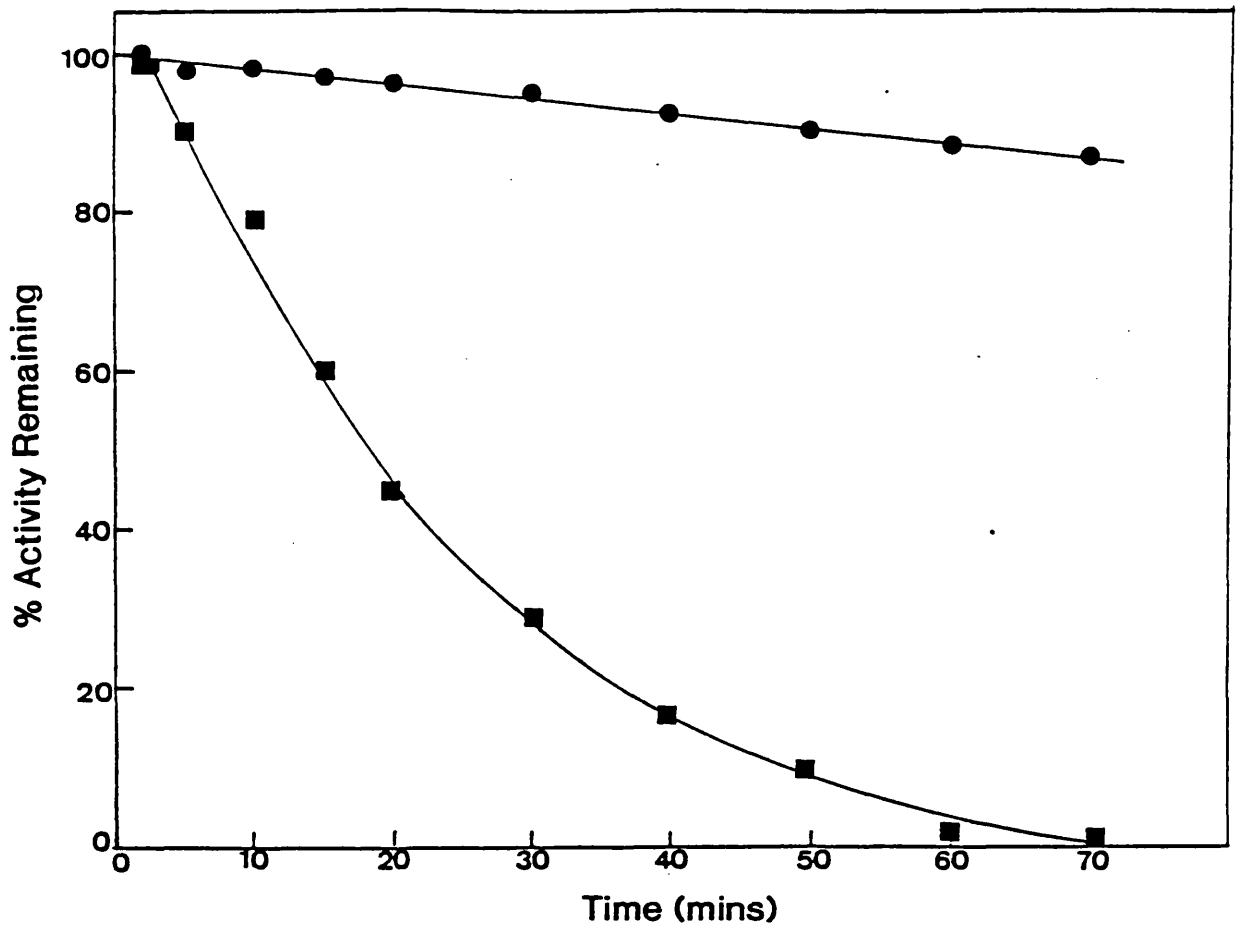


Figure 6.7 b

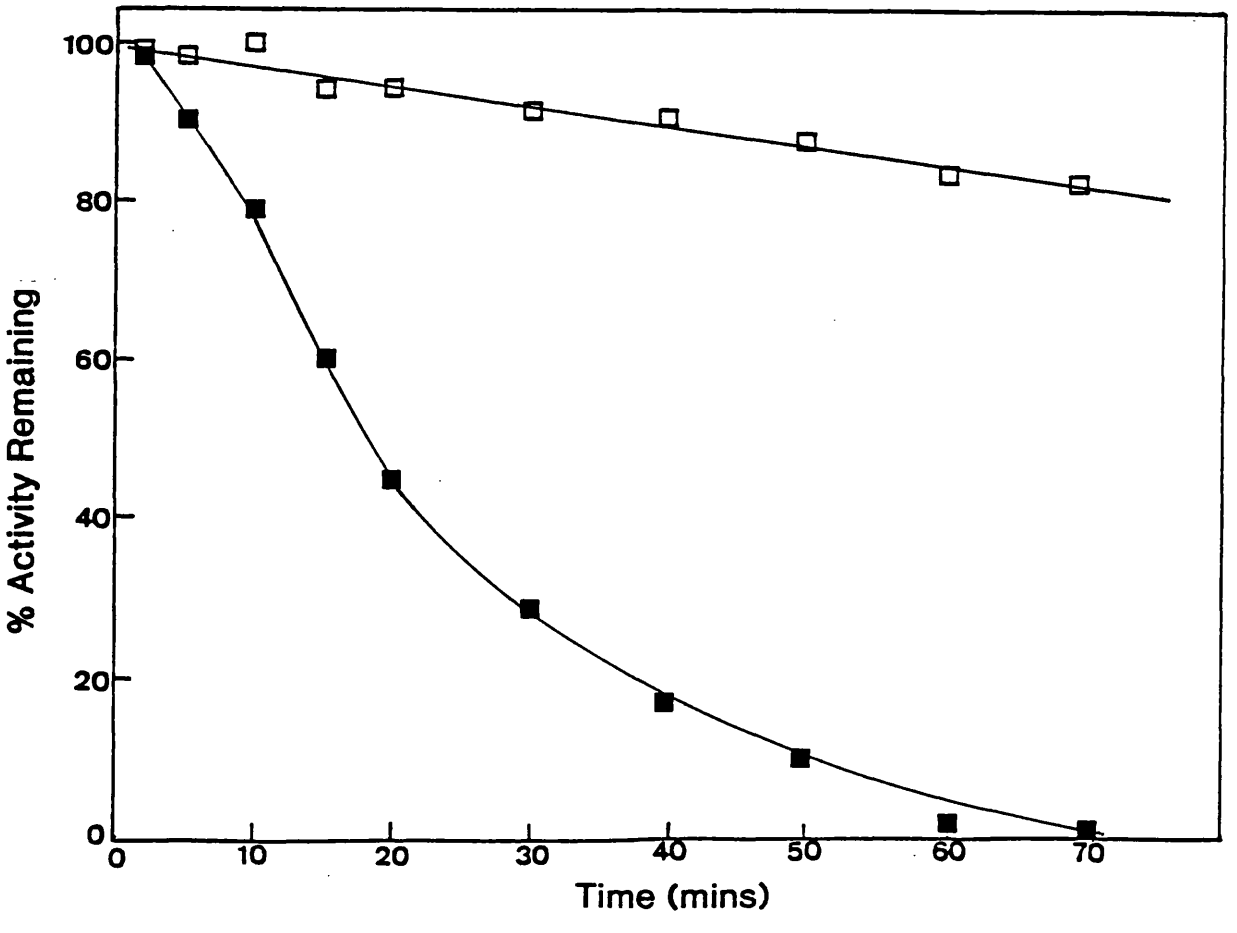


Figure 6.8

The Reversal of the Iron-activation of L-Serine Dehydratase by EDTA

L-Serine dehydratase (step 8, protein concentration 0.15 mg ml^{-1}) was preincubated at 37 °C with 100 mM tris-HCl, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$, until maximally activated, as described in Chapter 2, section 2.6.4. At zero time in the Figure, EDTA (pH 7.0) was added to the iron-activated enzyme, to a final concentration of 2 mM. Samples (10µl) of the incubation mixture were withdrawn at the times shown for assay in 100 mM tris-HCl, pH 8.0 and 50 mM L-serine or 100 mM tris-HCl, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. The results of a control experiment, in which the EDTA was replaced by an equal volume of water, is also shown.

Figure 6.8a The activity of iron-activated L-serine dehydratase treated with EDTA (■ ■ ■ ■) or an equal volume of water (● ● ● ●) when assayed in 100 mM tris and 50 mM L-serine.

Figure 6.8b The activity of iron-activated L-serine dehydratase treated with EDTA when assayed in 100 mM tris, pH 8.0 and 50 mM L-serine (■ ■ ■ ■) and 100 mM tris, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$ (□ □ □ □)

Figure 6.8 a

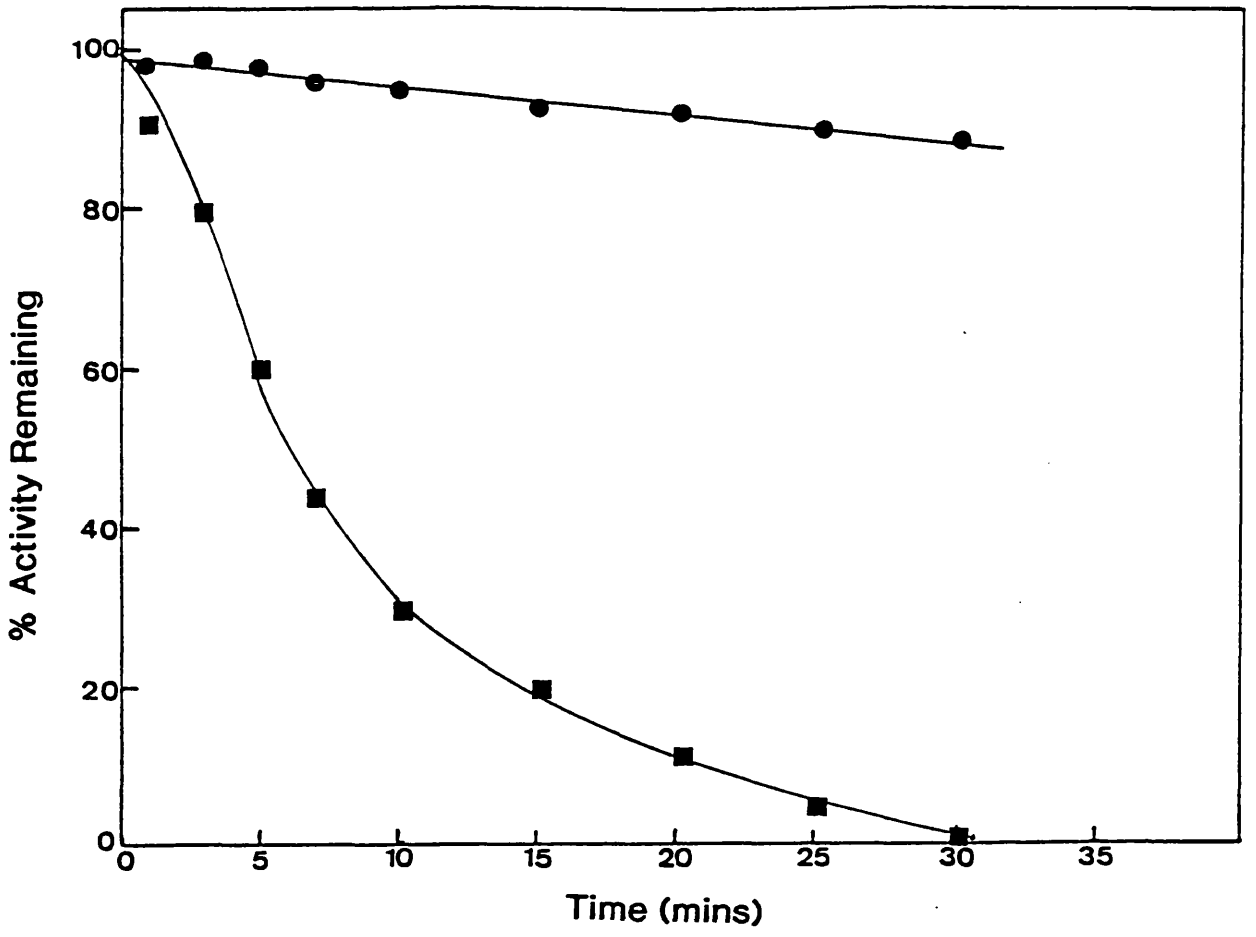
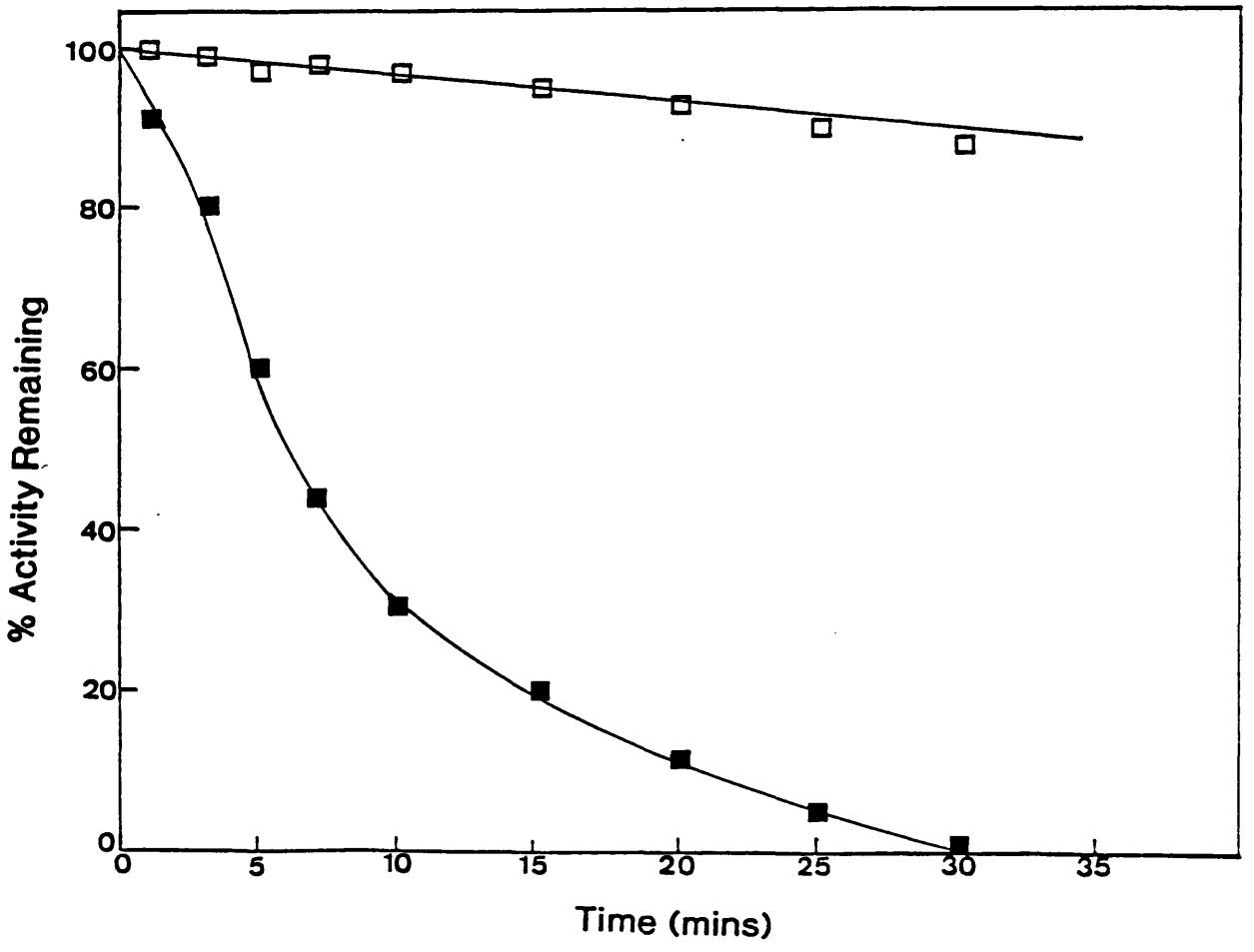


Figure 6.8 b



Both aconitase (Kennedy *et al*, 1983) and maleate hydratase (Dreyer, 1985) were found to be inhibited by metal chelators, although to somewhat differing degrees. Although EDTA was found to inhibit the activation of Fe-deficient aconitase by ferrous iron and DTT, by chelating the Fe^{2+} and preventing its uptake into the enzyme, it was less effective once activation had been achieved. A 5 hour aerobic incubation of iron-activated aconitase with 1 mM EDTA produced a 56 % inhibition of activity. This inhibition occurred as a consequence of the chelation of Fe released from the enzyme, and was fully reversible by a further incubation with iron and DTT. On the other hand, the FeS centre in maleate hydratase was much less stable, and it proved possible to use EDTA, NTA and o-phenanthroline to both prevent activation of maleate hydratase and to actually reversibly extract iron from the iron-activated enzyme. The characteristics of this chelator inhibition, and its reversal by further treatment with iron and reducing agents, are strikingly similar to those previously described for L-serine dehydratase.

6.4 pH-dependence of the Iron-activation Process

L-Serine dehydratase activity was shown earlier to be significantly influenced by the pH at which the enzymatic reaction was performed (Chapter 4, section 4.6), and so an examination was made of the importance of pH to the iron-activation of L-serine dehydratase. This was assessed by incubating L-serine dehydratase with 10 mM DTT and 1 mM $FeSO_4$ over a range of buffered pH values (6.0 to 9.5), and assaying at a constant pH (8.0) (Figure 6.9). The maximal level of activity achieved was then plotted as a function of the pH at which it was obtained.

Figure 6.9 shows that the activation of L-serine dehydratase by Fe plus DTT was significantly affected by the pH of the activation medium. Activation increased very rapidly between pH 7.0 and 7.75 and was maximal over the 8.0 to 8.75 pH range. Although activation was almost negligible below pH 7.0, a substantial activation of the dehydratase (up to 75 % of that obtained at pH 8.0) was still possible at pH 9.5.

The activation by iron and DTT of aconitase was also found to be influenced by pH (Kennedy *et al*, 1983), with the optimal pH for activation occurring between pH 7.5 and 8.5. The iron-activation of the L-serine dehydratase from *Cl. acidiurici* (Carter and Sagers, 1972) was also pH-dependent, with maximal activation only being reached between pH 7 and pH 8.

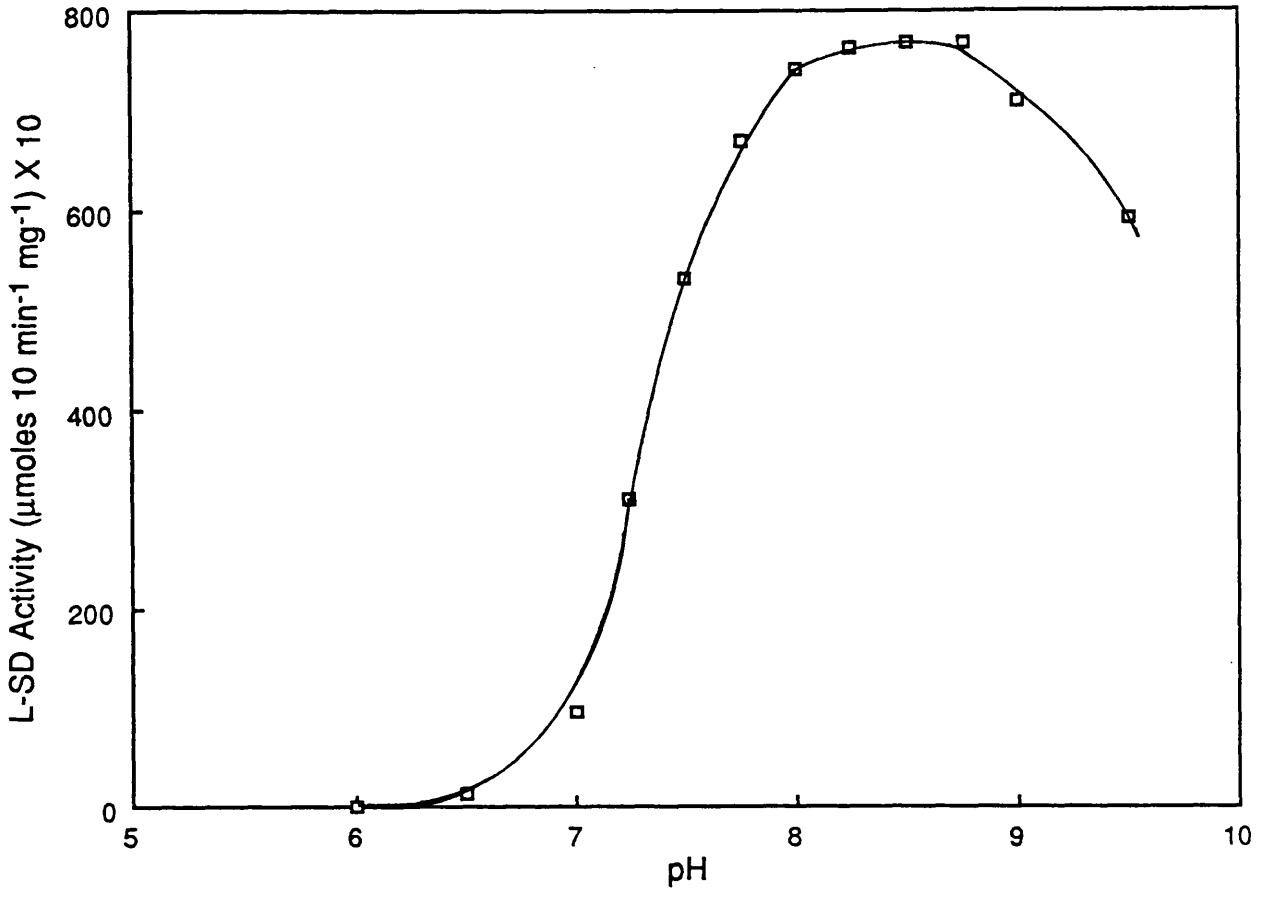


Figure 6.9

The pH-dependence of the Iron-activation of L-Serine Dehydratase

L-Serine dehydratase (step 8, enzyme concentration 0.12 mg ml^{-1}) was incubated at 37 °C in mixtures containing 10 mM DTT and 1 mM $FeSO_4$, which had been adjusted to the pH values shown in the Figure with 100 mM glycylglycine-bis-tris-propane buffer. The timecourse of the iron-activation of each of these preincubations was then measured as described in the legend to Figure 6. 5. The maximal levels of activity achieved were then plotted as a function of the pH at which they were obtained.

6.5 Temperature-dependence of the Iron-activation Process

An examination was also made of the importance of incubation temperature to the activation of L-serine dehydratase by Fe^{2+} and dithiothreitol. The activation of L-serine dehydratase by 10 mM DTT and 1 mM $FeSO_4$ was followed over a range of temperatures. Figure 6.10 shows that the iron-activation of the dehydratase was markedly affected by temperature. At 0^o C, no activation was observed. With increasing temperature, the final extent of the activation of the dehydratase was also increased, becoming maximal at 37 ^oC. At higher temperatures (above 42 ^oC), the level of activation achieved decreased, possibly as result of heat denaturation of the enzyme.

Although no activation appeared to occur when the incubation of L-serine dehydratase with iron and DTT took place at 0 ^oC, raising the temperature of the activation mixture to 37 ^oC resulted in a marked increase in the activity of the dehydratase. The final level of activity reached was similar to that achieved by enzyme maintained at this temperature from the start of the incubation. Similarly, full activation was also observed when the temperatures of the mixtures preincubated at 10 and 20 ^oC were increased to 37 ^oC.

6.6 The Binding of Iron to L-Serine Dehydratase

A considerable amount of indirect evidence has been presented which suggests that activation of L-serine dehydratase by iron (and DTT) involves the actual binding of this metal, as it did for the FeS enzymes aconitase and maleate hydratase (Kennedy *et al*, 1983, Dreyer, 1985). Section 6.3 showed that agents which chelate iron are able to inactivate Fe-independent or iron-activated dehydratase, and that ferrous iron and DTT were able to reverse this inactivation. Further, the observation that iron-activated L-serine dehydratase retained a substantial proportion of its activity (up to 90 %) when separated from its activating agents by passage through either a Sephadex G-25 desalting column, or G-100 spun column, would also seem to indicate that iron had been bound to the enzyme. However, to provide an unequivocal answer to the question of whether activation by iron and DTT involves a direct binding of the iron, experiments were performed to determine if iron was taken up by the dehydratase during the iron-activation process, and if so, how much. These involved both chemical estimations with $^{56}FeSO_4$ and isotope binding studies with $^{59}FeSO_4$

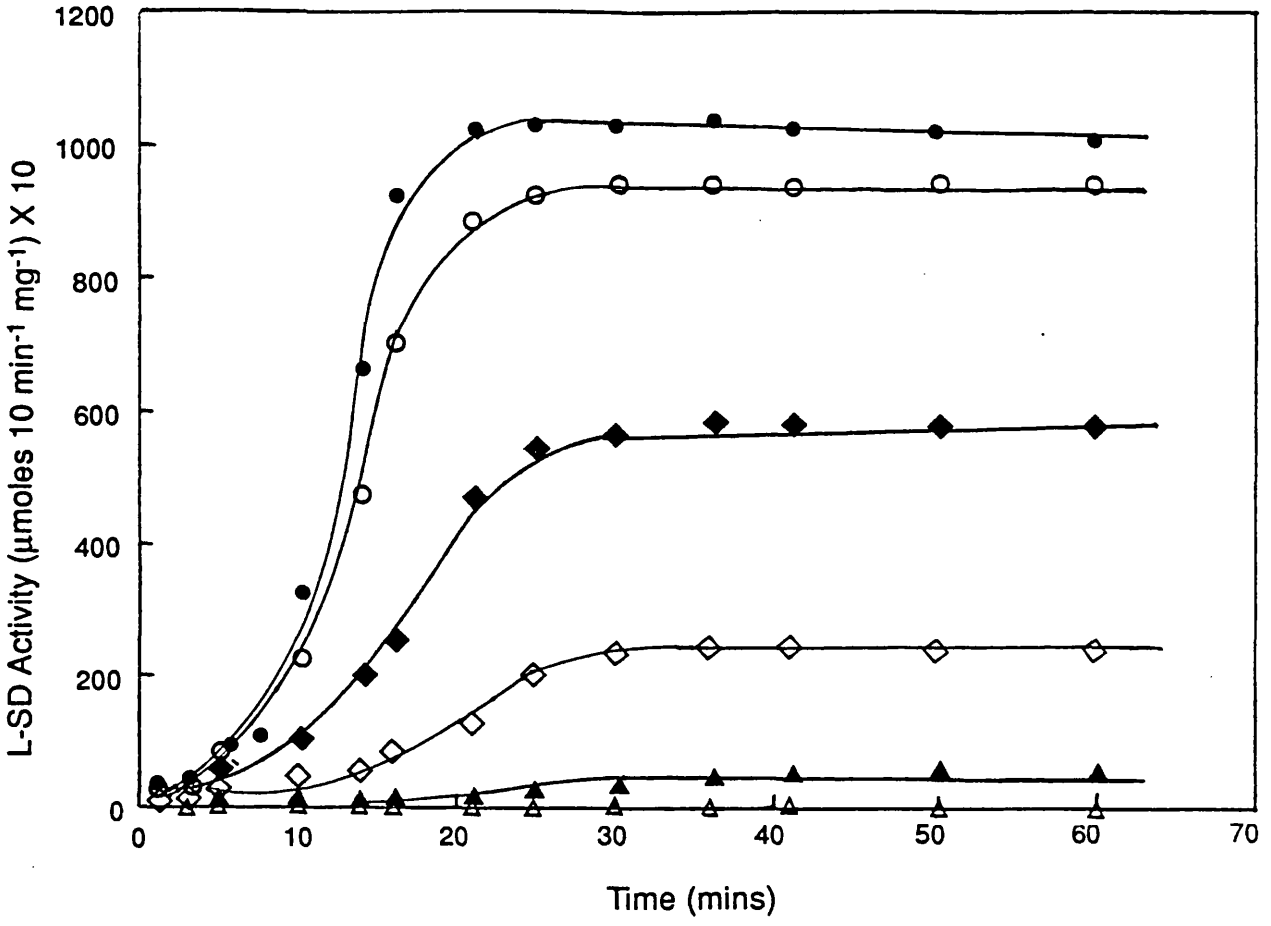


Figure 6.10

The Temperature Dependence of the Iron-activation of L-Serine Dehydratase

L-Serine dehydratase (step 8, enzyme concentration 0.10 mg ml^{-1}) was incubated with 100 mM glycylglycine-NaOH buffer, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$ at the temperatures indicated below. At the times shown in the Figure, samples (10 µl) of the incubation mixtures were removed for assay at 37 °C in 100 mM glycylglycine-NaOH , pH 8.0 containing 50 mM L-serine. Pyruvate formed during the assays was measured as described in Chapter 2, section 2.6.1.

△ △ △ △ 0 °C

▲ ▲ ▲ ▲ 10 °C

◇ ◇ ◇ ◇ 20 °C

◆ ◆ ◆ ◆ 25 °C

○ ○ ○ ○ 30 °C

● ● ● ● 37 °C

6.6.1 Chemical Analyses with $^{56}Fe^{2+}$

An estimation was made of the amount of ^{56}Fe bound to iron-activated L-serine dehydratase using an adaptation of the o-phenanthroline chelation method of Massey (1957). Triplicate samples of concentrated L-serine dehydratase (step 8, enzyme concentration 0.56 mg ml^{-1}) were activated by preincubation with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$, using the protocol described in Chapter 2, section 2.6.4. Excess $FeSO_4$ was removed from samples (150 µl) of the activated enzyme mixtures by centrifugation through a G-100 spun column equilibrated in 50 mM Tris-HCl buffer, pH 7.8. The protein concentration of each column eluate was measured by the A_{280}/A_{260} method of Warburg and Christian (1942), and L-serine dehydratase activity by the lactate dehydrogenase-coupled assay (Chapter 2, section 2.6.2). The amount of Fe^{2+} bound by the dehydratase was measured by incubating a 100 µl sample of the desalted enzyme solution with 5 mM o-phenanthroline at 25 °C for 45 to 60 minutes. The samples were then read at 510 nm against a suitable blank. The amount of ferrous iron bound to the enzyme was estimated by reference to a standard curve previously prepared with $FeSO_4$. Corrections for the unbound iron were made using the carryover of $FeSO_4$ from a control preincubation mixture consisting of 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$. This value was usually less than 0.5 %.

Measurements of the quantities of ferrous iron bound to several different preparations of L-serine dehydratase from step 8 of the purification produced results suggesting a ratio of 2-4 µmole of Fe (average 3.0) per µmole of enzyme. No iron was detected by this method in the non-iron-activated protein.

6.6.2 The Binding of $^{59}FeSO_4$ to L-Serine Dehydratase

A number of experiments examining the binding of iron to L-serine dehydratase were also performed using radiolabelled $^{59}FeSO_4$. These were designed to determine if activation by ^{59}Fe occurred in a similar way to activation by ^{56}Fe, and to further examine the amount of Fe bound to the dehydratase during the activation process.

L-Serine dehydratase was incubated with 100 mM glycylglycine-NaOH pH 8.0, 10 mM DTT and either 1 mM $^{59}FeSO_4$ or 1 mM $^{56}FeSO_4$ until maximally activated. The enzyme was then further processed as described in the legend to Table 6.3.

Table 6.3

The Binding of ^{59}Fe to L-Serine Dehydratase

Triplicate samples of L-serine dehydratase (step 8, protein concentration 0.35 mg ml^{-1}) were incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and either 1 mM $^{59}FeSO_4$ or 1 mM $^{56}FeSO_4$, as described in Chapter 2, section 2.6.4 ,except that the incubation time was extended to 40 minutes to ensure that maximal activation had occurred. Samples of the activation mixtures (10 µl) were removed after 5, 10, 15, 20, 30 and 40 minutes for assay at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 and 50 mM L-serine. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1

When the enzyme had become fully activated, 50 µl samples of each of the incubation mixtures were loaded onto 1 ml columns of sephadex G-100 equilibrated in 50 mM tris, and spun at room temperature at 1500 *g* for 3 minutes in an MSE bench top centrifuge, to remove unbound ^{59}Fe. The eluate from each column was then added to 5 ml of LKB Optiphase Safe scintillation fluid and counted on a Canberra Packard 2000CA Liquid Scintillation Counter. A control consisting of duplicate mixtures containing 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $^{59}FeSO_4$ was used to estimate the carryover of unbound ^{59}Fe.

Activation Mixture	**L-Serine Dehydratase Activity (μmoles 10 min^{-1} mg^{-1} enzyme) x 10**	**^{59}Fe-bound (cpm)**
Enzyme-^{56}Fe	1301	27
Enzyme-^{56}Fe	1310	41
Enzyme-^{56}Fe	1296	50
Enzyme-^{59}Fe	1300	4358
Enzyme-^{59}Fe	1269	3863
Enzyme-^{59}Fe	1282	3906
Controls		
Buffer-^{56}Fe	-	36
Buffer-^{59}Fe	-	76
Buffer-^{59}Fe	-	96

It can be seen that L-serine dehydratase incubated with $^{59}FeSO_4$ reached approximately the same level of activity as the control sample of dehydratase activated with $^{56}FeSO_4$, suggesting that activation of the dehydratase by ^{59}Fe occurred in the same manner as activation by ^{56}Fe. The high levels of radioactivity associated with the ^{59}Fe-activated enzyme preparations indicate that the ^{59}Fe in the samples was not the result of a non-specific carryover of unbound radiolabel. The amount of labelled iron taken up by the protein was equivalent to an average of 1.9 μmole of ^{59}Fe per μmole of L-serine dehydratase, which is within the range of the values calculated for ^{56}Fe incorporation obtained by chemical analysis.

6.6.3 The Effects of Variation in the Concentration of $^{59}FeSO_4$ on the Amount of Fe Incorporated into L-Serine Dehydratase

The binding of ^{59}Fe into L-serine dehydratase was also found to vary with the concentration of labelled ferrous sulphate present in the activation medium. Table 6.4 shows that there is a correlation between enzyme activation and iron incorporation. The binding of ^{59}Fe to L-serine dehydratase and the activity of the enzyme became maximal at the same $FeSO_4$ concentration (0.75 mM). Higher concentrations of the labelled iron failed to produce a further increase in either activation or incorporation, a feature also observed with the control experiment of enzyme incubated with unlabelled ferrous sulphate.

6.6.4 The Timecourse of the Iron-Activation of L-Serine Dehydratase by $^{59}FeSO_4$ in the Absence of L-Serine

In this experiment, L-serine dehydratase (step 8, 0.35 mg ml^{-1}) was incubated at 37 ^oC with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $^{59}FeSO_4$ for a total incubation time of 40 minutes. At the intervals indicated in Figure 6.11, samples were removed from the activation mixture for enzyme assay (10 μl) and estimation of ^{59}Fe incorporation (35 μl), as described above.

Figure 6.11 shows the close parallel between increasing activity and increasing iron uptake. The timecourse of the activation of the dehydratase and of the binding of ^{59}Fe to the enzyme follow a very similar pattern, with maximal activity and maximal radiolabel incorporation occurring after the same time interval (20 minutes). This suggests that the two phenomena are closely related, and that the activation of L-serine dehydratase by iron involves the direct incorporation of this metal, as it did for aconitase (Kennedy *et al*, 1983). Figure 6.11 also shows that the amount of iron bound to the dehydratase did not increase once maximal

Table 6.4

The Effects of Variations in the Concentration of $^{59}FeSO_4$ on Activation and Incorporation of ^{59}Fe on L-Serine Dehydratase

L-Serine dehydratase (Step 8, protein concentration 0.4 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and either $^{59}FeSO_4$ or $^{56}FeSO_4$ at the concentrations shown in the Table (0.05 to 1.5 mM $FeSO_4$). The timecourse of iron-activation and ^{59}Fe binding for each iron concentration was determined as described in the legend to Table 6.3.

The enzyme activities quoted in the Table refer to the maximal level of activation obtained at that particular iron concentration. Each of the values for ^{59}Fe incorporation are the average of two determinations per incubation mixture. Each incubation was corrected for carryover of unbound label. The control preparation of L-serine dehydratase incubated with unlabelled $FeSO_4$ did not show a radioactivity incorporation greater than that caused by background sources, and so the scintillation counts for this experiment have been omitted from the Table.

FeSO4 Concentration [^{56}Fe or ^{59}Fe] (mM)	**L-Serine Dehydratase Activity (μmole 10 min^{-1} mg^{-1}) X 10 in:**		**^{59}Fe-Bound (cpm)**
	$^{56}FeSO_4$	**$^{59}FeSO_4$**	
0.05	71.5	71.6	260
0.10	143.1	200.3	630
0.25	446.3	500.6	1226
0.50	979.8	1040.2	2106
0.75	1304.5	1364.7	4206
1.00	1287.5	1361.8	4806
1.50	1293.1	1299.0	4690
Controls			
Buffer-^{59}Fe	-	-	67
(1 mM)	-	-	90

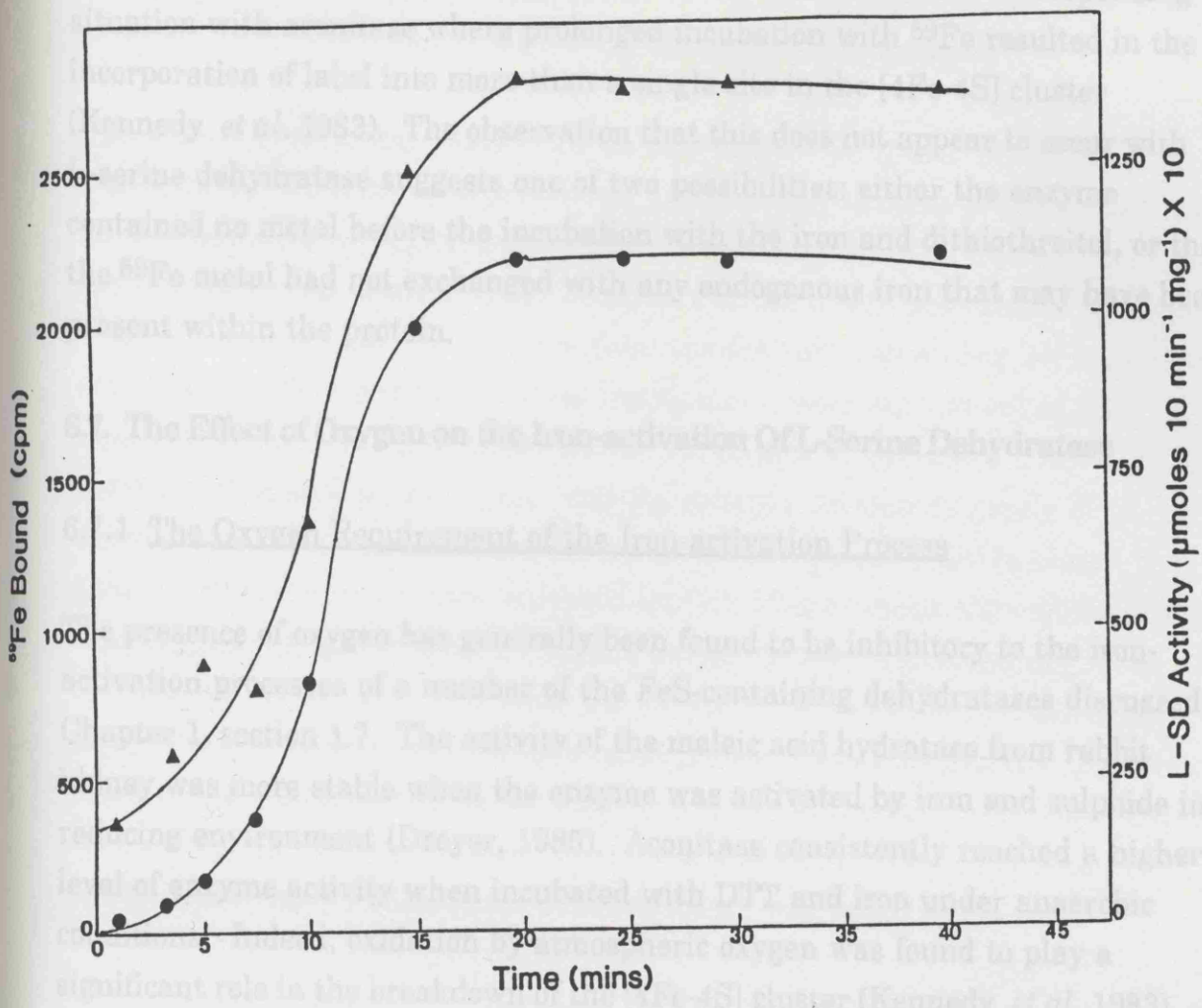


Figure 6.11

The Timecourse of Iron-activation by $^{59}FeSO_4$ in the Absence of L-Serine

L-Serine dehydratase (step 8, 0.35 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, 10 mM DTT and 1 mM $^{59}FeSO_4$ for a total incubation time of 40 minutes. At the intervals indicated in the Figure, samples were removed from the activation mixture for enzyme assay (10 μl), and estimation of ^{59}Fe bound (35 μl), as described in the legend to Table 6.3

▲ ▲ ▲ ▲ ^{59}Fe bound (cpm)

● ● ● ● ● L-Serine Dehydratase activity (μmoles pyruvate 10 min^{-1} mg enzyme) x 10

activation of the enzyme had been achieved. This is unlike the corresponding situation with aconitase where prolonged incubation with ^{59}Fe resulted in the incorporation of label into more than a single site in the [4Fe-4S] cluster (Kennedy *et al*, 1983). The observation that this does not appear to occur with L-serine dehydratase suggests one of two possibilities: either the enzyme contained no metal before the incubation with the iron and dithiothreitol, or that the ^{59}Fe metal had not exchanged with any endogenous iron that may have been present within the protein.

6.7. The Effect of Oxygen on the Iron-activation Of L-Serine Dehydratase

6.7.1 The Oxygen Requirement of the Iron-activation Process

The presence of oxygen has generally been found to be inhibitory to the iron-activation processes of a number of the FeS-containing dehydratases discussed in Chapter 1, section 1.7. The activity of the maleic acid hydratase from rabbit kidney was more stable when the enzyme was activated by iron and sulphide in a reducing environment (Dreyer, 1985). Aconitase consistently reached a higher level of enzyme activity when incubated with DTT and iron under anaerobic conditions. Indeed, oxidation by atmospheric oxygen was found to play a significant role in the breakdown of the [4Fe-4S] cluster (Kennedy *et al*, 1983). The presence of oxygen caused the destruction of the [3Fe-4S] centre from the E2 subunit of lactoyl Co A dehydratase (Kuchta *et al*, 1986). Anaerobic conditions were also favoured by the L-serine dehydratase from *Cl. acidiurici*. The thiol requirement of this dehydratase was reduced from 10 mM DTT under aerobic conditions to 0.2 mM DTT when the enzyme was activated in the absence of oxygen (Carter and Sagers, 1973).

The evidence presented so far has revealed considerable similarities in the iron-activation of the *E. coli* L-serine dehydratase with those of aconitase and maleate hydratase. These similarities are suggestive of the presence of an iron sulphur centre. In view of the general reaction of this class of FeS (de)hydratases to oxygen, and the indirect evidence which suggested that L-serine dehydratase might also be a member, it was expected that iron-activation of this dehydratase under anaerobic conditions would result in either a significant increase in the level of activation of the enzyme, or a reduction in the amount of DTT required for optimal activation.

To examine these possibilities. a preparation of L-serine dehydratase was anaerobically incubated with ferrous iron and dithiothreitol under an inert

atmosphere of nitrogen, as described in the legend to Figure 6.12. Care was taken to avoid the introduction of air during the sampling procedure.

The results of a typical anaerobic iron-activation experiment are shown in Figure 6.12. Contrary to expectations, L-serine dehydratase preincubated under an atmosphere of nitrogen showed only a slight activation by iron and DTT, while enzyme incubated under aerobic conditions achieved full activation. However, when the septum cap was removed from the the tube containing the anaerobic activation mixture after 40 minutes incubation - twice the amount of time required for maximal activation of a control sample of enzyme activated in the presence of atmospheric oxygen - and the mixture aerated by gentle shaking, activation of the dehydratase proceeded at the normal rate, and to almost the same level as the aerobically activated control. These results show quite clearly that oxygen is required for the iron-activation process. The small degree of activation that was observed when the dehydratase was preincubated under anaerobic conditions was possibly a consequence of the introduction of a small amount of air into the incubation tube as a result of the sampling procedure. When fewer samples were taken from the anaerobic mixture, and as a consequence less air introduced, less activation was seen.

These results unequivocally show that oxygen is needed for activation of L-serine dehydratase by Fe^{2+} and DTT. However, once iron-activation had occurred, oxygen no longer appeared to be required for the activity of the dehydratase. When assayed by the continuous method of pyruvate estimation, aerobically activated L-serine dehydratase showed very little difference in its activity under either anaerobic or aerobic assay conditions. The specific activity of enzyme assayed in the presence of air was 1250 μmoles pyruvate min^{-1} mg^{-1}, compared to 1210 μmoles pyruvate min^{-1} mg^{-1} for the activity of the same preparation of dehydratase measured under an atmosphere of nitrogen. These results are as expected, since the L-serine dehydratase reaction mechanism is not thought to involve any oxidation or reduction steps.

6.7.2 Inhibition of the Iron-activation of L-Serine Dehydratase by Superoxide Dismutase and Catalase, and its Enhancement by Hydrogen Peroxide

The roles played by ferrous iron and DTT in the activation of aconitase have been well-characterised. Dithiothreitol is required to reduce the iron-deficient [3Fe-4S] centre, a step shown to be an essential pre-requisite for the incorporation of

Figure 6.12

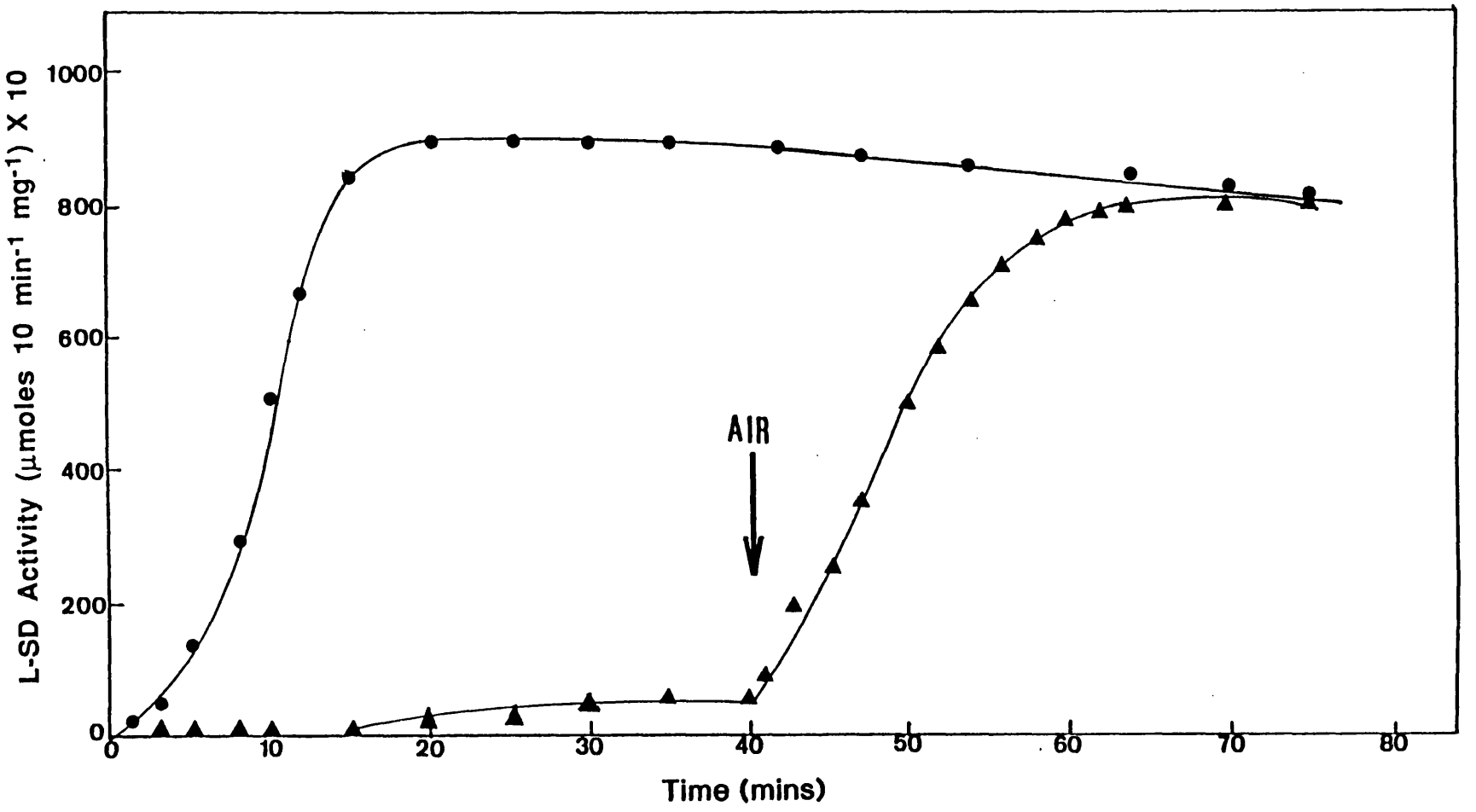
The Activation of L-Serine Dehydratase by Ferrous Sulphate and Dithiothreitol Under Anaerobic Conditions

A preparation of L-serine dehydratase (445 µl of Step 8 enzyme, protein concentration 0.15 mg ml^{-1} containing 100 mM glycylglycine-NaOH, pH 8.0) in a 3 ml glass tube fitted with an air-tight rubber septum was deoxygenated by repeated, gentle flushing with a stream of low pressure nitrogen gas introduced through a 1.1 mm bore syringe needle (nitrogen pressure approximately 1 psi). When the deoxygenation procedure was completed (a 10 to 15 minute treatment was usually sufficient to flush out the oxygen from the dehydratase solutions), the needles were removed from the septum and the dehydratase solution equilibrated at 37 °C. Dithiothreitol (50 µl of a 0.1 M solution) and $FeSO_4$ (5 µl of a 0.1 M preparation) which had been freshly prepared in deoxygenated water, and then treated with nitrogen as described for the enzyme solution, were warmed to 37 °C and then carefully injected through the septum using a gas-tight Hamilton syringe. The components of the incubation medium were carefully mixed, and samples (10 µl) withdrawn at the intervals shown in the Figure using a 10 µl Hamilton syringe, and assayed in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine, as described in Chapter 2, section 2.6.1. After 40 minutes had elapsed, the septum was removed from the tube, and the contents aerated by gentle shaking. Samples of the activation mixture were removed and assayed as already described.

A control experiment was also performed, in which L-serine dehydratase was incubated with 1 mM $FeSO_4$ and and 10 mM DTT under aerobic conditions. The assays of enzyme activity were performed as described above.

● ● ● ● Activity under aerobic conditions

▲ ▲ ▲ ▲ Activity under anaerobic conditions


L-SD Activity (μmoles 10 min-1 mg-1) X 10
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
AIR
Time (mins)

ferrous iron and the construction of the active [4Fe-4S] cluster (Kennedy *et al*, 1983). The oxygen dependence of the L-serine dehydratase iron-activation process suggests that the iron and dithiothreitol may be playing a somewhat different role. When in solution and exposed to atmospheric oxygen, Fe^{2+} and DTT have been shown by Kim *et al* (1985) to promote the formation of reactive oxygen species, such as hydrogen peroxide, the superoxide anion, or hydroxyl radicals. A possible scheme for the generation of such molecules involving iron and dithiothreitol is shown below:

$$Fe^{2+} + O_2 \text{ ------> } Fe^{3+} + O_2^- \quad (1)$$

$$2\,O_2^- + 2\,H^+ \text{ ------> } H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \text{ -------> } Fe^{3+} + OH^- + OH\cdot \quad (3)$$

(adapted from Kim *et al*, 1985).

Oxidation of the ferrous iron by molecular oxygen (equation 1) results in the formation of the superoxide anion (O_2^-), which can then undergo the dismutation reaction (equation 2) to form hydrogen peroxide. The hydrogen peroxide produced can in turn react with the Fe^{2+} via the Fenton reaction (equation 3), generating a hydroxyl radical (OH·). Ferrous iron can be re-generated from Fe^{3+} using DTT, thus allowing the recycling of the metal and the continued production of oxidants.

If one or other of these oxygen species were involved in the activation produced by iron and DTT, then the prevention of their formation should inhibit the iron-activation of L-serine dehydratase. Thus, if the superoxide radical was involved in the reaction of L-serine dehydratase with ferrous iron and DTT, then superoxide dismutase, which catalyzes the conversion of superoxide anions into hydrogen peroxide and molecular oxygen should be able to inhibit or prevent the activation. Similarly, if the activation process involves either peroxide, or a hydroxyl radical, then catalase, which decomposes H_2O_2 into O_2 and water, should be able to prevent the activation of the dehydratase.

To test these possibilities, L-serine dehydratase (step 8, protein concentration 0.13 mg ml^{-1}) was incubated in separate experiments in mixtures containing 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$ and catalase or superoxide dismutase each at concentrations ranging from 0.1 to 10 mg ml^{-1}. Care was taken to ensure that the addition of the enzymes was made before those of the iron or dithiothreitol. The iron-activation timecourses for L-serine

dehydratase in the presence of catalase and superoxide dismutase are shown in Figures 6.13 and 6.14, respectively.

It can be seen that both catalase and superoxide dismutase were able to cause a reduction in the activation of the dehydratase, although high concentrations of the two enzymes were needed to achieve this. The level of inhibition of the iron-activation process produced by catalase was significantly greater than that produced by superoxide dismutase. At a catalase concentration of 10 mg ml^{-1} the activation process showed a greater than 95 % inhibition, while the same concentration of superoxide dismutase produced an inhibition of less than 35 %. When both catalase and superoxide dismutase were included in the iron-activation medium together at concentrations of 0.1 and 1.0 mg ml^{-1} and 1.0 and 10.0 mg ml^{-1}, respectively, the activation profiles shown in Figure 6.14 were obtained. The combined effects of catalase and superoxide dismutase (at 10 and 1 mg ml^{-1}, respectively) did not produce a reduction in the extent of the activation by Fe^{2+} and DTT significantly beyond that achieved by preincubating the dehydratase with catalase (10 mg ml^{-1}) alone (Figure 6.15). These results show that the effect of superoxide dismutase on the iron-activation process is small, and suggest that the interaction between iron, DTT and L-serine dehydratase does not involve a superoxide anion.

An attempt to identify which oxygen species might be involved in the iron-activation reaction was also made using the hydroxyl (OH.) radical scavenger mannitol. The effect of this sugar alcohol on the activation of L-serine dehydratase was tested by its inclusion into incubation or assay mixtures containing iron and DTT, as described in the legend to Table 6.4. It can be seen that the inhibitory effects of this free radical scavenger appear to be fairly limited. Even at the highest concentration tested (100 mM), the dehydratase was only inhibited by about 25 % when mannitol was included in preincubation mixtures (column A). Even less inhibition of L-serine dehydratase activity was seen when the same concentrations of mannitol were included in assay mixtures containing iron and dithiothreitol (column B). In this experiment, 100 mM mannitol caused only a 15 % inhibition of the iron-activation process. These results appear to indicate that the effect of mannitol on the iron-activation of L-serine dehydratase is quite small, and suggests that the interaction of the dehydratase with iron, DTT and oxygen does not involve the production of hydroxyl radicals.

Positive evidence for the involvement of reactive peroxide molecules in the iron-activation process would seem to be provided by the observation that hydrogen peroxide was able to increase the extent of the activation produced by Fe^{2+} and

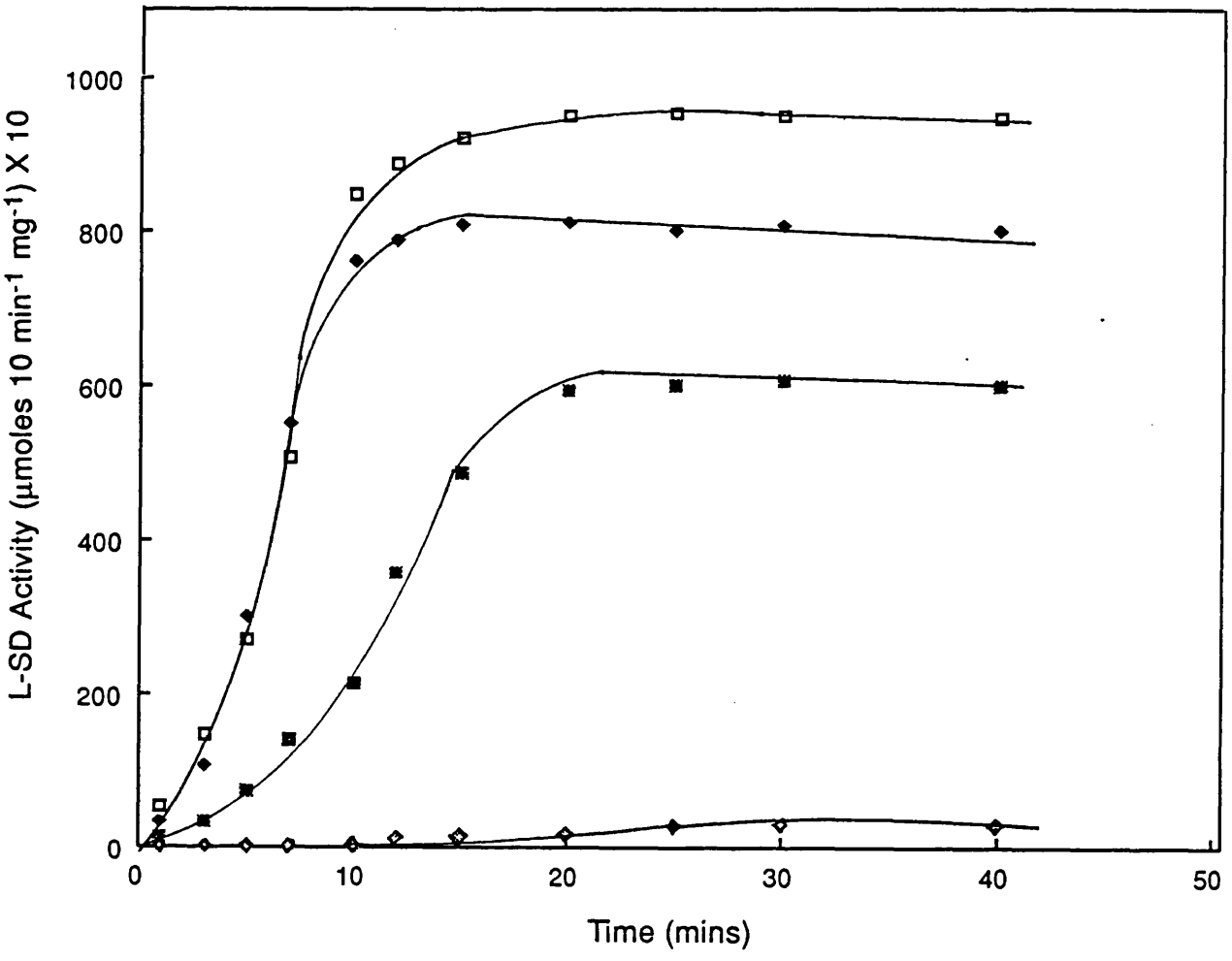


Figure 6. 13

The Inhibition of the Iron-activation of L-Serine Dehydratase by Catalase

L-Serine dehydratase (Step 8, protein concentration 0.13 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT, 1 mM $FeSO_4$, and catalase at the concentrations indicated below.

At the times shown, samples (10 µl) of the incubation mixtures were removed for assay at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1

□ □ □ □ 0 mg ml^{-1} Catalase

◆ ◆ ◆ ◆ 0.1 mg ml^{-1} Catalase

■ ■ ■ ■ 1.0 mg ml^{-1} Catalase

◇ ◇ ◇ ◇ 10.0 mg ml^{-1} Catalase

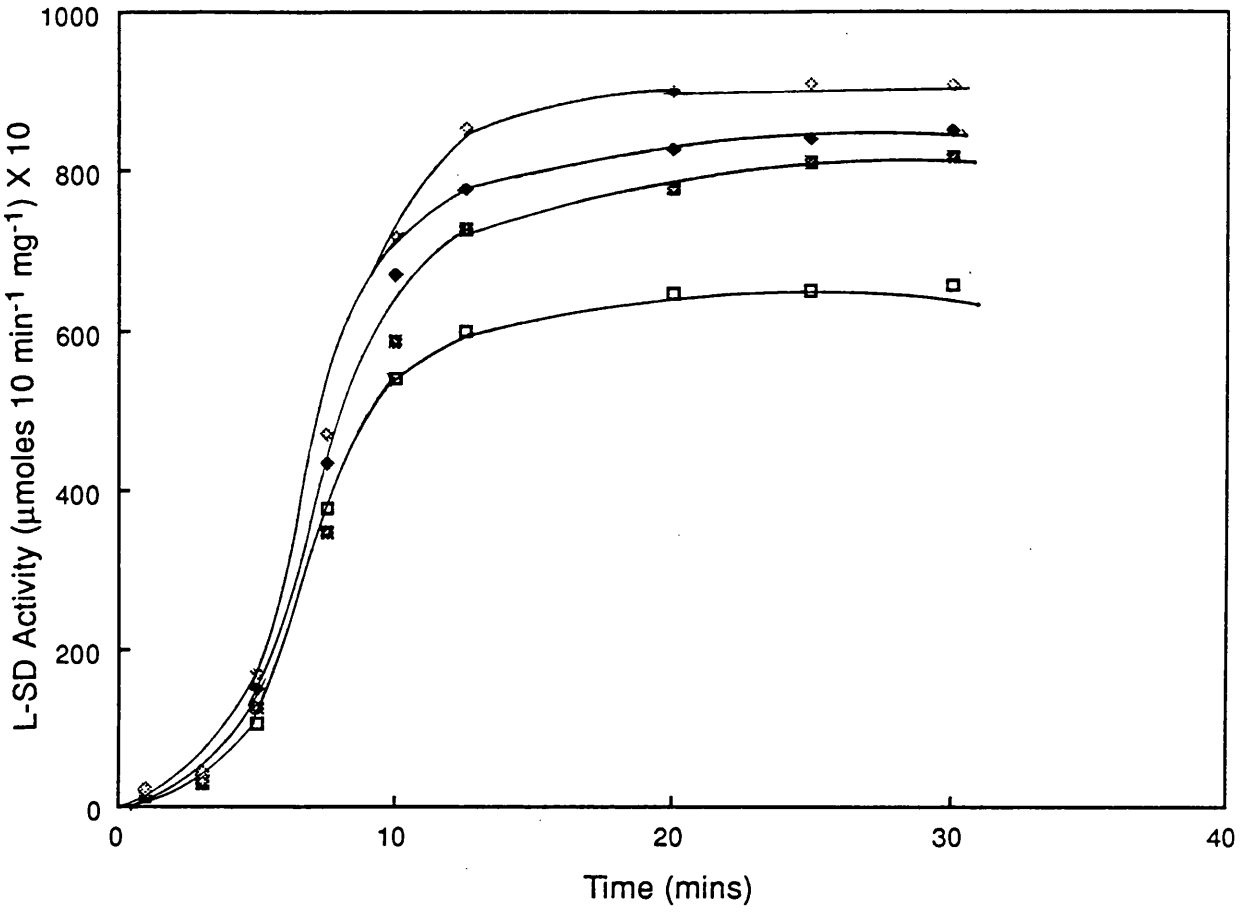


Figure 6.14

The Inhibition of the Iron-activation of L-Serine Dehydratase by Superoxide Dismutase

L-Serine dehydratase (Step 8, protein concentration 0.13 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$, and superoxide dismutase at the concentrations indicated below.

At the times shown, samples (10 µl) of the incubation mixtures were removed for assay at 37 °C in 100 mM glycylglycine-NaOH, pH 8.0 containing 50 mM L-serine. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1

◇ ◇ ◇ ◇ 0 mg ml^{-1} Superoxide Dismutase

◆ ◆ ◆ ◆ 0.1 mg ml^{-1} Superoxide Dismutase

■ ■ ■ ■ 1.0 mg ml^{-1} Superoxide Dismutase

□ □ □ □ 10.0 mg ml^{-1} Superoxide Dismutase

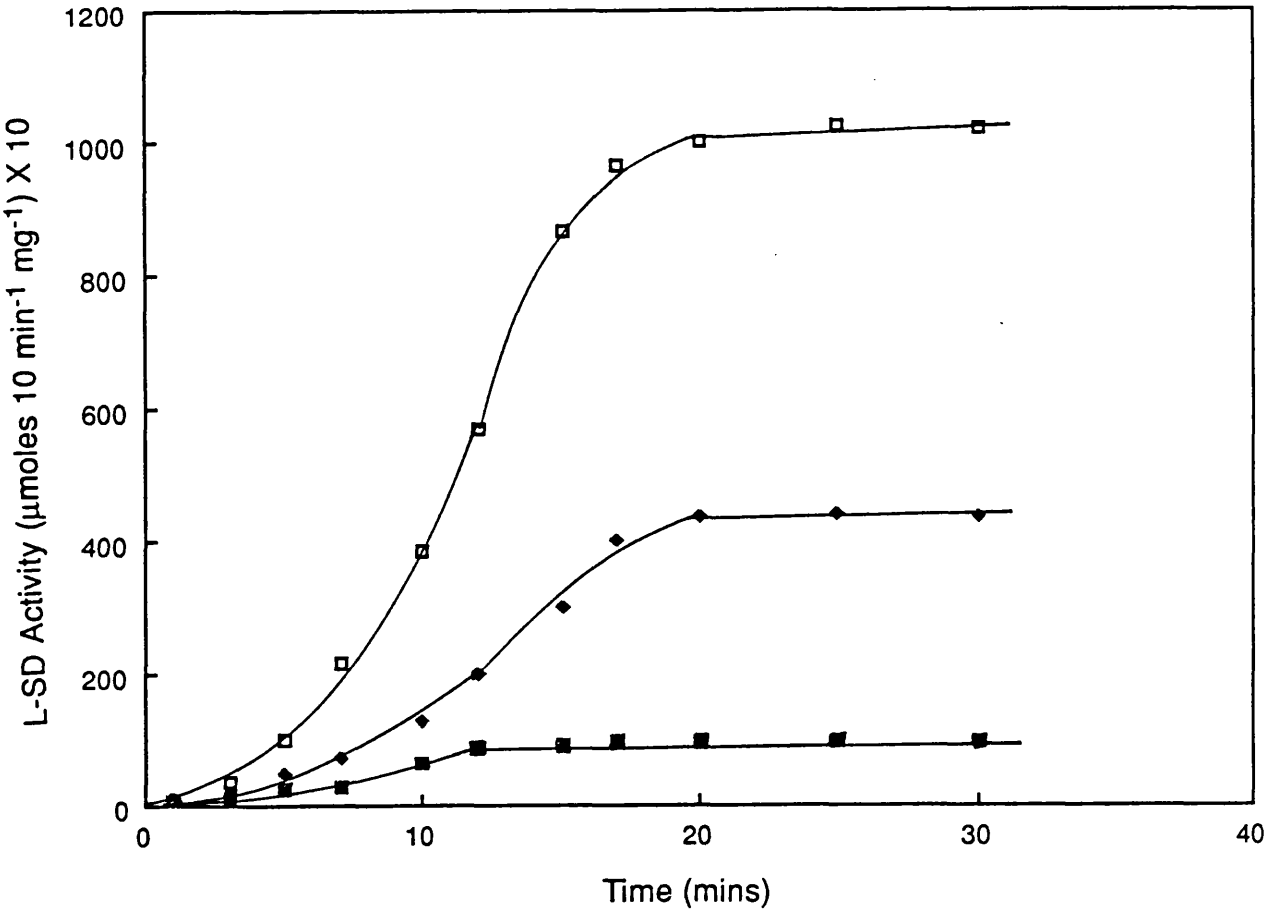


Figure 6.15

The Inhibition of the Iron-activation of L-Serine Dehydratase by Catalase and Superoxide Dismutase

L-Serine dehydratase (step 8, protein concentration 0.12 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT, 1 mM $FeSO_4$, plus catalase and superoxide dismutase at the concentrations indicated below. At the time intervals shown in the Figure, samples (10 µl) of the preincubation mixtures were removed for assay in 100 mM glycylglycine-NaOH, pH 8.0 and 50 mM L-serine. Pyruvate formed during the assays was measured using the 2,4-dinitrophenylhydrazine method, as described in Chapter 2, section 2.6.1.

□ □ □ □ 0 mg ml^{-1} Catalase,
0 mg ml^{-1} Superoxide Dismutase

◆ ◆ ◆ ◆ 1.0 mg ml^{-1} Catalase
1.0 mg ml^{-1} Superoxide Dismutase

■ ■ ■ ■ 10.0 mg ml^{-1} Catalase
10.0 mg ml^{-1} Superoxide Dismutase

Table 6.5

The Effect of Mannitol on the Iron-activation of L-Serine Dehydratase

L-Serine dehydratase (step 8, protein concentration 0.12 mg ml^{-1}) was either assayed with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT, 1 mM $FeSO_4$, 50 mM L-serine and the concentrations of mannitol shown below (column A); or preincubated with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT, 1 mM $FeSO_4$, and the concentrations of mannitol indicated below (column B). The iron-activation timecourse of the preincubation mixtures were followed by assaying samples of the mixtures (10 µl) in 100 mM glycylglycine-NaOH, pH 8.0 and 50 mM L-serine, as described in the legend to Figure 6.5. The results shown below represent the maximal level of activity reached by each preincubation.

Mannitol Concentration (mM)	**L-Serine Dehydratase Activity (µmoles 10 min^{-1} mg^{-1}) X 10**	
	A Assay	**B Preincubation**
0	1630.5	1610.0
0.10	1598.0	1530.0
10.0	1510.3	1450.0
100.0	1385.5	1203.5

dithiothreitol (Figure 6.16). L-Serine dehydratase which was aerobically iron-activated in the presence of 100 μM hydrogen peroxide showed a 40 % increase in activity compared to enzyme activated in its absence. This stimulation of the iron-activation process by hydrogen peroxide did not seem to be particularly dependent upon concentration, since a ten-fold higher peroxide concentrations produced only a 10 % further increase in activation.

The inhibition of the reaction between iron and DTT and L-serine dehydratase by catalase and its stimulation by hydrogen peroxide would seem to indicate that a species of oxygen radical might be involved in the iron-activation process, possibly involving, or formed from, hydrogen peroxide. Signals corresponding to (oxygen) free radicals were observed on all EPR traces of iron-activated L-serine dehydratase. However, since the potential for a Fenton reaction exists where there are even minute (sub-micromolar) traces of Fe^{2+} (and a reducing agent to re-cycle the oxidised iron), it is possible that these free radical signals were the product of a system unconnected with L-serine dehydratase.

6.8 Spectroscopic Studies of L-Serine Dehydratase

The results presented in section 6.7 have shown that the activation of L-serine dehydratase by iron and DTT requires oxygen, and that the interaction of these molecules may result in the production of a reactive oxygen species of some kind, possibly peroxide. Such a requirement for oxygen is not shared by the FeS (de)hydratases mentioned earlier, which would suggest that iron and DTT are not acting on L-serine dehydratase to re-plenish an Fe-deficient iron sulphur centre. It may be that it is some kind of reactive oxygen species generated by the interaction of these reagents that is responsible for the activation of L-serine dehydratase (a possibility that is considered at greater length in section 6.9). However, a requirement of ferrous iron, DTT and oxygen for activity was also shown by the ribonucleotide reductase from *E. coli*, an enzyme which possesses a centre in which the iron is complexed to oxygen, rather than to sulphur (Chapter 1, section 1.5.1), and it is therefore possible that L-serine dehydratase may also contain such a group.

In an attempt to clarify the structural form in which iron exists in L-serine dehydratase, the following investigations of the spectroscopic properties of this enzyme were undertaken.

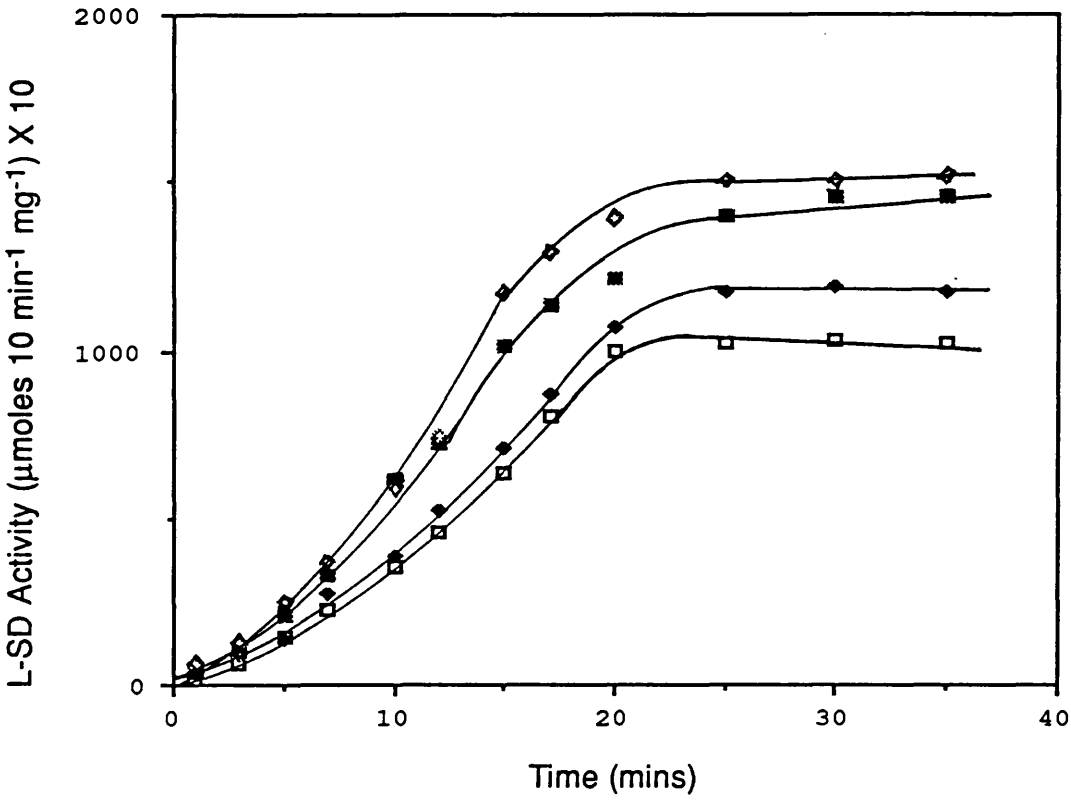


Figure 6.16

The Iron-activation of L-Serine Dehydratase In the Presence of Hydrogen Peroxide

L-Serine dehydratase (step 8, enzyme concentration 0.13 mg ml^{-1}) was incubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT, 1 mM $FeSO_4$ and the concentrations of hydrogen peroxide indicated below. At the times shown in the Figure, samples (10 µl) were removed and assayed at 37 °C in mixtures containing 100 mM glycylglycine-NaOH buffer, pH 8.0 and 50 mM L-serine. Pyruvate formed during the assays was measured as described in Chapter 2, section 2.6.1.

□ □ □ □ 0 µM H_2O_2

◆ ◆ ◆ ◆ 10 µM H_2O_2

■ ■ ■ ■ 100 µM H_2O_2

◆ ◆ ◆ ◆ 1000 µM H_2O_2

6.8.1 UV/Visible Spectrum of L-Serine Dehydratase

The UV/visible spectrum of L-serine dehydratase scanned over the 260 to 600 nm range was shown previously (Figure 5.1). The spectrum of the unactivated, inactive dehydratase appeared to have no significant absorbance in the visible range of the spectrum, and the dehydratase did not show the characteristic 410 to 435 nm peak typical of pyridoxal phosphate-containing enzymes. Iron-activation of the dehydratase resulted in a small, but consistent increase in absorbance over the 400 to 500 nm range. This increase in absorbance did not resemble the visible spectra produced by the FeS centres within either aconitase (Emptage *et al*, 1985), maleic acid hydratase (Dreyer, 1985) dihydroxyacid dehydratase (Flint and Emptage, 1988), nor the oxo bridged binuclear Fe centre in ribonucleotide reductase (Petersson *et al*, 1980). The addition of 100 mM D- or L-serine to unactivated L-serine dehydratase produced no obvious changes in the spectroscopic properties of the protein (trace not shown).

When L-serine (100 mM) was added to iron-activated enzyme, the only obvious change in the spectrum was a time dependent increase in absorbance at about 330 nm. This was not observed when an equivalent concentration of D-serine was added to activated L-serine dehydratase. An analysis of the L-serine-treated enzyme with 2,4-dinitrophenylhydrazine showed the presence of a 2,4-dinitrophenylhydrazone derivative that absorbed maximally at 445 nm. This suggested that the 330 nm peak was probably due to the accumulation of the product of the dehydratase reaction, pyruvic acid, which has a λ_{max} of 320 nm.

6.8.2 The EPR Spectrum of L-Serine Dehydratase

EPR spectra were carried out with the assistance of Dr. Nigel Deaton, of the Department of Chemistry, University of Leicester.

Proteins which contain FeS or Fe-O-Fe clusters usually produce EPR spectra which contain one or more distinctive *g* values, usually less than, or in the region of, 2.00. For example, reduced, iron-activated aconitase shows a 3-component signal consisting of *g* values of 2.06, 1.93 and 1.86, typical of a $[4Fe\text{-}4S]^{1+}$ centre (Emptage *et al*, 1983), while the binuclear centre in ribonucleotide reductase possesses *g*-average values in the 1.7 to 1.8 range (Que and Scarrow, 1988). Consequently, a careful search was made of the electron paramagnetic spectrum of L-serine dehydratase for the presence of such iron-containing centres. To maximise the likelihood of the detection of such a centre, the EPR properties of the enzyme were examined at high microwave power levels (5 to 20 mW), in order

to amplify the signals which might have been produced by any FeS, or Fe-O-Fe centres present, and under a wide variety of experimental conditions.

L-serine dehydratase which had not been activated by iron and DTT was EPR silent. When the spectrum of the enzyme was examined at liquid nitrogen temperatures (90 to 100 K), no signals were seen that could be attributed to either an iron-sulphur, or iron-oxygen cluster (Figure 6.15). However, this could be a consequence of the oxidation state of the iron, since only certain iron valencies (+1 and +3) are paramagnetic, and produce EPR signals. To test this possibility, the spectrum of unactivated dehydratase reduced by incubation with 100 μM sodium dithionite was examined (this concentration of dithionite produced a small stimulation of the activity of the enzyme). As before, no signals suggestive of any type of Fe-S or Fe-O centre were detected. Purified L-serine dehydratase is inactive, and possibly devoid of iron (a series of chemical analyses of the Fe-content of the unactivated dehydratase, described in section 6.6, were unable to detect iron within the protein). Therefore, it may be that the EPR-silence of the unactivated enzyme was a consequence of this lack of iron. However, the EPR spectrum of L-serine dehydratase activated by preincubation with Fe^{2+} and DTT, and then separated from its activators by spun column chromatography, as described in section 6.6.1, also failed to show the characteristic signals of either 2Fe-, 3Fe- or 4Fe-containing FeS centres, or oxo-bridged di- or poly-iron Fe-O centres.

The $[4Fe\text{-}4S]^{2+}$ centre in aconitase, produced by anaerobic incubation of the inactive native enzyme with Fe^{2+} and DTT is diamagnetic, and thus EPR-silent. Reduction of the centre by one electron by sodium dithionite converts the iron into a paramagnetic state, and therefore detectable by EPR (Emptage *et al*, 1983). Such a situation was envisaged for L-serine dehydratase, and so the spectrum of iron-activated enzyme which had been similarly reduced by dithionite was investigated. As before, no signals characteristic of iron-sulphur or iron-oxygen containing complexes were discovered. The addition of 100 mM L-serine to preparations of the iron-activated or iron-activated and reduced enzyme also failed to produce any significant change in the EPR properties of the dehydratase.

The spectra of both FeS and FeO proteins are both enhanced and better resolved at low temperatures (of the order of 30 K or less). Thus signals originating from any iron-containing centres within L-serine dehydratase might not have been detectable at the temperatures at which the initial set of EPR spectra were determined. To examine this possibility, measurements were made of the EPR spectrum of the dehydratase at lower temperatures (8 to 10 K) and under the

same conditions as those described above. As before, no signals were detected that might have been produced by either an FeS, or an oxo-bridged di- or poly-iron centre.

6.9 Discussion

L-Serine dehydratase, in contrast to the L-threonine dehydratases and the D-serine dehydratase of *E. coli*, does not appear to utilise pyridoxal phosphate as a cofactor. Instead, this dehydratase has an absolute and highly specific requirement for iron and a reducing agent, most effectively met by dithiothreitol. The results presented in this chapter have shown that the activation of L-serine dehydratase by these reagents is a process of considerable complexity which is influenced by a variety of factors.

It was seen that both iron and DTT were needed for the activation of the purified dehydratase. Either factor alone was unable to restore the activity of the dehydratase. The activation produced by Fe^{2+} and dithiothreitol was found to be slow. L-Serine dehydratase incubated with 10 mM DTT and 1 mM $FeSO_4$ only required up to 20 minutes to reach a state of maximal activity. However, the presence of L-serine in the iron-DTT activation mixture considerably reduced the amount of time required to achieve full activation of the dehydratase. This suggests that serine-activation and iron-activation reactions are processes which may be inter-related, a possibility that is considered at greater length in the next chapter.

The activation of L-serine dehydratase by ferrous iron and dithiothreitol was also found to be influenced by both temperature and pH. The requirement of L-serine dehydratase for iron was further shown by the fact that metal binding agents such as EDTA and o-phenanthroline were able to prevent, or reverse the iron-activation of the enzyme. Chelators were also able to inactivate Fe-independent L-serine dehydratase. In both cases, the inhibition caused by the chelators could be fully reversed by treating the inactivated enzyme with iron and DTT. The work described in section 6.6 demonstrated that the activation by iron appeared to involve the actual binding of the metal. A combination of chemical analyses and $^{59}Fe^{2+}$-binding experiments suggested that iron was incorporated into the dehydratase at a ratio of 2-3 μmoles of iron per μmole of enzyme.

These characteristics of the L-serine dehydratase iron-activation process are very similar to those possessed by FeS-containing (de)hydratases such as aconitase and maleate hydratase, which suggested in the early course of this work that

L-serine dehydratase may also have contained an iron sulphur centre. However, the subsequent discovery that molecular oxygen was required for the activation process began to cast some doubt upon this possibility, since oxygen was usually found to be inhibitory to FeS enzymes. Further, when a series of experiments which examined the UV/visible and EPR properties of L-serine dehydratase were undertaken, no evidence for the presence of an FeS centre in this dehydratase was found. Collectively, these results strongly suggest that L-serine dehydratase does not contain an iron sulphur centre.

A requirement for iron, a thiol and oxygen for activity similar to that of L-serine dehydratase was also shared by the ribonucleotide reductase from *E. coli*, an enzyme which contains a centre in which two iron atoms are complexed to oxygen (Reichard, 1979). To determine if L-serine dehydratase also contained such a group, an examination was made of the UV/visible and EPR properties of the enzyme. However, none of these investigations produced spectra which contained evidence for the presence any type of oxo bridged centre. In fact, the dehydratase did not appear to possess any significant light absorption or EPR characteristics at all. If iron does exist in L-serine dehydratase in a distinct molecular arrangement, it must exist in an oxidation state which renders it EPR-silent.

The requirement of an L-serine-specific dehydratase for oxygen for iron-activation has also been independently demonstrated by Newman and colleagues (Newman *et al*, 1990). These workers found that preincubation of the *E. coli* K12 L-serine dehydratase with iron and DTT under anaerobic conditions did not result in the activation of the enzyme. They also found that DETPAC, a metal chelating agent which binds Fe^{3+} particularly strongly (Brawn and Fridovich, 1981), was able to prevent the iron-activation when included in preincubation media. These facts suggested to Newman and co-workers that during the activation process, the iron was undergoing a cycling between Fe^{2+} and Fe^{3+} oxidation states. Since this cycling has the potential for generating reactive oxygen radicals, as discussed earlier (section 6.7), they concluded that the involvement of iron in the activation produced by Fe^{2+} and DTT was an indirect one, and that it was an oxidant generated during the interaction of these molecules that was responsible for the activation of L-serine dehydratase. A series of experiments were undertaken to determine the identity of this oxygen radical. No evidence was found to suggest that the reactive species was a superoxide anion, since superoxide dismutase was not found to inhibit the activation. Newman *et al* also found that catalase and hydrogen peroxide had no significant effect on the activation produced by Fe^{2+} and DTT, and so excluded a

role for peroxide in the iron-activation process. They did find, however, that hydroxyl radical scavengers such as mannitol and L-histidine were able to inhibit the activation of the dehydratase when included in activation mixtures from the start of the preincubation. For instance, mannitol concentrations of 50 and 250 mM caused a 23 % and 44 % inhibition of L-serine dehydratase, while a L-histidine concentration of 25 mM caused an 85 % inhibition of activity. Once activation by iron and DTT had occurred, the effect of mannitol and L-histidine on the activity of L-serine dehydratase was comparatively small, with the concentrations of mannitol and L-histidine quoted causing inhibitions of 1 %, 6 % and 10 %, respectively.

Newman and co-workers suggest that the activation of the *E. coli* K12 L-serine dehydratase by iron and DTT involves a proteolytic cleavage at a specific serine-serine bond within the dehydratase, in a manner analogous to the activation of the histidine decarboxylase from *Lactobacillus* (Recsei *et al*, 1983). They propose that it is a hydroxyl radical generated by the cycling of the iron between oxidation states that is the agent which is responsible for this cleavage and activation, and that iron plays no direct role in the iron-activation process. Since the activation reaction also requires DTT, Newman and co-workers suggest that the cut enzyme would exist in a reduced state, and that maintenance of this state would be required for continued activity. They base their evidence for this suggestion on the observation that the activity of iron-activated enzyme gradually decays upon standing, and that part of this lost activity can be restored by treatment with DTT alone. Thus, cleaved L-serine dehydratase would exist in two forms: reduced and active, or oxidised and inactive. Reduction of the latter would regenerate the former.

Unfortunately, the investigations of Newman *et al* (1990) were published when the work described in this thesis was completed, and so it has not proved possible to repeat or extend their investigations. The experiments described in this Chapter agree with Newman's work on a number of points. L-Serine dehydratase, in its Fe-dependent form requires DTT and oxygen, as well as iron for activity, which would seem to support Newman's suggestion that iron cycling, or at least iron oxidation, is involved in the iron-activation reaction. Evidence has also been obtained in the current study which suggests that the interaction of iron, DTT, O_2 and L-serine dehydratase involves the production of a reactive oxygen species, which also agrees with the work of Newman *et al*. However, the two studies do not entirely agree on the likely identity of this reactive oxygen molecule. The work in section 6.7.2 showed that catalase was able to inhibit the iron-activation of L-serine dehydratase, and that hydrogen peroxide was able to

stimulate it. In contrast, Newman *et al* found that the effect of these factors on the activation process was insignificant, and instead suggested, based on the evidence that hydroxyl radical scavengers, such as mannitol and L-histidine were inhibitory, that the iron-activation of L-serine dehydratase involved a hydroxyl radical. However, very high concentrations of mannitol were required to cause even a partial inhibition of the dehydratase: a mannitol concentration of 250 mM produced only a 44 % inhibition of activity. L-Histidine is an efficient chelator of Fe, and it is therefore possible it inhibited the iron-activation reaction by virtue of its ability to chelate Fe, rather than as a consequence of its ability to scavenge hydroxyl radicals, a point conceded by Newman and colleagues.

It is in the mechanism by which Newman *et al* (1990) propose that iron and DTT activate L-serine dehydratase that this study and the current work most strongly disagree. The work in section 6.6 showed a strong correlation between Fe-uptake and iron-activation, suggesting that the involvement of iron in the deamination reaction was rather more direct than that proposed by Newman. If L-serine dehydratase was activated by a mechanism involving serinolysis at a specific serine-serine bond, then the gradual loss of enzyme activity that was observed during the course of its purification, which is suggestive of the loss of a native cofactor, and its subsequent restoration by iron and dithiothreitol, should not be possible. The finding that iron-activated L-serine dehydratase can be separated from its activators, and still retain activity is more supportive of a model involving iron-binding, rather than iron-catalysed cleavage. Further, since cleavage reactions are usually irreversible molecular events, it should not be possible to re-activate Fe-dependent enzyme by incorporating iron, to remove it using chelators, and then to restore activity by re-adding the metal, as has been shown in section 6.5. The sucrose gradient studies in Chapter 7, section 7.3.1 show that iron-activation of L-serine dehydratase does not result in the cleavage of the enzyme, a finding that is also confirmed by the fact that native and SDS gels of L-serine dehydratase before and after iron-activation revealed no noticeable change in its molecular weight. Although it is possible that the mechanism by which iron and DTT activate L-serine dehydratase involves the production of an oxygen radical of some kind, it is most improbable that a proteolytic cleavage is involved in these events.

Although the L-serine dehydratase iron-activation process has been shown to involve iron-incorporation, the form in which the iron exists in the dehydratase and its role in the deamination reaction are as yet unclear. It is highly unlikely, for reasons that have already been discussed, that the incorporated Fe is completing an iron-deficient FeS centre, as occurred with aconitase. It is also

unlikely that the iron is used to build up an oxo-bridged binuclear cluster of the type utilised by ribonucleotide reductase. Further possible roles for the involvement of iron in the activation of L-serine dehydratase are discussed in Chapter 8, section 8.3.

CHAPTER 7 - STUDIES OF THE SERINE-ACTIVATION OF L -SERINE DEHYDRATASE

Chapter 7

Studies of the Serine-Activation of L-Serine Dehydratase

It was shown in Chapter 4, section 4.2 that the timecourse of the purified L-serine dehydratase from *E. coli* B is non-linear. This non-linearity is a consequence of the slow activation of the dehydratase by its substrate, L-serine. The presence of this lag phase allows the L-serine dehydratase to be classified as a hysteretic enzyme. Hysteretic enzymes, as defined by Frieden (1971) exist in two or more forms, interconversion of which is slow with respect to the rate of catalysis. Non-linear timecourses result when the substrate displaces the inter-conversion equilibrium towards the more or less active form of the protein (as described in Chapter 1, section 1.3.4.1).

Roberts (1983) presented evidence which suggested that the molecular basis of the slow activation of the *E. coli* B L-serine dehydratase by L-serine, was a process which involved the dimerisation of relatively inactive enzyme monomers to give fully active dimers. Roberts studied the serine-activation reaction using a variety of approaches, including analyses of the kinetic order of the activation process, and direct measurements of the molecular weight of the enzyme before and after serine-activation. The former approach produced somewhat conflicting results, which suggested that the activation process, with respect to enzyme concentration, was both second and zero order, depending on the method of analysis used. Gel-filtration of the dehydratase before and after activation by D-, or L-serine gave a clearer indication of the nature of the activation process. The molecular weight of activated L-serine dehydratase was found to be double that of the non-activated enzyme, leading Roberts to suggest that serine-activation and dimerisation were facets of the same process. However, to demonstrate unequivocally a causal relationship between serine-activation and dimerisation, a direct measurement of the molecular weight of the enzyme during the serine-activation process is required. Techniques used to measure such changes, such as laser light scattering, require purified enzyme. Roberts was unable to undertake these investigations because of the instability and heterogeneity of the dehydratase preparations with which she worked. However, with the highly purified L-serine dehydratase that is currently available, experiments which directly measure the molecular weight of the activated form of the enzyme can now be considered.

7.1 Definitions

The following terms will be used during this discussion of the 'hysteretic' response of L-serine dehydratase to its substrate:
(i) Fe-Independence This is a measure of the ability of L-serine dehydratase to deaminate L-serine in the absence of ferrous iron or DTT. It is the initial *in vivo* activity possessed by dehydratase in crude or slightly purified extracts, that is gradually lost during the course of its purification (Chapter 3, section 3.1). Purified L-serine dehydratase possesses no Fe-independent activity and is inactive unless supplied with Fe^{2+} and dithiothreitol, either in an assay mixture or in the form of a preincubation treatment with these reagents. Enzyme which exists in this form has been termed Fe-dependent. Chapter 4, section 4.1 contains a more detailed definition of the Fe-independent activity of L-serine dehydratase.

(ii) Iron-activation of L-Serine Dehydratase This terms describes the process which occurs when the activity of Fe-dependent L-serine dehydratase is restored by preincubation with ferrous iron and dithiothreitol (as described in Chapter 2, section 2.6.4 and Chapter 6). Once activation by these compounds has occurred, they are no longer required for activity (Chapter 6, section 6.1.4). The activation produced by iron and DTT is also distinct from that produced by D- or L-serine, since L-serine dehydratase which has been iron-activated is still subject to further activation by L- or D-serine.

(iii) Lag Period This is the period between the start of the enzyme-catalysed reaction and the attainment of the steady state rate, during which enzyme activity increases with time.

(iv) The Activation of L-Serine Dehydratase In this chapter, the terms 'activation', and 'serine-activation', are used to describe the transition which occurs during the lag phase, when L-serine dehydratase is converted from the non-(serine)-activated form of the enzyme, which is characterised by a non-linear continuous timecourse of activity and sigmoidal substrate saturation profile to the (serine)-activated form, which shows a linear timecourse of pyruvate formation and hyperbolic substrate saturation curve. Reagents which can cause this change are termed activators, or activating-ligands - they do not increase the linear catalytic rate, but are responsible for converting non-activated dehydratase to the activated form. In this sense of the use of the term 'activator', substances which inhibit the linear catalytic rate, but which are able to induce the transition to the serine-activated form of the dehydratase, such as D-serine, are also referred to as activators.

(v) The Index of Activation This is a semi-quantitative measure of the degree to which a sample of L-serine dehydratase (Fe-independent or iron-activated) has been activated by D- or L-serine at the time of its addition to an assay mixture. It is the ratio of initial rate : final rate of enzyme activity. The final rate is the rate observed when the rate of pyruvate production is linear and before any of the components in the assay mixture become limiting. The initial rate is the rate obtained by drawing a tangent at a point 0.5 cm after the start of the assay trace on the chart recorder. It was not possible to obtain reliable tangents at earlier points on the trace. The index of activation of dehydratase which has been fully activated by L- or D-serine was 1.0, while typical index of activation values for purified dehydratase which had not been activated before assay were 0.3 to 0.4.

7.2 The Activation of L-Serine Dehydratase by D-Serine

Gannon (1973) observed that L-cysteine and D-serine were able to substitute for L-serine in the the activation of the L-serine dehydratase from *A. globiformis*. Later work by Roberts (1983) showed that D-serine could also activate the L-serine dehydratase from *E. coli* B. Enzyme activated by D-serine showed all of the properties possessed by dehydratase which had been activated by L-serine, including a linear timecourse of activity and hyperbolic substrate saturation profile. Gel filtration studies showed that activation by D-serine also resulted in a doubling of the molecular weight of the enzyme. The ability of D-serine to catalyse the conversion of L-serine dehydratase from the non-activated to the activated form of the enzyme was an important discovery which allowed the activation process to be studied independently of the catalytic process. For this reason, D-serine was chosen as the activating ligand for the study of the serine-activation of purified L-serine dehydratase in the present study. Although it is possible to activate the purified dehydratase with L-serine, the use of D-serine, which is not deaminated by the dehydratase, is technically useful, since it avoids the problem of pyruvate production which occurs when L-serine is used as activator. This is particularly relevant for gel filtration and sucrose gradient work, where high levels of pyruvate in the enzyme fractions would complicate the analysis of the activity of the dehydratase.

7.2.1 The Timecourse of Activation of L-Serine Dehydratase by D-Serine

To test the ability of D-serine to activate purified L-serine dehydratase, samples of the purified enzyme (step 8, protein concentration 0.10 mg ml^{-1}) were iron-activated as described in Chapter 2, section 2.6.4. D-serine (100 mM) was then

added to the incubation mixture, and samples (10 µl) were removed at intervals over a 60 minute period for assay by the lactate dehydrogenase method (Chapter 2, section 2.6.2). The carryover of D-serine into the assay mixture (1mM) was not sufficient at the concentration of L-serine used (50 mM) to cause a significant inhibition of the activity of the dehydratase. The ratios of initial to final rates (the index of activation) were calculated from the reaction trace for each sample in order to quantitate the activation process.

The results shown in Table 7.1 reveal that D-serine (100 mM) was able to activate the dehydratase fully within 10 minutes. This is longer than the usual time required for L-serine-activation (1 - 2 minutes). Figure 7.1 shows that the timecourse of D-serine-activated L-serine dehydratase was linear. The concentration of L-serine used to obtain this timecourse was 50 mM. If, however, the D-serine-activated enzyme was assayed at a subsaturating concentration of L-serine (5 mM or less) a timecourse of the type shown in Figure 7.2 was seen. As the assay proceded there was a slow and progressive decrease in the rate of pyruvate production until a second linear rate was established. This phenomenon was also observed by Roberts (1983), who presented evidence to suggest that it was caused by a reversal of the serine-activation process, as some of the fully activated enzyme slowly reverted to the non-activated form. Roberts showed that the extent of this loss of activity was determined by the concentration of L-serine in the assay mixture: the higher the level of L-serine, the greater the proportion of enzyme which could be maintained in the serine-activated state, and the less deactivation seen during the course of the assay.

7.2.2 The Substrate Saturation Curve of D-Serine-activated L-Serine Dehydratase

It was seen in Chapter 4, section 4.3, that the substrate saturation curve for L-serine dehydratase which was not serine-activated was sigmoidal. This sigmoidicity was a consequence of variations in the extent to which the dehydratase was activated by sub-saturating concentrations of L-serine (Roberts, 1983). When iron-activated L-serine dehydratase (step 8, protein concentration 0.12 mg ml^{-1}) was preincubated with 100 mM D-serine until fully activated, and assayed in mixtures containing different concentration of L-serine, the hyperbolic substrate saturation profile shown in Figure 7.3 was obtained. As previously mentioned, when the L-serine concentrations in the assays were subsaturating (5 mM, or below), timecourses of the type shown in Figure 7.2 were obtained. For this reason, only the initial rates of the timecourses of serine-activated enzyme were plotted against L-serine concentration. A Hill plot of the

Table 7.1

The Timecourse of Activation of Iron-activated L-Serine Dehydratase by D-Serine

L-Serine dehydratase (step 8, protein concentration 0.1 mg ml^{-1}) was iron-activated by preincubation at 37 °C with 100 mM glycylglycine-NaOH buffer, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$, as described in Chapter 2, section 2.6.4. D-Serine, (100 mM) was then added to the iron-activated enzyme, and the preincubation continued for a further 60 minutes. At the times shown in the Table, samples (10 µl) of the activated enzyme were assayed at 37 °C in lactate dehydrogenase-coupled assay mixtures containing 100 mM glycylglycine-NaOH, pH 8.0 and 50 mM L-serine. The ratios of initial rate : final rate were calculated from the resulting timecourses.

Length of Preincubation (minutes)	**Initial Rate / Final Rate**
0	0.59
0.33	0.68
2.50	0.80
5.0	0.95
10.0	0.98
15.0	0.99
20.0	1.00
25.0	1.00
30.0	1.00
60.0	1.00

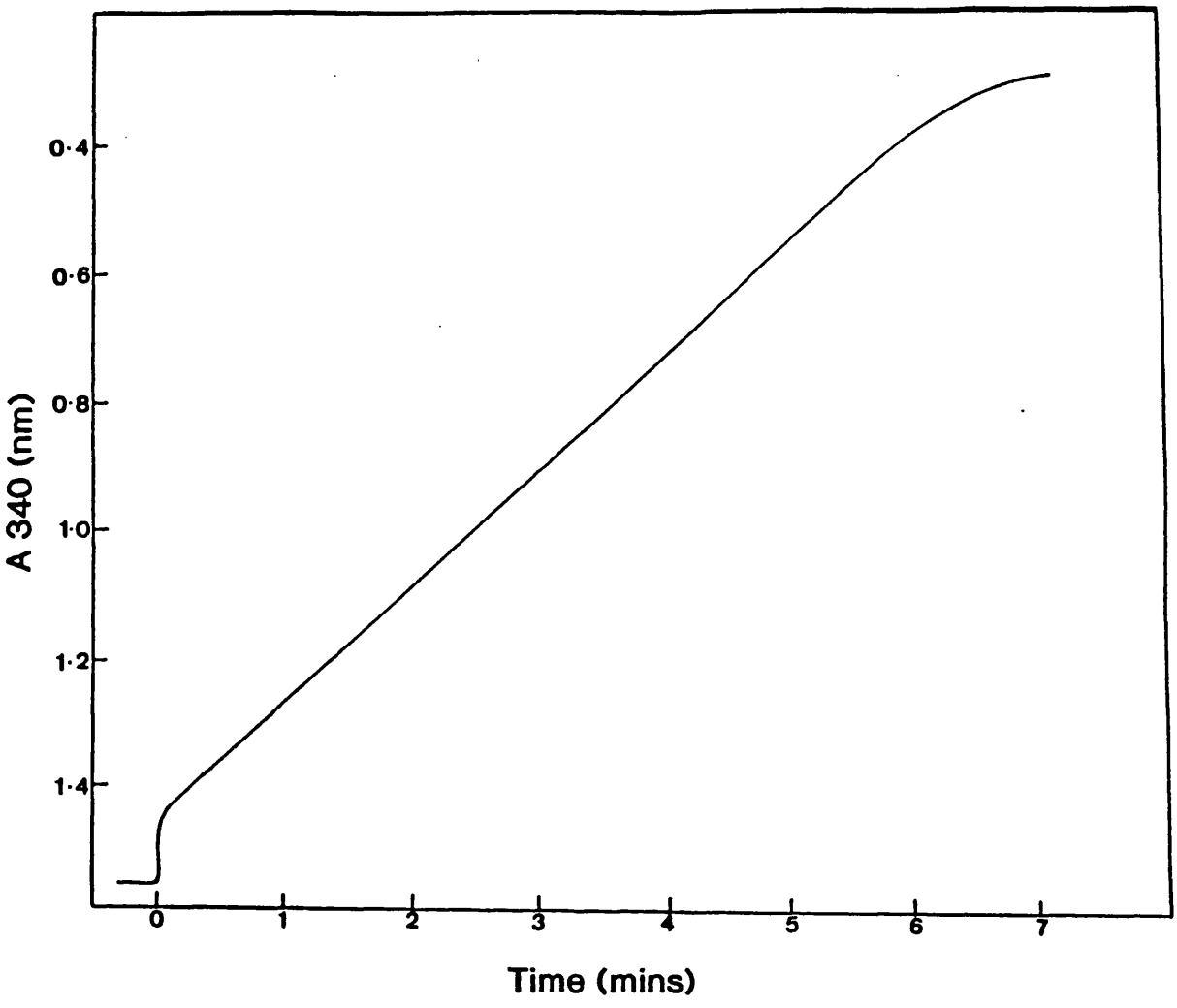


Figure 7.1

The Timecourse of Pyruvate Formation of Serine-Activated L-Serine Dehydratase

L-Serine dehydratase (step 8, 0.25 units) was iron-activated, as described in Chapter 2, section 2.6.4. D-Serine was added to the iron-enzyme preincubation mixture to a final concentration of 100 mM, and the incubation continued for a further 20 minutes. Samples (0.025 units) of the activated dehydratase were then added to lactate dehydrogenase-coupled assay mixtures containing 50 mM L-serine.

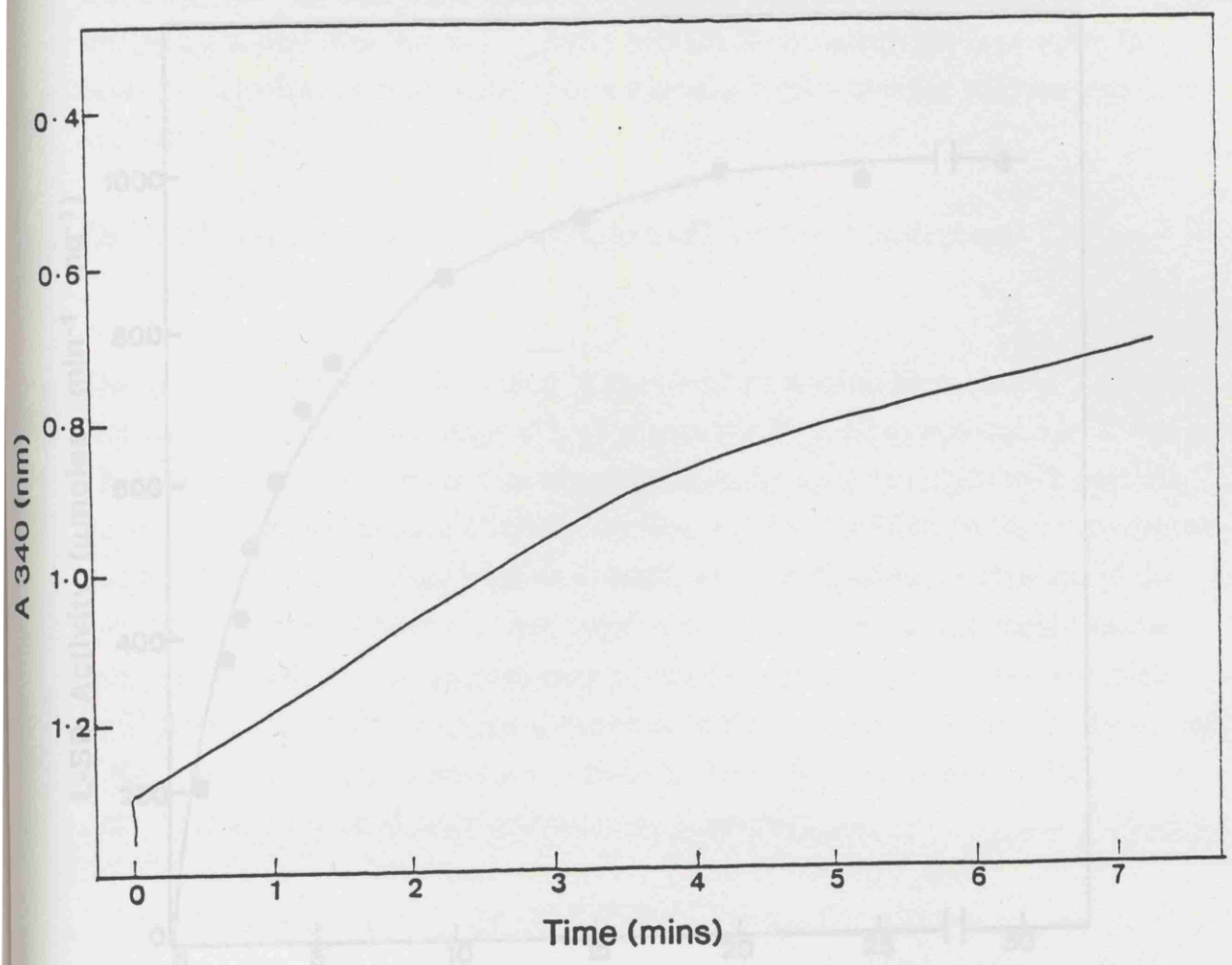


Figure 7.2

The Reversal of Activation Occurring on Addition of Fully Activated L-Serine Dehydratase to an Assay Containing a Subsaturating Concentration of L-Serine (2.5 mM)

L-Serine dehydratase (step 8, 0.25 units) was iron-activated, as described in Chapter 2, section 2.6.4. D-Serine was added to the iron-enzyme preincubation mixture to a final concentration of 100 mM, and the incubation continued for a further 20 minutes. Samples (0.025 units) of the activated dehydratase were then added to lactate dehydrogenase-coupled assay mixtures equilibrated at 37 °C containing 100 mM glycylglycine-NaOH, pH 8.0 and 2.5 mM L-serine. The rate of pyruvate formation was then followed at 340 nm.

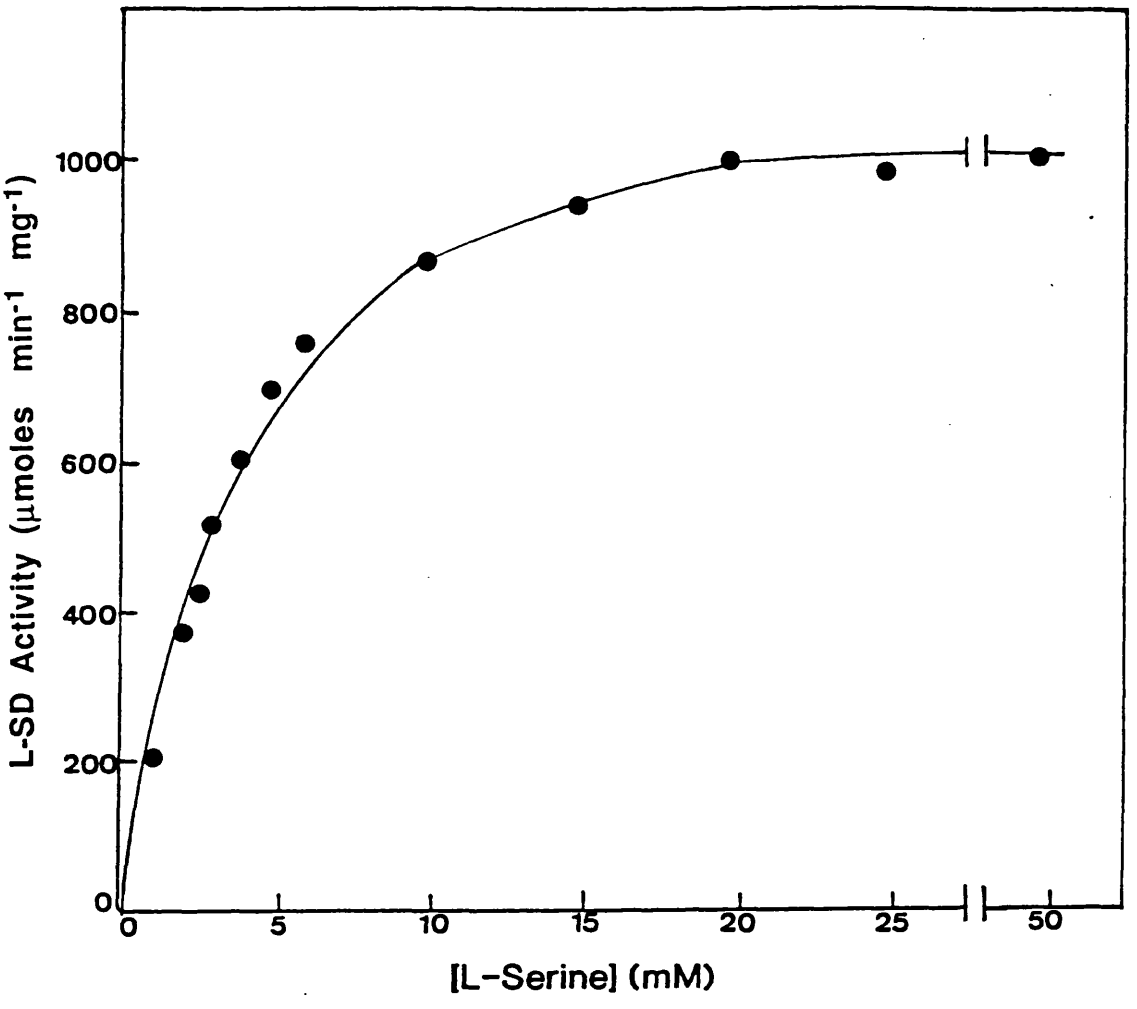


Figure 7.3

The Substrate Saturation Curve of L-Serine Dehydratase Fully Activated by Preincubation with D-Serine

L-Serine dehydratase (step 8, enzyme concentration 0.10 mg ml^{-1}) was iron-activated, as described in Chapter 2, section 2.6.4. D-Serine, (100 mM) was then added to the iron-activated enzyme and the incubation continued for at 37 °C for a further 20 minutes, until the dehydratase had attained an index of activation of 1.0. Samples of the activated enzyme (10 μl) were then assayed at 37 °C in lactate dehydrogenase-coupled assay mixtures containing 100 mM glycylglycine-NaOH, pH 8.0 and the concentrations of L-serine shown in the Figure. The initial, linear rates of pyruvate formation were then plotted against the substrate concentrations at which they were obtained.

data from the substrate saturation curve in Figure 7.3 revealed an $S_{[0.5]}$ value of 3.08 mM L-serine, and a Hill coefficient of 1.10. The low value of the Hill coefficient shows that the cooperativity evident in the substrate saturation for L-serine dehydratase was reduced to a very low level when the enzyme was fully activated.

7.3 The Molecular Basis of the Activation of L-Serine Dehydratase By D-Serine

The kinetic experiments described in the previous section have shown that iron-activated L-serine dehydratase of high purity can be further activated by D-serine. These results confirm the earlier kinetic studies by Roberts (1983) with partially purified D-serine-activated enzyme. In view of Robert's findings that dehydratase activated by D- or L-serine became dimeric, and the clear demonstration of the fact during the present study that purified L-serine dehydratase could become serine-activated, it was expected that purified enzyme which had been activated by D-serine would also undergo a doubling in its molecular weight. To determine if this was so, measurements were made of the molecular weight of the dehydratase before and after activation by D-serine.

7.3.1 Molecular Weight Studies of Purified L-Serine Dehydratase

7.3.1.1 Gel Filtration Studies

Gel filtration on Sephadex G100 columns by the method of Andrews (1964) were used to examine the molecular weight of the activated and non-activated forms of the dehydratase.

The Sephadex G100 columns used in the gel filtration studies were calibrated with standard proteins for every buffer used in their elution, and internal standards were included in every analytical column (Chapter 2, section 2.9.1). The presence of L-serine dehydratase in the column fractions was determined using iron-containing assay mixtures, as described in Chapter 2, section 2.6.1.

When Fe-dependent, non-serine-activated L-serine dehydratase (step 8, 20 units) was placed on a Sephadex G100 column equilibrated in 100 mM glycylglycine-NaOH, pH 7.8 containing 100 mM KCl and 2 mM DTT, the enzyme was eluted to a position corresponding to a molecular weight of 52 000 +/- 3000. This value agrees well with the measurements of 52 000 +/- 2000 made by SDS PAGE and was taken to represent the subunit, or monomeric molecular weight of the enzyme.

When the same preparation of dehydratase was preincubated for 30 minutes with 100 mM D-serine and eluted from a column equilibrated in the same buffers plus D-serine (100 mM), the position of elution of the enzyme also corresponded to a molecular weight of 52 000 +/- 3000. This experiment would appear to indicate that the enzyme had not dimerised in the presence of D-serine. However, the L-serine dehydratase preparation used in this set of molecular weight determinations had not been preincubated with ferrous iron and DTT prior to the treatment with D-serine, and was therefore inactive. Roberts (1983), who was able to demonstrate that dimerisation followed activation by D-serine, used active, Fe-independent enzyme that was only slightly purified. It is possible, therefore, that L-Serine dehydratase may need to be catalytically active, that is Fe-independent or iron-activated, before it can become dimeric.

To test this possibility, L-serine dehydratase (step 8, 20 units) was iron-activated, as described in Chapter 2, section 2.6.4. The continuous timecourse of this preparation of L-serine dehydratase was non-linear. D-Serine (100 mM) was then added to the iron-activated enzyme and the preincubation continued for 15 minutes until the dehydratase showed a clearly linear timecourse of activity, indicating that full serine-activation had occurred. The activated enzyme was then placed on the Sephadex G100 column, and eluted with D-serine-containing glycylglycine buffer, as described above. When the column fractions were assayed for L-serine dehydratase activity no change in the elution profile of the enzyme was observed, and the dehydratase was found to have eluted to a position corresponding to a molecular weight of 54 000 +/- 3 000. The eluted enzyme was no longer active, and required a further incubation with ferrous iron and dithiothreitol for activity.

The failure of the purified dehydratase to double its molecular weight under conditions of activation which had been shown by Roberts (1983) to result in the dimerisation of the enzyme, was unexpected. The techniques used in the present study for the estimation of molecular weights did not significantly deviate from the methods used by Roberts, and so the reason for the non-dimerisation of the activated, purified dehydratase must have originated from some property of this enzyme not shared by the L-serine dehydratase preparation that she used. The dehydratase preparation that Roberts used was catalytically active both before and after elution. Gannon (1973), who was able to demonstrate for the purified *A. globiformis* L-serine dehydratase that dimerisation followed activation by D-serine, also used enzyme that had retained its Fe-independent activity after gel filtration. In contrast, the purified iron-activated, serine-activated enzyme used

in the present study was inactive after its passage down a gel filtration column. It may be that L-serine dehydratase must be catalytically active, that is iron-activated or Fe-independent, before it can dimerise. The results presented in Chapter 6, section 6.6 suggest that iron binding may play an important role in the functioning of the dehydratase. If the presence of iron was necessary for the enzyme to become dimeric and to remain as a dimer it is therefore possible, given the conditions of prolonged and extensive dilution that the dehydratase would have undergone during its passage down the Sephadex column, that the enzyme would have been unable to retain its iron long enough to exist as a dimer for the duration of the gel filtration experiment. If iron was needed for dimerisation, then iron-activated, serine-activated L-serine dehydratase which was eluted in the presence of iron, dithiothreitol and D-serine should elute as a dimer.

Accordingly, L-serine dehydratase (step 8, 20 units) was preincubated at 37 °C with 100 mM glycylglycine-NaOH, pH 8.0, 100 mM KCl, 10 mM DTT and 1 mM $FeSO_4$ until fully active. D-Serine (100 mM) was then added to the iron-activated L-serine dehydratase, and the incubation continued until the enzyme had attained an index of activation of 1.0. The iron-activated, serine-activated dehydratase, plus internal standards, was then placed on a column of Sephadex G100 equilibrated in 50 mM glycylglycine-NaOH buffer, pH 7.8 containing 100 mM D-serine, 10 mM DTT and 1 mM $FeSO_4$. Under these conditions L-serine dehydratase was eluted to a position corresponding to a molecular weight of 55 000 +/- 3 000, indicating that dimerisation had not taken place. The relative positions of elution of the standard proteins were unaffected by the presence of the iron and dithiothreitol. When the enzyme fractions were analysed using continuous assay mixtures containing L-serine (50 mM), the timecourses obtained were fully linear, indicating that the dehydratase was still both iron- and serine-activated. The substrate saturation profile of L-serine dehydratase eluted from this column also showed a considerably reduced level of cooperativity, with a Hill coefficient 0f 1.20 +/- 0.05, confirming that the dehydratase was close to a state of full serine-activation; the $S_{[0.5]}$ of the enzyme was 3.4 +/- 0.11 mM L-serine. The Hill coefficient and $S_{[0.5]}$ for the same preparation of iron-activated dehydratase before it had undergone activation by D-serine and gel filtration analysis were 2.20 +/- 0.09 and 3.0 +/- 0.10 mM L-serine, respectively.

Glycylglycine, (50 mM, pH 7.8) was the choice of elution buffer for the gel filtration experiments described above, because L-serine dehydratase is most stable when prepared and stored in this buffer. Glycylglycine does not stabilise the dehydratase by preserving its Fe-independent activity, which would suggest that it must exert its protective effect by some other mechanism, possibly by

interacting with a catalytically or structurally important site within the enzyme. It is possible that this interaction may have prevented the association and binding of the dehydratase monomers and the formation of the dimeric complex. If this was the case, L-serine dehydratase which was serine-activated and eluted in a buffer other than glycylglycine, such as Tris-HCl, would be expected to undergo a doubling in its molecular weight.

Accordingly, a sample of purified L-serine dehydratase (step 8, 40 units) which had been exchanged into 100 mM Tris-HCl buffer, pH 7.8 was preincubated at 37 °C with 10 mM DTT and 1 mM $FeSO_4$ until fully active (20 minutes) and then further incubated with 100 mM D-serine, until the enzyme showed a linear timecourse of activity. The activated dehydratase, plus internal standards, was then eluted from a column of Sephadex G100 equilibrated in 100 mM Tris-HCl, pH 7.8 containing 100 mM KCl, 10 mM dithiothreitol, 1 mM $FeSO_4$ and 100 mM D-serine. Under these conditions the enzyme again eluted to a position corresponding to the molecular weight of the monomeric form of the dehydratase (52 000 +/- 5 000). Although dimerisation did not appear to have taken place, an examination of the kinetic properties of the enzyme showed that the dehydratase was fully activated. The enzyme possessed a linear timecourse of activity and a hyperbolic substrate saturation profile. A Hill plot of the substrate saturation data revealed an $S_{[0.5]}$ value of 2.9 +/- 0.15 mM L-serine, and a Hill coefficient of 1.13 +/- 0.16. These results show that the inability of serine-activated L-serine dehydratase to undergo a doubling of its molecular weight is not a consequence of the buffer in which it is eluted.

7.3.1.2 Sucrose Density Gradient Centrifugation Studies

A series of molecular weight studies of activated L-serine dehydratase analogous to the gel filtration experiments described above, were also performed using sucrose density gradient centrifugation (Martin and Ames, 1961), as described in Chapter 2, section 2.9.2 D-Serine was used to activate the dehydratase since it was not consumed during the ultracentrifugation analysis. Internal standards (lysozyme and lactate dehydrogenase) were included in all sucrose gradient analyses.

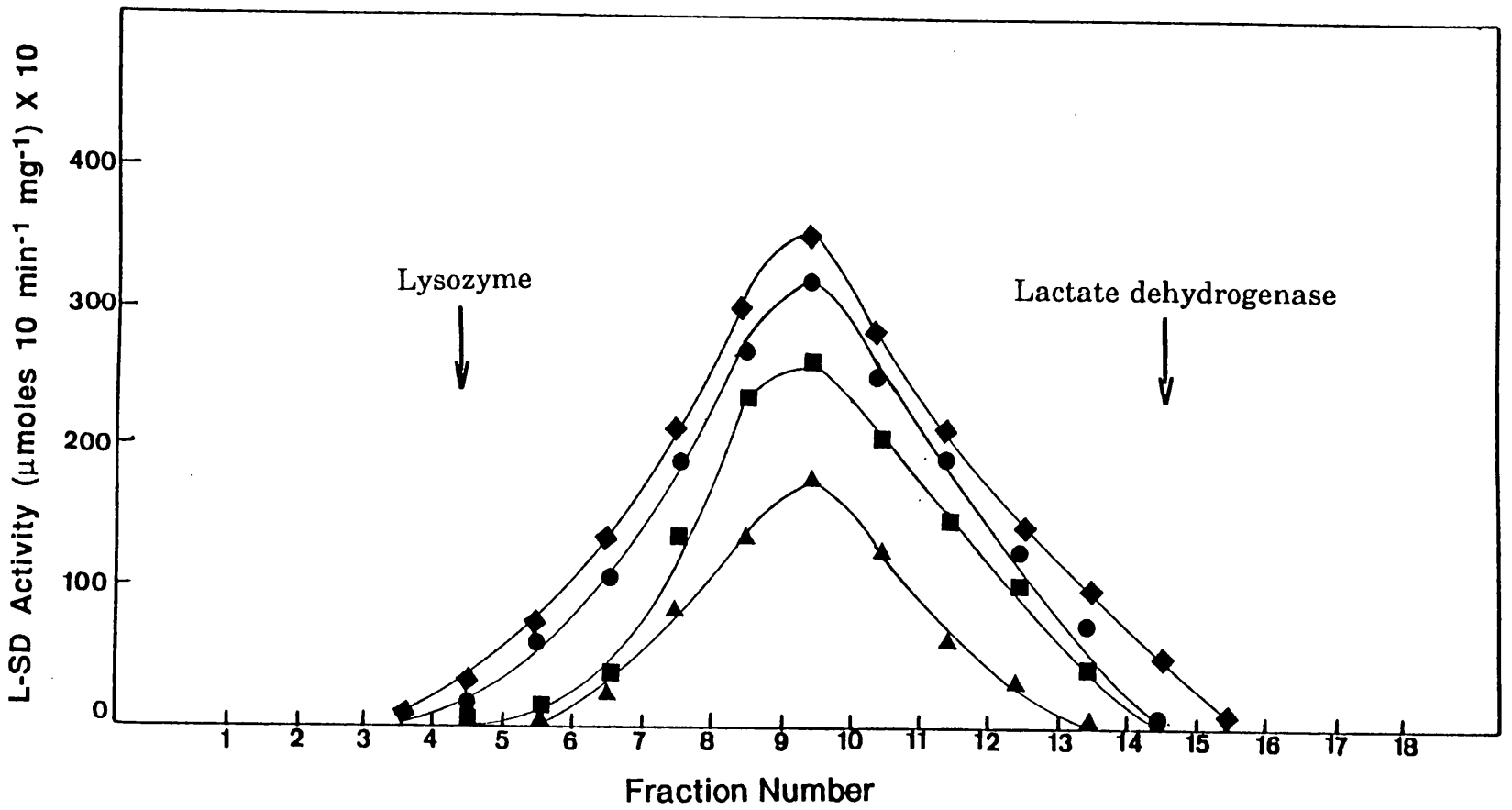
L-Serine dehydratase (step 8, 10 units) which was neither iron- nor serine-activated was analysed on a sucrose gradient in 100 mM glycylglycine-NaOH, buffer, pH 7.8 containing 100 mM KCl, as described in the legend to Figure 7.4. When the position on the gradient of this enzyme was compared with the position

Figure 7.4

The Sedimentation Profile of L-Serine Dehydratase from Sucrose Density Gradients

L-Serine dehydratase preparations (step 8, protein concentration 0.15 mg ml^{-1}) were treated as described in the Table below. Samples of the enzyme incubation mixtures (10 units of enzyme activity plus internal standards lysozyme and lactate dehydrogenase in a final volume of 0.15 ml) were carefully loaded on to the sucrose gradients of the composition described in section 2.9.2 plus the additional components shown below. The gradients were then centrifuged for 16 hours at 78 000 *g*, as described in Chapter 2, section 2.9.2. Fractions (0.15 ml) were collected from the tubes and assayed for L-serine dehydratase activity in the presence of 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method. The position on the gradient of the standard proteins are indicated on the abscissa of the sedimentation profiles.

Enzyme Treatment	**Experimental Conditions**	**Additions to Gradients**
Unactivated	No treatment **Key:** ▲ ▲ ▲ ▲	None
Iron-activated	Dehydratase preincubation with 1 mM $FeSO_4$ and 10 mM DTT, as described in section 2.6.4 **Key:** ● ● ● ●	10 mM DTT + 1 mM $FeSO_4$
Serine-activated	Unactivated dehydratase preincubated with 100 mM D-serine to an index of activation of 1.0. **Key:** ■ ■ ■ ■	100 mM D-Serine
Iron-activated, serine-activated	Iron-activated dehydratase with 100 mM D-serine to an index of activation of 1.0. **Key:** ◆ ◆ ◆ ◆	10 mM DTT, 1 mM $FeSO_4$, plus 100 mM D-Serine


L-SD Activity (μmoles 10 min-1 mg-1) X 10
400
300
200
100
0
Lysozyme
Lactate dehydrogenase
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
Fraction Number

of the standard proteins lactate dehydrogenase and lysozyme, a mean s_{20w} of 4.67 S was obtained. If the dehydratase was preincubated for 20 minutes with 100 mM D-serine, and then spun down a sucrose gradient containing 100 mM D-serine, no change in the s_{20w} of the enzyme was seen and the sedimentation profile shown in Figure 7.4 was obtained. When iron-activated L-serine dehydratase was sedimented through a sucrose gradient containing 100 mM glycylglycine-NaOH, pH 7.8, 10 mM DTT and 1 mM $FeSO_4$, the s_{20w} value of the dehydratase also remained unchanged at 4.67 S. L-Serine dehydratase obtained from this gradient was active in the absence of added iron and dithiothreitol, and showed a non-linear timecourse of activity when assayed continuously, indicating that the enzyme was still iron-activated but not serine-activated. When enzyme which was both iron- and serine-activated was spun down a sucrose gradient containing 100 mM glycylglycine-NaOH, pH 8.0, 10 mM DTT, 1 mM $FeSO_4$ and 100 mM D-serine, no significant shift in the position of the enzyme on the gradient was observed. Although the unchanged s_{20w} value showed that the dehydratase had not undergone an increase in its molecular weight, the enzyme was still iron-activated and showed a linear timecourse of activity. The substrate saturation curve showed very little cooperativity: the Hill plot of the substrate saturation data showed an $S_{[0.5]}$ of 2.83 +/- 0.2 mM L-serine and a Hill coefficient of 1.16 +/- 0.10, indicating that the dehydratase was serine-activated.

The experiments shown in sections 7.3.1.1 and 7.3.1.2 would appear to indicate that serine-activation and dimerisation are not facets of the same process. Although the purified dehydratase can be activated by both D- and L-serine, the results described above showed quite clearly that purified L-serine dehydratase which had been serine-activated was did not dimerise. These findings are markedly different from those obtained by Gannon (1973) for the highly purified L-serine dehydratase from *A. globiformis* and Roberts (1983) for the slightly purified enzyme from *E. coli* B, both of whom were able to demonstrate a clear correlation between serine-activation and dimerisation. However,the enzyme preparations used by both of these workers were Fe-independent, that is, catalytically active in the absence of ferrous iron and dithiothreitol. This is in contrast to the purified *E. coli* L-serine dehydratase used in the present study, which was inactive unless preincubated with these reagents. It may be that iron-activated dehydratase can undergo activation by serine, but only Fe-independent enzyme which is still in possession of its native cofactor can dimerise. If dimerisation is dependent upon Fe-independence, then the ability of L-serine dehydratase to dimerise should be lost at the same stage of the purification in

which all of the original Fe-independent activity of the enzyme is lost, a possibility that is examined in the next section.

7.3.2 Molecular Weight Studies of L-Serine Dehydratase from Successive Stages of Its Purification

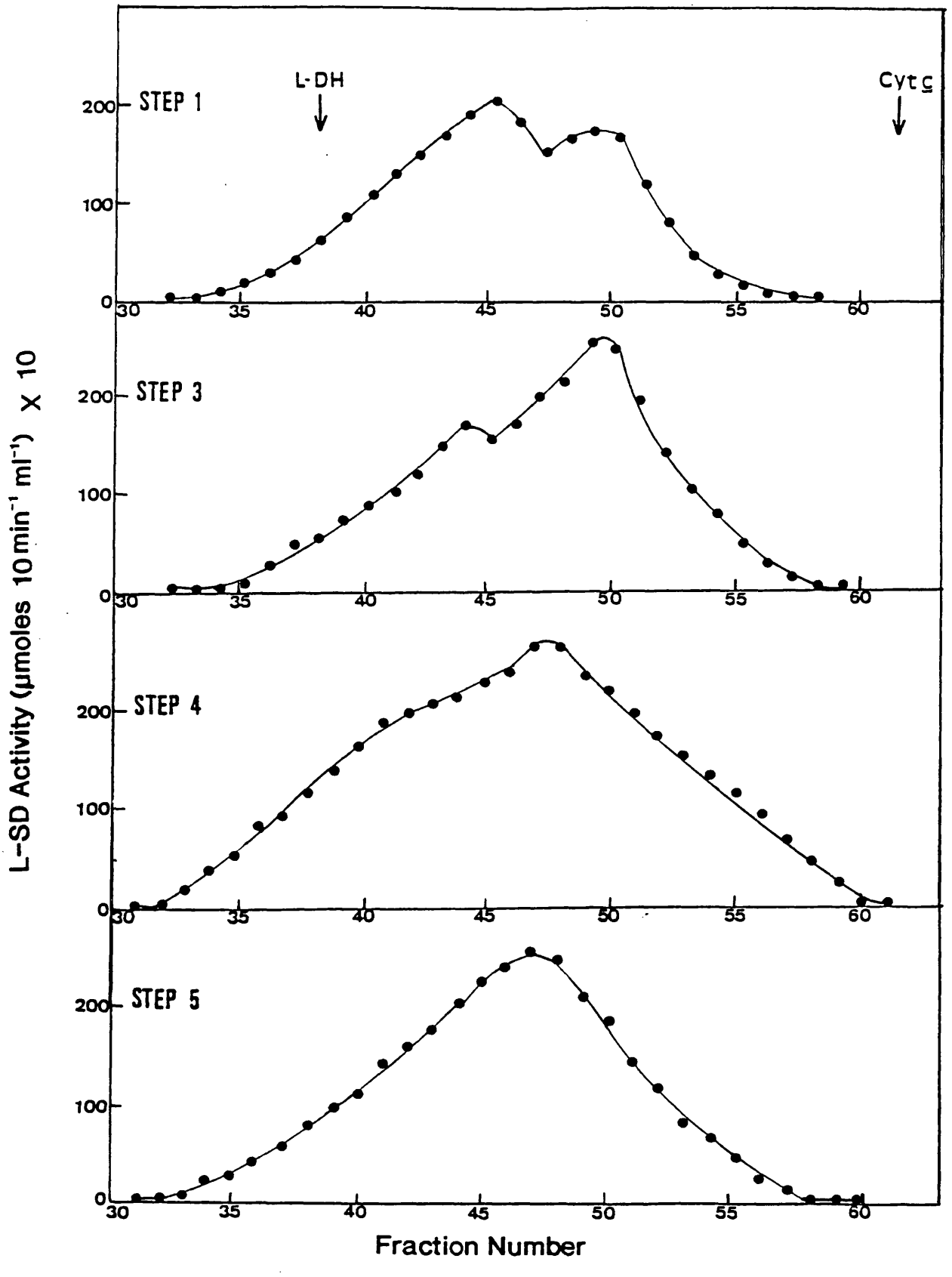
L-Serine dehydratase preparations from the successive steps of a single purification [steps 1 (crude extract), 3 (ammonium sulphate), 4 (DEAE cellulose) and 5 (pentyl agarose)] were preincubated for 30 minutes at 37 °C with 100 mM D-serine, as described in the legend to Figure 7.5. Samples of the D-serine-activated enzyme (40 units plus internal standards) were placed on a Sephadex G100 column, equilibrated in 100 mM Tris-HCl, pH 7.8 containing 100 mM KCl, 2 mM DTT and 100 mM D-serine and eluted with buffer of the same composition. The specific activity and degree of Fe-independence of each of these preparations of enzyme are shown in Table 7.2

Figure 7.5 shows the patterns of elution of each of the dehydratase preparations from the successive stages of a purification. It can be seen that L-serine dehydratase from steps 1, 3 and 4 of the purification scheme produced elution profiles which showed evidence of two peaks, both possessing L-serine dehydratase activity, and corresponding to two distinct molecular weights: 110 000 +/- 7 000 and 55 000 +/- 3 000, the former value representing the expected molecular weight of the dimeric form of the dehydratase, and the latter, the monomer. Neither of these two forms of the enzyme were able to retain a significant degree of Fe-independent activity after their elution from the Sephadex columns, and so were assayed in iron-containing assay media. The sizes, relative to one another, of the two peaks of enzyme activity were also seen to change as the dehydratase became more purified, and less Fe-independent: for enzyme from a crude extract (step 1), which was only moderately stimulated by iron and dithiothreitol, the peak corresponding to a molecular weight of 110 000 was larger than the peak corresponding to the lower molecular weight form of the dehydratase. This order was reversed for L-serine dehydratase from step 3 of the purification, which showed less Fe-independent activity and a greater degree of iron-activation than the enzyme from step 1, and appeared to dimerise to a considerably lesser extent. The pattern was less clear for step 4 dehydratase, which showed very little activity in the absence of iron and DTT, but was still able to achieve a small degree of dimerisation. However, enzyme from the pentyl agarose stage, which had no Fe-independent activity and was completely inactive in the absence of added iron and DTT, existed solely as a monomer. L-Serine dehydratase from the remaining steps of the purification also showed elution

Figure 7.5

The Elution Pattern of L-Serine Dehydratase from Successive Stages of its Purification from a Sephadex G-100 Column in the Presence of 100 mM D-Serine

L-Serine dehydratase preparations from the successive stages of a single purification - steps 1 (crude extract), 3 (ammonium sulphate), 4 (DEAE cellulose) and 5 (pentyl agarose) - were preincubated with 100 mM D-serine for 30 minutes at 37 °C. Samples of the preincubation mixtures (20 units of enzyme activity plus internal standards cytochrome c and lactate dehydrogenase in a final volume of 0.5 ml) were eluted from a column of Sephadex G-100 equilibrated with 100 mM Tris-HCl pH 7.8, 100 mM KCl and 100 mM D-serine, as described in Chapter 2, section 2.9.1. Fractions (2 ml) were collected and assayed for L-serine dehydratase activity in media containing 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM DTT, 1 mM $FeSO_4$, 10 mM L-isoleucine and 1 mM hydroxylamine. Pyruvate formed during the assays was measured by the 2,4-dinitrophenylhydrazine method, as described in section 2.6.1. The position of elution of the standard proteins are indicated on the abscissa.


L-DH
Cyt c
STEP 1
STEP 3
STEP 4
STEP 5
200
100
0
30
35
40
45
50
55
60
L-SD Activity (μmoles 10 min⁻¹ ml⁻¹) X 10
Fraction Number

profiles consisting of a single peak of activity corresponding to the molecular weight of the monomer form of the enzyme.

The molecular weight studies described above, and in the previous two sections were performed with enzyme preparations obtained from *E. coli* B/S53, the variant possessing elevated levels of L-serine dehydratase activity (Chapter 3, section 3.2). It is possible that the dehydratase from this source, in addition to being produced in higher amounts, was also structurally different from the enzyme in wild-type *E. coli* B, in that it could become activated by D- or L-serine, but was not able to dimerise. This possibility was investigated by purifying L-serine dehydratase obtained from extracts of cultures of wild-type *E. coli* B grown on the minimal medium of Davis and Mingioli (1951), by the method described in Chapter 2, section 2.2.2. Enzyme samples from each stage of the purification were then preincubated with D-serine and subjected to molecular weight analysis by gel filtration as described above. When the activity of the eluted enzyme was plotted as a function of its elution volume, a set of profiles almost identical to those obtained with dehydratase obtained from variant S53 were seen. These revealed a two-peak spread of dehydratase activity corresponding to the molecular weights associated with the monomeric and dimeric forms of the enzyme (55 000 +/- 3 000 and 108 000 +/- 6 000, respectively). As the wild-type dehydratase became more purified, and less Fe-independent, the peak of activity of the high molecular weight form the enzyme was seen to decrease in size, until the pentyl agarose step of the purification, when all of the dehydratase was found to exist as a monomer.

These results would appear to indicate that L-serine dehydratase in crude, or slightly purified extracts, exists as two distinct species of enzyme, only one of which, the species with Fe-independent activity, is able to dimerise. Although Roberts (1983) observed the presence of only dimeric enzyme following serine-activation of L-serine dehydratase, Gannon (1973) found that a similar type of two-peak elution profile was produced when the purified L-serine dehydratase from *A. globiformis* was activated, and eluted, with a sub-saturating concentration of D-serine. Gannon showed that enzyme which had been partially activated to an index of 0.6 by D-serine (40 mM, as opposed to the standard 150 mM), and eluted from a Sephadex G100 column equilibrated in buffer also containing 40 mM D-serine, eluted to positions corresponding to molecular weights of the dimer and monomer forms of the enzyme. The timecourse of pyruvate formation of the dimer species was linear while that of the monomeric form of the enzyme was found to be non-linear. Gannon demonstrated that the ratio of monomer to dimer in the column eluate was determined by the level to

which the dehydratase was activated by the D-serine. Enzyme which was preincubated with a saturating concentration of this activator (150 mM) to an index of activation of 1.0 was eluted solely as the dimer, while subsaturating levels of activator produced index values of less than 1 (typically 0.6 to 0.7), and elution profiles showing a combination of monomer and dimer.

In the present study, the presence of two distinct forms of enzyme in the elution profile of the serine-activated, unpurified *E. coli* B L-serine dehydratase are unlikely to be a consequence of an incomplete activation by the D-serine. The reasons for this are two-fold: firstly, the concentration of D-serine used in the activation mixture and elution buffers (100 mM) was twice that needed to bring about a full activation of the dehydratase; secondly, the index of activation of the step 1 and step 3 enzyme preparations was 1.0, indicating that the enzyme was fully activated before the gel filtration analysis.

All extracts of Fe-independent L-serine dehydratase are capable of further activation by iron and DTT (Table 7.2), which suggests that there are two species of enzymes in these preparations, one form which is Fe-independent and another which needs iron and DTT for activity. The results presented indicate a considerable degree of correlation between Fe-independence and the ability to dimerise. They appear to indicate that only the Fe-independent form is able to dimerise. Consequently, enzyme which elutes with the molecular weight of the dimer should not have a requirement for Fe^{2+} and DTT for activity, while Fe-dependent enzyme, which is unable to dimerise, would require these cofactors. Unfortunately, as already stated, it did not prove possible to confirm this since the eluted dehydratase was too unstable to allow its activity to be measured using the lactate dehydrogenase method, and enzyme fractions were, *per force*, measured in iron-containing assay media.

Neither Roberts (1983) nor Gannon (1973) were able to detect the presence of monomeric L-serine dehydratase in the elution profiles of serine-activated enzyme. However, the L-serine dehydratase preparations that these workers used were both Fe-independent, and were able to retain this Fe-independent activity during their gel filtration elution. In addition, the activity of the eluted enzyme in both Roberts and Gannon's studies was measured using lactate dehydrogenase coupled assays which do not contain iron and DTT, and which therefore detect Fe-independent activity only. As a consequence these workers would have only detected the activity of the Fe-independent dimer.

Table 7.2

The Degree of Fe-Independence Possessed by L-Serine Dehydratase from Successive Stages of its Purification

Samples of L-Serine dehydratase (0.1 units) from preparations from each of the successive stages of a single purification - steps 1 (crude extract), 3 (ammonium sulphate), 4 (DEAE cellulose) and 5 (pentyl agarose) - were assayed in both 100 mM glycylglycine-NaOH, pH 8.0, and 50 mM L-serine or 100 mM glycylglycine-NaOH, pH 8.0, 50 mM L-serine, 10 mM dithiothreitol and 1 mM $FeSO_4$.

The 'degree of iron-activation' of the enzyme preparations is the ratio of the activity of the dehydratase when assayed in glycylglycine, iron and DTT : the activity of the dehydratase when assayed in glycylglycine only

Purification Step	**L-Serine Dehydratase Activity (μmole Pyruvate 10 min^{-1} mg^{-1}) X 10 when assayed in:**		**Degree of Iron-activation (b/a)**
	(a) Glycylglycine only	**(b) Glycylglycine + Fe^{2+} + DTT**	
(1) Crude Extract	0.48	1.36	2.8
(2) Streptomycin Sulphate	0.46	1.34	2.9
(3) Ammonium Sulphate	0.21	2.89	13.8
(4) DEAE Cellulose	0.020	13.7	368.5
(5) Pentyl Agarose	0	54.95	-

7.4 The Relationship Between Iron-activation and Serine-activation

The changes in properties that the dehydratase undergoes when activated by D- or L-serine indicate that considerable structural changes must have taken place within the enzyme. If the underlying mechanism of this activation does not result in an association of enzyme subunits, it must involve a molecular rearrangement of the dehydratase molecule. The results of the following experiments suggest that serine-activation produces a considerable change in the conformation of the dehydratase.

7.4.1 The Serine-activation of Iron-activated L-Serine Dehydratase

It was seen in Chapter 6, section 6.2, that the activation of L-serine dehydratase by ferrous iron and dithiothreitol occurred more rapidly when L-serine was present within the preincubation medium. Enzyme which was incubated with 10 mM DTT, 1 mM Fe^{2+} and 50 mM L-serine became fully active within the space of 4 to 5 minutes (Figure 6.3). Dehydratase preincubated with iron and dithiothreitol in the absence of L-serine required up to 25 minutes to reach a similar level of maximal activity (Figure 6.5). This suggests that the presence of L-serine in the incubation medium induced the dehydratase to adopt a conformation which favoured activation by the iron and reducing agent.

L-Serine was also found to protect the activity of both iron-activated purified L-serine dehydratase and Fe-independent enzyme from crude extracts. In the experiment described in Figure 7.6, L-serine dehydratase (step 8, protein concentration 0.050 mg ml^{-1}) was incubated at 37 °C with 100 mM Tris-HCl, pH 7.8, 10 mM DTT and 1 mM $FeSO_4$ until fully active. Samples of the iron-activated enzyme (0.10 ml) were then diluted ten-fold into 1.0 ml preincubation mixtures containing either 100 mM Tris-HCl, pH 8.0, 100 mM Tris-HCl, pH 8.0 containing 2.5 or 40 mM L-serine or, 100 mM Tris plus additions of 10 mM DTT and 1 mM $FeSO_4$ (Tris buffer was used in preference to glycylglycine because the latter compound possesses metal chelating abilities. In the absence of effective stabilising agents such as iron or L-serine, glycylglycine is capable of extracting iron from iron-activated dehydratase which has been extensively diluted into this buffer). At the times shown in the Figure, 30 µl aliquots of the preincubation media were transferred to 1 ml assay mixtures consisting of 100 mM Tris-HCl, pH 8.0 plus 50 mM L-serine. Pyruvate formed during the assays was measured as described in Chapter 2, section 2.6.1. A correction for the carryover of pyruvate from those preincubation mixtures which contained L-serine was made by

Figure 7.6

The Ability of L-Serine to Protect the Activity of Iron-activated, Purified L-Serine Dehydratase

L-Serine dehydratase (step 8, protein concentration 0.050 mg ml^{-1}) was incubated at 37 °C with 100 mM Tris-HCl, pH 8.0, 10 mM DTT and 1 mM $FeSO_4$ until fully active. Samples of the iron-activated enzyme (0.10 ml) were then diluted ten-fold into duplicate 1.0 ml preincubation mixtures containing either 100 mM Tris-HCl, pH 8.0 only, 100 mM Tris-HCl, pH 8.0 containing 2.5 or 40 mM L-serine or, 100 mM Tris plus additions of 10 mM DTT and 1 mM $FeSO_4$. At the intervals shown in the Figure, 30 µl aliquots of the preincubation media were transferred to 1 ml assay mixtures consisting of 100 mM Tris-HCl, pH 8.0 plus 50 mM L-serine. All assays and preincubations were performed at 37 °C. Pyruvate formed during the assays was measured as described in section 2.6.1. A correction for the carryover of pyruvate from those preincubation mixtures which contained L-serine was made by adding 30 µl of the medium to 0.33 ml of 0.1 % 2,4-dinitrophenylhydrazine plus 0.97 ml of water.

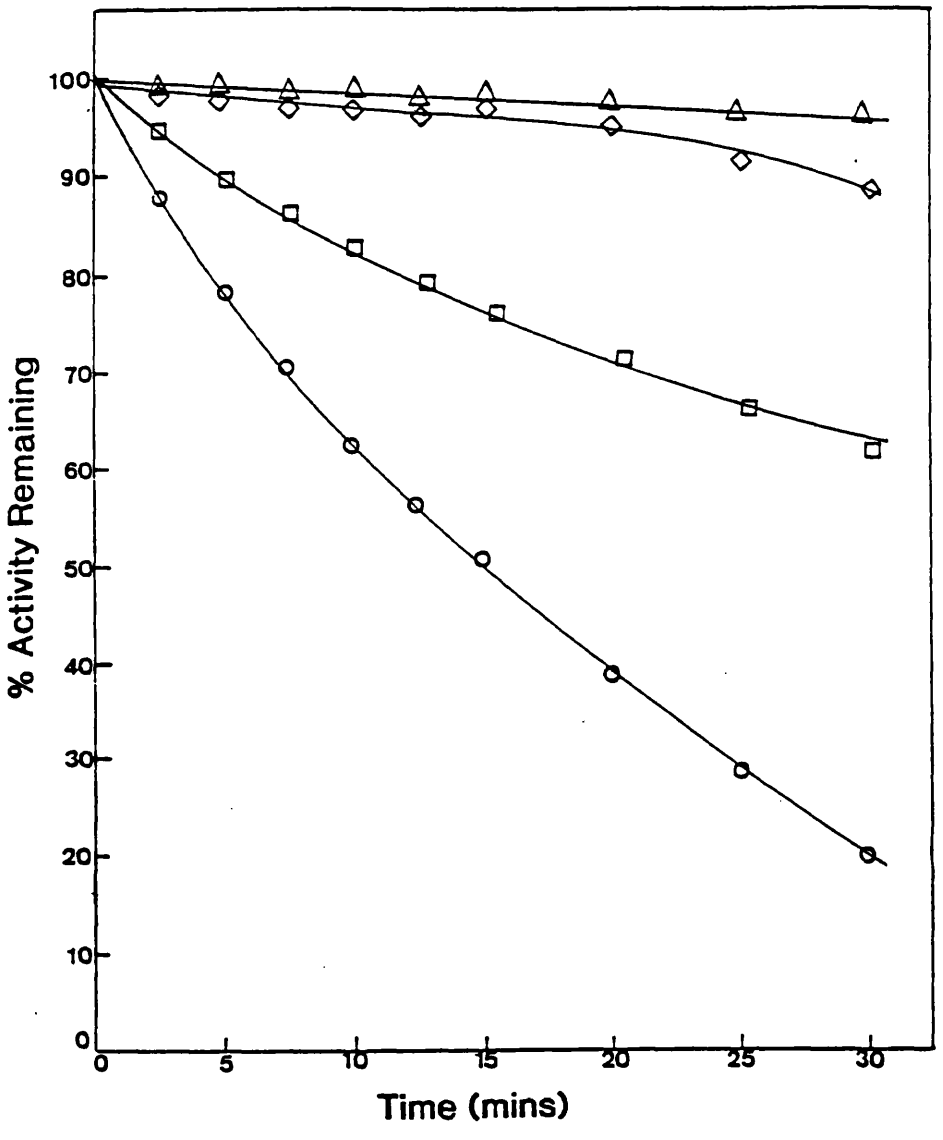
Preincubation media composition:

○ ○ ○ ○ 100 mM Tris-HCl, pH 8.0 only

□ □ □ □ 100 mM Tris-HCl, pH 8.0 containing 2.5 mM L-serine

◇◇◇◇ 100 mM Tris-HCl, pH 8.0 containing 40 mM L-serine

△ △ △ △ 100 mM Tris-HCl, pH 8.0 plus 10 mM DTT and 1 mM $FeSO_4$.


% Activity Remaining
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (mins)

adding 30 µl of the medium to 0.33 ml of 0.1 % 2,4-dinitrophenylhydrazine plus 0.97 ml of water.

Figure 7.6 shows that at a sufficiently high concentration (40 mM), L-serine was almost as effective as iron and dithiothreitol in its ability to protect the activity of the iron-activated dehydratase. Enzyme which was preincubated in Tris buffer only lost over 80 % of its initial activity over the 30 minutes of the experiment. Over the same time interval, iron-activated L-serine dehydratase which had been added to preincubation media containing 40 mM L-serine lost only 20 % of its starting activity. Optimal stabilisation of the iron-enzyme complex was provided by 10 mM DTT and 1 mM $FeSO_4$. Enzyme preincubated in this medium retained over 90 % of its initial activity.

The extent of the protection afforded by L-serine was found to be dependent on its concentration in the preincubation medium, with 40 mM L-serine producing a greater stabilisation of the iron-activated dehydratase than 2.5 mM L-serine. This is probably a consequence of the extent to which the dehydratase had become activated by the L-serine. Less activity was lost with a saturating concentration of this activator because under this set of conditions most of the dehydratase would have existed in the more stable serine-activated form of the enzyme. The results presented in Figure 7.6 suggest that activation by L-serine causes a change in the conformation of the dehydratase, to a form which protects the iron-activated state of the enzyme. Iron-activation appears to involve iron uptake (Chapter 6, section 6.6) and it maybe that the conformation which the dehydratase adopts following serine-activation causes the iron within the enzyme to be maintained in a more stable configuration. If this was so, L-serine dehydratase which was diluted into media lacking L-serine would lose activity because it was not in the conformation which is most effective at retaining the iron.

7.4.2 The Serine-activation of Fe-Independent L-Serine Dehydratase

L-Serine was also found to protect the activity of Fe-independent L-serine dehydratase. In the experiment shown in Figure 7.7, L-serine dehydratase (step 1, 1.0 units ml^{-1}) was preincubated in mixtures containing 100 mM Tris-HCl buffer, pH 8.0 and 1 mM hydroxylamine plus either 0, 2.5 or 40 mM L-serine, or 10 mM DTT and 1 mM $FeSO_4$. Samples (0.10 ml) were removed over a period of 15 minutes from those preincubations containing L-serine and added to 0.33 ml of 0.1 % 2,4-dinitrophenylhydrazine in 2 M HCL and 0.9 ml of water, to give the carryover of pyruvate into the preincubation mixtures. Further samples (0.20 ml)

Figure 7. 7

The Ability of L-Serine to Protect the Activity of Fe-Independent L-Serine Dehydratase

Duplicate samples of L-Serine dehydratase (step 1, 0.1 units) were preincubated in assay mixtures containing 100 mM tris-HCl buffer, pH 8.0, 1 mM hydroxylamine, and 0, 2.5 or 40 mM L-serine. Samples (0.10 ml) were removed over a period of 15 minutes from these preincubations and added to 0.33 ml of 0.1 % 2,4-dinitrophenylhydrazine in 2 M HCL and 0.9 ml of water. The pyruvate content of each sample was then determined, as described in Chapter 2, section 2.6.1, to give the carryover of pyruvate into the preincubation mixtures. Samples (0.20 ml) were also removed from the preincubations at timed intervals and added to assay mixtures containing 100 mM tris, 40 mM L-serine and 1 mM hydroxylamine, with, and without, 10 mM DTT and 1 mM $FeSO_4$. These reactions were stopped after 10 minutes using the assay technique described above.

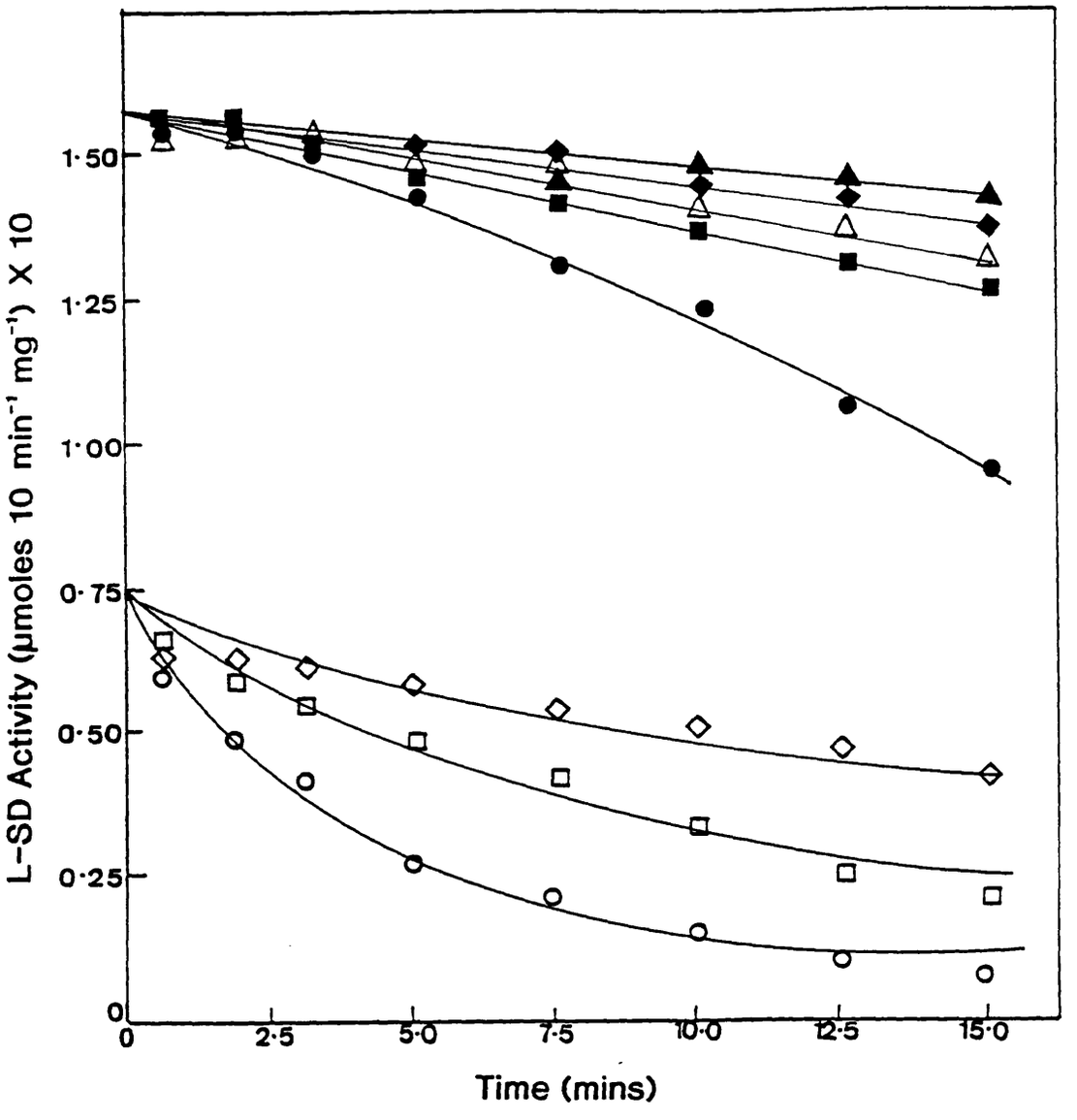
'+' refers to assay mixtures which contain 10 mM DTT and 1 mM $FeSO_4$.
'-' refers to assay mixtures which do not contain these reagents.

○ ○ ○ ○ Transferred from 0 mM L-serine preincubation to - assay
● ● ● ● Transferred from 0 mM L-serine preincubation to + assay

□ □ □ □ Transferred from 2.5 mM L-serine preincubation to - assay
■ ■ ■ ■ Transferred from 2.5 mM L-serine preincubation to + assay

◇ ◇ ◇ ◇ Transferred from 40 mM L-serine preincubation to - assay
◆ ◆ ◆ ◆ Transferred from 40 mM L-serine preincubation to + assay

△ △ △ △ Transferred from Fe^{2+} plus DTT preincubation to - assay
▲ ▲ ▲ ▲ Transferred form Fe^{2+} plus DTT preincubation to + assay


L-SD Activity (μmoles 10 min⁻¹ mg⁻¹) X 10
1·50
1·25
1·00
0·75
0·50
0·25
0
0
2·5
5·0
7·5
10·0
12·5
15·0
Time (mins)

were removed from all of the preincubations at the intervals shown in the Figure, and added to assay mixtures containing 100 mM Tris-HCl, pH 8.0, 40 mM L-serine and 1 mM hydroxylamine, with, and without, 10 mM DTT and 1 mM $FeSO_4$. Pyruvate formed during the assays was measured as described above.

The lower set of curves in Figure 7.7, show the residual activity of dehydratase from the preincubation mixtures which had been assayed without additions of iron and dithiothreitol. It can be seen that the Fe-independent activity of L-serine dehydratase which had been preincubated with L-serine (2.5 and 40 mM) was considerably more stable than enzyme which had undergone preincubation in the absence of L-serine. The protection afforded by the inclusion of L-serine was, again, concentration dependent, there being comparatively little activity lost from dehydratase preincubated with 40 mM L-serine. The inclusion of 1 mM Fe^{2+} and 10 mM DTT into the preincubation medium afforded almost complete protection of the dehydratase, a phenomenon that was also observed with the iron-activated, purified enzyme.

When the L-serine dehydratase samples from the preincubation media were also assayed in the presence of Fe^{2+} and DTT, the upper set of lines in Figure 7.7 was obtained. These suggest that the inactivation seen at low substrate concentrations was the result of a reversible loss of a catalytically essential cofactor from the dehydratase, possibly iron.

These results confirm the experiment described in Figure 7.6. They show that activation by L-serine resulted in a structural change in L-serine dehydratase, such that the Fe-independent activity of the dehydratase was more stably maintained, possibly by protecting the native cofactor of the enzyme. The gel filtration analyses described in section 7.3.2 suggest that the formation of enzyme dimers may, at least in part, underlie the structural rearrangement of which follows serine-activation of unpurified Fe-independent L-serine dehydratase. In the case of the Fe-dependent purified enzyme, which does not dimerise when serine-activated, this structual change is more likely to be a consequence of a rearrangement of the molecular configuration of the monomeric form of the dehydratase.

7.4.3 The Relationship Between Serine-activation and Iron-activation

It has been shown that the *E. coli* L-serine dehydratase can undergo two distinct forms of activation: its activity can be increased by ferrous iron and dithiothreitol,

and by serine-activating agents, such as D- or L-serine. Each form of activation is characterised by certain distinctive features, which have been described earlier, in Chapter 6, and Chapter 4, sections 4.2 and 4.3, and in the preceding sections of the current Chapter. It has been seen that, while each form of activation is characterised by certain distinctive features, the iron-activation and serine-activation processes are not mutually exclusive reactions. Thus, L-serine dehydratase which has been iron-activated can also become serine-activated (Chapter 7, section 7.2), and enzyme which is serine-activated (in permeabilised cells), can also be further activated by iron and DTT (Chapter 4, section 4.8.1). The relationship between serine-activation and iron-activation is considered further in Table 7.3, which examines the effect of these two types of activation on the substrate saturation kinetics of Fe-independent, iron-activated and serine-activated L-serine dehydratase. The Fe-independent and purified preparations of enzyme shown were obtained from the same purification.

It can be seen that non-serine-activated L-serine dehydratase showed a considerable amount of cooperativity in its substrate binding, and that activation by iron and DTT had little effect upon this. The iron-activation of Fe-independent dehydratase served to increase its catalytic activity, but affected neither the cooperativity of substrate binding, nor the affinity of the enzyme for L-serine. The Hill coefficients for iron-activated and non-iron-activated Fe-independent L-serine dehydratase remained relatively constant at 2.23 +/- 0.11 and 2.14 +/- 0.08, respectively, with the $S_{[0.5]}$ for L-serine remaining unchanged at 2.4 +/- 0.05 mM. Extensive purification and iron-activation of the same enzyme preparation had little effect upon these kinetic constants: Fe-dependent L-serine dehydratase which had been re-activated by iron and DTT had a Hill coefficient of 2.53 +/- 0.10 and and an $S_{[0.5]}$ of 2.6 +/- 0.15 mM L-serine.

Iron-activation of Fe-independent L-serine dehydratase also did not appear to have any effect on the kinetics of its subsequent activation by D-serine. Fe-independent dehydratase which had been preincubated with iron and dithiothreitol was catalytically more active than non-iron-activated enzyme, but otherwise showed no significant change in either its affinity for its substrate or the cooperativity of its binding of L-serine. This would seem to suggest that the Fe-independent and iron-activated states are functionally equivalent, irrespective of the molecular form in which the two activity states may occur within the L-serine dehydratase protein. The iron-activation of L-serine dehydratase in whole cells, which exists in a state of natural serine-activation, also did not significantly affect the kinetics of the interaction of the enzyme with its substrate. Apart from increasing the V_{max} of the dehydratase-catalysed reaction, there was

Table 7.3

The Effect of Iron-activation and Activation by D-Serine on the Substrate Saturation Kinetics of Fe-independent L-Serine Dehydratase

Samples of Fe-independent L-serine dehydratase (step 1, enzyme concentration 5.0 units of unactivated activity ml^{-1}) were subjected to the treatments described below. Samples of the dehydratase preparations (5 to 10 µl, depending on the level of activity of the enzyme) were then added to lactate dehydrogenase assays containing different concentrations of L-serine. Hydroxylamine (1 mM) and 10 mM isoleucine were included in the assay mixtures to inhibit L-threonine dehydratase activity. The linear rates of pyruvate production for each substrate concentration were then plotted on a Hill plot, and the $S_{[0.5]}$ values and Hill coefficients calculated as described in Chapter 4, section 4.3

For comparison purposes, the kinetic constants for unactivated and iron-activated L-serine dehydratase from permeabilised cells (Chapter 4, section 4.8.1) and for iron-activated and iron-activated, serine-activated purified dehydratase (Chapter 4, section 4.3.2 and Chapter 7, section 7.2.2) are also included.

Enzyme Treatment	**Experimental Conditions**
Unactivated	No pre-treatment
Iron-activated	Dehydratase preincubated with 1 mM $FeSO_4$ and 10 mM DTT, as described in Chapter 2, section 2.6.4
Serine-activated	Unactivated dehydratase preincubated with 100 mM D-serine to an index of activation of 1.0
Iron-activated and Serine-activated	Iron-activated dehydratase preincubated with 100 mM D-serine to an index of activation of 1.0.

Enzyme Treatment	$S_{[0.5]}$ (mM)	V_{max} (μmoles min^{-1} mg protein/μmoles min^{-1} mg^{-1} dry wt. cells)	Hill Coefficient
Non-purified, Fe-independent Enzyme			
Unactivated	2.40 +/- 0.05	0.66	2.14 +/- 0.08
Iron-activated	2.40 +/- 0.05	1.49	2.23 +/- 0.11
Serine-activated	2.67 +/- 0.10	0.89	1.19 +/- 0.10
Iron-activated, serine-activated	2.61 +/- 0.08	2.25	1.13 +/- 0.08
Permeabilised Cells			
Unactivated	3.20 +/- 0.10	0.97	1.13 +/- 0.05
Iron-activated	3.00 +/- 0.20	2.30	1.18 +/- 0.10
Purified, Fe-dependent Enzyme			
Iron-activated	2.60 +/- 0.15	1230.0	2.53 +/- 0.10
Iron-activated, serine-activated	3.08 +/-0.16	1250.0	1.10 +/- 0.05

almost no change in either the $S_{[0.5]}$ or Hill coefficient values between iron-activated and non-iron-activated enzyme.

On the basis of the data in Table 7.3, the effects of iron-activation on the activity of L-serine dehydratase appear to be distinct and separate from those which follow when the enzyme becomes serine-activated. Iron-activation of Fe-independent L-serine dehydratase produced no noticeable changes in the kinetics of the enzyme's subsequent activation by D- or L-serine. Almost identical $S_{[0.5]}$ and Hill coefficient values were obtained for both iron-activated and non-iron-activated dehydratase. Nor did activation by iron and DTT have a significant effect on the interaction with L-serine of serine-activated L-serine dehydratase from whole, permeabilised cells. These observations all suggest that iron-activation and serine-activation are separate processes that may act at different sites on the enzyme. However, this does not mean that the two processes may not be inter-related, and it is possible that L-serine dehydratase must be enzymatically active, that is Fe-independent or iron-activated, before it can become serine-activated.

7.5 Discussion

Roberts (1983) showed that the L-serine dehydratase from *E. coli* B possessed a number of distinctive properties that had their origin in the slow response of the dehydratase to its substrate, L-serine. The most notable of these included a non-linear timecourse of activity, and a sigmoidal substrate saturation profile. Enzyme activated by L-serine showed a linear timecourse and a hyperbolic substrate saturation profile. Roberts also showed that the dehydratase could also be activated by the competitive inhibitor, D-serine. Enzyme preincubated with D-serine showed a linear timecourse and a much reduced level of cooperativity in its substrate saturation kinetics.

The results described in this chapter, and in sections 4.2 and 4.3, have shown quite clearly that the purified *E. coli* B L-serine dehydratase responds to both L- and D-serine in a fashion which bears many similarities to Roberts' earlier work with the slightly purified enzyme. Although the enzyme used in the present study differed from the dehydratase preparation used by Roberts in that it required an initial preincubation with ferrous iron and dithiothreitol for activity, it still showed all of the kinetic properties characteristic of the substrate activation response described above (section 7.2). It is in the molecular basis of the serine-activation reaction, however, that Roberts findings and the present work apparently disagree. Roberts presented evidence from a series of gel filtration experiments with partially purified enzyme which suggested that the

dimerisation of inactive enzyme monomers formed the basis of the serine-activation process, as it did for the purified L-serine dehydratase from *A. globiformis* Gannon, 1973, Gannon *et al*, 1978). In contrast, the results from the molecular weight studies in section 7.3 show quite clearly that although purified L-serine dehydratase could become serine-activated, serine-activation did not result in dimerisation.

A possible reason for this may lay in the nature of the enzyme preparation used in the present study. Purified *E. coli* L-serine dehydratase differs from the dehydratase preparations used by Roberts in that it has lost all of its initial Fe-independent activity. Molecular weight studies of serine-activated Fe-independent enzyme suggest that L-serine dehydratase must be in possession of this native activity in order to dimerise. The gel filtration analyses described in section 7.3.2 show a considerable degree of correlation between Fe-independence and the ability to dimerise. L-Serine dehydratase which possessed a good level of Fe-independent activity was able to dimerise following serine-activation. As the dehydratase became pure, and less Fe-independent, the ability to dimerise was gradually lost. At the pentyl agarose stage of the purification, when the enzyme had become completely inactive in the absence of added iron and dithiothreitol, the dehydratase appeared to completely lose its ability to dimerise. This was also true for enzyme from the remaining steps of the purification.

In contrast to the present study, under the conditions of a saturating concentration of D-serine Roberts was only able to demonstrate the presence of dimeric enzyme following serine-activation of L-serine dehydratase. She did not find any evidence for the existence of monomeric dehydratase. However, this may be a consequence of the way in which Roberts measured the activity of her column fractions. The enzyme that this worker used was an Fe-independent preparations which was able to retain its Fe-independent activity following its gel filtration column elution. The activity of the eluted enzyme in Roberts' study was measured using lactate dehydrogenase coupled assays which do not contain iron and DTT, and which therefore measures Fe-independent activity only. The assay procedure used in the present work is capable of detecting both forms of L-serine dehydratase activity. Consequently, although Fe-dependent monomeric enzyme might have been present its activity would not have been detected because no iron was available in the assay medium to activate the enzyme, and so only dimeric enzyme would have been noted.

Roberts was able to demonstrate dimerisation with L-serine dehydratase that had been partially purified by nuclease-treatment, salt precipitation and DEAE cellulose chromatography. She was unable to purify the dehydratase beyond this point because of the enzyme's apparent instability. Roberts investigations of the serine-activation process were therefore limited to the equivalent of enzyme from step 4 of the purification from the present study. Her work showed that enzyme of this purity dimerised following activation by D- or L-serine. The work described in section 7.3.2 shows that although step 4 enzyme has little Fe-independent activity, unlike the equivalent preparation made by Roberts, it is still able to dimerise, although to a limited extent. Thus, on this point, Roberts and the present study are in agreement: Fe-independent L-serine dehydratase is able to dimerise. The characteristics of the serine-activation process for the *A. globiformis* dehydratase and the *E. coli* enzyme share many similarities (Chapter 1, section 1.3.3.1). Because of these similarities, and the fact that Roberts was unable to take the purification of L-serine dehydratase beyond the stage where the activity of the enzyme became Fe-dependent, it is not unreasonable that she should conclude that dimerisation and serine-activation were also causually linked. However, the current extension work to Roberts' preliminary study has shown that L-serine dehydratase of higher purity does not dimerise, although it can become serine-activated. Thus, the connection between serine-activation and dimerisation would now appear to be more casual than causal.

The results shown in section 7.3 suggest that Fe-independent unpurified L-serine dehydratase is able to dimerise following serine-activation, and that iron-activated purified L-serine dehydratase is not. Thus the dehydratase appears to exist in two separate molecular forms, dimeric and monomeric. Yet, the kinetic constants for the two enzyme forms were almost identical (section 7.4.3). No significant difference was observed in either the S[0.5] or Hill coefficient values for either Fe-independent, serine-activated un-purified enzyme and iron-activated, serine-activated purified enzyme, despite the fact that the former preparation of dehydratase was probably dimeric in nature, and the latter unequivocally monomeric. These observations would suggest that monomeric serine-activated L-serine dehydratase interacts with its substrate in the same way as dimeric enzyme, and that the relationship between serine-activation and dimerisation is incidental.

In terms of its Fe-independent activity, the *A. globiformis* L-serine dehydratase is quite stable, a property which allowed it to be purified to near homogeneity with most of this initial activity intact (Gannon, 1973, Gannon *et al*, 1977). If the ability

to dimerise is dependent upon Fe-independence, this may explain why dimerisation was observed with the purified enzyme from this organism. For the *A. globiformis* L-serine dehydratase the association of enzyme monomers may well be the cause of the cooperativity which this enzyme exhibits when it interacts with L-serine.

For the purified *E. coli* L-serine dehydratase, dimerisation is clearly not the underlying mechanism which is responsible for the slow activation of this by its substrate. However, enzyme which is preincubated with either D- or L-serine shows characteristic changes in its kinetic properties, indicating that a significant rearrangement of the tertiary structure of the dehydratase must have occurred. The experiments described in sections 7.4.1 and 7.4.2 showed that Fe-independent or iron-activated L-serine dehydratase was showed greater stability when incubated with L-serine. This suggests that serine-activation resulted in a conformational change in the dehydratase which protected the activity of the enzyme, possibly by maintaining the iron within the iron-activated enzyme, or the native cofactor in Fe-independent L-serine dehydratase in a more stable configuration.

The results described in this chapter have shown that, for the purified L-serine dehydratase from *E. coli* B, dimerisation is not the process responsible for the molecular changes which occur during the transitional phase when the dehydratase is slowly activated by its substrate. Frieden (1971) has shown that slow responses of hysteretic enzymes to changes in substrate or other ligand concentrations can be a consequence of a variety of mechanisms, including changes in the molecular arrangement of the enzyme. On the basis of the evidence presented, it is likely that it is a conformational change that is responsible for the slow activation of L-serine dehydratase by L-serine, and not, as Roberts (1983) suggested, a dimerisation reaction.

CHAPTER 8 - GENERAL DISCUSSION

Chapter 8

General Discussion

8.1 Properties of L-Serine Dehydratase

8.1.1 The Purification of the L-Serine Dehydratase from *E. coli* B

The L-serine dehydratase from *E. coli* B has been purified by a factor of between 1300 and 1700-fold, at an overall yield of between 2 and 4 % of the initial activity (Chapter 3, section 3.3). At the end of its isolation scheme, the enzyme has a specific activity of 1450 to 1700 μmoles pyruvate min^{-1} mg^{-1}, and, as judged by SDS PAGE, is between 70 and 90 % pure. This is the most highly purified preparation of *E. coli* L-serine dehydratase that has so far been obtained, and is only the third member of the class of L-serine-specific dehydratases to have been purified to a level approaching homogeneity.

8.1.2 General Properties of L-Serine Dehydratase

In terms of its general properties (Chapter 4), the purified *E. coli* enzyme resembles L-serine dehydratases from other microbial sources: it has the same highly specific requirement for L-serine as substrate, it has a similar K_m value for L-serine, and is inhibited competitively by D-serine and L-cysteine, with similar K_i values. The *E. coli* L-serine dehydratase is also inhibited by mercurial compounds, and other agents which react with protein sulphydryl groups, has a broad pH optimum, is rather unstable and has a relatively low molecular weight.

Newman and Kapoor (1980) and Roberts (1983) had also demonstrated similar properties for the *E. coli* L-serine dehydratase in crude or slightly purified extracts. However, the purification of this enzyme has allowed a number of studies that were previously not possible to be undertaken. The first of these provides an unequivocal answer to the question of whether or not the *E. coli* L-serine dehydratase contains pyridoxal phosphate.

8.1.3 Pyridoxal Phosphate

In light of the well-characterised dependence of the microbial D-serine-specific dehydratase on pyridoxal phosphate for activity (Umbarger, 1973), it has long been assumed that the microbial L-serine-specific dehydratase also utilised this cofactor, even though its presence has been demonstrated in only one L-serine

dehydratase, that of *Cl. acidiurici* (Carter and Sagers, 1972). Considering the structural similarity between the substrates of these two enzymes, one might have expected that similar cofactors would be employed for the deamination of D- and L- serine. Never the less, the work described in Chapter 5 shows quite clearly that the *E. coli* L-serine dehydratase, unlike the *E. coli* D-serine dehydratase, does not contain pyridoxal phosphate.

The evidence for the absence of a vitamin B6-derived coenzyme is very strong. The investigative techniques described in Chapter 5 did not find any evidence for the presence of pyridoxal phosphate. L-Serine dehydratase was neither stabilised nor activated by pyridoxal phosphate. It could not be resolved by extensive dialysis against inhibitors which might be expected to remove such a cofactor, and showed little sensitivity to concentrations of carbonyl reagents which were able to completely inactivate PLP-dependent enzymes such as the *E. coli* biosynthetic and degradative L-threonine dehydratases (Umbarger and Brown, 1957). An examination of the spectroscopic properties of L-serine dehydratase also failed to find any evidence for the presence of pyridoxal phosphate. The UV/visible spectrum of the dehydratase did not show the 412 nm absorption maximum which is indicative of protein-bound PLP, and the enzyme did not fluoresce when activated at the wavelengths which normally cause fluorescence in pyridoxal phosphate-containing enzymes. All of these results show quite clearly that the *E. coli* L-serine dehydratase does not contain pyridoxal phosphate. This suggests that the L-serine-specific dehydratase from this microoorganism must deaminate its substrate via a mechanism that is quite different to that used by the D-serine-specific dehydratase

8.1.4 The N-Terminal Sequence of L-Serine Dehydratase

The availability of purified L-serine dehydratase has also allowed the sequencing of the amino terminus of this enzyme to be undertaken (Chapter 4, section 4.7). Two determinations were performed, yielding identical results, and producing a sequence covering the first 20 amino acids of the dehydratase. This sequence, with the exception of one unassigned residue, shows a high level of homology with the predicted amino terminal sequence of the *E. coli* K12 L-serine dehydratase, deduced by Su *et al* (1989) from the nucleotide sequence of the *sdaA* gene. Although this confirms that the gene isolated by these workers is indeed the structural gene for L-serine dehydratase, quite marked differences were found to exist between the apparent initiation codons of the two enzymes. The measured *E. coli* B N-terminal sequence begins 18 bases earlier than the predicted *E. coli* K12 sequence, with a GTG rather than ATG codon. This could

represent a genuine difference in the translational start sites of the two dehydratases. It is also possible, given the relative infrequency in *E. coli* of GTG to ATG as an initiation codon (approximately 3 %, Watson *et al*, 1988), that Su *et al* (1989) failed to recognise the GTG sequence as the initiating methionine, and that the N-terminal sequences of the *E. coli* B and K12 L-serine dehydratases are infact identical.

The similarities in properties which the *E. coli* K12 and *E. coli* B L-serine dehydratases share, most notably the requirement for iron and DTT for activity (Newman *et al*, 1985) and the fact that neither enzyme appears to contain pyridoxal phosphate (Newman and Kapoor, 1980), plus the high level of homology between the N-terminal sequences of the two dehydratases, suggests that these enzymes may also have a number of structural features in common. This is likely to be reflected in quite considerable similarities in the primary structures of the two dehydratases. The predicted primary sequence of the E. coli K12 L-serine dehydratase could therefore be used to search for homologies between the *E. coli* B L-serine dehydratase and other proteins. Thus, the sequence of the 448 amino acids of the *E. coli* K12 L-serine dehydratase was fed into a primary sequence data base and a search undertaken for notable homologies, most particularly with the structural motifs which are common to enzymes which contain pyridoxal phosphate or FeS centres.

However, the primary structure derived from the *sdaA* gene did not reveal any significant homologies with the sequences of either pyridoxal phosphate-dependent amino acid dehydratases or iron-sulphur-containing proteins. For instance, although the product of the *sdaA* gene possesses 9 cysteine residues, there is no apparent clustering of these cysteines such as is found in Fe-S proteins such as ferredoxins, hydrogenases and the glutamine phosphoribosylpyrophosphate amidotransferase of *Bacillus subtilis* (Yasunobu and Tenaka, 1973; Voordouw and Brenner, 1985 and Makaroff *et al*, 1983). The positioning of cysteines in L-serine dehydratase resembles that found in the Fe-S proteins of nitrogenase (Lundell and Howard, 1981), and the aconitase from beef heart (Nakamura and Veyama, 1988), where the cysteines are distributed irregularly throughout the primary sequence. However, both of these enzymes possess at least two cysteines with a primary sequence distance between them that is similar to the -Cys-X-X-Cys- sequence found in ferredoxins. No such motif was found within the *sdaA* gene product.

Extensive homologies have also been reported in the amino acid sequences of the pyridoxal phosphate attachment sites of the threonine synthase from *B. subtilis,*

(Parsot, 1986), the threonine dehydratase from *Saccharomyces cerevisiae* (Kielland-Brandt *et al*, 1984) and the D-serine dehydratase from *E. coli* (Schiltz and Schmitt, 1981). The sequences flanking the pyridoxyl-lysine residue common to these three enzymes are shown below:

-Pro-X-Thr/Ser-Ser-**Lys**-X-Arg-Gly-X-

X represents no amino acid homology.

The primary sequence surrounding each of the 16 lysine residues in L-serine dehydratase was examined for any resemblance to this sequence. No significant similarities were found.

The discovery that no significant homologies were found with the primary sequences of known PLP or FeS-proteins cannot be regarded in itself as conclusive evidence for the absence of these cofactors in L-serine dehydratase. However, taken in conjunction with the investigations described in Chapters 5 and 6, it does provide confirmatory evidence for the fact that L-serine dehydratase contains neither an iron sulphur centre, nor pyridoxal phosphate.

8.2 The Serine-activation of L-Serine Dehydratase

8.2.1 Serine-activation and Dimerisation

The results in Chapter 4, sections 4.2 and 4.3, and Chapter 7 have shown that the purified *E. coli* L-serine dehydratase is subject to a form of activation which appears to be distinct from the process which results from the interaction of this enzyme with iron and DTT (Chapter 6). This activation is a consequence of the slowness of the response of L-serine dehydratase to its substrate, L-serine, and manifests itself in the form of a non-linear timecourse of activity and sigmoidal substrate saturation profile. In addition to L-serine, the dehydratase was also found to undergo activation of this type by the optical isomer D-serine. Preincubation of L-serine dehydratase with either amino acid resulted in the activation of this enzyme and the production of significant changes in its activity, most notably characterised by a linear timecourse of activity, and hyperbolic substrate saturation curve.

The serine-activation of L-serine dehydratase has also been studied by Roberts (1983). Using slightly purified enzyme she demonstrated the existence of kinetic phenomena very similar to the above results, which were obtained using L-serine

dehydratase of high purity. Roberts further showed that enzyme which had been serine-activated, whether by D- or L-serine, also appeared to undergo a doubling of its molecular weight. She therefore concluded that a dimerisation of inactive enzyme monomers to give catalytically active dimers was the cause of the slow step in the serine-activation of L-serine dehydratase.

In contrast, the present study suggests that dimerisation is not the molecular event which underlies the serine-activation process. An extensive series of molecular weight studies (section 7.3.1) showed quite clearly that, although purified L-serine dehydratase could become serine-activated, it did not dimerise, unlike the relatively unpurified enzyme that Roberts used. This was quite unlike the corresponding situation for the L-serine dehydratase from *A. globiformis*, which underwent a doubling of its molecular weight following serine-activation irrespective of whether it was from a crude, or intermediately or highly purified enzyme preparation (Gannon, 1973). A number of possible reasons for the disparity between Roberts (1983) study and the present work were investigated, and a strong correlation was found to exist between the activity state of the dehydratase (that is, whether it was Fe-independent or iron-activated), and the molecular form in which it existed following serine-activation (section 7.3.2). L-Serine dehydratase which was in possession of its native, Fe-independent activity was able to dimerise following activation by D-serine. However, this ability diminished as the dehydratase became more purified, and less Fe-independent, and was eventually lost at the stage of the purification (step 5) at which the enzyme became fully dependent upon iron and DTT for activity. L-Serine dehydratase from the subsequent steps of the purification required a preincubation with iron and dithiothreitol for activity, and did not dimerise, although it could clearly become serine-activated. Kinetic analyses of serine-activated monomeric and dimeric enzyme revealed no significant differences in the manner in which these enzyme species interacted with L-serine, which suggests that the link between the ability to dimerise and serine-activation is incidental. It therefore appears that dimerisation is not the mechanism which underlies the serine-activation reaction.

The activation of L-serine dehydratase by L- or D-serine results in quite profound changes in the kinetics of the enzyme, which would suggest that some kind of structural rearrangement must have taken place within the dehydratase. If this rearrangement does not have its origin in the polymerisation of enzyme monomers, then it must involve a change in the actual conformation of the dehydratase molecule. Evidence for this latter suggestion was provided in Chapter 7, section 7.4, which showed that activation by L-serine protected the

activity of Fe-independent or iron-activated L-serine dehydratase when diluted into media lacking iron and DTT. The presence of L-serine was also found to considerably enhance the rate at which Fe-dependent dehydratase was activated by iron and DTT (Chapter 6, section 6.2). These experiments suggest that a change in structual form occurred following serine-activation which caused the dehydratase to adopt a conformation which favoured both iron uptake and retention.

Since serine-activation does not result in an overt physical change such as a molecular weight increase, the serine-activated state in L-serine dehydratase was monitored by following changes in the kinetic properties of the enzyme. This approach by necessity required that the dehydratase be active, that is iron-activated, or in possession of its Fe-independent activity. However, it is possible that L-serine dehydratase can become serine-activated without having to be catalytically competent. To investigate this possibility, and to study the structural rearrangement which occurs during serine-activation, an investigative procedure is needed which responds to conformational changes in the dehydratase. One possible method would be to monitor the tryptophan fluorescence of the dehydratase during its interaction with L-serine, and to correlate changes in fluorescence (and therefore changes in conformation) with the corresponding timescale of the serine-activation process. Because such molecular events tend to be very brief in duration, the reaction would be monitored using a fluorimeter suitably adapted to record over a short timescale, such as a stopped flow instrument. This possible approach was investigated by following the changes in tryptophan fluorescence which occurred when iron-activated L-serine dehydratase (step 8) was mixed with L-serine. Although it was only possible to undertake a brief number of investigations, the results obtained were able to show that changes in tryptophan fluorescence did occur during the serine-activation of L-serine dehydratase. These studies were very preliminary, and so it would be unjustified to assign more detailed interpretations to them. However, they do provide a possible route by which the changes which take place during serine-activation and, indeed during iron-activation, might be studied.

8.2.3 The Relationship Between Fe-independence, Iron-activation and Serine-activation

It has been seen that the *E. coli* L-serine dehydratase can undergo two distinct forms of activation: its activity can be increased by Fe and DTT (Chapter 6), and by interaction with its substrate, L-serine, or with serine-activators, such as D-serine (Chapter 7). While each form of activation produces quite distinctive

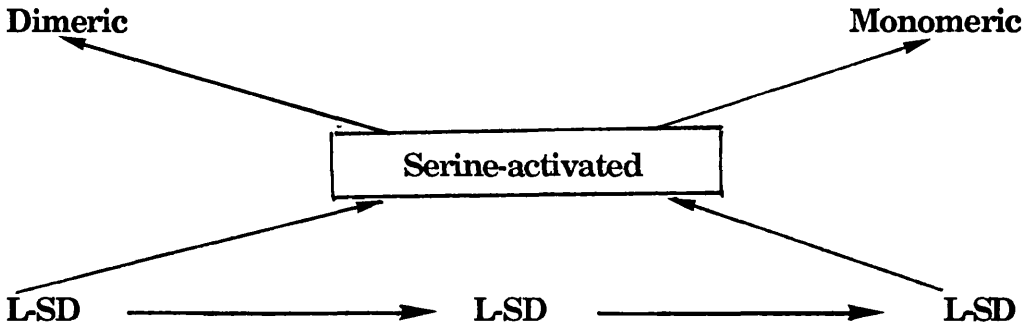
changes in L-serine dehydratase activity, Chapter 7, section 7.4.3 showed that the iron- and serine-activation processes were not mutually exclusive reactions. Enzyme which had been iron-activated could become further activated by L- or D-serine, and serine-activated dehydratase (in permeabilised cells) could be further activated by iron and DTT without affecting the kinetics of its subsequent interaction with L-serine. These observations suggest that the changes in activity which L-serine dehydratase undergoes when it interacts with L-serine, and with Fe and DTT, involve quite separate reactions, which may even be occurring at different sites on the dehydratase molecule. This is not meant to imply, however, that the iron-activation and serine-activation processes are completely unrelated events, and it is possible that achievement of the latter activated state is dependent upon first attaining the former.

Section 7.3.4 also showed that while iron-activation of Fe-independent L-serine dehydratase increased the activity of the enzyme, it had no obvious effect upon its subsequent activation by L- or D-serine. Further, the same preparation of dehydratase, iron-activated and purified by more than a thousand-fold, displayed no significant differences in the kinetics of its interaction with L-serine from the unpurified Fe-independent enzyme. These results suggest that the Fe-independent and iron-activated states are functionally equivalent. However, they may not be physically equivalent. The chelator studies described in Chapter 6, section 6.3 showed that over a similar timescale, the Fe-independent activity of L-serine dehydratase was more resistant to inhibition by EDTA than the iron-activated activity of the enzyme. If the molecular basis of the Fe-independent activity is a cofactor of some kind, possibly iron, these studies suggest that the binding of this native cofactor to L-serine dehydratase must be stronger than the binding of iron which occurs during the *in vitro* iron-activation of the enzyme.

Chapters 6 and 7 have shown that L-serine dehydratase can exist in a number of activity states, all of which possess certain characteristic features. These activity states are summarised in the model shown below.

The experiments described in Chapter 7, section 7.3 showed that while iron-activated, serine-activated purified L-serine dehydratase was unequivocally monomeric, Fe-independent, serine-activated unpurified enzyme was able to dimerise. This suggests that the functional equivalence which the Fe-independent and iron-activated states of L-serine dehydratase appeared to possess with regards to the kinetics of their interaction with L-serine, does not necessarily extend to the molecular form which these enzyme species can adopt once serine-activation has occurred. It is possible that the activity state which is

formed during the iron-activation of purified L-serine dehydratase is different to the native Fe-independent activity of the enzyme in crude extracts. Alternatively, it is also possible that the ability of unpurified enzyme to dimerise is a consequence of a feature quite unconnected to Fe-independence. However, it is quite clear that the ability to dimerise is incidental rather than causal to the serine-activation of L-serine dehydratase.



Fe-independent Time, chelators other factors **Fe-dependent** Fe, DTT **Iron-activated**

(active) (inactive) (active)

8.3 The Role of Iron in the Iron-activation of L-Serine Dehydratase

Purified L-serine dehydratase has an absolute and highly specific requirement for iron and a thiol reducing agent for activity. The experiments described in Chapter 6 showed that the activation of the dehydratase by these reagents was influenced by a variety of factors, including temperature, pH and metal chelating agents. The iron-activation reaction was found to involve iron binding, and a quantitative relationship was seen between iron uptake and increasing enzyme activity (sections 6.6.3 and 6.6.4). The activation of L-serine dehydratase by iron and DTT was also found to be an oxygen-dependent reaction, which appeared to involve the production of a reactive oxygen molecule (section 6.7). The observation that catalase could inhibit the iron-activation process, and that hydrogen peroxide could stimulate, it suggested that the identity of this reactive species might be a peroxide anion, or possibly some molecule derived from it.

These results show that the iron-activation of L-serine dehydratase is a process of considerable complexity. Although the binding of iron has been shown to be an integral part of the activation reaction, the form in which it exists once bound, and its role in the catalytic reaction are uncertain. The iron-requirement of L-serine dehydratase was seen to be similar to those of the FeS-containing (de)hydratases aconitase (Kennedy *et al*, 1983) and maleate hydratase (Dreyer, 1985). This initially suggested that an iron sulphur centre might also exist in the *E. coli* enzyme. However, the oxygen-dependence of the iron-activation process, coupled with the absence of any spectroscopic evidence for the presence of such an iron sulphur centre (section 6.8) suggested that this was not the case. Iron can also complex with oxygen in proteins to form oxo-bridged iron centres (Lippard, 1988). The possibility that such a centre might be present in L-serine dehydratase was examined, since the iron-activation of ribonucleotide reductase, which contains a binuclear iron-oxo centre (Petersson *et al*, 1980), was seen to show some resemblance to the activation process of this enzyme. However, neither UV/visible, nor EPR investigations were able to provide any evidence for the existence of an Fe-O centre in L-serine dehydratase.

Newman *et al* (1990) have also investigated the oxygen-dependence of L-serine dehydratase iron-activation process. They suggest that the activation produced by iron and DTT involves a proteolytic cleavage at a specific serine-serine peptide bond, in a manner which parallels the zymogen activation of the *Lactobacillus* prohistidine decarboxylase (Recsei *et al*, 1983). Newman *et al* (1990) argue that the involvement of iron in this reaction is indirect, and that it is an oxidant (a hydroxyl radical) generated during the cycling of Fe between the ferrous and ferric oxidation states which is responsible for the cleavage and activation of the dehydratase.

The results obtained during the present study suggest that this hypothesis is incorrect. The reasons for this have already been discussed in section 6.9. The fact that iron is bound to L-serine dehydratase in a quantitative and specific manner suggests a much more direct involvement in the enzymatic process. However, what form the iron might take once has it has bound to the dehydratase is currently uncertain, since this enzyme does not appear to contain any recognised Fe-containing centre. The nature and role of the reactive oxygen radical which appears to be essential to the iron-activation of L-serine dehydratase have also to be elucidated.

8.4 Future Developments

The availability of nearly homogeneous preparations of the *E. coli* L-serine dehydratase has allowed the undertaking of investigations which were previously not possible. These have provided answers to a number of the questions posed at the beginning of this thesis, and have revealed much about the activity of this enzyme. However, as the study of L-serine dehydratase has unfolded a new set of questions have been arisen, which now need to be answered. These new areas of enquiry include:

> the elucidation of role played by iron (and oxygen) in the iron-activation of L-serine dehydratase;
>
> the molecular characterisation of the Fe-independent and iron-activated states and the role they play in the reaction catalysed by L-serine dehydratase;
>
> the nature of the conformational changes which occur during serine-activation and their relationship with the iron-activation process.

In order to undertake these investigations, a source of L-serine dehydratase would be required which is much richer than that which was available during the present study. This could most readily be achieved by the cloning and over-expression of the L-serine dehydratase gene, as described in section 3.4.5. The availability of an amino terminal sequence with which to construct an oligonucleotide probe, and the complete gene sequence of a closely related L-serine dehydratase (the *E. coli* K12 *sdaA* gene, Su *et al*, 1989) with which to verify the identity of the isolated *E. coli* B gene would hopefully make such an undertaking relatively straight forward.

The production of large amounts of L-serine dehydratase would also enable biochemical studies additional to those shown above to be carried out. For example the growth of protein crystals for X-ray crystallography, or the identification of the residues which make up the active site, and their role in the deamination reaction. From such studies it should be possible to characterise the L-serine dehydratase reaction mechanism, and to see how it differs from the mechanism of the dehydratase which works on the D-isomer of serine.

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