VARIOUS ASPECTS OF L-SERINE DEAMINASE ACTIVITY IN CRUDE CELL EXTRACTS OF E. COLI K_{12}

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ABSTRACT

VARIOUS ASPECTS OF L-SERINE DEAMINASE ACTIVITY IN CRUDE CELL EXTRACTS OF *E. COLI* K_{12}

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L-Serine deaminase has been shown to be unusually unstable in crude cell extracts of *E. coli* K_{12} prepared by sonication. These extracts can, however, be stored frozen for some time (days). Various factors which might stabilize, inhibit, or increase L-serine deaminase activity have been studied. Possible factors in the instability of the enzyme have been discussed.

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INTRODUCTION

L-Serine deaminase of Escherichia coli (E. coli) K_{12} is unstable under both in vivo (Isenberg and Newman, 1974) and in vitro conditions. The aim of this study was to determine which physiological and physical factors affect the in vitro instability of L-serine deaminase. It was hoped that elucidation of such factors would assist in the purification of the enzyme thereby enabling studies on the metabolic function of L-serine deaminase to be performed.

It should be noted that L-serine deaminase is also commonly called L-serine dehydratase [L-serine hydro-lyase (deaminating), EC4.2.1.13]. Throughout this thesis, the enzyme will be referred to as L-serine deaminase.

(i) Biosynthesis and Metabolism of L-Serine

L-Serine is synthesized from a member of the Embden-Meyerhof-Parnas (EMP) pathway (Figure I). The formation of L-serine from 3-phosphoglyceric acid, an intermediate of EMP, involves three different enzymes. 3-Phosphoglyceric acid is oxidized to 3-phosphohydroxy-pyruvic acid by phosphoglycerate dehydrogenase. Phosphoserine amino-transferase carries out a transamination reaction between glutamate and 3-phosphohydroxypyruvic acid to form 3-phosphoserine. L-Serine is then formed via hydrolysis by phosphoserine phosphatase.

L-Serine is a precursor of many molecules of biosynthetic importance (Figure II) (Mahler and Cordes, 1971). L-Cysteine can be synthesized from L-serine and H_2S via the enzyme serine sulfhydrase or from L-serine and L-homocysteine. L-Cysteine can then be used to

synthesize L-methionine in the presence of L-homoserine through a multistep process. Tryptophan synthetase can catalyze the formation of L-tryptophan and glyceraldehyde-3-phosphate from indole-3-glycerol-phosphate and L-serine. In the presence of tetrahydrofolate (THF), L-serine can be converted by serine transhydroxymethylase to glycine and $N^{5,10}$-methylene THF. The $N^{5,10}$-methylene THF, so generated, can donate C-1 units in other reactions. The β-carbon of L-serine can thus be utilized as a C-1 unit. Various derivatives of THF participate in a variety of reactions giving rise to methionine, thymine, and purines. Purines, in turn, can be involved in the formation of L-histidine. Carbon 2 and nitrogen 1 of the imidazol ring of L-histidine are derived from carbon 2 and nitrogen 1 of the purine nucleus. The carboxyl and amino group joined to the α carbon (as a unit) can be used in the formation of glycine. Glycine can be incorporated into purines. Carbons 4,5, and 7 of the purine ring system are derived from glycine. This amino acid is also an important building block in the formation of tetrapyrroles which are precursors to porphyrins. Glycine can be incorporated into proteins through the normal biosynthetic processes of protein formation.

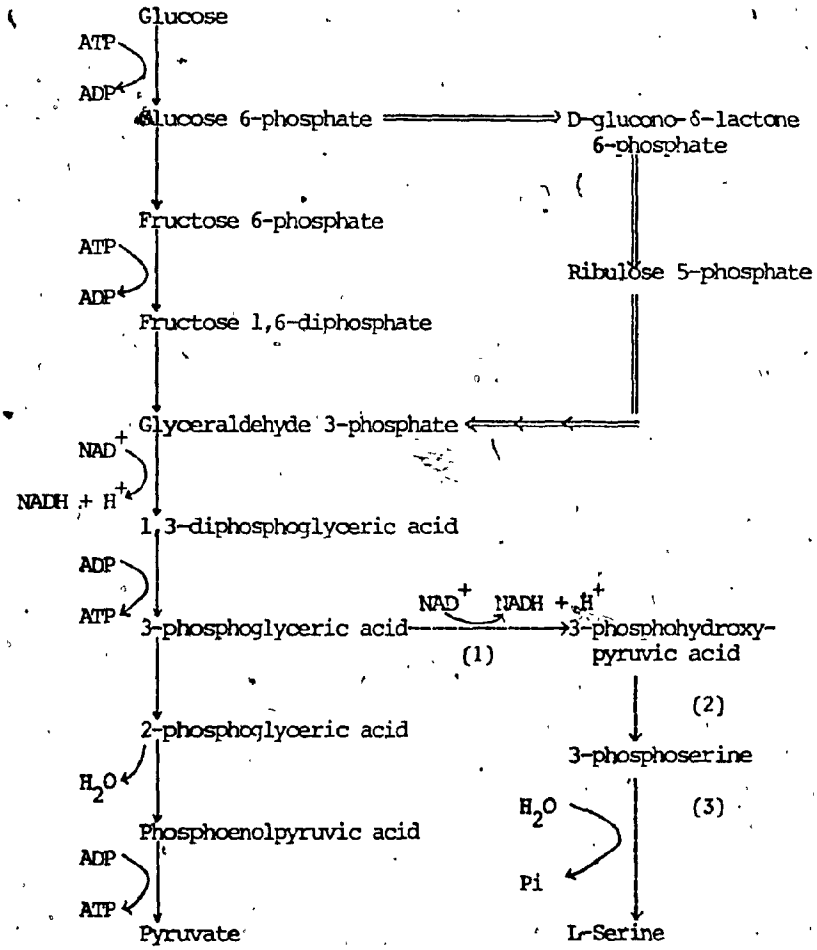
All of the above mentioned reactions appear to have a clear role in bacterial metabolism. Why then should there be an enzyme for the non-oxidative deamination of L-serine to pyruvate and ammonia (Figure III)?

An E. coli cell performing glycolysis via the EMP pathway would show the following overall reaction:

$$\text{Glucose} + 2\text{ATP} + 2\text{NAD}^+ \longrightarrow 2\ \text{Pyruvate} + 4\text{ATP} + 2\text{NADH} + 2\text{H}^+$$

It is then not obvious why a cell grown on glucose would produce

Figure I Biosynthesis of L-Serine in E. coli



HMP (Hexose monophosphate) Shunt ═══

EMP Pathway ————

(1) Phosphoglycerate Dehydrogenase

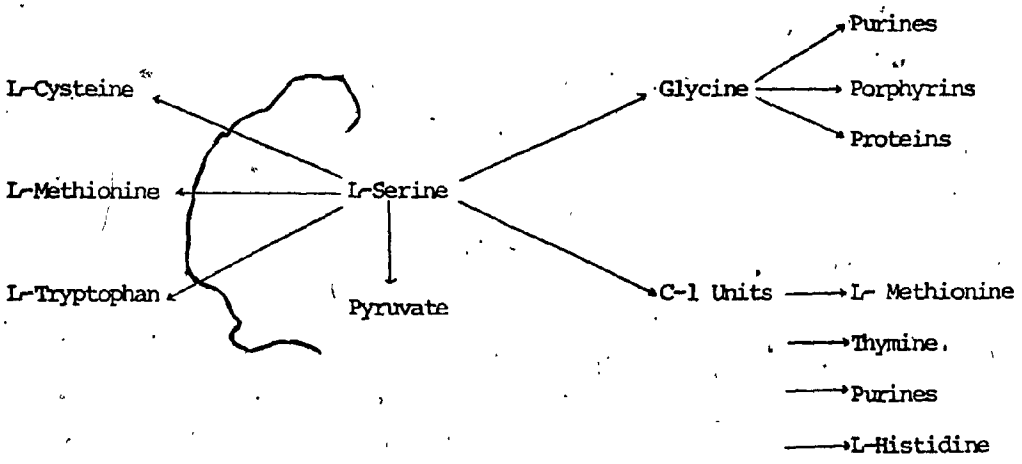
(2) Phosphoserine Aminotransferase

(3) Phosphoserine Phosphatase

Doelle, 1975

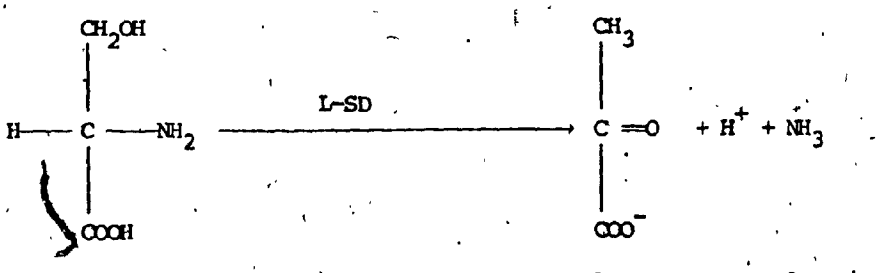
Dawes and Sutherland, 1976

Figure II Metabolism of L-Serine in E. coli



Mahler and Cordes, 1971

Figure III Non-Oxidative Deamination of L-Serine in E. coli



L-SD = L-Serine Deaminase

Doelle, 1975

pyruvate from L-serine via phosphoglyceric acid with the formation of two less ATP's as compared to when glucose is metabolized directly to pyruvate through the EMP pathway (Figure I).

(ii) Demonstration of L-Serine Deaminase as a Discrete Enzyme

For sometime, it was thought that L-serine deaminase activity was a function of an enzyme having either D-serine deaminase activity or L-threonine deaminase activity. D-Serine deaminase catalyzes the deamination of D-serine to pyruvate and ammonia and L-threonine. deaminase catalyzes the deamination of L-threonine to α-ketobutyrate and ammonia. The controversy was in part put to rest once racemic mixtures of D- and L-serine, which had originally been used by Gale and Stephenson (1938) in their studies on bacterial deamination in two strains of E. coli and by Boyd and Lichstein (1955) in their studies on serine deaminases from various microorganisms (e.g. several strains of E. coli (e.g. Crookes, Texas, Tennessee, mutabilis, and Davis), Bacterium cadaveris (e.g. Gale), and two strains of Aerobacter aerogenes (e.g. T and D - 1)), were no longer used as substrates in assessing D- or L-serine deaminase activity. It then became clear that there were two enzymes each very specific for its substrate, L-serine deaminase for L-serine and D-serine deaminase for D-serine (Pardee and Prestidge, 1955; McFall, 1964). Recently, a mutant of E. coli K_{12}, strain Cu 1008, which carries a deletion in ilv A gene and therefore does not produce L-threonine deaminase has been shown to produce L-serine deaminase (Beeraj et al., 1978). This demonstrated conclusively that the cell makes two enzymes, one recognized as L-threonine deaminase and the other recognized as L-serine deaminase.

In conclusion, L-serine deaminase, D-serine deaminase, and L-threonine deaminase all exist as separate enzyme molecules in E. coli.

(iii) Metabolic Role of L-Serine Deaminase

The possible metabolic role of L-serine deaminase in glycolyzing cells of E. coli has been reviewed previously by Isenberg (1974) in some detail. Since that time, no advancement in this area has been achieved.

For many years, there was little mention in the literature of the metabolic role of L-serine deaminase from E. coli. This resulted from the fact that L-serine deaminase activity and L-threonine deaminase activity were believed to be associated with the same protein molecule until 1955 (Pardee and Prestidge, 1955). Once it became clear that L-serine deaminase was a unique enzyme molecule, investigators (Alfoldi and Rasko, 1970; Isenberg and Newman, 1974) became more eager to explore the possible role of L-serine deaminase in E. coli metabolism.

From the previous discussion entitled 'Biosynthesis and Metabolism of L-Serine', it seems unlikely that L-serine deaminase is involved in carbon metabolism. It appears much more economical for the cell to form pyruvate from glucose rather than from L-serine (Figure I).

Nitrogen is required for the growth of E. coli cells. Since non-oxidative deamination of L-serine via L-serine deaminase produces both pyruvate and ammonia, the end product of this reaction, ammonia, may be used by the cell to compensate for any lack of or insufficient

presence of exogenous nitrogen in the growth media.

L-Serine has been found to be toxic to E. coli K_{12} cells (Cosloy and McFall, 1970). L-Threonine added to the medium can reverse this inhibition. The addition of L-threonine and L-isoleucine can completely overcome the L-serine effect (Rasko and Alfoldi, 1971). Isenberg and Newman (1974) proposed that L-serine deaminase could be acting as a detoxifying agent. L-Serine deaminase would then provide the cell with means for keeping the L-serine concentration low within the cell. In this way, the cell would avoid the inhibition of L-threonine deaminase by L-serine (Rasko and Alfoldi, 1971). If this hypothesis were true one would expect that L-serine could induce L-serine deaminase. However, this amino acid cannot induce L-serine deaminase (Isenberg and Newman, 1974) thus giving less credibility to this hypothesis.

E. coli requires ammonia for the synthesis of all amino acids (Doelle, 1975). Two enzymes, glutamine synthetase and glutamic acid dehydrogenase, convert inorganic nitrogen, ammonia, to organic forms, L-glutamine and L-glutamic acid, respectively. These two amino acids serve as substrates for the synthesis of the remaining amino acids. L-Serine deaminase may then provide ammonia for these two reactions under appropriate conditions. However, the strain of E. coli used during this study was already grown on excess ammonia (i.e. 0.2% (w/v) $(NH_4)_2SO_4$) and exhibited unusually high levels of L-serine deaminase activity making it unlikely that this enzyme would be providing the ammonia for glutamine synthetase and glutamic acid dehydrogenase.

In other microorganisms, a variety of roles have been proposed for this enzyme. Benziman et al. (1960) suggested that L-serine

deaminase from Clostridium acidiurici is one of the enzymes of the uric fermentation pathway. This was indicated when L-serine deaminase activity in extracts containing partially purified enzyme was found to be comparable to the uric acid fermentation rate in whole cells. In 1965, Bridgeland and Jones examined L-serine deaminase activity in toluene-treated whole cells of Arthobacter globiformis. L-Serine deaminase was present in high activity when cells were grown with glycine, where glycine functioned as the sole source of nitrogen and carbon. L-Serine deaminase is thought to be the key enzyme in the metabolism of glycine under the above stated conditions. Klein and Sagers (1961) believe L-Serine deaminase from Diplococcus glycinophilus to be the second enzyme in the conversion of glycine to acetate. They found that the overall rate of glycine fermentation by whole cells was approximately 9 μ moles per hour per mg of protein. The conversion of L-serine to pyruvate by L-serine deaminase in crude cell extracts occurred at about 180 μ moles per hour per mg of protein. This rate was above the glycine fermentation rate of whole cells. However, when all the individual enzyme reactions were coupled together, the overall rate accounted for the fermentation of glycine as performed by whole cells. It, therefore, appears that L-serine deaminase has several roles in metabolic pathways that involve L-serine. Its role in cells of E. coli which are undergoing glycolysis is, however, still unclear.

(iv) in vivo Instability of L-Serine Deaminase

Though the enzyme has been studied in E. coli for the past forty years little information is available about it. This is due to the fact that L-serine deaminase cannot as yet be purified to homogeneity from E. coli cells. It is unusually unstable under both in vivo

and in vitro conditions.

In 1955, Pardee and Prestidge reported that L-serine deaminase was unstable in toluene-treated whole cells as well as in intact cells of E. coli, strain B. After two hours at low temperature (i.e. 0°C) the activity had declined by 60% when the enzyme was incubated in phosphate buffer (0.01M, pH 8.0) containing ethylenediaminetetraacetic acid (EDTA) (0.2 mg/ml). The enzyme activity was lost much more rapidly in the absence of EDTA and in preparations where the concentration of cells was very low. In intact cells, L-serine deaminase was almost completely inactive within 30 to 60 minutes at 37°C in minimal medium, pH 7.0 to 7.5. The authors failed to give the experimental design used to obtain this latter result . Two other strains of E. coli (strain ML and strain C) showed the same pattern of instability of L-serine deaminase. Various compounds (e.g. Na_2S, yeast extract, Mg^{++}, vitamin B_{12}, pyridoxal phosphate, glutathione, formate, 5' adenylic acid, biotin, and β-mercaptoethanol) were examined for their effect on the stability of the enzyme. None were able to stabilize L-serine deaminase; although, formate appeared to help slightly. These authors, therefore, added 0.005 M Na formate into the assay mixture. To this mixture, they also added 0.2 mg/ml of adenylic acid. No explanation for this addition was made.

L-Serine deaminase in toluene-treated whole cells of Arthobacter globiformis (Bridgeland and Jones, 1965) has been shown to produce pyruvate linearly with time (i.e. for 30 minutes) and to have no apparent requirement for univalent or divalent cations (e.g. Mg^{++}, Ca^{++}, K^+, or NH_4^+). There was no mention of the stability of the enzyme in these cells.

L-Serine deaminase in toluene-treated whole cell preparations from Bacillus alvei has been demonstrated by Griffiths and DeMoss

(1970) to be unstable in the presence of tris (hydroxymethyl) aminomethane (Tris) buffer. The enzyme was more stable in the presence of phosphate buffer. In phosphate buffer, it had a pH optimum of 7.5. The three cations, K^+, NH_4^+, and Na^+, stimulated L-serine deaminase activity. The enzyme was specific for L-serine. However, D-serine, in the presence of L-serine, was found to be a competitive inhibitor of L-serine deaminase. The authors, therefore, proposed that the D-isomer can occupy the active site on the enzyme. L-Serine deaminase was also active with some β-substituted serine analogues (e.g. β-phenylserine).

Isenberg and Newman (1974) showed that in toluene-treated whole cells of E. coli K_{12}, L-serine deaminase was unstable in the presence of glycine and L-leucine, the inducers of the enzyme. However, the enzyme appeared much more stable in their absence. When these cells were incubated at 37°C (without glycine and L-leucine) 55% of the L-serine deaminase activity was lost within one hour and 86% of the activity was lost within two and one-half hours. The addition of L-serine (without inducers) to the enzyme preparation prevented any loss of enzyme activity for the first hour at 37°C. The substrate was, therefore, a potent protector of enzyme activity at least for the first hours. Recently, Beeraj et al. (1978) demonstrated that the factors involved in determining L-serine deaminase stability were quite complex. The instability of the enzyme was related to the nitrogen content of the medium in which the cells were grown. L-Serine deaminase was unstable when the cells were grown with organic nitrogen (i.e glycine and L-leucine). This instability was markedly increased by incubating the cells in the presence of inducers of L-serine deaminase.

When cells were grown with inorganic nitrogen the enzyme activity was somewhat more unstable than when cells were grown with organic nitrogen. The instability of L-serine deaminase in cells grown with inorganic nitrogen increased when these same cells were incubated with inorganic nitrogen (i.e. ammonium sulfate). Glycine and L-leucine were without effect.

(v) *in vitro* Studies on L-Serine Deaminase

L-Serine deaminase of bacterial origin has been found to be unusually unstable. This enzyme has been reported in crude cell extracts or has been partially purified from *E. coli* (Wood and Gunsalus, 1949; Artman and Markenson, 1956; Alfoldi *et al.*, 1968; Rasko and Molnar, 1971), *Neurospora crassa* (Yanofsky and Reissig, 1953), *Streptomyces rimosus* (Szentirmai and Horvath, 1963), and *Salmonella typhimurium* and *Bacillus cereus* (Rasko *et al.*, 1969), but only the enzyme from *Clostridium acidiurici* (Carter and Sagers, 1972), *Corynebacterium* sp (ATCC 21050) (Morikawa *et al.*, 1974), and *Arthrobacter globiformis* (Gannon *et al.*, 1977) has been purified a 100 -fold or greater. In each case, the enzyme activity was lost rapidly in crude cell extracts.

In 1949, Wood and Gunsalus reported that they could liberate L-serine deaminase from cells of *E. coli* Crookes strain by autolysis and that they could partially purify the enzyme by ammonium sulfate precipitation and adsorption on calcium phosphate gel. This isolated enzyme was unstable becoming inactivated within 5 to 15 minutes at 37°C. Adenylic acid and glutathione were required for activity.

Other nucleotides and nucleosides (e.g. ATP, adenosine-3-phosphate and adenosine) and other reducing agents (e.g. cysteine, sodium thioglycolate and ascorbic acid) were without effect. Incubating the enzyme with L-serine in the absence of adenylic acid and glutathione could not prevent the inactivation of the enzyme.

Artman and Markenson (1956) prepared crude cell extracts from E. coli strain B/r by sonication. These extracts were subject to the purification steps previously used by Wood and Gunsalus (1949). The partially purified extracts maintained about 70% of the enzyme activity of the crude cell extracts. Adenylic acid and glutathione had no effect on the activity of L-serine deaminase. The authors made no mention of the final stability of the enzyme.

In 1968, Alfoldi et al. demonstrated that L-serine deaminase from E. coli K_{12} was sensitive to dilution and to sonication. At saturating concentrations of D- or L-serine the enzyme was protected against spontaneous inactivation both during sonication and in dilute enzyme preparations. The addition of various cofactors to crude cell extracts or to diluted enzyme preparations (e.g. adenosine triphosphate, nicotinamide adenine dinucleotide, pyridoxal phosphate, glutathione, and vitamin B_{12}) did not affect the enzyme activity. Rasko and Molnar (1971) reaffirmed that L-serine deaminase is a very labile protein in crude cell extracts by using two mutant strains of E. coli K_{12} with very high L-serine deaminase activity [Strains E. coli Hfr C (Met^-) and E. coli Hfr C (Met^-, $Ileu^-$), S-2]. The rate of inactivation of the enzyme was again a function of dilution.

L-Serine deaminase from Neurospora crassa (Yanofsky and Reissig, 1953) has been purified 15- to 20-fold via two ammonium sulfate

precipitation steps and two calcium phosphate gel treatments. The optimum pH for L-serine deaminase activity was about 9.3 in pyrophosphate buffer and about 9.5 in borate buffer. The enzyme demonstrated an absolute requiremint for pyridoxal phosphate. Adenylic acid and glutathione stimulated the activity of L-serine deaminase when added to assay mixtures either alone or together. There was no mention of the final enzyme stability.

In 1963, Szentirmai and Horvath reported partial purification of L-serine deaminase from Streptomyces rimosus. The cells were broken by sonication. The crude cell extract was subjected to streptomycin sulfate treatment followed by ammonium sulfate fractionation and calcium phosphate gel treatment. The purified enzyme was found to be unstable. The inactivation was slowest in phosphate buffer pH 7.5 at 0°C but even under these conditions 50 to 70% of the enzyme activity was lost overnight. The enzyme was specific for L-serine. D-Serine acted as a competitive inhibitor of L-serine deaminase. This enzyme was also able to attack DL-β-chloralanine. L-Serine deaminase activity was inhibited by heavy metal ions (Ag^{++}, Cu^{++}, Pb^{++}, and Fe^{++}) at 10^{-6}M. The enzyme showed no apparent cofactor requirement, however, aminothiols and ketonic reagents were able to inhibit L-serine deaminase activity suggesting that pyridoxal phosphate may be the prosthetic group of this enzyme.

L-Serine deaminase from Salmonella typhimurium and Bacillus cereus (Rasko et al., 1969) have been shown to be sensitive to sonication and dilution. The inactivation of the enzyme from Salmonella typhimurium could be partially inhibited by the presence of D- or L-serine at the time of sonication or at the time of dilution.

However, the presence of L-serine at the time of sonication was without effect in crude cell extracts of Bacillus cereus. The rate of inactivation of the enzyme from Salmonella typhimurium and Bacillus cereus was a function of its dilution. L-Serine deaminase from Bacillus cereus was less sensitive to dilution than L-serine deaminase from Salmonella typhimurium. The inactivation of the enzyme from Bacillus cereus by dilution was not affected by the presence of L-serine. The optimum pH for L-serine deaminase from both microorganisms was 8.5 in phosphate buffer. The enzyme from both sources was specific for L-serine and showed no apparent cofactor requirement. L-Cysteine and D-serine acted as competitive inhibitors of the enzyme in either case.

L-Serine deaminase has been purified a 100-fold or greater from three microorganisms. Carter and Sagers (1972) purified L-serine deaminase from Clostridium acidiurici 400-fold. This was accomplished by ammonium sulfate precipitation and column chromatography on DEAE-cellulose and Sephadex G-200. L-Serine deaminase was sensitive to dialysis. Studies with a variety of small molecules (AMP, ADP, ATP, L-isoleucine, pyridoxal phosphate, L-threonine, glycine, and L-serine) showed that either L-serine or glycine could stabilize the enzyme during dialysis, possibly by occupying the active site (Carter and Sagers, 1972). None of the small molecules tested either stimulated or inhibited the enzyme activity. L-Serine deaminase was activated by catalytic amounts (1mM) of ferrous ion (Fe^{++}) and a thiol reducing agent prior to the addition of L-serine. The reducing agent used during this study was dithiothreitol. The reaction was performed at pH 8.4 in phosphate

buffer. Through spectroscopic methods, L-serine deaminase was shown to contain pyridoxal phosphate as coenzyme. It was tightly bound to the enzyme.

L-Serine deaminase from Corynebacterium sp (ATCC 21050) (Morikawa et al., 1974) has been purified 100-fold by ammonium sulfate precipitation, heat treatment, DEAE-cellulose and hydroxyapatite chromatography. The enzyme was specific for L-serine. It was inhibited by L-cysteine, L-alanine, D-serine, and L-tryptophan and was activated by magnesium ions (Mg^{++}). The enzyme reaction was optimal in Tris-HCl buffer at pH 9.0. Kinetic studies on the purified enzyme showed L-serine deaminase to behave in a manner described by the authors to be allosteric. Cooperative interactions between the enzyme and the substrate were observed. The degree of cooperativity was affected by the reaction pH and the concentration of Mg^{++} (Morikawa et al., 1974). Of the variety of compounds examined including nucleotides (ATP, ADP, AMP, cAMP, or ADP-ribose) and amino acids (L-cysteine, D-serine, or L-alanine), none affected the kinetics of L-serine deaminase (i.e. none were allosteric effectors of L-serine deaminase). The enzyme could not be purified to homogeneity.

Recently, Gannon et al. (1977) purified L-serine deaminase from Arthrobacter globiformis 970-fold. The enzyme was purified from crude extract via heat treatment, ammonium sulfate precipitation, and DEAE-cellulose and Sephadex G-100 chromatography. L-Serine deaminase was specific for L-serine. D-Serine, L-threonine, and L-cysteine were not degraded by the enzyme. L-Serine deaminase demonstrated a non-specific requirement for a univalent or bivalent

cation. Addition of KCl, NaCl, $(NH_4)_2SO_4$, Tris-hydrochloride, $MgCl_2$, $MnCl_2$, or $CaCl_2$ stimulated the enzyme activity. The addition of pyridoxal phosphate (1mM) did not affect this activity. L-Cysteine and D-serine behaved as competitive inhibitors of L-serine deaminase. L-Cysteine at concentrations greater than 4.9 mM caused a slowly developing irreversible inhibition of the enzyme. The time course of pyruvate formation by the purified enzyme, in common with the enzyme from crude extracts, was non-linear. The reaction rate increased progressively for several minutes before becoming constant. In contrast, toluene-treated whole cells formed pyruvate from L-serine at a constant rate from the initiation of the reaction, showed hyperbolic substrate-saturation curve, and did not require a cation for activity. The properties shown in in vitro studies were all properties that have been proposed to play a regulatory role in metabolic pathways of living cells (Gannon et al., 1977). Since the properties were absent in in vivo studies, the authors concluded that they were accidental and did not play a regulatory role.

L-Serine deaminase has been characterized in part from Clostridium acidiurici (Carter and Sagers, 1972), Corynebacterium sp (ATCC 21050) (Morikawa et al., 1974), and Arthrobacter globiformis (Gannon et al., 1977). However, characterization of L-serine deaminase from E. coli has been much less successful. L-Serine deaminase from Crookes strain of E. coli (Wood and Gunsalus, 1949), E. coli K_{12} (Alfoldi et al., 1968; Beeraj et al., 1978), and E. coli strain B/r (Artman and Markenson, 1956) has shown no obvious cofactor requirement. Alfoldi and Rasko (1970) did, nonetheless, add 5 µg of pyridoxal phosphate to assay tubes containing L-serine

deaminase. No explanation for this addition was given. None of the E. coli studies were carried out on purified enzyme preparations. The interpretation of the results then presents some problem. In preparations were the enzyme is not pure, the cofactor may be tightly bound to the enzyme and may not be released from the enzyme despite the enzyme's extraction from the cell; the cofactor may be present in the extract at high enough concentrations to maintain the enzyme in active form; or the cofactor may not be required by the enzyme for the enzyme to express enzyme activity. In such studies, it is difficult to distinguish between the above stated possibilities.

Though the bacterial enzyme appears to be unstable in most species studied, purification of L-serine deaminase of mammalian origin has been very successful. The enzyme has been purified to homogeneity from rat liver (Nakagawa et al., 1967; Hoshino and Kroger, 1969; Inoue et al., 1971; Suda and Nakagawa, 1971; Simon et al., 1973).

The mammalian enzyme is very stable. Indeed, instability of L-serine deaminase has never been mentioned. Hopgood and Ballard (1974) reported that the inactivation rate of L-serine deaminase from rat liver in in vitro preparations (i.e. liver homogenates) was similar to the rate constant of degradation of this enzyme in the intact animal. The enzyme was very stable both under in vivo and in vitro conditions. L-Serine deaminase retained enzymatic activity up to three hours when the liver homogenate was incubated at 37°C.

Nakagawa et al. (1967) were the first to succeed in purifying L-serine deaminase from rat liver to homogeneity. This was accomplished by two ammonium sulfate fractionation steps, two acetone

fractionation steps, DEAE-cellulose column chromatography, and by finally allowing crystallization of the enzyme at 4°C. L-Serine deaminase was found to be specific for L-serine, to require pyridoxal phosphate as coenzyme, and to be a sulfhydryl (-SH) enzyme. Consequently, it could be inhibited by carbonyl reagents (Hunter and Harper, 1976) and -SH reagents. No effect of nucleotides was observed. A molecular weight of 63,500 was determined.

By using a modification of the method of Nakagawa et al. (1967), Inoue et al. (1971) separated the crystalline enzyme into two homogeneous components. Each estimated at having a molecular weight of 34,000. L-Serine deaminase I and II (isozymes) had similar amino acid composition. Form I contained one more lysyl residue and between one and two less prolyl residues as compared to form II. No difference in the cofactor (pyridoxal phosphate) requirement was observed.

L-Serine deaminase in rat liver is involved in gluconeogenesis (Bhatia et al., 1975). The two forms of the enzyme were found by Inoue et al. (1971) to be under different metabolic regulatory controls. Form I, the more electropositive isozyme, was controlled by corticosteroids and form II, the more electronegative form, was controlled by glucagon. The two isozymes were apparently regulated by different hormones. The regulation of their synthesis at the genetic level (i.e. translation) is still unclear.

Simon et al. (1973) used a novel method to isolate L-serine deaminase from rat liver. This method involved heat treatment, ammonium sulfate fractionation, gel filtration on Sephadex G-25 column, and DEAE-cellulose chromatography. Two isozymes were isolated

each having a molecular weight of approximately 35,000. This was in good agreement with the studies previously reported by Inoue et al. (1971).

Simon et al. (1973) proposed that L-serine deaminase was composed of two subunits, each having a molecular weight of 35,000. The subunits were much less active than the intact dimer. The dissociation into subunits was counteracted by addition of K^+ and NH_4^+. Investigators (Hoshino et al., 1972; Simon and Kroger, 1974) previously observed that K^+ and NH_4^+ increased the pyridoxal phosphate enzyme binding constant. Simon et al. (1973) therefore concluded that the monovalent cations probably induced a slight conformation change in the subunits of L-serine deaminase.

In conclusion, L-serine deaminase of mammalian origin can be purified to homogeneity and is well characterized. However, L-serine deaminase of bacterial origin is very unstable, can be purified from certain bacteria, and is much more poorly characterized.

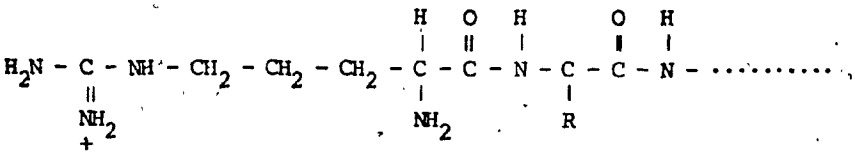
(vi) L-Leucyl-, L-phenylalanyl-Transfer RNA (tRNA) Protein Transferase

A single gene, wyb, in the E. coli K_{12} genome has been shown to influence the production of L-serine deaminase and L-leucyl-, L-phenylalanyl-tRNA protein transferase (Tam et al., 1978). This latter enzyme is one of a class of soluble enzymes known as amino-acyl-tRNA protein transferases found in bacterial and mammalian cells which catalyze the transfer of certain amino acids from a tRNA into peptide linkage with a specific NH_2-terminal residue of a protein acceptor (Soffer, 1974). L-Leucyl-, L-phenylalanyl-tRNA protein transferase catalyses the transfer of either L-leucine or L-phenyl-

Figure IV General Reaction for AminoAcyl-tRNA Protein Transferase

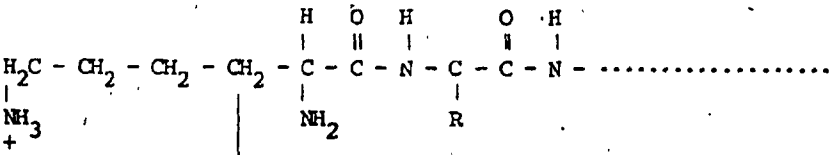
Protein Acceptor + AminoAcyl-tRNA $\xrightarrow[\text{Transferase}]{}$ Protein Acceptor — Amino Acid

SPECIFICALLY: the reaction for L-leucyl-,L-phenylalanyl-tRNA protein transferase



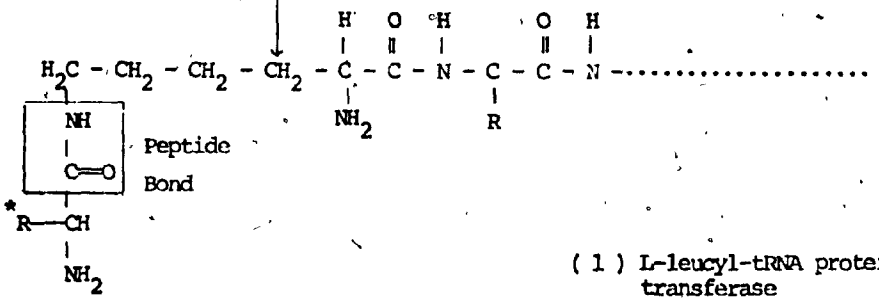
Arginine Protein Acceptor

OR



Lysine Protein Acceptor

(1)



(1) L-leucyl-tRNA protein transferase

*R = group of L-leucine or L-phenylalanine depending on which amino acid the tRNA carries.

alanine to the free basic ε-amino group of arginine or lysine (Leibowitz and Soffer, 1971; Momose and Kaji, 1966) (Figure IV). Since this enzyme modifies an already made protein and since some factor in its regulation also regulates L-serine deaminase (Tam et al., 1978), it then seemed reasonable to suggest that L-serine deaminase may be a substrate for this aminoacyl-tRNA protein transferase. The E. coli K_{12} strain, strain MS845, which was primarily studied in this thesis lacked L-leucyl-, L-phenylalanyl-tRNA protein transferase (Deutch et al. 1977). An attempt was made to determine whether there is any association between the instability and/or increased activity of L-serine deaminase observed in the strain and the lack of this protein transferase enzyme.

(vii) Summary

The historical review of L-serine deaminase from E. coli demonstrates that the enzyme is a unique and very labile molecule whose metabolic role is as yet undefined. The object of this study was to determine some of the factors responsible for this instability. It was hoped that the identification of such factors would aid in its later purification and thus enable studies on its metabolic function to be performed.

MATERIALS AND METHODS

(i) Bacterial Strains

Two strains of E. coli K_{12} were obtained from Dr. R.L. Soffer of Albert Einstein College of Medicine; strain W4977, and strain MS845, Each strain is a proline auxotroph. Strain W4977 is an F^- strain from which strain MS845 lacking in L-leucyl-, L-phenylalanyl-tRNA protein transferase was isolated after nitrosoguanidine treatment (Soffer and Savage, 1974). Strain MS845 expresses L-serine deaminase activity at unusually high levels. All of the strains were maintained on yeast extract-tryptone-agar slants at 4°C.

(ii) Growth Media

The bacterial cells were grown on liquid medium composed of 0.54% (w/v) K_2HPO_4, 1.26% (w/v) KH_2PO_4, 0.2% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.001% (w/v) $CaCl_2$, buffered at pH 6.4. 0.2% (w/v) $(NH_4)_2SO_4$ was omitted when minimal medium was required in the absence of inorganic nitrogen. In all cases, a final concentration of 0.2% (w/v) glucose was added to the growth media providing the energy and carbon source. Other compounds were added depending on the requirements of the particular strain and the conditions called for by the experiment. L-Proline was added to a final concentration of 2 mg/ml. L-Valine, L-leucine, L-isoleucine, and glycine were added to a final concentration of 20 µg/ml.

The bacterial strains were kept on yeast extract-tryptone-agar slants which contained 1% (w/v) yeast extract, 1% (w/v) tryptone, 2% (w/v) agar, 0.25% (w/v) K_2HPO_4, and 0.01% (w/v) glucose.

The cultures were periodically transferred to new slants.

(iii) Growth of Cells

Pre-cultures of bacterial cells from yeast extract-tryptone slants were prepared in 200 ml of minimal medium containing nitrogen with 2 mg/ml of L-proline (and/or other amino acids if required) and with 0.2% (w/v) glucose as the single energy and carbon source in 1000 ml Erlenmeyer flask. The cells were grown overnight at 37°C in a gyrotory water bath shaker manufactured by New Brunswick Scientific Co., N.J., Model G76 at 180 rpm (approximation). The cells were then subcultured to an optical density of 30 Klett Units into four 1000 ml Erlenmeyer flasks, each containing 100 ml of media and allowed to grow until they reach an optical density of 100 Klett Units. The cells were then chilled in ice water and collected by filtration (multi-tube filter, Cat # MT-VFA-19-2000-.27 obtained from Selas Corporation of America, Heat and Fluid Processing. Dresher, Pennsylvania.). They were washed once with minimal medium without nitrogen and centrifuged at 6,000 rpm for 10 minutes at 0°C in a refrigerated centrifuge manufactured by International Equipment Co., Needham Hts., Mass., Model B-20. In this and all subsequent centrifugations, the fixed angle rotor IEC (410) was used. These cells were then used to make a crude cell extract.

For ammonium sulfate precipitation experiments, a pre-culture of strain MS845 was used to inoculate a large volume closed flask (obtained as a present from Dr. R. Verschingel) containing 4 liters or more of minimal medium with nitrogen, 2 mg/ml of L-proline and 0.2% (w/v) glucose. This flask was allowed to sit in a 37°C non-shaking

water bath until the growth reached an optical density of 100 or more Klett Units. In order to facilitate adequate aeration, one sparger sat in the growth media and was attached to a compressed air outlet. Another outlet provided for the release of air from the flask. The cells were then collected by filtration, washed once with minimal medium without nitrogen, and the pellet was used to make a crude cell extract.

(iv) Measurement of Bacterial Growth

Bacterial cell growth was assessed by using filter # 42, whose spectral range is between 400 to 460 mμ in a Klett-Summerson colorimeter, produced by Klett Mfg. Co. Inc., New York, Model 800-3.

(v) Preparation of Crude Cell Extract

Cells grown in liquid medium were collected, washed twice with minimal medium without nitrogen and centrifuged at 6,000 rpm for 10 minutes at 0°C. The pellet was resuspended to approximately 20% w/v in 0.05M potassium phosphate buffer (pH 7.2). The cells were broken by sonciation for one minute with a 100 Watt ultrasonic disintegrator manufactured by MSE Ltd. London S.W.I. at top power. The homogenate was then centrifuged at 10,000 rpm for 20 minutes at 0°C and the supernatant was used for the enzyme assay.

(vi) Preparation of Whole Cell Suspension

Cells grown in liquid medium were collected, washed twice with minimal medium without nitrogen and centrifuged at 6,000 rpm for 10 minutes at 0°C. The pellet was resuspended to 100 Klett Units using a

Klett-Summerson colorimeter with # 54 filter (spectral range 525 to 580 mμ) in Tris buffer pH 8.5, 0.05M. This cell suspension was used in the enzyme assay.

(vii) L-Serine Deaminase Assay

The L-serine deaminase assay measures the amount of α-keto acid produced from L-serine by the enzyme, L-serine deaminase (L-SD). It was assumed that the major α-keto acid derived from L-serine is pyruvate. This assay is a modification of the method employed by Pardee and Prestidge (1955). The method varies from the original in that cell extract was used in place of toluene-treated whole cells. The incubation mixture contained 0.1 ml of 0.05M Tris buffer pH 8.5, 0.1 ml of L-serine (20 mg/ml) and 0.05 ml of crude cell extract. This mixture was incubated in a 37°C water bath for 10 minutes followed by the addition of 0.9 ml of 0.025% 2,4-dinitrophenylhydrazine (DNPH) in 4.1% (v/v) hydrochloric acid. The color was then allowed to develop for 20 minutes at room temperature followed by the addition of 1.7 ml of 10% (w/v) NaOH. The amount of pyruvate produced was measured on a Klett-Summerson colorimeter with # 54 filter (spectral range between 525 and 580 mμ). Pyruvate was used as the standard. In addition, blanks were prepared which contained no substrate (L-serine). It was replaced by 0.1 ml of distilled water. DNPH was then added immediately (at zero time). This allowed the determination of the amount of pyruvate present in the cell extract prior to the assay. The second set of blanks contained L-serine. DNPH was added immediately (at zero time). This allowed the determination of the amount of pyruvate produced in the time needed to add DNPH. The third set of blanks

contained no substrate (L-serine). It was replaced by 0.1 ml of distilled water. DNPH was then added after the 10 minute incubation period. This allowed the determination of the amount of pyruvate produced within 10 minutes by the extract in the absence of addition of exogenous substrate.

Toluene-treated whole cells were examined for L-SD activity according to the method previously described by Isenberg and Newman (1974).

(viii) Toluene Extraction of Pyruvate Dinitrophenylhydrazone

Since pyridoxal phosphate reacts with DNPH, toluene extraction of the DNPH derivative of pyruvate was performed (Friedemann and Haugen, 1943). The incubation mix containing 0.1 ml of 0.05M Tris buffer pH 8.5, 0.1 ml of L-serine (20 mg/ml), 0.1 ml of pyridoxal phosphate (100 µg/ml) and 0.05 ml of crude extract was incubated at 37°C for 10 minutes followed by the addition of 0.9 ml of DNPH in 4.1% (v/v) HCl. The color was allowed to develop for 20 minutes at room temperature. A 5 ml aliquot of toluene was then added. The sample was vortexed well. Four ml of the toluene phase were removed and added to another tube containing 3 ml of 10% (w/v) sodium carbonate. Again, the sample was vortexed well. Two ml of the sodium carbonate layer were removed and added to a tube containing 0.5 ml of 20% (w/v) KOH. The sample was read on the # 54 filter using a Klett-Summerson colorimeter. A pyruvate standard and blanks having no substrate, with distilled water in its place, both containing 0.1 ml pyridoxal phosphate (100 µg/ml), were preapred in the same manner.

(ix) Protein Concentration

Protein was determined by the method of Lowry et al. (1951), with crystalline serum albumin or trypsin as a standard. When crystalline serum albumin was used as standard, it gave at most, a 15 Klett Unit increase (i.e. 20 µg/ml of protein) in the readings as compared to when trypsin was used. The protein concentration of concentrated cell extracts was 16.0 $\pm$ 2.0 mg/ml. Concentrated cell extracts which had been diluted 1:20 contained 0.8 $\pm$ 0.2 mg/ml of protein and concentrated cell extracts which had been diluted 1:10 continued 1.6 $\pm$ 0.3 mg/ml of protein.

(x) Ammonium Sulfate Preciptation

Cells were grown overnight in a 37°C non-shaking water bath (as described previously) in a large volume flask containing 4 liters of minimal medium with nitrogen, 2 mg/ml of L-proline and 0.2% (w/v) glucose. The cells were then cooled in ice water and collected by filtration and centrifugation (6,000 rpm for 10 minutes at 0°C). They were washed once with cold minimal medium without nitrogen. The cells were resuspended to 20% w/v in cold 0.05M phosphate buffer pH 7.2. They were then disrupted by sonciation and the crude cell extract was subjected to treatment with 1% (w/v) cold protamine sulfate (5 ml/gram of protein). This sample was centrifuged for 20 minutes at 10,000 rpm at 0°C and the supernatant was collected. Solid ammonium sulfate was added to a 20 ml cell extract contained in a 50 ml prechilled beaker surrounded by ice. The sample was brought to the desired ammonium sulfate saturation by the gradual addition of solid ammonium sulfate. It was stirred slowly with a magnetic stirrer for 20 minutes, and then

the suspension was transferred to a prechilled 50 ml centrifuge tube and centrifuged for 10 minutes at 10,000 rpm. After centrifugation, the supernatant was decanted into a 50 ml beaker and the pellet was resuspended in 0.05M potassium phosphate buffer pH 7.2. The supernatant solution from the foregoing centrifugation was further saturated with the desired amount of solid ammonium sulfate and allowed to stir for 20 minutes on ice. The suspension was again centrifuged at 10,000 rpm for 10 minutes at 0°C and the supernatant was decanted. The pellet was resuspended in cold 0.05M potassium phosphate buffer pH 7.2. This procedure was continued for as many times as the ammonium sulfate saturations were desired.

(xi) Chemicals

Chemicals of particular interest which were used in this work and their origin are listed below:

crude DNA (calf thymus); Aldrich Chemical Company.

ethylene glycol; Canadian Aniline and Extract Co., Ltd..

glycerol; Canlab.

ethylenediaminetetraacetic acid (EDTA) (disodium salt, standard); Fisher Chemicals.

crude RNA (pine), arginine rich histones; Nutritional Biochemicals Corporation (NBC).

ammonium sulfate (ultrapure); Schartz/Mann.

bovine serum albumin (BSA) (crystalline, lyophilized), dithiothreitol, (2,3-dihydroxy 1,4-dithiolbutane), dithioerythritol, soybean trypsin inhibitor (type 1-S), aprotinin from bovine lung containing 10^4 KIU/ml (KIU = KALLIKREIN inactivator units), protamine sulfate (grade II from

salmon], phenylmethylsulfonylfluoride; Sigma Chemical Company. All other chemicals were purchased from Sigma or Fisher Chemical Co.

(xii) Definitions and Abbreviations

The abbreviations used during this study are the same as those previously described by Isenberg (1974) and Isenberg and Newman (1974). MM + N was used by these authors to refer to minimal medium containing inorganic nitrogen. While, MM - N was used to refer to minimal medium in the absence of inorganic nitrogen (ammonium sulfate), +N and -N, respectively, were the shortened versions of the above abbreviations. Therefore, in this study +Np will mean minimal medium with inorganic nitrogen and with L-proline.

Enzyme activity (E.A.) is expressed as μmoles of pyruvate produced/ 10 minutes/ ml of cell extract or μmoles of pyruvate produced/ 35 minutes/ ml of toluene-treated whole cells. Specific activity (S.A.) is expressed as μmoles of pyruvate produced/ 10 minutes/ mg protein. Appropriate blanks were subtracted each time.

(xiii) Other Considerations

The initial S.A. of prepared crude cell extracts varied (example Table I). This difference depended on the time that elapsed between the collecting of cells and the assaying of the crude cell extract for L-SD activity. L-SD was very unstable. The initial loss of activity was rapid even when whole cell pellets were allowed to sit in ice water for some period of time. However, each experiment was performed several times in the same manner and the initial activities then obtained were well comparable. The decay curves, as far as it was

possible to determine, indicated the presence of only one population of enzyme. The behaviour of the cells collected and assayed for L-SD activity within the shortest possible time showed no difference in behaviour to those cells collected a short time thereafter, except that the inital S.A. varied.

There was also a variation in the temperature at which the experiments were performed. Since the enzyme was very unstable, it was decided to expose L-SD in the presence and absence of various compounds to room temperature for x minutes rather than to 37°C (ref. to Figure IX). The room temperature in the laboratory fluctuated between 20°C and 27°C. When the experiment was designed, it was not realized that there would be such a large variation in temperature.

RESULTS

DEVELOPMENT OF AN ASSAY FOR L-SD ACTIVITY IN EXTRACTS OF *E. COLI* K_{12}

The enzyme assay developed in this study is based on the method employed by Pardee and Prestidge (1955). The method varies from the original primarily in that cell extract was used in place of toluene-treated whole cells.

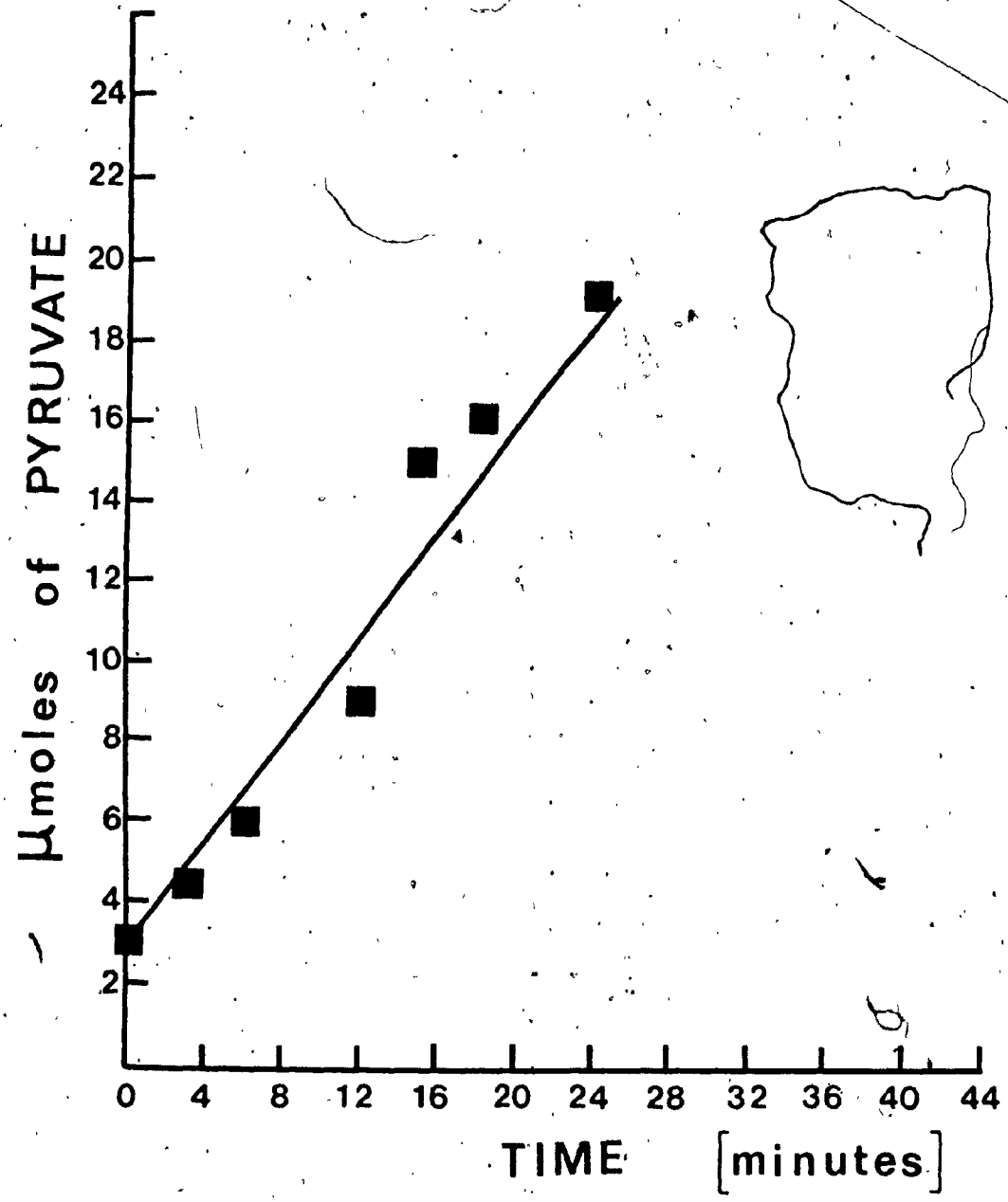
L-Serine deaminase (L-SD) of animal origin is known to be a pyridoxal phosphate dependent enzyme (Goldstein *et al.*, 1962; Sayre and Greenberg, 1956). However, the cofactor requirement of L-SD in *E. coli* is unclear. The enzyme from extracts of *E. coli* K_{12} is rountinely assayed without the addition of pyridoxal phosphate (Section II, part (vi)).

To show that the enyzme assay used in this study is valid the following conditions were examined: (i) the linearity of enzyme activity with respect to time; (ii) the linearity of enzyme activity with respect to enzyme concentration, (iii) the formation of product (α Keto acid) as a function of substrate concentration and (iv) the disappearance of pyruvate with respect to time on incubation with crude cell extract. The determination of these conditions (Section I, part (i), (ii), and (iii)) is analogous to that used in this laboratory for the determination of the validity of the L-SD assay when toluene-treated whole cells were used (Isenberg, 1974).

For any enzyme assay, the amount of product formed increases linearly with time. At later times the substrate becomes exhausted, and the reaction rate declines. The selection of a time in the

Figure V L-SD Assay: Linearity of Enzyme Activity with Respect to Time

Aliquots of crude cell extract of E. coli K_{12}, strain MS845, were incubated with L-serine for times varying from 0 to 24 minutes at 37°C. The amount of pyruvate produced at each time was determined. The assay was performed at an L-serine concentration of 0.076M. The ordinate is expressed as μmoles of pyruvate/ml of cell extract.


μmoles of PYRUVATE
24
22
20
18
16
14
12
10
8
6
4
2
0
4
8
12
16
20
24
28
32
36
40
44
TIME [minutes]

linear portion of the time course of an enzymatic reaction then ensures that the amount of substrate available will be non-limiting for the enzyme. This also prevents end-product inhibition and inactivation of the enzyme (Dixon and Webb, 1964; Isenberg, 1974). Furthermore, the initial rate of an enzymatically catalyzed reaction is directly proportional to the number of enzyme molecules present at substrate saturation. Thus, by increasing the enzyme concentration, the rate of the enzyme reaction is also increased (Dixon and Webb, 1964; Isenberg, 1974). However, until the saturation level is approached, the rate of the enzyme reaction should be a function of the amount of substrate present (Dixon and Webb, 1964; Isenberg, 1974).

Section I

VALIDITY OF ASSAY FOR L-SD ACTIVITY IN EXTRACTS OF E. COLI K_{12}

(i) Linearity of Enzyme Activity with Respect to Time

To determine whether the reaction is linear with time and what length of time is most appropriate for use in the enzyme assay with crude cell extract, duplicate assay tubes were incubated for 0, 3, 6, 12, 15, 18, and 24 minutes at 37°C and the amount of pyruvate produced each time was determined in μmoles of pyruvate/ml of cell extract. The results are given in Figure V. As illustrated, the reaction is linear for 24 minutes. The time chosen for the L-serine deaminase reaction was 10 minutes, where the amount of pyruvate produced as a function of time was linear.

(ii) Linearity of Enzyme Activity with Respect to Enzyme Concentration

In order to test the linearity of enzyme activity with respect to enzyme concentration for this assay, various amounts of crude cell

Figure VI L-SD Assay: Linearity of Enzyme Activity with Respect to Enzyme Concentration

Varying amounts of crude cell extract of E. coli K_{12}, strain MS845, were incubated for 10 minutes with 0.076M L-serine . The amount of pyruvate produced at each enzyme concentration was determined.

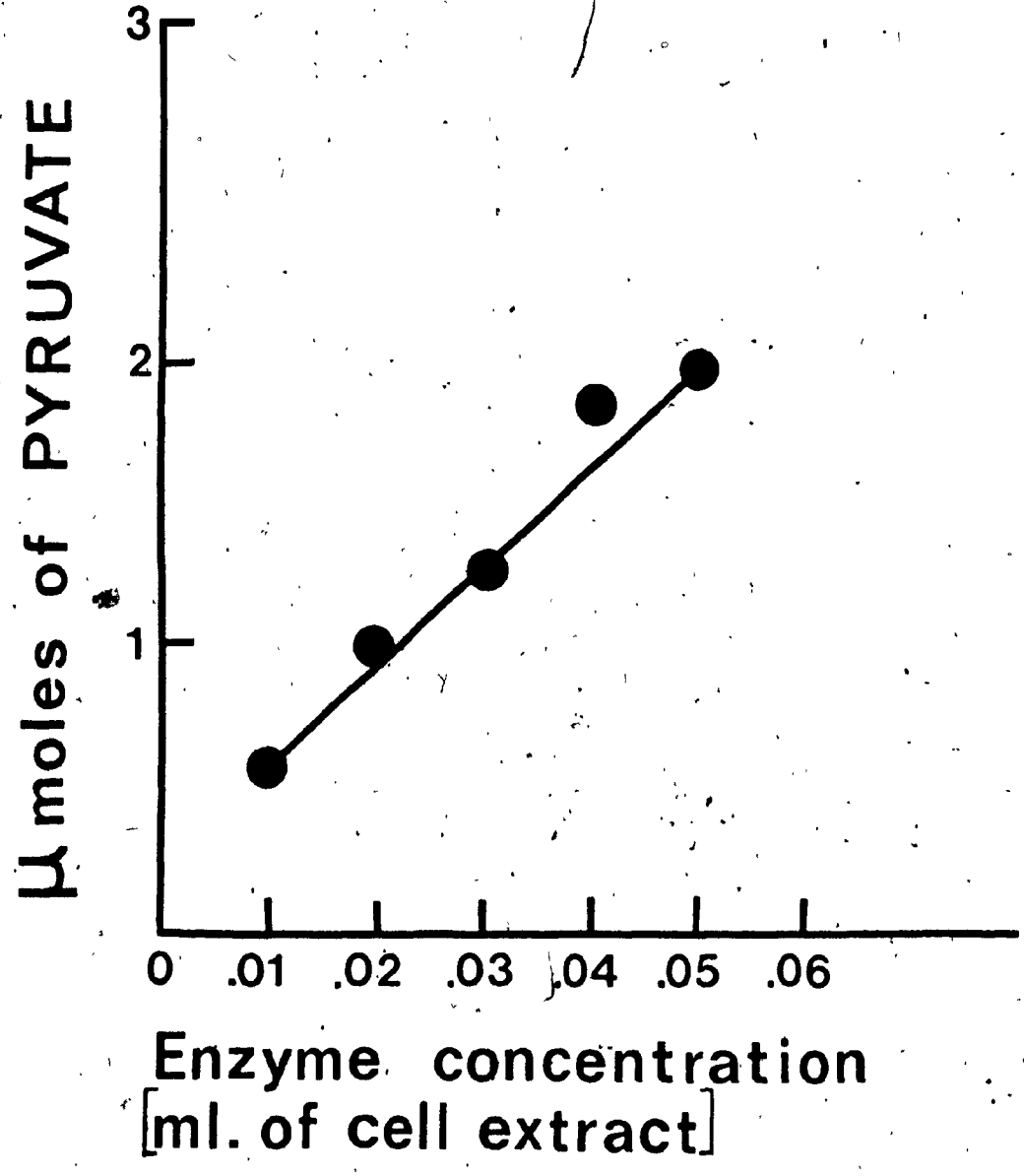
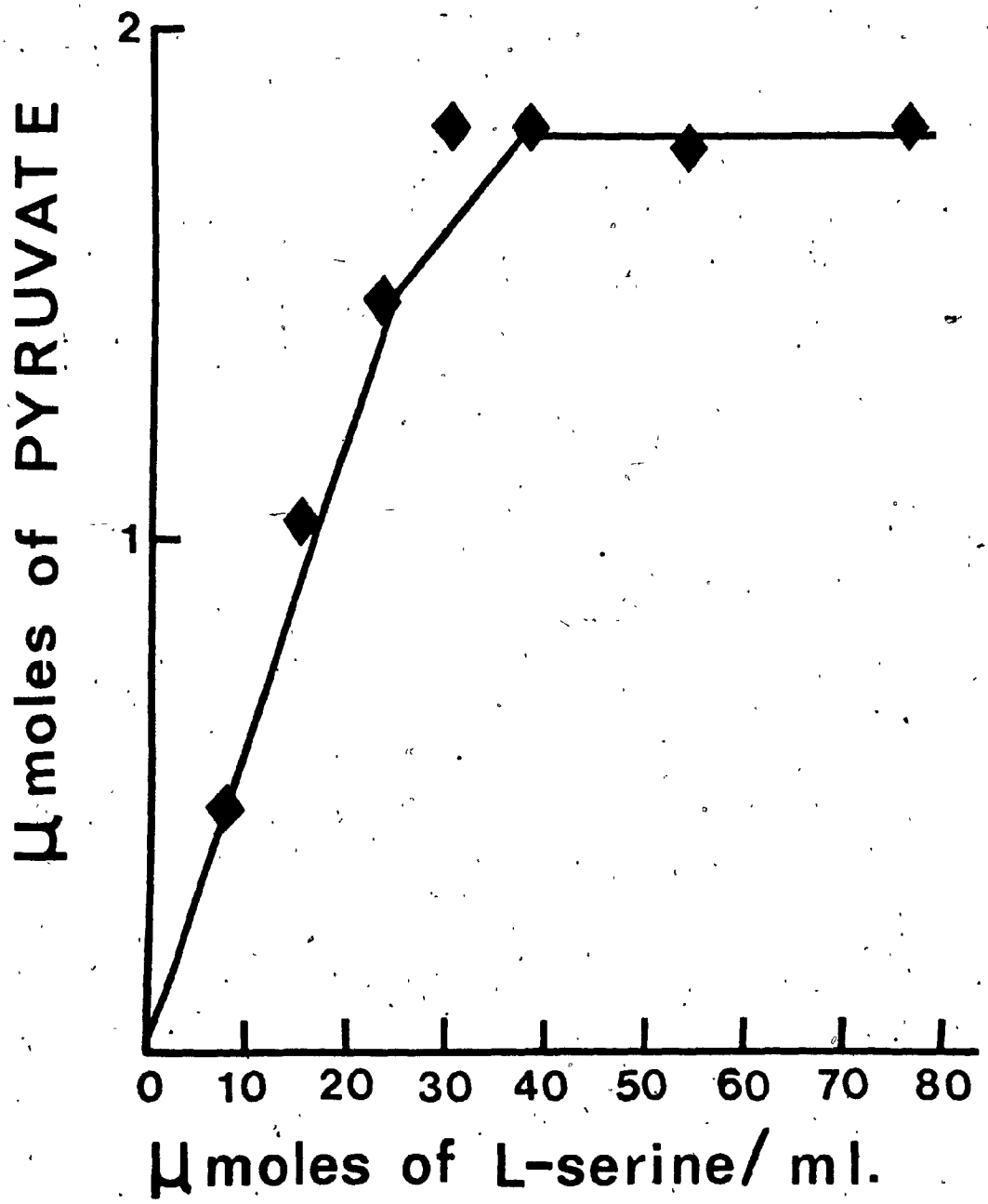

μ moles of PYRUVATE
3
2
1
0
.01
.02
.03
.04
.05
.06
Enzyme concentration
[ml. of cell extract]

Figure VII L-SD Assay: Formation of Product as a Function of Substrate Concentration

Duplicate samples containing curde cell extract of E. coli K_{12}, strain MS845, were incubated with 8, 15, 23, 30, 38, 54, and 76 µmoles of L-serine/ ml. The amount of pyruvate (µmoles) formed in each assay tube after 10 minutes was determined.


2
1
μ moles of PYRUVATE
0 10 20 30 40 50 60 70 80
μ moles of L-serine/ ml.

extract were incubated for 10 minutes at 37°C and the amount of pyruvate produced was determined. As Figure VI indicates, the amount of product formed is directly proportional to the number of enzyme molecules present. The initial velocity of the enzymatic reaction is then proportional to the amount of enzyme added.

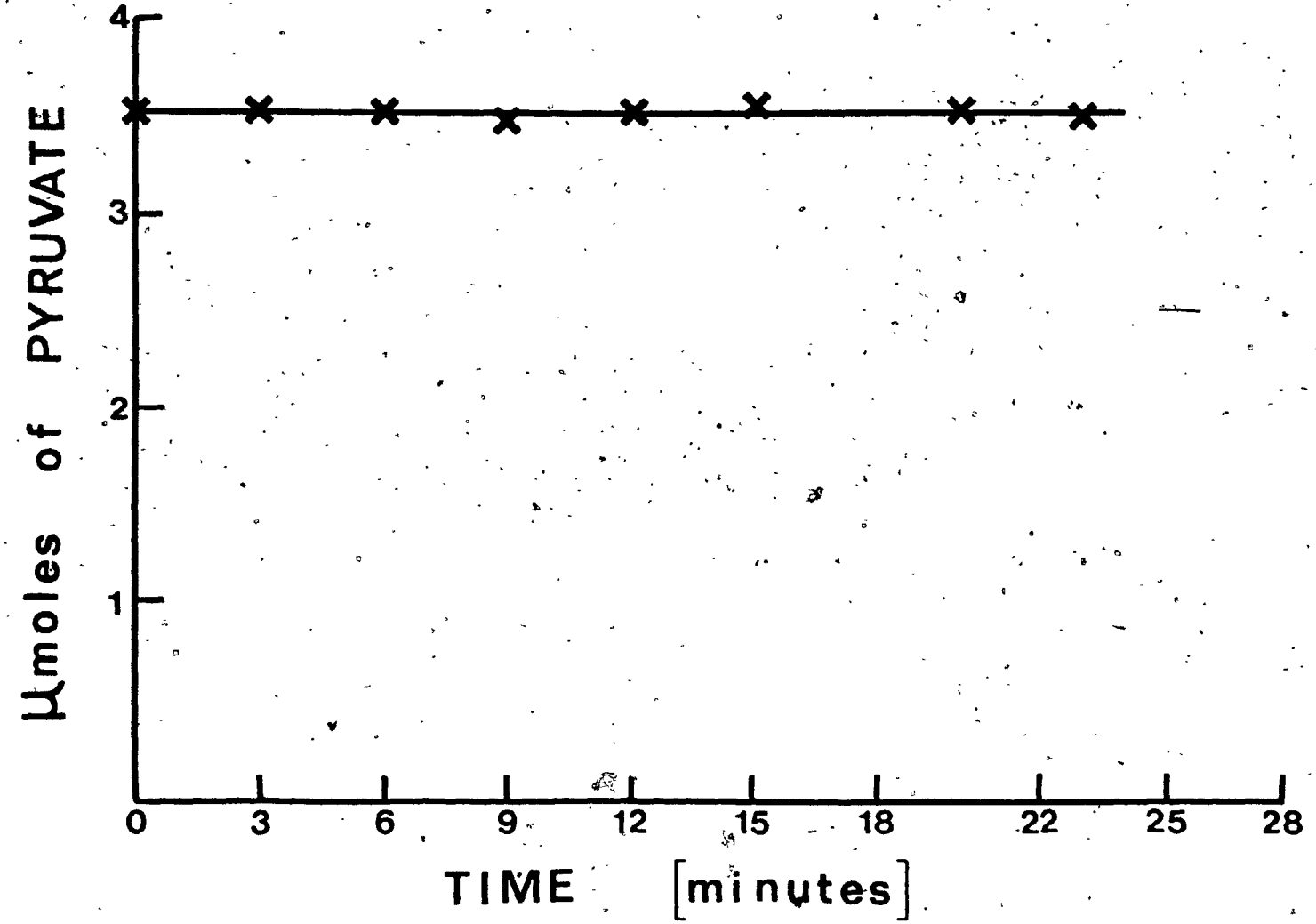
It should be stressed that comparison of enzyme activity from different experiments is difficult because of the instability of the enzyme activity in crude extracts. It was impossible to control to any degree of accuracy the amount of activity produced by any given extract under normal experimental conditions though the protein concentration varied no more than by 2 mg/ml for concentrated crude extract preparations.

(iii) Formation of Product as a Function of Substrate Concentration

The formation of product as a function of substrate concentration was examined for the L-serine deaminase reaction used during this study by incubating samples containing crude cell extract with varying concentrations of L-serine (i.e. 8, 15, 23, 30, 38, 54, and 76 μ moles of L-serine/ml). The amount of pyruvate produced at each substrate concentration after 10 minutes was determined. As presented in Figure VII, the formation of product as a function of substrate concentration increases until a concentration of 30 μ moles of L-serine/ml is reached. The concentration used throughout the experiments, 76 μ moles of L-serine/ml, was apparently saturating. In contrast, other investigators (Alfoldi et al., 1968; Alfoldi and Rasko, 1970; Rasko and Molnar, 1971) in their studies on L-SD from E. coli added L-serine at a concentration of 44 μmoles/ml to the reaction mixture containing 0.1 ml of their crude cell extract.

Figure VIII L-SD Assay: Disappearance of Pyruvate with Respect to Time on Incubation with Crude Cell Extract

Crude cell extract of *E. coli* K_{12}, strain MS845, was incubated with 20 µg of pyruvate / 0.05 ml of cell extract in the absence of L-serine for times varying from 0 to 23 minutes at 37°C. The amount of pyruvate remaining at each time was determined. The ordinate is expressed as µmoles of pyruvate / ml of cell extract.


μmoles of PYRUVATE
4
3
2
1
0
3
6
9
12
15
18
22
25
28
TIME [minutes]

(iv) The Disappearance of Pyruvate with Respect to Time on Incubation with Crude Cell Extract

It has been suggested that since a single point assay was being used and a very crude cell extract that the end-product, pyruvate, may be consumed by some component in the cell extract. To ensure that at least relatively large and measureable amounts of pyruvate were not disappearing, duplicate assay tubes were prepared containing 20 µg of pyruvate/0.05 ml of cell extract in the absence of L-serine. They were incubated for 0, 3, 6, 9, 12, 15, and 23 minutes at 37°C and the amount of pyruvate remaining was measured in µ moles of pyruvate/ml of cell extract. Figure VIII, clearly indicates that there was no measurable decrease in the amount of pyruvate initially present.

In summary, the enzyme assay employed in this study is valid. Experiments were performed that showed the assay to be linear with respect to enzyme concentration and time. The enzymatic reaction was carried out at apparently saturating concentration of substrate and the end-product (pyruvate) of the reaction was shown to be stable.

STATEMENT OF PROBLEM AND EXPLANATION OF EXPERIMENTAL PLAN

Preliminary experiments showed L-serine deaminase (L-SD) to be very unstable in extracts of E. coli K_{12}. The experiments in this thesis are intended to elucidate physiological and physical factors which may modify the extent of this instability. It is hoped that identification of such factors may aid in the purification of the enzyme so that studies on its metabolic function could be performed.

As discussed in 'Method' the standard assay for L-SD involves the incubation of an extract with L-serine for a convenient period of time (10 minutes) and a determination of the amount of product

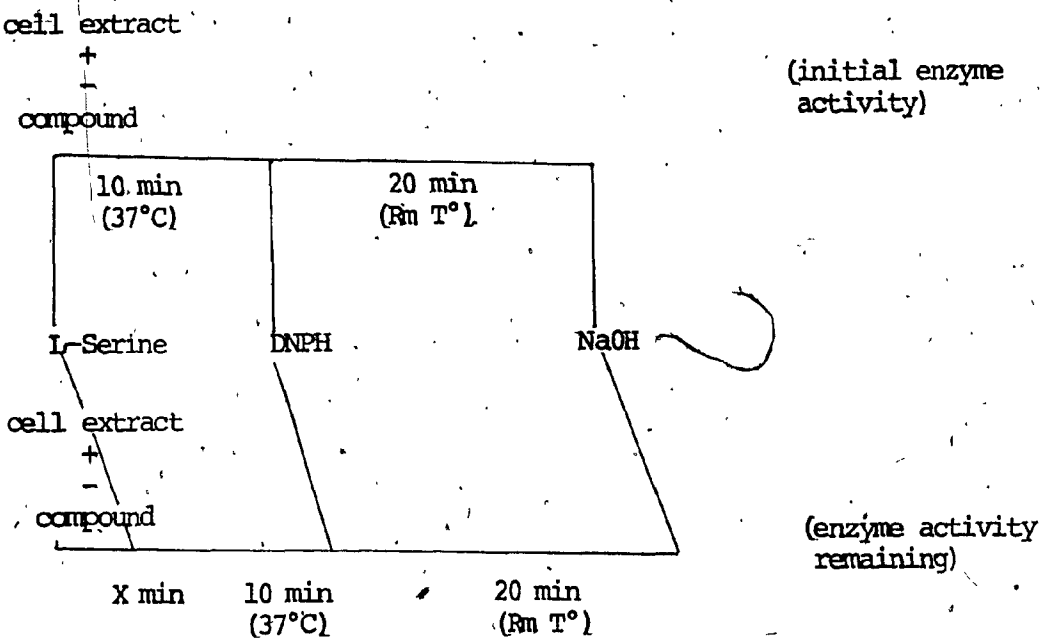
(pyruvate) formed. This gives a measure of the amount of L-SD activity present in any given extract and is referred to as 'initial enzyme activity'.

The purpose of the experiments described herein is to study the stability of L-SD activity in extracts. This involves incubating the extract WITHOUT substrate under various test conditions, and then adding L-serine, incubating the sample for 10 minutes and determining the amount of pyruvate formed. This measures the L-SD activity still present after the test incubation, and is referred to as 'enzyme activity remaining'.

A typical experiment then is carried out in the following manner. An extract is prepared from log phase cells at 20% w/v in cold phosphate buffer pH 7.2, 0.05M, and kept in crushed ice. This 'concentrated' extract is diluted to a convenient concentration (1:10 or 1:20) in the same buffer just prior to assay. A 0.05 ml aliquot of this 'diluted' extract is added to 0.2 ml of Tris buffer pH 8.5, 0.025M, in cold 13 x 100 tubes. L-Serine (1 ml) is added to one set of tubes to give a final concentration of 0.076M. This allows determination of the initial enzyme activity. An equivalent volume of distilled water (0.1 ml) is added to the other set of tubes which are then incubated under whatever experimental conditions are desired, after which 0.1 ml of L-serine (0.19M) is added to a final concentration of 0.054M. This final concentration is still saturating and the pH of the preparation is unaltered. One can then determine the enzyme activity remaining, which can be expressed as a percentage of the initial enzyme activity. The experimental plan is represented schematically in Figure IX.

In many experiment, compounds are added to the extract to try

Figure IX Experimental Plan



to stabilize the enzyme. In this case, the same compound is added to both sets of tubes. This shows whether the compound affects the initial enzyme activity or the enzyme activity remaining, or both, or neither.

Section II

FACTORS AFFECTING L-SD STABILITY IN EXTRACTS OF E. COLI K_{12}, STRAIN MS845

(i) Substrate and Protein Concentration

To determine the effect of substrate on the stability of L-SD, an extract of strain MS845 was diluted 1:20 and incubated at 22°C and 37°C. The amount of enzyme activity remaining was determined at various times between 0 and 30 minutes. As can be seen in Figure X most of the enzyme activity is lost within the first 4 minutes at both temperatures. The 5% remaining at 22°C is retained for some time

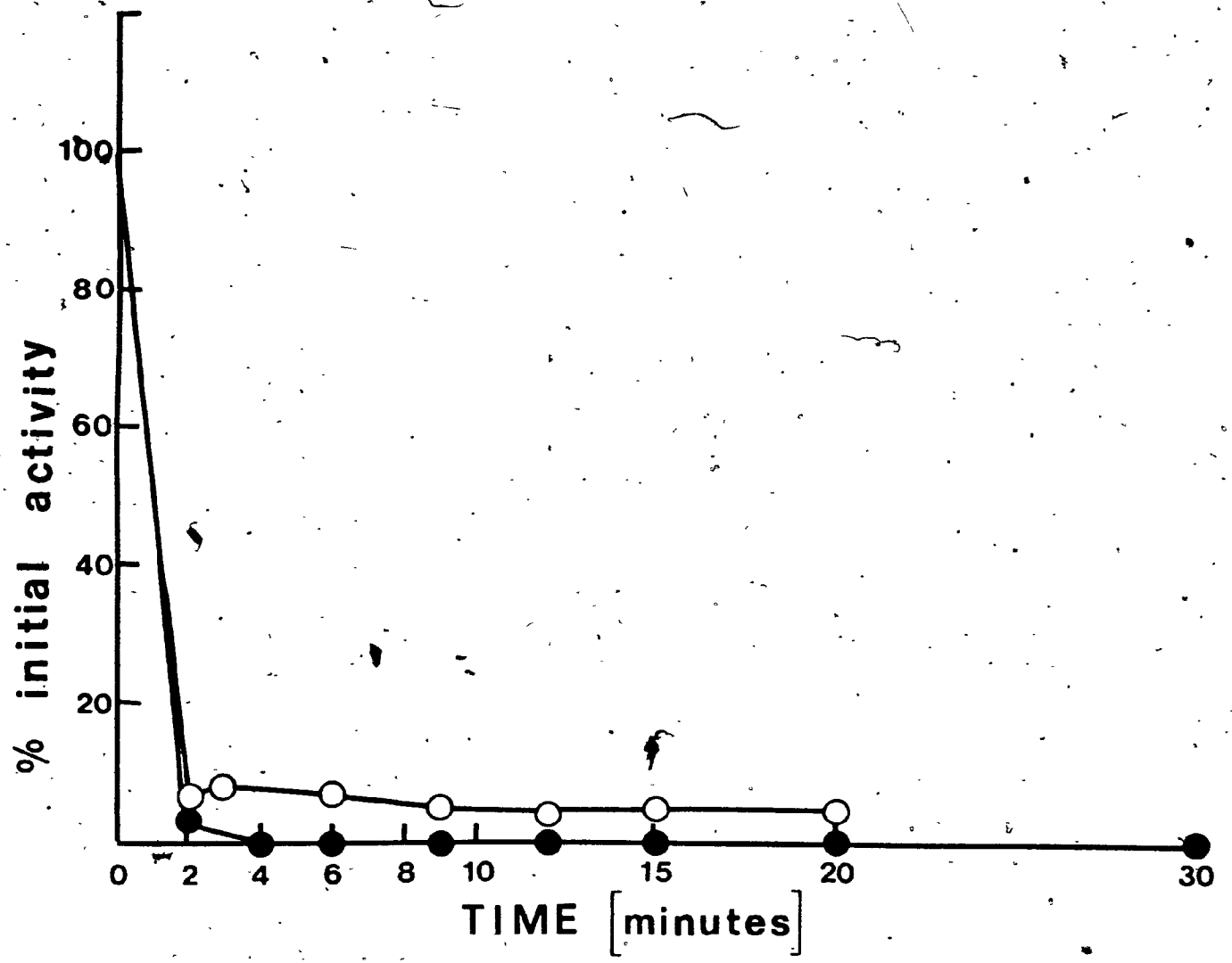
Figure X Effect of Incubation at 22°C and 37°C on L-SD Activity

Cell extracts of *E. coli* K_{12}, strain MS845, were prepared in the usual manner and diluted 1:20. These extracts were incubated for times varying from 0 to 30 minutes at 22°C and 37°C. The amount of pyruvate formed in 10 minutes following the addition of L-serine (0.076M final concentration) was determined.

$$\% \text{ initial activity} = \frac{\text{S.A. (x min)}}{\text{S.A. (0 min)}} \times 100$$

○—○ Decay of L-SD activity at 22°C

●—● Decay of L-SD activity at 37°C


% initial activity
100
80
60
40
20
0
2
4
6
8
10
15
20
30
TIME [minutes]

(20 minutes), thereafter. It can, therefore, be concluded that the enzyme is exceedingly unstable in the absence of its substrate at various temperatures. Since the reaction with L-serine at 37°C is linear for 24 minutes (see 'Method') and without L-serine the enzyme activity is lost in 4 minutes, the substrate is clearly a potent protector of L-SD activity.

The effect of protein concentration on the stability of L-SD was also examined. Figure XI compares the stability of L-SD at two different protein concentrations both incubated at 22°C. When an extract was diluted 1:20, almost all (93%) of the enzyme activity is lost in the first 4 minutes. An extract which was diluted 1:10, on the other hand, loses about 30% of its activity. It is, therefore, clear that both substrate and protein concentration affect the stability of L-SD in strain MS845.

Most enzyme purifications involve manipulation of concentrated cell extracts at low temperature. In order to determine the stability of these cell extracts at low temperature, concentrated cell extracts from strain MS845 were incubated in ice water for 0 hours, 2 hours, and 4 hours. The amount of L-SD activity present was determined by using a 1:20 dilution of the concentrated cell extract. After 2 hours, 72% (Figure XII) of the initial enzyme activity remained, and after 4 hours 40% of the initial enzyme activity remained. It is evident, therefore, that concentrated cell extracts can be maintained for considerable periods at 0°C, but even under these conditions the enzyme is unstable.

(ii) Various Amino Acids

The ability of various compounds to affect L-SD activity was

Figure XI Effect of Protein Concentration on L-SD Activity

Cell extracts of *E. coli* K_{12}, strain MS845, were prepared in the usual manner. The extracts were diluted 1:10 and 1:20. They were then incubated without L-serine for times varying from 0 to 30 minutes at 22°C. L-Serine (0.076M final concentration) was added and the amount of pyruvate produced in 10 minutes after the addition of L-serine was determined.

Decay of L-SD activity with a 1:10 dilution

Decay of L-SD activity with a 1:20 dilution

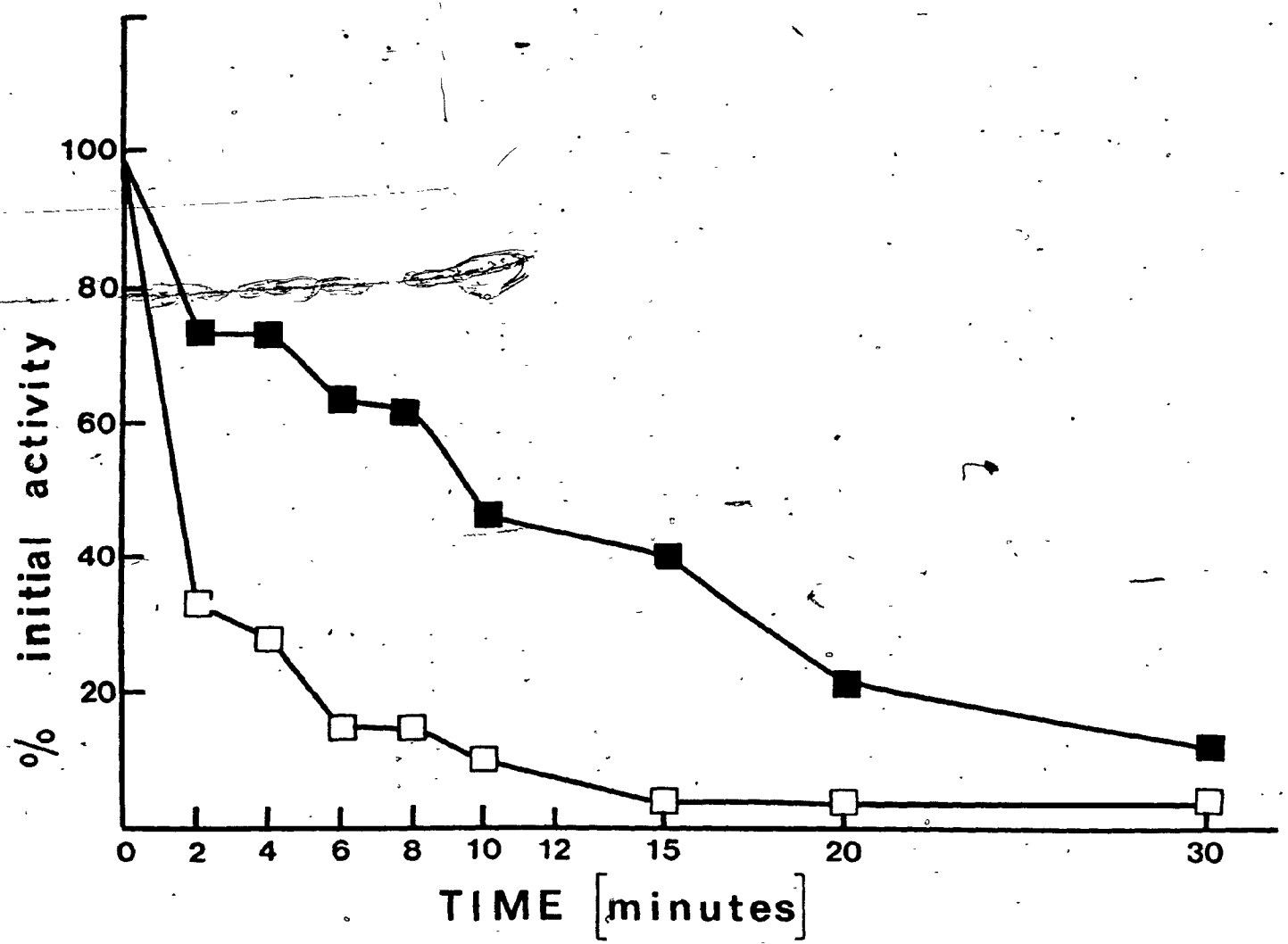
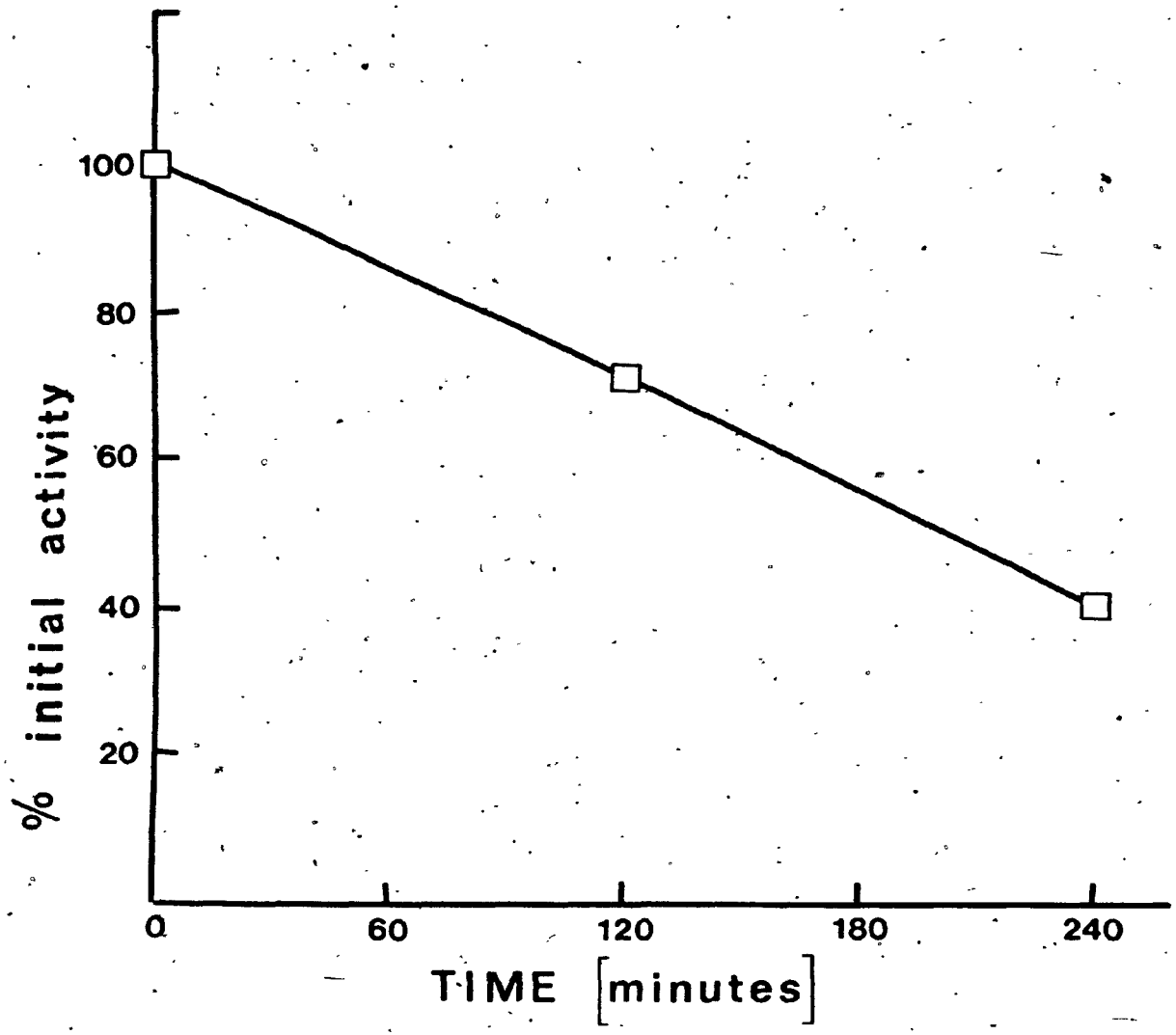

100
80
60
40
20
% initial activity
0
2
4
6
8
10
12
15
20
30
TIME [minutes]

Figure XII Effect of Incubation at 0°C on Concentrated Cell Extracts

Cell extracts of *E. coli* K_{12}, strain MS845, were prepared in the usual manner. These concentrated cell extracts were incubated in ice water for 0, 2, and 4 hours. The extracts were diluted 1:20 just prior to the assay and the L-SD assay was carried out immediately.


100
80
60
40
20
% initial activity
0
60
120
180
240
TIME [minutes]

tested by incubating diluted cell extracts with and without the compound for 3 minutes at 37°C followed by the addition of substrate. The amount of L-SD activity remaining was determined. For comparison, the L-SD assay was performed, prior to incubation, on samples containing substrate and diluted cell extract with or without the compound. The activity remaining after a 3 minute incubation with and without the compound is compared to the activity seen on assaying with the compound prior to incubation and is expressed as % protection (Table I).

Of the 23 compounds tested, several allowed the retention of 7 to 24% of the enzyme activity. These include D-serine (18%), allothreonine (8%), L-alanine (8%), L-isoleucine (11%), L-threonine (11%), L-leucine (7%), L-valine (15%), L-proline (19%), L-glutamine (14%), L-methionine (12%), L-phenylalanine (8%), L-tryptophan (8%), L-histidine (24%), L-asparagine (22%), L-tyrosine (15%), L-aspartic acid (22%), L-glutamic acid (13%), and L-lysine (7%). The extent of protection is not strictly comparable since some compounds were tested with cell extracts diluted 1:10 prior to assay and some were tested with cell extracts diluted 1:20 (as indicated in Table I). Preliminary experiments did not indicate any significant difference in the pattern of protection observed with 1:10 and 1:20 dilutions. Glycine, sodium acetate, L-arginine, L-cysteine, and DL-serine methylester failed to protect the enzyme.

The reaction with L-serine at 37°C is linear for 24 minutes. Without substrate the enzyme activity is lost in 4 minutes. This indicates that L-serine is a very effective protector of the enzyme. That D-serine protects then may not be surprising, though many enzymes

Table I Effects of Various Amino Acids of L-SD Activity

Compound Added	Conc. in Assay (X 10^{-3}M)	Initial S.A. of Extract	S.A. after 3 min	% Protection
0	-	5.01	0.00	-
D-serine	54	-	0.87	18 **
allothreonine	48	-	0.42	8 **
L-alanine	64	-	0.37	8 **
sodium acetate	70	-	0.00	0 **
glycine	76	-	0.00	0 **
0	-	2.89	0.00	- *
L-isoleucine	12	3.08	0.35	11 *
L-threonine	31	2.80	0.31	11 *
L-leucine	12	3.03	0.22	7 *
L-ileu/L-thr	12/31	2.81	0.26	9 *
L-leu/L-thr	12/31	3.05	0.29	9 *
L-leu/L-ileu	12/12	3.15	0.00	0 *
L-leu/L-ileu/L-thr	12/12/31	3.12	0.04	1 *
0	-	2.14	0.00	-
L-valine	45	2.11	0.32	15
L-arginine	33	1.58	0.00	0
L-proline	50	2.13	0.40	19
L-glutamine	39	2.14	0.30	14
L-cysteine	47	0.03	0.00	0
L-methionine	38	2.11	0.25	12
DL-serine methylester	48	2.08	0.00	0

Compound Added	Conc. in Assay (X 10^{-3}M)	Initial S.A. of Extract	S.A. after 3 min	% Protection
0	-	2.46	0.00	-
L-phenylalanine	17	2.44	0.20	8
L-tryptophan	0.8	2.39	0.20	8
L-histidine	14	2.53	0.61	24
L-asparagine	3.8	2.55	0.56	22
L-tyrosine	0.7	2.61	0.38	15
L-aspartic acid	26	2.64	0.57	22
L-glutamic acid	24	2.59	0.34	13
L-lysine	20	2.40	0.20	7

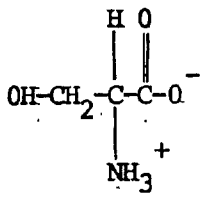
0.05 ml of diluted cell extract from E. coli K_{12}, strain MS845, was incubated for 3 minutes at 37°C in the presence of the compound to be studied. This involved the addition of 0.05 ml of diluted enzyme to 0.3ml of Tris buffer pH 8.5, 0.014M rather than to 0.2 ml of Tris buffer pH 8.5, 0.025M as in the previous experiments. L-Serine was added after a 3 minute incubation in the presence of compound and the amount of pyruvate formed was determined.

$$\% \text{ protection} = \frac{\text{S.A. (3 min) + comp} - \text{S.A. (3 min) - comp}}{\text{S.A. (0 min) + comp}} \times 100$$

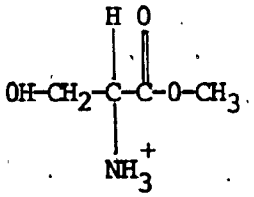
* Cell extracts diluted 1:10, rest diluted 1:20 prior to assay

** $\left[\text{S.A. (0 min) + comp}\right]$ was taken as being 5.01

do discriminate very strongly among stereoisomers. Apparently, almost any amino acids (allothreonine an isomer of L-threonine, L-alanine, L-isoleucine, L-threonine, L-leucine, L-valine, L-glutamine, L-methionine, L-phenylalanine, L-tryptophan, L-histidine, L-asparagine, L-tyrosine, L-aspartic acid, L-glutamic acid, L-proline and L-lysine) can interact with the enzyme. DL-Serine methylester fails to protect the enzyme. It differs from serine only in that it has a methyl group attached at the carboxyl end (see figure below). This suggests that the carboxyl group may be involved in binding L-serine to the active site.



serine



serine methylester

That sodium acetate and glycine fail to protect the enzyme is not surprising since they lack the β-carbon, one of the carbons on which the enzyme probably acts. L-Arginine and L-cysteine also fail to protect the enzyme. Since L-arginine inhibits the initial enzyme activity by 26% and L-cysteine by 99%, their inability to protect the enzyme is not surprising.

L-Leucine and L-isoleucine interact with the enzyme. Neither compound resembles L-serine other than by having an α-amino group. When saturating concentrations of L-leucine and L-isoleucine were tested, no further increase in protection was observed. L-Threonine

also protects the enzyme. It differs from L-serine in that it has a methyl group attached to the β-carbon.

Combinations of L-isoleucine, L-leucine, and L-threonine were also tested. A combination of L-threonine with either L-isoleucine or L-leucine has about the same effect as any of these tested alone. However, if both L-isoleucine and L-leucine are present with or without L-threonine, no protection is seen. Thus, the combination of L-isoleucine and L-leucine cancels the effect produced by either of these compounds alone. No other combinations of the various amino acids were attempted.

(iii) BSA and EDTA

As indicated in Section II, part (i), L-SD stability is much affected by protein concentration. It might then be expected that increasing the protein concentration by the addition of a protein that has no enzyme activity may stabilize L-SD. To investigate this, bovine serum albumin (BSA) was added to cell extracts of E. coli K_{12}, strain MS845. Diluted enzyme was added to test tubes containing BSA (0, 1.43 mg/ml, 2.86 mg/ml). In one set of assay tubes, L-serine was present from the time the enzyme was added, and in the other set L-serine was added after a 3 minute incubation at 37°C. The amount of pyruvate produced after 10 minutes was determined and the % protection calculated (Table II).

When the results are expressed, as has been done previously, they indicate that extensive protection, 31% and 28%, is given by both 'high' and 'low' BSA concentrations, respectively. The activity of L-SD measured with BSA at 2.86 mg/ml is higher than the activity measured

Table II Effects of BSA and EDTA on L-SD Activity

Compound Added	Conc. in Assay (mg/ml)	Initial S.A. of Extract	S.A. after 3 min	% Protection	Increase in Initial S.A. (%)
0	-	5.05	0.00	-	-
BSA	1.43	5.09	1.42	28	1
BSA	2.86	6.99	2.14	31	39
EDTA	0.06	4.94	1.48	30	0
EDTA	0.28	5.24	2.00	38	4
EDTA/BSA	0.06/2.86	6.23	1.62	26	23

A 0.05 ml aliquot of diluted enzyme, 1:20, from E. coli K_{12}, strain MS845, was added to test tubes containing BSA (0, 1.43 mg/ml, 2.86 mg/ml) and/or EDTA (0.06 mg/ml, 0.28 mg/ml). In one set of assay tubes, 0.1 ml of L-serine (0.19M) was present from the time the enzyme was added, and in the other set 0.1 ml of L-serine (0.19M) was added after a 3 minute incubation at 37°C. The amount of pyruvate produced after 10 minutes was determined.

$$\text{\% increase in initial S.A.} = \frac{\text{S.A. (0 min) - S.A. (0 min)}}{\text{+ comp} \qquad \text{- comp}} \times 100$$

$$\text{S.A. (0 min)}$$

$$\text{- comp}$$

without BSA - that is, BSA at the higher concentration increases the apparent L-SD activity. This increase is expressed as '% increase in initial S.A.' (Table II). There is a 39% increase in initial S.A. of the enzyme when 2.86 mg/ml of BSA is present per assay tube.

Each test tube contains on the average 160 µg/ml of protein. The addition of 10 times more of inert protein (BSA: 1.43 mg/ml, 2.86 mg/ml) may be enough to stabilize L-SD in so far as exogenous protein can. That higher concentrations of protein (>1.43 mg/ml) then do not further increase the protection is not surprising. The 39% increase in initial S.A. observed at the higher concentration of BSA may well be a result of the increase in the total protein present during dilution and/or inhibition by BSA of some component toxic to the enzyme.

Another compound which stabilized the enzyme is ethylenediaminetetraacetic acid (EDTA). This compound is an active chelating agent and is commonly added to crude enzyme preparations. It forms complexes with most metal ions, thus protecting any sensitive enzyme present.

The EDTA experiments were carried out in the same manner as the BSA and amino acid experiments. Cell extracts were incubated at 37°C with EDTA in concentrations ranging from 0.06 mg/ml to 6 mg/ml. As can be seen in Table II, the presence of EDTA allowed the retention of a considerable amount of enzyme activity: between 30 to 40%. Higher levels of EDTA did not increase this effect.

EDTA and BSA, separately, were able to protect L-SD. One might not a priori expect that they act by the same mechanism. What, then, would be the result of using them simultaneously in the cell extract? EDTA (0.06 mg/ml) and BSA (2.86 mg/ml) were added to the assay mix.

26% of the enzyme activity remained after a 3 minute incubation at 37°C. This is approximately the same as that given by either compound alone. In addition, as when only BSA was added, the initial activity in the presence of these compounds was increased by 23%.

In summary, both EDTA and BSA can protect the enzyme by about 30%. However, the two effects are not additive. This result is rather surprising. It is, nonetheless, possible that the BSA preparation may contain some component that binds EDTA or metal ions and thus only the BSA effect is observed.

(iv) Sulfhydryl Reagents

A common cause of enzyme inactivation is oxidation of sulfhydryl groups (-SH) to disulfide-cross-links (S-S) or to sulfate ($-SO_4$). This is frequently avoided by the addition of sulfhydryl compounds to cell extracts. To examine if this holds true for L-SD, cell extracts were incubated with reducing agents, β-mercaptoethanol (MSH), dithiothreitol (DTT), and dithioerythritol (DTE). The experiments were carried out in the same manner as for various amino acids.

The effects of DTT and DTE on L-SD activity could not be determined. In fact, both compounds seemed to inhibit the enzyme, DTE (2×10^{-4}M) by 30% and DTT (2×10^{-4}M) by 79%. That is, both compounds reduced the activity of the enzyme when it was tested with L-serine from zero time (Table III). To determine whether the decrease observed in initial activity is a result of the compounds reducing DNPH, assay tubes containing pyruvate (3.64 μ moles/ml), without enzyme, were incubated with DTT and DTE. Both compounds decrease the color produced with DNPH (Table IV). This results from the fact that these compounds have a reducing effect on DNPH in alkaline solutions

Table III Effects of Sulfhydryl Reagents on L-SD Activity

Compound Added	Conc. in Assay (X 10^{-4}M)	Initial S.A. of Extract	S.A. after 3 minutes	% Protection
0	-	3.15	0.00	-
DTT	2	0.67	0.00	0
DTE	2	2.19	0.03	1
MSH	36	2.89	0.00	0

An extract of E. coli K_{12}, strain MS845, diluted 1:20 was added to assay tubes containing DTT (2 X 10^{-4}M), DTE (2 X 10^{-4}M), and MSH (36 X 10^{-4}M). One set of test tubes contained substrate from the time the enzyme was added and in the other set, L-serine was added after 3 minutes at 37°C. The amount of pyruvate present was calculated.

Table IV Effect of DTT and DTE on DNPH

Pyruvate Conc. per Assay (µmoles/ml)	Compound Added	Conc. in Assay (X 10^{-4}M)	µmoles of pyruvate/ml
3.64	-	-	3.60
3.64	DTT	2	0.76
3.64	DTE	2	2.44

and thus cause a decrease in its color (Carter and Sagers, 1972). This effect could not be overcome by using smaller concentrations of either DTT or DTE.

MSH (36×10^{-4}M) had no effect on the assay or on the stability of L-SD.

(v) Ferrous Ion

Ferrous ion (Fe^{++}) has been shown as a requirement for the activity of L-SD in Clostridium acidiurici (Carter and Sagers, 1972). The effect of ferrous ion on the activity of L-SD in E. coli K_{12}, strain MS845, was examined. Cell extracts were prepared with and without ferrous ion (0.1M $FeSO_4$) and assayed with (6×10^{-3}M or 17×10^{-3}M $FeSO_4$) and without ferrous ion added to the assay mix. The experiments were carried out as usual, using 1:10 dilution of crude cell extract. The results are given in Table V.

Ferrous ion was seen to have an effect on L-SD determinations. Adding ferrous ion (6×10^{-3}M or 17×10^{-3}M) into the assay mix increased the initial activity of the enzyme between 7 and 18%. Some enzyme activity was retained (8%) when cell extracts in the absence of substrate but in the presence of ferrous ion (17×10^{-3}M) were incubated for 3 minutes at 20°C.

The addition of ferrous ion during the extraction procedure had little further effect. The time of addition of ferrous ion (17×10^{-3}M), whether it be present when the extracts are made (Table V, exp. 3) or whether it be added only to the assay tube (Table V, exp. 2) does not appear to affect the % protection. The initial activity, however, is increased by 34% when cell extracts are prepared in the

Table V Effects of Fe^{++} on L-SD Activity

Exp.	Fe^{++} Conc. in Extract (X 10^{-3}M)	Final Conc. in Assay (X10^{-3}M)	Initial S.A. of Extract	S.A. after 3 min	% Protection	Increase in Initial S.A. (%)
	0	0	2.32	0.18	-	-
1	0	6	2.48	0.18	0	7
2	0	17	2.74	0.41	8	18
3	100	17	3.10	0.37	6	34
4	100	23	2.89	0.57	13	25
5	100	33	3.17	0.55	12	38

Cell extract of E. coli K_{12}, strain MS845, diluted 1:10 was prepared with and without 0.1M $FeSO_4$ and assayed with and without 6 X 10^{-3}M or 17 X 10^{-3}M $FeSO_4$ added to the assay mix. One set of test tubes contained substrate from the time the enzyme was added and in the other set, L-serine was added after 3 minutes at 20°C. The amount of pyruvate formed in 10 minutes was determined.

presence of ferrous ion as compared to 18% when ferrous ion is added to the assay mix (Table V, exp. 2, 3).

Assay tubes containing both ferrous ion and cell extracts prepared in ferrous ion (Table V, exp. 4, 5) did not further increase the initial specific activity of L-SD but did increase the % protection to approximately 13%.

In summary, ferrous ion appears to be involved in the expression of L-SD between 7 and 38% and gives at most about 13% protection.

(vi) Pyridoxal Phosphate and Pyridoxine

L-SD of animal origin is known to be a pyridoxal phosphate dependent enzyme (Goldstein et al., 1962; Sayre and Greenberg, 1956). However, the cofactor requirement of L-SD in E. coli is as yet unclear. The enzyme is assayed routinely without the addition of pyridoxal phosphate. This does not, however, exclude an involvement of pyridoxal phosphate in the L-SD reaction.

To investigate this matter, the activity of cell extracts diluted 1:20 from E. coli K_{12}, strain MS845, incubated with or without pyridoxal phosphate (33 µg/ml) at 20°C was examined. The usual L-SD assay could not be used because pyridoxal phosphate reacts with DNPH. Therefore, after the 20 minute incubation with DNPH, the pyruvate dinitrophenylhydrazone was extracted into toluene, in which the pyridoxal phosphate dinitrophenylhydrazone is not soluble. The pyruvate dinitrophenylhydrazone was then re-extracted into Na_2CO_3 (Pardee and Prestidge, 1955). The amount of pyruvate formed in the presence of pyridoxal phosphate (eg. 2.99 µm pyruvate/mg protein) did not differ from the amount formed in its absence (eg. 3.01 µm pyruvate/mg

Table VI · Effects of Pyridoxine on L-SD Activity

Pyridoxine Conc. in growth media (µg/ml)	Pyridoxine Conc. in dilution tube (µg/ml)	Pyridoxine Conc. added to assay (µg/ml)	Initial S.A. of Extract	Increase in Initial S.A. (%)
0	0	0	1.99*	0
		6	1.99	0
		20	2.07	4
		100	2.07	4
0	20	0	1.90	0
		6	1.86	0
		20	2.02	0
		100	1.90	0
20	0	0	5.45	174
		6	5.45	174
		20	5.36	169
		100	5.41	172
20	20	0	5.24	163
		6	4.89	146
		20	4.81	142
		100	5.24	163

continued

Table VI Effects of Pyridoxine on L-SD Activity

Cell extracts of *E. coli* K_{12}, strain MS845, grown with or without pyridoxine monohydrochloride (20 µg/ml) were diluted 1:20 with or without pyridoxine monohydrochloride (20 µg/ml) and added to assay tubes with or without pyridoxine monohydrochloride (6 µg/ml , 20 µg/ml, 100 µg/ml). The test tubes contained L-serine (0.076M) from the time the enzyme was added. The amount of pyruvate formed in 10 minutes was determined.

* To obtain the % in the last column, all S.A. values are compared to 1.99 which is taken as 100%

NOTE: No protein difference per assay tube when cells are grown with or without pyridoxine was observed.

protein). Thus pyridoxal phosphate has no effect on the initial L-SD activity. It was also unable to stabilize the enzyme.

In conclusion, no pyridoxal phosphate requirement could be shown by addition of exogenous pyridoxal phosphate. This does not, however, exclude the fact that pyridoxal phosphate may be tightly bound to the enzyme and is not released despite extraction from the cell.

It was also considered that by growing cells in the presence of pyridoxine, the size of the internal pool of pyridoxal phosphate could then be affected. Pyridoxine, being a precursor of pyridoxal phosphate and an unphosphorylated form of vitamin B_6, can be taken up and utilized by E. coli cells whereas pyridoxal phosphate, a phosphate form of vitamin B_6, cannot unless it is dephosphorylated (Yamada et al., 1977). To determine the effect of pyridoxine on L-SD activity, cell extracts were prepared from cells grown with and without pyridoxine (20 µg/ml), were diluted with and without pyridoxine (20 µg/ml) and added to assay tubes with and without pyridoxine (6 µg/ml, 20 µg/ml, 100 µg/ml). The results are given in Table VI.

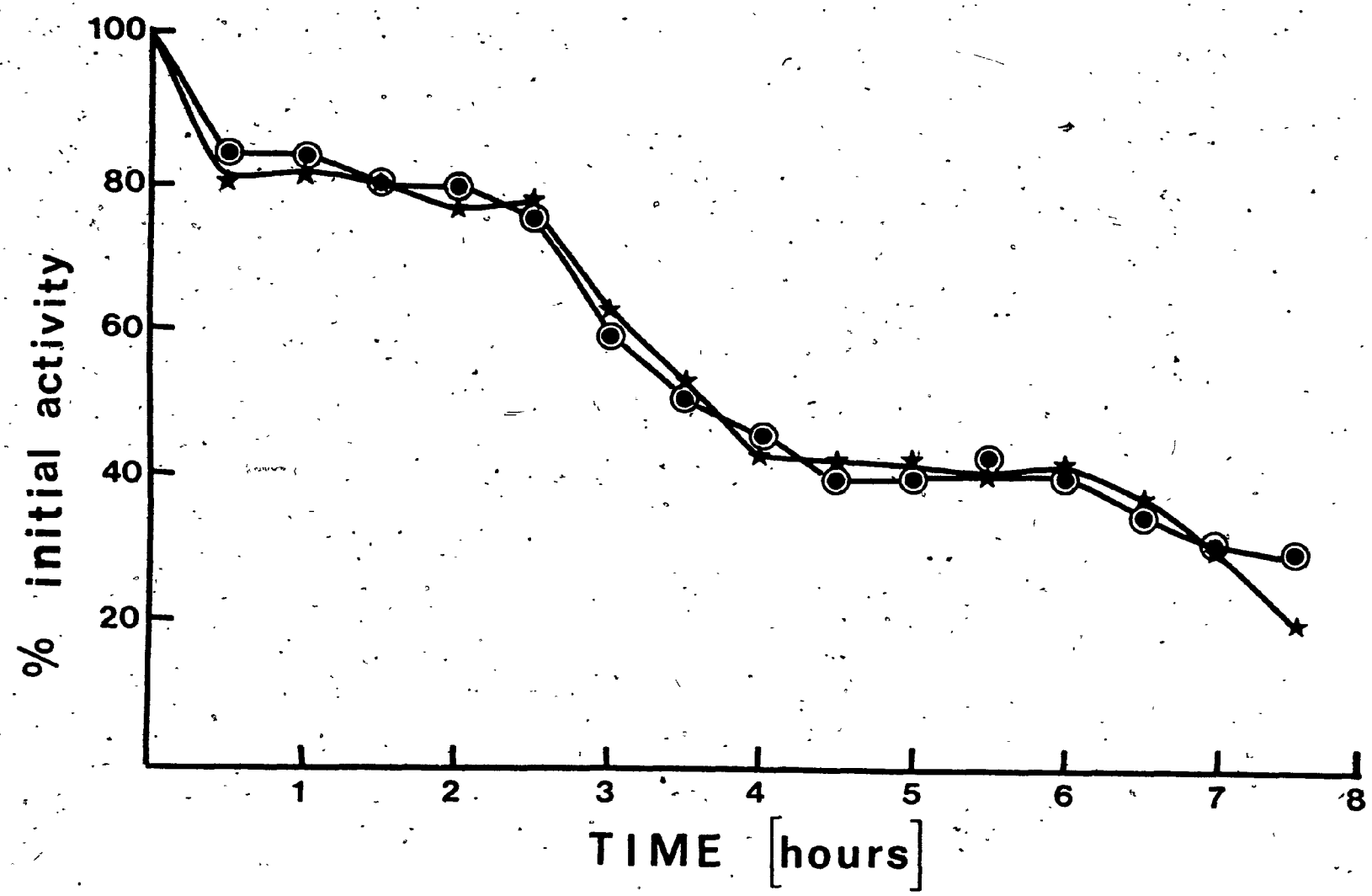
When cells are grown in the presence of pyridoxine, the initial S.A. is tremendously increased (S.A. without pyridoxine, 1.99; S.A. when cells are grown with pyridoxine, 5.45). This is the most striking effect on enzyme activity observed in this work and would seem to imply that pyridoxal phosphate is involved in the expression of L-SD activity. From the experimental design, it is clear that growth of cells in pyridoxine is alone responsible for the increase in enzyme activity observed. Adding pyridoxine either at the time of dilution or into the assay mix has no effect on the enzyme activity.

Figure XIII Effect of Pyridoxine on the Decay of L-SD Activity

Cell extracts of E. coli K_{12}, strain MS845, were prepared from cells grown with and without pyridoxine monohydrochloride (20 µg/ml) in the usual manner. The concentrated cell extracts were allowed to sit on ice water for 0, 1/2, 1, 1 1/2, 2, 2 1/2, 3, 3 1/2, 4, 4 1/2, 5, 5 1/2, 6, 6 1/2, 7, and 7 1/2 hours. The extracts were diluted 1:20 prior to the assay and the L-SD assay was performed immediately.

★——★ Cells grown without pyridoxine

◉——◉ Cells grown with pyridoxine


100
80
60
40
20
% initial activity
1
2
3
4
5
6
7
8
TIME [hours]

Because this increase in initial specific activity of L-SD is so marked, it was hoped that the decay in L-SD activity would be less rapid and less extensive when the cells are grown in the presence of pyridoxine. For this reason, concentrated cell extracts prepared from cells grown with or without pyridoxine (20 µg/ml) were incubated in ice water for times varying between 0 and 7 1/2 hours. The extracts were diluted 1:20 just prior to assay and the amount of pyruvate formed after 10 minutes was determined. No difference in the decay patterns of extracts grown with or without pyridoxine could be observed (Figure XIII). It is evident, therefore, that pyridoxine cannot stabilize the enzyme, even though it does increase the initial specific activity of L-SD.

Preliminary experiments indicated no increase in initial S.A. of the enzyme when toluene-treated whole cells grown with or without pyridoxine (20 µg/ml) were assayed for L-SD activity. This is surprising since crude extracts of these cells give very different L-SD values. An experimental scheme was then designed to enable us to compare the L-SD assay when toluene-treated whole cells (referred to as 'whole cell assay') and cell extracts (referred to as 'cell extract assay') were used (Figure XIV and Method). All values were expressed in µ moles of pyruvate produced per gram (wet weight) of cells.

Table VII shows the results of a comparison made between cells grown with and without pyridoxine (20 µg/ml) using both cell extracts and whole cells for detection of L-SD activity. The L-SD assay using toluene-treated cells was performed on E. coli K_{12},

Figure XIV Comparison of Whole Cell and Cell Extract Assay in the Pyridoxine Experiments

grow *E. coli* K_{12}, strain MS845, in

+Np | +Np pyridoxine (20 µg/ml)

collect and weigh pellets

resuspend to OD of 100 Klett Units (#54 filter)
in Tris buffer pH 8.5, 0.05M

perform whole cell assay (1)

recentrifuge, collect pellets

sonicate

perform whole cell assay (2) and the cell extract assay* (3) on sonicated suspensions both prepared 20% w/v in Tris buffer pH 8.5, 0.05M

spin down debris

perform cell extract assay (4) on supernatants

resuspend pellets in Tris buffer pH 8.5, 0.05M
perform whole cell assay (5) and cell extract assay (6)

* all dilutions were carried out in Tris buffer pH 8.5, 0.05M

Table VII Effects of Pyridoxine on L-SD Activity Using Toluene-Treated Whole Cells and Cell Extracts

Experiment (see Figure XIV)	Condition of Growth (20 µg/ml)	µmoles of pyruvate/ gram of cells	% Total Activity
(1)	-pyridoxine	643	100
	+pyridoxine	633	100
(2)	-pyridoxine	420	65
	+pyridoxine	600	95
(3)	-pyridoxine	171	27
	+pyridoxine	302	48
(4)	-pyridoxine	165	26
	+pyridoxine	285	45
(5)	-pyridoxine	220	34
	+pyridoxine	250	39
(6)	-pyridoxine	11	2
	+pyridoxine	21	3

$$\% \text{ total activity} = \frac{\text{Exp. (2) or (3) or (4) or (5) or (6) +pyridoxine}}{\text{Exp. (1) + pyridoxine}} \times 100$$

Similarly for -pyridoxine

strain MS845, grown with or without pyridoxine prior to sonication. The μ moles of pyruvate produced was then related back to the wet weight (grams) of the cells. This was taken to be the total enzyme activity present in each set of cells. After the cells were sonicated, the whole cell assay was used to detect L-SD activity (Table VII, exp. 2). Cells grown with pyridoxine show as much enzyme activity after sonication as before. However, cells grown without pyridoxine lose 1/3 of their total L-SD activity. When the cell extract assay was performed on the same sonicated suspension (Table VII, exp. 3), cells grown with pyridoxine maintain 1/2 of their total enzyme activity whereas cells grown without pyridoxine maintain only about 1/3. This seems to indicate that sonication affects only some 50% of the cells. Spinning down the debris did not significantly alter these results (Table VII, exp. 4). The pellets contained about 40% (Table VII, exp. 5) of the total enzyme activity when the whole cell assay was performed. However, very small amounts (2 and 3%) of the enzyme activity were detected for both growth conditions when the cell extract assay was used (Table VII, exp. 6). The pellet, therefore, consisted largely of whole cells. Some enzyme molecules did become trapped within or between the debris and were as a result spun down.

These results may be explained in two ways. The cells grown with pyridoxine may be more easily susceptible to breakage, due to a difference in their cell wall, by sonication, or more likely, the enzyme in the presence of pyridoxine is less labile to sonication. The observation was made that when the whole cell assay is performed on the sonicated suspension containing cells grown without

pyridoxine, all the enzyme activity is recovered whereas those grown without pyridoxine lose about 1/3 of their activity. If one then considers the value 171 μmoles of pyruvate/gram of cells for cells grown without pyridoxine to already include the 1/3 loss in enzyme activity (when the cell extract assay is performed) then in actuality sonication is effective in breaking open about 1/2 the cells in both cases. This is further supported by the fact that both pellets contain about 40% of the total enzyme activity when they are examined with toluene. If the cells grown with pyridoxine were more susceptible to breakage, one would expect that the pellet value would be less in samples grown with pyridoxine than in samples grown without pyridoxine. This, however, is not the case. The difference in activity observed is then probably due to L-SD being more labile to sonication when cells are grown without pyridoxine.

Increasing the pyridoxine concentration to 50 μg/ml in the growth media of E. coli cells did not alter any of the results observed in this section.

(vii) Nucleic Acids and Histone

Commercial preparations of calf thymus DNA, pine RNA and arginine rich histones were tested as possible protective agents in E. coli K_{12}, strain MS845. The experiments were performed in the same manner as for the various amino acids.

All the polyelectrolytes were able to increase the initial

Table VIII Effects of Nucleic Acids and Histones on L-SD Activity

Compound Added	Conc. in Assay (µg/ml)	Initial S.A. of Extract	S.A. after 3 min.	% Protection	Increase in Initial S.A. (%)
0	0	3.16	0.25	-	-
crude DNA	167	4.16	0.25	0	32
crude RNA	167	3.87	0.25	0	22
histones (arginine)	83	4.16	0.31	1	32

Cell extract of E. coli K_{12}, strain MS845, diluted 1:10 was added to tubes containing crude DNA (167 µg/ml), crude RNA (167 µg/ml), and arginine rich histones (83 µg/ml). One set of assay tubes contained substrate from the time the enzyme was added and to the other set L-serine was added after a 3 minute incubation at 20°C. The amount of pyruvate formed after 10 minutes was calculated.

activity of the enzyme by about 30% (Table VIII). None were able to stabilize the enzyme. This increase in initial activity observed may well result from an ability of these compounds to inhibit some component toxic to the enzyme and/or these preparations may contain some element that enhances the initial activity of L-SD.

(viii) Compounds Affecting Proteolytic Enzymes

Since L-SD in crude cell extracts of E. coli K_{12}, strain MS845, is very unstable the possiblity was considered that a protease may be activated upon lysis of the cell. This protease would then quickly digest the enzyme. To test this, various known protease inhibitors were added to crude enzyme preparations and their effects were noted.

None of the protease inhibitors examined stabilized the enzyme. Soybean trypsin inhibitor was tested at concentrations from 1 µg/ml to 28 µg/ml, aprotinin at concentrations from 10 to 280 KIU/ml, and phenylmethylsulfonylfluoride at only one concentration of 570 µg/ml. Even when cell extracts were made in the presence of aprotinin, no difference in L-SD stability was observed.

In summary, it appears that if there is a protease acting on this enzyme in strain MS845 it is not susceptible to soybean trypsin inhibitor, aprotinin, or phenylmethylsulfonylfluoride at the concentrations tested.

(ix) Ethylene Glycol and Glycerol

Some of the enzymes which are particularly sensitive to freezing and thawing can be stabilized by the addition of 10 to 50% (v/v) ethylene glycol or glycerol. These reagents have been shown to have different effects, all of which are not clearly understood, depending on the temperature at which the enzyme is stored. They can, however, provide protection simply by lowering the freezing point of the solution containing the enzyme from -15°C to -20°C.

When 20% (v/v) ethylene glycol and 20% (v/v) glycerol were added to crude cell extracts of E. coli K_{12}, strain MS845, both compounds were found to interfere with the enzyme assay. They were, therefore, not used.

Section III

FACTORS AFFECTING L-SD STABILITY IN EXTRACTS FROZEN AND THAWED OF E. COLI K_{12}, STRAIN MS845

(i) Amount of Enzyme Activity Remaining After Freezing

In the course of the study, it was noted that crude cell extracts of strain MS845 could be frozen at -15°C for various time intervals with considerable recovery of enzyme activity, after thawing at 0°C.

To determine the actual amount of enzyme activity recovered after freezing and thawing, crude cell extracts were prepared 20% w/v in phosphate buffer pH 7.2, 0.05M. These were

immediately frozen in 1 ml aliquots at -15°C. At various time intervals (days), an aliquot was thawed in ice water and diluted 1:20 in cold phosphate buffer pH 7.2, 0.05M. The L-SD assay was then performed immediately. This protocol tested the resistance of concentrated cell extracts to freezing and thawing.

From Figure XV, it is clear that L-SD can be frozen. Enzyme activity is lost progressively over the first five days. The 30% remaining at day 5 is retained for several days, thereafter.

(ii) Comparison of L-SD Stability in Extracts Freshly Prepared and Extracts Frozen and Thawed

Concentrated extracts were prepared in the usual manner. Immediately after they were made, aliquots of these extracts were frozen. The remaining extract was diluted (1:10 or 1:20 as stated) and the stability of the enzyme was determined. After a certain period of time, the frozen sample was thawed in ice water, diluted (1:10 or 1:20) to the same extent as the freshly prepared extract, and the L-SD stability was determined. The two conditions are referred to as 'fresh cell extracts' and 'frozen cell extracts.'

In Figure XVI, the stability of the enzyme between fresh and frozen cell extracts (24 hours) at a 1:10 dilution at 22°C is compared. It can be seen that the frozen cell extract is much less stable. It loses 67% of its activity in two minutes whereas the fresh cell extract loses only 27%, in the same time period. These numbers refer to the total activity measured on the day the assay was performed. Some activity is lost after freezing and thawing (Section III, part (i)). In this experiment the frozen cell extract retained about 80%.

Figure XV Amount of Enzyme Activity Remaining After Freezing

Cell extracts of *E. coli* K_{12}, strain MS845, were prepared in the usual manner. These concentrated cell extracts were frozen for 0, 1, 3, 4, 5, 7, and 11 days. An aliquot of the crude cell extract was then thawed in ice water and diluted 1:20. The L-SD assay was carried out immediately.

$$\text{\% original activity remaining after freezing} = \frac{\text{S.A. of 'frozen cell extract'}}{\text{S.A. of 'fresh cell extract'}} \times 100$$

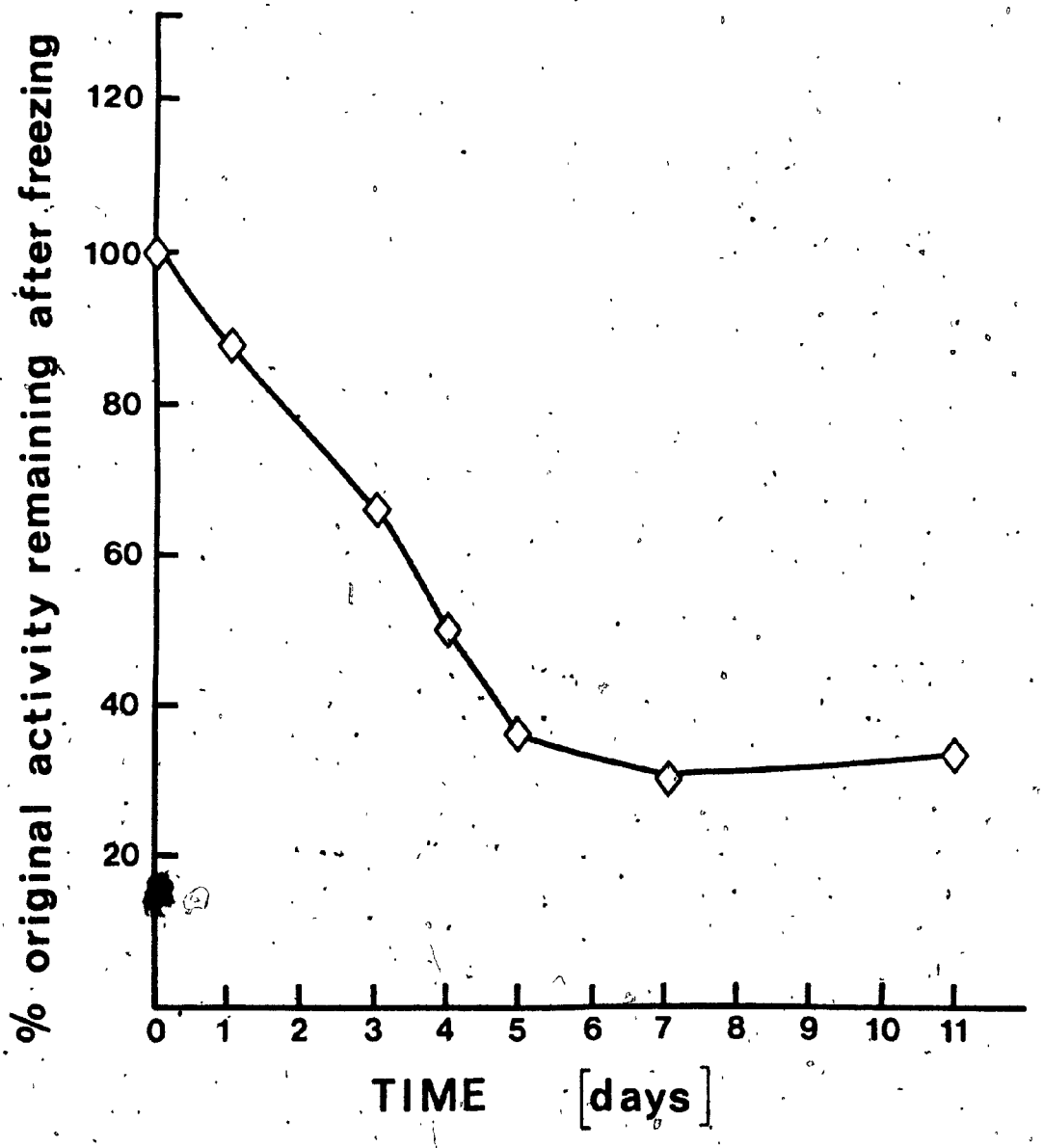
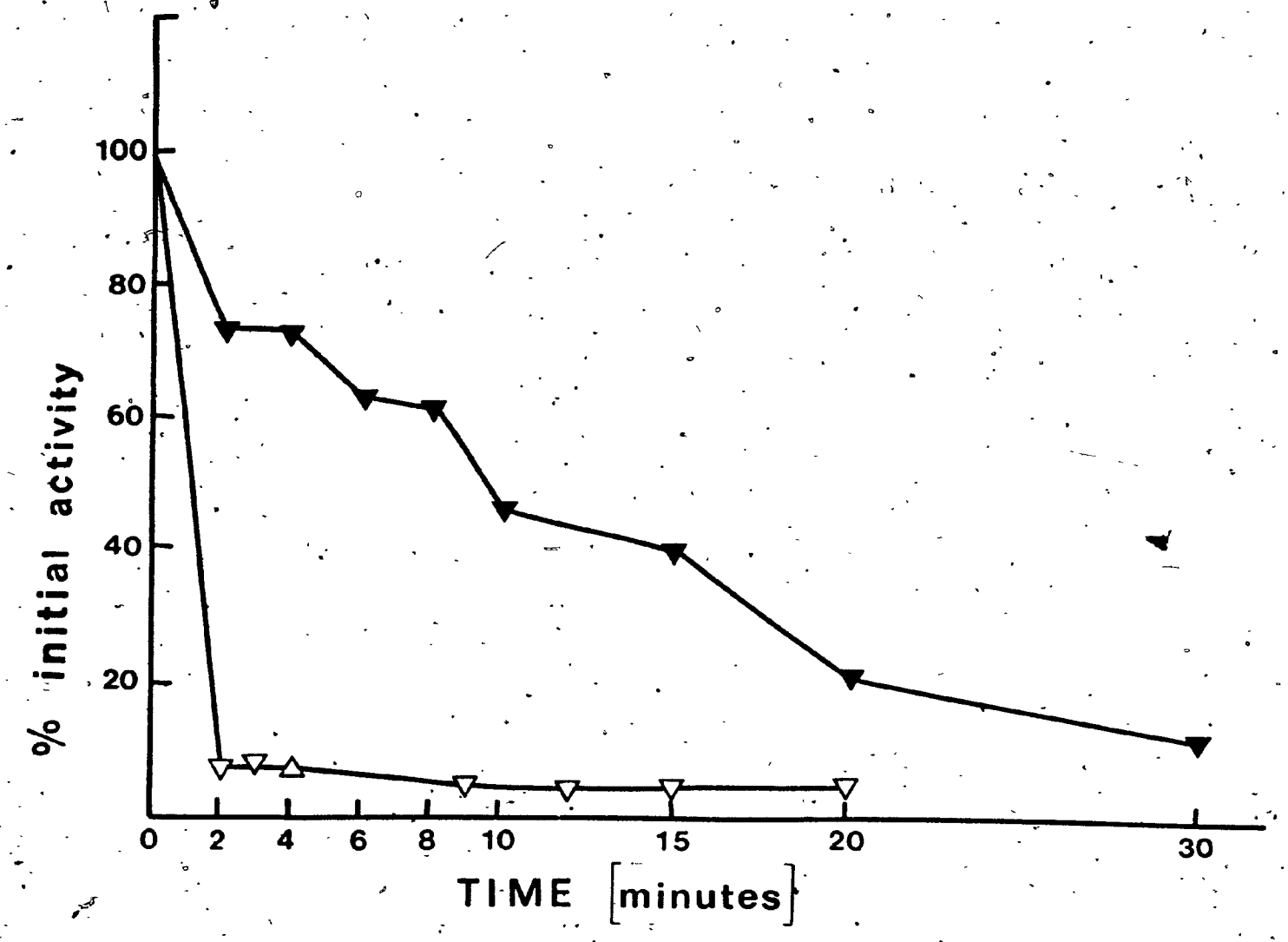

% original activity remaining after freezing
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
TIME [days]

Figure XVI Comparison of L-SD Stability in Fresh and Frozen Cell Extracts

A cell extract of *E. coli* K_{12}, strain MS845, was prepared in the usual manner. Aliquots of the concentrated cell extract were frozen. After one day, the frozen sample was thawed in ice water, diluted 1:10, and incubated for times varying from 0 to 30 minutes at 22°C. On the remaining freshly prepared extract, the L-SD assay was performed immediately. This fresh cell extract was also diluted 1:10 and incubated for times varying form 0 to 30 minutes at 22°C. The amount of pyruvate produced in 10 minutes after the addition of L-serine (0.19M) was determined for fresh and frozen cell extracts.

▼——▼ Decay of L-SD in fresh cell extract

▽——▽ Decay of L-SD in frozen cell extract


100
80
60
40
20
% initial activity
0
2
4
6
8
10
15
20
30
TIME [minutes]

of its activity after freezing and thawing, but this 80% is much less stable.

(iii) Various Amino Acids

Some of the same amino acids which stabilized L-SD in fresh cell extracts were examined for their effect on frozen cell extracts. These included L-isoleucine, L-leucine, L-threonine, and their combinations (Table IX). All were able to stabilize L-SD. The % protection observed was, however, greater in frozen cell extracts (L-isoleucine 22% vs. 11%, L-threonine 28% vs. 11% and L-leucine 19% vs. 7%). Moreover, since the fresh cell extracts were tested at a 1:10 dilution and the frozen cell extracts at a 1:20 dilution, this difference in % protection may be even greater (Table I vs. Table IX).

These amino acids in combination showed much the same pattern as with freshly prepared cell extracts. The combination of L-isoleucine and L-leucine with or without L-threonine cancelled each others effects in fresh cell extracts. When tested on frozen cell extracts, however, they allowed some protection but less than either alone.

The freezing and thawing process may well alter the conformation of the enzyme in such a manner so that it can more readily interact with these compounds. This change may then be reflected in the % protection observed.

(iv) BSA and EDTA

L-SD in frozen cell extracts was stabilized by BSA and EDTA. The initial activity of the enzyme was also increased by both concentrations of BSA tested (Table X). These results are similar to

Table IX Effects of Various Amino Acids on L-SD Activity

Compound Added	Conc. in Assay ($X\ 10^{-3}M$)	Initial S.A. of Extract	S.A. after 3 min	% Protection
0	0	2.11	0.00	-
L-isoleucine	12	1.94	0.44	22
L-threonine	31	2.28	0.64	28
L-leucine	12	2.24	0.43	19
L-ileu/L-thr	12/31	1.69	0.19	11
L-leu/L-thr	12/31	2.07	0.55	26
L-leu/L-ileu	12/12	1.67	0.24	14
L-leu/L-ileu/L-thr	12/12/31	1.88	0.28	14

An aliquot of concentrated cell extract of E. coli K_{12}, strain MS845, which was frozen for one day, was thawed in ice water and diluted 1:20. A 0.05 ml aliquot of this diluted enzyme was then added to test tubes containing L-isoleucine ($12\ X\ 10^{-3}M$), L-threonine ($31\ X\ 10^{-3}M$), L-leucine ($12\ X\ 10^{-3}M$) and their combinations. In one set of assay tubes, 0.1 ml of L-serine (0.19M) was present from the time the enzyme was added, and in the other set, 0.1 ml of L-serine (0.19M) was added after a 3 minute incubation at 37°C. The amount of pyruvate produced after 10 minutes was calculated.

Table X Effects of BSA and EDTA on L-SD Activity

Compound Added	Conc. in Assay (mg/ml)	Initial S.A. of Extract	S.A. after 3 min	% Protection	Increase in Initial S.A. (%)
0	-	1.94	0.00	-	-
BSA	1.43	3.10	0.83	27	59
0	-	2.40	0.00	-	-
BSA	2.86	3.08	1.05	34	28
0	-	2.40	0.00	-	-
EDTA	0.06	2.46	0.68	28	-
0	-	2.33	0.00	-	-
EDTA	0.28	2.30	0.69	30	-
0	-	2.38	0.00	-	-
EDTA/BSA	0.06/2.86	2.18	0.55	25	-

Concentrated cell extracts of *E. coli* K_{12}, strain MS845, were frozen and thawed in ice water. A 0.05 ml aliquot of a 1:20 dilution of the concentrated enzyme preparation was added to assay tubes containing BSA (1.43 mg/ml, 2.86 mg/ml) and/or EDTA (0.06 mg/ml, 0.28 mg/ml). One set of test tubes contained substrate from the time the enzyme was added, and in the other set, L-serine was added after 3 minutes at 37°C. The amount of pyruvate formed in 10 minutes was determined.

those obtained with fresh cell extracts except that 1.43 mg/ml of BSA increased the initial activity of L-SD in frozen cell extracts but not in fresh cell extracts. This may be related to the fact that the enzyme is less stable after freezing and thawing (Figure XVI) and may, therefore, be more sensitive to protein concentration.

The effects of EDTA and BSA in combination were once again not additive. The combination did not increase the initial activity of L-SD in frozen cell extracts as it did in fresh cell extracts. The % protection was about the same in both cases.

(v) Ferrous Ion

In frozen cell extracts, the initial activity of L-SD was again increased (Table XI). However, this increase was about 30% greater than that observed in fresh cell extracts. The % protection was also 7% greater when ferrous ion was added, alone, to the assay mix in frozen cell extracts as compared to fresh cell extracts. When cell extracts were frozen with ferrous ion (0.1M), the % protection was about the same as that of fresh cell extracts made with ferrous ion (0.1M). It appears then that ferrous ion is very much involved in the expression of L-SD after the enzyme preparation has been frozen and thawed.

(vi) Pyridoxal Phosphate

Various concentrations of pyridoxal phosphate (1.7 µg/ml to 430 µg/ml) were added to frozen cell extracts. Pyridoxal phosphate had no effect on L-SD in frozen cell extracts just as it did not in fresh cell extracts.

Table XI Effects of Ferrous Ion on L-SD Activity

Compound Added	Fe^{++} Conc. in Extract ($X\ 10^{-3}M$)	Final Conc. in Assay ($X\ 10^{-3}M$)	Initial S.A. of Extract	S.A. after 3 min	% Protection	Increase in Initial S.A. (%)
0	-	-	2.49	0.17	-	-
$FeSO_4$	0	17	3.96	0.76	15	59
$FeSO_4$	100	33	4.08	0.73	14	64

Concentrated cell extract of E. coli K_{12}, strain MS845, were frozen with and without $FeSO_4$ (0.1M) and thawed in ice water. A 0.05 ml aliquot of a 1:10 dilution of the concentrated cell extract was added to assay tubes containing 17 $X\ 10^{-3}M$ $FeSO_4$. One set of test tubes contained substrate from the time the enzyme was added and to the other set L-serine was added after 3 minutes at 20°C. The amount of pyruvate produced after 10 minutes was determined.

(vii) Compounds Affecting Proteolytic Activity

The same concentrations of soybean trypsin inhibitor and aprotinin were tested with frozen cell extracts as with fresh cell extracts. None were able to affect the stability of L-SD in frozen cell extracts.

Section IV

COMPARISON OF L-SD STABILITY IN TWO STRAINS OF E. COLI K_{12}

(i) L-SD Stability in Strain W4977 and Strain MS845

The strain of E. coli K_{12} (i.e. strain MS845) studied in the preceding sections of this thesis was chosen because it expressed L-SD activity at an unusually high level. This high level is not further increased by growing the cells with the two amino acids, L-leucine and glycine, which usually induce L-SD (Beeraj et al., 1978; Isenberg and Newman, 1974). The increased L-SD activity and its lack of inducibility are accompanied by a lack of a protein modifying enzyme, L-leucyl-, L-phenylalanyl-tRNA protein transferase. This enzyme carries either an L-leucine or an L-phenylalanine to a specific protein acceptor molecule which has a free basic ε-amino group of arginine or lysine (Leibowitz and Soffer, 1971). This post-translational addition of an amino acid to an already made protein could then serve as a rapid regulatory system for that protein if the activity of the acylated form were different from that of the non-acylated form.

Strain W4977 has normal levels of L-SD; L-SD activity is increased when cells are grown with glycine or L-leucine. It is also

known to possess an L-leucyl-, L-phenyalanyl-tRNA protein transferase (Soffer and Savage, 1974). Strain MS845 is a mutant of strain W4977 obtained by nitrosoguanidine treatment (see 'Method'). If L-SD were an acceptor for the protein trasnferase enzyme, it could then be that the L-SD would be acylated in strain W4977 but not in strain MS845. If this acylation affects the conformation of the enzyme, this might be reflected in a difference in the enzyme stability in the type of experiment used in this work.

L-SD stability in two strains of E. coli K_{12} which express L-SD activity at very different levels was compared (Table XII). Fresh cell extracts from strain W4977 and strain MS845 were made and diluted 1:10 in the usual manner. A 0.05 ml aliquot of this diluted sample was then added to the assay mix. One set of test tubes contained substrate from the time the enzyme was added and in the other set, L-serine was added after 3 minutes at 27°C. The activity remaining after the 3 minute incubation in the absence of substrate was compared to the activity seen on incubation with substrate and is expressed as % remaining after 3 minutes (Table XII).

The L-SD of strain W4977 appears to be considerably more stable than that of strain MS845. Strain W4977 retains approximately 62% of the initial enzyme activity after exposure to 27°C for 3 minutes in the absence of substrate, whereas, the transferase negative strain, strain MS845, retains only 26%. The data, therefore, clearly shows that L-SD is more stable in strain W4977 at this temperature. However, strain W4977 loses almost all (94%) of its activity in 3 minutes at 37°C as does strain MS845 when tested at the same protein concentration. Both enzymes are extremely labile at higher temperatures.

Table XII Comparison of L-SD Stability in Strain W4977 and Strain MS845

Strain	Initial S.A. of Extract	S.A. after 3 min	% Remaining after 3 min
W4977	1.45	0.90	62
MS845	3.38	0.89	26

Fresh cell extracts from E. coli K_{12}, strain W4977 and strain MS845, were made and diluted 1:10 in the usual manner. A 0.05 ml aliquot of this diluted sample was added to 0.2 ml of Tris buffer pH 8.5, 0.05M. After 3 minutes at 27°C, 0.1 ml of L-serine (0.19M) was added and the amount of pyruvate produced was determined. The protein concentration of both the diluted samples were found not to differ by more than $\pm$ 0.02 mg/ml.

$$\text{\% Remaining after 3 min} = \frac{\text{S.A. (3 min)}}{\text{S.A. (0 min)}} \times 100$$

Cells from both strains were grown on +Nplg.

No definite answer to the question of whether acylation of L-SD is responsible for the differences observed in L-SD activity in the two strains can be obtained until the enzyme has been isolated and purified to homogeneity. This has as yet not been possible since the enzyme is so unstable.

(ii) Various Amino Acids

Some of the same amino acids which stabilized L-SD in fresh cell extracts of strain MS845 also stabilize fresh cell extracts of strain W4977. These include L-isoleucine, L-leucine, L-threonine, and their combinations (Table XIII). These amino acids alone and in combination show much the same pattern as that seen with strain MS845 (Table I). This data, therefore, does not give evidence that the structure of L-SD in the two strains is different.

(iii) 'Mixing' Experiments Performed with Strain W4977 and Strain MS845

L-SD from strain W4977 is considerably more stable (40%), than L-SD from strain MS845 at 27°C (Section IV, part (i)). The stability of L-SD that is observed at this temperature in strain MS845 could well arise as a result of the fact that L-SD in strain MS845 is inherently less stable (i.e. is structurally different from L-SD in strain W4977), or that there is some factor which destabilizes L-SD in strain MS845 but which is absent in strain W4977, or that there is some factor which stabilizes L-SD in strain W4977 but which is absent in strain MS845. An experiment was designed which might have been able to show which of these possibilities is responsible for the instability

Table XIII Effects of Various Amino Acids on L-SD Activity

Compound Added	Conc. in Assay ($X\ 10^{-3}M$)	Initial S.A. of Extract	S.A. after 3 min	% Protection
0	0	1.04	0.04	-
L-isoleucine	12	0.96	0.15	11
L-threonine	31	0.86	0.13	10
L-leucine	12	0.91	0.17	14
L-ileu/L-thr	12/31	0.91	0.10	6
L-leu/L-thr	12/31	0.88	0.12	9
L-leu/L-ileu	12/12	1.03	0.05	1
L-leu/L-ileu/L-thr	12/12/31	0.84	0.00	0

Concentrated cell extract of E. coli K_{12}, strain W4977, was made and diluted 1:10, in the usual manner. A 0.05 ml aliquot of this diluted sample was then added to test tubes containing L-isoleucine ($12 \times 10^{-3}M$), L-threonine ($31 \times 10^{-3}M$), L-leucine ($12 \times 10^{-3}M$), and their combinations. In one set of assay tubes, substrate was present from the time the enzyme was added and in the other set, L-serine was added after a 3 minute incubation at 37°C. The amount of pyruvate produced after 10 minutes was determined.

Table XIV Mixing Experiments Performed with Strain W4977 and Strain MS845

Time of Adding L-Serine (min)	% Initial S.A.			
	Strain MS845	Strain W4977	Strains MS845 : W4977 1 : 1	Strains MS845 : W4977 1 : 2
0	100	100	100	100
2	2	17	1	4
4	1	6	0	2
6	0	4	0	1

E. coli K_{12}, strain W4977 and strain MS845, were grown on +Nplg. Concentrated cell extracts were prepared in the usual manner. They were then diluted 1:10 or mixed (1:1 or 1:2) and then diluted 1:10, as usual. The decay of L-SD from 0 to 6 minutes at 37°C was determined. The initial S.A. of the enzyme preparation (0 minutes) for strain MS845 was 3.50; for strain W4977, 1.06; for strains MS845 : W4977 (1:1), 2.36; for strains MS845 : W4977 (1:2), 2.24.

of L-SD in strain MS845.

Concentrated fresh cell extracts of strain MS845 and strain W4977 were prepared. Various amounts of these two concentrated cell extracts were mixed (Table XIV) and the mixture was diluted 1:10 in the ususal manner. The remaining extract from each strain was also diluted 1:10. The stability at 37°C was determined for each sample. These are referred to as 'mixing' experiments.

As can be seen in Table XIV, the addition of cell extract from strain W4977 to that of strain MS845 did not, in any way, decrease the decay of L-SD from strain MS845, although cell extracts from strain MS845 may have an effect on the decay of L-SD from strain W4977. No clear cut answer could be obtained from this data. The extracts from strain MS845 and strain W4977 were sonicated separately. The homogenates were then centrifuged (see'Method') and the supernatants were used for the mixing experiments (i.e. the extracts were first mixed and then diluted). This procedure requires the manipulation of the enzyme over extended periods of time during which time the enzyme is spontaneously inactivating. Thus the decay of L-SD in the mixing experiments is not directly comparable to the decay of L-SD observed from either strain MS845 or strain W4977. This could have been prevented, in part, if each strain after washing was first measured out in wet weight added to the same beaker resuspended in phosphate buffer and then sonicated simultaneously. This would certainly have circumvented the need for the extract to sit in ice water for any length of time.

If the first mixing experiment, containing one part of L-SD from strain W4977 and one part of L-SD from strain MS845, had given an

additive effect for L-SD activity when the mixture was exposed for times varying from 0 to 6 minutes at 37°C then the cell extract of strain W4977 would have contained some factor which stabilized L-SD in strain MS845. If one-half of the activity remained, then the L-SD of strain W4977 would be structurally different from that of strain MS845. Only, the L-SD activity from strain W4977 would have been seen. If, however, no activity was observed, the cell extract from strain MS845 would have contained some factor which destabilized L-SD in strain W4977. Unfortunately, this experimental procedure was unable to provide any clear answers. Furthermore, mixing experiments, when they were performed at 27°C in the same manner, were no more successful at giving information as to why L-SD in strain W4977 appears to be more stable than L-SD in strain MS845.

Section V

AMMONIUM SULPHATE PRECIPITATION OF L-SD FROM E. COLI K_{12}, STRAIN MS845

(i) Effect of Ammonium Sulphate Precipitation on L-SD activity

Certain enzymes, which are unstable in crude cell extracts, are much more stable after purification. The first most commonly used step in purifying enzymes is precipitation by ammonium sulphate since it is highly soluble and also effective. As a first attempt at purifying L-SD from E. coli K_{12}, strain MS845, several ammonium sulphate fractions were prepared and the L-SD activity in each was assayed.

Cell extracts from strain MS845 were made 20% w/v in phosphate

Table XV. Effects of Ammonium Sulfate Precipitation on L-SD Activity

$(NH_4)_2SO_4$ Fraction (%)	g of $(NH_4)_2SO_4$ per 20 ml of Cell Extract	Total Enzyme Activity (μmoles of pyruvate per ml of extract)	Recovery of Enzyme (%)	Total Protein (mg)	Protein Pecipitated (%)	S.A.
0	0.00	78.40	-	15.60	-	5.03
0-10	1.12	1.38	2	0.20	1	6.90
10-25	1.72	19.86	25	3.36	22	5.91
25-50	3.16	24.60	31	11.40	73	2.16
50-75	2.56	0.20	0	0.60	4	0.33

E. coli K_{12}, strain MS845, was grown and collected as described in 'Method'. Concentrated cell extract was prepared 20% w/v in phosphate buffer pH 7.2, 0.05M, containing 0.026M $FeSO_4$. This was then treated with protamine sulfate (see 'Method'). Various amounts of ammonium sulfate [$(NH_4)_2SO_4$] were added to cell extract kept in ice water. The amount of L-SD activity present in each fraction was determined, as was the protein present.

buffer pH 7.2, 0.05M, containing 0.026M $FeSO_4$. These were then treated with protamine sulphate, as described in methods, to remove nucleic acids. The protamine sulphate precipitation of nucleic acids did not affect (lower) the initial amount of the enzyme activity present.

Various amounts of solid ammonium sulphate (Table XV) were added to crude cell extract kept in ice water. The ammonium sulphate did not interfere with the L-SD assay. However, only 50 to 60% of the L-SD activity was recovered after ammonium sulphate ($(NH_4)_2SO_4$) treatment. Most of the enzyme precipitated between 10 to 50% (w/v) $(NH_4)_2SO_4$ saturation. As can be seen in Table XV, the 10 to 15% (w/v) $(NH_4)_2SO_4$ fraction contained 25% of the initial enzyme activity and the 25 to 50% (w/v) $(NH_4)_2SO_4$ fraction contained 31% of the initial enzyme activity. Most of the cell protein (73%) also precipitated in the 25 to 50% (w/v) $(NH_4)_2SO_4$ fraction. No L-SD activity was present in the supernatant from the 50 to 75% (w/v) $(NH_4)_2SO_4$ fraction..

The proteins that precipitated out at 10 to 25% (w/v) $(NH_4)_2SO_4$ saturation (containing 3.36 mg of protein) and 20 to 50% (w/v) $(NH_4)_2SO_4$ saturation (containing 11.40 mg of protein) were resuspended in phosphate buffer pH 7.2, 0.05M, containing 0.026M $FeSO_4$. These samples were then dialyzed overnight at 0°C against two liters of phosphate buffer pH 7.2, 0.05M, containing 0.026M $FeSO_4$. No enzyme activity was present in either sample after dialysis. These experiments were then not pursued further.

DISCUSSION

The work presented in this thesis deals with various aspects of L-serine deaminase (L-SD) activity in crude cell extracts of E. coli K_{12}. Although this enzyme has been studied since 1938 (Gale and Stephenson, 1938), little is known about it. To date, purification of L-SD to homogeneity from cells of E. coli has not been achieved. The enzyme is extremely labile, both under in vivo (Isenberg and Newman, 1974) and in vitro conditions.

Various aspects of L-SD activity in crude cell extracts of E. coli K_{12} have been examined. These include its in vitro instability, its requirement for cofactor(s), and compounds which act as inhibitors, as activators, or as protectors of enzyme activity. These studies will be discussed in detail in the following sections.

(i) In vitro Instability of L-SD

(a) Influence of Protein Concentration

During this work, it has been possible to develop a crude cell extract system which maintained L-SD activity at a level at which it could be studied. This crude cell extract system was used to investigate various aspects of the enzyme activity.

The stability of L-SD activity is markedly dependent on protein concentration (Results: Section II, part (i)). Extracts diluted 1:10 lose only about 30% of the enzyme activity within 4 minutes whereas extracts diluted only two-fold more (diluted 1:20) lose most (93%) of the activity. Both experiments were performed at 22°C. However, the most concentrated cell extract studied even when allowed to sit in ice water, loses 40% of the enzyme activity within 4 hours. It is

clear, therefore, that extracts can be manipulated in the cold when concentrated but even under these conditions the enzyme activity is unstable.

Concentrated cell extracts are clearly unstable even at 0°C. They can, however, be stored frozen (Results: Section III, part (i)) but lose 70% of their activity in 5 days. The enzyme activity in extracts frozen and thawed is much less stable (Results: Section III, part (ii)). The frozen cell extract diluted 1:10 loses 67% of its activity in 2 minutes at 22°C whereas the fresh cell extract loses only 27% within the same time period. Some activity is lost after freezing and thawing (20%). The remaining enzyme activity is much less stable.

Since L-SD activity is more stable at high protein concentration, the fact that BSA protects the enzyme is perhaps not surprising (Results: Section II, part (iii) and Section III, part (iv)). Each test tube contained approximately 160 µg/ml of protein. The addition of 10 times more of inert protein (BSA: 1.43 mg/ml, 2.86 mg/ml) may be enough to stabilize L-SD (approximately 30%) in so far as this exogenous protein can. Higher concentrations of protein (> 1.43 mg/ml) did not further increase the protection observed. Adding BSA to test tubes containing diluted enzyme (diluted 1:20) also increases the initial S.A. (39% for fresh cell extract and 28% and 59% for frozen cell extract) observed. It is then possible that we are not measuring the total cellular activity present in the crude cell extracts.

Why is the enzyme activity in a concentrated preparation more stable? Possible explanations exist from what is known about controls

of enzyme activity. Control of enzyme activity can occur either by a change in the quantity or catalytic efficiency of the enzyme. The quantity of an enzyme can be regulated by a change in either its rate of synthesis or its rate of degradation. The former involves regulatory mechanisms that may act at the transcriptional level (i.e. induction and repression) and the latter involves regulatory mechanisms that act at the level of protein turnover.

We shall first be concerned here with regulatory mechanisms that reversibly alter the catalytic properties of enzymes. Following this discussion, we shall examine mechanisms that lead to irreversible alterations of enzymatic proteins. Both topics will be discussed as they relate to the data obtained on L-SD stability.

Regulatory mechanisms that reversibly alter the catalytic properties of enzymes can be catagorized into (1) protein-protein interactions (i.e. non-covalent and reversible interactions with similar or different polypeptides) and (2) covalent conversions (i.e. enzyme-catalyzed covalent changes).

(1) Protein-Protein Interactions

Protein-protein interactions can be found in a wide variety of biological processes (Frieden, 1971). The interactions are of two kinds: homomeric protein interactions and heteromeric protein interactions. In homomeric protein interactions only similar subunits of one protein are involved (i.e. the subunits are involved in changing their degree of dissociation or polymerization). Examples of such enzymes are acetyl-CoA carboxylase (Numa, 1974), CTP synthase (Levitzki, 1975), and beef-liver glutamate dehydrogenase (Engle and Dalziet, 1969).

Acetyl-CoA carboxylase (Numa, 1974) is under allosteric control because polymerization of the inactive monomeric form to a filamentous structure or active homomeric form is triggered by the binding of an allosteric effector, citrate or isocitrate. The substrates, ATP and UTP, dimerize CTP synthase (Levitzki, 1975). Beef-liver glutamate dehydrogenase (Engel and Dalziet, 1969) undergoes a concentration dependent polymerization-depolymerization. In dilute solutions the latter enzyme exists primarily in the dissociated inactive form. In concentrated solutions where beef-liver glutamate dehydrogenase exists in a polymerized active form, the presence of GTP or NADH (allosteric inhibitors) changes the degree of polymerization of the enzyme back to the inactive monomeric form. In heteromeric protein interactions two different proteins are involved. Their association alters the overall nature of the catalytic activity of the aggregate (Frieden, 1971). Heteromeric protein interactions are classified into two catagories: molecular conversions and non-covalent interactions. In the case of molecular conversions (Monod and Jacob, 1969) the association of two proteins, where both proteins may or may not be enzymes (i.e. have catalytic activity), bestows new catalytic properties onto the complex. Examples of such complexes where both proteins have enzyme activity are tryptophan synthetase (Kirschner and Wiskocil, 1972), anthranilate synthetase (Zalkin, 1973), and mammary gland fatty acid synthetase (Libertini and Smith, 1978). Examples of complexes where only one protein has enzyme activity and the second is a non-enzymatic polypeptide which acts as a 'specifier' of the enzyme activity (i.e. specifies substrate specificity) are RNA polymerase of prokaryotes (Duffy and Geiduschek, 1973; Horvitz, 1973;

Chamberlin, 1974) and Qβ-replicase (Wahba et al., 1974; Thelander and Reichard, 1979). In non-covalent interactions only one of the proteins which associate has enzyme activity. This enzyme activity is increased or decreased (activated or inhibited) in the presence of a second, non-enzymatic protein (Frieden, 1971). Often, if the enzyme has more than one substrate, the non-enzymatic protein component determines which substrate will bind to the active site (Frieden, 1971). Some examples of such interactions include lactose synthetase (Ebner and Magee, 1975; Powel and Brew, 1975), aspartate transcarbamylase (Schachman, 1972), and protein kinase (Reimann et al., 1971; Krebs, 1972). For a thorough discussion of each individual enzyme the reader is asked to refer to the references given herein.

Since the activity of L-SD from E. coli K_{12} (Results: Section II, part (i) and (iii)) is markedly dependent on protein concentration and since adding BSA to diluted enzyme preparations increases the initial S.A. observed it is then likely that protein-protein interactions are stabilizing L-SD. L-SD from E coli K_{12} may be a polymer composed of two or more subunits. Upon dilution, the enzyme may dissociate into its subunits, being inactive or much less active than the holoenzyme. The addition of BSA to assay tubes could prevent depolymerization of the enzyme thus stabilizing the enzyme against depolymerization and against inactivation. The increase in initial S.A. observed would then result from increased polymerization thus raising the initial S.A. observed over that found under assay conditions without this inert protein. This concentration dependent polymerization-depolymerization phenomenon is not uncommon in the literature. As stated previously, it has been observed with beef-

liver glutamate dehydrogenase (Engle and Dalziet, 1969). The enzyme is inactivated by dilution. In concentrated preparations this enzyme exists as a polymer and is active. L-SD from rat liver shows a similar inactivation pattern (Simon et al., 1973). Upon dilution this mammalian L-SD dissociates into two subunits. These subunits show no or very little enzyme activity. The dissociation by dilution can be prevented by the addition of K^+ and NH_4^+ to the enzyme preparation. The cations probably reassemble the subunits to the native enzyme thus activating the enzyme at low enzyme concentrations (Simon et al., 1973). Several investigators have independently reported that L-SD in some microorganisms is unstable upon dilution. In Bacillus cereus (Rasko et al., 1969) and in E. coli K_{12} (Alfoldi et al., 1968; Rasko and Molnar,1971) the inactivation of L-SD was found to be a function of its dilution. To date, no mechanism for this inactivation has been discerned.

The activity of L-SD from E. coli K_{12} in extracts frozen and thawed is much less stable than this same activity in fresh cell extracts (Results: Section III, part (ii)). The process of freezing and thawing may alter the conformation of the enzyme in such a way so as to make it more sensitive to denaturants. The subunits of L-SD after the process of freezing and thawing may more readily dissociate or the process of freezing and thawing, itself, may partly depolymerize the enzyme so that little extra depolymerization is necessary to completely inactivate the enzyme. This could then account for the decrease in stability observed. The addition of exogenous protein (BSA) should stabilize L-SD (Results: Section III, part (iv)) and increase the initial S.A. of the enzyme for the same reasons as the

ones given for L-SD obtained from fresh cell extracts.

(2) Covalent Conversions

Krebs and Fischer (1956) were the first to discover covalent conversions when they observed that mammalian glycogen phosphorylase can exist in two interconvertible forms, phosphorylated a (active) and dephosphorylated b (inactive). To date, several enzyme systems have been discovered which undergo enzyme-catalyzed covalent conversions. So far, five types of conversions have been found. These include phosphorylation (Krebs and Beavo, 1979), nucleotidation (adenylylation and uridylylation) (Shrake et al., 1978), ADP-ribosylation (Moss and Vaughan, 1979), methylation (Springer et al., 1979) and thiol oxidation (S-S/-SH conversions) (Buchanan et al., 1979). Each of the five covalent conversions is reversible by an appropriate enzyme(s).

Modification of peptide acceptors by NH_2- terminal addition of aminoacyl residues (Soffer, 1974; Deutch and Soffer, 1975), tyrosylations (Raybin and Flavin, 1975), and proteolysis can be included as examples of covalent conversions; however, these processes are unidirectional. Deutch and Soffer (1975) believe that the purpose of some of these alterations (aminoacylations) may be to prepare the proteins for proteolytic degradation. If this proves to be true, then these alterations are not involved in regulating the activities of enzymatic proteins. At the present time, the role of these aminoacylations and tyrosylations in living cells is unclear.

During this study, no attempt was made to determine whether L-SD from E. coli K_{12} was modified. However, we did attempt to analyze the differences in stability of L-SD from two different strains

(strain MS845 vs. strain W4977) of E. coli K_{12} differing in a particular enzyme (i.e. L-leucyl-, L-phenylalanyl-tRNA protein transferase).

Strain MS845 obtained by nitrosoguanidine treatment from strain W4977 lacks the tRNA protein transferase (Tam et al., 1978) and shows particularly high levels of L-SD activity. This high level is not further increased when cells are grown with glycine or L-leucine which normally induce the enzyme (Beeraj et al., 1978). If L-SD was an acceptor for the protein transferase molecule then L-SD may be acylated in strain W4977 and not in strain MS845. If this acylation then affects the conformation of the enzyme this might be reflected in a difference in enzyme stability.

L-SD from strain W4977 is more stable (about 40%) than L-SD from strain MS845. However, the type of experiments performed in this work (Results: Section IV, part (ii) and (iii)) were unable to show that the lack of the aminoacyl-tRNA protein transferase is in fact responsible for the difference in the L-SD stability observed in the two strains or if some other factor(s) stabilized L-SD from strain W4977.

Next, we shall examine mechanisms that lead to irreversible alterations of enzymatic proteins. Following this discussion, we shall examine how these alterations may affect the activity and stability of L-SD from E. coli K_{12}. Irreversible changes may result from limited proteolysis such as with proenzyme-enzyme and prohormone-hormone conversions and from protein degradation. Examples of limited proteolysis are the formation of serine proteases from their corresponding zymogens (Neurath et al., 1973), the cleavage of proalkaline phosphatase to alkaline phosphatase of E. coli (Inouye

and Beckwith, 1977), and the conversion of fibrinogen to fibrin (Davie and Fujikawa, 1975). Most of the above mentioned systems are well understood. The regulation in these cases is functionally, but not operationally, the same as induction of enzyme synthesis and is not involved with the control of enzyme activity. Much less well understood than the irreversible changes discussed in the above systems is protein degradation.

Degradation of proteins has been studied in some detail under protein turnover. It was believed for some time that enzymes in microbial systems unlike those in mammalian cells were completely stable or turned over slowly, that enzyme concentrations were a result of their rate of synthesis, and that the concentration of a protein could only be altered by dilution through repeated cell division rather than by protein turnover (Holzer et al., 1975). These ideas were dispelled once radioactive amino acids were used to label proteins and determine quantitatively their different stabilities. Generally, the rates of total protein turnover are lower in growing (1-8% per hour) than in resting microorganisms (5-20% per hour) (Holzer et al., 1975; Goldberg and St. John, 1976). The highest protein turnover thus far calculated (8% per hour for growing cells) is not enough to account for the L-SD instability observed in this study unless L-SD belongs to a set of enzymes that turnover particularly rapidly.

Turnover has been described as a dynamic process of intracellular protein breakdown and resynthesis (Goldberg and St. John, 1976). In mammalian cells there is a constant balance of proteins which is adjusted according to changing environmental conditions without extensive cell growth. The turnover then reflects the equilibrium

between the synthesis and degradation of the protein(s), both processes being separately controlled (Goldberg and St. John, 1976).

In 1970, Schimke suggested that the rate of degradation is different for each protein (eg. ornithine decarboxylase, hydroxymethylglutaryl CoA reductase, and actin have half-lives ($t_{1/2}$) of 11 minutes, 2 hours, and 120 days, respectively (Goldberg et al., 1976)). He proposed that the degradation rate is determined by conformation. Proteins can be degraded only if they assume one of several conformations. Different degradation rates would arise depending on the conformation of the enzyme. This would be determined by the number and nature of labile peptide bonds present in a given conformation and/or by the interaction of the enzyme with some molecule that would alter its conformation.

A wide variety of factors have been found to determine the degradation rate of a given protein. There appears to be a correlation between the size and charge of proteins and their relative rates of degradation (Schimke, 1973). The proposed correlation between the size of a protein and its rate of degradation is based on the overall greater chance of a large protein being hit by a proteolytic enzyme. It has been found that the lower the isoelectric point of a protein the higher is its turnover rate. Therefore, highly negatively charged and large proteins are essentially designed for high turnover. There are, however, many exceptions to this model so that structural (physical) features do not necessarily disclose the reason for the varied rate of turnover observed for different proteins. It is generally believed that if a protein has substrate, cofactor, or coenzyme bound to it, the protein will be protected against degradation (Katunuma, 1973;

Goldberg et al., 1976) and that denatured proteins are much more sensitive to proteolytic attack than native ones (Goldberg, 1972). These aberrant proteins are selectively eliminated from the cell.

Proteases are thought to be responsible for the degradation of proteins (Mandelstam and Halverson, 1960; Goldberg, 1972; Roberts et al., 1977). In mammalian systems three types of proteases (i.e. acidic (cathepsins), neutral, and alkaline proteases) are found (Segal, 1976). The acidic proteases are located in the lysosome and are active at acidic pH's. Proteins are degraded in the lysosome and the amino acids are transferred to the cytosol. Neutral proteases are located in the cytosol as are alkaline proteases. The latter activity is also associated with membrane fractions. Only alkaline and neutral proteases are present in E. coli cells (Goldberg and St. John, 1976). Their localization within the cell appears analogous to that of alkaline and neutral proteases of mammalian cells .

Since proteases are responsible for protein degradation and since the stability and activity of L-SD from E. coli K_{12} in crude cell extracts is noticeably affected by protein concentration then BSA or protein concentration can help to stabilize L-SD by protecting this enzyme from being attacked or degraded by a protease(s). With increasing amounts of protein present in the preparations (Results: Section II, part (i) and (iii)) the protease(s) has more protein to act on and is less likely to attack this enzyme. This can account for the increase in L-SD stability and activity observed with increased protein concentration.

When these same extracts of E. coli K_{12} are frozen and thawed the enzyme activity is much less stable (Results: Section III, part (ii)).

The process of freezing and thawing may alter the conformation of L-SD in such a way so that it is now more susceptible to protease attack. The addition of exogenous BSA (Results: Section III, part (iv)) would stabilize L-SD and increase the initial S.A. of the enzyme for the same reason as the one given for fresh cell extracts.

(b) Influence of Protease Inhibitors

Since L-SD in crude cell extracts of E. coli K_{12} is very unstable the possibility was considered that masked proteolytic activity may be activated upon disintegration of whole cells by sonication. For this reason protease inhibitors were examined for their effects on L-SD stability in crude cell extracts of E. coli K_{12}. None of the protease inhibitors at the concentrations tested stabilized L-SD.

During this study, aprotinin, soybean trypsin inhibitor, and phenylmethylsulfonylfluoride were examined for their effect on L-SD stability in crude cell extracts of E. coli K_{12} (Results: Section II, part (viii) and Section III, part (vii)). Aprotinin is known to inhibit kallikrein (Fritz et al., 1967; Fritz et al., 1979), trypsin (Colman and Bagdasarian, 1976; Vogel and Werle, 1970; Fritz et al., 1979), and chymotrypsin (Fritz et al., 1979). Kallikreins are serine proteases found in the pancreas, submandibular glands, urine (kidney), colon etc. of mammals. They exist in these tissues as inactive proenzymes and are activated upon limited proteolysis. Soybean trypsin inhibitor (type 1 - S also called Kunitz soybean trypsin inhibitor (SBTI)) is known to inhibit trypsin but not chymotrypsin (Birks, 1976) and phenylmethylsulfonylfluoride inhibits serine proteases (Proutry and Goldberg, 1972). Of the three protease inhibitors

added to crude cell extract preparations only soybean trypsin inhibitor was tested at comparable concentrations to those used in other studies on protein degradation in E. coli (Pacaud and Uriel, 1971; Pacaud et al., 1976; Pacaud and Richaud, 1975; Strongin et al., 1979). In fact, there was no mention of the use of aprotinin in any study involving degradation of proteins in microorganisms. A large variety of serine protease inhibitors (e.g. diisopropyl fluorophosphate, phenylmethane sulfonyl fluoride and toluenesulfonyl fluoride) and trypsin inhibitors (e.g. aromatic diamidines, dibromopropamidine, pentamidine, and tosyllysine chloromethylketone) are available, several of which could have been tested for their effects on L-SD stability in crude cell extracts of E. coli K_{12}. It is not uncommon for a protease to respond to different degrees to each protease inhibitor from a given class of protease inhibitors. Protease I (Pacaud and Uriel, 1971), protease II (Pacaud and Richaud, 1975), and ISP-A-Eco (Strongin et al., 1979) of E. coli are all inhibited by diisopropylfluorophosphate but not by phenylmethylsulfonylfluoride. Both chemicals are serine protease inhibitors. They react covalently with a serine at the enzyme active site (Gladner and Laki, 1958). During this study, it would have been worthwhile to prepare the crude cell extracts of E. coli K_{12} in the presence of any of a broad variety of protease inhibitors presently available (Colowick and Kaplan, 1976). Certainly more than one protease inhibitor should have been examined in this regard. In summary, one must conclude that the experiments in this section were incomplete.

The existence of proteolytic activity in bacterial cells has

been known for sometime (Goldberg and St. John, 1976), however, very few studies on purified bacterial intracellular proteases can be found in the literature. Intracellular serine proteases have been purified from Bacillus sp (e.g. Bacillus subtilis, strain A-50, (Stepanov et al., 1977) and Bacillus thuringiensis var. berliner (Lecadet et al., 1977)). Their involvement in protein turnover, cellular distribution, and other properties have been determined in these species. Although E. coli is the most widely studied microorganisms, limited knowledge is available about its proteolytic system. This is due to the fact that the isolation of proteases from E. coli cells presents some problems. The proteases are present at extremely low concentrations within the E. coli cells and there is little knowledge about their substrate specificities. To date, some five proteases have been purified to homogeneity from extracts of E. coli. The chymotrypsin-like protease I (Pacaud and Uriel, 1971; Pacaud et al., 1976) and trypsin-like protease II (Pacaud and Richaud, 1975) have been studied in some detail. The three other proteases, ISP-A-Eco, ISP-L-Eco, and protease III have been only recently purified to homogeneity and little information is available about them. ISP-A-Eco (Strongin et al., 1979) is a serine protease. It has been found to be similar in its properties to protease II (Pacaud and Richaud, 1975). Both enzymes are completely inactivated by diisopropylfluorophosphate whereas phenylmethylsulfonylfluoride is without any inhibitory effect. Chelating agents (EDTA and EGTA (ethyleneglycol-bis (β-aminoethylether) N, N'-tetraacetic acid) and soybean trypsin inhibitor do not affect their activities. It has been suggested that protease II and ISP-A-Eco may be one and the same enzyme

(Strongin et al., 1979). ISP-L-Eco is also a serine protease and is similar in properties to the serine protease isolated from Bacillus subtilis, strain A-50 (Strongin et al., 1979). Both proteases show the same substrate specificity (i.e. rapidly hydrolyse N-benzoyl-DL-arginine-p-nitroanilide) and both are completely inactivated by diisopropylfluorophosphate or phenylmethylsulfonylfluoride and EDTA or EGTA. ISP-L-Eco has a strict requirement for Ca^{++}. This protease is stable and can be stored for sometime (weeks) at 4°C. Protease I (Pacaud and Uriel, 1971) hydrolyses N-acetyl-DL-phenylalanine-2-naphthyl ester, a chymotrypsin substrate. The enzyme is inhibited by diisopropylfluorophosphate but is resistant to phenylmethylsulfonyl-fluoride. Metal chelating agents, sulfhydryl agents, and several metal ions (Ca^{++}, Cu^{++}, Hg^{++}, Zn^{++}, Pb^{++}, Mn^{++}, Fe^{++}, or Mg^{++}) have no effect on the enzyme activity. Protease III (Cheng and Zipser, 1979) requires a divalent metal ion (Zn^{++}, Co^{++}, or Mn^{++}) for activity and is very active against small molecular weight proteins (< 7,000 MW) (e.g. auto α and insulin B chain). The enzyme is sensitive to dithiothreitol. It has been proposed that protease III may be the major enzyme involved in the degradation of small proteins in crude cell extracts (Cheng and Zipser, 1979). Another protease, protease A, has been isolated and partially purified by Regnier and Thang (1975) from crude cell extracts of E. coli. The enzyme is most active at pH 9. Its enzyme activity is sensitive to EDTA and resistant to dithiothreitol. The EDTA-inhibited protease A can be reactivated by Mg^{++}, Mn^{++} or Ca^{++} but not by Zn^{++}. These same authors (Regnier and Thang, 1972; Regnier and Thang, 1979) have been able to demonstrate that proteolytic activity is greatly enhanced by sonication and that

this activity is primarily (70 to 90%) localized in the outer membrane of E. coli. It is not known what membrane-bound protease(s) is responsible for this proteolytic activity. Another interesting feature of intracellular protein degradation is its apparent energy requirement (Murakami et al., 1979; Goldberg and St. John, 1976). Recently, Murakami et al. (1979) examined the rapid degradation of abnormal proteins in E. coli by an energy-dependent process. ATP was found to stimulate the hydrolysis of methylapohemoglobin. EDTA and diisopropylfluorophosphate strongly inhibit this degradation process. N-ethylmaleimide, a sulfhydryl inhibitor, causes some inhibition. This inhibition is increased in the presence of ATP. It is not known what proteolytic enzyme(s) is involved in this degradation system. Although several proteases have been purified from E. coli cells their role in the proteolytic system of these cells is unclear. At the present time, there is no correlation between the degradation of a given protein and the types or number of proteases involved in that degradation. In fact, it is not known how many proteases are present within an E. coli cell.

(c) Influence of Various Amino Acids

Twenty-three amino acids were examined for their ability to affect L-SD stability in crude cell extracts of E. coli K_{12}. Several allow the retention (from 7 to 24%) of the enzyme activity. L-Serine is a very effective protector of the enzyme since the reaction with L-serine at 37°C is linear for 24 minutes; without substrate the enzyme activity is lost in 4 minutes. That D-serine then protects may not be surprising though many enzymes do discriminate strongly among

stereoisomers. The presence of the α-amino group (the α -imino group of L-proline) of the various amino acids (Results: Section II, part (ii)) appears to be sufficient for the enzyme to interact with them. The inability of DL-serine methylester to protect L-SD suggests that the carboxyl group may be involved in the binding of L-serine to the active site since this compound differs from serine only in that it has a methyl group attached at the carboxyl end. That sodium acetate and glycine fail to protect the enzyme may not be surprising since they lack the β-carbon one of the carbons on which the enzyme probably acts.

When combinations of L-isoleucine, L-leucine, and L-threonine are used in cell extract preparations, an interesting and consistent result is obtained (both for fresh and frozen cell extracts). A combination of L-threonine with either L-isoleucine or L-leucine has about the same effect as any of these tested alone (Results: Section II, part (ii)). When both L-isoleucine and L-leucine are present with or without L-threonine, no protection is observed. Therefore, the combination of L-isoleucine and L-leucine cancels the effect produced by either of these compounds alone.

This event may be attributed to any one of several explanations. Both L-isoleucine and L-leucine may bind to the enzyme in such a manner that the conformation of L-SD is so altered that the enzyme is no longer stabilized by these amino acids; neither bind to the enzyme because both are competing for the same binding site (as in the case of competitive inhibition) and are therefore antagonistic to one another; or since the study deals with crude cell extract, the possibility cannot be dismissed that these amino acids (any of the

23 tested) may be affecting some other component other than L-SD which in turn affects L-SD activity.

Since L-serine is the most effective stabilizer of L-SD, crude cell extracts can be prepared in the presence of L-serine. The enzyme may then possibly be purified by passing the crude cell extract through a L-serine-agarose affinity column. The enzyme should bind to the immobilized substrate-agarose particles. The protein could then be eluted specifically with L-serine in phosphate buffer. Although such an experiment was never performed during this study, it may be used for future purification studies on L-SD.

The ability of various amino acids to affect L-SD stability from various sources has been examined in a number of studies. In 1968, Alfoldi et al. reported that L-SD from E. coli was sensitive to dilution and sonication. Saturating concentrations of D- or L-serine were found to protect the enzyme from inactivation. However, these two amino acids could not protect the enzyme during purification procedures. Rasko and Molnar (1971) reaffirmed that when L-SD from E. coli [Strains E. coli HfrC (Met^-) and E. coli HfrC (Met^-, $ILeu^-$). S - 2] was incubated in an assay mixture containing high L-serine concentration (40 - 50 µg/ml), the diluted enzyme (diluted 100 to 200-fold) was protected against inactivation. Subsequently, Carter and Sagers (1972) were able to show that L-serine or glycine could stabilize L-SD from Clostridium acidiurici during dialysis, possibly by occupying the active site. All these studies were performed on preparations which contained different degrees of purity of the L-SD molecule. Studies on preparations where the enzyme is not pure are subject to the same limitations as described for our studies on crude

cell extracts.

(ii) Cofactor Requirement

The maintenance of enzyme activity and/or conformation may require the presence of various cofactors (Lehninger, 1975). A cofactor can be a metal ion (e.g. Fe^{++} of carbonic anhydrase (Gibbons and Edsall, 1963)) or an organic molecule referred to as a coenzyme (Lehninger, 1975) (e.g. lipoic acid and thioctic acid which participate in the enzymatic oxidative decarboxylation of α-keto acids between thiamine pyrophosphate and coenzyme A (Guirard and Snell, 1964)). A distinction between a prosthetic group and a coenzyme (i.e. cofactor) is often made. Prothetic group refers to a cofactor which is tightly bound to the enzyme (Lehninger, 1975). Its loss may render the protein inactive and would then be an absolute requirement for enzyme activity (e.g. pyridoxal phosphate in L-SD of Clostridium acidiurici (Carter and Sagers, 1972)). Some enzymes require the involvement of both a cofactor and a coenzyme for expression of the enzyme's catalytic activity. Serine hydroxymethyltransferase is an example of such an enzyme. It requires both pyridoxal phosphate and tetrahydrofolate (THF) for activity (Schirch and Mason, 1963). A cofactor may be used (a) to stabilize the conformation of the enzyme thus rendering it catalytically active; (b) to bind substrate and enzyme; or (c) may be part of the catalytic center (Lehninger, 1975). In the case of uridine diphosphogalactose-4-epimerase, uridine diphosphate glucose acts both as coenzyme and substrate (Kalckar, 1957). Coenzymes generally serve as acceptors or donors of functional groups, of atoms, or of electrons that are

transferred in the overall reaction (Lehninger, 1975).

The cofactor requirement of L-SD has been studied in many systems; unfortunately, not always on purified enzyme preparations making any clear deductions sometimes hazardous. In non-homogeneous systems, it is difficult to determine whether (a) the cofactor is tightly bound to the enzyme and is not released despite its extraction from the cell; (b) the cofactor is present in the extract at a sufficient concentration to maintain enzyme activity; or (c) the enzyme does not require a cofactor for activity. For example, while L-SD from mammalian cells (Selim and Greenberg, 1959; Goldstein et al., 1962; Simon et al., 1973; Hunter and Harper, 1976) and from Clostridium acidiurici (Carter and Sagers, 1972) requires pyridoxal phosphate as a cofactor, it is not a requirement in the assay for L-SD from E. coli. Part of our intention is to clarify the role of pyridoxal phosphate with respect to L-SD from E. coli K_{12}.

While the addition of exogenous pyridoxal phosphate has no effect on L-SD activity, it may nonetheless be tightly bound to the enzyme. Pyridoxal phosphate may not be released from the molecule despite the enzyme's extraction from the cell. This is a common enough phenomenon with transaminases and decarboxylases (Guirard and Snell, 1964). When an enzyme is available in purified form, various reagents (aminothiols, sodium borohydride, ketonic, and reducing agents) can be added which release pyridoxal phosphate from the protein molecule. If the enzyme is pyridoxal phosphate requiring, it is inactivated by these compounds (Guirard and Snell, 1964). Since L-SD cannot as yet be purified, no such attempts were made here.

The addition of pyridoxal phosphate (1 mM) to the assay mix

containing L-SD purified 970-fold from Arthrobacter globiformis (Gannon et al., 1977) did not increase the activity of the enzyme. The same result was obtained when inactivated enzyme was preincubated with pyridoxal phosphate for 30 minutes prior to assay. The activity of L-SD from Clostridium acidiurici (purified 400-fold) (Carter and Sagers, 1972) did not increase or decrease upon the addition of pyridoxal phosphate (1 mM) to the assay tube. However, spectroscopic methods indicated the presence of pyridoxal phosphate within the enzyme. Treating L-SD with sodium borohydride resulted in the loss of most (90%) of the activity of the enzyme. Sonicated samples from E. coli K_{12} (Alfoldi et al., 1968) containing L-SD activity showed no apparent cofactor requirement. The addition of pyridoxal phosphate or other possible cofactors (e.g. adenosine triphosphate, nicotinamide, adenosine dinucleotide, glutathione, or vitamin B_6) did not alter the enzyme activity.

Several other studies were able to show a pyridoxal phosphate involvement in L-SD activity. In Neurospora crassa (Yanofsky and Reissing, 1953) the addition of 4×10^{-7} M pyridoxal phosphate to inactivated enzyme was required to restore the activity of the enzyme to one-half maximal velocity. Griffiths and deMoss (1970) added 40 n moles of pyridoxal phosphate to each assay tube containing toluene-treated whole cells of Bacillus alvei, although providing no explanation for this addition.

During this study, cells were grown in the presence of pyridoxine, (Results: Section II, part (vi)). In this way, the internal pool of pyridoxal phosphate might be affected. Pyridoxine being an unphosphorylated form of vitamin B_6 can be taken up by

E. coli cells whereas pyridoxal phosphate cannot unless it is dephosphorylated (Yamada et al., 1977). Cells grown in the presence of pyridoxine show a tremendous increase in initial S.A. (approximately 2.6 times). This is the most striking result observed in this work and would then seem to imply that pyridoxal phosphate is involved in the expression of L-SD activity. Though pyridoxine is able to increase the initial S.A. of L-SD, it is unable to stabilize it.

A comparison of L-SD activity from toluene-treated whole cells and cell extract was made. No difference in the initial S.A. of cells grown with or without pyridoxine can be observed when toluene-treated whole cells are used in the L-SD assay. A comparison of the two assays (using toluene-treated whole cells and cell extract) reveals that the increase in initial S.A. observed with cell extract probably results from the fact that L-SD obtained from E. coli cells grown without pyridoxine is more labile to sonication (Results: Section II, part (vi)). By comparing % recovery (in S.A.) of another enzyme (e.g. phosphofructokinase) to that of L-SD when E. coli cells grown with and without pyridoxine and subsequently subjected to sonication, one can determine if L-SD is indeed less labile to sonication when grown with pyridoxine. While such a comparison was never made during this study, it could, however, be used in future examinations of the pyridoxine phenomenon.

It is then inviting to postulate the following: pyridoxal phosphate must be incorporated into the enzyme (L-SD) during assembly and is not released from the molecule despite its extraction from the cell. Pyridoxine would then increase the amount of pyridoxal phosphate available within the cell for assembly into L-SD. If this indeed were

true, one would expect L-SD activity to also increase in toluene-treated whole cells grown with pyridoxine. However, this is never observed. Since the study deals with a crude cell extract, pyridoxine may affect some component other than L-SD which in turn affects the initial activity of the enzyme at the time of sonication. Addition of pyridoxine or pyridoxal phosphate to crude cell extracts at any time after sonication has no effect on L-SD activity or on L-SD stability.

The E. coli cells used during this study cannot be pyridoxine auxotrophs since they grown normally in the absence of pyridoxine. Their growth rate is not increased by addition of exogenous pyridoxine to the growth media. At the moment, this inconsistance cannot be resolved.

Many enzymes require a metal ion for activity. In some cases the requirement is specific for a particular metal (e.g. carbonic anhydrase for Zn^{++} and pyruvate phosphokinase for Mg^{++} and K^{+}). None of the deaminases from microorganisms thus far studied show an absolute requirement for metal ions; however, many of the enzymes are stimulated by monovalent or divalent cations. Mn^{++} and Mg^{++} increase D-serine deaminase activity from chicken kidney (Grillo et al., 1965) and NH_4^{+}, K^{+}, Li^{+}, and Na^{+} increase L-threonine deaminase activity from Salmonella typhimurium (Burns and Zarlengo, 1968). L-SD from Arthrobacter globiformis (Gannon et al., 1977) shows a nonspecific requirement for a univalent or bivalent cation (e.g. Na^{+}, K^{+}, Mg^{++}, and Ca^{++}). This enzyme may simply require a higher ionic strength for stability. In Clostridium acidiurici, ferrous ion (Fe^{++}) has been shown as a requirement for L-SD activity (Carter and Sagers, 1972).

With the last point in mind, the effect of ferrous ion on L-SD activity from E. coli K_{12} was then examined (Results: Section II, part

(v)). The metal ion was found to protect L-SD activity (about 13%) and to increase the initial S.A. (between 7 and 35%). Its greatest effect appears to be when cell extracts are made in the presence of ferrous ion. Adding ferrous ion to the assay tube does not affect L-SD activity to any great extent (the % protection increases from about 8% to some 13%).

A low concentration of ferrous ion ($6x10^{-3}$M to $17x10^{-3}$M as compared to 0.1M (Carter and Sagers, 1972) is needed to affect L-SD activity. This may indicate that the role of ferrous ion is to activate or participate at the catalytic center rather than to stabilize the tertiary structure of L-SD. Since crude cell extract was used, it is also possible that ferrous ion is affecting some other component other than L-SD which modifies the activity of L-SD.

In frozen cell extracts, the initial activity of L-SD is again increased (about 60%) (Results: Section III, part (v)). This increase is, however, greater (30%) than that observed in fresh cell extracts. The % protection is also greater (8% vs. 15%). The process of freezing and thawing possibly alters the conformation of the enzyme in such a manner so that L-SD becomes more susceptible to interaction with ferrous ion or perhaps the ferrous ion effectively counteracts the dissociation of L-SD into subunits (refer to '<u>in vitro</u> Instability', part (a)). This study was performed with crude cell extract. The possibility then cannot be excluded that ferrous ion is acting on some component other than L-SD which affects L-SD activity. This component may then be affected by freezing and thawing.

Since these studies are dealing with crude cell extracts, saturating levels of cofactor(s) may already be available in our preparations or the cofactor may remain bound to the enzyme despite its extraction

from the cell. That there should be different cofactor requirements for L-SD in various organisms is not surprising. Conclusive evidence about cofactor requirements for L-SD from E. coli K_{12} cannot be obtained until the L-SD molecule has been purified to homogeneity.

(iii) Inhibitors of L-SD activity

Studies of inhibitors can be used to determine what compounds inhibit the enzyme activity of a given protein and then such studies can be used to characterize the means of that inhibition (Plowman, 1972). In this discussion, inhibition of enzyme activity will be taken to mean that the initial S.A. of the enzyme in the cell extract preparation is less in the presence of compound as compared to without compound.

There are two kinds of enzyme inhibition, reversible and irreversible (Lehninger, 1975). Reversible inhibition can be divided kinetically into three types: competitive, un-competitive, and non-competitive. In competitive inhibition, the inhibitor competes with the substrate for binding to the active site of the enzyme. This inhibition can readily be reversed by increasing the substrate concentration. In un-competitive inhibition, the inhibitor combines rapidly and reversibly with the enzyme-substrate complex. This inhibition is, however, not reversed by elevating the substrate concentration. Un-competitive inhibition is commonly seen with two-substrate reactions but not with a one-substrate reaction. For this reason, we will not be concerned, here, with this type of inhibition. In non-competitive inhibition, the inhibitor combines with either the free enzyme or enzyme-substrate complex. Such inhibitors bind to a site other than the substrate binding site (i.e. allosteric site).

The activity of the enzyme cannot be restored by increasing the substrate concentration. Irreversible inhibitors (i.e. irreversible inhibition) inactivate the enzyme molecule by permanently modifying one or more functional groups which are required for activity.

Metal ions play an important role in biological processes (Mahler and Cordes, 1971). They are capable of associating with variously sized molecules. Oxygen, nitrogen, and sulfur are the atoms to which metal ions typically bind in proteins. All amino acids are capable of binding metal ions. In particular, cysteine and histidine are capable of forming in the presence of a metal ion a variety of metal complexes. These two amino acids are, therefore, thought to be involved in metal ion-protein complex formation (Mahler and Cordes, 1971). In many cases (i.e. many proteases (e.g. carboxypeptidase)) the loss of a metal ion from a protein renders that protein enzymatically inactive (e.g. metalloproteins). In other cases, enzymes are inactivated by metal ions (e.g. pyruvate dehydrogenase$_a$ (PDH_a) kinase by Mg^{++}, Ca^{++}, and K^+ (Krebs and Beavo, 1979)). There is no general model of action of metal ions that can be used to conclusively predict the effect of metal ions on a given protein. At present, it is not known whether the L-SD molecule from E. coli is directly affected by a metal ion(s).

EDTA which is an active chelating agent of metal ions was found in this study (Results: Section II, part (iii)) to protect L-SD activity in extracts of E. coli K_{12}. This suggests that the L-SD molecule may be sensitive to some metal ion(s). No attempt was made to determine which metal ion(s) may be involved. When EDTA is added to cell extracts in the presence of BSA, the extract behaved as it does when only BSA is present. This result is rather surprising. It may

be that the BSA preparation contains some component which binds EDTA so that only the BSA effect is observed or it may be that BSA, like EDTA, binds metal ions - BSA is a polyelectrolyte. Perhaps a more likely explanation is that EDTA is affecting a metalloprotease. If BSA protects L-SD by providing more protein for a protease to act on thus making it less likely that it will attack the L-SD molecule and if this same protease requires a metal ion for activity then applying both BSA and EDTA to the crude cell extract should yield an additive effect. However, EDTA and BSA were added to the preparations at concentrations that produced maximum protection of L-SD (approximately 30% each). If BSA and EDTA affect the same enzyme (i.e. a protease) to the maximum extent each can (i.e. each compound protects L-SD from the protease a 100%) then adding them simultaneously into the cell extract preparation would not produce an additive effect. We should have attempted combinations of EDTA and BSA at concentrations where each compound protects L-SD to a lesser degree (say 15% each). If these two compounds then affect the same enzyme and if they do not act by the same mechanism their addition to the preparation should yield an additive effect (i.e. 30%). There is ample evidence in the literature which supports the idea that many proteases found in E. coli may be metalloenzymes. Certainly, the recently isolated ones, such as ISP-L-Eco and ISP-A-Eco (Strongin et al., 1979), protease I (Pacaud and Uriel, 1971; Pacaud et al., 1976), protease II (Pacaud and Richaud, 1975), and protease III (Cheng and Zipser, 1979) all appear to be metalloproteases. Several metal ions (i.e. Cu^{++}, Pb^{++}, or Hg^{++} at 0.1 μ moles/ml) inhibit L-SD activity from Clostridium acidiurici (Benziman et al., 1960) The metal ion inhibition was

completely overcome with the addition of EDTA to cell extracts which were prepared by crushing frozen cells in a Hughes press at -20°C and contained partially purified L-SD. L-SD from Arthrobacter globiformis (Gannon et al., 1977) is inhibited by $HgCl_2$ (5 μM). The inhibition is not instantaneous but rather it occurs progressively over a period of 4 minutes. This inhibition could be partially overcome by L-cysteine (1 mM). Concentrations above 40 mM $MgCl_2$ were also inhibitory. The activity of L-SD decrease 50% of its maximum value at 80 mM $MgCl_2$. Ag^+, Cu^+, and Hg^{++} (at 10^{-5}M) inhibit L-SD activity from Crookes strain of E. coli and the enzyme is not reactivated by glutathione (Wood and Gunsalus, 1949). None of these studies could determine the relationship between the metal ions and L-SD activity.

Since a common cause of enzyme inactivation is oxidation of sulfhydryl groups (-SH) to disulfide-cross-links (S-S) or to sulfate ($-SO_4$) , three sulfhydryl reagents were tested for their effect on L-SD activity (Results: Section II, part (iv)). These reagents interact with thiol groups that are necessary to maintain the enzyme-active conformation (Cleland, 1964). DTT and DTE appear to inhibit the enzyme. These compounds, however, were found to reduce DNPH (Carter and Sagers, 1972). Their effect on the L-SD activity could, therefore, not be assessed with the type of assay used in this work. MSH has no effect on L-SD activity at the concentration tested.

The inhibitors (EDTA and sulfhydryl reagents) mentioned above affect the enzyme activity of a wide variety of enzymes. Other inhibitors which are more specific for L-SD have been examined in this study and by other investigators. Glycine and D-serine inhibit L-SD activity in Crookes strain of E. coli (Wood and Gunsalus, 1949), in

E. coli strain B (Pardee and Prestidge, 1955), and in E. coli K_{12} (Alfoldi et al., 1968; Isenberg and Newman, 1974). L-Cysteine has an inhibitory effect in extracts of E. coli K_{12} (Alfoldi et al., 1968). L-SD from Corynebacterium sp (ATCC 21050) (Morikawa et al., 1974) is inhibited by L-cysteine, L-alanine, D-serine, and L-tryptophan. In Arthrobacter globiformis (Gannon et al., 1977) L-cysteine and D-serine act as competitive inhibitors of L-SD. L-Cysteine at concentrations greater than 4.9 mM caused a slow irreversible inhibition of L-SD.

In this study, L-cysteine and L-arginine inhibited L-SD activity (Results: Section II, part (ii)). L-Cysteine differs from the substrate, L-serine, in that it has a sulfhydryl group attached at the β-carbon in place of a hydroxyl group. Almost all (99%) of the enzyme activity is lost in the presence of L-cysteine. This amino acid may reduce a sulfhydryl group at the active site which is essential for maintaining the enzyme-active conformation or it may be competing with L-serine for the active site on the enzyme. L-Arginine inhibits L-SD activity by 26%. It resembles L-serine only in that it has the α-amino group. It, therefore, seems unlikely that this amino acid would bind at the active site on L-SD. L-Arginine may interact with the enzyme at a site other than the active site (i.e. at an allosteric site). By the binding of this amino acid the conformation of L-SD may be altered in such a way so that the enzyme can no longer utilise its substrate, L-serine, with the same efficiency as the native enzyme. As these studies were performed in crude cell extracts, the effect of the amino acids or metal ions (susceptibility to EDTA) could then be on some component other than L-SD which in turn affects L-SD activity. Definite answers as to how L-cysteine and L-arginine

interact with the L-SD molecule can only be obtained once the enzyme has been purified to homogeneity.

(iv) Activators of L-SD activity

In the context of this thesis, the terms activation of enzyme activity (activators), increase in initial S.A. of the enzyme or increase in enzyme activity, and stimulation of enzyme activity are meant to include specific and nonspecific reagents which increase enzyme activity either by converting or maintaining the enzyme in its active form.

The well known role of metal ions as necessary components for enzyme activity was discussed previously. Deaminases from several microorganisms are stimulated by monovalent and divalent ions. L-Threonine deaminase activity from Acetobacter suboxydans is increased when Mg^{++} (10 μ moles) is present in the assay system (Kerwar et al., 1964). In E. coli, D-serine deaminase activity is stimulated by K^{+} (10 mM) and NH_4^{+} (10 mM) (Dupourque et al., 1966). D-Serine deaminase from chicken kidney (Grillo et al., 1965) is activated by Mg^{++} (1.4×10^{-2}M) and Mn^{++} (1.1×10^{-4}M). L-SD activity from Arthrobacter globiformis (Gannon et al., 1977) is increased by a univalent or bivalent cation and L-SD activity from Clostridium acidiurici (Carter and Sagers, 1972) is increased by ferrous ion (1 mM). In this study, L-SD from E. coli K_{12} also shows increased activity in the presence of ferrous ion. A detailed discussion of this result can be found in the 'Cofactor Requirement' section.

Some of the recorded activations of enzyme activity by nonspecific reagents will be discussed. In 1951, Cori et al. reported that

phosphofructokinase in liver homogenates is activated by glutamate. Veibel (1937) noted that toluene activated β-galactosidase. The activation of nitrate reductase by inorganic phosphate (Kinsky and McElroy, 1958), and of dehydroascorbic reductase by inorganic phosphate (Pi) and by arsenate (Yamaguchi and Joslyn, 1951) have been described. The well known protection of enzyme activity in dilute solution by BSA, so that an increase in enzyme activity is observed, can also be included here (e.g. glyoxylase (Racker, 1959)).

In this study, several nonspecific reagents were found to increase the initial S.A. of L-SD from extracts of E. coli K_{12}. BSA, when added to diluted extracts, increases L-SD activity. A thorough discussion of this phenomenon can be found under 'in vitro Instability, part (a)'. Various polyelectrolytes also increase the enzyme activity but are unable to stabilize the L-SD molecule. Ethylene glycol and glycerol are commonly added to crude enzyme preparations during purification procedures. These compounds appear to be most effective with enzymes that are particularly sensitive to temperature and protein concentration (Thach and Newberger, 1972). Both compounds were found to interfere with the assay and could not therefore be assessed for their effect on L-SD activity with the type of assay used in this work.

Because ethylene glycol, glycerol, and the sulfhydryl reagents, DTT and DTE (Results: Section II, part (iv)), were found to interfere with the L-SD assay used in this study, an alternative assay must be used to examine the effect these compounds have upon L-SD activity and stability. To circumvent the interference, the L-SD assay used in this study could be substituted for by one coupled to L-lactate

dehydrogenase (LDH) (Von Korff, 1969). LDH catalyzes the following reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$$

In such an assay, L-SD activity is measured indirectly by monitoring the reduction of pyruvate to lactate by following the oxidation of NADH to NAD^+ as NADH but not NAD^+ absorbs strongly at 340 mu (i.e. decrease in absorbance at 340 mu).

SUMMARY

The work in this thesis deals with various aspects of L-SD activity in crude cell extracts of E. coli K_{12}. L-SD catalyzes the conversion of L-serine to pyruvate and ammonia. Although the enzyme has been studied for some time, limited information is available about it. This is due to the fact that this enzyme is unusually unstable both under in vivo and in vitro conditions.

During this study, an enzyme assay system was developed which maintained L-SD activity at a level at which it could be studied. This assay was used to investigate various aspects of the enzyme activity.

L-SD was found to be extremely unstable in crude cell extracts of E. coli K_{12}. These cell extracts could, however, be stored frozen for some time (days). Various factors were found to stabilize, inhibit, or activate L-SD activity. Several factors stabilized L-SD (i.e. concentration, substrate, certain amino acids, EDTA, and ferrous ion). Other components (i.e. pyridoxal phosphate, β-mercaptoethanol, polyelectrolytes, and certain protease inhibitors) had no effect but somehow when cells were grown in pyridoxine the activity of L-SD was increased but not stabilized. The increase in initial S.A. observed with crude cell extracts most likely resulted from the fact that L-SD obtained from cells grown with pyridoxine was less labile to sonication. Some factors were found to inhibit (i.e. L-cysteine, L-arginine) and activate (i.e. pyridoxine, ferrous ion) the initial S.A. of L-SD. The in vitro instability of L-SD was considered in terms of the organizational structure of the enzyme

(i.e. possible subunit structure) and in terms of proteolytic activity (i.e. protease(s) acting on L-SD). Studies of inhibitors and activators were considered in terms of alterations in protein structure of L-SD upon its association with various ligands.

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