STUDIES ON THE CHARACTERISTICS AND METABOLIC ROLE OF L-SERINE DEAMINASE IN ESCHERICHIA COLI

by

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ABSTRACT

L-serine deaminase has been shown to be a distinct entity, different from L-threonine deaminase. The enzyme has been shown to be specific for L-serine. Its pH optimum, cofactors, and inhibitors have also been studied.

L-serine deaminase activity exists in substantial amounts in cells grown in minimal medium. It is increased by growth in the absence of inorganic nitrogen and in the presence of certain amino acids, in particular L-glycine and L-leucine. The enzyme is unstable *in vivo* as well as *in vitro*. In vivo instability is seen most markedly in the presence of the inducers of the enzyme.

These results are analyzed in terms of the possible metabolic role of the enzyme as well as the possible mechanisms of its induction.

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INTRODUCTION

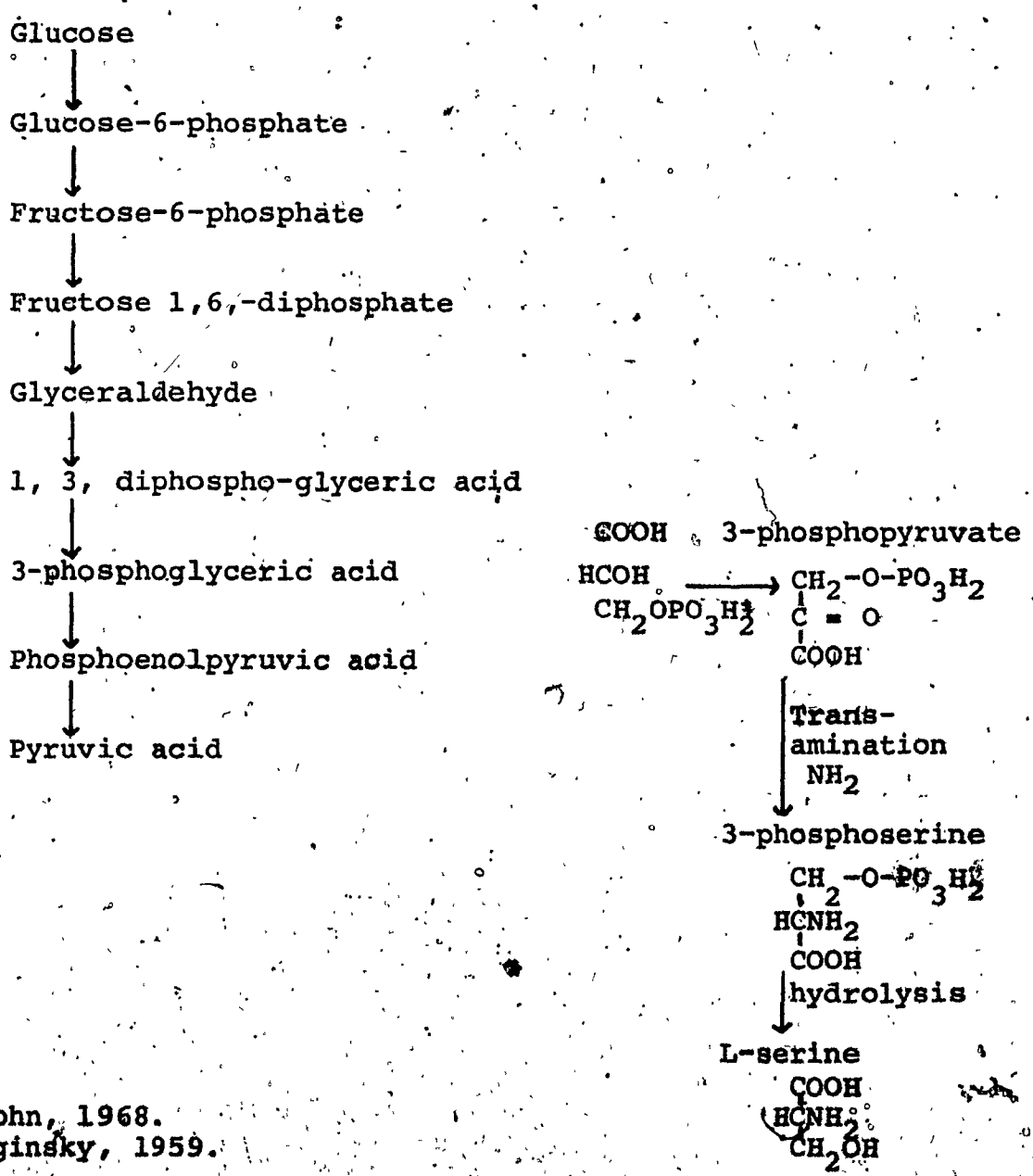
This thesis is concerned with the role of L-serine deaminase in the metabolism of E. coli K_{12}. The biosynthesis and further metabolism of L-serine is reasonably well understood. However, why there should be an enzyme which converts L-serine to pyruvate is not obvious.

L-serine is synthesized from an intermediate of the Embden-Myerhoff pathway, 3-phosphoglyceric acid. This is reduced to 3-phosphopyruvic acid, which in turn undergoes transamination to form 3-phosphoserine. Hydrolysis by a phosphatase produces L-serine. (Figure I).

L-serine may then be converted, as an intact molecule, to cysteine, tryptophane, and methionine. (Figure II). When cleaved by serine transhydroxymethylase into α and β carbons L-serine is used in the synthesis of glycine and C-1 units. Glycine is incorporated into purines, and poryphrins and proteins. C-1 units are essential for the synthesis of purines, tyrosine, histidine, methionine, and thymine.

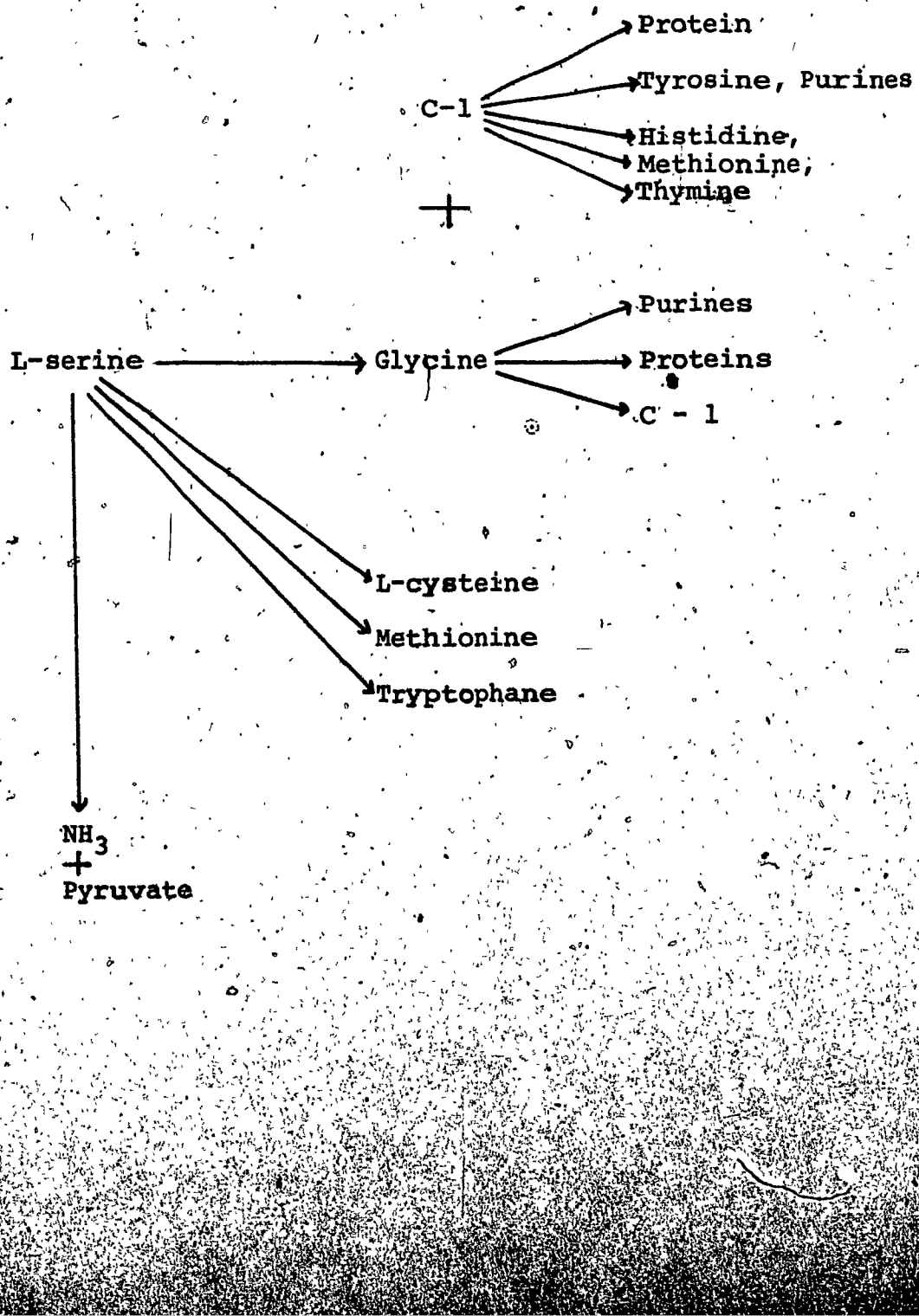
C-1 units also have a more general role in protein synthesis. It has been found that N-formylmethionine is the starting amino acid in the synthesis of all bacterial polypeptides (Clark and Marcker, 1966). Initiation of protein synthesis thus requires the presence of C-1 units for formylating methionine. Therefore, the availability of L-serine will influence the rate of formation of new protein.

Figure I EMBDEN MYERHOFF Pathway and Serine Biosynthesis in E. coli.



Cohn, 1968.
Oginsky, 1959.

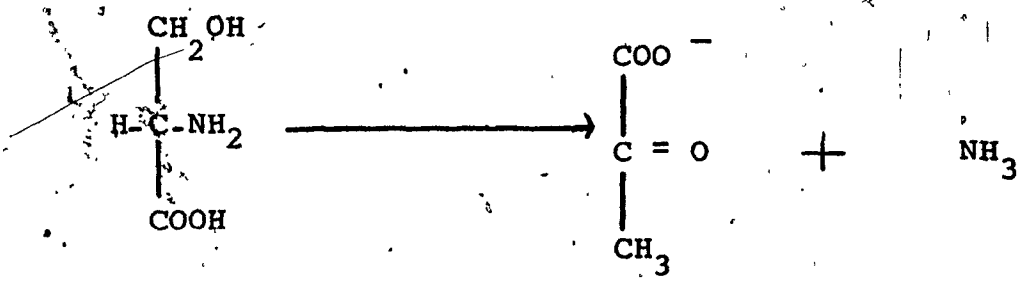
Figure II Metabolism of L-serine in *E. coli*



In addition to all these reactions, each of which seems to have an obvious role in metabolism, L-serine can also undergo non-oxidative deamination to form pyruvate and ammonia. (The reaction is diagrammed in Figure III). The enzyme catalyzing it is one of the least studied in this area of metabolism. Perhaps the main reason for this neglect is the fact that in the past, L-serine deaminase activity has not been attributed to the action of a distinct enzyme, L-serine deaminase, but rather to a subsidiary activity of some other enzyme(s) whose characteristics were well known. Two enzymes in particular were suggested to be responsible for L-serine deaminating activity. One was D-serine deaminase, which catalyzes the deamination of D-serine to pyruvate and ammonia; the other was L-threonine deaminase, which catalyzes the deamination of L-threonine to α-ketobutyrate.

Proof that L-serine deaminase and D-serine deaminase are different enzymes is relatively simple. In early reports on serine deamination, the methods used did not even permit a distinction between L- and D-serine deaminase. The substrate used was a racemic mixture of D- and L-serine, and there was no way of discovering whether one or two enzymes were involved (Gale and Stephenson, 1938; Boyd and Lichstein, 1955; Lichstein and Christian, 1956). However, when the isomers of serine were used separately, it became obvious

Figure III L-serine Deamination Reaction



L-Serine Pyruvate Ammonia

that there are two enzymes involved in serine deamination, a D-serine deaminase, and an L-serine deaminase. D-serine deaminase is synthesized only in the presence of D-serine and is specific for D-serine and D-threonine and is inactive against L-serine; L-serine deaminase is specific for L-serine. (Pardee and Prestidge, 1955; McFall, 1964).

The possibility still remained that L-serine deamination and threonine deamination are properties of the same enzyme. Perhaps the first to show evidence for this was Wood and Gunsalus (1949) studying Crookes Strain of E. coli. They partially purified the enzymes and showed that the same cofactors (adenylic acid and glutathione) were needed for both activities. Moreover, the rate of deamination of a serine-threonine mixture was lower than the sum of the rates obtained for each substrate alone. Because the activities were not additive, a competition for a single enzyme was suggested. The enzyme assayed was very unstable in the presence of serine. The rate of inactivation of the two activities were also compared. They were lost simultaneously. This indicated that threonine deaminase could not function independently of serine deaminase, thus suggesting that they were in fact one enzyme.

In a study of Neuospora crassa (Yanofsky and Reissig, 1953), it was also suggested that serine deaminase and threonine deaminase were one enzyme. Both activities had

the same strict requirement for pyridoxal phosphate, and the same pH optimum at about pH 9. Activities against substrate mixtures were not additive, again suggesting competition for a single enzyme.

A more recent report on the serine and threonine deaminase activities of rat liver (Goldstein et al. 1962) also provides evidence that a single enzyme is responsible for both of these activities. This was again based on similar pH optimum and non-additivity of activities. Further evidence was obtained during enzyme purification. The ratio of activity against serine and threonine remained constant during 8-fold purification, heat inactivation, UV irradiation, and tryptic and chymotryptic digestion.

It therefore seems that there is considerable evidence that in Crookes Strain of E. coli, Neurospora crassa, and rat liver, there is only one enzyme catalyzing L-serine and L-threonine deamination. While it may be that a single enzyme was involved in the systems described above, it is more likely that the separation was prevented by lack of proper distinguishing criteria. In any case, the evidence was accepted at the time it was published, and this understandably would have temporarily prevented the description of a separate L-serine deaminase enzyme, or the realization of its significance.

Pardee and Prestidge (1955) suggested that the evidence

given for a single enzyme deaminating both L-serine and L-threonine was not conclusive, and attempted to show the existence of separate enzymes for each activity. They did this by trying to find conditions in which one was present and the other was not. This was done by growing the cells in a variety of conditions expected to induce one or the other of the activities. Since the ratio of L-serine and L-threonine deaminase activity varied widely (0.15-2.3) with the conditions of growth, it could be concluded that more than one enzyme was involved.

Using the same method, Sayre and Greenberg (1956) demonstrated that L-serine and L-threonine deaminases were distinct enzymes in the sheep liver system, and went on further to separate them by controlled heat denaturation and ammonium sulfate fractionation.

Additional proof of the existence of distinct enzymes for L-serine and L-threonine deamination exists in the E. coli B/r system studied by Artman & Markenson (1956). This study reported that the organism used possessed no L-threonine deaminase, while two other deaminases, L and D serine deaminases were still present.

It seems therefore, that L-serine deaminase and L-threonine deaminase are two different enzymes. Earlier statements to the contrary probably are due to

insufficiencies in the experimental design. In any case, in the present study the question of the existence of a distinct L-serine deaminase has been considered in detail and successfully demonstrated.

Thus, for a long time, it was thought that there was no distinct L-serine deaminase. It is therefore not surprising that there is little mention in the literature of its possible metabolic role. Once it became clear, however, that this activity belongs to a distinct enzyme, its metabolic role became of interest. The products of the reaction being pyruvate and ammonia, two possible roles, i.e. in either carbon or nitrogen metabolism could be immediately suggested. However, a role in carbon metabolism seems unlikely since pyruvate can be produced directly from glucose via the Embden-Myerhoff pathway (Figure 1). The formation of pyruvate from L-serine involves the conversion of phosphoglyceric acid to L-serine and thence to pyruvate. It is difficult to understand what advantage a glucose grown cell would find in producing pyruvate via L-serine rather than directly from glucose.

If the enzyme is not involved in carbon metabolism, it might be involved in nitrogen metabolism. Since the nitrogenous reaction product is ammonia, and inorganic nitrogen is required for the growth of microorganisms,

one conceivable role of L-serine deaminase could be in providing the cell with a nitrogen source when another is absent or insufficient.

E. coli can synthesize all amino acids starting from ammonia (Lehninger 1970). Inorganic nitrogen, as ammonia, can be converted to organic form directly by two enzymes, glutamine synthetase and glutamate dehydrogenase. These are the only ways of transfer of inorganic nitrogen into the cell. However, once incorporated into either glutamine or glutamate the nitrogen can be utilized in many different ways.

Glutamine synthetase catalyzes the formation of glutamine from glutamic acid and ammonia, as follows:

$$\text{L-glutamate} + NH_3 + ATP \longrightarrow \text{L-glutamine} + ADP^+ \; P$$

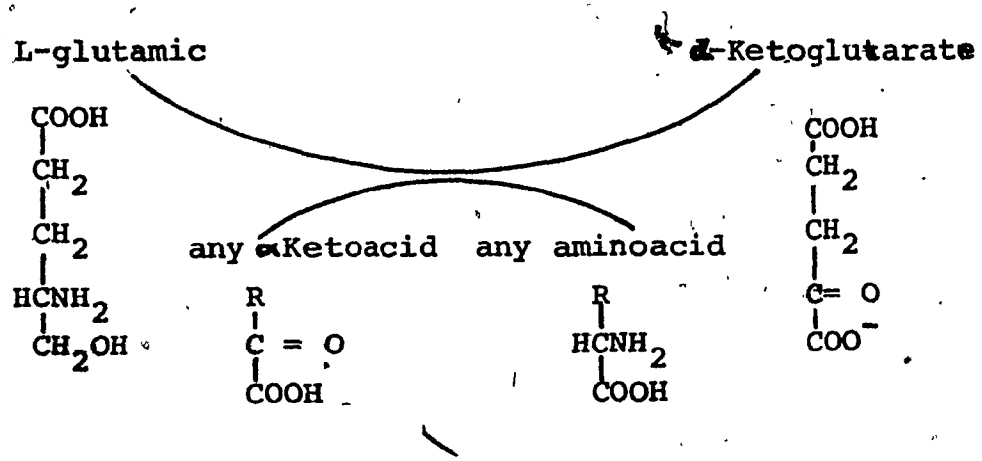
It is the glutamine amide group which in turn supplies the nitrogen atoms of tryptophane, adenylic acid, cytidylic acid, glucosamine-6-phosphate, histidine, and carbamyl phosphate (Cohn 1968).

Glutamate dehydrogenase catalyzes the synthesis of L-glutamic acid from α-ketoglutarate and ammonia, as follows:

$$NH_3 + \alpha\text{-ketoglutarate} + NADPH + H \longrightarrow \text{glutamic acid} + NADP^+ + H_2O$$

Transamination of α-ketoacids with glutamic acid as the amino group donor represents the major pathway of the

introduction of the α amino group in the biosynthesis of most other amino acids (figure below) (Lehninger 1970).



This reaction is reversible and therefore can either use available nitrogen, for synthesis, or can be a source of it. L-serine deaminase activity may in some circumstances be a source of the ammonia for the above reactions.

Alternatively, L-serine deaminase could be of use to the cell by providing a mechanism to rid itself of excess organic nitrogen, for whatever reason this condition may arise. This would be done by converting organic nitrogen, in the form of L-serine, to inorganic nitrogen as ammonia, i.e. a detoxifying agent. Since inorganic nitrogen has a limited way of entering cellular metabolism it may also be that organic nitrogen has a limited way of leaving, i.e. via specific deaminases.

Studies of L-serine deaminase activity in other

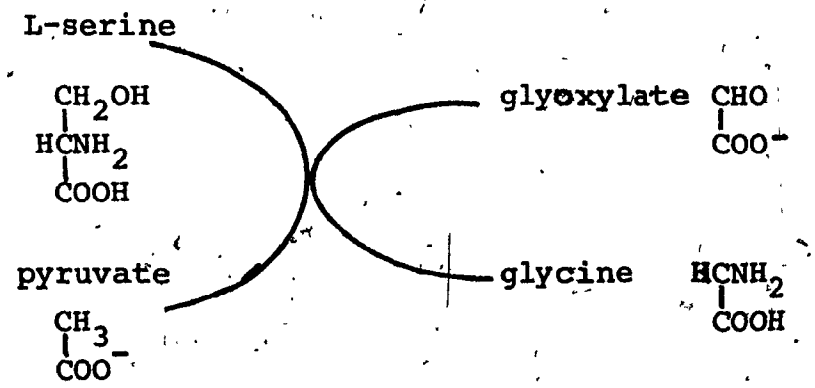
microorganisms provide further proposals as to its role. For example, in Clostridium acidi urici (Benziman 1960) which can grow on uric acid, it has been suggested that the high level of L-serine deaminase* activity indicates that L-serine acts as an intermediate in the overall uric acid fermentation. It was seen that at its maximum rate, L-serine deaminase activity in crude extracts was comparable to the overall rate of uric acid fermentation by the whole cells of Clostridium. It was therefore suggested that L-serine deaminase could be part of the same system.

In Arthrobacter globiformis (Bridgeland and Jones 1965) the conversion of L-serine to pyruvate is thought to be the key step in the utilization of glycine as a sole carbon and nitrogen source for growth. This was also described for Pseudomanas (Morris 1963). The pathway is the following: glycine is converted to L-serine and then deaminated to pyruvate. Pyruvate can then be carboxylated to give oxaloacetate or oxidized to acetyl CoA, which in turn condenses with glyoxylate to give malate. Thus, essential tricarboxylic acid cycle intermediates are synthesized from the 2-carbon compound, glycine.

*This author and several others call this enzyme 'L-serine dehydratase'. In this thesis, any enzyme converting L-serine to pyruvate and ammonia is termed 'L-serine deaminase.'

These reactions are also involved in photorespiration (Bird et al. 1972) and gluconeogenesis from 2-carbon compounds (Wang and Waygood 1962).

If the mechanism of pyruvate production from L-serine were transamination rather than deamination, a role could be imagined for the enzyme in a pathway of glycine production similar to that recently described in Pseudomonas by Quayle (1971). This pathway involves a L-serine-glyoxylate transaminating enzyme which catalyzes the following reaction, with L-serine as the amino donor:



A survey of the literature thus indicates that L-serine deaminase is a distinct enzyme of unknown metabolic role. The present study was undertaken in the hope of determining the extent of L-serine deaminase activity of an E. coli cell and investigating its role in the cell's metabolism.

MATERIALS & METHODS

Bacterial Strain:

The strain used in these experiments was a mutant of *Escherichia coli* K_{12}, a L-proline-requiring auxotroph ($K_{12}P^-$) obtained from Dr. E. McFall at New York University. It was maintained on slants of yeast-extract-tryptone agar at 4°C. The composition of this and other growth media is described below.

Growth Media:

Bacterial cells were grown in liquid minimal medium containing 0.54% K_2HPO_4, 1.26% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.001% $CaCl_2$, buffered at pH 6.4. When a minimal medium excluding inorganic nitrogen was required, 0.2% $(NH_4)_2SO_4$ was omitted from the rest of the medium contents.

The carbon and energy sources, glucose, glycerol, and succinate, were added to the medium to a final concentration of 0.2%. Other additions to the growth medium were made according to the requirements of the particular experiment. All amino acids were added to the growth medium at 50 ug/ml except glycine at 25 ug/ml and L-serine at 100 ug/ml.

Cell cultures were kept by frequent transfer on slants of yeast-extract-tryptone agar containing 1% yeast extract, 1% tryptone, 2% agar, and 0.25% K_2HPO_4. Glucose was added to a final concentration of 0.01%.

Chemicals: All chemicals used were purchased from either Calbiochem of Los Angeles, California, or Schwartz Mann of Orangeburg, New York.

Growth of Bacteria:

Cells from yeast-extract-tryptone slant cultures grown at 37°C, were inoculated into one-liter Erlynmeyer flasks, each containing 100 ml of medium and a single carbon and energy source. Cells were grown at 37°C in a gyrotory water bath shaker (New Brunswick Scientific Co., N.J., Model G76) at approximately 180 rpm, until the culture reached the desired absorbance. Cells were then chilled in ice water and salt, centrifuged at 6000 rpm for 5 minutes at 0°C in a refrigerated centrifuge (International Equipment Co., Needham Hts., Mass., Model B-20), washed once by resuspension in minimal medium without ammonium sulfate, recentrifuged, resuspended again in minimal medium without ammonium sulfate, and then assayed for enzyme activity.

Cells requiring subculture were harvested as described above, and then resuspended into 100 ml. of fresh

medium, and incubated shaking at 37°C until the culture reached the desired absorbance.

Measurement of Cell Growth

Cell growth was followed by measuring the absorbance using a Klett-Summerson colorimeter (Klett Mfg. Co. Inc., New York, Model 800-3), filter #42 (spectral range 400-465 mu).

Assay of L-serine Deaminase Activity

The assay for l-serine deaminase activity used here is based on the production of pyruvate according to a slight modification of the method of Pardee and Prestidge (1955). The incubation mixture contained 0.3 ml. of washed cells, 0.02 ml. of toluene, and 0.1 ml. of a substrate solution at either 10 or 20 mg/ml. This mixture was incubated for 35 minutes at 37° ; 0.9 ml. of 2,4 dinitrophenylhydrazine (DNPH) in 4.1% hydrochloric acid was added, and the mixture incubated 20 minutes at room temperature. 1.7 ml. of 10% NaOH were then added, and the absorbance determined with a Klett-Summerson colorimeter #54 filter using pyruvate as standard. (The pyruvate reacts with the DNPH to produce a yellow colour. This assay is a general one for α keto acids and what is measured therefore is the production of α keto acid from

L-serine. The assumption is made that the principal α-keto acid produced is pyruvate. This method differs from the original in that it uses 0.3 ml rather than 0.2 ml of cell suspension, and in that substrate and toluene are added simultaneously.

Also included in this assay was one set of tubes to which no substrate was added and one set containing boiled cells. The former set allowed a measure of the endogenous production of pyruvate. The latter was included to determine the amount of pyruvate in the cells before the assay was performed. This value was always negligible. All assays were done in triplicate and average values used for calculations. Activity is expressed as the difference between the amounts of pyruvate formed with and without substrate, related to the amount of protein in the cells: μmoles pyruvate produced per mg protein (μm pyr/mg prt).

Note:

Activity against any substrate is expressed as μmoles pyruvate formed per mg. protein used in the standard assay time of 35 minutes. When L-threonine was used as substrate, α-ketobutyrate was presumably formed. However, pyruvate was always used as the standard. No attempt was made to convert these units to corresponding amounts of α-ketobutyrate, or to use α-ketobutyrate as a standard.

Note:

In the earlier experiments described below, the concentration of L-serine used as substrate was 952 um/ml (.1 ml. of 10 mg/ml solution). However, it was later learned that a concentration of 19.04 um (0.1 ml of 20 mg/ml solution) was closer to enzyme saturation, and therefore in subsequent experiments, this latter concentration was used in the assays. The concentration used in each experiment will therefore be noted with each table of results.

Cell Extract Preparation

A 400 ml. aliquot of cells grown to an absorbance of 60, was centrifuged at 6,000 rpm for 5 minutes at 0°C, washed once with minimal medium without ammonium sulfate, and then resuspended to about 20% weight/volume in potassium phosphate buffer (pH 7.0). The cells were disrupted by sonic oscillation with a 100 Watt ultrasonic disintegrator (MSE Ltd. London S.W. 1). The homogenate was clarified by centrifugation at 8,000 rpm for 18 minutes, and the supernatant fluid collected for the enzyme assay.

Protein Determination

Protein was determined by the method of Lowry et al. (1951) using trypsin as standard.

Definitions

Medium containing inorganic nitrogen (as ammonium sulfate) was referred to as MM+N. However, medium to which inorganic nitrogen was not added was referred to as MM-N. When the discussion requires that the other organic components of the medium be specified, these abreviations were shortened to +N and -N respectively, and the other components specified by their first initial. Thus, -Npg is the abreviation for minimal medium without the addition of ammonium sulfate, but with L-proline and glycine. Similarly, MM-N refers to any medium to which ammonium sulfate has been omitted, and does not specify what other additions were made.

Validity of the Enzyme Assay

A study of the type conducted for this thesis requires a valid assay of enzyme activity. The presence of L-serine deaminase is detected by the occurence of the specific reaction: L-serine ⟶ pyruvate + ammonia, and the measurement of the pyruvate produced. To verify that the assay used gave accurate measurements of the amount of enzyme activity, studies were made on the amount of α-ketoacid produced as a function of (a) time, (b) enzyme concentration, and (c) substrate concentration, as well as (d) the reproducibility of the assay.

(a) Amount of Pyruvate Produced as a Function of Time

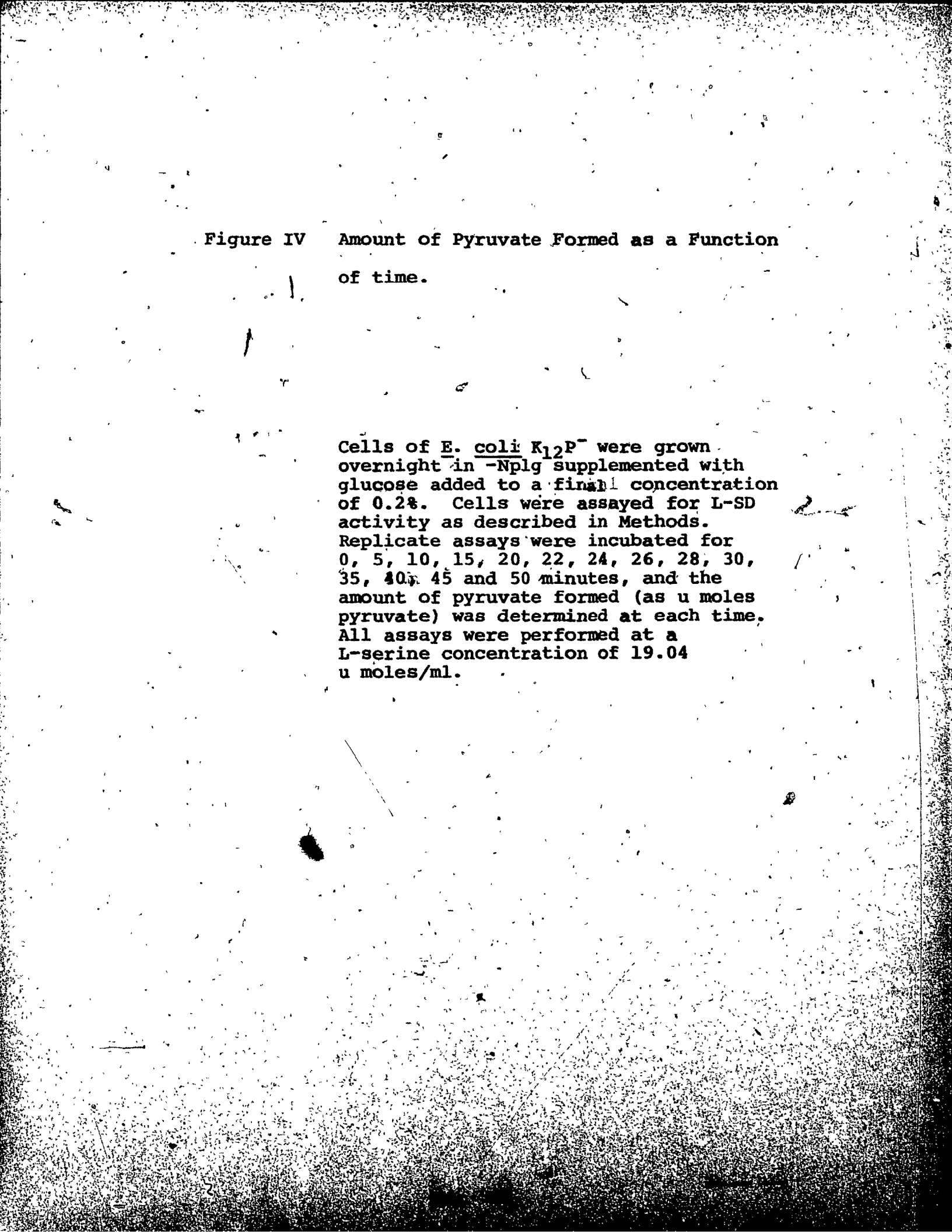
Generally, for an enzyme assay, the amount of substrate converted should be proportional to the time allowed for the reaction, at least for short time periods. The time chosen for an assay must allow enough time to permit a measureable amount of product to be formed. However, it must not be so long as to reduce the substrate concentration to less than saturating level for the enzyme. Further, the enzyme must be stable during the time period chosen, (Dixon and Webb) 1964).

To test whether the L-serine deaminase reaction was indeed proportional to the time allowed, and to select a time period for further assays, replicate assays were incubated for 0, 5, 10, 15, 20, 22, 24, 26, 28, 30, 35, 40, 45 and 50 minutes, and the amount of pyruvate formed (as um pyruvate) was determined at each time. It can be seen (Figure (IV) that the reaction rate was linear with the time over the period of 20-35 minutes. The initial rate was slow, probably due to the time required for toluene to make the cell wall permeable to the substrate, before the reaction could begin. At 35 minutes, the time chosen for the L-serine deaminase reaction, the rate was still linear. The selection of a time within the linear portion of the curve, albeit at the upper limit, excluded the possibility that the end-product might accumulate and inhibit the enzyme, as well as the possibility of enzyme inactivation.

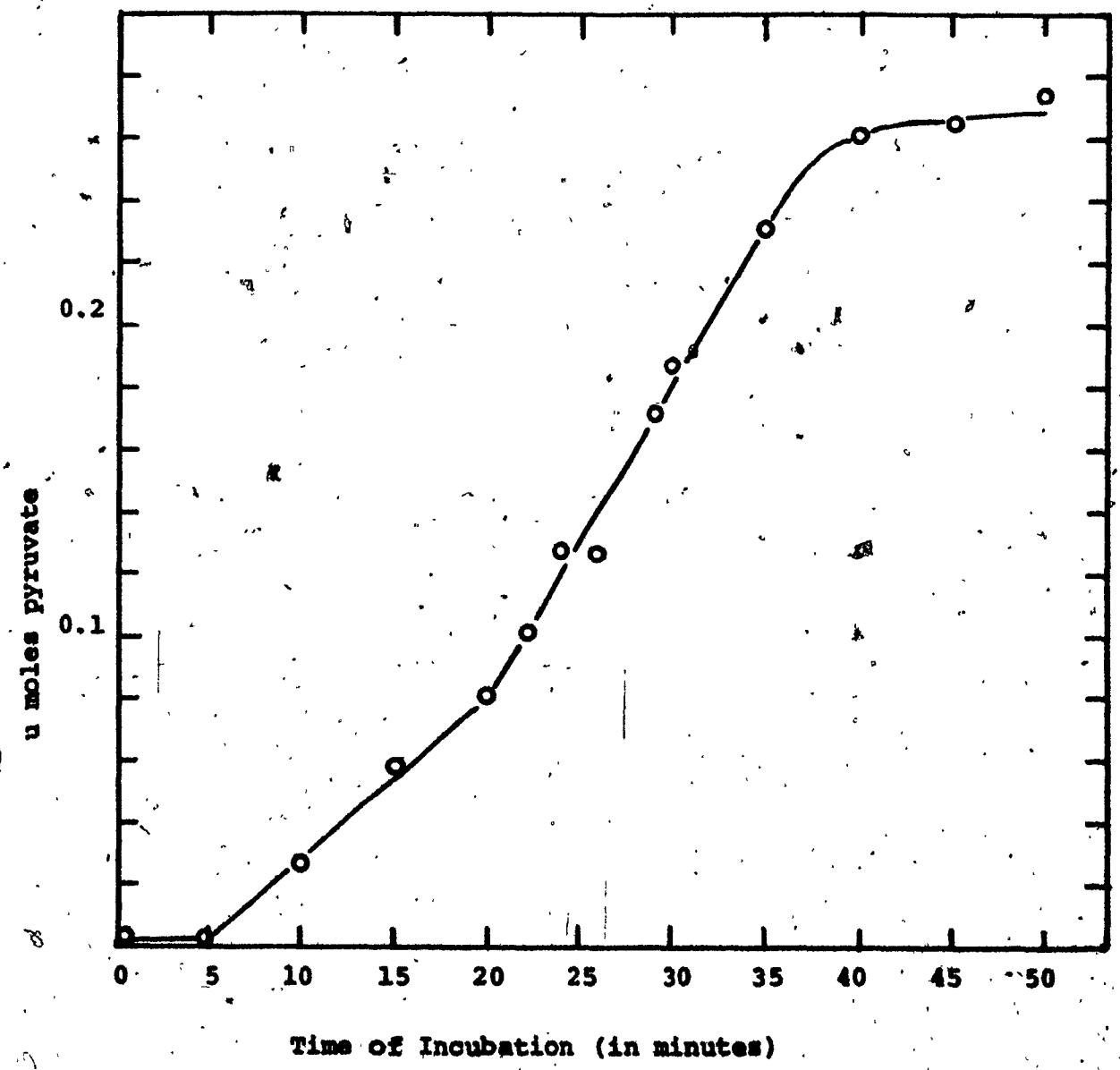
(b) Amount of Pyruvate Produced as a Function of Enzyme Concentration.

The rate of an enzymatic reaction is generally found to increase with increasing enzyme concentration, provided other conditions are optimal (Dixon and Webb, 1964). To test if this were true for L-serine

Figure IV Amount of Pyruvate Formed as a Function of time.



Cells of E. coli $K_{12}P^-$ were grown overnight in -Nplg supplemented with glucose added to a final concentration of 0.2%. Cells were assayed for L-SD activity as described in Methods. Replicate assays were incubated for 0, 5, 10, 15, 20, 22, 24, 26, 28, 30, 35, 40, 45 and 50 minutes, and the amount of pyruvate formed (as u moles pyruvate) was determined at each time. All assays were performed at a L-serine concentration of 19.04 u moles/ml.


0.2
0.1
u moles pyruvate
0
5
10
15
20
25
30
35
40
45
50
Time of Incubation (in minutes)

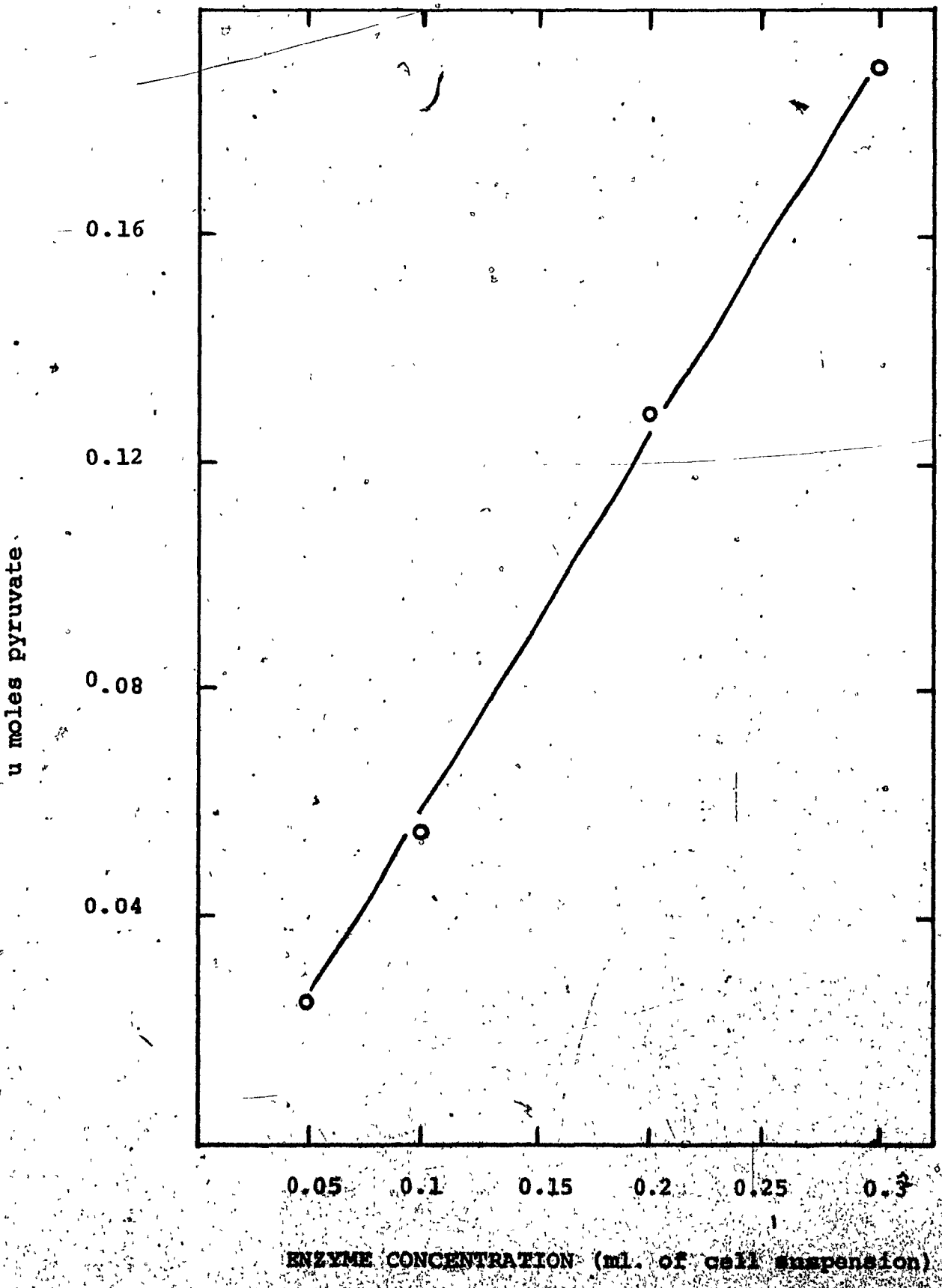
deaminase, various concentrations of enzyme (as whole cells) were incubated with toluene and L-serine for 35 minutes, as described above. Figure (V) shows that the relationship between enzyme activity (as um pyruvate) and enzyme concentration tested, was approximately linear.

(c) Amount of Pyruvate Produced as a Function of Substrate Concentration

A further generalization for enzyme assays is the rate of the enzyme reaction should be proportional to the concentration of substrate, at least until a saturation level is reached. To test if this were true for the L-serine deaminase reaction, replicate assays were incubated containing 0, 4, 9, 14, 19, 38, 57, 76, 90, 114, 143, 152, 171 and 190 um serine/ml., and the amount of pyruvate (in um) formed in each after 35 minutes was determined. It can be seen in Figure (VI) that the relationship between enzyme activity (as um pyruvate produced) and substrate concentration tested (as um L-serine/ml.) was approximately linear, until a concentrations of 76 um serine/ml. The concentration used (9.52 and 19.04 um serine/ml) were lower than saturation, as stated in the description of the assay. The lower concentration used here (9.52 um serine/ml) is the same concentration used by Pardee and Prestidge 1955).

Figure V Amount of Pyruvate Formed as a Function of Enzyme Concentration.

Cells of *E. coli* $K_{12}P^-$ were grown overnight in -Nplg supplemented with glucose added to a final concentration of 0.2%. Cells were assayed for L-SD activity as described in 'Methods'. Replicate assays containing various concentrations of enzyme (measured as ml of whole cell suspension) were incubated with toluene and L-serine for 35 minutes. L-SD activity was measured as u moles pyruvate produced. All assays were performed at at L-serine concentration of 9.52 u moles ml.


0.16
0.12
0.08
0.04
u moles pyruvate
0.05
0.1
0.15
0.2
0.25
0.3
ENZYME CONCENTRATION (ml. of cell suspension)

(d) Reproducibility of Assay

Included in this work is a comparison of the activity of L-serine deaminase in cells grown under different conditions. The assay has been shown to be a valid one. However, it was necessary also to determine the variation between assays on cells grown under the same conditions.

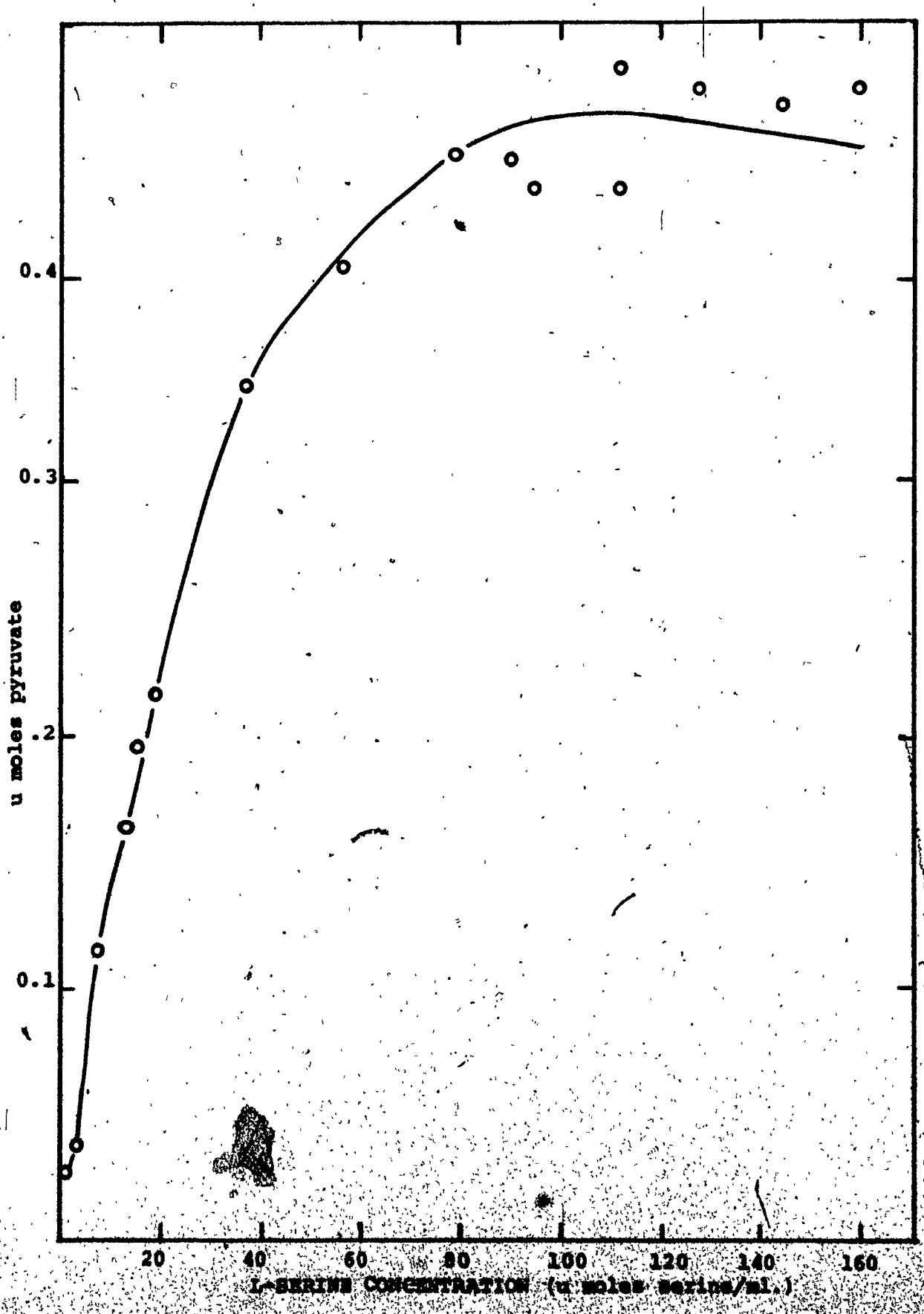
Enzyme activity is expressed in a ratio of u moles pyruvate/mg. protein. However, there appears to be no explicit expression for the variance of a ratio, although confidence intervals can be determined (Goldstein 1964), and on the basis of these confidence intervals an approximate estimation of the standard error of each mean can be made. This was how the data presented here were analyzed. The median standard error was taken since it seemed to be the most representative. Multiplying the median standard error by $\sqrt{2}$ gives an estimate of the standard error of the difference between two means. This gives a critical value for the difference between two means of 0.427 u moles pyruvate/mg. protein, at the 95% level of confidence.

In other words, any treatment value which differs by less than 0.427 u moles pyruvate/mg. protein from its control cannot be considered significantly different.

This value was calculated using data from assays on cells grown in -Nplg and therefore only applies to the variation present for that medium. It has been assumed that the variation between assays using other media is the same.

Figure VI Amount of Pyruvate Formed as a Function of Substrate Concentration

Cells of *E. coli* $K_{12}P^-$ were grown overnight in -Nplg supplemented with glucose added to a final concentration of 0.2%. Cells were assayed for L-SD activity as described in 'Methods' Replicate assays were incubated containing 0, 4, 9, 14, 19, 38, 57, 76, 90, 114, 143, 152, 171 and 190 u moles serine/ml. Enzyme activity was measured as u moles pyruvate formed in each assay after 35 minutes.


0.4
0.3
.2
0.1
u moles pyruvate
20
40
60
80
100
120
140
160
L-SERINE CONCENTRATION (u moles serine/ml.)

Note:

A note may be added at this point on the term 'L-serine deaminase' being used throughout this thesis. The activity that is being studied is the conversion of L-serine to pyruvate. The mechanism by which this reaction occurs is not of immediate concern. However, other possibilities besides deamination could include transamination or dehydration. Whereas deamination involves the conversion of the amino group of an animo acid to free ammonia, the process of transamination is the transfer of the amino group of one amino acid, via an acceptor molecule, to another carbon skeleton to form a new amino acid. The dehydration reaction also results in free ammonia, but proceeds by an initial loss of the elements of water, although water is later added back during the loss of ammonia from an unstable imino intermediate. Dehydration is very similar to deamination in that free ammonia results from both, and the two terms are often used interchangeably. Both dehydrase and transaminase enzymes require pyridoxal phosphate as cofactor; transaminases also require an acceptor molecule. None of the papers reviewed discuss the different mechanisms which might be responsible for L-serine deaminase activity. Thus, all published reports have actually studied the production of pyruvate from L-serine, with no attempt to discern the mechanism. Thus, the same convention is used here. It

It should, however, be remembered that the assay is done on toluenized whole cells which might very well contain saturating amounts of any cofactor or acceptor molecule.

RESULTS

Although an enzyme converting L-serine to pyruvate has often been mentioned in the literature, it has not been studied in detail, so, little is known about its characteristics or metabolic role. It was decided therefore, to characterize the enzyme in terms of the following properties: substrate specificity, cofactor requirement, enzyme inhibitors, and enzyme stability. The results of these studies form Part I of this presentation.

Once the enzyme was characterized, various attempts were made, within the limitations of an unpurified preparation, to define its metabolic role. This is presented in Part II of the 'Results' section.

ENZYME CHARACTERISTICS

1. Specificity

The results of this study indicate that L-serine deaminase shows considerable substrate specificity. Assays were performed to test the ability of cells possessing L-serine deaminase (L-SD) activity to act on several molecules structurally or metabolically quite

similar to L-serine, Those tried were L-threonine, D-serine, L-isoleucine, L-leucine, glycine and L-cysteine. All cells were grown in the same medium (-Nplg) and all substrates tested were added at the same concentration normally used for assay against L-serine (i.e. 2.5 mg./ml.). As can be seen in Table I, though activity against L-serine was high (2.05 um pyruvate/mg protein), there was little or no activity against any of the other substrates tried.

2. Cofactor Requirement

Although the L-SD of animal origin is known to have pyridoxal phosphate as cofactor (Goldstein et al 1962, Sayre and Greenberg 1956), the cofactor requirement of the E. coli L-SD is still unclear. Since a toluenized cell assay, rather than an extract, was being used in this study, pyridoxal phosphate might already have been present in saturating concentrations and therefore its importance might not be apparent. However, in such a toluene based system, it is also possible that some pyridoxal phosphate could leak from the cells. Therefore to see whether the addition of pyridoxal phosphate (PP) had any effect, toluenized cells were incubated with PP (38 ug/ml.) and the amount of

Table I SUBSTRATE SPECIFICITY OF L-SD

Substrate	L-SD Activity*(as um pyr/mg. prot.)
L-serine	2.05
D-serine	0.02
L-threonine	0.03
L-isoleucine	0.04
L-leucine	0.10
glycine	0
L-cysteine	0

* Assays were performed at D and L-serine concentration of 9.52 um/ml; L-threonine concentration of 8.40 um/ml; L-isoleucine and L-leucine concentration of 7.63 um/ml; glycine concentration of 13.30 um/ml; and L-cysteine concentration of 4.17 um/ml.

pyruvate formed was determed. As can be seen in Table II, (in experiment No. 1 for example) the amount of pyruvate formed in the presence of PP (e.g. 1.13 um pyr./mg. prot.) did not differ from the amount formed in its absence (1.18 um pyr./mg. prot.). Thus exogenous PP had no effect on L-SD activity.

It was also considered that the intracellular concentration of PP might be limiting in the assay, even if exogenous PP was without effect. For example, the permeability barrrier of the cell might be sufficiently changed by toluene to allow some PP to leak out of the cell but it might still be impossible for PP to enter. The only way of testing this would be to try to affect the size of the internal pool during growth. For this reason, cells were grown in the presence of pyridoxine and assayed for activity against L-serine alone or with pyridoxine, as pyridoxal phosphate (PP), added to the assay mixture.

In comparison with activity in cells grown without the addition of pyridoxine (above mentioned experiment) the results seen in Table II show that pyridoxine had no significant stimulatory effect. There is therefore, no evidence for a role for PP in this enzyme. However, PP might be tightly bound to the enzyme at all times, so a role for PP cannot be completely excluded.

Table II COFACTOR REQUIREMENT OF L-SD ACTIVITY

Experiment No.	Growth Medium	L-SD ACTIVITY (as um pyr/mg. prot) No additions	+ pyridoxal phosphate	+ pyridoxine
1	-Nplg	1.18	1.13	1.11
2	-Nplg	1.65	1.73	1.61
3	-Nplg	1.21	1.62	-
	-Nplg pyridoxine	1.48	1.77	1.68

* Assays were performed with an L-serine concentration of 9.52 um/ml.

3 Effect of pH .

E. coli used in this study was routinely grown in phosphate-based medium buffered at pH 6.4. This was also the pH of the assay.

The pH of whole cells does not depend on that of the external medium (within a considerable variation in external pH). However, once the membrane of the cells are disrupted by toluene, the cells probably cannot maintain their constant pH, and in this respect a toluenized preparation can be considered more like an extract than a whole cell. It was therefore important to determine whether the pH of the assay affected the enzyme activity.

For this purpose, cells were assayed at a range of pH from 5.8 to 8.0. As shown in Table III enzyme activity did in fact increase with increasing pH. These results are susceptible to different interpretations. They may indicate that L-SD activity is increasingly active as the pH of incubation increases. However, they are also consistent with the idea that at the higher pH's other deaminases become active. It is clear that at pH 6.4 L-SD activity is quite specific and that other deaminases do not function (Section 1 of 'Results'). Those deaminases might however function at the higher pH's.

Table III. EFFECT OF pH ON ABILITY TO DEAMINATE L-SERINE AND L-THREONINE

pH	Activity against L-serine (um pyr/mg. prot)	Activity against L-threonine (um pyr/mg. prot)
5.8	0.23	0
6.4	1.70	0.05
7.0	2.16	0.43
7.5	2.77	0.76
8.0	3,69	1.26

* Assays were performed at L-serine concentration of 19.04 um/ml.

One of the enzymes which could very possible be contributing to activity measured against L-serine would be L-threonine deaminase (Section B (7)). This enzyme converts L-threonine to ∝ketobutyrate but is known to be able to also deaminate L-serine (Davis et al 1961, Umbarger and Brown 1957, Nishimura et al 1961, Alfoldi and Rasko 1968).

To demonstrate the extent of L-threonine deaminase (L-TD) participation in this system, activity against L-threonine as substrate was measured. Figure VII shows that at pH 6.4 or lower, L-TD was not active, i.e. little (0.05 um pyr./mg. prot.) or no activity against L-threonine was detectable. Activity against L-serine was high (1.70 um pyr./mg. prot.). This is consistent with the results of Section 1 in which it was seen that at pH 6.4 activity against L-threonine or other amino acids besides L-serine was not observed. However, above pH 6.4, e.g. at pH 7.5, activity against L-threonine can be measured (0.76 um pyr./mg. prot.). Activity against L-serine at pH 7.5 is also increased (2.77 um pyr./mg. prot.). With increasing pH, therefore, activities against both L-serine and L-threonine increased, but the activity against L-serine was always much greater.

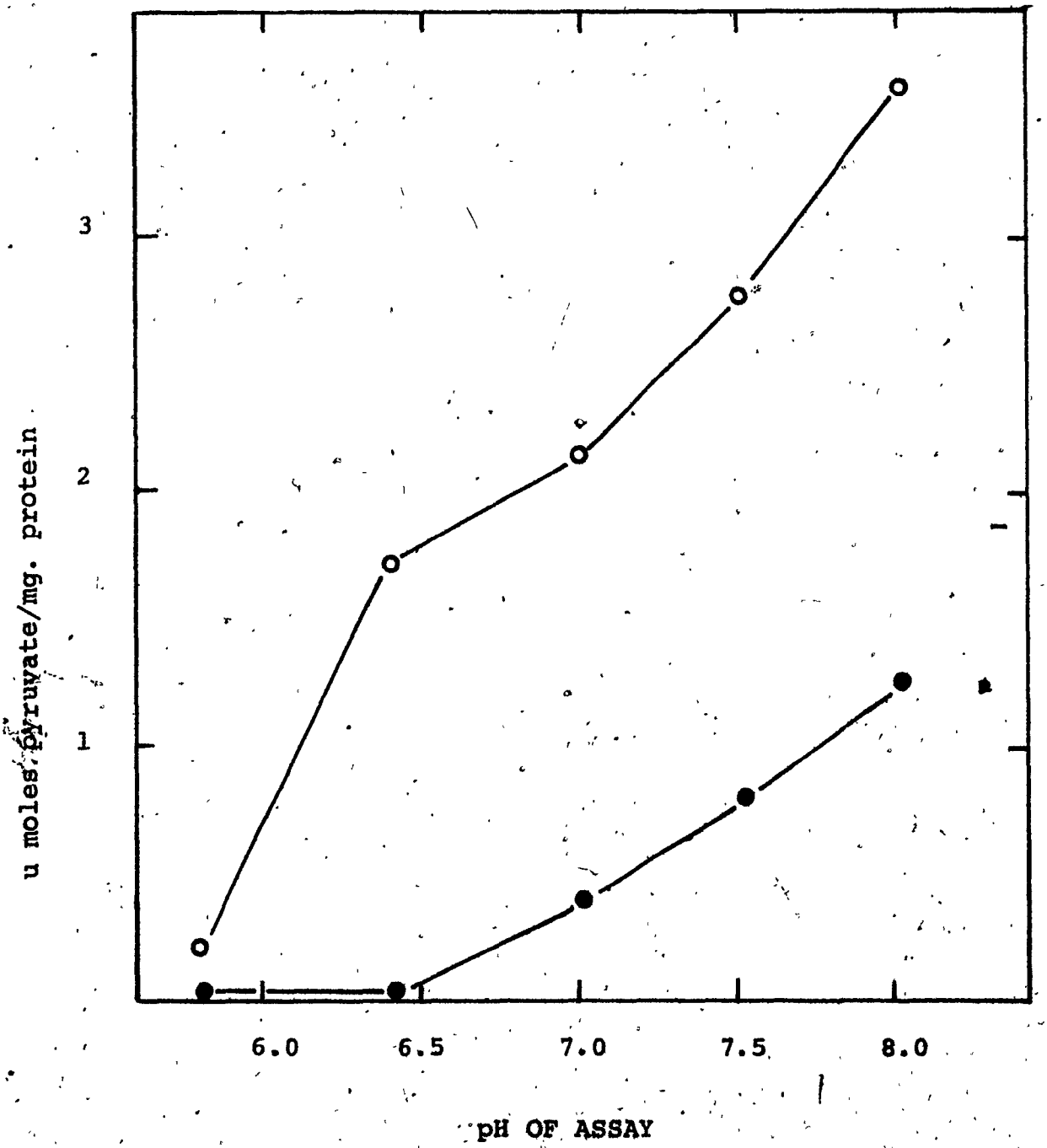
It therefore appeared that there was an enzyme

Figure VII The Effect of pH on L-Serine and L-threonine Deaminating Activity.

Cells of *E. coli* $K_{12}P^-$ were grown overnight in -Nplg supplemented with glucose added to a final concentration of 0.2%. Cells were assayed for enzyme activity as described in 'Methods'. Replicate assays were performed at pH 5.8, 6.4, 7.0, 7.5, and 8.0 with L-serine and L-threonine as substrate. Assays were performed at a L-serine concentration of 19.04 u moles/ml and L-threonine concentration of 16.80 u moles/ml.

o—o Activity against L-serine

●—● Activity against L-threonine


u moles pyruvate/mg. protein
3
2
1
6.0
6.5
7.0
7.5
8.0
pH OF ASSAY

active at higher pH's which was not active at pH 6.4 although a pH dependent conformational change is not entirely ruled out. This would be a L-threonine deaminase, active against L-threonine and also active against L-serine.

At pH 6.4 considerable amounts of activity could still be measured against L-serine as substrate. Since activity against L-threonine was absent, this activity was presumeably due to the action of L-serine deaminase (L-SD) itself. In order to measure a maximum amount of serine deamination specific to L-serine without including the activities of other enzymes, pH 6.4 was adopted as the pH of the assay used in this work.

4 Enzyme Inhibition

Various compounds have been reported to inhibit the activity of L-serine deaminase. These include D-serine (Pardee and Prestidge 1955, Alfoldi et al 1968 Griffiths and deMoss 1969, Rasko et al 1969, Szentirmae et al 1963, Wood and Gunsalus 1949), L-glycine (Pardee and Prestidge 1955), L-cysteine (Alfoldi et al 1968, Rasko et al 1969, Selim and Greenberg 1959); D-threonine (Wood and Gunsalus 1949).

To determine the characteristics of L-serine deaminase (L-SD) in the system used here, and to compare it with preparations of other investigators, the effects of D-serine, glycine, L-cysteine, L-threonine and L-isoleucine on enzyme activity were tested.

Cells were grown in -Nplg (except for the effect of L-glycine which was tested in cells grown in -Npg) and incubated with L-serine. Compounds were tested for inhibitory capacity by adding them to the incubation mixture with L-serine and at the same concentration as L-serine (2.5 mg./ml.). The results of such an experiment are presented in Table IV. It can be seen that glycine, L-isoleucine, and D-serine were inhibitory (L-glycine inhibited 37%; D-serine 48%), D-serine being slightly more effective, (L-isoleucine had a smaller inhibitory effect (about 10%, 20%). L-threonine and L-cysteine had no observeable effect.

5 Lability of L-serine Deaminase 'in vitro'

L-serine deaminase has been reported to be unstable 'in vitro' (Pardee and Prestidge 1955, Alfoldi et al 1968). Indeed, three investigators have reported activity in crude extracts (Alfoldi et al 1968, Artman and Markenson 1956 and Wood and Gunsalus 1959), but all with considerable loss of activity. In order to see if L-SD was equally labile in the strain used here, activity was compared in toluene treated whole cells and a sonicated extract of the untreated cells.

Cells were grown in -Nplg to mid-log phase, chilled, centrifuged, and washed once with cold MM-N.

Table IV EFFECT OF AMINO ACIDS ON L-SD ACTIVITY

Amino Acid Added to Assay Mixture (in addition to L-serine)	% Inhibition
D-serine	48
glycine	37
L-cysteine	0
L-threonine	0
L-isoleucine**	10 20

** 2 determinations were made for effect of L-isoleucine

* Assays were performed at L-serine concentration of 9.52 um/ml; D-serine concentration of 9.52 um/ml; glycine concentration of 13.3 um/ml; L-cysteine concentration of 4.17 um/ml; L-threonine concentration of 8.40 um/ml and L-isoleucine concentration of 7.63 um/ml.

One portion of the cells was resuspended as usual and assayed in the presence of toluene. Another portion was resuspended in potassium phosphate (pH 7.0) and exposed to the action of a sonic disintegrator. The supernatant after centrifugation to remove debris, was assayed for L-SD activity.

As shown in Table V, activity measured in the toluene-based assay was 1.70 um pyr./mg. prot., while the activity measured in the sonicated cells indicated only 0.31 um pyr./mg. prot., Thus, extraction treatment had resulted in a loss of 82% of L-SD activity. Purification of the enzyme would have been of obvious use in determining its characteristics. However, in view of its lability, and in view of the amount of information that could be obtained from toluenized cells, the attempt to purify the enzyme was not continued.

PART II ROLE(S) OF L-SERINE DEAMINASE

The few studies previously done on L-serine deaminase have not concerned themselves with the metabolic role of the enzyme. Several possible roles have been suggested in the Introduction of this Thesis. A series of experiments designed to elucidate the role of serine deaminase will be presented in this section of the 'Results'.

Table V LABILITY OF L-SD 'IN VIVO'

Cell Treatment	L-SD Activity (um pyr/mg. prot)
Toluene	1.70
Sonication	0.31

* Assays were performed at L-serine concentration of 9.52 um/ml.

** Assays were performed on cells grown in -Nplg.

A L-Serine Deaminase Activity in Cells Grown in Non-supplemented Medium

If L-serine deaminase has a role in the basic metabolism of the cell, one would expect it to be present in cells grown in non-supplemented minimal medium as are the various biosynthetic and glycolytic enzymes (Lehninger 1970). If it does not have such a role, one would expect it to be absent under these conditions, as is β-galactosidase.

To determine the basal level of L-SD, cells were grown in MM+N with glucose as carbon and energy source[1] (as described in 'Materials'). The overnight cells had reached stationary phase of growth and were then sub-cultured into fresh medium of the same kind and harvested in early log phase[2] (absorbance of 63 on Klett-calorimeter). The cells were then assayed for L-SD activity. In this way the basal level has been measured at an average of 0.65 um pyr./mg. prot. (range of 0.36 to 0.95 um pyr./mg. prot.) (Table VI). This is about ½ the level later measured in inducing conditions (Section B (3)), and as will be described below, this level was higher than expected. These results gave some indication that L-SD was in fact involved in the cells basic metabolism.

1 L-proline, the cells nutritional requirement, was included in all media unless otherwise stated.

2 It was considered desirable to use log phase cells since subsequent experiments indicate an effect of phase of growth on enzyme level.

Table VI DETERMINATIONS OF BASAL LEVEL OF L-SD

L-serine Concentration (um/ml)	L-SD Activity	Average
9.52	0.48	0.40
	0.32	
19.04	0.95	0.65
	0.36	
	0.85	
	0.59	
	0.59	
	0.37	
	0.74	
	0.42	
	0.59	
	0.66	

B L-Serine Deaminase Activity as a Function of Altered Growth Conditions.

It is clear from the preceeding results that L-SD did exist in apparently significant levels even in the noninduced cells. However, its metabolic role was by no means evident. Thus, to gain insight into its metabolic role, the following areas were investigated: the effects of inorganic nitrogen levels and cell growth phase on enzyme level; enzyme inducibility; effect of growth on different carbon sources, i.e. possible glucose effect; and enzyme stability '*in vivo*'.

B(1) Effect of Inorganic Nitrogen

It has been mentioned in the Introduction of this Thesis that a possible role of L-serine deaminase could be to provide the cell with an inorganic nitrogen (N) source, in the form of NH_3, at least when other sources were absent or inadequate. If this were true, one would expect that cells grown in the absence of inorganic N would have higher enzyme levels than cells grown in its presence.

To test this hypothesis, experiments were conducted in which cells were grown overnight in medium, MM+N and MM-N, and also supplemented with L-glycine and L-leucine. The results of such an experiment can be seen in Table VII.

Cells grown in MM-N generally had greater amounts of L-SD than cells grown in MM+N. The differences varied

TABLE VII EFFECT OF GROWTH IN MM+N ON L-SD ACTIVITY

Concentration of L-serine (um/ml)	L-SD ACTIVITY			
	In cells Grown in +Nplg	Average of +Nplg values	In cells Grown -Nplg	Average of -Nplg values
9.52	0.30, 0.36, 0.56, 0.57, 0.59, 0.59, 0.60, 0.71, 0.78, 0.88, 0.88, 0.88, 1,38, 1.60, 1.89	0.78	0.84, 0.91, 0.92, 0.99, 1.00, 1.14, 1.16, 1.18, 1.21, 1.22, 1.24, 1.26, 1.27, 1.33, 1.31, 1.47, 1.65, 1.66, 1.70, 1.78, 1.83, 2.31, 2.24	1.34
19.04	1.96 2.01	1.98	2.10, 2.25, 2.42, 2.46, 2.58, 2.60, 2.61, 2.77, 2.82, 2.87	2.55

from 0 to 6 times (average about twice) depending on other conditions such as absorbance or stage of growth (see section B (2)). These results indicated that the presence of inorganic N in the medium did in fact decrease the level of L-SD.

To examine this further, cells were grown in the presence of various concentrations of inorganic N (as ammonium sulfate) added to the medium. Overnight cultures were grown in the usual MM+N medium and then subcultures were made into 5 separate flasks containing 500, 1,000, 2,000, 4,000 and 8,000 ug ammonium sulfate/ml. respectively. The concentrations chosen represented levels of inorganic N above and below the normal level of 2000 ug/ml ammonium sulfate found in the MM+N usually used. It was found that for this range of concentrations tested, the amount of inorganic N made no difference in the enzyme level for example, 0.31 um pyr./mg. prot. at 500 ug./ml. ammonium sulfate and 0.30 pyr./mg. prot. at 8,000 ug./ml. ammonium sulfate (Table VIII).

The above experiment was designed to determine the effect of limiting and excess amounts of inorganic N on the L-SD level, however, no effect was seen over the range tested. However, it might be that no concentration in the range tested was in fact limiting. It was therefore decided to determine the actual concentration of ammonium

Table VIII EFFECT OF GROWTH IN VARIOUS CONCENTRATIONS OF AMMONIUM SULFATE ON L-SD ACTIVITY

Concentration of Ammonium Sulfate (ug/ml)	L-SD Activity (um pyr/mg. prot)
500	0.31
1,000	0.31
2,000	0.36
4,000	0.21
8,000	0.30

* Assays were performed at L-serine concentration of 19.04 um/ml.

sulfate that is growth limiting, and to use this concentration to determine the effect of N limitation on enzyme level.

In order to do this, cells were grown overnight in 300 ug./ml. ammonium sulfate, then subcultured into 13 sidearm flasks containing MM-N, with the addition of 0 to 300 ug/ml. ammonium sulfate, and one other flask containing +Np. The flasks were incubated at 37°, with shaking, and the growth of the cells was followed by measuring the absorbance (in a Klett-Summerson colorimeter) every 30 minutes for 7 hours. Growth in 300 ug/ml was just as rapid and as extensive, as growth in +Np. However, levels of ammonium sulfate lower than 250 ug/ml were in fact limiting in both respects. It can also be noted that even in the flask where no ammonium sulfate was added, the bacteria were able to grow to a final absorbance of 70, which probably indicates that the medium is still not totally lacking in inorganic N (Figure VIII)

With the information as to limiting ammonium sulfate available, the effect of N limitation on L-SD activity could be investigated. Cells were grown overnight again in 300 ug/ml ammonium sulfate, and then subcultured into 4 flasks containing +N; -N + 60 ug/ml ammonium sulfate; -N +90 ug/ml ammonium sulfate and -N +120 ug/ml ammonium sulfate. Cells were harvested,

Figure VIII Growth of *E. coli* $K_{12}P^-$ with Low Levels of Inorganic Nitrogen

Cells of *E. coli* $K_{12}P^-$ were grown overnight in MM-N containing 300 ug/ml ammonium sulfate. Subcultures were then made into 4 sidearm flasks containing MM-Np with the addition of 0, 30, 120, and 240 ug/ml ammonium sulfate, and 1 sidearm flask containing +Np. The flasks were incubated at 37°, with shaking, and the growth of the cells was followed by measuring the absorbance in a Klett Summerson, colourimeter every 30 minutes for 7 hours.

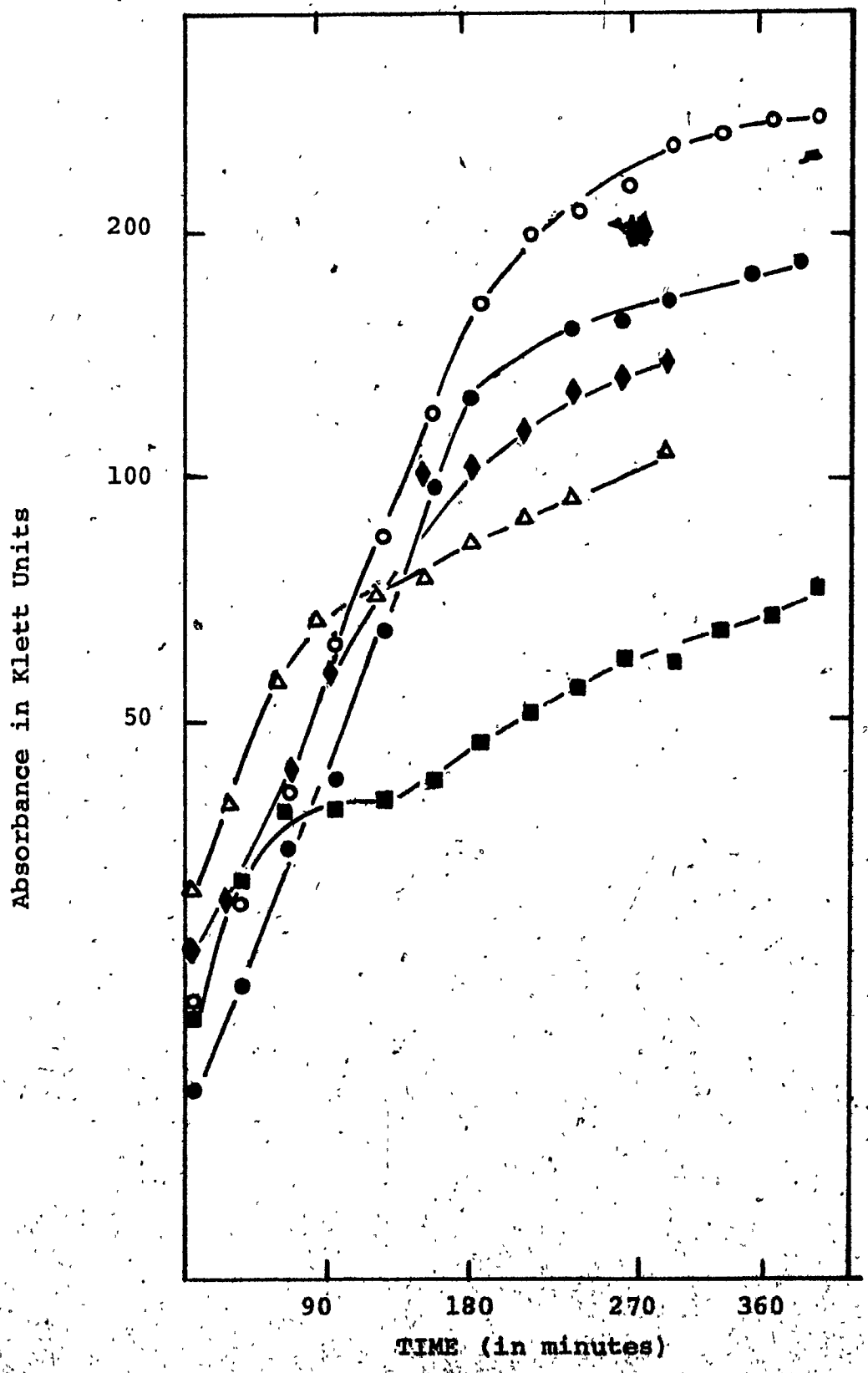
◇—◇ Growth in +Np

●—● Growth in 240 ug/ml ammonium sulfate

◆—◆ Growth in 120 ug/ml ammonium sulfate

△—△ Growth in 30 ug/ml ammonium sulfate

■—■ Growth in -Np


200
100
50
Absorbance in Klett Units
90
180
270
360
TIME (in minutes)

and assayed for L-SD activity at 120, 180 and 275 minutes after subculture (Table IX). Although levels of enzyme were generally higher in the -N cultures, no increase in enzyme level was seen between 120 and 275 minutes, i.e. at the time the N supply would be supposed to be depleted. Thus, in the culture incubated in 120 ug/ml. ammonium sulfate, enzyme levels changed very little and certainly do not increase (0.72, 0.52, 0.64 um pyr./mg. prot.). On the other hand, 7 of the 9 values measured in -N (0.59 - 1.08 um pyr./mg. prot) are higher than the values measured in+ N (0.48, 0.55 um pyr/mg. prot).

It is thus clear that the decrease in N concentration does result in increased L-SD activity. No difference can be seen between the enzyme level at late stages of growth in limiting N. This may simply mean that all concentrations of ammonium sulfate below 300 ug/ml result in higher enzyme levels.

B(2) <u>The Effect of Phase of Growth of Cells on L-SD Activity</u>

Another factor which appeared to have an effect on the level of L-SD in the cell was the phase of growth of the cells assayed. Table Xa provides data from a variety of experiments done on different days for a variety of purposes. These results indicate that cells

Table IX EFFECT OF GROWTH IN LOW CONCENTRATIONS OF AMMONIUM SULFATE ON L-SD ACTIVITY

Concentration of Ammonium Sulfate (ug/ml)	Time of Harvesting (after culture) min.	Absorbance at time of harvesting	L-SD Activity (um pyr/ mg prot)
60	120	61	0.80
	180	77	1.08
	275	105	0.38
90	120	59	0.83
	180	80	0.81
	275	120	0.59
120	120	57	0.72
	180	88	0.52
	275	120	0.64
200 (+N)	120	57	0.48
	180	125	-
	275	220	0.55

* Assays were performed at L-serine concentration of 19.04 um/ml.

Table X(a) EFFECT OF PHASE OF GROWTH OF CELLS GROWN IN +Nplg ON L-SD ACTIVITY

Absorbance (Klett Units)	L-SD Activity (um pyr/mg. prot)
36	0.90
93	0.88
108	1.38
153	0.13
157	0.30
165	0.43
190	0.47
236	0.69
292	0.60
290	0.22

* Assays were performed at L-serine concentration of 9.52 um/ml.

grown until early log phase tended to have higher enzyme levels than cells grown to later log or stationary phases. For example, cells harvested from +Nplg at an absorbance of 36 had 0.90 um pyr./mg. prot. activity while cells at an absorbance of 153 had an activity level of only 0.13 um pyr./mg. prot.

In order to test the validity of this generalization, the effect of absorbance on L-SD level was tested in one experiment. Cells were grown overnight in either +Np or +Nplg, and subcultured into several flasks containing the same medium as the preculture. Cells were harvested from both media as a function of time after subculture, the absorbance measured, and an assay for L-SD activity performed. As can be seen in Table Xb, in +Np, contrary to expectations, the absorbance or stage of growth in the range from 54 - 206 seemed to have little or no effect (absorbance of 54, 0.85 um pyr/mg. prot; absorbance 206, 0.42 um pyr./mg prot). In contrast, in +Nplg, a more definite effect of phase of growth of the cells was seen. In this case, log phase cells, absorbance 108, had a level of enzyme at 1.38 um pyr./mg. prot. while later log or stationary phase cells, absorbance 190, had an activity of only 0.47 um. pyr/mg. prot.

Table X(b) EFFECT OF PHASE OF GROWTH OF CELLS GROWN IN +Np AND +Nplg ON L-SD ACTIVITY

Growth medium	Absorbance of culture (in Klett Units)	L-SD Activity (um pyr/mg. prot)
+Np	54	0.85
	83	0.59
	110	0.59
	135	0.37
	168	0.74
	206	0.42
+Nplg*	36	0.90
	108	1.38
	190	0.47

* Assays were performed at L-serine concentration of 9.52 um/ml.

** Assays were performed at L-serine concentration of 19.04 um/ml.

Thus, the effect of phase of growth of cells assayed on L-SD activity is not completely clear. It appears to have an effect on enzyme level in the presence of glycine and L-leucine, but not in cells grown in their absence. This will be discussed below.

B(3) Effect of Growth in the Presence of Amino Acids on L-SD Activity

In Section A it was shown that the cell always maintains a basal level of L-SD. However, this does not exclude the possibility that there are some conditions in which the cell synthesizes more or less of the enzyme. Indeed, earlier reports (Pardee and Prestidge 1955 and Alfoldi et al 1968) have shown that L-SD is an inducible enzyme i.e., that growth in the presence of certain compounds results in a higher level of enzyme. Both Alfoldi, using E. coli K_{12}, and Pardee using Strain B, showed that growth in rich medium results in higher level of L-SD, and Pardee further demonstrated that it was the amino acids glycine and L-leucine that were responsible for the induction.

To test for an effect of growth in rich medium, cells of E. coli $K_{12}P^-$ were grown to an absorbance of 115 in yeast extract 0,1%, tryptone 1%, and glucose 0.04%, harvested, and assayed for L-SD activity. The level found, 0.83 um pyr./mg. prot., was only about basal level.

When cells were grown to an absorbance of 58 in the same medium without glucose, the enzyme level was 1.08 um pyr/mg prot. It seems therefore, that this strain of E. coli makes less enzyme in this medium than does that of either Alfoldi or Pardee.

Several amino acids, alone and in combination, were then tested for their ability to induce L-SD. These included L-serine, L-isoleucine, L-valine, glycine, L-leucine, L-aspartate, L-glutamic, and L-threonine, each added at a concentration of 50 ug/ml. except for -glycine, which was added at 75 ug/ml. Cells were grown in the presence of each amino acid until log phase (the absorbance of each culture is indicated in Table XI) at which time they were harvested and tested for activity against L-serine in the usual way.

In MM+N medium, -glycine and L-leucine induced but L-serine, L-threonine, L-aspartate, and L-glutamate did not. A combination of L-serine, L-isoleucine, and L-valine also failed to induce. In MM-N medium L-serine alone or in combination with L-isoleucine and L-valine still failed to induce. The level of enzyme measured in -Np serine (0.60 um pyr/mg. prot.) was in fact two times higher than the level in +Np serine (0.25 um pyr/mg prot) but this appears to be due to the effect of growth in MM-N (Section B(1).

Table XI INDUCTION OF L-SD

Exp. #	Growth Medium	Concentration of L-serine in Assay (um/ml)	Absorbance of culture (Klett units)	L-SD Activity (um pyr/ mg. prot)
1	Yeast-extract-tryptone+ glucose	9.52	115	0.83
	Yeast-extract- -glucose		58	1.08
2	+Np serine	9.52	72	0.25
	-Np serine		61	0.60
	+Np serine isoleucine valine		86	0.35
	-Np serine isoleucine valine		68	0.59
3	+Np threo-nine	19.04	124 100	0.22 0.32
	+Np aspartic		220 87	0.10 0.31
	-Np aspartic		67	0.29
	+Np glutamic		185 79	0.29 0.30
4	-Nplg	9.52	92 69	1.21 1.18
	-Nplg isoleucine valine		122 114	2.05 1.82
5	-Nplg	9.52	90	1.14
	-Nplg		30	0.93
	-Nplg		93	0.88
6 7	$^{+}Npl_{50}{}^{g}{}_{75}$	19.04 19.04	92 82	2.01 1.95
6 7	$^{+}Npl_{50}{}^{g}{}_{2000}$		107 97	1.82 2.56
6 7	$^{+}Npl_{0}{}^{g}{}_{2000}$		78 123	0.62 1.13
6 7	$^{+}Npl_{2000}{}^{g}{}_{75}$		65 -	1.59 -
6 7	$^{+}Npl_{2000}{}^{g}{}_{0}$		80 -	0.23 -

Although L-isoleucine and L-valine do not seem to be inducers at least in the presence of L-serine, when added to the growth medium along with -glycine and L-leucine, i.e. -Nplg. isoleucine valine, they resulted in an increase in the L-SD level (2.05 vs 1,21 and 1.82 vs 1.18 um pyr/mg prot. on 2 occasions) (Table XI).

As Pardee showed, a combination of -glycine and L-leucine has the greatest inducing ability. In +Nplg an average activity of 0.78 um pyr/mg. prot. (Table VII) was measured, and in -Nplg, an average activity of 1.34 um pyr/mg. prot (Table VII) in order to see whether -glycine and L-leucine were both needed, a comparison was made between cells grown in -Nplg, -Npl, and -Npg. The enzyme levels measured were 1.14, 0.93, 0.88 um pyr/mg. prot. respectively (Table XI). The increase in activity seem in the presence of the 2 amino acids did not appear to be significantly different from the activity measured in the presence of either amino acid alone.

However, certain strains of E. coli K_{12} appear to have difficulty in transporting -glycine into the cell (Newman 1970). For this reason, the effect of -glycine and L-leucine at much higher concentrations was tested. Cells were grown in MM+N at the usual concentrations of -glycine and L-leucine (75 and 50 ug/ml respectively) (+Nplg) in -glycine 2000 ug/ml, L-leucine 50 ug/ml (+Nplg) in -glycine 2000 ug/ml

without L-leucine (+NpG); in glycine 75 ug/ml., 2000 ug/ml (+NpgL); and in L-leucine 2000 ug/ml, without glycine (+NpL), (Table XI). The cells were assayed with twice the L-serine concentration used in the preceeding experiments (in 19.04 um/ml), and therefore, the numbers obtained are not strictly comparable.

As long as both amino acids were present, i.e., +Nplg, +NpLg, +NplG, the enzyme levels assayed was about the same (1.98, 1.59, 1.19 um pyr/mg. prot) respectively. The omission of either amino acid resulted in greatly decreasing activity (0.87 um pyr/mg. prot +NpG and 0.23 um pyr/mg. prot +NpL). Growth in L-leucine 2000 ug/ml without glycine resulted in even lower levels than growth without either amino acid (+Np 0.65 um pyr/mg. prot). It proved to be impossible to grow cells in medium containing both glycine 2000 ug/ml and L-leucine 2000 ug/ml.

Since glycine and L-leucine together at high concentration appeared to be inhibitory, it seemed possible that even at the usual physiological concentrations, they might have an effect on the growth rate. To test this, cells were grown overnight in +Np, chilled, centrifuged, washed and subcultured into sidearm flasks containing +Np with a variety of supplements. Growth was followed in the Klett with a 42 filter.

Doubling time was calculated from the linear part of an exponential plot of absorbance vs time. In +Np, +Np ser, +Npg, and +Npg ser, the doubling time was 55 minutes (figure 3). In +Npl, +Nplg, +Nplg ser, and +NpLser the doubling time was increased to about 95 minutes (Figure [illegible]). Thus, L-leucine appeared to decrease the growth rate; additions of L-serine and/or glycine with the L-leucine did not alter this. However, addition of L-isoleucine and L-valine along with L-serine, glycine and L-leucine completely reversed the inhibition seen in L-serine glycine L-leucine.

Thus, glycine and L-leucine are the best inducers for L-SD; and as Pardee showed originally, the induction is best in the presence of both, as opposed to each separately. L-isoleucine and L-valine, added with glycine and L-leucine, induce even further. This effect may be related to the fact that L-leucine inhibits growth and L-isoleucine and L-valine reverse that inhibition.

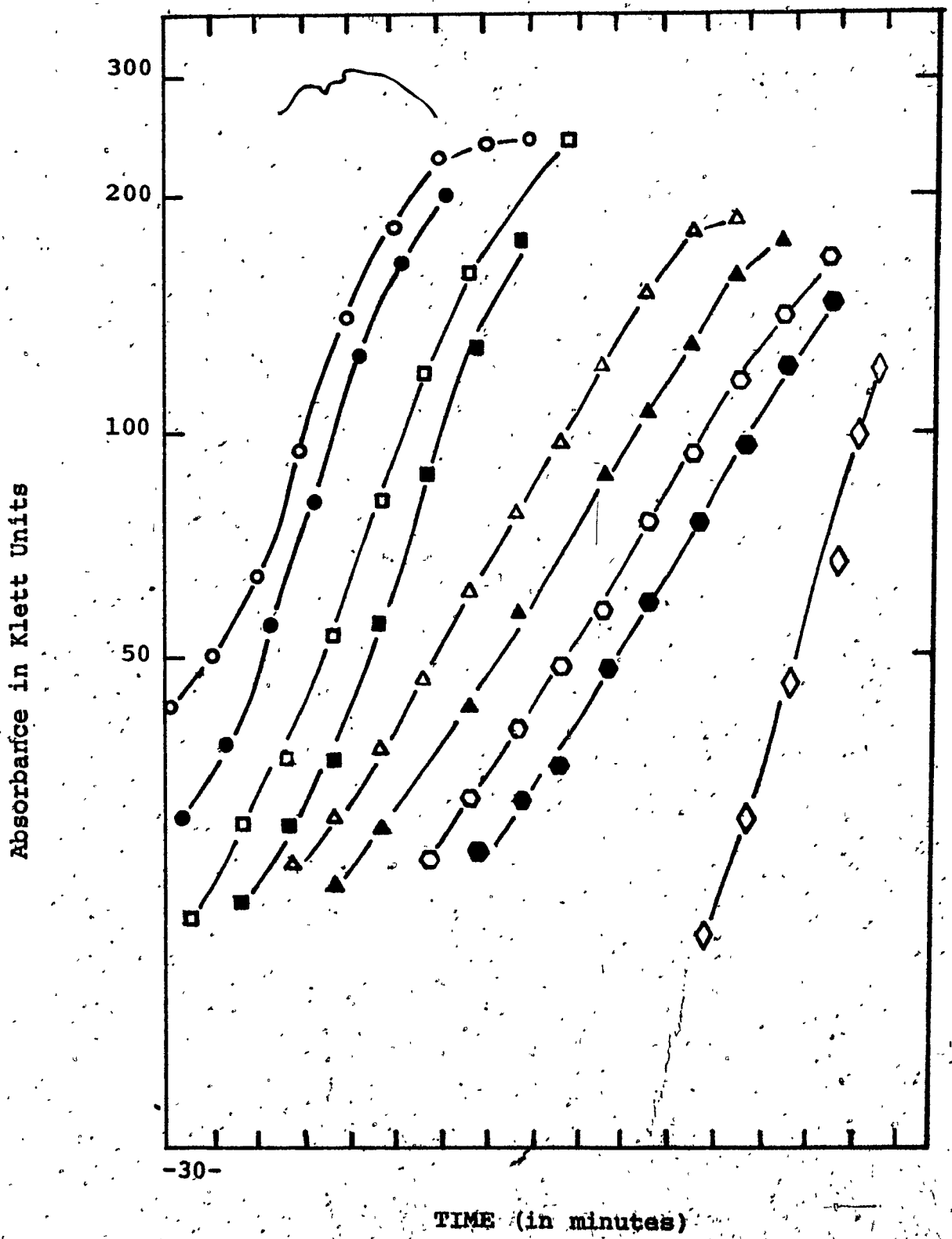
B(4) Growth on Different Carbon sources - The Glucose Effect

In experiments considered up until this point, the cells assayed were alsways grown with glucose as the carbon and energy source. However, glucose is known to

Figure IX Effect of Various Amino Acids on the growth of E. coli $K_{12}P^-$

Cells of E. coli $K_{12}P^-$ were grown overnight in +Np, and then sub-cultured into 9 sidearm flasks containing +Np supplemented with L-serine; glycine; L-serine+ glycine; L-leucine; L-leucine + glycine; L-serine+ glycine+ L-leucine; L-serine+L-leucine; and L-serine+ glycine+L-leucine +L-isoleucine+L-valine. Growth was followed by measuring the absorbance in a Klett Summerson colourimeter every 30 minutes for 3 hours. The doubling time of the culture was calculated from the linear part of the exponential plot of absorbance vs time.

	Growth medium	Doubling time (in minutes)
o—o	+Np	57
●—●	+Nps	53
□—□	+Npg	55
■—■	+Npgs	52
Δ—Δ	+NpL	90
▲—▲	+NpGL	95
O—O	+NpGLS	100
●—●	+NpLS	90
◊—◊	+NpSGLIV	55


300
200
100
50
Absorbance in Klett Units
TIME (in minutes)

have a repressing effect (catabolite repression) on several enzymes (Magasanik 1961). Those enzymes repressed by glucose are often catabolic (Magasanik 1961) i.e. energy producers. If there were an effect of glucose on L-SD, this might indicate a catabolic role for the enzyme.

To determine whether glucose was having any effect on L-SD production, the enzyme level was measured in cells grown on succinate or glycerol as carbon and energy sources. Each was tested in three situations: +Np (noninducing) +Nplg and -Nplg (both inducing). Cells were grown until log phase (absorbance about 80) and assayed in the usual manner. The results of this experiment are presented in Table XII. In both glycerol and succinate grown cells, the noninduced or basal level was very low at most (0.07 um pyr/mg. prot. and 0.11 um pyr/mg. prot. respectively) much lower than that of glucose grown cells (0.65 um pyr./mg. prot).

In the presence of L-glycine and L-leucine, in both MM±N medium, results for glycerol-and succinate-grown cells were very different.

In glycerol-grown cells, in MM+N, the presence of glycine and L-leucine had little or no inducing effect. The enzyme level was at best 2 times greater

Table XII EFFECT OF CARBON SOURCE ON L-SD ACTIVITY

Carbon Source	L-SD activity (um pyr/mg. prot) Growth Medium +Np	+Nplg	-Nplg
Glucose	0.65	1.98	2.55
Glycerol (Exp.1)	0.07	0.14	0.28
(Exp.2)	0.04	0.02	0.72
Succinate (Exp.1)	0.11	0.99	0.39
(Exp.2)	0.05	0.70	0.24

* Assays were performed at L-serine concentration of 19.04 um/ml.

(0.14 um pyr/mg. prot. vs 0.07 um pyr/mg. prot) than the basal level on the same carbon source. In MM-N in the presence of glycine and L-leucine however, the amount of enzyme measured on one occasion was 4 times and on another 20 times the noninduced level. It therefore seems that in glycerol-grown cells, glycine and L-leucine are less effective as inducers than they were in glucose grown cells, but the influence of inorganic N is still very apparent.

In succinate grown cells, in MM+N, glycine and L-leucine were very good inducers, causing 9 to 15 fold increases over the basal level measured in the same carbon source. Surprisingly, however the level in -Npgl was only 4 to 5 times above basal, and far below the level in +Nplg. In this case therefore, it seems that glycine and L-leucine acted well as inducers but that the presence of inorganic N produced the opposite result to what has been seen in all previous experiments.

From the above results, it can be noted that neither the basal nor induced L-SD levels as measured in glycerol- or succinate-grown cells was as high as the basal level determined in glucose grown cells. This indicates that there is no glucose repressing effect but rather that L-SD may be more active during non-oxidative (glucose) metabolism than during oxidative (glycerol or succinate) metabolism.

B(5) Demonstration of the 'in vivo' Instability of L-SD

In the cultures so far described, two levels of L-SD have been seen - a basal level of about 0.65 um pyr/ mg. prot. and an induced level of up to 2.55 um pyr./ mg. prot. in cells grown in the presence of glycine and L-leucine. Certain experiments were performed in order to determine the kinetics of the change from a noninduced (basal) to an induced state. These experiments did not in fact give information on the question originally asked. However, they have proved to be of considerable interest for other purposes.

For the first of these experiments, $K_{12}P^-$ cells were grown overnight in inducing media (-Nplg). They were then chilled and washed and 4 subcultures were made into inducing and noninducing media: +Np, -Np, +Nplg, -Nplg. A fifth subculture was made into inducing medium (-Nplg) to which chloramphenicol (20 ug/ml) was added in order to inhibit any further protein synthesis (Mahler and Cordes 1966, Trant and Munro 1964). All subcultures were incubated for 6 hours at 37°C. L-SD activity was determined in aliquots of each subculture after 3 and 6 hours, as well as in the overnight culture. The results of this experiment are presented in Table XIII and in the following diagram.

Table XIII DEMONSTRATION OF 'IN VIVO' INSTABILITY OF L-SD

Culture	Growth Medium	Absorbance (Klett Unit)		L-SD Activity (um pyr/mg. prot)	
Overnight	-Nplg	90		1.91	
		3 hrs. after subculture	6 hrs after subculture	3 hrs. after	6 hrs. after subculture
Subculture	+Np	87	257	0.28	0.28
Subculture	+Nplg	82	197	0.78	0.86
Subculture	-Np	53	62	0.86	0.84
Subculture	-Nplg	70	106	0.92	1.03
Subculture	-Nplg chloramphe-nicol	38	38	0.11	0.12

* Assays were performed at L-serine concentration of 9.52 um/ml

		Activity in um pyr/mg. prot. after 3 hrs.	after 6 hrs.
Overnight -Nplg subculture (1.91)	→ +Np	(0.28)	(0.28)
	→ +Nplg	(0.78)	(0.86)
	→ -Np	(0.86)	(0.34)
	→ -Nplg	(0.92)	(1.03)
	→ -Nplg chloramphenicol	(0.11)	(0.12)

In the overnight (ON) culture, enzyme activity was at a characteristic high level of 1.91 um pyr./mg. prot. The level was lower in every subculture tested, inducing or noninducing MM+N or MM-N. Thus, in inducing conditions with glycine and L-leucine, the level of activity in um pyr./mg. prot. at 3 hours was 0.78 in MM+N and 0.92 in MM-N. It slightly increased in the next 3 hours, 9% in MM+N and 12% in MM-N. In noninducing conditions, at 3 hours the level was slightly lower than in inducing media, i.e. 0.86 vs 0.92 in MM-N, moreover, by 6 hours the enzyme activity had decreased to 30.3 or 33% of the activity in inducing conditions. The subculture in noninduced MM+N reached this low level within 3 hours (0.28) and activity remained at that level thereafter (0.28 at 6 hours).

The most striking result of this experiment however is the final subculture in chloramphenicol in which no protein synthesis and no growth could occur. That the subculture did not grow is shown by the fact that at the time of innoculation the absorbance was 30, after three hours 38, and after six hours still 38. The slight increase seen can be attributed to changes in cell conformation during incubation at 37° from the conformation of the chilled cell used at the time of innoculation. In this subculture, L-SD activity fell to the lowest level of all, 0.11 after 3 hours. This remained the same (0.12) after 6 hours.

These results are clearly inconsistent with a classical induction system. If one postulates that all induced enzyme synthesis stops when the inducer is removed, one would expect a dilution of enzyme activity at the rate of 50% per generation in growing cultures. However, despite the fact that no dilution of enzyme by synthesis of new noninduced cells was possible, the one non-growing culture, i.e., the one with chloramphenicol added, loses activity even faster than that. It is clear that L-SD is unstable even within the cell. If L-SD is unstable inside the cell, the enzyme activity at any time will depend on the rate of synthesis of the enzyme, the rate of degradation and the presence or

absence of any stabilizing factors. Thus, the interpretation of these results is much less straightforward than was expected when the experiments were begun.

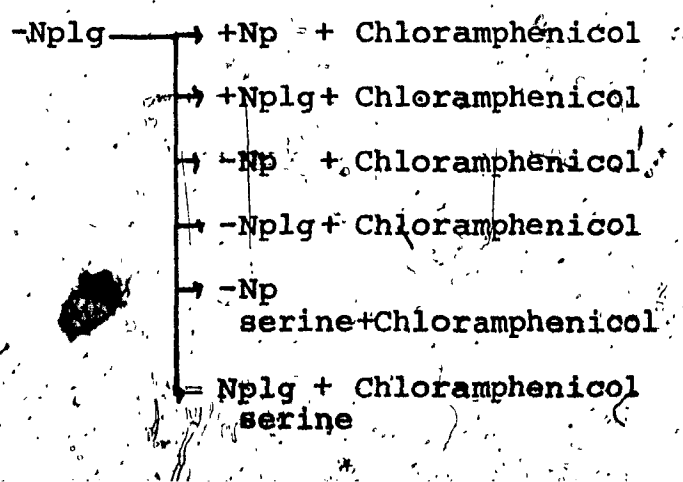
In chloramphenicol, enzyme was lost to the extent tht 92% was inactive by 3 hours, the remaining 8% stable thereafter. If one assumes that the same rate of loss occurs in -Nplg without chloramphenicol, to reach a level of 0.92 um pyr/mg. prot., enzyme must be synthesized to replace the degraded enzyme i.e. the different enzyme levels might not represent different rates of loss by dilution, but may represent different rates of synthesis in the different conditions. The meaningful comparison thus may be with a level of 0.11 and not 1.91 um pyr/mgprot.

One might expect the cells in noninduced media to loose enzyme activity and indeed they do, and at about the expected rate for dilution due to cell division. If the doubling time for the culture is about one hour, in three hours the enzyme level should decrease to about 12% of the original level. In the +Np culture the enzyme level is 0.28 um pyr/mg. prot. i.e. 14.7% of the original. Though the proceeding is consistent with a dilution model, the fact that the same loss is seen in inducing conditions complicates the analysis. Is the enzyme also unstable during growth in +Np? In

the presence of chloramphenicol no new protein synthesis occurs, but loss of activity due to instability is seen. Since no new enzyme is formed, the instability of pre-existing enzyme is seen to its largest extent. In all other conditions, the enzyme is still unstable, but growth and protein synthesis proceed normally and therefore enzyme was resynthesized and the degree of loss seen is less. Generally in a growing cell, whatever degradation of protein occurs is matched by resynthesis, and thus enzyme instability is masked by new synthesis. (Pine 1972, Shimke 1969)

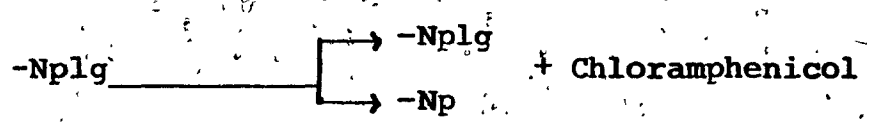
B(6) Rate of loss of L-SD Activity

It was clear from the preceeding results that L-SD is extremely labile even 'in vivo'. To further characterise this loss of enzyme activity, the rate of loss was observed in several situations - in inducing and noninducing media, in MM+N and in MM-N, as well as in the presence of L-serine, (see figure below). In all cases, chloramphenicol was added as inhibitor of protein synthesis.



B(6)a The Effect of Glycine and L-leucine on Enzyme Activity During Chloramphenicol Incubation

To observe the effect, if any, of the inducers, glycine and L-leucine on the loss of L-SD activity, a comparison was made between enzyme activity in cells shaken in inducing (+ glycine and L-leucine) and noninducing (- glycine and L-leucine) media, (see figure below) in both MM+N and MM-N.



B(6)a_1 Effect of Incubation in MM-N

Cells were grown overnight in inducing conditions, -Nplg, in order to achieve a high initial level of enzyme activity. They were then harvested, washed and subcultured into MM-N+ inducers (glycine and L-leucine), all in the presence of chloramphenicol at a concentration of 20 ug/ml. Five flasks of -Np (series A) and five flasks of -Nplg (series B) were incubated with shaking at 37°. At 2, 30, 60, 120, and 150 minutes after subculture, one flask of each series was removed from the shaker and assayed for L-SD activity in the usual manner. The results of such an experiment are presented in Table XIVa.

Table XIV(a) LOSS OF L-SD ACTIVITY DURING INCUBATION IN -Np CHLORAMPHENICOL AND -Nplg CHLORAMPHENICOL

Incubation Medium	Time of Assay	L-SD ACTIVITY (um pyr/mg. prot) Exp.#1	Exp.#2	Exp.#3	Exp.#4
-Nplg	After overnight growth	1.31	1.26	0.86	1.47
-Nplg chloramphe-nicol	2 min. after subculture	1.77	1.20	0.85	1.00
	30 min. after subculture	0.65	0.83	0.60	0.53
	60 min. after subculture	0.54	0.43	0.69	0.44
	120 min. after subculture	0.32	0.26	-	-
	150 min. after subculture	0.15	0.25	-	-
-Nplg	After overnight growth (same culture as above)	1.31	-	0.86	1.47
-Np chloramphe-nicol	2 min. after subculture	1.61	-	0.77	1.32
	30 min. after subculture	1.44	-	1.23	1.39
	60 min. after subculture	1.33	-	0.83	1.02
	120 min. after subculture	1.43	-	-	-
	150 min. after subculture	1.29	-	-	-

* Assays were performed at L-serine concentration of 9.52 um/ml.

An experiment of this type involves repeated centrifugation and washing of the cells. Thus, a sample assayed 2 minutes after subculture has been manipulated as follows: the overnight culture is centrifuged, the cells are resuspended in cold medium, recentrifuged, and resuspended again and innoculated into warm medium for 2 minutes; they are then chilled, once again centrifuged, resuspended and recentrifuged and finally resuspended for the enzyme assay.

That this extensive manipulation per se does not alter the enzyme level is surprising, but in fact true, can be seen from the fact that in 11 such experiments, the level of enzyme assayed at 2 minutes after subculture was only on two occasions more than 10% below the level of activity in the overnight culture (Tables XIV(b) and Table XVI(a). Table XIV(b) provides a comparison of the levels of activity measured in cells of overnight cultures and after 2 minute subculture.

After 2 minutes, however, the enzyme level in series A (-Nplg) decreased rapidly. In Experiment No. 1, the activity seen at 30 minutes was only 36.7% of that measured at 2 minutes. After this point, the rate of decrease in activity was slower. At 60 minutes, the levels of activity was 30.3% of that measured at 2 minutes,

Table XIV LOSS OF L-SD ACTIVITY DURING INCUBATION IN -Np CHLORAMPHENICOL AND -Nplg CHLORAMPHENICOL

(b) Comparison of levels of Activity in cells from overnight culture and 2 minute subculture

Exp.#	Incubation Medium	L-SD ACTIVITIY (um pyr/mg. prot) In overnight culture	In 2 min. subculture	Activity in 2 min. subculture as % of overnight culture
1	-Nplg chloramphenicol	1.31	1.77	134.7
2		1.26	1.20	96.3
3		0.86	0.85	98.7
4		1.47	1.00	68.2
1	-Np chloramphenicol	1.31	1.61	122.6
2		-	-	-
3		0.86	0.77	86.8
4		1.47	1.32	89.9

* Assays were performed at L-serine concentration of 9.52 um/ml.

and by 150 minutes, only 0.15 um pyr/mg.prot. or 9.2% of the 2 minute level of activity could be detected. When this experiment was repeated (Experiment No. 2 Table XIV(c)) the activity measured at 30 minutes was 65.7% of that at 2 minutes i.e. the rate of loss was slower than in the first experiment, however, by 60 minutes, only 33.3% of the 2 minutes level was left as had been seen in Experiment No. 1 and only 20% left by 150 minutes.

It is thus clear that in MM-Nplg L-SD activity is lost from the start of the subculture; no significant lag period during which the enzyme was stable could be detected. However, the rate of loss of activity appears to decrease with time.

The situation is not the same in the absence of inducers i.e. in -Np (series B). In this case, no significant loss of L-SD activity is seen. In all assays done, i.e. a total of 10 individual measurements at various time intervals, the level of L-SD activity was only once less than 75% of the 2 minute value.

Table XIV(c) indicates the percentage of the 2 minutes value seen at the various time intervals. It is obvious that in -Np, L-SD activity does not 'decay' and that loss of activity is therefore associated with the presence of inducers, glycine and L-leucine.

Table XIV LOSS OF L-SD ACTIVITY DURING INCUBATION IN -Np CHLORAMPHENICOL AND -Nplg CHLORAMPHENICOL

(c) Loss of L-SD Activity as % of Activity Measured in Cells of 2 minute subculture

Incubation Medium	Time of Assay (after sub-culture) (min.)	L-SD ACTIVITY (as % of Act measured at 2 min)			
		Exp.#1	Exp.#2	Exp.#3	Exp.#4
-Nplg chloramphenicol	30	36.7	65.7	70.6	53.3
	60	30.3	33.3	82.1	44.3
	120	19.3	21.7	-	-
	150	9.2	20.0	-	-
-Np chloramphenicol	30	90	-	157	105
	60	84	-	107	51
	120	90	-	-	-
	150	80	-	-	-

* Assays were performed at L-serine concentration of 9.52 um/ml.

B(6)a_2 Effect of Incubation in MM+N

From the above, it is clear, if paradoxical, that the presence of the inducers L-glycine and L-leucine results in a loss of L-SD activity. An induction effect has also been seen in MM-N as opposed to MM+N (Section B(1). Therefore, to determine if the presence of inorganic N would have any additional effect on the loss of L-SD activity, the experiment presented in Section B(6)a was repeated in MM+N, The results of this experiment are presented in Table XV.

Cells were grown overnight in -Nplg, but sub-cultured into Series A (+Nplg chloramphenicol) and Series B (+Np chloramphenicol). In this experiment, the level of activity measured after 2 minutes subculture was less than 1% below that of the overnight culture. After 2 minutes, the level of activity in Series A (+Nplg) decreased continuously. The activity seen at 30 minutes was 56.4% of that at 2 minutes. At 60 minutes, 44% remained, and at 150 minutes only 0.02 um pyr/mg. prot. i.e. about 1% of the 2 minute level could be measured.

In the absence of inducers (+Np Series B) the results were different. No significant loss of activity was observed. Of the individual determinations made between 30 and 150 minutes, the level of activity was never

Table XV LOSS OF L-SD ACTIVITY DURING INCUBATION IN +Np CHLORAMPHENICOL AND +Nplg CHLORAMPHENICOL

Incubation Medium	Time of Assay	L-SD ACTIVITY	
		um pyr/mg prot.	as % of 2 min. Level
+Nplg	after overnight growth	1.34	-
+Nplg chloramphenicol	2 min. after subculture	1.25	100
	30 min. after subculture	0.75	56.4
	60 min. after subculture	0.60	44.0
	120 min. after subculture	0.27	20.9
	150 min. after subculture	0.02	1.5
+Np	after overnight growth (same as above)	1.34	-
+Np chloramphenicol	2 min. after subculture	1.38	100
	30 min. after subculture	0.97	70.2
	60 min. after subculture	1.29	93.3
	120 min. after subculture	1.07	77.6
	150 min. after subculture	0.83	60.3

Assays were performed at L-serine conc. of 9.52 um/ml.

less than 60% of the initial 2 minute value. Thus levels may decrease or vary but are never very low and no trend of 'decay' was observed (See Table XV).

It is therefore clear that, as was seen in MM-N no significant loss of enzyme activity occurs in +Np (noninducing) but extensive loss is seen in the presence of the inducers (+Nplg). As well, the extent of loss in +Nplg appears to be even greater than in -Nplg.

B(6)b Effect of L-Serine on Loss of L-SD Activity

Previous studies have reported that under various conditions of loss of enzyme activity, the addition of the enzyme substrate has a 'protecting' effect i.e. can decrease or prevent the loss of activity (Alfoldi et al 1968, Grisolia 1964). It was therefore of interest to determine whether the loss of L-SD activity could be affected by the presence of its substrate L-serine. Therefore, the experiment presented in B(6)a was repeated, however in this case, further subcultures were made with the addition of L-serine (50 ug/ml.) to the media. The experimental design can be seen in the figure below and results are presented in Tables XVI(a) and XVI(b).

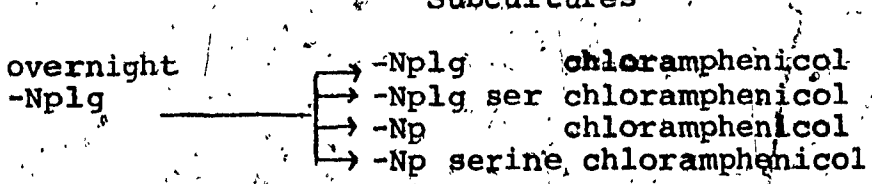


Table XVI(a) EFFECT OF L-SERINE ON LOSS OF L-SD ACTIVITY

Exp. #	Time of Assay	L-SD ACTIVITY (um. pyr/mg. prot) Incubation Medium (all+chloramphenicol			
		-Nplg.	-Nplg serine	-Np	-Npserine
1	after overnight growth	0.86	0.86	0.86	0.86
	2 min. after subculture	-	0.74	-	-
	30 min. after subculture	-	0.76	-	0.98
	60 min. after subculture	0.45	0.61	1.20	0.76
	120 min. after subculture	-	0.39	-	-
	150 min. after subculture	-	0.22	-	-
2	after overnight growth	0.84	0.84	0.84	0.84
	2 min. after subculture	-	0.98	-	-
	30 min. after subculture	-	0.86	-	1.04
	60 min. after subculture	0.44	0.77	1.19	0.52
	120 min. after subculture	-	0.41	-	-
	150 min. after subculture	-	0.43	-	-

* Assays were performed at L-serine concentration of 9.52 um/ml.

Table XVI(b) EFFECT OF L-SERINE ON LOSS OF L SD ACTIVITY

: ACTIVITY EXPRESSED AS % OF ACTIVITY MEASURED AT 2 MINUTES AFTER SUBCULTURE

Exp. #	Time of Assay (minute after subculture)	L-SD ACTIVITY (As % overnight value) Incubation Medium (all chloramphenicol)			
		-Nplg	-Nplg serine	-Np	-Np serine
1	30	-	87	-	113
	60	50	92	140	84
	120	-	45	-	-
	150	-	25	-	-
2	30	-	101	-	81
	60	52	70	71	70
	120	-	36	-	-
	150	-	39	-	-

* Assays were performed at L-serine concentration of 9.52 um/ml.

L-Serine did to some extent protect against the inactivation seen in inducing conditions. In this experiment, figures must be considered relative to the overnight values since no 2 minute sample was taken for the flasks without L-serine. On this basis, after 60 minutes, enzyme activity in two duplicate experiments was 92% and 70% with L-serine and 50% and 52% without L-serine. The values for the flasks with L-serine relative to the 2 minute culture are 80% and 77% which may be related to 30% and 33% without L-serine in Section B(6)a. However, in both the presence and absence of L-serine, after 60 minutes, enzyme activity was rapidly lost as before. After 150 minutes 39% in Experiment No. 1 and only 25% in Experiment No. 2 was measureable (as compared to the overnight values). Thus, it appears that addition of L-serine had some protecting effect against loss of enzyme activity but that this effect only lasted up to 60 minutes.

The situation in -Np was examined in less detail and only within the first 60 minutes after subculture. On the basis of previous experiments, no loss of enzyme activity was expected in -Np and none was seen by 60 minutes. In -Np serine, there was no loss within the first 30 minutes in two experiments. However, at 60 minutes 14% and 30% of the activity was lost. The

effect of L-serine in noninducing medium is thus less clear, but the subject was not pursued further.

It has therefore been observed that L-SD is unstable in vivo, especially in the presence of L-glycine and L-leucine the inducers. This effect has been seen in both MM+N and MM-N but occurs to a slightly greater degree in MM+N. In addition, L-serine can 'protect' against loss of activity seen in inducing conditions, however, only for a period of about 60 minutes. Its effect in noninducing conditions is not clear. These phenomenon are not well understood but will be discussed below.

B(7) Investigation into the Contribution of Enzymes, Other than L-SD, to Serine Deaminating Activity

In has been shown (Section 1) that L-SD is specific for L-serine, i.e. it will not deaminate other substrates under the conditions used here. However, other enzymes are known which are less specific and which in addition to their major activity, possess the ability to deaminate L-serine. If the extracts studied here contained such enzymes, the total L-serine deaminating activity measured might not be due to the enzyme presently under study, i.e. what appears to be L-serine deaminase activity might be the combination of L-serine deaminase and e.g. L-threonine deaminase activity on L-serine.

L-threonine deaminase is in fact the enzyme most likely to present such problems. Though its primary activity is the deamination of L-threonine to ∝ketobutyrate, it has often been reported to also have activity against L-serine (Davis and Metzer 1961, Umbarger 1957, Lessie et al 1969, Nishimura et al 1961 Alfoldi 1969). Indeed, several workers have considered that L-SD and L-TD might be names for the same enzyme molecule.

Even though the extracts studied appeared to have activity only against L-serine, it seemed important to be sure that no other enzyme was being measured or misread as L-SD. A variety of experiments were therefore designed to determine if L-TD was in fact active in this assay system, and if it were, to devise a method to distinguish and separate the activity of L-TD[1] from that of L-SD and determine if the two were different enzyme molecules.

In section I(3) it was shown that toluene treated cells show activity against both L-serine and L-threonine at pH 7.5 but only against L-serine at pH 6.4. The experiments about to be described were performed before this was discovered. In order to determine if L-TD was active in this assay system, a

1 (There are in fact at least 2 L-TD's in E. coli)

control against L-threonine as substrate was included in every L-serine assay reported in this thesis. Table XVII provides representative results showing that no activity was ever observed against L-threonine as substrate at pH 6.4.

Because L-TD is known to require pyridoxal phosphate (PP) as cofactor (Umbarger and Brown 1949, Alfoldi et al 1969, Nishimura et al 1961 and Wood 1969), PP at 38 ug/ml. was added to the assay mixture, but still no activity was detected. (Table XVII). These results seemed to indicate that L-TD was not functioning in this system and therefore could not contribute to the activity against L-serine.

However, L-TD is well known to exist in E. coli and therefore the possibility was considered that it may in fact have been present and contributing to the activity measured against L-serine, but that it could not be detected by the assay being used. One therefore sought a more indirect way of determining whether L-TD activity was involved.

Methods are known which can specifically alter the activity of L-TD i.e. inhibit, induce or repress the enzyme. If the use of such methods resulted in a change

Table XVII A COMPARISON BETWEEN ACTIVITY AGAINST L-SERINE AND L-THREONINE

Exp.#**	Activity against L-Serine (um pyr/mg. prot)	Activity against L-threonine (um pyr/mg prot)	Activity against L-threonine in the presence of pyridoxal phosphate (um pyr/mg prot)
1	1.22	0.08	0.03
2	1.11	0	0.19
3	2.05	0.02	0.51
4	1.82	0	0.03

** Assays of experiments #1 and #2 were done on cells grown in -Nplg ,

Assays of experiments #3 and #4 were done on cells grown in -Nplg isoleucine and valine

* Assays were performed at L-serine concentration of 9.52 um/ml and L-threonine concentration of 8.40 um/ml.

in the level of activity against L-serine being measured, it would be clear that L-TD was indeed contributing to the L-serine deaminating activity being measured, and the extent of change would indicate the extent of L-TD participation. The details of such experiments are described below.

B(7)a The Effect of Conditions Known to Repress L-TD

L-isoleucine is a known repressor of biosynthetic L-TD (Wood 1969) i.e. the addition of L-isoleucine to the growth medium results in the decreased synthesis of this enzyme. Therefore, the activity of L-TD against L-serine could be clearly indicated if growth in the presence of L-isoleucine resulted in a decrease in the activity being measured against L-serine. Such an experiment was conducted as shown in the Figure below.

Growth medium	Assay Conditions
-Nplg	Serine ± PP threonine ± PP
-Nplg isol. val.	Serine ± PP threonine ± PP

Cells were grown in inducing conditions (-Nplg) with the further addition of L-isoleucine at a concentration of 50 ug/ml. L-valine was also included in this

medium, also at 50 ug/ml., since L-isoleucine alone inhibits the growth of E. coli K_{12} (Umbarger 1969). As a control, cells were grown in the same inducing medium (-Nplg) excluding L-isoleucine and L-valine. This experiment was repeated twice. The cells of both of these cultures were grown overnight, harvested in log phase of growth (absorbance 92 - 120) and assayed for activity against both L-serine and L-threonine in the usual way. Since previous experiments had not given conclusive results on the PP requirement, assays were also performed against L-serine and L-threonine with the addition of PP to the assay mixture to prevent this being a limiting factor. Enzyme activity was unaffected or very slightly increased by the presence of PP.

Cells grown in the presence of L-isoleucine and L-valine in addition to glycine and L-leucine had higher levels of activity against L-serine than cells grown in their absence (-Nplg) i.e. 2.05, 1.82 vs 1.22, 1.11 um pyr./mg. prot. respectively (Table XVII(a). The same pattern was seen in assays to which PP was added, i.e. 2.40, 1.89 vs 1.61, 1.13 um pyr/mg. prot. in presence and absence of L-isoleucine and L-valine respectively. Thus, far from repressing the enzyme, L-isoleucine and

Table XVIII(a) EFFECT OF GROWTH IN THE PRESENCE OF L-ISOLEUCINE AND L-VALINE ON L-SD ACTIVITY

Exp.#	Growth Medium	L-SD ACTIVITY (um pyr/mg. prot) Substrate			
		L-Serine	L-serine +PP**	L-threonine	L-threonine +PP
1	-Nplg	1.22	1.61	0.08	0.03
	-Nplg isoleucine valine	2.05	2.40	0.02	0.51
2	-Nplg	1.11	1.13	0	0.19
	-Nplg isoleucine valine	1.82	1.89	0	0.03

** Pyridoxal phosphate

* Assays were performed at an L-serine concentration of 9.52 um/ml and L-threonine concentration of 8.40 um/ml.

L-valine induce it at least in the presence of glycine and L-leucine.

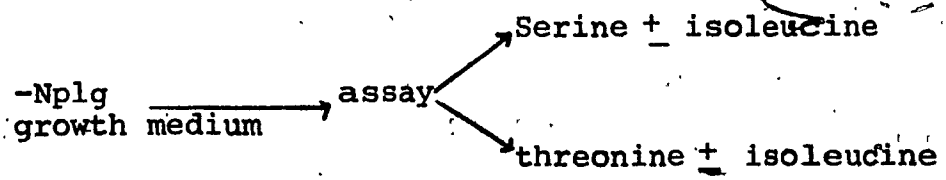
In the absence of PP no activity was seen against L-threonine (0.02, 0, vs 0.08, 0 um. pyr/mg. prot. respectively). In the presence of PP, in some cases, a slight amount was seen in both cultures (0.51, 0.03 vs 0.03, 0.19 um pyr/mg. prot. respectively). The effect of PP is unclear since it stimulated activity against L-serine and L-threonine, but only in some cases - the variability of this effect therefore allows no conclusion.

In any case, the results of this experiment appear to indicate the lack of L-TD participation in L-serine deaminating activity since, had it been contributing, the level of activity against L-serine should have decreased under the conditions that repress L-TD, but it did not.

B(7)b Effect of Conditions Known to Inhibit L-TD

As well as being a repressor of L-TD, L-isoleucine is known to inhibit the activity of L-TD (Lessie et al 1961, Umbarger and Brown 1957, Umbarger 1956, and Wood 1969 and Alfoldi et al 1969), i.e. the addition of L-isoleucine to the assay mixture results in

a decrease in the activity of this enzyme. Thus, if L-TD were contributing to activity measured against L-serine, addition of L-isoleucine to the assay should result in a decrease in the activity measured against L-serine. An experiment designed to determine this was conducted as diagrammed below.



Cells were grown overnight in inducing medium (-Nplg), harvested in log phase (absorbance 70 - 90) and assayed for activity against L-serine and L-threonine in the usual manner except with the addition of L-isoleucine (at same concentration as the substrate i.e. 2.5 mg/ml.). A control assay was done without the addition of L-isoleucine.

In the presence of L-isoleucine, activity measured against L-serine on three occasions was not significantly lowered as compared to that measured in its absence (Table XVIII(b)). Thus, L-isoleucine does not inhibit the enzyme activity to the extent expected of a feedback inhibitor (Alfoldi et al 1969).

The effect of L-isoleucine was also studied in cells grown in the presence of other metabolites: (a) L-threonine 50 ug/ml., (b) L-isoleucine and L-valine (each 50 ug./ml.) and (c) pyridoxine (30 ug/ml.). In

Table XVIII(b) EFFECT OF L-ISOLEUCINE ON L-SD ACTIVITY

Growth Medium		L-SD ACTIVITY Substrate				
		L-serine	L-serine + L-isoleucine	L-serine + L-isoleucine / L-serine (as %)	L-threonine	L-threonine + L-isoleucine
-Nplg	(a)	1.22	1.03	85	0.08	0
-Nplg	(b)	1.18	0.90	76	0	0
-Nplg	(c)	1.65	1.36	82	0	0
-Nplg threonine		1.42	1.31	93	0	0.03
-Nplg isoleucine valine	(a)	2.05	1.80	88	0.02	0
	(b)	1.82	1.77	97	0	0
-Nplg pyridoxine		1.47	1.64	111	0.09	0.05

* Assays were performed at L-serine concentration of 9.52 um/ml and L-threonine concentration of 8.40 um/ml and L-isoleucine concentration of 7.63 um/ml.

these cases, the activity seen against L-serine in the presence of L-isoleucine was again not significantly altered.

With respect to L-threonine as substrate, no activity could be measured in cells from any of the above cultures (Table XVIII(b) and therefore no effect of L-isoleucine could be observed.

It has thus been seen that the activity of L-TD against L-serine could not be demonstrated by the use of repression or inhibition of that enzyme. However, L-TD is well known to exist in E. coli and therefore should have been assayable under some conditions, if not under the ones thus far used. It therefore became important to find the appropriate conditions in which L-TD could be measured and studied. For this purpose, the following experiments were designed.

B(7)c Effect of Conditions Known to Induce L-TD

L-TD activity has thus far been undetectable. In case this was due to the fact that only low levels of this enzyme exist under the various growth conditions that had been used, attempts were made to induce i.e. increase the level of L-TD in the cell.

L-Threonine Deaminase (degradative) has been demonstrated to be inducible (Wood 1969) by L-threonine

Table XVIII(c) EFFECT OF CONDITIONS EXPECTED TO INDUCE L-TD, ON L-SD ACTIVITY

Growth Media	L-Serine Deaminating Activity (um pyr/mg. prot) Substrate	
	L-Serine	L-threonine
-Nplg	1.75	0
-Nplg threonine	1.42	0
-Nplg pyridoxine	1.48	0.09
-Nplg threonine (without glucose)	0.50	0

* Assays were performed on L-serine concentration of 9.52 um/ml and L-threonine concentration of 8.40 um/ml.

(Sayre et al 1956). Therefore, to increase L-TD levels, cells were grown in inducing medium (-Nplg) with the further addition of L-threonine, at a concentration of 50 ug/ml. This was compared with cells grown in the absence of L-threonine, i.e. -Nplg. As Table XVIII(c) indicates, still no activity could be measured against L-threonine in cells from either culture. In cells grown in the presence of L-threonine, activity against L-serine was not significantly increased from the level of those grown in the absence of L-threonine. If L-TD had been induced and contributing to activity against L-serine, this activity should have increased. This therefore seemed to be further evidence against the involvement of L-TD.

In a second experiment, cells were grown with the addition of pyridoxine (30 ug/ml), the cofactor of L-TD, to the growth medium (-Nplg). If this cofactor had been in limiting amounts, this could have explained the absence of L-TD activity. Results compared with those grown in the absence of pyridoxine. Still, activity against L-threonine was essentially absent (0.09 um pyr/mg. prot.) and the effect on activity against L-serine was again insignificant.

Another method tried to induce the L-TD level in the cells was the following: cells were grown overnight in -Nplg and then subcultured into fresh -Nplg medium in the presence of largely excess amounts of L-threonine (2000 ug/ml), and only a small amount of glucose (0.004%) and grown to log phase (absorbance 80). In this way, it was hoped that cells would be forced to use L-threonine, rather than glucose, as the carbon source (Wood 1969). The use of L-threonine as carbon source requires that the cell must first deaminate the L-threonine, and therefore one might expect high levels of L-TD in cells grown in these conditions. The small amount of glucose was added to allow the cells to start growing and adapt to using L-threonine as carbon source. Unfortunately, as Table XVIII(c) shows, even this method was unsuccessful at inducing measureable amount of L-TD as activity against L-threonine as substrate or as increased activity against L-serine.

The approach thus far used seemed to indicate that L-TD was not involved in this assay system. Though L-TD could not be measured, it must have been varied under the various conditions tried, and therefore if it had been responsible for the L-serine deaminating activity (in part or in total) this measurement would also have varied, but it did not. Therefore, all conclusions made up to this point about L-SD should still be valid.

However, it had also become quite clear that rather than some fault in the design of the experiments, something about the conditions of the assay or procedure themselves had resulted in L-TD being unassayable or inactive while still not affecting L-SD activity.

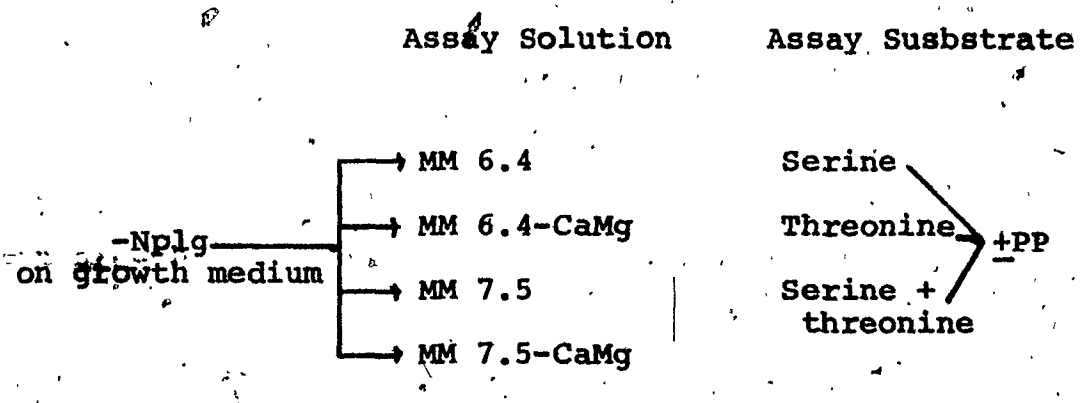
Two factors which might account for the inability to measure L-TD are the pH at which the assay was conducted and the presence of inorganic ions, Mg^{++} and Ca^{++}, in the solutions used to resuspend the cells after harvesting (Feigelson, personal communication). That the pH of the assay is important has already been demonstrated (Section I(3), though it was not known when the experiments described in the preceeding sections were performed. The presence of ions in the resuspension solutions could have been important because of their ability to bind to cofactors (Feigelson). With the cell being made more permeable due to the toluene treatment, these ions could enter, bind to the cofactor (e.g. PP), and thus inactivate L-TD. In the experiments about to be described, both of these factors were indeed found to influence the enzyme assay.

B(7)d Effect of pH and Ions

The L-SD assay was routinely performed on cells suspended in minimal medium (MM) pH 6.4. This medium

contains phosphate buffer, $CaCl_2$, and Mg SO_4, and depending on the circumstances, also ammonium sulphate. To study the effect of pH, a similar medium was prepared in which the phosphate was buffered at pH 7.5. These are referred to as MM 6.4 and MM 7.5 respectively in order to study the effect of ions on the assay the same media were prepared omitting the $CaCl_2$ and $MgSO_4$. These are referred to as MM6.4 - CaMg and MM7.5 - CaMg respectively but it should also be noted that they are also missing the chloride and sulphate ions.

Thus, to test the effect of pH and ions on the L-SD assay, the following experiment was conducted (figure below).



Cells were grown to log phase (absorbance 68) in inducing medium (-Nplg). The culture was then divided into four parts which were harvested by centrifugation and then resuspended, one part in each of the following solutions: MM6.4, MM6.4 - CaMg, MM7.5, and MM7.5 - CaMg. Cells resuspended in the above solutions were assayed in the usual way for activity against L-serine, L-threonine, and L-serine and L-threonine together. All of these were assayed in the presence and absence of PP. (See Table XIX).

A comparison of results at pH 6.4 with and without ions shows that activity against L-serine is greater in the absence of ions (2.25 vs 3.83 and 0.99 vs 2.92 um pyr/mg. prot). Thus, one can conclude that the ions interfer in some way with the measurement of L-SD activity. If the effect of ions was indeed by binding to endogenous PP, addition of exogenous PP should counteract this effect and restore activity. In fact, the addition of PP to MM6.4 increased activity quite noteably (0.99 vs 1.54 and 2.25 vs 4.67 um pyr/mg. prot). These are however the only experiments in which PP had this effect (see Section I(2). The addition of PP to MM6.4 - CaMg increased activity in one experiment (3.83 vs 5.53) but not in another (4.04 vs 4.28 um pyr/mg. prot). This is consistent

Table XIX EFFECT OF IONS AND pH ON DEAMINATING ACTIVITY AGAINST L-SERINE AND L-THREONINE

Exp.#	SUBSTRATE	DEAMINATING ACTIVITY (um pyr/mg. prot) Resuspension Solution MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
1	L-serine	2.25	3.83	5.28	5.90
	L-serine+PP**	4.67	5.53	6.80	7.62
	L-threonine	0.21	0.20	2.14	3.44
	L-threonine+PP	0	0.11	3.95	4.66
	L-serine+ L-threonine	2.30	4.27	5.76	7.60
	L-serine+ L-threonine+PP	4.33	6.08	7.21	9.60
2	L-serine	0.99	4.04	3.79	2.37
	L-serine+PP	1.54	4.28	3.98	1.93
	L-threonine	0	0	1.80	2.83
	L-threonine+PP	0.29	0.72	2.38	2.14
	L-serine+ L-threonine	-	-	-	-
	L-serine+ L-threonine+PP	1.49	4.03	4.66	4.21

** Pyridoxal phosphate

* Assays were performed at L-serine concentration of 9.52 um/ml., and L-threonine concentration of 8.40 um/ml.

with a hypothesis that PP is required for L-SD activity and is bound by ions. One could then assume that the quantity present in MM6.4 - CaMg was just sufficient to saturate thesystem in one experiment (4.04 vs 4.28) but not quite enough in the other (3.83vs 5.53). However, because this effect of PP in MM6.4 was seen only on this occasion, the role of PP remains uncertain and the effect of ions may not be related to PP at all.

With respect to the effect of pH, very significant differences were seen at the two pH's. Activity against L-threonine was seen only at pH 7.5, 2.14 vs 0.21 and 1.80 vs 0 um pyr/mg. prot. at pH 6.4 and 7.5 respectively. Thus it appeared that the pH normally used for assay in this study, i.e. 6.4 was not in the range for the activity of L-TD.

An ion effect was also seen in the assays against L-threonine. Thus, omission of ions at pH 7.5 resulted in an increase in activity against L-threonine (3.44 vs 2.14, and 2.83 vs 1.80 um pyr/mg. prot). Again this could be due to binding of PP. However, the effect of the addition of PP to the assay mixture is again not quite clear. In the presence of ions MM 7.5 addition of PP did increase activity against L-threonine (1.80 vs 2.38 and 2.14 vs 3.95 um pyr/mg prot.)

However, in the absence of ions, MM 7.5 - CaMg, PP did increase activity in one experiment (3.44 vs 4.66) but not in another (2.83 vs 2.14). This is consistent with the idea that cells normally contain enough PP so that none need be added to the assay. In the presence of ions, the free PP is sufficienty reduced so that the addition of more PP does increase activity. If one assumes that the level of PP in the cell was just saturating, one could explain the variability in response to PP in cells assayed in the absence of ions as being due to the variations in cellular PP. However, no definite conclusion can be made on the basis of these experiments.

Conducting the assay at pH 7.5 also had an effect on the activity measured against L-serine. In MM, activity against L-serine at pH 7.5 increases over that at pH 6.4 (3.79 vs 0.99 and 5.28 vs 2.25 um pyr/ mg. prot). This could be due to an increase in activity of L-SD at the higher pH, but might rather be due to the contribution to activity against L-serine by L-TD which is also active at this higher pH, or even both.

At pH 7.5, the omission of ions resulted in an increase in activity against L-serine in one experiment

(3.79 vs 2.37) but not in another (5.28 vs 5.90 um pyr/mg. prot). Similarly, the addition of PP, as usual, did not give consistant results, causing an increase in one experiment in both MM and MM - CaMg (5.28 vs 6.80 and 5.90 vs 7.62 um pyr/mg. prot. respectively), but not in another experiment (3.79 vs 3.98 and 2.39 vs 1.93 um pyr/mg. prot. respectively). The effect of ions and PP on activity against L-serine at pH 7.5 are not clear for the following reasons: At pH 7.5 these factors are probably affecting the activity of at least two enzymes on L-serine, i.e. L-SD and L-TD. But, the effects of these factors on each enzyme assayed separately were not themselves completely clear and the combined effect that changes in both enzymes caused by ions or PP would have on activity against L-serine are that much more uncertain.

Another part of this experiment involved the assay of L-serine and L-threonine together at pH 6.4 and 7.5 and ± ions. At pH 7.5, where both L-SD and L-TD are active, it was observed that the activity measured against both substrates together was not as high as the sum of the activity measured against each separtely (5.76 vs 7.42 respectively Table XIX). The

effect was the same upon the omission of Ca and Mg, however, both values were higher (7.60 vs 9.34 um pyr/ mg. prot. respectively). If there were a competition between the substrates for the active site of one or both of the enzymes, or if either enzyme was inhibited by the other substrate this result might be expected.

Thus, these experiments have demonstrated several points. It is now clear that the cells of K_{12} P^- do contain L-TD although it is only apparent if the assay is conducted at a high pH, i.e. 7.5 as opposed to 6.4 It also appears that a competition between L-serine and L-threonine, or an inhibition effect of one of the substrates on L-SD or L-TD exists. The presence of ions generally has some kind of inhibitory effect on both L-SD and L-TD. The effect of PP on L-SD is less clear.

B(8) Separation of L-SD and L-TD Activity: Demonstration of the Presence of Two Distinct Enzymes - L-SD and L-TD

In the preceeding sections, it has been shown that L-serine deaminating activity can be assayed with no involvement of L-threonine deaminase, by conducting the assay at pH 6.4. However, since L-TD is well known to exist in E. coli and to deaminate L-serine, and since L-SD is much less well documented, the question still

arises as to whether these activities are due to one or two separate enzyme molecules. One could, after all, imagine a single protein molecule which might be active against L-serine at pH 6.4 and active against both L-serine and L-threonine at pH 7.5. This question was approached using the 'enzyme decay' experiment described in Section B(6).

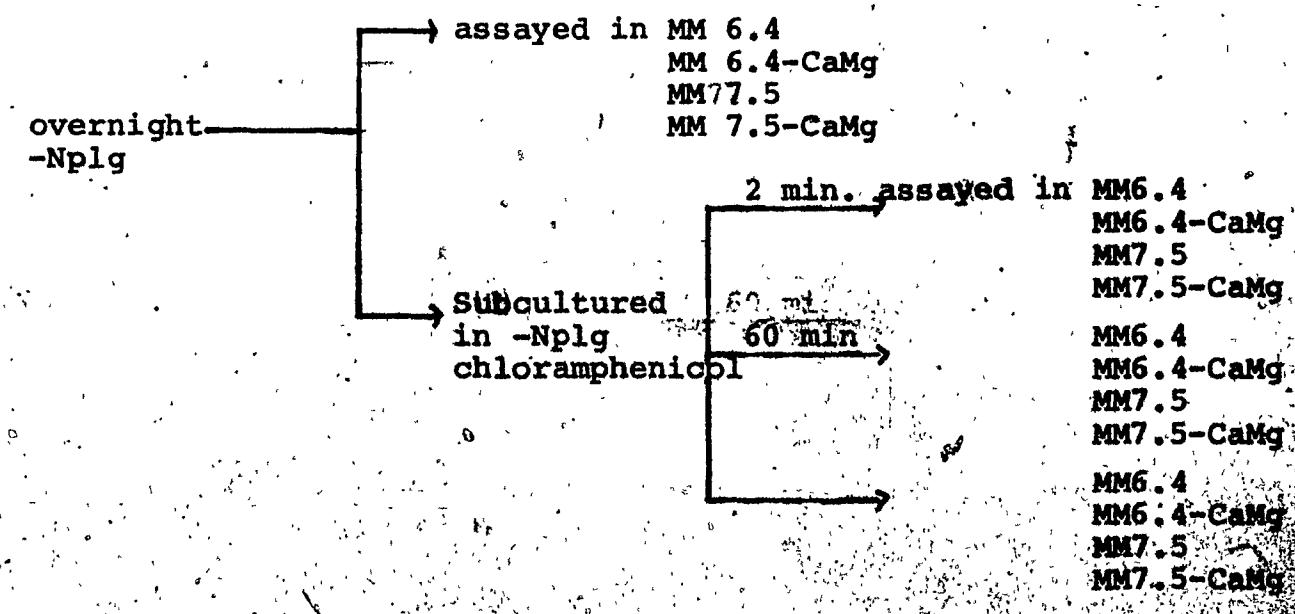
It has been established (Section B(6)) that in cells incubated at pH 6.4, activity against L-serine is unstable and decays. If L-TD and L-SD were both the activities of one enzyme protein, activity against L-threonine as measured at pH 7.5, should simultaneously decay with activity against L-serine, also at pH 7.5. If two enzyme molecules were involved (and activity were measured at pH 7.5 where both substrates are deaminated), activity against L-serine would decay as seen before, but activity against L-threonine would not.[1]

Thus, as diagrammed in figure below, cells were grown overnight in inducing conditions (-Nplg) till early log phase (absorbance about 55). Cells were then centrifuged, washed and subcultured into three flasks each containing -Nplg + chloramphenicol, which

[1] L-TD is known to be stable and has even been partially purified (Davis et al 1961)

were then incubated shaking at 37°. At 2, 60 and 150 minutes after subculture, one flask was removed from the shaker and assayed for L-SD activity. The assay was conducted in 4 media as described in Section B(7)d i.e. each subculture was divided into 4 parts which were resuspended in one of the following solutions: MM6.4, MM6.4 - CaMg, MM7.5 and MM7.5 - CaMg (Figure below).

As above, the overnight culture was assayed in the same manner as was each subculture after 2 minutes, as a check on the effect of the manipulations made during preparation of the assay. The change in this case after two minutes was at most a decrease of about 7% from the overnight values. All subsequent values were compared with the 2 minute value.



The results of 2 such experiments are presented in Figure X and Tables XX and XXI.

Activity against L-serine, as measured at pH 6.4 decayed as expected. In MM, by 60 minutes only 42% or 31% of the activity at 2 minutes still remained, and by 150 minutes, only 8% or 16% remained. In the absence of ions, the results were similar: 41% and 68% at 60 minutes, and 13% and 6% at 150 minutes. The the molecule carrying L-SD activity appears to be unstable in these conditions.

At pH 6.4 activity against L-threonine is low in any case, and a clear picture does not emerge. Such loss of activity as is seen may simply indicate that L-SD has some, though very little L-TD activity.

The results at pH 7.5 are very different. Activity against both L-serine and L-threonine occurs at high levels and a clearer change can therefore be observed. Activity against L-serine was once again rapidly lost. In MM 7.5, by 60 minutes only 57% or 35% of the 2 minute value remained, and by 150 minutes only 34% and 15% respectively remained. The absence of ions had little effect: 53% or 35% remaining after 60 minutes, and 38% or 12% at 150 minutes.

Activity against L-threonine was much more stable.

Figure X Deaminating Activity Measured in Different Assay Conditions as a Function of Time of Incubation in Chloramphenicol containing Medium

Cells of E. coli $K_{12}P^-$ were grown overnight in -Nplg and then sub-cultured into -Nplg chloramphenicol which were incubated shaking at 37°C. At 2, 60 and 150 minutes after subculture, cells were assayed for L-SD activity. The assay was conducted in the following 4 media: MM6.4; MM6.4-CaMg; MM7.5; and MM7.5-CaMg. Activity was measured against both L-serine (at 19.04 u moles/ml) and L-threonine (at 16.80 u moles/ml) as substrates.

Activity against L-serine	o—o	Activity Measured in MM7.5
	□—□	Activity Measured in MM7.5-CaMg
	●—●	Activity Measured in MM6.4
	■—■	Activity Measured in MM6.4-CaMg
Activity against L-threonine	O-O	Activity Measured in MM7.5
	Δ—Δ	Activity Measured in MM7.5-CaMg
	⬢-⬢	Activity Measured in MM6.4
	▲—▲	Activity Measured in MM6.4-CaMg

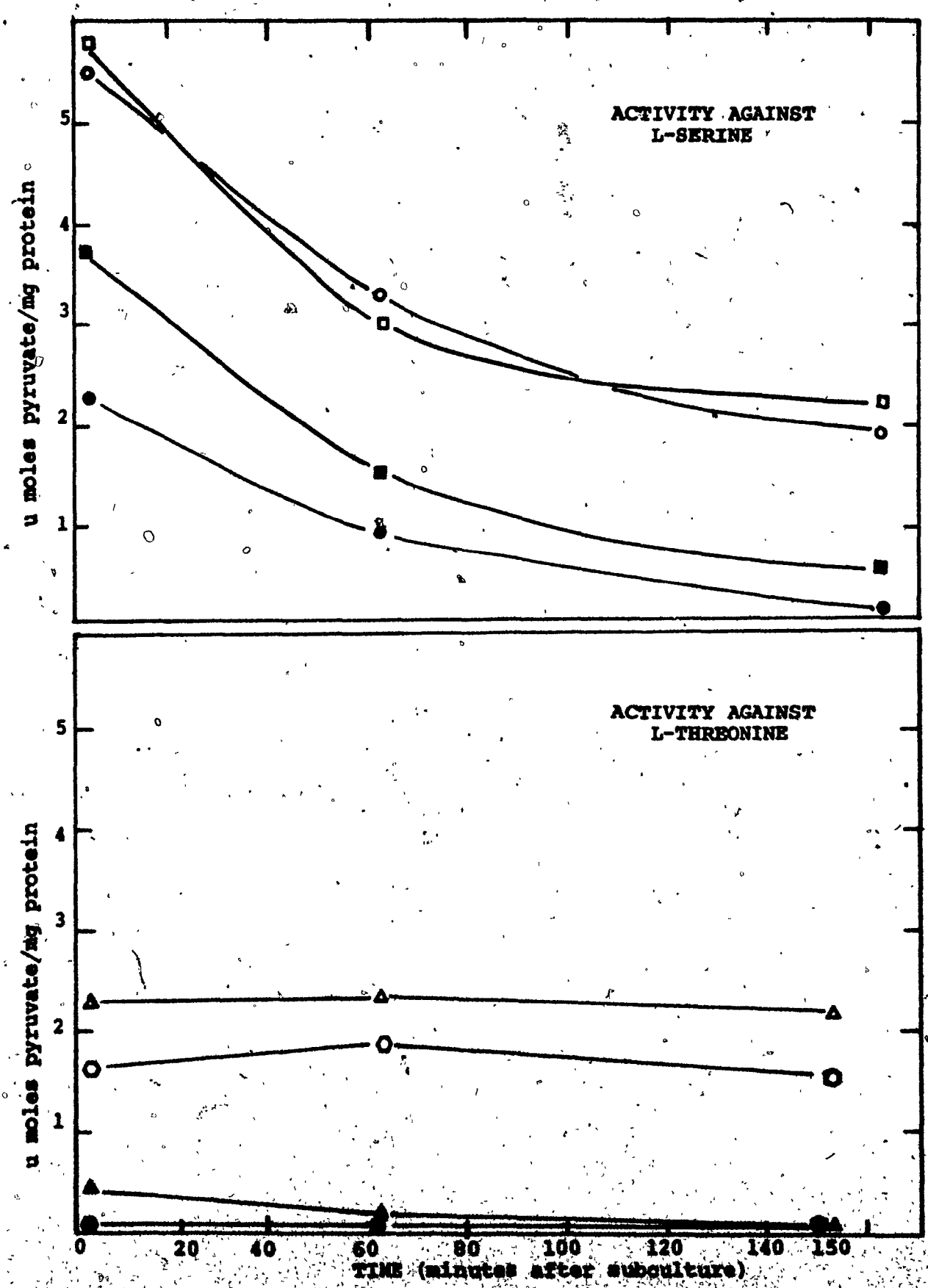

ACTIVITY AGAINST
L-SERINE
u moles pyruvate/mg protein
1
2
3
4
5
ACTIVITY AGAINST
L-THRBONINE
u moles pyruvate/mg protein
1
2
3
4
5
0
20
40
60
80
100
120
140
150
TIME (minutes after subculture)

Table XX DEAMINATING ACTIVITY MEASURABLE IN DIFFERENT ASSAY CONDITIONS AS A FUNCTION OF TIME OF INCUBATION IN CHLORAMPHENICOL-CONTAINING MEDIUM

Exp. #1(a) Activity against L-serine (um pyr/mg. prot)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
After overnight growth	2.42	3.54	5.24	5.95
2 min. after subculture	2.26	3.74	5.54	5.89
60 min. after subculture	0.96	1.56	3.33	3.10
150 min. after subculture	0.20	0.61	1.95	2.25

Exp. #1(b) Activity against L-threonine (um pyr/mg. prot)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
After overnight growth	0.30	0.57	2.33	2.54
2 min. after subculture	0.14	0.46	1.66	2.27
60 min. after subculture	0.16	0.18	1.91	2.38
150 min. after subculture	0	0.09	1.55	2.21

Exp. #2(a) Activity against L-serine (um pyr/mg. prot)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
After overnight growth	2.46	4.57	6.28	7.55
2 min. after subculture	3.10	4.47	9.23	10.76
60 min. after subculture	1.10	3.06	4.92	5.81
150 min. after subculture	0.50	0.28	1.44	1.29

Exp. #2(b) Activity against L-threonine (um pyr/mg. prot)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
After overnight growth	0.37	0.59	2.44	2.80
2 min. after subculture	0.31	0.58	3.13	3.26
60 min. after subculture	0	0.38	3.13	3.00
150 min. after subculture	0.19	0.48	1.54	1.52

* Assays were performed at L-serine concentration of 19.04 um/ml and L-threonine concentration of 16.80 um/ml.

Table XXI DEAMINATING ACTIVITY MEASURABLE IN DIFFERENT ASSAY CONDITIONS AS A FUNCTION OF TIME OF INCUBATION IN CHLORAMPHENICOL-CONTAINING MEDIUM

Activity Expressed as Percentage Remaining of Activity in 2 Minutes Subculture

Exp. #1(a) Activity against L-serine (% remaining of 2 minutes value)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
60 min. after subculture	42	41	57	53
150 min. after subculture	8	13	34	38

Exp. #1(b) Activity against L-threonine (as % remaining 2 minutes value)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
60 min. after subculture	116	37	115	104
150 min. after subculture	0	21	93	97

Exp. #2(a) Activity against L-serine (as % remaining of 2 minutes value)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
60 min. after subculture	31	68	54	35
150 min. after subculture	16	6	15	12

Exp. #2(b) Activity against L-threonine (as % remaining of 2 minutes value)

Time of Assay	Assay Conditions			
	MM6.4	MM6.4-CaMg	MM7.5	MM7.5-CaMg
60 min. after subculture	0	64	100	92
150 min. after subculture	59	80	49	47

* Assays were performed at L-serine concentration of 19.04 um/ml and L-threonine concentration of 16.80 um/ml.

In MM 7.5, by 60 minutes, 115% or 100% of the activity remained, and at 150 minutes 93% or 49% respectively. In the absence of ions, the results were similar: at 60 minutes 104% or 92% was left, and at 150 minutes 97% and 45% respectively.

Thus, when cells were incubated for 60 minutes in conditions inhibiting protein synthesis, there was a 65% loss of L-SD activity and at most a 10% loss of L-TD activity, By 150 minutes, 80% of L-SD activity was lost, and in one experiment 50% of L-TD activity was lost, while in the other, all L-TD activity was still present at 150 minutes. It is thus clear that if all the activity against L-threonine could be assayed while almost all the activity against L-serine was lost, that the two activities must be due to the action of two distinct enzymes.

It has been previously mentioned that both L-SD and L-TD may have activity against the opposite substrate. In MM6.4 by 60 minutes 37% of L-TD activity and 42 or 31% of L-SD activity remains. This would seem to indicate that such L-SD activity as is measured at pH 6.4 is a property of L-SD. However, this is not very clear since in MM 6.4 - CaMg, 80% of the L-TD activity and only 13or 6% of L-SD remains. It is not therefore

clear whether L-SD has some activity against L-threonine. However, at pH 7.5, one seems that by 60 minutes 46% L-SD and 100% L-TD remains, whereas at 150 minutes 25% L-SD and 71% L-TD is left. This might indicate that some 25% of L-TD activity is accounted for by the L-SD molecule and a similar amount of L-SD activity by the L-TD molecule. This data is not good enough to give this conclusion much strength, but these are certainly upper limits and the real values are likely much lower.

DISCUSSION

This thesis is concerned with the characteristics and metabolic role of L-serine deaminase. Though the enzyme was described over 20 years ago, it has been little studied and there is little definite information about it available. Indeed, the study of the enzyme is of considerable difficulty. The enzyme cannot so far be purified, therefore many of its characteristics cannot be studied at all. Its role is not obvious, and as shown here, it is extremely labile *in vivo*, maintains a 'high' basal level, and can be induced by growth in the presence of L-glycine and L-leucine.

The discussion will take the following form. It will first consider the properties of L-serine deaminase revealed in this study as well as in previous investigations; then it will discuss the possible roles for the enzyme as suggested from experimental evidence. It will then consider possible reasons for its '*in vivo*' instability, and the final differentiation between L-serine deaminase and L-threonine deaminase.

A Enzyme Characteristics

Among the enzyme characteristics studied in this report were substrate specificity, enzyme inhibitors, pH

optimum, cofactor requirement, and 'in vitro' stability. Since so little is known about L-serine deaminase (L-SD), these studies are still quite preliminary. However, data on such enzyme properties can provide information on the nature of the functional groups at the active site, participation of certain functional groups in maintaining the enzymes specific conformation, and eventually, the enzyme's mechanism of action.

A(1) 'In Vitro' Instability

Normally, to study the characteristics of an enzyme, it must first be isolated and purified. However, we have found that impossible in the case of L-SD. An attempt made here to prepare a cell-free extract by sonication resulted in almost total (82%) loss of enzyme activity. This problem has been reported by many investigators of L-SD in microorganisms. The L-SD of animal origin is much more stable and can survive purification (Sayre and Greenberg, 1956).

Pardee and Prestidge (1955) using E. coli Strain B, found that L-SD activity was rapidly lost in whole or toluenized cells; e.g. 60% of the activity was lost in 2 hours at 0^{o} and almost all the activity was lost in 30 to 60 minutes in intact cells at 37^{o}. He could find no

effective stabilizing conditions, although the addition of formate helped slightly. He therefore found it necessary to use toluene treated cells rather than an extract.

L-SD activity has been demonstrated in cell-free extracts of Salmonella typhimurium, Bacillus cereus (Raskoz et al, 1969), Clostridium acidi urici (Benziman et al 1960) and Streptomyces rimosus (Szentirmae and Horvath 1963) but in all cases, the extraction procedure resulted in considerable loss of activity. Various attempts to stabilize activity were made. In Salmonella, the inactivation could be inhibited by the presence of L- or D- serine. In Bacillus, the rate of inactivation varied with the enzyme concentration and a concentrated solution was considerably more stable. The Streptomyces enzyme showed a relation between pH and stability - the rate of inactivation was lowest at pH 7.5, but even at pH 7.5, the enzyme was so unstable that at 0^{o} activity decreased 50 to 70% overnight.

In 1949, Wood and Gunsalus reported a partial purification of L-SD using cells of E. coli Crookes Strain, which were broken not by sonication, but by a cycle of freezing, thawing and autolysis. The purified enzyme was extremely labile. During the assay, enzyme activity was totally lost by 10 minutes and indeed almost totally lost

in 5 minutes. Because Wood and Gunsalus believed that L-threonine deaminase and L-serine deaminase were activities of a single protein, they concluded that L-SD activity caused the destruction of the enzyme. According to our present view, however, they probably had 2 enzymes, an unstable L-SD and a stable L-TD. The implication of these experiments will be taken up later. Partial purification with a 70% yield was reported by Artman and Markenson (1956). No comment as to stability was included.

In the report by Alfoldi et al (1969) on E. coli K_{12} it was shown that L-SD was very sensitive to sonication, especially at low enzyme concentrations. It was also found that the enzyme could be somewhat protected during sonication by the presence of D- or L-serine after which loss of activity was still rapid. The rate and final level of this spontaneous inactivation was proportional to the dilution. It was found, however, that the enzyme in extract would be stable enough to study if assayed immediately after any necessary dilutions, and if not kept in diluted state any longer than 30 seconds.

Alfoldi proposed two possible explanations for this extreme lability of L-SD. First, the L-SD of E. coli K_{12} might be a polymer molecule - made up of subunits

whose disassociation causes irreversible inactivation. Sonication treatment and dilution could favour this dissociation. D- or L-serine could protect the polymer state but could not affect reassociation. The second possibility is that L-SD is associated with some unknown protective molecule or structure which stabilizes it. Without such stabilization, the enzyme molecule is in an extremely labile conformation and is easily inactivated. Sonication could result in a detachment of the enzyme from its stabilizer. Similarly, such a dissociation could be the cause of rapid inactivation in the diluted state. Again, no reassociation could occur but D- or L-serine could protect against the original inactivation.

It has been obviously extremely difficult to develop a cell-free extract system and maintain a reasonable level of L-SD activity in it. Purification has thus far been impossible. No extensive efforts were made here to develop a working cell-free extract since, for the purposes of this study, the L-serine deaminase assay can be successfully and well done in the toluenized cell system. However, if a more sophisticated level of study is to be done on the enzyme, isolation and purification is essential.

A (2) Other L-Serine Deaminating Enzymes

It is clear that the toluenized extracts contain L-serine deaminating activity. However, since the enzyme could not be purified, it was important to show that L-serine deaminating activity could properly be attributed to an L-serine deaminase. This is particularly important because several other enzymes are known to have activity against L-serine, and the existence of a separate L-SD enzyme has been questioned. Among the enzymes that have been implicated are L-threonine deaminase (L-TD), tryptophane synthetase, tryptophanase, and cystathione synthetase. In this thesis, L-SD has been shown to be distinct from L-TD. The possibility that one of the otherenzymes is responsible for the L-SD activity measured here is discussed below.

Apart from L-SD itself, the enzyme which has most frequently been reported to be responsible for the deamination of L-serine is L-TD. It is the most important to be considered since, as described in the Introduction of this thesis, it is thought by many that L-SD and L-TD are the same enzyme protein.

L-TD catalyzes the conversion of L-threonine to α-ketobutyrate and ammonia, but, in many microorganisms and animal systems, it is able to deaminate L-serine as well (Davis and Meltzer, 1961, Umbarger and Brown, 1957, Lessie and Whitely 1969, Nishimura and Greenberg, 1961,

Wood and Gunsalus, 1949, Sayre and Greenberg, 1956 and Rasko and Alfoldi, 1971). This is understandable because of the similarity in the structures of the two molecules, i.e. they differ by only one carbon and therefore it would be extremely difficult for an enzyme to absolutely distinguish between the two. The extent to which different L-TS's can deaminate L-serine varies.

Thus, Lessie and Whitely, studying Pseudamonas, describe an enzyme with much greater affinity for L-threonine than for L-serine (10 fold more). Similarly, Sayre and Greenberg report only slight activity of L-TD against L-serine. However, the L-TD enzyme described by Rasko and Alfoldi, Lessie and Whitely, Wood and Gunsalus, and Davis and Melzer, have such an affinity for L-serine that L-serine acts as an inhibitor of the activity against L-threonine. This is indeed the explanation for L-serine inhibition of the growth of E. coli K_{12} (Cosley and McFall 1970). The inhibition is reversed by L-isoleucine, the first enzyme of L-isoleucine biosynthesis being the L-serine-inhibited L-TD.

It has been shown in this thesis that two distinct enzymes - a L-SD and a L-TD are responsible for the activity against L-serine and L-threonine respectively. This has been demonstrated by the fact that under

conditions in which rapid decay of activity against L-serine was observed, (at either pH 6.4 or 7.5), little or no decrease in activity against L-threonine (at pH 6.4 or 7.5) was seen. Had one protein been catalyzing the deamination of both substrates, the activity against both should have simultaneously decreased.

E. coli in fact makes two different L-TD's, a biosynthetic one (described above as inhibited by L-isoleucine) and a degradative one (Wood 1969). However, because all the L-TD activity measured here is stable, it appears that neither is involved in measurements of L-SD activity.

Others have also tried to differentiate L-SD and L-TD. Alfoldi and Rasko (1969) differentiated three enzymes in E. coli K_{12}, a biosynthetic L-TD (bTD) which is entirely inhibited by L-isoleucine; a degradative L-TD (dTD) which is not inhibited by L-isoleucine; and an L-SD. Cells grown in minimal medium contained only bTD and not dTD. Since only half of the L-SD activity in minimal grown cells was inhibited by L-isoleucine, he was able to conclude that the other half represented a specific L-SD. In yeast extract-tryptone only dTD and no bTD was formed. In this rich medium, L-SD activity was 10 fold the L-TD activity, therefore, only 10% could be attributed to side reaction of L-TD.

In this study, dTD could not be measured even in yeast-extract-tryptone medium. Therefore, an attempt to induce high levels of dTD made use of the fact that dTD is induced in conditions of limited energy supply (Wood 1969). Cells were therefore grown with L-threonine as sole carbon and energy source. Though these conditions were expected to induce dTD, no increase in activity against L-serine was seen. It was therefore concluded that the L-serine deaminating activity measured was of L-serine deaminase.

It is therefore clear that L- SD and L- TD, both biosynthetic and degradative, are different molecules. But, is L-SD different from other molecules? The three other enzymes which might be involved were tryptophane synthetase (Crawford and Ito 1964,) tryptophanase (Newton and Snell 1964, Griffiths and DeMoss 1969) and cystathione synthetase (Selim and Greenberg 1959).

Recently it has been shown that the B protein of E. coli tryptophane synthetase deaminates L-serine (Crawford and Ito 1964). The enzyme tryptophane synthetase consists of 4 polypeptide chains made up of 2 α (A protein) and 2 β (B protein) chains. When complexed, this enzyme catalyzes the synthesis of tryptophane from indole-3-glycerophosphate and L-serine. The reaction takes place in the following two steps:

(1) Indole-3-glycerophosphate → Indole + D-glyceraldehyde-3-phosphate

(2) Indole + L-serine ------→ Tryptophane

The A protein alone can inefficiently catalyze Reaction (1), while the B protein can catalyze reaction (2) at various rates depending on the environment. When A and B are combined, the enzyme efficiently catalyzes the overall reaction of tryptophane synthesis. Recently, the B protein has been isolated and shown to possess the second activity of L-serine deamination. It appears that the same site that catalyzes the tryptophane formation also catalyzes the deamination of L-serine. In any case, the B protein does not exist free in the cell to any great extent, i.e. it is complexed with the A protein and is thus effective in the overall tryptophane synthesis reaction but quite ineffective for deamination of L-serine.

Another enzyme which has been reported to have L-serine deaminating activity in E. coli is tryptophanase, which catalyzes the degradation of L-tryptophane to indole + pyruvate + ammonia, as well as several other related reactions, including the deamination of L-serine (Newton et. al. 1955, Newton and Snell, 1964). However, it was found that the rate of L-serine deamination was only 12% of the rate of the main reaction (i.e. tryptophane

degradation). A similar comparison (15% as efficient) was reported for the tryptophanase of Bacillus alvei (Griffiths and DeMoss 1969).

No determination of tryptophanase activity against L-serine was made in this present study. However, it can be noted that tryptophanase is an inducible enzyme, i.e. synthesized in the presence of tryptophane, which was not added to the media used in this study. It is therefore unlikely that this enzyme contributed to any extent to the L-serine deaminase activity being measured.

The third enzyme which is reported to have L-SD activity is the cysteine synthetase of rat liver (Selim and Greenberg, 1959) which catalyzes the synthesis of cysteine from methionine. The reaction is:

L-methionine+ATP+methyl acceptor+H_2O+L-serine $\longrightarrow$ Methylated acceptor (e.g. homocysteine or nicatinamide) + adonosine + αketobutyrate + NH_3 + PP_i + P_i + cysteine

Evidence has been provided that in the rat liver system, both activities, i.e. L-serine deamination and cysteine synthesis were performed by a single enzyme protein, cysteine synthetase. However, in bacteria, the above pathway is replaced by one from serine --> cysteine --> methionine and the enzyme cysteine synthetase does not exist. Therefore, contribution by this enzyme to L-SD

activity in the system studied here is highly unlikely. It has therefore been shown in this thesis that L-SD is distinct from L-TD. According to other papers reviewed, tryptophane synthetase and tryptophanase, as well, cannot be responsible for the L-SD activity measured. To the extent that no other candidates exist, it can be concluded that L-serine deaminating activity is due to the activity of the unique, but unstable L-serine deaminase.

A(3) Substrate Specificity

Enzymes vary in the degree of specificity they show with respect to substrate. Some enzymes show relatively little specificity and can attack a wide range of molecules. Thus all esterases hydrolyze many different aliphatic esters (Lehninger 1970). Other enzymes work on a much more restricted range of molecules, e.g. aspartase adds NH_3 to fumerate but not to methylfumaric acid. Some enzymes recognize only one stereoisomer thus lactate dehydrogenase is specific for L-lactate and will not attack D-lactate. Others show geometric specificity, thus aspartase will not add NH_3 to maleate, the cis geometric isomer of fumerate (Lehninger, 1970).

The results described in Section I (1) indicate that L-SD of E. coli $K_{12}P^-$ is a very specific enzyme with L-serine alone as substrate. Experiments of the type described in this thesis can show specificity, but could not show non-specificity. If toluenized cells contained several deaminases, it would not be possible to distinguish between one enzyme with wide specificity and several enzymes each with a narrow specificity. The chance decision to assay at pH 6.4 provided the conditions in which L-serine but not glycine, L-threonine, L-leucine, L-isoleucine, L-cysteine, or D-serine was

deaminated. Thus it is clear that L-SD is not active against the stereoisomer D-serine, or against the closely related molecule (only one carbon more) L-threonine. The highest activity seen at pH 6.4 was against L-leucine, which showed only 5.1% of the activity against L-serine. It is thus clear that the cell contains an enzyme with a specificity for L-serine.

In other investigations on the L-SD of several E. coli strains, as well as other microorganisms, different degrees of specificity were found. Some studies showed strict specificity of L-SD for L-serine. Thus Alfoldi et al (1969) using E. coli K_{12}, found that of the 20 L-amino acids and several D isomers tested, only activity against L-serine could be measured. Similarly, the L-SD of Salmonella typhimurium, Bacillus cereus (Rasko et al 1969) Streptomyces rimosus (Szentirmae and Horvath 1963) and Clostridium acidi urici (Benziman et al 1960) were all strictly specific for L-serine.

However, in a study done on Bacillus alvei (Griffiths and DeMoss 1969), the L-SD of that organism appears to be less specific. It was reported that of the L-serine analogues, the β- phenyl derivative, βφ DL serine, could be deaminated also, but only 54% as efficiently as L-serine itself. This lower efficiency might be a

characteristic of the enzyme or might be due to inhibition by the D isomer.

Some reports indicate L-SD to be sufficiently less specific that it can act on both L-serine and L-threonine. This was the case in both Crookes Strain of E. coli (Wood and Gunsalus 1949) and in Neurospora (Yanofsky and Rassig 1953). It must however be noted that the authors of both these studies considered that there was only one enzyme, a deaminase which acted on both L-serine and L-threonine, since the activities of both were inseparable. This was also once thought true for other organisms including E. coli, in which, as has been shown in this thesis, the two activities can now be separated as two distinct enzymes. Therefore, if a method of separation could be developed in Crookes Strain of E. coli and Neurospora, it might be seen that L-SD is specific for L-serine, as seems to be generally true in microorganisms.

In contrast, the L-SD of animal origin can usually deaminate L-threonine as well as L-serine, e.g. in rat liver (Goldstein et. al. 1962) but there are some, e.g. sheep liver (Sayre and Greenberg 1956) in which the enzyme is L-serine specific.

One can therefore conclude that most microbial L-SD's show specificity, mammalian enzymes do not. Two microbial L-SD are reported to act on both L-serine and

L-threonine. These however may yet prove to be separable possibly even by using the phenomenon of differential decay described here.

Apart from its interest as an aid in characterizing an enzyme, an enzyme's substrate specificity gives some indication as to its probably mechanism of action. A generally accepted hypothesis is that the substrate molecule, or part of it, binds to a complementary surface (the active site) on the enzyme molecule. For this to be possible, several structural features are important to both substrate and enzyme. The substrate molecule must possess the specific chemical bond or linkage that can be attacked by the enzyme, as well as some functional group(s) which bind to the enzyme and properly orientates the substrate molecule on the catalytic site. The enzyme protein molecule must be held in a folded configuration in such a way as to bring together the essential groups (often from different parts of the molecule) to form the active site.

The 'Induced Fit' theory (Lehninger 1970), based on a study with carboxypeptidase proposes that the enzyme's 3-D conformation changes as it binds to the substrate. This 'induced fit' produces the precise positioning of the catalytic site and binding groups needed to cause the reaction. Thus, it has been shown

using phosphoglucomutase (Yankeelov and Koshland 1965) that the substrate itself, in this case glucose-6-phosphate, induces conformational changes in the enzyme protein.

The above evidence could help explain substrate specificity. Enzymes can usually exist in several different conformations, yet generally only one is catalytically active. Since the achievement of the exact conformation is so crucial, and since certain molecules (the substrate) could determine this conformation, it is understandable that an enzyme might be extremely specific for one substrate, if, i.e. this substrate were the only one which could bring about the formation of the precise catalytically active conformation. Where an enzyme is active against more than one substrate, its relative efficiency against each can be related to the differences in chemical structure between the substrates. This can be used to analyze the mechanism of action of the enzyme. Unfortunately however, this was impossible in the case of L-SD since the enzyme was found to be strictly specific for one substrate, L-serine.

A(4) Cofactor Requirement

The function or maintenance of the enzyme-substrate complex may also involve other compounds known as cofactors. This cofactor may be a metal ion (e.g. Mo^{+} in nitrate reductase) or a complex organic molecule (coenzyme) as lipoic acid. More than one may be involved, e.g. pyridoxal phosphate (PP) and THF (tetrahydrafolate) in serine transhydroxymethylase (STHM). The cofactor may serve to bind substrate and enzyme or may be part of the catalytic group itself and may therefore be an absolute requirement (e.g. iron atoms of catalase which decomposes hydrogen peroxide). Coenzymes usually function as intermediary carriers of electrons, of specific atoms, or of functional groups that are transferred in the overall reaction, e.g. the heme group of cytochrome C (Lehninger 1970). Some coenzymes are very tightly bound to the enzyme molecule and are usually called prosthetic groups.

Examples of both cofactors and coenzymes involved in the activity of L-SD in different organisms will be seen below. Cofactor studies present the same difficulties as specificity studies. In a nonpurified system, it is difficult to distinguish between a nonrequirement for cofactors and a requirement for a cofactor which is so

tightly bound to the enzyme that the requirement cannot be demonstrated. Thus PP has been shown to be a cofactor for the mammalian L-SD (Goldstein et al 1962, Sayre and Greenberg 1956) and was therefore tested in the present study. PP is the coenzyme of the transaminases and other reactions including amino acids. It functions as the carrier of the amino group from an amino acid to a keto acid (Lehninger 1970).

No effect of PP addition either to the growth medium or the assay could be seen. This could mean simply that PP is not involved in the reaction. Other experiments reported in Section B(7)(d) allowed no clear conclusion. In those experiments it could be seen that the level of L-SD assayable depended on both the pH and the ions present. At pH 6.4 in the presence of Ca^{++} and Mg^{++}, a decrease in activity was seen. Activity could be restored by exogenous PP. It therefore seemed likely that L-SD is a PP requiring enzyme. However at pH 7.5 the effect of ions and exogenous PP on activity against serine were not consistent. This was probably due to the additional involvement of other deaminases (e.g. L-TD).

However, other coenzymes could also be anticipated, and in addition to PP have been tested in various organisms. The L-SD of several organisms appears to have no cofactor requirement. In toluenized cells of

E. coli Strain B, adenylic acid, glutathione, Vitamin B_{12}, biotin, PP and Mg^{++} were all without effect (Pardee and Prestidge 1955).

In sonically treated cells of E. coli K_{12} (Alfoldi et al 1969) ATP, NAD, PP, glutathione, and Vitamin B_{12} had no effect. (A point of confusion exists here since the author concluded no PP requirement, yet a subsequent report by the same author (Alfoldi and Rasko 1971) includes PP in the assay mixture, apparently believing it to be a requirement, but giving no experimental reference as to why). Similarly, sonically treated cells of Salmonella typhimurium and Bacillus cereus (Rasko et al 1969) and a partially purified L-SD of E. coli Strain B/r (Artman and Markenson 1958) had no apparent cofactor requirement. However, since all of the above studies did not use purified enzyme systems, the results are therefore open to the previously stated objections.

On the other hand, several studies have been able to show L-SD requirement of various cofactors. PP has most widely been tested. Above were mentioned several enzymes in which no PP requirement could be demonstrated. This was also true for Clostridium (Benziman et al 1960). However, in both the toluene

treated preparation of Bacillus alvei (Griffiths & deMoss 1969) and in Neurospora (Yanofsky and Reissig 1953) addition of PP did stimulate L-SD activity.

The PP requirement of Streptomyces rimosus (Szentimae and Horvath 1963) is not clear. The fact that the L-SD activity of this organism was inhibited by aminothiols and ketonic reagents indicated that PP was the coenzyme. However, exogenous addition of PP had no effect. It was therefore suggested that (as discussed previously above) no PP requirement could be shown due to the tight binding of endogenous PP to the enzyme that was not released despite extraction.

The effect of glutathione (GSH) on L-SD activity has also been investigated. Above it was seen that the L-SD of E. coli Strain B (Pardee and Prestidge 1955) and Strain K_{12} (Alfoldi et al 1969) had no GSH requirement. This was true as well for Neurospora (Yanofsky and Reissig 1953).

However, the L-SD of both E. coli Crookes Strain (Wood and Gunsalus 1949) and Clostridium (Benziman et al 1960) did show a GSH requirement for activity. Glutathione forms complexes with heavy metals, and also acts as a reducing agent. In the case of Crookes Strain, since other complex-forming agents (bipyridyl,

8-hydroxyguinonline, histidine, pyrophosphate, and gum arabic) were without effect, it was therefore suggested that glutathione was acting in its role as a reducing agent, rather than a heavy metal inhibitor. This indicated the presence of oxidized functional sulfhydryl groups on the enzyme. This was further supported by the fact that the enzyme was inhibited by 10^{-5}M mercuric, silver, and cupric ions which could not be reactivated by glutathione.

In Clostridium, glutathione was found to activate L-SD at high concentrations. Activity was greatly increased by hydrosulfite, (an enzyme reducing agent (Dixon and Webb 1964) which also strongly suggested that reducing groups were essential for L-SD activity. This requirement, together with the fact that L-SD was inhibited by sulfhydryl blocking agents (Section A(b) suggested the presence of functional sulfhydryl groups on the active enzyme.

In several studies on L-SD, a requirement for various ions has also been found. In a toluene treated preparation of B. alvei (Griffiths and deMoss 1969) the monovalent cations K^+, NH_4^+, Na^+ were required for enzyme activity. In a similar preparation of

Arthrobacter globiformis (Bridgeland and Jones 1965) no cations were needed however, in a cell free extract of that organism, L-SD activity required the addition of Mg^{++}, Ca^{++}, K^{+}, or NH_4^{+} . This again indicates the importance of the type of preparation used for demonstration of cofactor requirement. The L-SD of Clostridium (Benziman et al 1960) was activated by ferrous iron. Mn was only half as effective as iron, while Zn and Mg had no effect.

Compounds also tested have been biotin, which had no effect in Neurospora as well as E. coli B; AMP (adenylic acid) which was required for activity in E. coli Crookes Strain and adenosine-5-phosphate which had no effect in Neurospora.

It can therefore be seen that in the range of microorganisms reviewed, a variety of different cofactor requirements has been shown and used to determine some features of the enzyme molecule. It has however been pointed out that there are disadvantages in such a study to using crude or even partially purified enzymes because of the possibilities that saturation levels of cofactor may already exist in the preparation, or that cofactors may have remained bound to the enzyme despite extraction treatment. It is conceivable that the cofactor requirement of various organisms can be different.

However, it is felt that definite answers to the questions of which cofactors are required cannot be attained until the enzyme has been isolated and purified. This, as has been discussed above, has as yet been impossible because of the enzyme's extreme lability.

A(5) The Effect of pH

The activity of any given enzyme varies considerably with the pH of its environment. Enzymes are proteins containing many ionizable groups and can thus exist in a series of different states of ionization. The amount of each state present depends on the pH. Most enzymes have a characteristic pH at which activity is greatest, above or below this, the activity declines. It is therefore important to establish the proper pH at which activity can be maximally measured.

The experiments described here do not allow the determination of the optimum pH for L-SD. The problem is the same for epecificity or cofactor studies. As the pH increases from pH 6.4 to pH 8.0, activity against L-serine does increase. However, a new activity against L-threonine (LTD) is also measurable at the higher pH. In the crude extract system, there is no way of distinguishing the extent to which each enzyme is active at the higher pH. At pH 7.5, both L-SD and L-TD could be measured. At pH 6.4, which is also the pH at which the cells were grown, only activity against L-serine could be measured. Cells growing at pH 6.4 may well have both activities.

Indeed, the internal pH of growing cells may well be different from the pH of the medium in which they are suspended. However, the toluenized cells no longer can maintain a different pH and are in fact more like an extract in this respect. Moreover, the optimum pH of an enzyme is not necessarily the same as the pH of its normal intracellular environment, which may be at a point where a particular enzyme would be far below or approaching the activity at its optimum. This, in fact, may be a factor in intracellular control of enzyme activity (Lehninger 1970).

Other reports on several strains of E. coli have indicated a range of optimum pH for L-SD activity from pH 7.2 to pH 9 (Alfoldi et al 1969, Pardee and Prestidge 1956 and Wood and Gunsalus 1949). A very similar range has been shown for the L-SD of other microorganisms (Griffiths and deMoss 1969, Rasko et al 1969, Szentimae and Horvath 1963, Benziman et al 1960, Yanofsky and Reissig 1953) as well as the L-SD of animal origin (Goldstein et al 1962, Selim and Greenberg 1956). None of the above reports considered the possible participation of L-TD in the activity being measured. It must, however, be noted that this present study and previous reports (Davis and Metzler 1961, Goldstein et al 1962,

(Sayre and Greenberg 1956) have shown that in that pH range (i.e. above pH 6.4) L-TD is active, and further, it has previously been shown that L-TD can act on L-serine as well as L-threonine. (See Section A(2)).

The question therefore arises as to whether the reports quoted above actually represent descriptions of L-SD activity alone. It is possible that an overlap of the two activities has been measured since their since their similar properties have made it extremely difficult to separate them. It is because of this that they have often been considered as one enzyme protein (Introduction of this thesis, Wood and Gunsalus 1949, Yanofsky and Reissig 1953, Goldstein et al 1962)

A method however will be described below which uses the information obtained here (i.e. that L-TD is inactive at pH 6.4 while L-SD is still very active) to separate the two activities and distinguish them as two separate enzyme proteins. This method could be very useful in any subsequent studies of L-SD.

A(6) Inhibition of L-SD Activity

Inhibitor studies can be used in the same way as specificity studies to analyze the mechanism of action of the enzyme. Recently, they have also taken on a physiological importance in terms of regulation of cell metabolism. Thus one would wish to determine what compounds inhibit a given enzyme and characterize the nature of the inhibition.

Inhibitors are known to be irreversible and reversible. Irreversible inhibition usually involves the destruction or modification of one or more of the functional groups of the enzyme. Reversible inhibition can be competitive, where substrate and inhibitor compete for the same site on the enzyme, and inhibition can be reversed by increasing the substrate concentration; or noncompetitive, where the inhibitor presumably binds at a site on the enzyme other than the substrate binding site, but a site close enough to reduce the affinity of the enzyme for the substrate. The noncompetitive inhibitor may bind to the free enzyme, the enzyme-substrate complex, or both, and cannot be reversed by increasing the substrate concentration.

Some inhibitors influence a wide variety of enzymes, e.g. heavy metals and compounds reacting with

SH groups. These tend to be compounds which react generally with protein molecules. Other inhibitors are more specific to a particular enzyme. This class of inhibitors is more useful for physiological studies.

The mechanism of metal inhibition is not entirely clear. Many enzymes are known to be inhibited by metal ions. One would therefore expect that the metal ions react with protein molecules generally. Metal ions are known to react with thiol and carboxyl groups and even amino acid residues. No general rule can be formulated and the site of inhibition in the LΘSD studies is not known. (Lehninger 1970, Dixon and Webb 1964).

In Streptomyces rimosus (Szentimae and Horvath 1963) Ag, Pb, Cu and Fe, at a concentration of 10^{-6}M were inhibitory. In Neurospora (Yanofsky and Reissig 1953) Cu^{++}, and Zn^{++} at a concentration of 10^{-3}M were partially inhibitory. The L-SD of Clostridium (Benziman et al 1960) was also inhibited by heavy metals. As stated above, the mechanism of the inhibition is not clear. In Crookes Strain (Wood and Gunsalus 1949) L-SD was inhibited by 10^{-5}M mercuric, silver and cupric ions. This was discussed in Section A(4) in relation to cofactors.

L-SD activity has also been shown to be affected

by SH blocking reagents (Benziman et al 1960). These reagents reversibly combine with the SH of cysteine residues that are essential for the catalytic action of some enzymes. This was discussed in Section A(4) since it led to the conclusion that PP is a cofactor of this enzyme.

The preceeding inhibitors are not specific for L-SD, but, are known to affect many enzymes. More specific inhibitors have also been described. In various strains of E. coli L-SD was shown to be inhibited by glycine (in Strain B, Pardee and Prestidge 1955) D-serine (Pardee and Prestidge 1955) Strain B; Alfoldi et al 1969 (K_{12}; Wood and Gunsalus 1949, Crookes Strain) L-threonine, (Wood and Gunsalus 1949, Crookes St.) and L-cysteine (Alfoldi et al 1969 K_{12}). In the most comprehensive report, Alfoldi studied the effect of all L-amino acids, some D-isomers, DL-homoserine and ethanolamine. Only D-serine and L-cysteine had an effect, glycine was one of the compounds which had no effect.

In this study, D-serine, and glycine have all been shown to inhibit the enzyme. Similar results were found in other microorganisms. L-SD activity was inhibited by D-serine in Salmonella typhimurium

and B. cereus (Rasko et al 1969) and in B. alvei (Griffiths and deMoss 1969); and L-cysteine was found to be inhibitory in Streptomyces rimosus (Szentirmae and Horvath 1963) and B. cereus (Rasko et al 1969). The L-SD of B. alvei was also inhibited at high substrate concentration, and this was shown to be directly due to substrate inhibition rather than indirectly via product accumulation.

Thus, most authors agree on the effects of D-serine and L-cysteine however, there is no concensus with glycine or L-serine itself. In all cases, a mechanism of competitive inhibition was suggested, i.e. because of the similarity in chemical structure with L-serine the inhibitory effect observed by for example D-serine or L-cysteine or L-glycine, can be understood in terms of a competition with L-serine for the active site on the enzyme. Moreover, no regulatory role is obvious.

B Metabolic Role(s) of L-SD

Since L-SD has been studied so little, its metabolic role has not thus far been known. In this thesis, to investigate the possible roles of this enzyme, studies were made on the effect of inorganic N, different carbon sources, and specific compounds (for example amino acids) on the enzyme level. The significance of these results, in terms of possible metabolic role for L-SD, will now be discussed.

B(1) Basal Level of L-SD

E. coli can grow in an inorganic medium supplemented only with glucose. The particular strain of *E. coli* studied here requires, in addition, only L-proline. This means that all the cell's metabolites, except L-proline, and all the cell's energy requirements can be met from glucose. Cells grown in these minimally supplemented conditions do not contain the enzyme β-galactosidase. It is therefore concluded that this enzyme is not involved in the cells 'basic metabolism'. A determination of L-SD activity in cells grown in minimal medium with glucose and L-proline in the same way can indicate whether L-SD is involved in the cells 'basic metabolism'.

In this study, the basal level varied from 0.35 to 0.94 um pyr./mg.prot. (average 0.65 um pyr./mg prot.) Therefore significant amounts of the enzyme seems to exist. One would like to compare these results with those of other investigators. However, only one other report on L-SD (Pardee and Prestidge 1955) determined the uninduced enzyme levels in E. coli. Pardee made use of glycerol-grown cells, but the results were no different from glucose-grown cells. Moreover, values from that study cannot be directly compared with those obtained here due to the fact that the units used in the former (mu moles/min) did not include an accurate measurement of the amount of protein involved and therefore can not be interconverted with the units used here (um pyr/mg.prot./35 min.). Nevertheless, some indication of the relation between the results of both studies can be obtained by comparing the differences between the noninduced (basal) and induced levels in each case.

In this study, the average noninduced level was 0.65 um pyr/mg. prot., and the average induced level was 2.54 um pyr./mg. prot., giving a noninduced/induced ratio of about $\frac{1}{4}$. In the study by Pardee, the basal level was 0.7 mu moles keto acid/min., and the induced

level was 4.6 - 8.8 giving a noninduced/induced ratio of about 1/12 to 1/7.

It is clear then that at least the variation between noninduced and induced levels in these two studies is about the same, though the absolute values cannot be compared. The extent of this variation has further significance. This can be seen by comparing these results to a 'typically inducible'enzyme such as D-serine deaminase or β-galactosidase.

The report by Pardee included a study of D-serine deaminase, which deaminates D-serine to pyruvate and ammonia, and is induced by D-serine. It was found to have a noninduced level of 0.08 mu moles keto acid/min. and an induced level of 16.6 mu moles/min. giving a noninduced to induced ratio of 1/200. Even more striking is the example of β-galactosidase which cleaves β-galactosides such as lactose to glucose and galactose, and is induced by growth on β-galactosides. Its noninduced to induced ratio is about 1/1000 (Watson 1970). Thus a 'typically inducible' enzyme would be expected to have a basal level that was almost negligeable in comparison to its induced level. It is probably because of this that a discussion of the basal level of an inducible enzyme is rarely found.

It therefore appears that significant amounts of L-SD do exist in the celland that there is relatively little variation between noninducing and inducing conditions. This may mean that the best inducing conditions have not yet been found, or, that the enzyme is so essential that its level can never drop very far. Such variations as do exist in the different growth conditions can however shed light on the metabolic role of the enzyme, as will be seen in the following discussion.

B(2) The Effect of Inorganic Nitrogen

It has been shown in this thesis, that various conditions affect the level of L-SD measured. One of these involves nitrogen nutrition. It is clear that cells grown in medium to which no inorganic N is added contain more L-SD activity than cells grown in an excess of inorganic N. Thus, the level of L-SD activity in -Nplg grown cells was up to 5 times higher than cells grown in +Nplg. Since one product of the L-SD reaction is ammonia, it seems reasonable that the enzyme might have a role in N metabolism. This might be to provide the cell with an inorganic N source, at least when other sources were absent or insufficient. It might also act as a valve to get of excess organic N.

Two attempts were made to show a direct relationship between the levels of inorganic N available to the cell, and L-SD activity. In the first, cells were grown overnight with 500-8000 ug/ml. ammonium sulfate. This range was chosen because MM+N contains 2000 ug/ml. In the range tested, little variation in enzyme activity was seen. It later became obvious that even 500 ug/ml. ammonium sulfate is in excess of the cells requirements. The results plotted in Figure VII indicate that a level of about 250 ug/ml is somewhere near saturation for ammonium sulfate.

Moreover, the cell can efficiently scavenge even the trace amounts of N available when no ammonium sulfate is added.

The question was therefore investigated in another way. Cells were incubated with limiting ammonium sulfate and the amount of L-SD measured before and after the ammonium sulfate was exhausted. A simple hypothesis which says that as inorganic N is decreased, L-SD is formed, is excluded by the data obtained. However, almost all values obtained for L-SD activity in that experiment were higher than the value in the preculture and it appeared therefore that the enzyme was induced earlier after inoculation than was anticipated. Thus there is a clear relation between N nutrition and L-SD activity. L-SD may be regulated according to the availability of inorganic N.

It may also be that L-SD is correlated more with the level of organic N than that or inorganic N. This may be why glycine and L-leucine induce L-SD even in the presence of inorganic N. However, this cannot involve all organic N, since not all amino acids induce. It may then be that the level of L-SD is keyed to the concentration of some specific organic N compound and could even be that its role is in detoxification - i.e. the elimination of excess L-serine.

In this last experiment, some induction by -N was seen, but the final enzyme level was still quite low. There are no figures to compare this value to, since cells do not grow in -Np. To the extent that the value measured seems low, one could make the following argument.

It is obvious from Figure VIII that the growth of cells is limited by the amount of ammonium sulfate available, at least in the range from 0 to 150 ug/ml. This is clear from the fact that the growth curve is biphasic and the absorbance at which the shift to phase #2 is seen to be proportional to the ammonium sulfate given. However, slow growth in phase #2 is seen.

Bacteria such as E. coli are known to store N, i.e. to form endogenous reserves which can be utilized under conditions of starvation. Studies on E. coli have reported several compounds capable of serving as endogenous N supplies, for example the amino acid pools of glycine, L-alanine, L-serine, L-threonine, L-aspartate, L-glutamate, and L-arginine; RNA by its degradation; and protein via turnover. (Dawes and Ribbons 1962, 1964, 1965).

... It may be that the cell used the endogenous

sources first, using the normal levels of L-SD as well as other enzymes, and that the increase in L-SD would not be seen until endogenous reserves were used up, and therefore that the measurements of L-SD were made too early. The cell would eventually reach a point where endogenous sources would be depleted and exogenous sources would have to be utilized. However, synthesis of new enzymes would still require a source of N, and it is still possible that L-SD levels would increase in the presence of some organic compound. This could be tested by repeating the experiment illustrated in Figure VIII this time in the presence of glycine and L-leucine.

B(3) L-SD Induction

It is clear that the level of L-SD is in some way correlated to the N supply available, though a detailed description of the relationship has not been possible. As indicated in Section B(3), certain compounds are known to induce L-SD, i.e. L-SD activity is higher in cells grown in the presence of these compounds than in cells grown in their absence. The best inducers for this enzyme are glycine and L-leucine; its substrate L-serine is not an inducer, nor are the amino acids L-isoleucine and L-valine, at least in the presence of L-serine.

This is similar to the results of Pardee and Prestidge (1955). They described a high level of L-SD in cells grown in rich medium, and attempted to find the components of the rich medium that were required to produce high levels of activity. Cells grown in glycine and L-leucine showed the same level of activity as cells grown in rich medium (DLserine, casein hydrolysate, and yeast extract). Attempts to replace glycine, in the presence of L-leucine, showed that L-threonine, DL allothreonine, and suprisingly pyruvate as well all have an inducing activity, whereas L-serine does not. Similarly, L-leucine could be replaced by

DL-isoleucine, but not by L-valine, as long as glycine was also present. This may indicate that even the effect of glycine and L-leucine is not direct but involves a shift in the metabolic pattern.

The question of the effect of growth in complex media (i.e. yeast extract) was not pursued in detail here. It is clear though that growth on yeast extract-tryptone glucose medium is inducing in the strain used in the present study as well. This effect of growth in rich medium has been reported by many other authors working on a variety of organisms: Crookes Strain of E. coli (Wood and Gunsalus 1949), E. coli K_{12} (Alfoldi et al 1969), Salmonella typhimurium and Bacillus cereus (Rasko et al 1969).

There is general agreement that the substrate of the enzyme, L-serine is not an inducer. Pardee and Prestidge (1955) reported this in their paper. Artman and Markenson (1956) found the same thing in E. coli B/r. An L-SD of rat liver studied by Sayre et al (1956) is also not substrate inducible.

Pardee and Prestidge showed conclusively that the combination of glycine and L-leucine induces better than either amino acid alone. This was not studied in detail here, but it is reasonably clear that this is also the case for the strain used here. However, it is not

immediately obvious why glycine and L-leucine should induce L-SD. It was suggested earlier that the level of L-SD is keyed to the level of some organic N compounds, perhaps the endogenous level of L-serine itself, i.e. L-SD might be a L-serine detoxifying enzyme. Since L-serine itself does not induce the enzyme, this seems unlikely, unless, there is some reason to believe that the endogenous L-serine pool is higher in cells grown with exogenous glycine and L-leucine than in cells grown with exogenous L-serine.

Such a situation could exist if the rate of entry of L-serine into the cell was slow, or decreased as the intracellular concentration of L-serine increased. Then, the endogenous L-serine pool might be maintained at a low level even in the presence of exogenous L-serine. However, any compound which could enter the cell by another mechanism and be converted to L-serine might increase the L-serine pool. Thus, if glycine were provided exogenously, it might enter the cell and be converted to L-serine. It might also act indirectly by decreasing the conversion of L-serine to glycine via serine transhydroxymethylase and thus increase the L-serine pool. However, if this indirect mechanism were to exist, one would expect the L-serine to inhibit its own biosynthesis.

A relation between L-serine and L-glycine would be reasonable enough, but why L-leucine? L-leucine has various effects on the growth of bacteria. That it is inhibitory to this strain has been shown here (Section B(3). The growth rate in the presence of L-leucine is about ½ the rate in its absence. The fact that the inhibition is relieved by L-isoleucine and L-valine, which are the same amino acids that relieve L-serine inhibition in some strains (Cosloy and McFall 1970) may indicate that L-leucine acts by increasing the endogenous L-serine pool. These two amino acids (L-isoleucine and L-valine) also increase considerably the inducing effect of -Nplg (i.e.-Nplg isol. val. 2.25, 2.04 um pyr/mg port; -Nplg, 1.34 um pyr./mg prot)

L-serine inhibits growth by inhibiting the action of biosynthetic L-TD and starving the cell for L-isoleucine. If L-isoleucine and L-valine are provided to overcome the inhibitory effect of L-serine, the cells should be able to tolerate a high L-serine pool. One could then imagine the following complex situation: L-glycine and L-leucine act to inhibit certain routes of L-serine degradation, the presence of L-isoleucine circumvents the inhibition of biosynthetic L-TD, L-valine is provided to overcome the inhibition of L-valine

biosynthesis by L-isoleucine, the L-serine pool rises without being toxic, and L-SD is maximally induced. The only evidence which is not consistent with this picture is the fact that L-serine + L-isoleucine + L-valine does not induce. If L-leucine were also involved, one might expect that L-serine+ L-isoleucine+ L-valine+ L-leucine would induce, or even L-serine+ L-leucine.

According to such an explanation, L-SD is a detoxifying enzyme. It is not the only L-serine degrading enzyme. However, when other pathways of L-serine degradation are blocked, the L-serine pool increases and becomes toxic. Anything that blocks other degradation pathways partially might increase the L-serine pool slightly and thus induce L-SD. However, blocks in L-serine degradation would also lead to toxicity and thus anything relieving toxicity might also appear to induce, L-serine would normally be expected to shut off its own biosynthesis; it may be that there is some leeway in the system, and that the L-serine pool can increase considerably over normal before biosynthesis is inhibited.

The role of L-leucine remains unclear. L-leucine is known to increase the rate of growth of *E. coli* K_{10} using glycine as a N source, i.e. growth in -Ngl is

faster than growth in -Ng (Nichanj, unpublished results). Further, L-leucine spares glycine in a strain lacking serine transhydroxymethylase (STHM). L-leucine stimulates growth on L-serine in some STHM$^-$ strains and decreases the rate of growth in others. There is therefore obviously some hitherto unsuspected involvement of L-leucine in this area of metabolism, but the nature of the involvement is unknown. (Newman, unpublished results)

Whatever the reason, growth in -Nplg media results in higher levels of enzyme than growth in $^+$Np. However the difference between induced and noninduced levels is much less than for most inducible systems. The question then arises as to whether the phenomenon seen here results from a change in transcription, as is usually proposed for an induced enzyme, or whether controls are post-transcriptional. Because there is an added complication in the interpretation due to the discovery that L-SD is unstable in the cell, and unstable to different extents in different conditions, the discussion of mechanism of induction is postponed until later.

B(4) The Effect of Growth on Different Carbon Sources: The Glucose Effect

Part of this study was aimed at determining whether L-SD was subject to the 'glucose effect'. The synthesis of many enzymes is decreased in cells grown with glucose as carbon and energy source as compared to cells grown with other carbon and energy sources such as glycerol or succinate. This repression effect of glucose is known as the 'glucose effect'. The enzymes subject to this effect are usually amino acid deaminases and catabolic enzymes leading to energy production (Magasanik 1961). Because other amino acid deaminases have been shown to be subject to the glucose effect, it was considered that L-SD might be similarly controlled. A consideration of the physiological role of the glucose effect made this more likely.

All glucose-sensitive enzymes are capable of converting their substrates to metabolites which the cell can also obtain independently and more readily by the metabolism of glucose. For example, those enzymes which degrade sugars or sugar alcohols (lactose, galactose, glycerol, inositol) each can serve as sole carbon and energy source to many bacteria, but each is degraded to a mixture of catabolites which produce keto-compounds + NH_3 that can be converted to the same intermediate meta-

bolites produced from glucose and NH_3. On the other hand, the products of glucose insensitive enzymes cannot be produced independently by separate glucose pathways, e.g. acid production from penicillin by the inducible penicillinase cannot be formed by any other mechanism.

Generally, glucose is more rapidly metabolized than other carbon compounds and the rate at which the metabolites are formed is apparently more than sufficient to saturate the cells requirements of precursors and nucleic acids. Therefore, the synthesis of enzymes that provide the same service to the cell, but even less efficiently, would be uneconomical, and therefore it would be an advantage to the cell to be able to restrict the formation of these unneeded enzymes. Thus, the concept arose that the catabolites that are rapidly formed from glucose accumulate in the cell and repress the formation of enzymes whose synthesis would increase the already large pools of these compounds, and hence the term 'catabolite repression' (Magasanik 1961).

In the present study, it was seen that the non-induced level of L-SD in glucose grown cells (0.65 um pyr/mg. prot) is in fact much higher than the level in glycerol (0.07 um pyr/mg. prot) or succinate (0.11 um pyr/

mg. prot). grown cells. It appears then that either glucose is in fact stimulatory or the other carbon sources repressive.

As previously mentioned, enzymes subject to the glucose effect are commonly those whose products are also products of glucose metabolism. Since the product of the L-SD reaction, pyruvate, is in fact one of the central products of glucose metabolism, one might expect a pronounced glucose effect. This could indicate that the important role of L-SD is not in the production of pyruvate but rather the destruction of L-serine or the release of ammonia.

DeCrombrugghe et al (1969) showed similar results. Whereas several other inducible enzymes were repressed by glucose, L-SD was the only one in the group whose level was increased in glucose-grown cells and decreased by cyclic AMP. This effect could not be explained.

Earlier reports on glucose effects are harder to interpret because the authors used DL-serine as substrate, and their data does not differentiate a glucose effect on D-SD (which is known to be glucose sensitive (McFall and Mandelstam 1963, McFall and Bloom 1971), from one on L-SD. Thus Boyd and Lichstein

(1955) with E. coli Strain 86G and Bacterium cadaveris reported SD to be repressed by 2% glucose, while Dawes (1951) studying E. coli NCTC Strain 5928 reported no glucose effect. Both used DL-serine as substrate.

Pardee and Prestidge (1955) studied the effect of glucose in the presence of inducers only. In his experiments, cells grown with 0.5% glucose had much the same level of enzyme as cells grown with 0.5% glycerol, -glycine and L-leucine being present in both cases. However, when grown at higher concentration of glucose (2%) the level of enzyme was 30% lower. These results are considerably different from those reported here.

If preceeding arguments with respect to the L-serine pool were correct, one would have to assume that there is less L-serine in cells grown on glycerol or succinate than in glucose grown cells. There is some evidence, in fact, that L-serine biosynthesis in yeast proceeds by different routes depending on the carbon source used (Ogane and Ogur 1972). Similarly Hm_{100}, a strain of E. coli K_{12}, requires L-serine and L-glycine when grown on glucose, but not when grown on oxidizable substrates (Umbarger, punpublished results). At the moment this situation is not understood.

The present study concerned itself with the inducibility by glycine and L-leucine in cells grown on different carbon sources. With glycerol as carbon and energy source, glycine and L-leucine show little inducing ability, but the effect of decreased inorganic N (-Nplg) is still seen. With succinate as carbon and energy source, the situation is reversed. In this case, glycine and L-leucine induce in +N, but the level in -Nplg is actually lower than the level in +Nplg. From this point of view, one could even argue that -N conditions repress enzyme synthesis. In +Nplg, the level of enzyme is similar in succinate (0.99, 0.70 um pyr/mg. prot) or glucose (1.98 um pyr/mg. prot) grown cells. In glycerol-grown cells, the level is only 10% of the others, and is indeed even lower than in +Np glucose. If glycine and L-leucine do induce by virtue of their influence on the L-serine pool, it seems clear that this pool must be very different on glycerol.

In -Nplg, the level is much the same in glycerol and glucose grown cells, but lower in succinate grown cells. The usual inducing effect of MM-N as compared to MM+N medium, is simply not seen when succinate is the carbon source. Thus, there seems to be a difference

between glucose and succinate with respect to N metabolism, and between glucose and glycerol with respect to L-serine metabolism. The reactions involved are not known.

In this thesis, a report of the effect of growth phase on L-SD activity has also been made. It appears that the induced level of enzyme decreases greatly in the later stages of growth, but the noninduced level remains more or less constant. This effect has not been studied in great detail. If early log phase is accompanied by the fermentation of glucose and if the later log phase is accompanied by oxidation of metabolites formed from glucose, it might be that in late log phase the metabolic pattern used is similar to that of glycerol-grown cells, and the induction effect if glycine and L-leucine is no longer seen.

There is in the literature, one report of the levels of L-SD as a function of the phase of growth. This is a study done on the same enzyme studied here, but under the name of L-serine dehydratase in Bacillus alvei (Griffiths and deMoss, 1969). This study was done in medium containing 1% casein hydrolysate including either 1% glucose or 1% glycerol. With either carbon source, the enzyme level fluctuated greatly with

the phase of growth, but no pattern was obvious, other than very low levels after prolonged incubation. In both Griffiths' and deMoss's experiments and those reported here, the fall of enzyme activity might be better explained by the decay studies than by a carbon source effect. Decay, as described in this study, takes place in the presence of glycine and L-leucine. As the growth rate decreases towards stationary phase, one might expect the enzyme to decay if glycine and L-leucine were still present or if casein hydrolysate was in excess.

C Enzyme Decay: 'In Vivo' Instability

The other major concern in this thesis has been the demonstration that L-SD activity is unstable even within the cell. This was discovered during an attempt to study conditions necessary to induce L-SD. When cells were transferred from +Np to +Nplg, an increase in enzyme activity was expected due to the presence of the inducers - glycine and L-leucine. It was expected that this increase would be prevented in the presence of chloramphenicol. However, on incubation in chloramphenicol, the enzyme activity not only did not increase but decreased considerably. This loss of activity is here known as 'in vivo decay'.

Because this sort of 'decay' in vivo is a rare phenomenon, it was described in some detail. The rate and extent depended on the medium of incubation: decay was more rapid and more extensive in the presence of the inducers, glycine and L-leucine, than in their absence; when L-serine is also present, no loss of activity is seen for the first hour of incubation, i.e. L-serine seemed to protect or stabilize the enzyme for an hour at least. Decay was observed in noninduced medium (+Np) but only after a one hour stable period, and the extent of decay was very small in comparison

to that seen in inducing conditions; decay was more rapid in MM+N where 99% of the activity was lost by 150 minutes, than in MM-N where 90% was lost by 150 minutes. The rate of decay was not constant during the incubation period; being rapid at the beginning, and slower after the first hour; a certain proportion of activity remained stable even at 150 minutes; this proportion was greater in MM-N than in MM+N.

The first question which arises is whether the process of decay in enzyme levels is a passive or active one, i.e. is the L-SD molecule inherently unstable even within the cell, or are there other factors in the cell which are responsible for the decay? Since there is little or no decay in cells incubated in +Np, it would appear that the enzyme molecule itself must be relatively stable, at least within the cell, and one must look for other factors to explain the in vivo decay.

Enzyme activity can be modified by a number of factors external to the enzyme structure. Some of these result in physical changes such as conformational changes. Others result in chemical alterations, such as limited proteolysis or the class of chemical modifications described by Holzer (1969).

Physical alterations in enzymes have been extensively described in the literature. These alterations may either increase or decrease activity, and may be caused by small molecules (effectors), which are not substrates for the enzyme in question. Such alterations in activity are usually known as 'allosteric' and to the extent that they are involved in the control of metabolism, this is known as 'allosteric regulation'. The most fundamental characteristic of these allosterically controlled enzymes is their ability to be activated or inhibited by metabolites other than their substrates. Often, there is no structural similarity between the 'effectors' (activators or inhibitors) and the substrate. A well known example is the enzyme L-TD the first enzyme in the biosynthetic pathway to L-isoleucine. The activity of L-TD is decreased in the presence of L-isoleucine, and increased in the presence of L-valine, both of these operating by changes in the physical form of the enzyme. Because of the structural differences that can exist between the effectors and substrate, it has been postulated that the binding site for the effector must be a different one from the substrate binding site responsible for the enzyme's catalytic activity.

Substrate induced physical changes are also known, Substrates may activate or inhibit activity, probably due to the induction of conformational changes (Grisolia 1964, Yankeelov and Koshland 1965). Substrates are usually known to activate enzymes, as is true for malic dehydrogenase, isocitric dehydrogenase, glycogen synthetase, or aspartate semialdehyde. Substrates can also protect enzymes from inactivation as is true for lactic dehydrogenase, glutamine synthetase, ketoglutarate transamylase, or glycerokinases. However, substrate induced inhibition of enzyme activity is now being recognized, i.e. alcohol dehydrogenase, carbamyl p synthetase, hexokinase, and aspartate transaminase (Grisolia, 1964). These substrate induced conformational changes would not necessarily affect the catalytic site, but perhaps the rest of the protein molecule which may have to be retained in aparticular folding pattern for the catalytic site to be operational (Grisolia 1964).

Alterations in enzyme activity can also be done chemically, i.e. the primary structure ofthe enzyme can be modified by the formation or breaking of covalent bonds which in turn attaches or removes specific groups from a protein. These chemical modifications are catalyzed by modifying enzymes which are in turn

controlled by particular effectors.

One example is the enzyme glutamine synthetase of E. coli which catalyzes the ATP dependent formation of glutamine from glutamate and NH_4. This enzyme exists in an active form which can be inactivated by adenylation. This enzyme is sensitive to allosteric inhibition, but chemical modification appears to be the most important control of its activity. It can exist in a biosynthetically active 'a' form and a second 'b' form which has about 5% of the activity of the 'a' form.

The relative concentrations of forms 'a' and 'b' depend on the N source in the growth medium i.e. glutamine synthetase is rapidly inactivated upon the addition of NH_4^+ to the medium. The inaction is due to a specific enzyme, an adenytransferase, which in the presence of ATP, Mg^{++}, and glutamine (its effectors) catalyzes the conversion of form 'a' by adenylation to form 'b'.

Rapid conversion of glutaine synthetase 'b' to the deadenylated form 'a' i.e. reactivation of the enzyme, occurs in cells after transfer into medium free of NH_4^+. The important effectors that influence the interconversion of glutamine synthetase 'a' and

'b' are glutamine (which stimulates adenylation and inhibits deadenylation) and ∝-ketoglutarate (which has the reverse effects) (Holzer and Duntze 1971, Holzer 1969).

Other enzymes of E. coli which have also been found to be subject to chemical control are pyruvate lyase and leucyl-t-RNA synthetase; while examples of animal enzymes include glycogen phosphorylase, phosphorylase b kinase, fructose-i-6-diphosphatase, translocase, and xanthine oxidase . Enzymes regulated by chemical modification are usually key enzymes of metabolic pathways (Holzer 1969, Holzer and Duntze 1971)

Enzyme activity can also be modified by the process of limited proteolysis. This involves the enzymatic conversion of large precursor polypeptides into smaller functional products. Examples of this phenomenon are seen in the conversion of proinsulin to insulin (Steiner and Oyer 1967, Pine 1972), the activation of zymogens by chymotrypsinogen to enzymes e.g. chymotrypsin (Otterson 1967) in the process of assembly of mengovirus virions and Coxackie B virions; capsid subunit proteins result from cleavage of a large precursor protein which is again cleaved into smaller proteins (Holland and Kiehn 1968).

Jackson and Baltimore in 1968 showed that three procapsid proteins of poliovirus (VPO, VP_1, and VP_3) are formed from cleavage of a single large polypeptide. They suggest that all viral proteins may arise by cleavage of a single polypeptide that represents the total information of the viral genome.

A study by Summers and Maizel (1968) indicates that some of the polypeptides of Type I poliovirus are 'primary' translation products of the virus mRNA, while others are 'secondary' products which arise from conversion of the 'primary' proteins. Others studies suggest that the total genome of the poliovirus appears to be translated as a single cistron to give a giant polypeptide of 230,000 to 250,000 daltons which is cleaved in several stages to give as many as 16 different proteins (Pine 1972). Thus, limited proteolysis is interpreted as a maturation process for proteins and is therefore probably irreversible.

Activity of a given protein can thus be altered in different ways. Various small molecules, substrate of the enzyme or not, can alter the conformation of the protein and thus increase or decrease activity. Enzymes can be chemically altered by a variety of reactions including more or less extensive proteolysis.

Each of these is specific for the particular enzyme, and could be invoked as an explanation for decay in activity seen in this study.

The decay described here is demonstrated only in the presence of chloramphenicol. This is a technical problem. Unless new protein synthesis is inhibited, decay of preexisting proteins cannot be detected. If the enzyme were stable and easily isolated, new protein could be detected by incorporation of isotopes, as was done in a study of arginyl-t-RNA synthetase (Williams and Neidhardt 1969). In this system however, the enzyme is unstable in vitro as well as in vivo, experiments must therefore be done in the presence of chloramphenicol. The question then arises as to whether chloramphenicol is responsible or necessary for decay.

There is no indication that proteins become unstable in the presence of chloramphenicol. Pine 1972, reports that high concentrations of chloramphenicol which inhibit RNA synthesis or completely repress protein synthesis can lightly or moderately stimulate proteolysis. He estimated that about 1-7% of the total fraction can be stimulated in this way. This is suggested as only a minor control on proteolysis. However, here, only 20 ug/ml chloramphenicol was used, and decay of up to 99% of enzyme activity was seen,

therefore cannot likely be accounted for in this way.

Mandelstam (1958 and 1960) reports that chloramphenicol actually inhibits protein degradation by about 50%. This effect was not produced immediately, i.e. was delayed about one hour, and therefore, it was suggested that the effect was indirect, possibly by the prior accumulation of some metabolite. Willets (1967) reports no effect by chloramphenicol. Thus, there seems to be no basis in the literature for a general stimulation of protein breakdown in the presence of chloramphenicol.

Whether chloramphenicol promotes a breakdown of this specific enzyme is a harder question to answer, though it seems unlikely. When a -Nplg culture with an activity of 1.91 um pyr./mg. prot. was subcultured into +Np, the activity fell to 0.28 um pyr/mg. prot. in three hours (Table XIV). This could be accounted for by dilution only if the subculture began growing immediately and stopped synthesizing enzyme entirely in those first three hours. Both assumptions seem unlikely. In the same experiment, the enzyme level dropped from 0.86 to 0.34 um pyr/mg. prot. in non-growing conditions (-Np), and thus it would seem that chloramphenicol is not needed for decay. The question could be settled perhaps by a detailed comparison of growth and enzyme activity to differentiate dilution

by growth from dilution and decay, but this was not attempted.

The strongest evidence that chloramphenicol is not responsible for decay is the fact that there were conditions in which chloramphenicol was present but decay was not observed (e.g.+Mp chlor.). It is therefore assumed in this discussion that chloramphenicol does not cause decay.

'In vivo' decay in this system requires the presence of 'Inducers'. This is perhaps the most surprising aspect of the phenomenon. In the study of Williams and Neidhart (1969) the conditions were the opposite. In that study, arginyl-t-RNA synthetase of E. coli has a constant exponential decay yielding a half life of 50 minutes when growing under amino acid restriction (arginine) particularly in conditions allowing intermittant growth. The enzyme was however stable in inducing conditions.

In the present study, glycine and L-leucine act to stimulate decay as well as to 'induce' the enzyme. This raises some question as to the actual mechanism of induction, which will be discussed later. It also suggests that in glycine and L-leucine grown cells, the actual amount of enzyme synthesized must be considerably greater than the amount of activity

measured indicates. If the enzyme has a half life of 45 minutes and the cells are dividing every 50 minutes, one would need a rate of synthesis of about 165%/50 min. to maintain the enzyme level.

Considering the possible models of alteration in enzyme activity discussed previously, what could the role of glycine and L-leucine be? It is easy to postulate an allosteric interaction between either glycine or L-leucine with the L-SD molecule, which would convert it to a less stable conformation, and a conformational change in the presence of L-serine which would protect it. If glycine and L-leucine were not present, no protection would be necessary.

If L-SD is subject to chemical modification, glycine and/or L-leucine might be effectors of the modifying enzymes. They might also act to shift the metabolic state of the cell to one in which modification is induced. Thus, glutamine synthetase is adenylated in the presence of inorganic N and deadenylated in its absence. L-SD could be similarly modified in the presence of glycine and L-leucine. The reversibility of the inactivation has not been studied in our system. The same considerations apply to the possibility of limited proteolysis.

Degradation of proteins has been studied frequently under the aspect of turnover. At one time it was believed that proteins, once synthesized, were infinitely stable in the living cell. This was disproved with the advent of isotopes, and it was shown that significant fractions of the cell protein are subject to degradation in both growing and non-growing cells. Since the highest estimates of turnover are only 7%, however, this turnover in general cannot account for the instability of L-SD unless L-SD belonged to a class of enzymes that was particularly subject to turnover. In order to judge the likelihood of this, a survey of the literature on turnover follows.

Turnover is the dynamic process of degradation and resynthesis of cellular proteins. In the animal cell, continual dynamic equilibria of proteins is an effective means of adjusting enzyme levels to a changing environment without extensive cell growth. For unstable enzymes, the effective level at any one time reflects a balance between synthesis and degradation, each process separately controlled (Pine 1972).

In animals, the greatest amount of protein turnover takes place in the liver, since that organ is involved in the conversion of ingested nutrients to

a form that can maintain a constant internal environment for the organism, and therefore its enzyme profile must undergo major changes under various physiological and nutritional conditions. For example, the turnover rate in rat liver is fast and extensive, i.e. 50% of the protein is replaced in 4 to days; individual enzymes are degraded at different rates, from hours to days, e.g. tryptophane oxygenase, 2 to 4 hours; glutamine-alanine transaminase 2 to 3 days.

On the other hand, it is generally assumed that the growing microbial cell can adjust enzyme levels by outgrowth rather than turnover. However, in non-proliferating microorganisms (e.g. during starvation, diauxic growth, or differentiation), turnover has been recognized as an important process which will ensure a sufficient supply of material to allow the bacteria to synthesize inducible enzymes which may be useful in the changed chemical environment (i.e. allows adaption). (Mandelstam 1958, and Shimke 1969). Thus, an animal cell would be comparable to a non-growing bacterial cell in that its growth rate is slow or negligeable, and it must therefore depend on turnover for metabolic alterations, rather than on adaption through extensive growth.

Many studies have been done on the extent of protein turnover occuring in bacterial cultures growing under different conditions or in non-growing bacterial cultures. It has often been assumed that turnover is insignificant or nonexistant in growing bacterial cultures. Mandelstam (1958) reports no detectable turnover in rapidly growing cells. Koch and Levy (1955) estimate a minimum half life of 30 days for protein in growing bacteria.

In a review, Mandelstam (1960) includes a report that proteins of growing bacteria are stable ; or that when detected, the rate of degradation is less than 1% of the rate of synthesis in E. coli; 1.4%/hour in growing Bacillus cereus; and nondetectable in growing yeast. Another report on Bacillus alvei claims 1%/hour turnover in growing cultures and similar rates for yeast (Mandelstam and Halverson 1960).

Willets (1967) studying E. coli reports that protein degradation occurs in growing cultures, but very slowly. Following growth for 4 hours he found that the initial rate after resuspension was 1.3% in 30 minutes and then decreased and was maintained at 0.6%/hours

On the other hand, turnover is found quite generally in nonproliferating bacteria, and has been reported to occur at a rate of about 4 to 7%/hour under various conditions of starvation of carbon or nitrogen. The cell in starvation conditions would be at a clear advantage if it were able to degrade non-functional cell proteins or enzymes and use the materials thus derived to synthesize new proteins or enzymes which could allow the cell to adapt to its new environment. It has been sugeested (Mandelstam 1958) that bacteria always possess the mechanism(s) for degrading protein but that during growth this would be inhibited in some way.

Whereas Madelstam suggested that protein turnover is a general phenomenon caused by any condition that slows the growth rate or stops it completely (Mandelstam and Halversom 1960). Pine (1972) suggests that it is rather only a certain number of deficiencies which can activate proteolysis. He reports that proteolysis is actually lowered when growth is stopped by exposure to mytomycin C or p-fluorophenylanine, or thymidine starvation. These treatments allow normal ribosomal function, but stops protein synthesis by secondary regulatory mechanisms. A deficiency in some amino acids stimulates proteolysis (e.g. tryptophane, leucine,

histidine, tyrosine have weak or limited stimulation), while others do not. Overall proteolysis in E. coli appears to be regulated additively, but to unequal extents by cellular amino acid pools.

Proteins synthesized during starvation are considerably more unstable than the original cell protein, about 25-35% decompose within 20 minutes after pulse-labelling. These unstable proteins may have some function in initiating cellular rapport with the environment (e.g. permeases) or may simply be peptide fragments prematurely released at unfilled codon sites for a depleted amino acid.

Pine (1972) attempts to classify E. coli proteins and includes in this classification, an unstable fraction (about 10% of the cellular protein) which has a half life of 13-60 minutes, and breaks down in growing, starved, or chloramphenicol treated cells; a fraction making up 20-30% of the total cell protein which does not normally degrade during growth, but can be if starved; and the bulk of the cell protein (70%) which is not degraded under any conditions.

In animal cells where degradation may be a more widespread occurance, Shimke (1969) attempts to explain why rates of degradation are different for each protein.

He postulates that proteins theoretically exist in different thermodynamic states or conformations, and therefore a protein will be degraded only if it assumes one of a number of conformations. Different rates exist depending on the number and nature of particularly labile peptide bonds exposed to certain conformations or an interaction with other proteins or molecules that can alter the conformation. Rates also depend on the activity of the degrading system, and therefore may depend on activation, inhibition, or synthesis of degrading proteins (Shimke 1969).

Mandelstam and Halversom (1960) considered the possibility that turnover effects only nonenzyme proteins. They therefore compared the extent of turnover in the soluble and ribosomal fractions. They assumed that the soluble fraction contains the active enzymes, and that the ribosomal fraction was associated with little enzyme activity. However, they found that proteins of both ribosomal and soluble fractions were about equally labile and had a turnover rate of about 5%/hour.

It must be stressed that the above discussion on turnover refers to turnover of the total cell protein, including nuclear proteins (e.g. histones), mitochondrial proteins (e.g. outer membrance and

inducible enzymes), and ribosomal proteins. Reports of a significant rate of turnover of individual proteins is extremely rare. When individual proteins are investigated, they appear to be stable under the conditions of general protein turnover. In their review, Mandelstam and Halverson (1960) cite as examples nitrase, tetratonase, lysine-apo-decarboxylase, and β-galactosidase.

Recent studies reviewed here, revealed only two cases of turnover of individual enzymes, Willets (1967) tested E. coli ML 328c lac leuc⁻ for degradation of β-galactosidase, D-SD, and alkaline phosphatase, under conditions of NH_4^+, and leucine starvation. After 4 hours, D-SD and alkaline phosphatase were still stable, however, the β-galactosidase level had dropped by 30% (i.e. degradation rate of 8%/hour). As mentioned above, Williams and Neidhart (1969) report that arginyl-t-RNA synthetase has a constant exponential rate of decay giving a half life of 50 minutes when grown under arginine restriction, particularly in conditions allowing intermittent growth.

The 'in vivo' decay described here is thus a rare one. Its rapidity and extent exclude it from the class of phenomenon studied under the heading of protein turnover. Its mechanism is entirely unknown, the

though some possible mechanisms have been reviewed.

It has been assumed up to now that the inducing effect of glycine and L-leucine, i.e. the increase in activity in cells grown in their presence, is a classical induction phenomenon. This would mean that glycine and L-leucine or some compound made in their presence, interacts directly with the genome of the cell, to remove a repressor from the operator locus of the L-SD gene. This would thus be a transcriptional control, inhibiting or increasing the amount of mRNA transcribed from the DNA.

However, glycine and L-leucine are not very powerful inducers. The difference between basal and highly induced levels is at most five fold. This fact, taken with the decay phenomenon, suggests that L-SD may not be subject to classical induction at all, and the fluctuations in activity, both increases and decreases, may be post-transcriptional controls.

We have supposed that the enzyme can exist in different conformations or even different chemical forms (e.g. adenylated or deadenylated). It might also be that the enzyme is composed of subunits, and can exist with different degrees of association between its subunits. For the purpose of this discussion, no choice

need be made between these. The discussion will refer simply to changes in the enzyme state with respect to activity and/or stability. These changes would all be post-transcriptional.

-glycine and L-leucine increase activity and decrease stability. All this could be understood in terms of a single transition to an enzyme state that was both active and unstable (enzyme state I-ESI). ESI might be inherently unstable. Its stability might also depend on its environment. In growing cells, ESI might be perfectly stable. In nongrowing cells, proteolytic enzymes might exist and it might be sensitive to them. Other factors which might act on ESI also could be different in growing and nongrowing cells.

In the absence of -glycine and L-leucine, the enzyme is stable. It would then be in ES2. Since L-serine protects against -glycine and L-leucine, it may convert the enzyme to ES2, or a third still active but stable form, ES3. L-serine protects only for one hour. This might simply mean that the L-serine provided is degraded within one hour. The effect of higher concentrations or repeated provisions of serine was not tested.

Whatever the other conditions, there is more enzyme in MM-N than in MM+N media. This would imply that in MM-N the enzyme takes ESI. If the assumption is that ESI is not inherently unstable, one need only assume further that in MM-N without glycine and L-leucine the inactivating factors, whatever they are, are not made. Moreover, if the inactivating factors are enzymes or effectors of enzymes, one could explain the slowing of inactivation during incubation in terms of a chemical reaction with first order kinetics. As the level of L-SD remaining decreases, less substrate remains for the inactivating enzyme to work on and one would expect the rate of inactivation to decrease.

One could account for a basal level of enzyme by assuming that ES2 is stable, but at least somewhat active, or one could assume that some of the enzyme is made in a different and stable and active state of ES3.

In this formulation, we have not considered the facts that the rate of decay is not constant and that the percentage of the total activity that is unstable is not the same in all cases. This could be accounted for with further assumption as to the percentage of each form present under different circumstances, but this does not seem appropriate here.

It is thus possible to explain the effects of glycine and L-leucine in terms of post-transcriptional controls. This would invoke an ESI induced by glycine and L-leucine and by MM-N, inherently stable, and subject to modifiers; as well as an ES2, the basal form, and the form present when glycine and L-leucine and L-serine are all available; ES2 would be active, but not as active as ESI.

Whatever the mechanism of decay, the possibility of decay complicates the interpretation of all other experiments. For instance, activity decreases late in the exponential phase of growth. This has been explained in terms of metabolic regulation. It might however occur by the same phenomenon as decribed here, <u>in vivo</u> decay, the more so since it appears to happen only in the presence of glycine and L-leucine.

SUMMARY

The work in this thesis concerns the characteristics and the metabolic role of the enzyme L-serine deaminase, which catalyzes the conversion of L-serine to pyruvate and ammonia. This enzyme has not previously been studied in any great detail, and is so unstable in vitro that most studies, including this one, have used toluenized cells as assay material.

The enzyme has been shown to be specific for L-serine; no activity was seen against a variety of similar molecules including L-threonine, D-serine and glycine. It was most convenient to assay the enzyme at pH 6.4 because no other deaminating enzymes were active at that pH. However, total L-serine deaminating activity increased with pH, from pH 6.4 to pH 8.0.

To investigate the possible metabolic role of L-serine deaminase, studies were made of the enzyme level in growth conditions which were expected to influence it. The basal level of L-serine deaminase in the cell was found to be about 0.65 u moles pyruvate/mg. protein, which was considered high enough to indicate a significant role in cell metabolism. The level was increased in cells grown

in the presence of glycine and L-leucine which were previously reported to be inducers of the enzyme. It was also increased in the absence of inorganic nitrogen. Highest levels assayed were found in cells grown in the absence of inorganic nitrogen, and the presence of glycine, L-leucine, L-isoleucine and L-valine. No 'glucose effect' on the enzyme was seen, indeed, the level of enzyme was decreased on the other carbon sources tested.

The enzyme was found to be unstable within the cell. This instability was most marked in the presence of the "inducers", glycine and L-leucine, in which conditions L-serine deaminase has a half-life of about 45 minutes. This phenomenon has been called '*in vivo* decay'. In the absence of L-glycine and L-leucine, 'decay' was not observed. The enzyme substrate, L-serine, "protected" against the decay seen in the presence of L-glycine and L-leucine, but only for short periods.

Because the enzyme has been confused often with other enzymes, it was shown in some detail that L-serine deaminase is a distinct entity and that L-threonine deaminase is not responsible for the activity studied here.

The variation of enzyme levels with growth conditions was analyzed in terms of a possible metabolic role of the enzyme. The phenomenon of "*in vivo* decay" was considered in terms of certain models of induction and alterations in protein structure.

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