ASPARAGINE BIOSYNTHESIS AND UTILIZATION

A Thesis

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Doctor of Philosophy

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by

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SUMMARY

The biosynthesis and utilization of asparagine in seedlings and micro-organisms was examined.

From initial studies on the biosynthesis of asparagine in plants it was possible to demonstrate the *de novo* synthesis of asparagine in wheat seedlings and shoots grown under normal conditions. The rate of this synthesis was similar to that of glutamine, aspartic acid and glutamic acid. It was also possible to demonstrate the *in vivo* incorporation of label from [^{14}C]-U-aspartic acid into asparagine in these seedlings, but no *in vitro* synthesis of asparagine from aspartic acid and ammonia dependent on ATP and Mg^{++} could be detected.

It was not possible in studies on the utilization of asparagine in wheat seedlings and shoots to detect any *in vivo* or *in vitro* hydrolysis of the amide, nor was there any evidence of transamination reactions involving asparagine. Except for incorporation into protein, asparagine appears to be metabolically inactive.

Following these studies, the biosynthesis of asparagine by the cyanide pathway (which had been demonstrated previously in plants and *E. coli*) was examined with a view to determining its possible physiological significance in asparagine biosynthesis. A rapid *in vivo* incorporation of HCN into

asparagine in plants was observed and the *in vitro* synthesis of asparagine from cyanide and cysteine via β-cyano-alanine demonstrated. Some of the properties and the intracellular distribution of the crude wheat leaf β-cyano-alanine synthase and hydrolase were determined. β-cyano-alanine synthase activity was also detected in lupin shoot and root extracts, and in *E. coli* extracts, but not in any of the mammalian extracts examined. β-cyano-alanine hydrolase activity was detected in extracts of lupin leaves, *E. coli* and guinea pig liver and serum. Studies on the β-cyano-alanine synthase and hydrolase in *E. coli* raised doubts as to the significance of these enzymes in asparagine biosynthesis in this organism. In particular, purification and induction studies on the *E. coli* EC II asparaginase and β-cyano-alanine hydrolase showed that it is likely that the EC II asparaginase catalyzes both the hydrolysis of asparagine and β-cyano-alanine. Genetic studies in *E. coli*, although unfortunately not unequivocal supported the concept that in this organism the cyanide pathway enzymes are not physiologically significant in asparagine biosynthesis. An *E. coli* asparagine auxotroph was isolated and found to lack an ATP-dependent asparagine synthetase. This auxotroph was not impaired in its ability to synthesize or hydrolyze β-cyano-alanine and the asparagine requirement could not be replaced by cyanide at the concentration tested. Furthermore, in direct contrast to

plants, E. coli did not rapidly incorporate exogenous cyanide into asparagine in vivo. However, the significance of the rapid incorporation of cyanide into asparagine in plants was still speculative and became the object of further study.

It was found possible to induce the synthesis of asparagine in wheat seedlings in vivo by ammonium sulphate feeding. No significant increase of cyanide or β-cyano-alanine was associated with this increased asparagine synthesis, nor were the cyanide pathway enzymes induced. Unfortunately though, no definitive evidence is presented which suggests that the cyanide pathway is, or is not, physiologically significant in asparagine biosynthesis in plants. The results were, however, compatible with the pathway being a cyanide detoxification mechanism.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference is made in the text.

Kevin J.F. Farnden

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Special acknowledgement of the collaboration of Dr. Edith Lees in the initial studies on the biosynthesis and utilization of asparagine reported in this thesis in Chapter III is appreciably recorded. These collaborative initial studies were commenced during the author's (K.J.F. Farnden) fulfilment of the requirements for the degree B.Sc.(Hons.). Acknowledgement is therefore given of a preliminary account

of the results presented in this thesis in Chapter III, B, 4 and Chapter III, C, 1 and 2, which appeared in the thesis submitted for the B.Sc.(Hons.). The studies reported in these particular sections were repeated and extended during the work for this thesis and are therefore re-included for completeness.

ABBREVIATIONS

The following abbreviations have been used in this thesis.

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine 5' phosphate
PPi	Inorganic pyrophosphate
Pi	Inorganic phosphate
EDTA	Ethylenediaminotetra-acetate
GSH	Reduced glutathione
Tris	2-amino-2-hydroxymethylpropane-1:3-diol
DEAE-cellulose	Diethylaminoethylcellulose
β-CN-ala	β-cyano-alanine
Cbz-phe	N-carbobenzoxy-L-phenylalanine
NNG	N-methyl-N-nitroso-N'-nitroguanidine
TCA	Trichloroacetic acid
DONV	5-diazo-4-oxo-L-norvaline

ENZYMES

For the sake of simplicity, the enzymes have been referred to in the text of this thesis, by their trivial names. A list of the enzymes that have been mentioned follows, together with the numbers by which they are designated in the Report of the Commission on Enzymes of the International Union of Biochemistry (1964) where possible, or by a description of the reaction they catalyze.

Trivial name	EC No. or Reaction
Asparaginase	3.5.1.1.
Asparagine synthetase	6.3.1.1.
Glutamine synthetase	6.3.1.2.
Cysteine synthase	4.2.1.22.
Asparagine-ketoacid aminotransferase	2.6.1.14
Glutamine-ketoacid aminotransferase	2.6.1.15
β-cyano-alanine synthase	[cysteine / serine] + HCN → β-cyano-alanine + H_2S + H_2O
β-cyano-alanine hydrolase	β-cyano-alanine + H_2O → asparagine
γ-glutamyl transpeptidase	Glutathione + β-cyano-alanine → γ-glutamyl-β-cyano-alanine.

CHAPTER I
INTRODUCTION

INTRODUCTION

The work in this thesis was undertaken as part of a research programme into the biosynthesis and utilization of asparagine.

For many years it has been proposed in the literature on plant metabolism that asparagine functions specifically in the storage and translocation of nitrogen and carbon. Asparagine was postulated to be synthesized either from an outside source of nitrogen, represented by ammonia or nitrate, and carbohydrate or from the carbon and nitrogen supplied from the metabolism of proteins and amino acids. These concepts were derived mainly from extensive studies on the changes of asparagine concentration in plant tissue under different conditions of nitrogen metabolism. But, despite these studies, both the mechanism by which asparagine is synthesized in plants and its specific role in the biochemistry of the plants was not convincingly elucidated.

It seemed therefore important to establish at a biochemical level the mechanism of asparagine biosynthesis and utilization in plants.

It is convenient to divide this introduction into three sections. The first will generally review the concepts commonly held on asparagine biosynthesis and

utilization in plant metabolism. The second section will review the biochemistry of asparagine utilization in micro-organisms, higher organisms and plants, whilst the third will review the various proposed mechanisms of asparagine biosynthesis.

This introduction is restrictive in that only literature available at the commencement of this work is reviewed. During the progress of this work numerous reports from other groups working in this field have appeared in the literature. These will be acknowledged and discussed in the concluding discussions of individual chapters and in the Final Discussion.

A. THE BIOSYNTHESIS AND UTILIZATION OF ASPARAGINE.

HISTORICAL REVIEW.

The biosynthesis and the role of the acid amide, asparagine, in plants has attracted the attention of plant physiologists and biochemists ever since its isolation from asparagus juice by Vauquelin and Robiquet in 1806.

It was the subject of the now classical researches of Pfeffer, Schulz and Prianischnikov carried out in the late nineteenth and early twentieth centuries. A comprehensive review and re-interpretation of much of this early work has been made by Chibnall (1939a) and more recently by McKee (1962).

The essence of Pfeffer's views, published in 1872, was that asparagine arose from protein breakdown and transported nitrogen from the cotyledons to the growing points in the seedlings. These ideas came mainly from the microscopic observation of asparagine crystals in tissues treated with alcohol.

The extensive work of Schulz and Prianischnikov, although limited in that their data were obtained only from the measurement of seedling growth and the chemical analysis of the dried tissues, resulted in the formulation of the following tenets as interpreted by Chibnall (1939b).

" (i) On germination, the reserve proteins undergo enzymic digestion to give, as primary products of hydrolysis, asparagine, glutamine and the usual series of amino acids, including aspartic acid and glutamic acid if these be combined as such (i.e. in the unamidated form) in the intact protein molecule. The reserve carbohydrates also undergo enzymic breakdown to give simpler products including monoses.

(ii) The decomposition of the reserve proteins is controlled by the outflow from the cotyledons or endosperm, as the case may be, of either monoses ('active' carbohydrates) or amino acids, etc. ('active' ammonia), depending on which component is present in the lesser amount.

(iii) The primary products of reserve protein decomposition are transported to the growing parts, where they become available for protein regeneration.

(iv) Obligatory metabolic processes, which Prianischnikov connected with respiration rather than with growth, bring about a secondary decomposition of certain of the amino acids, which result in the formation of asparagine, glutamine or both of these amides.

(v) Protein synthesis in the growing parts takes place at the expense of the amino acids translocated from the reserve organs, and of the acid amides resulting from secondary change.

(vi) The proteins of the growing parts may have a different amino acid composition from those of the reserve organs. Protein regeneration, therefore, in the absence of external sources of nitrogen, may necessitate an interconversion of amino acids (really a transfer of nitrogen from one nitrogen-free acid to another), and this probably takes place through the agency of asparagine and glutamine."

These now assumingly classical views on the biosynthesis and utilization of asparagine have been and still are widely held even though the basis on which they were formulated is surely in need of re-evaluation. It is pointed out therefore, that any hypothesis of a specific role of

asparagine in plant metabolism must be supported by the unequivocal description of the enzyme systems responsible for its metabolic conversion.

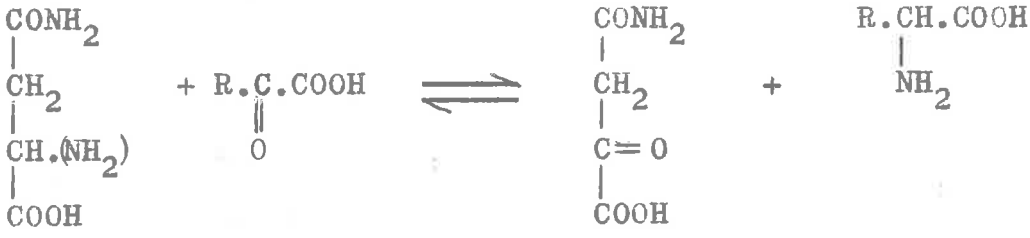
B. BIOCHEMISTRY OF ASPARAGINE UTILIZATION

1. The utilization of asparagine in animals

At the present time asparagine does not appear to have a function in animal tissues other than that of a protein constituent. It is known to be metabolized by the following mechanisms.

(a) Transamination and deamidation of asparagine.

Rat liver homogenates devoid of asparaginase activity following selective heat destruction have been shown to hydrolyze asparagine only in the presence of an α-keto acid (Meister et al., 1952). This reaction is due to the presence in the homogenates of an asparagine-α-keto-acid transamination-deamidation system. In this system a transamination between asparagine and the α-keto-acid is followed by the deamidation of the α-keto-succinamic acid formed to give oxalo-acetate (Equation I,1). It is not known whether the transamination and deamidation occur simultaneously or as a closely linked reaction.



asparagine α-keto-succinamate

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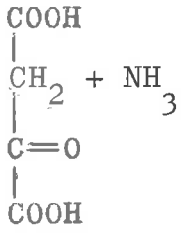


oxalo-acetate

(b) Asparaginases in higher-organisms, (excluding plants

Asparaginases, which catalyze the hydrolysis of asparagine to aspartic acid and ammonia are known to occur in various tissues of organisms (for review see Meister, 1965a and Zittle, 1951). The guinea-pig appears to be unique in possessing an active asparaginase in blood serum; no asparaginase activity has been found in the serum of the cat, rat, cow, dog, monkey, chicken, pig, sheep, horse and man (Meister, 1965a). The observation that guinea-pig serum but not the sera of animals, exerted an inhibitory effect on several transplantable mouse and rat lymphomas (Kidd, 1953) lead ultimately to the identification of

guinea-pig serum asparaginase as the tumour inhibitory factor (Broome, 1961).

2. The utilization of asparagine in micro-organisms

Asparagine is one of the more common nitrogen sources used in the culture of micro-organisms, especially fungi. But, apart from its hydrolysis by asparaginases to aspartic acid and ammonia its only function appears to be its involvement in protein synthesis.

(a) Asparaginases in micro-organisms.

Asparaginases are widely distributed in bacteria, fungi and yeasts and have been extensively studied (for review see Meister, 1965a and Zittle, 1951). The bacterial asparaginases from E. coli and S. marcescens have assumed great importance following the discovery of their anti-tumour activity (Schwartz, Reeves and Broome, 1966).

3. The utilization of asparagine in plants

Considering the apparent uniqueness of plants to accumulate large amounts of free asparagine it is somewhat perplexing to find that there is still no definitive evidence of a significant utilization of asparagine in plant metabolism.

(a) The utilization of asparagine in vivo.

Nelson, Krotkov and Reed (1953), vacuum infiltrated [^{14}C]-asparagine into Lupinus angustifolius seedlings and wheat leaves. A 10 - 20% incorporation of label into alcohol-insoluble material (presumably protein) was

observed with 80 - 90% of the label remaining in the alcohol extract. The evolution of labelled CO_2 was also detected. No further details are available on this work, but the utilization of asparagine in protein synthesis appears to have been demonstrated.

(b) α-Keto-acid dependent degradation of asparagine.

Yamamoto (1955) claims to have demonstrated the synthesis of glutamate and ammonia following the incubation of asparagine and α-keto-glutarate with a homogenate of Vigna sesquipedalis hypocotyls. No asparaginase activity could be detected in the homogenate. Yamamoto (1955) has therefore suggested that this conversion probably proceeds via the transaminase-deamidase system described by Meister et al. (1952) to occur in rat liver, rather than by an asparaginase-transaminase. No further studies, on the enzymes involved in this apparent degradation of asparagine have been reported.

(c) Plant asparaginases

Grover and Chibnall (1927) have described an asparaginase in the young rootlets of germinating barley. The hydrolysis of asparagine was, however, observed over a period of some three to twenty-seven days and the possibility of bacterial contamination was not eliminated. The physiological significance of this "asparaginase" must therefore be considered extremely questionable.

Kretovich, Evstigneeva and Makarenko (1954), claim to have demonstrated the presence of an asparaginase in a homogenate of lupin seedlings. However, no experimental results are available on this work.

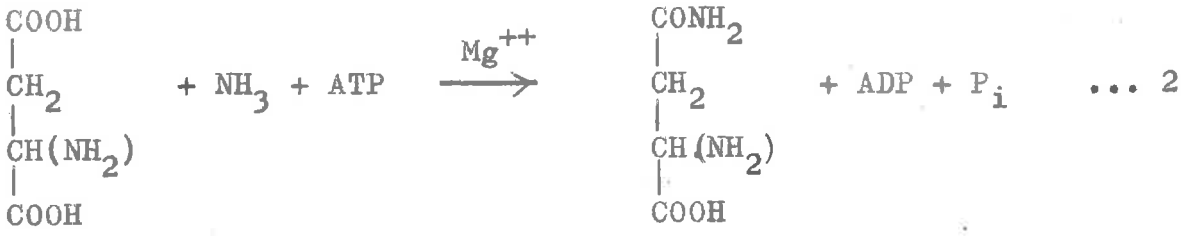
(d) Asparagine utilization in glycinamide ribonucleotide synthesis in plants

Kapoor and Waygood (1962) found that asparagine amide nitrogen was utilized in preference to any other donor by an enzyme preparation from wheat embryos for the synthesis of glycinamide ribonucleotide, the first step in purine biosynthesis. The quantitative significance of this reaction as a major metabolic role of asparagine is not clear.

C. BIOCHEMISTRY OF ASPARAGINE SYNTHESIS

There is evidence in the literature from various classes of organisms both for and against the existence of the following possible pathways for the biosynthesis of asparagine. They will be listed briefly and then discussed in more detail.

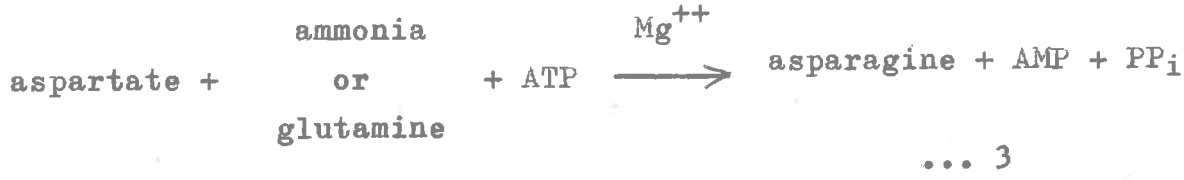
(i) Direct amidation of aspartic acid in the presence of ammonia, Mg^{++} and ATP, with the formation of ADP and P_i (Equation I,2).



aspartic acid asparagine

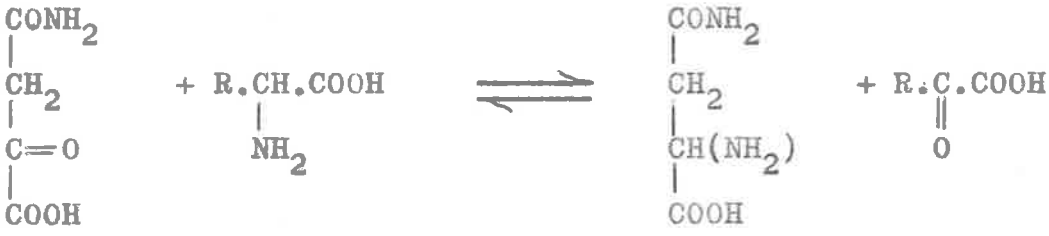
The conversion probably involves the formation of an enzyme-bound β-aspartyl phosphate with the nucleotide as a constituent of the active catalytic site of the enzyme, by analogy with the synthesis of glutamine.

(ii) Direct amidation of aspartic acid in the presence of ammonia or glutamine amide nitrogen as the preferred amide donor, ATP and Mg^{++}, with the formation of AMP and inorganic pyrophosphate (Equation I,3).



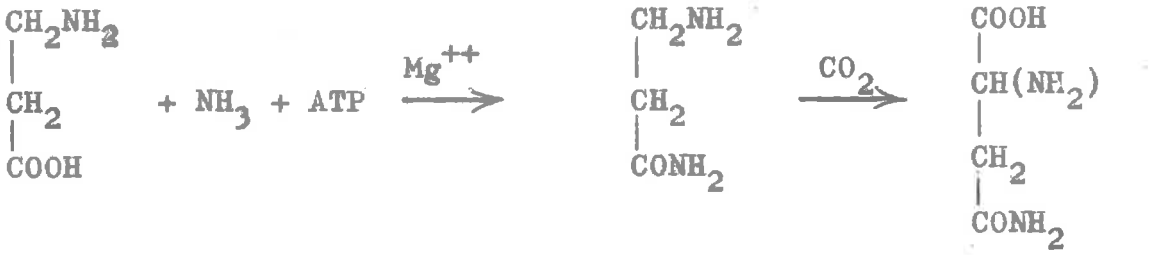
This mechanism probably involves the intermediate formation of an enzyme bound β-aspartyladenylate.

(iii) Transamination of α-keto-succinamate (Equation I,4).



α-keto-succinamate asparagine ... 4

(iv) Amidation of β-alanine followed by carboxylation (Equation I,5).



β-alanine β-alanylamide asparagine

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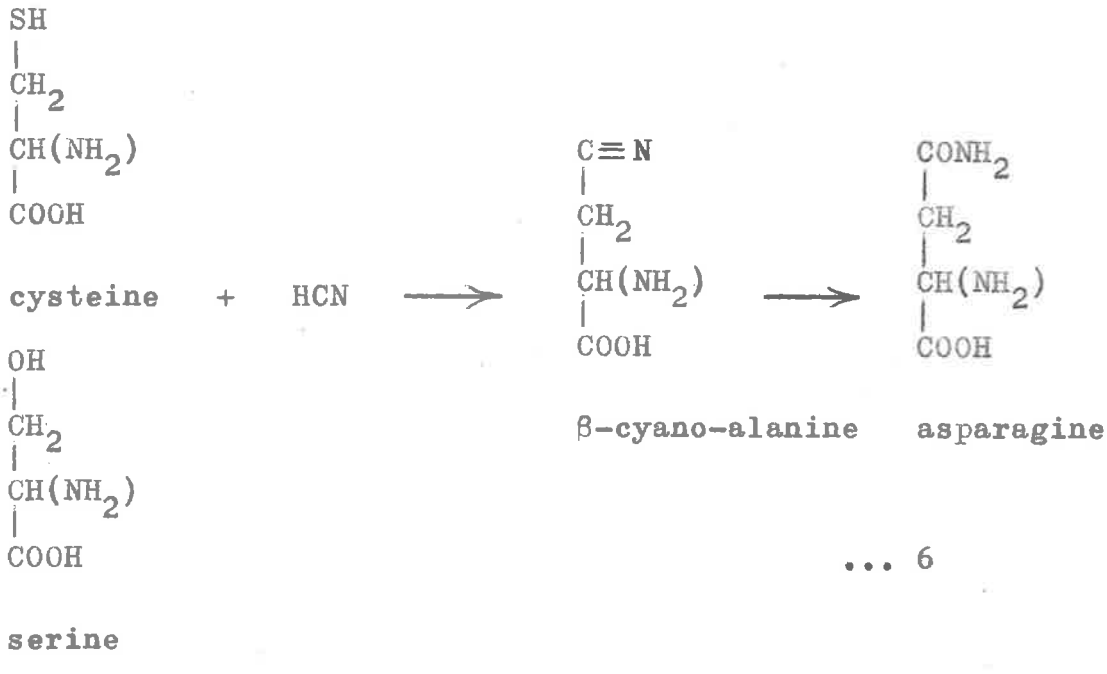
(v) Hydrolysis of β-cyano-alanine following its synthesis from serine or cysteine and cyanide (Equation I,6).



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1. Asparagine biosynthesis in micro-organisms

(a) The asparagine synthetases of yeast, Lactobacillus arabinosus and Streptococcus bovis.

Al-Dawody and Varner (1961) have reported the extraction of an asparagine synthetase from baker's yeast which catalyzes the formation of asparagine from aspartate, ammonia and ATP in the presence of Mg^{++} as indicated in Equation I,2. The enzyme was purified some 100-fold and assayed by measuring ammonia disappearance, P_i released and asparagine formed. The enzyme also catalysed an aspartate-dependent exchange of P_i into ATP. No asparagine

was synthesized if glutamine replaced ammonia.

In Lactobacillus arabinosus, Ravel, Norton, Humphreys and Shive (1962) have purified 10-fold an asparagine synthetase which catalyzes the conversion of aspartate to β-aspartyl hydroxamate in the presence of hydroxylamine and to asparagine in the presence of ammonia. ATP and a divalent metal ion (Mn^{++} or Mg^{++}) were required. The ATP was converted to AMP and inorganic PP_i during the reaction (Equation I,3) and an aspartate dependent exchange of ATP and PP_i was demonstrated. Asparagine was found to control its own synthesis in L. arabinosus through the repression of asparagine synthetase production and the inhibition of its activity.

Burchall, Reichelt and Wolin (1964) have isolated an asparagine synthetase from crude extracts of Streptococcus bovis which catalyzes the same reaction as that catalyzed by the L. arabinosus enzyme (Equation I,3), except that Mn^{++} cannot be substituted for Mg^{++}. This enzyme was similarly inhibited by asparagine but its synthesis was not subject to repression by asparagine.

(b) Asparagine biosynthesis from cyanide via β-cyano-alanine in Escherichia coli.

The biosynthesis of asparagine from serine or cysteine and cyanide via β-cyano-alanine (Equation I,6) was first discovered on young seedlings by Blumenthal-Goldschmidt, Butler and Conn (1963) and Ressler, Giza and Nigam (1963)

(see Chapter I, p.20). Dunnill and Fowden (1965) reported the demonstration of this pathway in E. coli extracts. The E. coli β-cyano-alanine synthase (which catalyzes the first reaction in the pathway) was found to have a pH optimum of 9.0. The Km values for the various substrates were as follows: cyanide ion, 1.3 x 10^{-2}M, serine 8 x 10^{-3}M, cysteine 5 x 10^{-3}M, The E. coli β-cyano-alanine synthase exhibited a partial dependence on ATP, a four-fold increase of reaction rate being observed in the presence of ATP at concentrations greater than 10^{-2}M. Dunnill and Fowden (1965) were hesitant in claiming physiological significance for this as a pathway of asparagine biosynthesis in E. coli and considered it likely that β-cyano-alanine formation could be catalyzed by the action of an enzyme that has some other role in the cell. Cysteine synthetase which catalyzes cysteine biosynthesis from serine and sulphide was regarded as one such possibility.

2. Asparagine biosynthesis in higher organisms (excluding plants)

(a) Enzymatic formation of asparagine by transamination

Meister and Fraser (1954) have demonstrated the formation of asparagine by transamination between α-keto-succinamic acid and a wide variety of α-amino acids catalyzed by a rat liver asparagine-keto acid aminotransferase (Equation I,4). This reversible conversion of asparagine

to α-keto-succinamic acid offered a possible pathway for the biosynthesis of asparagine. It is unlikely though that this reaction represents a physiologically significant pathway of asparagine biosynthesis in animals, since the formation of α-keto-succinamic acid by a pathway other than that of transamination or oxidative deamination of asparagine has not been found. Meister and Fraser (1954) further showed the synthesis of asparagine by the transamination between glutamine and α-keto-succinamic acid using a specific glutamine rat liver transaminase (glutamine-keto acid amino transferase).

(b) The asparagine synthetases of higher organisms.

Studies on the biosynthesis of asparagine in higher organisms have shown that the amide nitrogen atom of glutamine rather than ammonia is utilized in the reaction catalyzed by the asparagine synthetase.

This was first shown by Levintow (1957), who found in studies with ^{15}N-label in HeLa cells that the amide nitrogen atom of asparagine is derived from the amide group of glutamine rather than from ammonia, indicating a direct utilization of the amide nitrogen atom of glutamine. This study offered no clue to the mechanism of transfer of the amide nitrogen or to the nature of possible intermediates. It did, however, render unlikely the possibility that asparagine is synthesized in this system by the direct amidation of aspartic acid as observed in micro-organisms

(Chapter I, page 12).

The incorporation of [^{14}C]-aspartate into asparagine has now been demonstrated with chick embryo liver homogenates in the presence of glutamine (Arfin and Strecker, 1965 and Arfin, 1967). The glutamine requirement could not be replaced by iso-glutamine, glutamate or by ammonia and ATP. The synthesis of asparagine was inhibited by anaerobiosis, cyanide, sulphide, rotenone and 2,4-dinitrophenol suggesting a dependence upon an energy source. Such a requirement, however, could not be demonstrated directly with ATP and ATP-generating systems. Mg^{++} was found to give a stimulation of about 2-fold at concentrations of 1 mM, but became inhibitory at higher levels.

3. Asparagine biosynthesis in plants

(a) The biosynthesis of asparagine in vivo.

Nelson, Krotkov and Reed (1953), isolated labelled asparagine following the in vivo incorporation of [^{14}C]-CO_2 into Lupinus angustifolius seedlings for six days. Surprisingly, 55% of the label was found in the α-carboxyl group of asparagine. Under the same conditions glucose was found to be uniformly labelled within one hour. No further studies of the pathway by which asparagine is synthesized from CO_2 have as yet been reported by these authors.

Similarly, Al-Dawody, Varner and Webster (1960) demonstrated the synthesis of asparagine from [^{14}C]-4-aspartate and ammonium chloride by tissue sections of lentil stems, roots and cotyledons. [^{14}C]-Labelled glutamate and alanine were found to be incorporated about half as effectively as aspartate, and some incorporation of [^{14}C]-CO_2 was also observed. Degradation of the asparagine synthesized revealed little radioactivity (7%) in the α-carboxyl group after the incorporation of [^{14}C]-4-aspartate, suggesting a fairly direct conversion to asparagine. In contrast, asparagine synthesized from [^{14}C]-2-glutamate, [^{14}C]-1-alanine or [^{14}C]-CO_2 contained 44 - 54% of the radioactivity in the α-carboxyl.

Yamamoto (1955) has reported the <u>in vivo</u> synthesis of asparagine from aspartate and ammonium salt. The hypocotyls of three-day-old <u>Vigna sesquipedalis</u> plants were sliced into 3 mm discs and incubated with aspartate and ammonium salt for three hours at 30°. A significant increase in amide nitrogen was observed. Whether this was due to the direct amidation of the aspartic acid was not rigorously shown. Yamamoto (1955) also claimed (no experimental results were given) that a "hypocotyl extract was revealed to have a far weaker ability of asparagine formation from aspartate and ammonium salt".

(b) Attempts to elucidate the mechanism of asparagine synthesis in plants.

A pathway for the synthesis of asparagine that is analogous to the reaction catalyzed by glutamine synthetase described by Elliott (1951) in lupin seedlings (Equation I,2) has been described by Webster and Varner (1955). Elliott (1951) had reported the purification of an enzyme system from sheep brain which synthesized glutamine from glutamate and ammonia in the presence of Mg^{++} and ATP, with the liberation of P_i. Ammonia could be replaced by hydroxylamine, in which case glutamyl hydroxamate was synthesized. Elliott (1951) further reported the synthesis of glutamyl hydroxamate in crude extracts of Lupinus albus and Lupinus angustifolius, but was unable to detect any formation of aspartyl hydroxamate when glutamate was replaced by aspartate.

Webster and Varner (1955) confirmed this report of Elliott's (1951) but claimed that it was possible to demonstrate the formation of aspartyl hydroxamate in extracts of lupin seedlings, germinating peas and wheat germ if the concentrations of both the aspartate and the hydroxylamine were increased 10-fold. Webster and Varner (1955) further demonstrated the formation β-alanyl hydroxamate from β-alanine, hydroxylamine, Mg^{++} and ATP (Equation I,5). This raised the possibility of the carboxylative formation of asparagine from β-alanylamide. However, in carboxylation studies carried out by Webster and Varner (1955) the formation of

asparagine from β-alanylamide in these plant extracts was considerably less than the formation of asparagine from the carboxylation of free β-alanine. In fact, the label found in asparagine was only 7% of that found in aspartic acid. Webster and Varner (1955) therefore suggested that aspartate was probably the direct precursor of asparagine. Webster and Varner (1955) claim to have supported this suggestion by demonstrating the synthesis of asparagine from [^{14}C]-aspartate, ammonia, Mg^{++} and ATP. The asparagine synthesized was only 4% of the hydroxamate formed under the same conditions. Meister (1962 and 1965b) has since reported his inability to confirm this enzymic synthesis of asparagine.

Furthermore, the possibility remains that the syntheses of aspartyl- and alanyl hydroxamates observed by Webster and Varner (1955) were in fact catalyzed by an asparto-kinase. Black and Gray (1953) have demonstrated that yeast aspartokinase can catalyze the synthesis of aspartyl hydroxamate from aspartate and hydroxylamine but not asparagine from aspartate and ammonia.

Therefore, until independent confirmation of Webster and Varner's work is forthcoming, the existence of such a reaction at all must be regarded as tentative.

(c) The biosynthesis of asparagine from cyanide via β-cyano-alanine.

Blumenthal-Goldschmidt, Butler and Conn (1963) during their work on the biosynthesis of cyanoglucosides in plants observed that young seedlings of a number of plant species incorporated radioactivity from [^{14}C]-HCN into the amide carbon atom of asparagine. These authors proposed that asparagine was synthesized by a three-carbon plus one-carbon condensation where the one-carbon fragment was HCN. Preliminary experiments with [^{14}C]-serine suggested that serine or a related metabolite could be the three-carbon unit.

Ressler, Giza, and Nigam (1963) and Tschiersch (1964a) later established from *in vivo* feeding experiments that the neurotoxin β-cyano-alanine, (originally isolated from *Vicia sativa* seeds by Ressler, 1962) was a possible biosynthetic intermediate between cyanide and asparagine. Ressler, Giza and Nigam (1963) noted that labelled cyanide, β-cyano-alanine, and the dipeptide γ-glutamyl-β-cyano-alanine (the latter isolated from *Vicia sativa* by Ressler *et al.*, 1963) were effective precursors of asparagine in *Vicia villosa*, *Lathyrus odoratus* and *Lathyrus sylvestris*. In *Vicia sativa*, however, both cyanide and β-cyano-alanine were incorporated far less significantly into asparagine, and were found to accumulate instead as γ-glutamyl-β-cyano-alanine. This conversion of β-cyano-alanine to asparagine

or γ-glutamyl-β-cyano-alanine has been further investigated by Fowden and Bell (1965). These authors found that extracts of seedlings which converted HCN primarily into asparagine possessed an active β-cyano-alanine hydrolase but little γ-glutamyl transpeptidase activity. In contrast, Vicia sativa extracts readily transferred γ-glutamyl groups from reduced glutathione to β-cyano-alanine to give the γ-glutamyl-β-cyano-alanine, but possessed little β-cyano-alanine hydrolase activity.

Subsequently, serine (as originally proposed by Blumenthal-Goldschmidt, Butler and Conn, 1963) and cyanide were found to be efficient precursors in vivo of β-cyano-alanine, and in turn of γ-glutamyl-β-cyano-alanine, and of asparagine (Tschiersch, 1964b; Nigam and Ressler, 1964). The precursor role of serine in the synthesis of β-cyano-alanine from cyanide was confirmed in vitro by Floss, Hadwiger and Conn (1965) and Tschiersch (1965). In Lotus tenuis homogenates Floss, Hadwiger and Conn (1965) found an increased rate of β-cyano-alanine synthesis if serine was replaced by cysteine, suggesting that in the intact plant the synthesis of β-cyano-alanine may involve cysteine rather than serine as the three-carbon precursor. The activity of the Lotus tenuis β-cyano-alanine synthase was not enhanced by ATP as was reported for the E. coli enzyme (Dunnill and Fowden, 1965). The pH optimum of the Lotus β-cyano-alanine synthase was found to be 9.0,

the same as that reported for the E. coli enzyme. However, compared to the E. coli β-cyano-alanine synthase the Lotus enzyme showed vastly different affinities for cyanide and cysteine, (Km values for the cyanide ion of 5×10^{-6}M and 1.3×10^{-2}M and for cysteine of 9×10^{-5}M and 5×10^{-3}M were reported for the Lotus and E. coli enzymes respectively). The low Km value for cyanide determined for the plant enzyme suggested that this pathway to asparagine could have functional significance either in cyanide detoxification or asparagine biosynthesis for appreciable asparagine synthesis could occur at non-toxid cyanide concentrations.

Indeed, the physiological significance of this pathway in asparagine biosynthesis from cyanide has been repeatedly questioned in the literature. Dunnill and Fowden (1965) suggested that the synthesis of β-cyano-alanine may be catalyzed in fact by cysteine synthetase, an enzyme forming cysteine from serine and sulphide. Giza, Ratzkin and Ressler (1963) have proposed that the hydrolysis of β-cyano-alanine to asparagine could be mediated by the action of an asparaginase. Evidence, however, for a specific plant asparaginase is not definitive (Chapter I, p.8).

The work of Abrol and Conn (1966) and Abrol, Conn and Stoker (1966) on the in vivo conversion of amino acids to cyano-glucosides in which the nitrile moiety was further metabolized to cyanide and incorporated into asparagine,

established the pathway *in vivo* but did not determine the relative significance of the pathway between cyanide detoxification and asparagine biosynthesis. Obviously, any possible role of cyanide assimilation in asparagine biosynthesis is dependent upon a physiologically significant source of cyanide. The cyano-glycosides as indicated above could provide this source of cyanide but the same extensive incorporation of [^{14}C]-HCN into asparagine has been observed in plants which are classified as non-cyanophoric, *i.e.*, they contain little if any, organic nitriles (Blumenthal-Goldschmidt, Butler and Conn, 1963). Cyanide has also been shown to be produced from the methyl group of glycine by non-proliferating cells of *Chromobacterium violaceum* (Michaels, Hankes and Corpe, 1965).

It would be of interest to know if this mechanism of cyanide production has a more widespread distribution, in view of the absence of any definitive evidence of a ubiquitously distributed physiologically significant source of cyanide in plants.

D. SUMMARY EVALUATION OF THE LITERATURE ON THE BIOCHEMISTRY OF ASPARAGINE UTILIZATION AND BIOSYNTHESIS

In animals, asparagine is utilized in protein synthesis and metabolized by transamination with a variety of α-keto-acids, or is hydrolyzed by an asparaginase to aspartate and ammonia. Asparagine is synthesized in animals using the amide nitrogen of glutamine in the direct amidation of aspartate. The significance of the enzymatic formation of asparagine by transamination is doubtful.

In micro-organisms asparagine is utilized in protein synthesis and is hydrolyzed to aspartate and ammonia by various asparaginases. Asparagine is synthesized in micro-organisms from aspartate, ammonia, Mg^{++} and ATP with the liberation of either AMP and PPi or ADP and Pi depending on the micro-organism. E. coli possesses enzymes capable of forming asparagine from cysteine (or serine) plus cyanide via β-cyano-alanine, but the significance of this pathway is not clear.

In plants there is no definitive evidence of a significant utilization of asparagine, although of course one presumes it must be used for protein synthesis. The existence of a pathway for the synthesis of asparagine from aspartate, ammonia, Mg^{++} and ATP has not been confirmed. The biosynthesis of asparagine from cysteine or serine and cyanide via β-cyano-alanine has been confirmed both in vivo

and *in vitro*. The significance of this pathway in either asparagine biosynthesis or cyanide detoxification is speculative.

E. AIMS OF THE WORK IN THIS THESIS

As already mentioned at the beginning of this thesis, this work formed part of a research programme into the biosynthesis and utilization of asparagine.

From a study of the current literature it became obvious that both the mechanism by which asparagine is synthesized in plants and its specific role in the biochemistry of the plant was not understood.

Therefore, any knowledge gained at a biochemical level on the mechanism of asparagine biosynthesis and its utilization in plants would be a useful contribution to our basic understanding of the problem.

Following our initial studies reported in Chapter III this broad aim was interpreted more specifically as an investigation into the biosynthesis of asparagine by the cyanide pathway (reported to occur both in plants and *E. coli*) with a view to determining the significance of this pathway in either asparagine biosynthesis or cyanide detoxification.

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

A. MATERIALS

1. Chemicals

(a) β-cyano-alanine was purchased from the Cyclo Chemical Company, Los Angeles, California.

(b) ATP, the disodium and dipotassium salts of ATP were supplied by the Pabst Laboratories, Milwaukee, U.S.A.

(c) Amino acids were purchased from the British Drug Houses Ltd., Poole, England.

(d) Mann assay amino acids (asparagine and cysteine) and carbobenzoxy-phenylalanine were products of the Mann research laboratories, Orangeburg, N.Y., U.S.A.

(e) O-acetyl-serine was prepared by the method of Sheehan, Goodman and Hess (1956).

(f) Glutathione (reduced) was a product of Boehringer and Soehne, Mannheim, Germany.

(g) Ninhydrin was supplied by Hopkin and Williams Ltd., Essex, England.

(h) Penicillin was purchased from Evans Medical Australia Pty. Ltd., Melbourne, Australia.

(i) NNG was supplied by the Aldrich Chemical Company, Milwaukee, Wisconsin, U.S.A.

2. Radioisotopes

[^{14}C]-labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks., U.K., and the ICN Chemical and Radioisotope Division, Irvine, California, U.S.A.

(a) [^{14}C]-$NaHCO_3$ was prepared from [^{14}C]-$BaCO_3$ by distillation of [^{14}C]-CO_2 from perchloric acid into an equivalent amount of NaOH.

3. Chromatography materials

(a) Zeocarb 225 (H^+) was obtained from the Permutit Co. Ltd., London, England. Before use the resin was washed with 6N-HCl and water.

(b) Dowex AG 50W-X8 100-200 mesh (H^+) was obtained from the Bio-Rad laboratories, California, U.S.A. Before use the resin was washed with 1.0N-NaOH, 1.0N-HCl and water.

(c) DEAE-cellulose (Whatman) was washed with 1.0N-NaOH; 1.0N-HCl and water and finally equilibrated with the appropriate starting buffer.

(d) Sephadex G-100 (Pharmacia) was washed with 0.5N-NaOH, 0.5N-HCl and water and finally equilibrated with the appropriate starting buffer.

4. Materials for polyacrylamide gel electrophoresis

(a) Acrylamide monomer, NN'-methylenebisacrylamide and NNN'N'-tetramethylethylenediamine were obtained from Distillation Products, Rochester, New York, U.S.A.

(b) Ammonium persulphate was obtained from Canalco, Bethesda, Md., U.S.A.

(c) Riboflavin was the product of Fawns and McAllan, Melbourne, Australia.

(d) Amido Black (Naphthalene Black 10B) was the product of George T. Gurr Ltd., London, England.

5. Buffers

(a) Phosphate buffers were prepared from the sodium and potassium mono- and di-hydrogen phosphates.

(b) Crystalline tris was obtained from Sigma Chemical Co., as "Sigma 7-9". In all cell-free experiments, where maximum purity is essential "Trizma Base, Reagent Grade" was used. Solutions of tris were adjusted to the required pH value by the addition of HCl.

(c) Tris-glycine buffer, pH 9.5 contained tris, 0.3 g.; glycine, 1.44 g. per litre.

6. Bacterial strains

The bacterial strains used were from the collection of Dr. J. Barry Egan. They are shown with their collection number and original source.

E511	(C600)	K12	thr^-, leu^-, thi^-, $(\lambda)^+$. (Pittard, Melbourn
E547	(AB2882)	K12	ilv-7, arg-3, thi-1, pro-2, his-4, gal-2, lac-4, xyl-5, str-704, tsx-358, $(\lambda)^+$. (Pittard, Melbourne)
E551		B	wild type (Elliott, Adelaide)
E563		K12	wild type (Symons, Adelaide)
E586	(ECB6507)	B	wild type (Worthington, Biochemicals)
E596		K12	wild type (Fowden, England)
M36		K12	asn (Farnden, Adelaide)
E612	(AB2528)	K12	Hfr O-13, ilv D132, leu-6, his-1, lac-4, str-8 $(\lambda)^+$. (Pittard, Melbourne)

7. Constituents of bacterial growth media

Bacto tryptone, Bacto-Agar and Yeast extracts were obtained from the Difco Laboratories, Michigan, U.S.A.

8. Culture media

All media were sterilized by autoclaving at 120° for 20 minutes except where otherwise indicated. The per cent (%) sign indicates g per 100 ml of distilled water.

(a) Liquid media

(i) Tryptone broth (Complete) media contained 1.0% Difco Bacto-tryptone with 0.5% NaCl.

(ii) Tryptone broth + yeast extract contained 1.0% Difco Bacto-tryptone, 0.3% Difco Bacto-yeast extract with 0.5% NaCl.

(iii) Minimal medium contained 0.7% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl. In addition 1M-$MgSO_4$ (5 ml/l), 1.8×10^{-3}M-$FeCl_2$ (1.7 ml/l) and 20% glucose (9 ml/l) were added to the minimal salts after autoclaving. Amino acid supplements were sterilized by millipore filtration and added to the minimal medium as required.

(b) Solid media

(i) Tryptone broth was solidified for the preparation of plates by the addition of 1.2% Bacto-agar. Tryptone broth soft agar contained 0.7% agar.

(ii) Tryptone broth stabs were prepared by the addition of 1.2% Bacto-agar to tryptone broth. The 0.5% NaCl normally in tryptone broth was not included.

(iii) Minimal media was solidified by the addition of 1.2% Difco Bacto-agar. The minimal salts and the agar were autoclaved separately.

9. Millipore filters were obtained from the Millipore Filter Co., Bedford, Mass., U.S.A.

10. Dialysis tubing was a product of Visking Co., Chicago, Ill., U.S.A.

11. Bovine serum albumin was obtained from the Sigma Chem. Co., Miss., U.S.A.

12. Polyclar AT was a product of the General Aniline and Film Co., N.Y., U.S.A.

13. Penicillinase (Compenase) was purchased from the Commonwealth Serum Laboratories, Melbourne, Australia.

14. Scintillation fluid contained 1,4-bis-2(4-methyl-5-phenyloxaolyl)-benzene (POPOP), (0.03%, w/v) and 2,5, diphenyloxazole (PPO), (0.3%, w/v) in sulphur free toluene. PPO and POPOP were supplied by the Packard Instrument Co., Melbourne, Australia.

B. METHODS

1. Growth of seedlings

Wheat grain (Triticum var. unknown; local South Australian type) and Lupinus angustifolius seeds were surface sterilized by soaking in 1% sodium hypochlorite for 10 minutes and then washing well with distilled water. The grain or seeds were soaked overnight in either sterile distilled water or 0.1% ammonium sulphate before planting on sterilized vermiculite. Seedlings were grown at 22° with 12 hours illumination per day. Sterile distilled water or 0.1% ammonium sulphate was added as required.

2. Determination of protein

A modification of the method of Lowry, Rosebrough, Farr and Randall (1951) was used for estimations of specific activity. Bovine serum albumin was used as a standard.

3. Ninhydrin colour determinations

These were used to measure the amino acid content of protein hydrolysates and were performed by the method of Chibnall, Mangan and Rees (1958), using leucine as a standard. The concentration of the amino acids isolated from the soluble fraction of plant material was also determined by this method.

4. Preparation of plant samples for soluble amino acid analysis

Usually two wheat seedlings with shoots of appropriate length were dropped into boiling ethanol (10 ml) for 5 minutes. The seedlings were removed, ground and transferred to the original ethanol in an aqueous suspension to give a final mixture of 30% ethanol-water (v/v) which was then boiled for 1 minute. After centrifuging the mixture, the supernatant was passed through a Zeocarb 225 (H^+) column to adsorb the amino acids. The columns were washed with distilled water and the amino acids eluted with 2N-ammonia (25 ml). The eluate was evaporated to dryness on a rotary evaporator and the amino acids redissolved in water (2 ml). The amino acid solution was divided into two parts. One fraction was retained untreated. The second fraction was heated for 4 hours at 105° in 1N-HCl to hydrolyze asparagine (glutamine is also hydrolyzed). Amino acid analyses were carried out using a Technicon or Beckman Amino Acid Analyzer.

The recovery of the amino acids loaded in 30% ethanol-water mixtures from the Zeocarb 225 column and the Dowex AG 50W-X8 (100-200) was examined. Dowex AG 50W-X8 (100-200) was used in place of Zeocarb 225 in later work due to difficulties encountered in regenerating the Zeocarb 225.

The recovery of asparagine was routinely 98 to 100% and aspartic acid 88 to 90%. Threonine, serine, glutamic acid, proline, glycine, alanine, leucine, isoleucine and lysine showed recoveries of 90 to 100%, whilst 70-80% of the tyrosine, phenylalanine, histidine and arginine loaded was recovered. Methionine and half-cystine were only recovered 52% and 54% respectively.

5. Preparation of plant samples for total amino acid analysis

Wheat grain and wheat seedlings were ground with ethanol and dried to constant weight at 105°. Aliquots (20 mg) of the dry material were hydrolyzed with 6N-HCl (4 ml) at 105° for 24 hours under vacuum. The resulting hydrolysates were diluted with 20 volumes of distilled water, centrifuged for 10 minutes at 500 g and passed through columns of Zeocarb 225 (H^+). The columns were washed with distilled water and the amino acids eluted with 2N-NH_4OH (25 ml). The eluate was evaporated to dryness in a rotary evaporator and the amino acids dissolved in water (2 ml) prior to loading onto amino acid analyzer columns.

6. [^{14}C]-CO_2 and [^{14}C]-HCN plant incorporation experiments

These were carried out in a 100 ml quickfit conical flask fitted with an upper and a lower side-arm and an air leak fitted with a tap. Seedlings or excised shoots were placed in the flask in a small tube. [^{14}C]-labelled and non-radioactive $NaHCO_3$ solutions and KCN solutions were added to the flasks as needed. (All such solutions were made to 0.01N-NaOH to prevent premature release of radioactive label by traces of acid on the glass.) The flask was flushed with CO_2-free air for 10 minutes and then sealed off. Perchloric acid was injected through a rubber stopper in the lower side-arm. The flasks were illuminated by two 20W fluorescent tubes placed 5 cm on either side of the flask.

7. Extraction of the soluble amino acids from plant material

Following isotope incorporation experiments the soluble amino acids were extracted from the plant material using the procedure described for the preparation of samples for soluble amino acid analysis (Chapter II, page 34).

8. Isolation of labelled protein from plant material

The plant tissue was ground with 30% ethanol-water (v/v) and the suspension boiled for 5 minutes. After centrifuging the precipitate was resuspended in 5% trichloro-acetic acid (w/v) and collected by vacuum filtration onto Whatman No.5 filter paper. The precipitate was then successively washed with 5% trichloroacetic acid (w/v) containing 0.05% casamino acids (w/v), 5% acetic acid (v/v), ethanol and ether. Labelling in the protein was determined using a Nuclear Chicago Automatic Gas Flow Counter.

9. Separation and counting of labelled amino acid mixtures

Two dimensional separation of amino acids was carried out by two procedures.

(a) Descending chromatography firstly in n-butanol-n-butyl acetate-acetic acid-water (19 : 1 : 5 : 25 v/v) followed by phenol-water-ammonia (75 : 25 : 1 v/v).

(b) Electrophoresis in formic acid-water (4% v/v) adjusted to pH 2.2 with pyridine (3000 V for $1\frac{1}{2}$ hours) or acetic acid-water (3.1/3 v/v) adjusted to pH 3.6 with pyridine (2,500V for $1\frac{1}{2}$ hours) followed by descending chromatography in the butanol solvent described above.

A rapid one-dimensional separation of asparagine and aspartic acid was carried out by paper electrophoresis in 0.04 M-sodium acetate, pH 5.5 (2,000V, 15 minutes).

Amino acids were located and identified by spraying with ninhydrin (0.1% w/v in acetone). Spots were cut out and the radioactivity in each spot determined by counting in a Packard Tri-carb Scintillation Spectrometer. Marker amino acids were included in all separations and counts were corrected for quenching by the method of channel ratios (Herberg, 1965).

10. Preparation of enzyme extracts

(a) Cell-free plant extracts

Cell-free extracts of wheat and lupin seedlings, shoots and roots were prepared by grinding the tissue with 0.05M-potassium phosphate, pH 7.2 containing 10^{-3}M-GSH and 10^{-4}M EDTA at 4°, or with a modified buffer as indicated in the text. The supernatant obtained by centrifuging this extract at 5,000 g for 10 minutes was dialyzed against the respective buffer. β-Mercapto-ethanol and mercapto-benzothiazole were also used instead of GSH as thiol reagents.

Other cell-free plant extracts were prepared by stirring the juice extracted from the excised shoots or roots with a "Vacola" juice extractor at 4° for thirty minutes with Polyclar AT (10% w/v) to prevent browning and denaturation

(Loomis and Battaille, 1966). The supernatant obtained by centrifuging this extract at 5,000 g for 10 minutes was then dialyzed against the respective buffer.

Acetone powders, prepared either by adding the seedling or shoot juice directly to acetone at -15° or by grinding the seedlings or shoots with acetone at -15° were extracted with buffer to give further cell-free extracts.

(b) Wheat leaf mitochondrial and chloroplast extracts

Chloroplast and mitochondrial fractions were prepared in aqueous media by the method of James and Das (1957) except that a modified extraction medium was used (0.3 M-mannitol, 0.05M-tris HCl buffer, pH as indicated in the text).

Chloroplasts were also prepared by the non-aqueous method of Stocking (1959) which involved fractionation on the basis of the density of freeze-dried and ground plant material in hexane-carbon-tetrachloride mixtures.

Enzyme extracts prepared from mitochondria and from aqueous and non-aqueous prepared chloroplasts suspended in extraction buffer were disrupted by overnight dialysis against 0.05 M-tris-HCl buffer, pH as indicated in the text, to give the extracts used in the intracellular location experiments. Other extracts were prepared by first sonicating the

resuspended chloroplasts or mitochondria at 0° using a Dawe type 1130A Soniprobe at full power applied in three 10 second periods with cooling in between.

All other crude plant particulate extracts were prepared as described for the crude wheat extracts.

(c) Cell-free crude bacterial and animal extracts

Crude bacterial extracts were prepared by sonicating (as described above) resuspended log-phase bacteria in 0.05M-tris-HCl buffer, (pH as indicated in the text). The supernatant obtained by centrifuging this sonicate at 40,000 g for 30 minutes was dialyzed overnight against the respective buffer.

Animal cell extracts were prepared by homogenising the tissue with 0.05 M-tris HCl buffer (1 : 1 w/v), pH as indicated in the text, for 1 minute in a Potter-Elvehjem homogeniser. This homogenate was then centrifuged and the supernatant dialyzed as described above.

(d) Ammonium sulphate fractionation of crude E. coli extracts

Crude E. coli extracts prepared as described in Chapter II, page 39), in 0.02 M-sodium phosphate, pH 8.0 were treated with 0.05 volumes of 1 M-$MnCl_2$. After removal of the precipitated material by centrifugation the supernatant was brought to 2 M-ammonium sulphate by the addition of solid ammonium sulphate with stirring at room temperature. The suspension was adjusted to pH 8.0 with

ammonia.

The precipitated protein collected by centrifugation was redissolved in buffer to give the AS I extract when dialyzed against 0.02 M-sodium phosphate, pH 8.0.

The supernatant was brought to 4 M-ammonium sulphate again by the addition of solid ammonium sulphate and the pH adjusted to 8.0 with ammonia. The precipitated protein on centrifugation was redissolved in buffer to give the AS II extract when dialyzed against 0.02 M-sodium phosphate, pH 8.0.

11. Degradation of the [^{14}C]-labelled amino acids

Amino acids were decarboxylated using the ninhydrin method of Chibnall, Mangan and Rees (1958), but modified in that at the completion of the reaction, solid CO_2 was added to displace the [^{14}C]-CO_2 liberated. An aliquot of this reaction mixture was dried onto 1 inch squares of Whatman 3 MM filter paper and counted in a Packard Scintillation Spectrometer. Counts were corrected for quenching by the method of channel ratios (Herberg, 1965).

12. Preparation of [^{14}C]-β-cyano-alanine

Cysteine (150 μmoles) and potassium cyanide (100 μC, 2.2 μmoles) were incubated at 30° for 10 minutes with 10 ml of a crude wheat leaf extract in 0.05 M-tris HCl, pH 9.0 (15 mg protein/ml). The reaction was stopped by the

addition of 60% perchloric acid (0.5 ml) and the protein removed by centrifugation. The supernatant was neutralized with KOH, the potassium perchlorate removed by centrifugation and the supernatant passed through a Zeocarb 225 (H^+) column. The column was washed with distilled water and the amino acids eluted with 2 N-ammonia (20 ml). The eluate was evaporated to dryness on a rotary evaporator and the amino acids dissolved in water (1 ml). The labelled β-cyano-alanine and asparagine were separated and purified by paper electrophoresis at pH 2.2. The purified β-cyano-alanine was found to correspond to a β-cyano-alanine marker when subjected to all the two dimensional separations described in Chapter II, page 36.

13. Enzyme assays

(a) β-Cyano-alanine synthase assay

The standard incubation mixture contained in a final volume of 0.20 ml, plant enzyme preparation (1 mg protein/ml) in 0.05 M-tris-HCl, pH 9.0, 0.2 ml; [^{14}C]-potassium cyanide (22 mμmoles, 1 μC), cysteine, 5 μmoles. Mixtures were incubated in conical test tubes at 30° (37° for bacterial enzyme preparations) for various times and the reaction stopped by the addition of two volumes of ethanol. After removal of the precipitated protein by centrifugation, the supernatant was passed through a Zeocarb 225 (H^+) column. The column was washed with water

and the amino acids eluted with 2 N-ammonia. The eluate was evaporated to dryness on a rotary evaporator and the amino acids were separated two-dimensionally as in Chapter II, p.36.

This assay was subsequently modified in that during the incubation aliquots were removed at various times and applied directly to a Whatmann 3MM paper. The amino acids were separated one dimensionally using the phenol-water-ammonia (75:25:1 v/v) solvent and located, identified and counted as described in Chapter II, p.36.

(b) β-Cyano-alanine hydrolase assay

The standard incubation mixture had the following composition: plant enzyme preparation (5 mg protein/ml) in 0.05 M-tris-HCl, pH 7.8, 0.2 ml; [^{14}C]-β-cyano-alanine (1.07 μmoles, 10^5 cpm); total volume 0.25 ml. Mixtures were incubated at 30° in conical test tubes (37° for the bacterial enzyme preparations) for various times and the reaction was stopped by the addition of two volumes of ethanol. After the removal of the precipitated protein by centrifugation unconverted β-cyano-alanine in an aliquot of the supernatant was separated from the synthesized asparagine (or from aspartic acid if an asparaginase was present as in the case of E. coli extracts) by electrophoresis at pH 2.2, 3,000 V for 3 hours (buffer as in Chapter II, p.36).

This assay was subsequently modified in that during the incubation, aliquots were removed at various times and applied directly to the electrophoresis paper and rapidly dried.

The amino acids after electrophoresis were located, identified and counted as described in Chapter II, p.36).

(c) Asparaginase

The standard incubation mixture had the following composition; enzyme preparation in 0.05 M-tris-HCl, pH 7.8 or 0.02 M-sodium phosphate, pH 8.0 as indicated in the text, 0.18 ml; [^{14}C]-asparagine (0.2 μmole, 0.025 μC); total volume 0.2 ml. Mixtures were incubated at 30° in conical test tubes (37° for bacterial preparations) for various times and the reaction was stopped by the addition of two volumes of ethanol. After the removal precipitated protein by centrifugation the asparagine and aspartic acid were separated by electrophoresis at pH 5.5, 2,500V for 30 minutes using the buffer described in Chapter II, p.36.

This assay was subsequently modified in that during the incubation, aliquots were removed at various times and applied directly to the electrophoresis paper and dried.

The amino acids after electrophoresis were located, identified and counted as described in Chapter II, p.36.

(d) Asparagine synthetase

The standard incubation mixture contained in a total volume of 0.2 ml; enzyme preparation (0.1 ml), Mg^{++} (15 mM), ATP (5 mM), NH_4Cl (30 mM), [^{14}C]-aspartic acid (0.235 mM, 0.5 μC), Mixtures were incubated at 30° in conical test tubes (37° for the bacterial preparations) and aliquots were removed at various times and applied directly to an electrophoresis paper and dried. The amino

acids were separated by electrophoresis at pH 2.2, 3,000 V for 3 hours in the buffer described in Chapter II, B, 9(b). The amino acids were located after electrophoresis identified and counted as described in Chapter II, B, 9 .

14. Storage of bacterial strains

Ampoules of log-phase cultures grown in tryptone broth were stored frozen in 1% w/v non-fat milk solutions. Tryptone broth stabs of strains were also maintained. For daily inoculations bacteria were kept in tryptone broth at 4° for one month.

15. Growth of bacterial cultures

An aliquot (0.02 ml) of the stored strain was inoculated into 10 ml of the required medium and incubated overnight at 37° in a gyrotary water bath. This starter culture was then used to inoculate fresh medium. Growth was followed by measuring the OD at 600 mμ in a Shimadzu AQV-50 spectrophotometer. An OD_{600} of 1.5 was found to correspond to 1.0×10^9 bacteria per ml.

16. Sterilization by millipore filtration

Sterilization by millipore filtration was achieved by passing the solutions through a 13 mm millipore filter of pore size 0.45 μ using a sterile syringe and Swinney adaptor.

17. Lambda phage infection of E. coli

An aliquot of lambda phage (0.1 ml) prepared from E511 following the U.V. induction of a log-phase culture was mixed at various dilutions with 0.2 ml of a log-phase culture of bacteria grown in tryptone broth. The mixture was incubated for 30 minutes at 37° to allow phage absorption and then plated with 2.5 ml of tryptone broth soft agar on a tryptone broth plate. The plates were incubated for 16 hours at 37° and inspected for plaque formation.

18. NNG mutagenesis and penicillin auxotroph enrichment

E. coli E596 was grown to middle log phase in minimal medium, 1 x 10^8 cells were centrifuged and twice washed with 0.1 M-sodium citrate buffer, pH 5.5. Cells were resuspended in the citrate buffer (2 ml) and left at room temperature for 30 minutes before treatment with NNG (100 μg/ml) for 30 minutes at 37° without shaking. NNG was sterilized

before use by millipore filtration. NNG treated cells were filtered onto a millipore membrane filter and twice washed with citrate buffer. The membrane filter was then suspended in minimal medium supplemented with glucose (0.25 ml 20% glucose/100 ml) and asparagine (100 μg/ml) and shaken overnight at 37°. The culture (10 ml) was centrifuged and the cells resuspended in minimal medium containing sucrose (10% w/v). Cells were incubated in a large flask (maximal surface area) without shaking at 37° for one hour before the addition of penicillin (1,000 units/ml). Protoplast formation appeared maximal in about $3\frac{1}{2}$ to 4 hours (as observed by phase contrast microscopy) and cells were centrifuged and resuspended in cold 0.1 M-sodium phosphate buffer, pH 7.0. Penicillinase (40 units/ml) was added and the cells incubated for a further one hour to give the enriched auxotroph culture.

19. Genetic mapping of the asparagine auxotroph

The asparagine (asn) marker was mapped approximately by interrupted mating experiments using the Hfr 0-13 conjugation system (Taylor and Adelberg, 1960). Mating conditions were as described by Adelberg and Burns (1960). *E. coli* M36 was used as the F^- and E612 as the Hfr. Mating was interrupted by vigorous shaking in a test-tube on a Vortex-Genie for one minute. Recombinants were selected on minimal agar (salts and glucose only), the nutritive

requirements of the E612 being used to select against it.

20. Isolation of the protein and soluble amino acids from E. coli following the uptake of [^{14}C]-KCN in vivo

An aliquot of the E. coli cells incubating in minimal medium with [^{14}C]-KCN was added to two volumes of 1 N-NaOH at 0° and left for at least 30 minutes. The mixture was then incubated at 37° for 10 minutes, cooled and five volumes of cold TCA (10% w/v) added. The protein was allowed to precipitate overnight at 0°. The protein was precipitated by centrifugation and then resuspended and collected onto a millipore filter for counting as described in Chapter II, page 36. The supernatant was kept and the labelling in the soluble amino acids was determined as described in Chapter II, page 36.

21. Electrophoresis in polyacrylamide gel

The equipment and gel columns were made to the specifications of Ornstein and Davis (1961). The columns were stored in concentrated nitric acid and rinsed before use in distilled water. This procedure enabled easy removal of the gels after electrophoresis. Gels were prepared according to the 1965 formulae circulated by Canalco, Bethesda, Md., U.S.A., and contained 7% acrylamide in the separating gel. Samples were loaded in the column in 20%

sucrose and bromophenol blue was used as tracker dye. Carbon electrodes were used and the current maintained at 3 m amps per gel for 60 minutes. After electrophoresis the gels were removed from the columns and the protein detected by staining in 0.5% amido-black in 7% acetic acid. Excess stain was removed by washing the gels overnight in a large volume of 7% acetic acid.

CHAPTER III

INITIAL STUDIES ON THE BIOSYNTHESIS AND UTILIZATION OF ASPARAGINE IN SEEDLINGS

INITIAL STUDIES ON THE BIOSYNTHESIS AND UTILIZATION OF ASPARAGINE IN SEEDLINGS

A. INTRODUCTION

The classical views on the biosynthesis and utilization of asparagine in plants (see page 2) maintained that asparagine was derived both from the proteolysis of the reserve proteins and from the secondary decomposition of the other amino acids derived from this proteolysis. Asparagine was then considered to be translocated to the growing parts where it was both incorporated into protein and utilized as a carbon and nitrogen source for the synthesis of other amino acids.

The extensive studies from which these views were formulated dealt primarily with fluctuations in asparagine levels. It was not possible to deduce what fraction of the asparagine came from protein hydrolysis and what if any from de novo synthesis. Nor was it possible to determine whether in fact asparagine was utilized at the growing tip either in protein synthesis or in the synthesis of other amino acids.

In addition, the site(s) of asparagine synthesis had not been defined since asparagine appearing in a seedling shoot may have been translocated from the cotyledons or

endosperm where the amide is also found in large amounts, probably due to proteolysis.

In attempting to study the cell free synthesis of asparagine these difficulties were reinforced by the fact that it was uncertain at what rate a seedling did synthesize asparagine under normal conditions. Such information was essential for *in vitro* work both to determine the required sensitivity of the assay system employed, and to assess the possible physiological significance of any reactions discovered.

With these difficulties in mind, preliminary studies were carried out to determine whether seedlings and shoots grown under normal conditions did synthesize asparagine over and above that arising from proteolysis and to compare the rate of this synthesis with that of other amino acids. The main evidence that seedlings do in fact synthesize asparagine *de novo* still appears to rest on analyses by Schulz (1898). In addition the participation of asparagine in the overall metabolism of seedlings was examined.

B. EVIDENCE OF DE NOVO ASPARAGINE BIOSYNTHESIS

1. Total amino acid composition of wheat grain and wheat seedlings

In Table III.1 the total amino acids of the hydrolyzed proteins of dry wheat grains are compared with the total amino acids of six-day wheat seedlings. It can be seen from these figures that on germination some amino acids decrease while others increase in amount. However, the greatest increase is in the total aspartic acid content. In the soluble amino acid fraction of the seedling, asparagine (as determined by the difference in the aspartic acid content of the hydrolyzed and non-hydrolyzed samples) is the predominant free amino acid, amounting to one third of the total. Moreover, the asparagine in the soluble amino acid fraction of the seedling is double the total aspartic acid content of the dry grain. At least 50% of the free asparagine has therefore been synthesized in the wheat seedling de novo and not simply liberated by hydrolysis of the reserve protein.

2. Incorporation of $[^{14}C]$-CO_2 into whole wheat seedlings and excised wheat shoots

During photosynthesis $[^{14}C]$-CO_2 was found to be rapidly incorporated into amino acids by whole wheat seedlings (5 cm shoots). Over a 40 minute time period in three separate experiments the pattern of incorporation into amino acids was the same. Alanine and serine were invariably the most

TABLE III.1: AMINO ACID COMPOSITION OF WHEAT GRAIN AND SEEDLINGS

Amino acid	µmoles amino acid per grain (total)	µmoles amino acid per seedling (total)	µmoles amino acid per seedling (total)
Asp	0.26	1.30	0.40
Asp(NH_2)	-	-	0.50[a]
Thr	0.52	0.49	-
Ser	0.62	0.54	-
Glu	1.87	0.94	0.10
Glu(NH_2)	-	-	0.12[a]
Pro	1.42	0.91	0.14
Gly	1.07	1.57	0.04
Ala	0.77	1.57	0.25
Val	0.60	1.24	0.07
Ile	0.40	0.66	0.04
Leu	1.36	1.16	0.05
Tyr	0.18	0.12	0.02
Phe	0.26	0.40	0.03
Lys	0.31	1.15	0.02
His	0.16	0.13	0.02
Arg	0.19	0.41	0.01

[a]Determined as the increase in Asp or Glu on hydrolysis for 4 hours at 105° in 1.0 N-HCl.

Procedures are described on pp. 33 and 34.

rapidly labelled. Asparagine, aspartic acid, glutamine and glutamic acid were also labelled at approximately equal rates (Fig. III.1). The alanine and serine curves were essentially parallel to the others though at a level ten times higher.

Excised wheat shoots (5 cm in length) incorporated $[^{14}C]$-CO_2 into asparagine and the general pattern of labelling (Fig. III.2) was the same as that for whole wheat seedlings. The rate of incorporation per shoot was of the same order as for the whole seedling. Comparable patterns were also obtained when seedlings and shoots of Lupinus angustifolius were studied.

When excised wheat shoots (5 cm in length) were exposed to $[^{14}C]$-CO_2 for 20 minutes and then chased with normal air for varying times the radioactivity of serine and alanine decreased rapidly (Fig. III.3), while over a 30 minute period the activity in asparagine, aspartic acid, glutamine and glutamic acid either increased somewhat, or remained the same. This pattern was found consistently in a number of experiments.

3. In vivo biosynthesis of asparagine from $[^{14}C]$-U-aspartic acid in excised wheat shoots

When 5 cm long wheat shoots were placed in an aqueous solution (0.1 ml) of $[^{14}C]$-U-aspartic acid (1 μC) and illuminated for various times up to 20 hours, 78% of the label in an aliquot of the soluble fraction of the tissue

FIGURE III.1. THE INCORPORATION OF $[^{14}C]$-CO_2 (cpm/seedling) INTO THE SOLUBLE AMINO ACID FRACTION OF WHEAT SEEDLINGS

Administration of $[^{14}C]$-CO_2 (50 μC; 0.03% v/v) and the isolation of the amino acids was as described on p. 35, 36 .

(●) $Asp(NH_2)$; (○) $Glu(NH_2)$; (□) Asp; (▲) Glu.

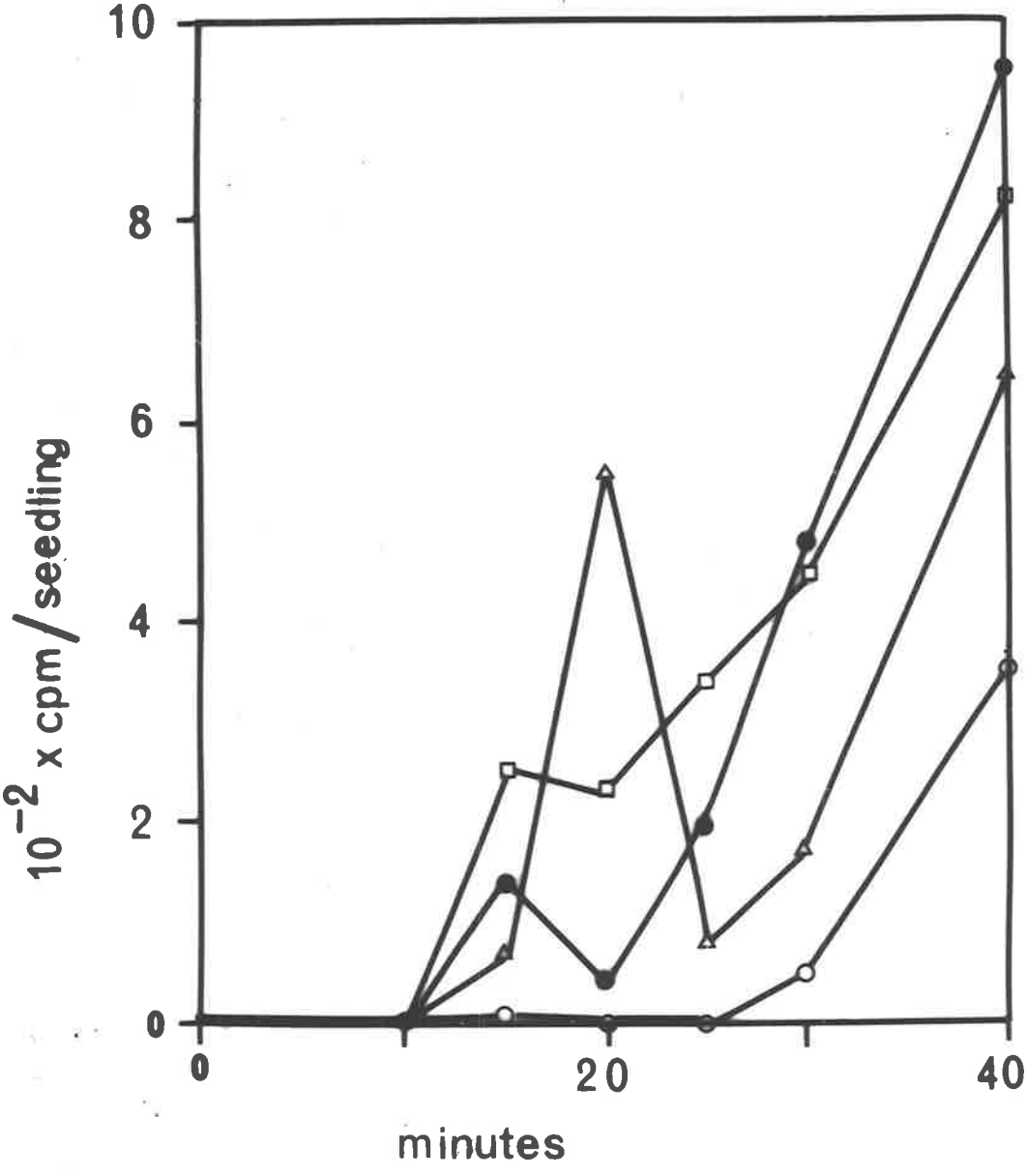

10
8
6
4
2
0
10^{-2} x cpm / seedling
0
20
40
minutes

FIGURE III.2. THE INCORPORATION OF $[^{14}C]$-CO_2 (cpm/shoot) INTO THE SOLUBLE AMINO ACIDS OF EXCISED WHEAT SHOOTS.

Administration of $[^{14}C]$-CO_2 (50 μC; 0.03% v/v) and the isolation of the amino acids was as described on pp. 35,36.

(•) $Asp(NH_2)$; (○) $Glu(NH_2)$; (□) Asp;

(△) Glu.

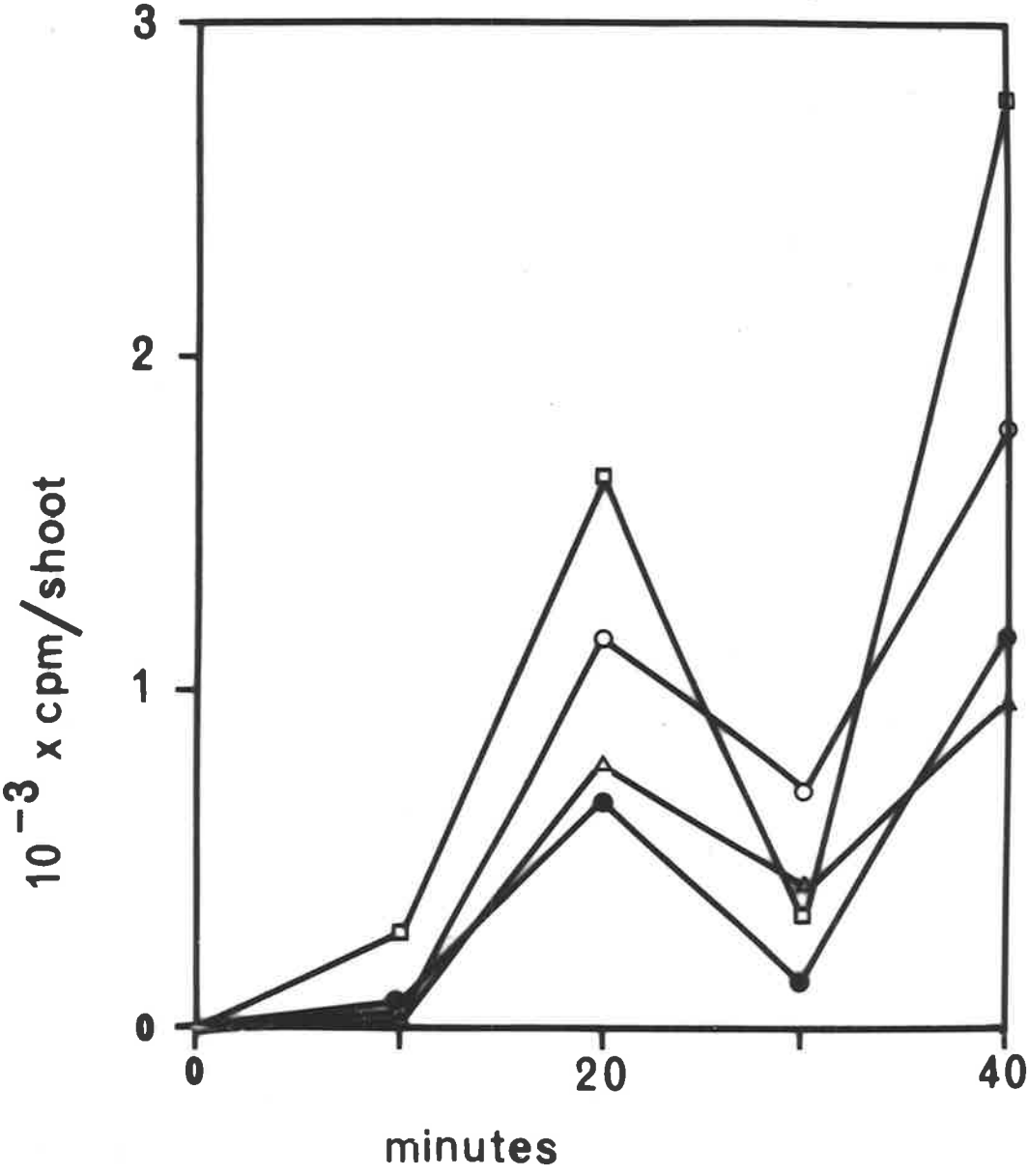
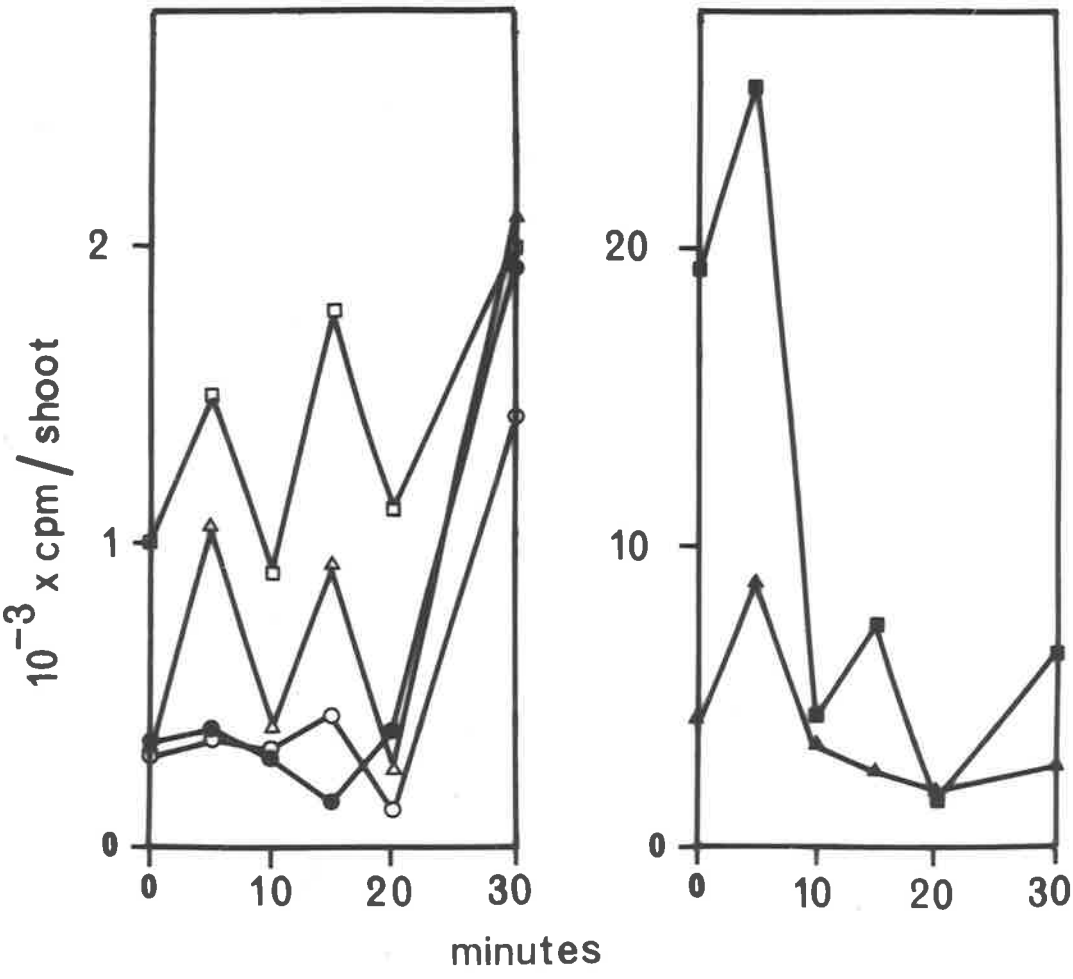

3
2
1
0
10^{-3} x cpm/shoot
0
20
40
minutes

FIGURE III.3. THE LEVEL OF RADIOACTIVITY (cpm/shoot) REMAINING IN THE SOLUBLE AMINO ACIDS OF EXCISED WHEAT SHOOTS FOLLOWING A 20 MINUTE EXPOSURE TO $[^{14}C]$-CO_2 FOLLOWED BY CHASING WITH NORMAL AIR FOR VARYING TIMES.

Excised wheat shoots were exposed to $[^{14}C]$-CO_2 (50 μC, 0.03% v/v) for 20 min. and then chased with normal air from time zero in figure. Methods as described on p.35,36.

(•) Asp(NH_2); (○) Glu(NH_2); (□) Asp;

(△) Glu; (■) Ser (▲) Ala.


10^{-3} x cpm / shoot
2
1
0
0
10
20
30
minutes
20
10
0
0
10
20
30

was found in asparagine at 20 hours (Fig. III 4 and 5). Smaller amounts of radioactivity were detected in glutamic acid (5%), glutamine (5%), and serine (3%) with 9% remaining in the aspartic acid at 20 hours (Fig. III.5).

However, the total label isolated in the soluble fraction was only 39% of that found incorporated into protein at twenty hours (Fig. III.6). A sample of this labelled protein was hydrolyzed, the amino acid fraction isolated and the amino acids separated and counted (Table III.2). In addition to labelling in glutamic acid and aspartic acid, alanine, leucine, serine, glycine, phenylalanine, valine, lysine, tyrosine, proline and histidine were found to be significantly labelled (Table III.2).

4. Attempted cell-free biosynthesis of asparagine

Cell-free extracts of wheat and lupin seedlings, excised shoots and roots prepared in either 0.05 M-potassium phosphate, pH 7.2 containing 10^{-3} M-GSH, 10^{-4} M-EDTA or 0.05 M-potassium phosphate, pH 7.2 containing 10^{-3} M-GSH, 10^{-4} M-EDTA and 0.35 M-mannitol in an attempt to maintain the integrity of chloroplasts and mitochondria (as described on page 37) were incubated for various times up to 3 hours at 30° with either [^{14}C]U-aspartic acid, [^{14}C]-2-pyruvate or [^{14}C]-$NaHCO_3$. In addition ATP, Mg^{++} and NH_4^+ were added to the incubation mixtures.

It was not possible in any of these incubations to

FIGURE III.4: THE INCORPORATION OF [^{14}C]-U-ASPARTIC ACID (cpm) INTO THE SOLUBLE AMINO ACID FRACTION OF EXCISED WHEAT SHOOTS.

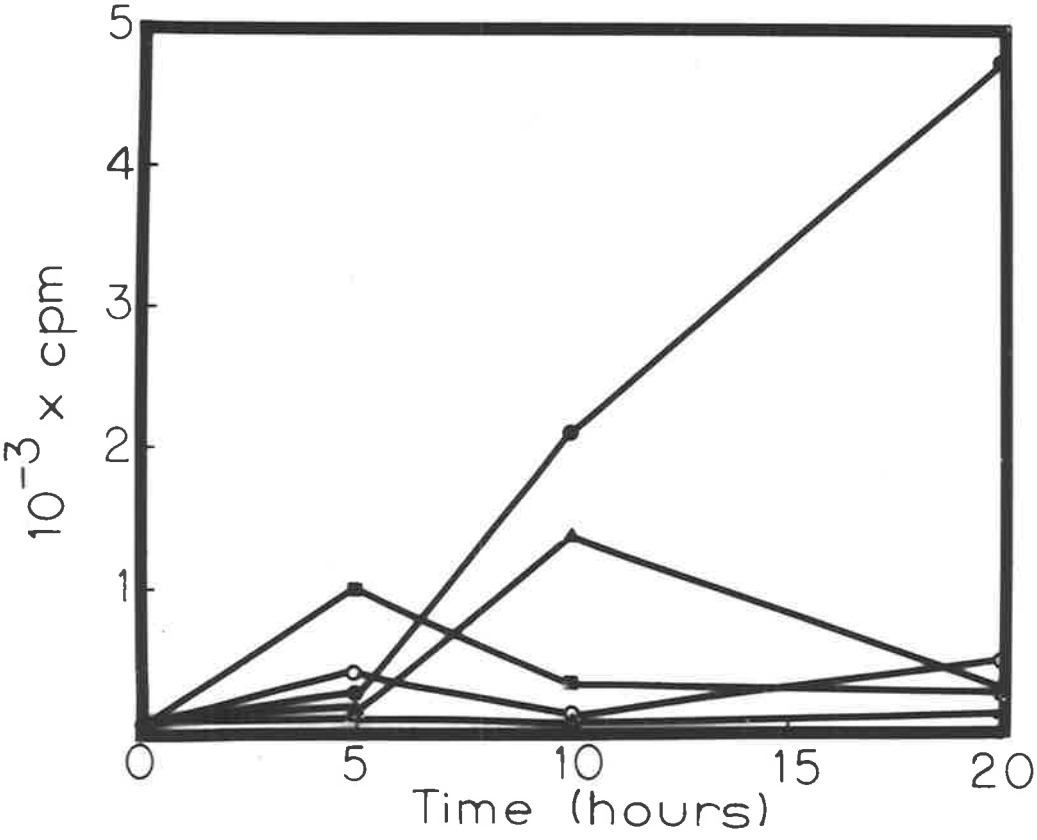
Following the uptake of [^{14}C]-U-aspartic acid (1 μC) by excised wheat shoots, the radioactivity (cpm) of the amino acids from the soluble fraction was determined as described on p.35,36 .

(•) Asp(NH_2); (○) Asp; (▲) Glu(NH_2);
(△) Ser; (■) Glu.

FIGURE III.5: THE INCORPORATION OF [^{14}C]-U-ASPARTIC ACID (% of total incorporation) INTO THE SOLUBLE AMINO ACID FRACTION OF EXCISED WHEAT SHOOTS.

The data from Figure III.4 has been replotted as the % of the total label incorporated found in each amino acid. Methods as in Fig. III.4.

(•) Asp(NH_2); (○) Asp; (▲) Glu(NH_2);
(△) Ser; (■) Glu.


5
4
3
2
1
10^{-3} x cpm
0
5
10
15
20
Time (hours)

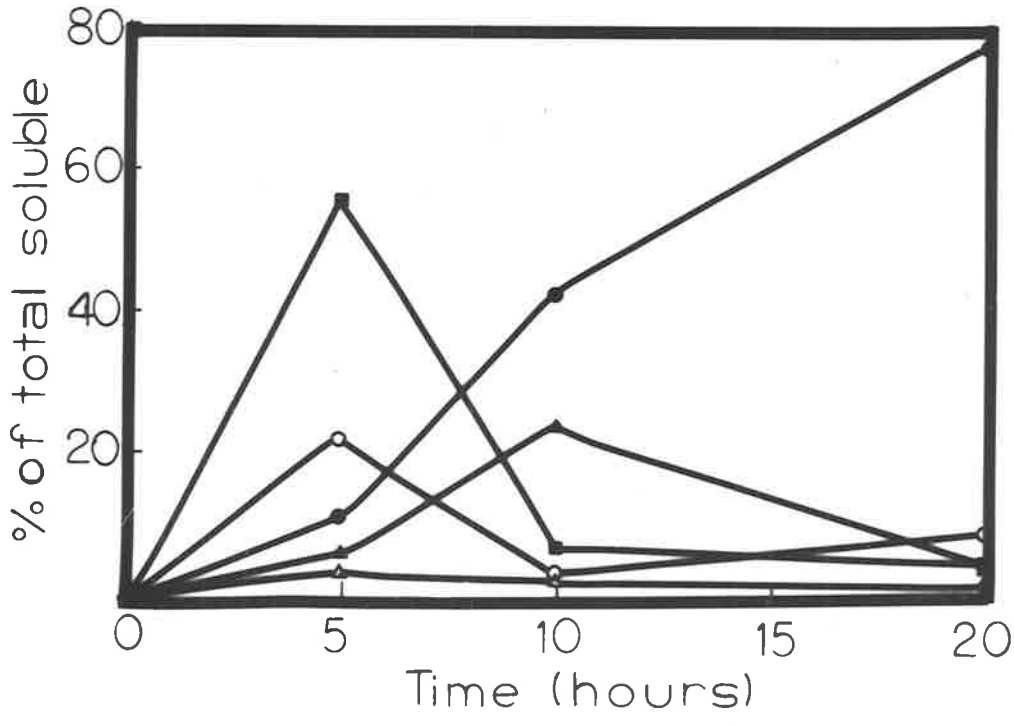

80
60
40
20
% of total soluble
0
5
10
15
20
Time (hours)

FIGURE III.6. THE INCORPORATION OF [^{14}C]-U-ASPARTIC ACID (cpm) INTO THE SOLUBLE AND PROTEIN FRACTION OF EXCISED WHEAT SHOOTS.

The labelling of the amino acids from the soluble fraction and protein fraction was determined at various times during the uptake of [^{14}C]-U-aspartic acid (1 μC) by excised wheat shoots. Methods as described on p. 35,36.

(•) Soluble; (○) Protein.

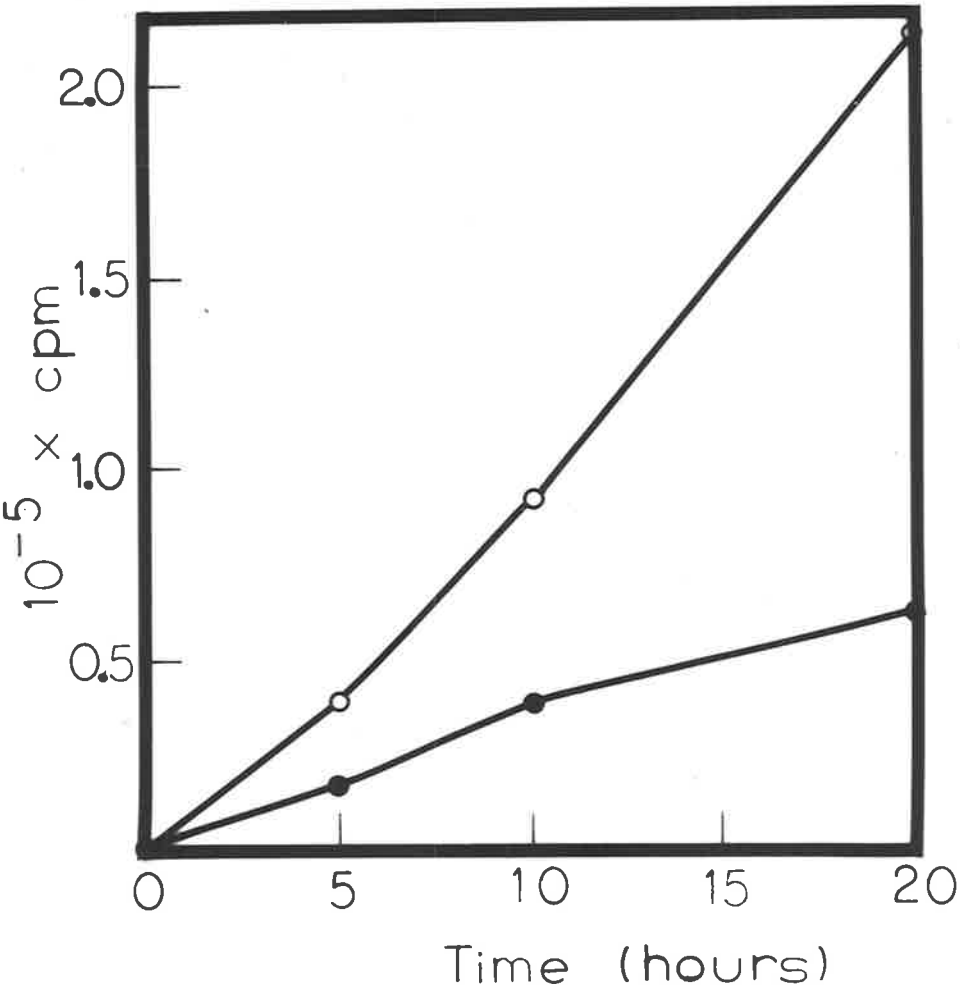

2.0
1.5
1.0
0.5
10^{-5} x cpm
0
5
10
15
20
Time (hours)

TABLE III.2: ASPARTIC ACID UTILIZATION

Amino acid	Radioactivity (cpm) Hydrolyzed protein	Amino acid	Radioactivity (cpm) Hydrolyzed protein
Glu	1139[a]	Gly	446
Glu(NH_2)	50	Phe	398
Asp	721[b]	Val	330
Asp(NH_2)	0	Lys	288
Ala	613	Tyr	265
Leu	519	Pro	221
Ser	472	His	201

[a]Glutamic acid and glutamine
[b]Aspartic acid and asparagine

After the 20 hour uptake of [^{14}C]-U-aspartic acid (1 μC) by 5 cm long wheat shoots the radioactivity (cpm) of the amino acids from aliquots of the hydrolyzed protein was determined as described on page 36.

demonstrate a significant synthesis of asparagine; a slight incorporation of pyruvate into asparagine occurred erratically. Repeated attempts to investigate this further were unfruitful. In the same experiments alanine was rapidly synthesized from pyruvate, showing that transaminase activity was present in these homogenates. [^{14}C]-Aspartic acid was also metabolized by the extracts, labelling again being found in alanine (Table III.3).

C. STUDIES ON THE IN VIVO AND IN VITRO UTILIZATION OF ASPARAGINE

1. Studies on the utilization of [^{14}C]-U-asparagine by wheat shoots

When 5 cm long etiolated wheat shoots were placed in an aqueous solution (0.1 ml) of [^{14}C]-U-asparagine (1 μC) and illuminated, the radioactive amino acid was incorporated into protein (Fig. III.7). The process was almost linear for 24 hours showing that the labelled compound readily entered the metabolic pool of the cells. When the soluble amino acids in the wheat shoots were examined after exposure to [^{14}C]-U-asparagine for 24 hours the radioactivity was present entirely in asparagine and not in any other amino acid (Table III.4). The protein labelled over a 24 hour period was hydrolyzed and the individual amino

TABLE III.3: TESTS FOR ASPARAGINE BIOSYNTHESIS IN HOMOGENATES

Label added	Incubation time (hours)	Amino acid[a]		
		Asp(NH_2)	Asp	Ala
[^{14}C]-U-aspartic acid	0	150	60360	290
	2	143	28723	23085
[^{14}C]-2-pyruvate	0	50	35	18
	2	373	1032	59060

[a]The activity of all other amino acids was less than 50 cpm.

The radioactivity (cpm) of an aliquot of the amino acids following the incubation of either [^{14}C]-U-aspartic acid or [^{14}C]-2-pyruvate with a dialyzed homogenate of 5 cm long wheat shoots for 2 hours at 30° prepared in 0.05 M-potassium phosphate, pH 7.2 containing 10^{-3} M-GSH, 10^{-4}M-EDTA, and 0.35 M-mannitol was determined. Incubation mixtures contained: homogenate (5 ml), Mg^{++} (15 µmole), dipotassium salt of ATP (10 µmoles), ammonium ion (10 µmole) and either [^{14}C]-U-aspartic acid (1 µC) or [^{14}C]-2-pyruvate (1 µC).

FIGURE III.7. RADIOACTIVITY (cpm) FOUND IN THE PROTEIN OF ETIOLATED WHEAT SHOOTS FOLLOWING THEIR UPTAKE OF [^{14}C]-U-ASPARAGINE.

Following the uptake of [^{14}C]-U-asparagine (1 μC) the protein was isolated and counted as described on p. 36 .

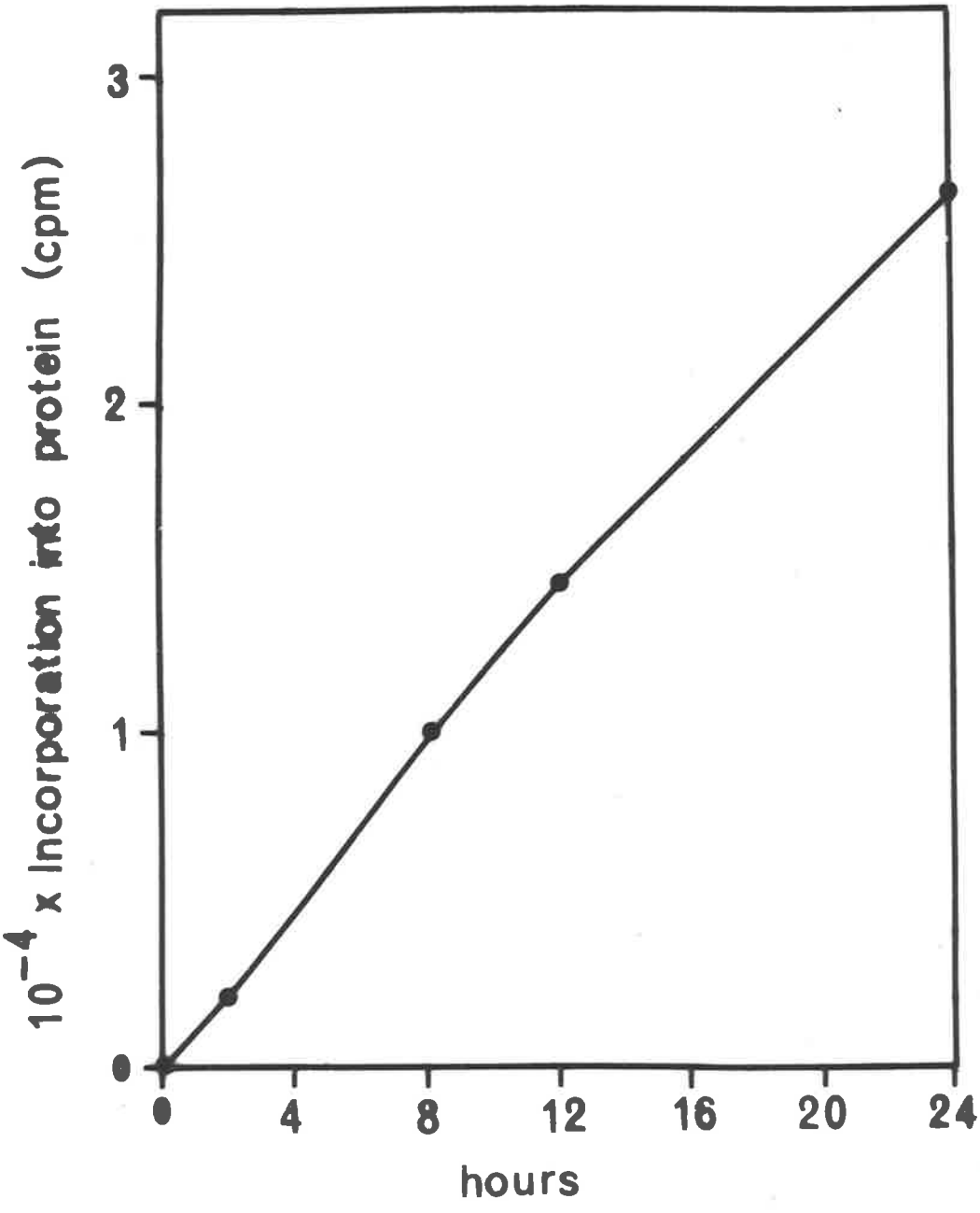

3
2
1
0
0
4
8
12
16
20
24
hours
10^{-4} x Incorporation into protein (cpm)

TABLE III.4: ASPARAGINE UTILIZATION

Amino acid[a]	Hydrolyzed protein	Soluble fraction
Glu	427[b]	59
Asp	3494[c]	90
Asp(NH_2)	70	5514

[a]Radioactivity of all other amino acids in both the soluble and protein fraction was less than 50 cpm.

[b]Glutamic acid and glutamine.

[c]Aspartic acid and asparagine.

Following the 24 hour uptake of [^{14}C]-U-asparagine (1 μC) by 5 cm long wheat shoots the radioactivity (cpm) of the amino acids from aliquots of the hydrolyzed protein and soluble fraction was determined as described on p.36.

acids examined. With the exception of a small amount of radioactivity in the glutamic acid plus glutamine fraction the entire label was present in the aspartic acid fraction (Table III.4), presumably this having arisen from the asparagine in the protein. The small amount of label in glutamic acid and glutamine is not explained but the results show a remarkable lack of conversion of asparagine to other amino acids.

2. Attempts to detect asparagine utilization in homogenates

Homogenates of wheat seedlings, excised shoots and roots were made under a variety of conditions as described on page 37. To render the assay system highly sensitive, homogenates were dialyzed overnight to remove free asparagine. [^{14}C]-U-Asparagine was incubated with these homogenates. The amino acid fraction was isolated and the amino acids separated and counted. All homogenates failed to show asparagine hydrolysis; a typical result is given in Table III.5. Freshly prepared undialyzed homogenates also failed to show asparagine hydrolysis. It is clear from these results that an asparaginase cannot be operating to any extent in these preparations. Homogenates of lupin seedlings were also exhaustively examined and again in no case could any significant asparagine hydrolysis be detected.

TABLE III.5: TESTS FOR ASPARAGINE HYDROLYSIS IN HOMOGENATES

Amino acid[a]	Time of incubation (hours)	
	0	3
$Asp(NH_2)$	33873	29979
Asp	189	130
Ala	167	297

[a]The activity of all amino acids was less than 50 cpm.

The radioactivity of an aliquot of the amino acids (cpm) following the incubation of $[^{14}C]$-U-asparagine with a dialyzed homogenate of 5 cm long wheat leaves for 3 hours at 30° prepared in 0.05 M-potassium phosphate, pH 7.2 containing 10^{-3} M-GSH, 10^{-4} M-EDTA, and 0.35 M-mannitol was determined. Incubation mixtures contained homogenate (5 ml), Mg^{++} (15 μmole), dipotassium salt of ATP (10 μmole) and $[^{14}C]$-U-asparagine (1 μC).

In addition these homogenates which can convert both pyruvate and aspartate to alanine (Table III.3), failed to incorporate label from asparagine into any other compound when incubated with [^{14}C]-U-asparagine and either α-keto-glutarate or pyruvate plus pyridoxal phosphate, indicating that asparagine does not participate in transamination reactions in these homogenates. This was confirmed by the fact that label was not incorporated under these conditions from asparagine into any other amino acid nor into any form which did not adsorb to Zeocarb 225 (H^+), (such as an α-keto acid).

D. DISCUSSION

The amino acid analyses of wheat grain and seedlings show that the latter accumulate free asparagine as the predominant amino acid. Part of this must come from proteolysis in the endosperm since free asparagine and most other amino acids can be detected there. However, since the total aspartic acid content of the wheat grain is only half that of the free asparagine of the seedling then clearly *de novo* synthesis has taken place which confirms the concept of a secondary production of asparagine to that derived from the decomposition of the reserve proteins, first proposed by Schulz in 1898.

The $[^{14}C]$-CO_2 incorporation studies confirm this. Asparagine synthesis from CO_2 occurs under normal conditions of photosynthesis in a whole wheat seedling or in a detached wheat shoot. Moreover, apart from the incorporation of $[^{14}C]$-CO_2 into alanine and serine which occurs ten times faster than that into any other amino acid, the rate of CO_2 fixation into asparagine is virtually identical with that into glutamine, glutamic acid, and aspartic acid.

The perplexing problem still unresolved is the pathway of asparagine biosynthesis and the factors determining its rate. Although $[^{14}C]$-U-aspartic acid was shown to be incorporated predominantly into asparagine *in vivo* this may simply be due to a large pool size of asparagine, coupled with its slow metabolism (which is substantiated by the $[^{14}C]$-CO_2 "chase" experiment) rather than to a direct and rapid conversion of aspartic acid to its amide. *In vitro* negative experiments are necessarily inconclusive, but no trace of such a conversion (as claimed by Webster and Varner, 1955) could be found in a large number of experiments.

The results obtained on the utilization of asparagine in these initial experiments, give no support to the concept that asparagine functions specifically in the translocation of either carbon or nitrogen from protein breakdown. Such a function would imply efficient

equilibration with other compounds in the metabolic pools of cells at the growing tip. This does not appear to happen to any significant degree. Asparagine, although incorporated into protein, is not converted to a significant extent to other amino acids. This conclusion is supported by the complete failure to detect any hydrolysis of labelled asparagine in dialyzed or crude extracts of both wheat and lupin seedlings. Additionally, homogenates which synthesized alanine from pyruvate and aspartic acid (and are therefore active in transamination) failed to incorporate label into any other compound when incubated with [^{14}C]-U-asparagine, α-keto-glutarate or pyruvate and pyridoxal phosphate. In other words, asparagine is neither hydrolyzed nor transaminated in these systems. The asparaginases described by Grover and Chibnall (1927) and Kretovich, Evstigneeva and Makarenko (1958) and the transaminase described by Yamamoto (1955) are presumably not of widespread importance. The existence of systems such as the utilization of asparagine rather than glutamine for purine biosynthesis (Kapoor and Waygood, 1962) may be either rare exceptions or else quantitatively of small significance with respect to asparagine utilization.

E. SUMMARY

1. The *de novo* synthesis of asparagine by wheat seedlings and shoots grown under normal conditions has been demonstrated.

2. Evidence from amino acid analyses and CO_2 fixation experiments has shown that asparagine is synthesized by wheat (and lupin) seedlings and shoots at a rate similar to that of glutamine, aspartic acid and glutamic acid.

3. The *in vivo* incorporation of label from [^{14}C]-U-aspartic acid into asparagine has been demonstrated. No *in vitro* synthesis of asparagine could be obtained from [^{14}C]-labelled aspartic acid, pyruvate or HCO_3^-.

4. Asparagine is incorporated into protein but it was not possible to demonstrate any *in vivo* or *in vitro* hydrolysis of asparagine, nor is there any evidence of transamination reactions involving asparagine.

CHAPTER IV

ASPARAGINE BIOSYNTHESIS FROM CYANIDE VIA β-CYANO-ALANINE

ASPARAGINE BIOSYNTHESIS FROM CYANIDE VIA β-CYANO-ALANINE

A. INTRODUCTION

As discussed in the last Chapter the pathway of asparagine biosynthesis in plants is still unresolved. There is no evidence to support the existence of a pathway by which asparagine is synthesized from aspartate, ammonia and dependent on Mg^{++} and ATP as claimed by Webster and Varner (1955).

However, the possibility of a new biosynthetic route to asparagine was raised by the finding of Blumenthal-Goldschmidt, Butler and Conn (1963) that young seedlings of a number of plant species incorporated [^{14}C]-HCN into the amide carbon atom of asparagine.

In vitro studies in plants (Floss, Hadwiger and Conn, 1965 and Fowden and Bell, 1965) have subsequently shown that asparagine can be synthesized from cyanide and either cysteine or serine via β-cyano-alanine.

It was decided therefore to further examine this pathway at the enzymic level with the aim of determining the significance of this cyanide pathway in either asparagine biosynthesis or cyanide detoxification.

B. ASPARAGINE BIOSYNTHESIS FROM CYANIDE VIA β-CYANO-ALANINE

1. In vivo incorporation of [^{14}C]-HCN into asparagine by excised wheat shoots

Excised wheat shoots exposed to [^{14}C]-HCN under normal conditions of growth incorporated label into asparagine (Table IV.1). The observation that there is a much greater incorporation of [^{14}C]-HCN into asparagine in the presence of CO_2 than in its absence is noteworthy (Table IV.1). This may be due to a limiting concentration of the precursor for cyanide fixation in the absence of CO_2.

Conversely the incorporation of [^{14}C]-CO_2 into asparagine was not reduced in the presence of unlabelled HCN, but the incorporation into all other amino acids except glycine was reduced. The effect of cyanide on the respiratory system of the plant would certainly complicate the issue preventing any conclusions being drawn about the pathway of CO_2 incorporation, namely whether CO_2 and cyanide proceed to asparagine by a common intermediate.

2. In vitro incorporation of [^{14}C]-KCN into asparagine via β-cyano-alanine in crude extracts of wheat leaf and E. coli cells

The synthesis of asparagine from [^{14}C]-KCN and cysteine via β-cyano-alanine has been demonstrated in crude wheat leaf extracts (Fig. IV.1). The label was found to be

TABLE IV.1. INCORPORATION OF $[^{14}C]$-HCN AND $[^{14}C]$-CO_2 INTO EXCISED WHEAT SHOOTS IN THE PRESENCE AND ABSENCE OF HCN AND CO_2

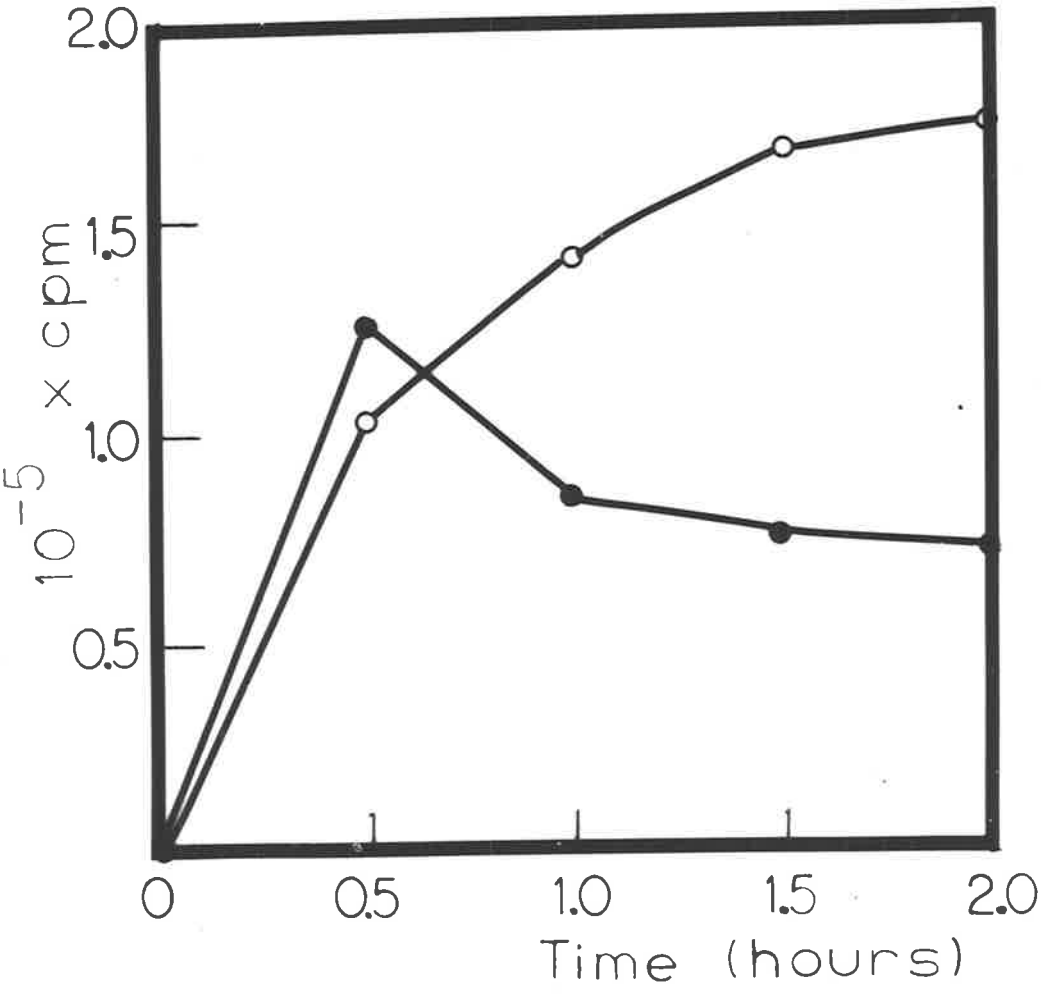
Amino acid	cpm/shoot from			
	$[^{14}C]$-HCN	$[^{14}C]$-HCN + CO_2	$[^{14}C]$-CO_2	$[^{14}C]$-CO_2 + HCN
Asp(NH_2)	583	6046	265	388
Glu(NH_2)	0	20	167	61
Gly	10	39	746	1467
Ser	0	183	2329	591
Asp	0	91	319	151
Ala	207	282	5317	2164
Glu	30	109	922	713
β-CN-ala	10	62	0	40

Radioactivity (cpm/shoot) from $[^{14}C]$-HCN (50 μC, 0.03% v/v) and $[^{14}C]$-CO_2 (50 μC, 0.03% v/v) incorporated into the soluble amino acids of excised wheat shoots after photosynthesis for 30 minutes. Non-labelled HCN and CO_2 when present were also at a level of 0.03% v/v.

FIGURE IV.1: THE INCORPORATION OF [^{14}C]-KCN INTO ASPARAGINE VIA β-CYANO-ALANINE IN A CRUDE WHEAT LEAF EXTRACT.

Following the incubation of [^{14}C]-KCN (1 μC, 22 mumoles) and cysteine (1.04 μmoles) with a dialyzed wheat leaf extract (5 ml, 15 mg protein/ml) in 0.05 M-tris HCl, pH 7.5 at 30°, the amino acids were separated and counted as described on p. 36 .

(•) B.CN.ala (○) Asp(NH_2).


2.0
1.5
1.0
0.5
10^{-5} x cpm
0
0.5
1.0
1.5
2.0
Time (hours)

incorporated exclusively into the nitrile group of β-cyano-alanine and the amide group of asparagine (Table IV.2). No asparaginase activity could be detected in this crude wheat leaf extract. However, in crude E. coli extracts incubated with [^{14}C]-KCN and cysteine, label was incorporated into β-cyano-alanine, asparagine and aspartic acid (Fig. IV.2).

3. Properties of the crude β-cyano-alanine synthase in wheat leaves

A standard assay for the crude β-cyano-alanine synthase in wheat leaves was developed as described on page 41 . Using this assay a linear response of enzymic activity to protein concentration up to 1 mg protein/ml was obtained (Fig. IV.3). A linear response was also found with respect to time of incubation up to 7 minutes (Fig. IV.4). β-cyano-alanine synthase activity was optimal at pH 8.9 but considerable activity was detected between pH 8.4 and pH 9.2 (Fig. IV.5). The synthesis of β-cyano-alanine from cysteine and cyanide in crude wheat leaf extracts was found to be dependent on native enzyme and independent of Mg^{++} (4 mM) and ATP (2 mM). A slight increase in activity (11%) was found with pyridoxal phosphate (25 μM) but this was not investigated further in the crude extracts.

TABLE IV.2: DEGRADATION OF THE [^{14}C]-LABELLED β-CYANO-ALANINE AND ASPARAGINE SYNTHESIZED FROM [^{14}C]-KCN AND CYSTEINE

	β-cyano-alanine (cpm)		Asparagine (cpm)	
	Unhydrolyzed	Hydrolyzed	Unhydrolyzed	Hydrolyzed
Control	9988	7043	363	632
Decarboxy-lated	10290	126	365	18
% Lost	0	98	0	97

Samples of the [^{14}C]-labelled β-cyano-alanine and asparagine synthesized from [^{14}C]-KCN and cysteine were hydrolyzed in 4N HCl for 48 hours. Samples of the hydrolyzed and unhydrolyzed amino acids were then decarboxylated as described on page 40 .

FIGURE IV.2. THE INCORPORATION OF [^{14}C]-KCN INTO ASPARTIC ACID ASPARAGINE AND β-CYANO ALANINE IN A CRUDE E. COLI EXTRACT.

Following the incubation of [^{14}C]-KCN (0.1 μC, 2.38 μmoles), cysteine (4.38 μmoles) and β-cyano-alanine (4.39 μmoles) with a dialyzed E. coli extract (1.0 ml, 20 mg protein/ml) in 0.1 M-potassium phosphate, pH 8.4 at 37° the amino acids were isolated and counted as described on p. 36 .

(■) β-CN-ala; (○) asp; (●) asp(NH_2).

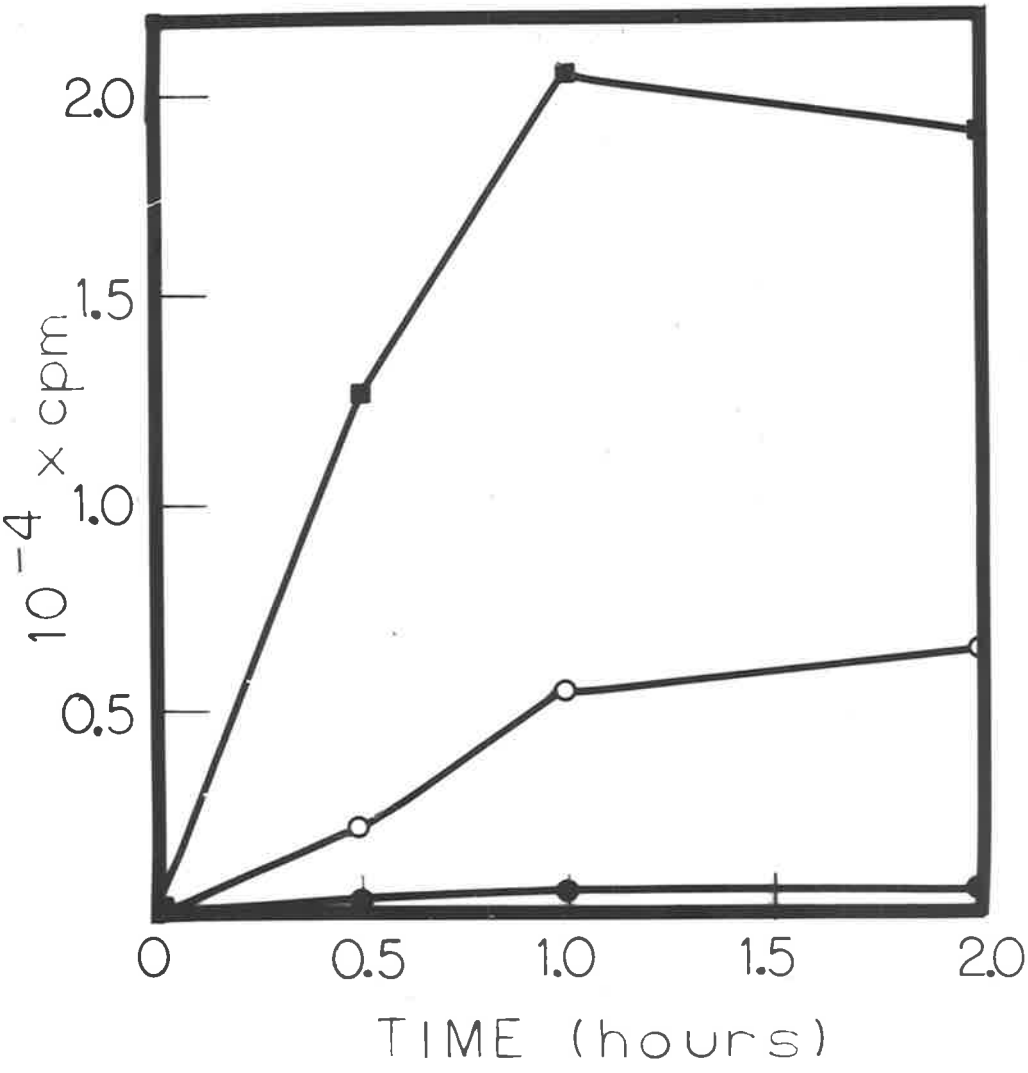
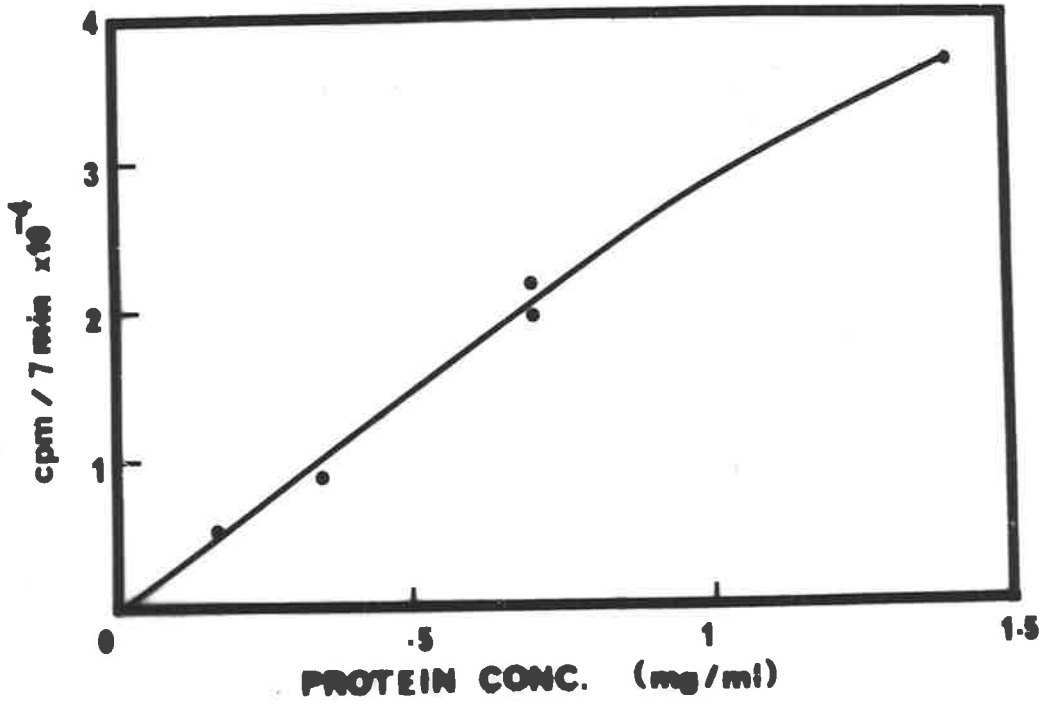

2.0
1.5
1.0
0.5
10 -4 x cpm
0
0.5
1.0
1.5
2.0
TIME (hours)

FIGURE IV.3: EFFECT OF ENZYME CONCENTRATION ON β-CYANO-ALANINE SYNTHESIS BY A CRUDE WHEAT LEAF EXTRACT.

The incubation mixture contained in a total volume of 0.2 ml; crude wheat leaf extract in 0.05M-tris HCl, pH 9.0, 0 - 0.28 mg protein; [^{14}C]-potassium cyanide, 22 mμmoles, 1 μC; cysteine, 5 μmoles. After incubation at 30° for seven minutes the reaction was stopped and the labelling determined in β-cyano-alanine and asparagine as described on p. 41 .

FIGURE IV.4: TIME COURSE OF β-CYANO-ALANINE SYNTHESIS BY A CRUDE WHEAT LEAF EXTRACT.

The experimental details are given in Figure IV.3.


4
3
2
1
0
.5
1
1.5
cpm / 7 min x10⁻⁴
PROTEIN CONC. (mg/ml)

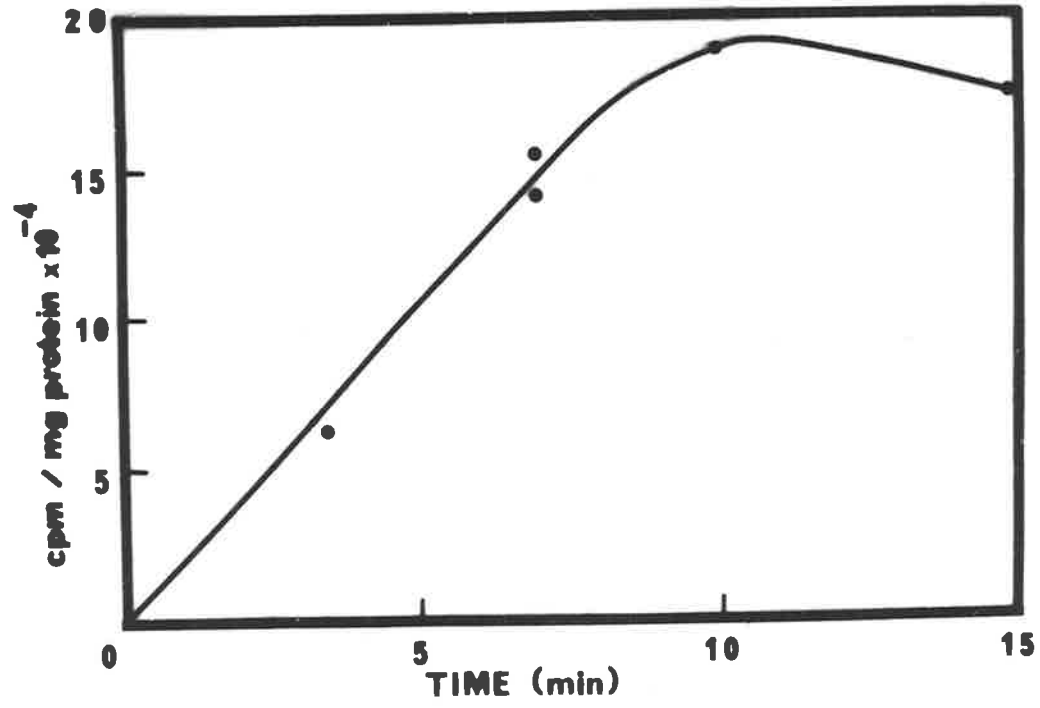
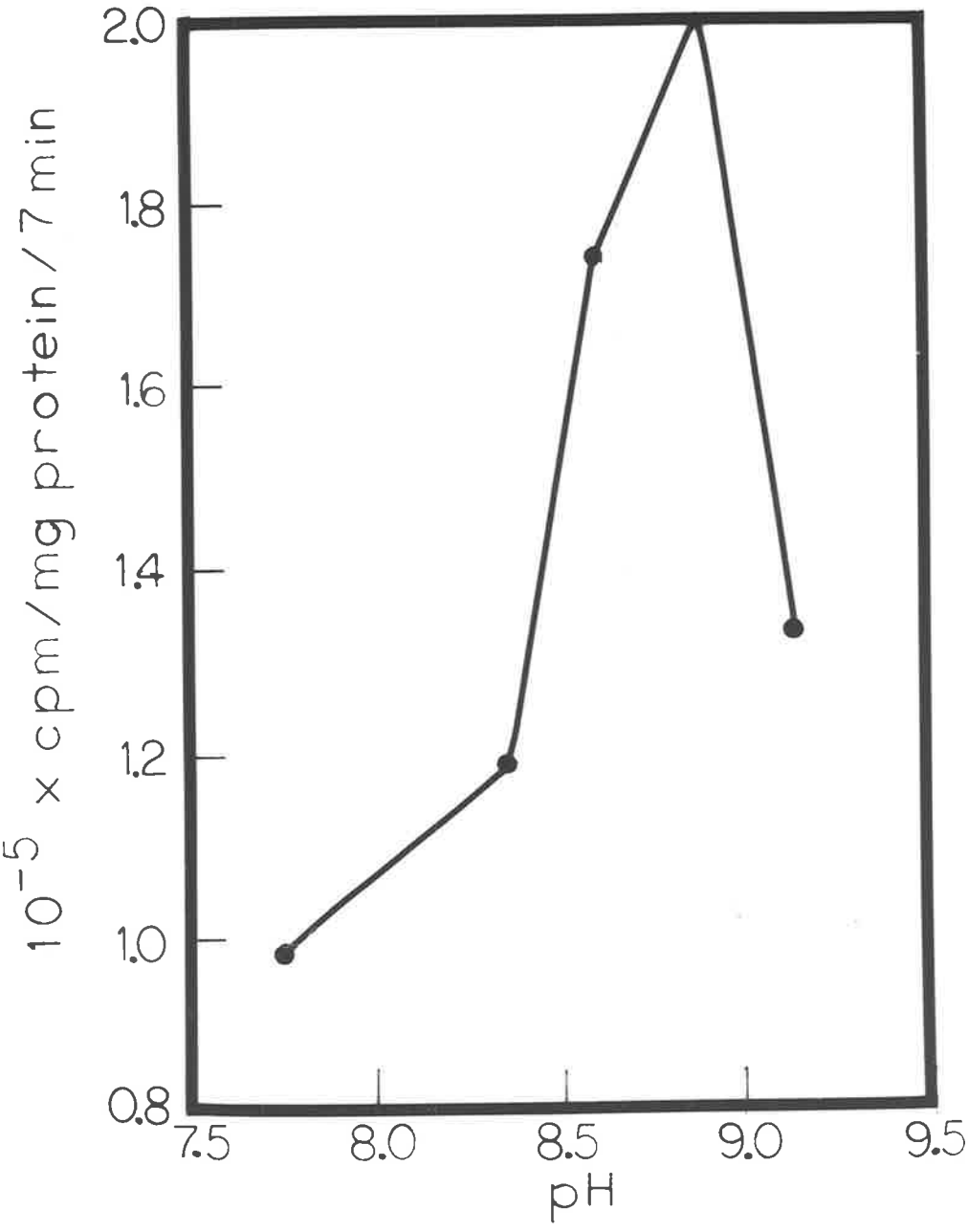

20
15
10
5
0
5
10
15
cpm / mg protein x10⁻⁴
TIME (min)

FIGURE IV.5: EFFECT OF pH ON THE β-CYANO-ALANINE SYNTHASE ACTIVITY OF A CRUDE WHEAT LEAF EXTRACT.

The experimental details are given in Figure IV.3. 0.05M-tris HCl buffers were used in the pH range indicated.


2.0
1.8
1.6
1.4
1.2
1.0
0.8
10^{-5} x cpm/mg protein/7 min
7.5
8.0
8.5
9.0
9.5
pH

4. Properties of the crude β-cyano-alanine hydrolase in wheat leaves

A standard assay for the crude β-cyano-alanine hydrolase in wheat leaves was developed as described on page 42 . Using this assay the hydrolysis of β-cyano-alanine to asparagine in crude wheat leaf extracts was found to be dependent on native enzyme; the addition of Mg^{++} (4 mM) or ATP (2 mM) were either inhibitory or had no effect. The results presented in Fig. IV.6 show that the β-cyano-alanine hydrolase shows an optimum pH of 7.8 and the activity greatly decreases below pH 7.5. The enzyme was inactivated at temperatures above 40° (Fig. IV.7). The apparent Km for β-cyano-alanine as substrate was estimated to be 2.2 mM (Fig. IV.8) by the method of Wilkinson (1961). Due to the difficulty and expense of obtaining [^{14}C]-β-cyano-alanine in large quantities (for method of preparation see page 40.) Routine assays contained only 4.28 mM β-cyano-alanine. However, a linear response of enzymic activity to protein concentration up to 5 mg protein/ml was obtained. (Fig. IV.9) A linear response was also found with respect to time of incubation up to thirty minutes (Fig. IV.10).

FIGURE IV.6: EFFECT OF pH ON THE β-CYANO-ALANINE HYDROLASE ACTIVITY OF A CRUDE WHEAT LEAF EXTRACT.

The incubation mixture contained in a total volume of 0.25 ml; crude wheat leaf extract in 0.05M-tris HCl buffers, pH as indicated, 1.0 mg protein; [^{14}C]-β-cyano-alanine, 1.07 μmoles, 10^5 cpm. After incubation at 30° for 30 min. the reaction was stopped and the labelling determined in β-cyano-alanine and asparagine as described on p. 42 .

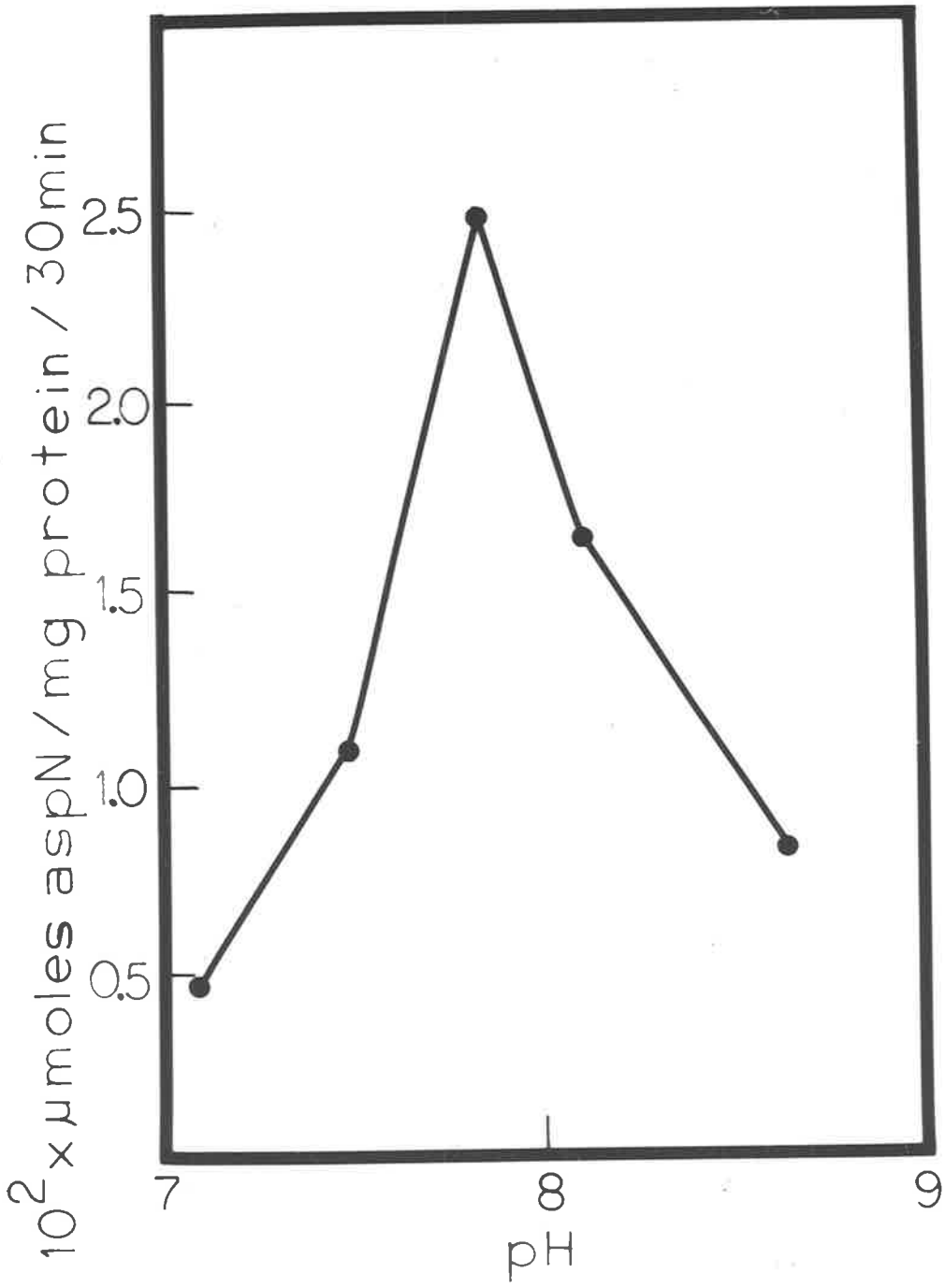

$10^2 \times \mu$moles aspN / mg protein / 30min
2.5
2.0
1.5
1.0
0.5
7
8
9
pH

FIGURE IV.7: EFFECT OF TEMPERATURE ON THE β-CYANO-ALANINE HYDROLASE ACTIVITY OF A CRUDE WHEAT LEAF EXTRACT.

The crude wheat leaf extracts in 0.05M-tris HCl, pH 7.8 were preincubated for 10 min. and assayed at the temperatures indicated. Assay details are given in Figure IV.6.

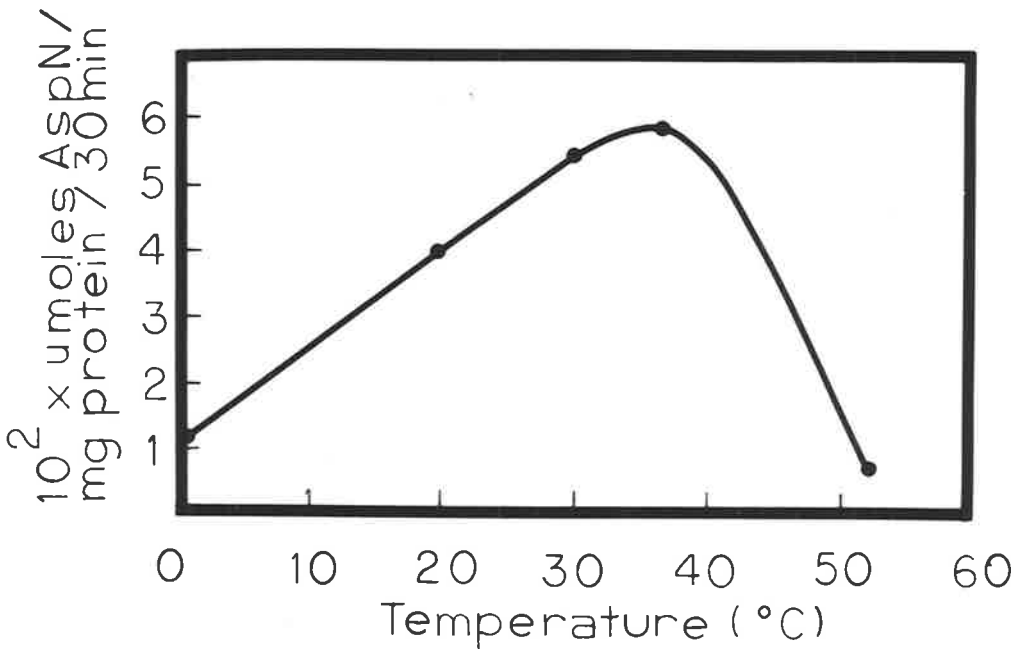
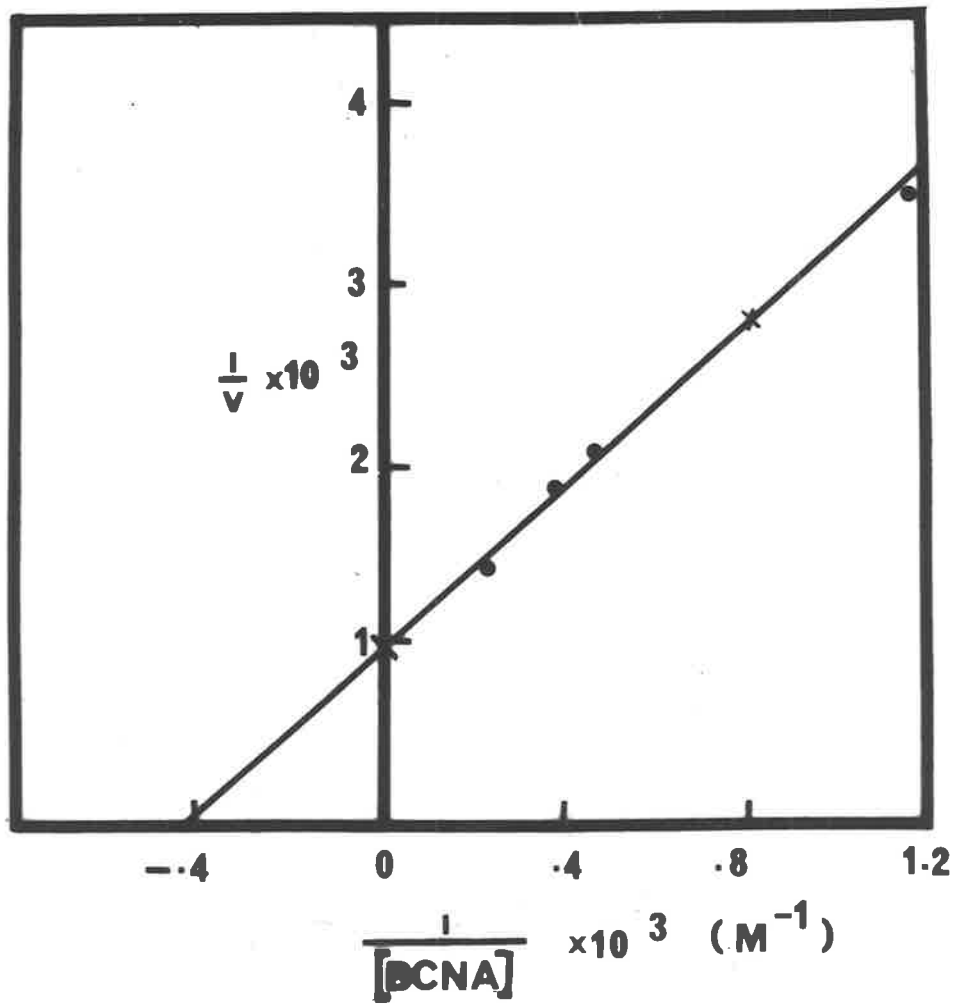

10^2 x umoles AspN/ mg protein / 30 min
1
2
3
4
5
6
0
10
20
30
40
50
60
Temperature (°C)

FIGURE IV.8: LINEWEAVER-BURK PLOT FOR β-CYANO-ALANINE.

The straight line drawn was obtained by connecting the two points (x) determined by calculating the intercept on the ordinate and the slope from the experimental points (•) by the method of least squares (Wilkinson, 1961). Assay details are given in Figure IV.6.



v = μ mole aspN / mg protein / min

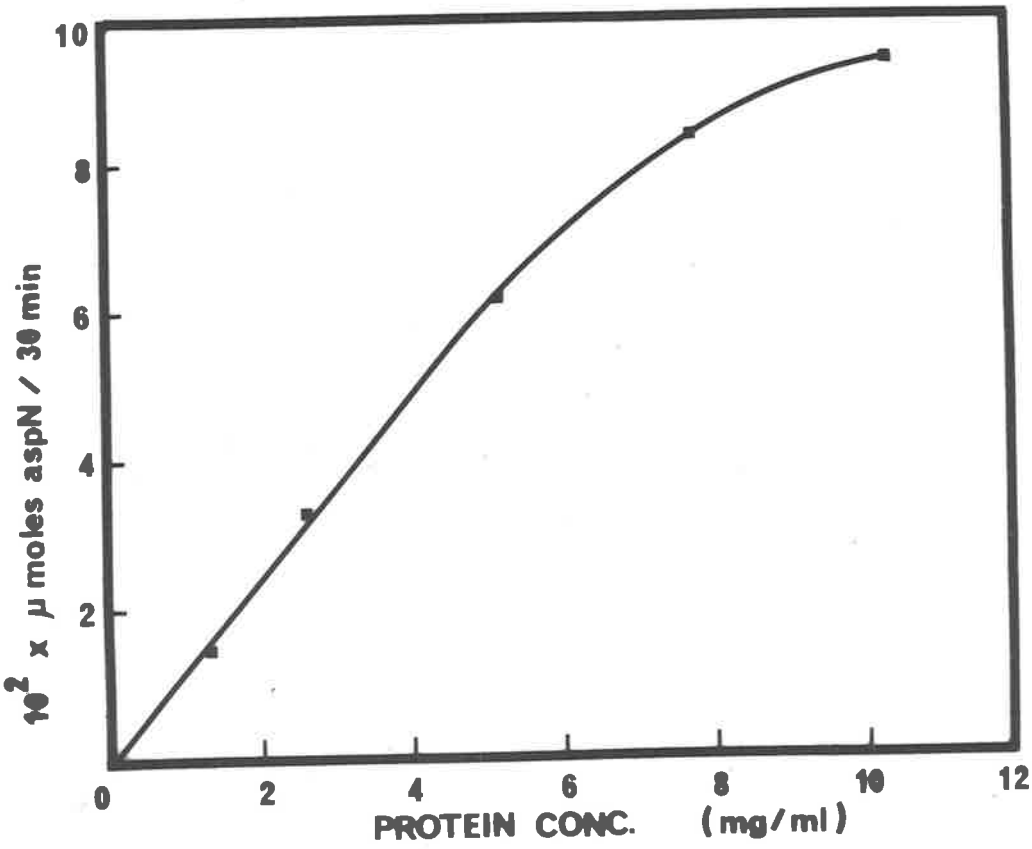
Km = 2·2 mM

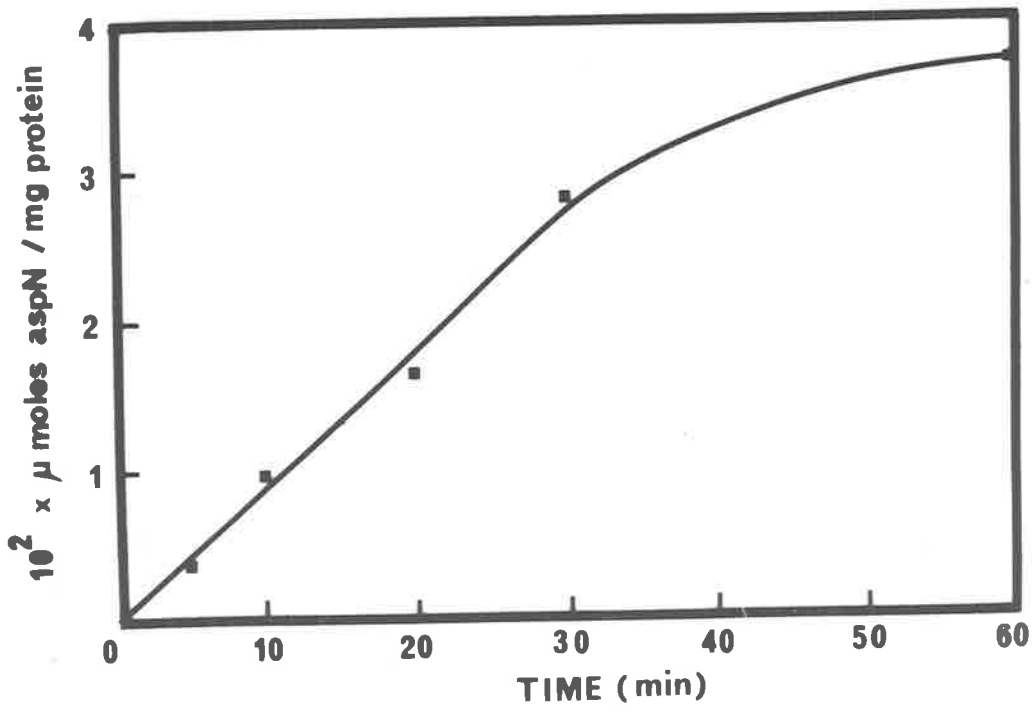
FIGURE IV.9: EFFECT OF ENZYME CONCENTRATION ON β-CYANO-ALANINE HYDROLYSIS BY A CRUDE WHEAT LEAF EXTRACT.

The crude wheat leaf extract in 0.05M-tris HCl, pH 7.8 (0 - 2.7 mg protein) was assayed for β-cyano-alanine hydrolase activity as described in Figure IV.6.

FIGURE IV.10: TIME COURSE OF β-CYANO-ALANINE HYDROLYSIS BY A CRUDE WHEAT LEAF EXTRACT.

Assay details are given in Figure IV.6; 0.05M-tris HCl buffer, pH 7.8 was used.


10
8
6
4
2
10^2 x μ moles aspN / 30 min
0
2
4
6
8
10
12
PROTEIN CONC. (mg/ml)


4
3
2
1
10^2 x μ moles aspN / mg protein
0
10
20
30
40
50
60
TIME (min)

5. Intracellular distribution of β-cyano-alanine synthase and hydrolase in wheat

Studies on the intracellular distribution of the β-cyano-alanine hydrolase in wheat have shown that this enzyme is located in the supernatant fraction of both the leaves and the roots, (Table IV.3). No β-cyano-alanine hydrolase activity could be detected in the chloroplast fraction, prepared either in aqueous or non-aqueous conditions, which indicates that the absence of this enzyme in the chloroplast is not due to leakage during the preparation. β-cyano-alanine hydrolase activity was not found in the mitochondrial fractions of either the leaves or the roots (Table IV.3).

β-Cyano-alanine synthase activity, however, was found in the mitochondrial fractions of the leaves and roots with considerable activity also present in the root and leaf supernatants and the chloroplast fractions (Table IV.3).

Sonication of the mitochondrial and chloroplast fractions before dialysis as described on page 38 , was found to decrease the β-cyano-alanine synthase activity by 70 to 80 percent but this effect was not examined further.

TABLE IV.3. INTRACELLULAR DISTRIBUTION OF β-CYANO-ALANINE SYNTHASE AND HYDROLASE IN WHEAT

	β-cyano-alanine synthase activity[a]	β-cyano-alanine hydrolase activity[b]
leaf supernatant	9.32	2.4
leaf mitochondria	33.50	0
chloroplast (aqueous)	3.59	0
chloroplast (non-aqueous)	5.58	0
root supernatant	19.84	0.45
root mitochondria	34.71	0

[a] cpm incorporated/mg protein/7 minutes x 10^{-4}. The specific activity of the potassium cyanide was 45.2 μC/μMole.

[b] μMoles asparagine/mg protein/30 minutes x 10^{2}.

Preparation of fractions and assays as described on pages 37, 41 and 42.

6. β-Cyano-alanine synthase and hydrolase activity in plant, bacterial and animal extracts

As well as the β-cyano-alanine synthase and hydrolase activity demonstrated in wheat and E. coli extracts (Table IV.4) β-cyano-alanine synthase and hydrolase activity has been found in Lupinus angustifolius leaf extracts (Table IV.4). With the lupin roots no hydrolase activity could be detected even if the sensitivity of the assay was increased by increasing the specific activity of the [^{14}C]-β-cyano-alanine 3-fold. However, considerable browning of this extract occurred which could have resulted in enzyme inactivation. The extract did however show synthase activity.

β-Cyano-alanine synthase activity was not found in any of the mammalian extracts examined (Table IV.4). β-Cyano-alanine hydrolase activity was found in guinea pig serum and liver but not in any of the other mammalian extracts (Table IV.4).

TABLE IV.4. β-CYANO-ALANINE SYNTHASE AND HYDROLASE ACTIVITY IN PLANT, BACTERIAL AND ANIMAL EXTRACTS

	β-cyano-alanine synthase activity[a]	β-cyano-alanine hydrolase activity[b]
wheat leaves	14.99	3.2
wheat roots	28.69	1.5
lupin leaves	16.44	3.8
lupin roots	11.65	0
E. coli	17.28	4.2
guinea pig serum	0	3.6
guinea pig liver	0	3.3
sheep liver	0	0
sheep kidney	0	0
sheep heart	0	0
rabbit liver	0	0
rabbit serum	0	0

[a] cpm incorporated/mg protein/7 minutes x 10^{-4}. The specific activity of the potassium cyanide was 45.2 μC/μmole.

[b] μmoles asparagine/mg protein/30 minutes x 10^{2}.

Preparation of extracts and assays are as described on pages 37, 39, 41 and 42.

C. DISCUSSION

The demonstration of the biosynthesis of asparagine from cyanide and cysteine via β-cyano-alanine both *in vivo* and *in vitro* in wheat and *Lupinus angustifolius* and *E. coli* extracts substantiates the existence of the cyanide pathway enzymes in plants and in *E. coli*. A preliminary account of this work has appeared (Farnden and Elliott, 1968). Blumenthal-Goldschmidt, Butler and Conn (1963) have previously reported the biosynthesis of asparagine from [^{14}C]-cyanide in a number of seedlings *in vivo*. Subsequently, the *in vitro* biosynthesis of β-cyano-alanine from cysteine or serine and cyanide was reported to occur in seedlings (Floss, Hadwiger and Conn, 1965) and *E. coli* (Dunnill and Fowden, 1965). The *in vitro* hydrolysis of β-cyano-alanine to asparagine in plants but not *E. coli* has also been demonstrated (Fowden and Bell, 1965).

The exclusive incorporation of the [^{14}C]-cyanide into the nitrile group of β-cyano-alanine and the amide group of asparagine reported here agrees with the preliminary assessment of the distribution of the label by Blumenthal-Goldschmidt, Butler and Conn (1963).

The β-cyano-alanine synthase of wheat was found to be similar to the *Lotus tenuis* enzyme (Floss, Hadwiger and Conn, 1965) in that it had an optimum pH around 9.0, was not ATP stimulated and was located in the mitochondrion. Significant β-cyano-alanine synthase activity was also found in the supernatant and chloroplast fractions of wheat.

This location of β-cyano-alanine synthase in the plant mitochondrion is of particular interest in view of the speculation that β-cyano-alanine synthase may have a role in the detoxification of cyanide produced in the plant following the degradation of cyanogenic glycosides. However, the demonstration here of β-cyano-alanine synthase activity in Lupinus angustifolius extracts makes this hypothesis somewhat less tenable since a possible source of cyanide such as the cyanogenic glycosides has not been reported in this species of plant.

The β-cyano-alanine hydrolase activity was found exclusively in the supernatant fractions. The pH optimum of 7.8 determined for the wheat β-cyano-alanine hydrolase is in agreement to reports for the Lathyrus odoratus enzyme by Fowden and Bell (1965).

The demonstration of the hydrolysis of the neurotoxin β-cyano-alanine, which is found free in some forage crops, e.g., vetch (Ressler, 1962) by the guinea pig serum and liver preparations but not the other mammalian extracts examined is noteworthy. It could represent a physiologically significant β-cyano-alanine detoxification mechanism. Both these guinea pig extracts which exhibited β-cyano-alanine hydrolase activity, however, had considerable asparaginase activity, and it is therefore possible that the hydrolysis of β-cyano-alanine to aspartic acid in this case, was actually catalyzed by the asparaginase, a possibility originally suggested by Giza, Ratzkin and Ressler

(1963). These authors demonstrated not only the hydrolysis of β-cyano-alanine to asparagine and aspartic acid by the guinea pig serum asparaginase but also the partial alleviation of the asparagine requirement of a Neurospora crassa auxotroph by β-cyano-alanine. Giza, Ratzkin and Ressler (1963) proposed that in both these cases the hydrolysis of β-cyano-alanine to asparagine was mediated by the action of an asparaginase.

The complete absence of any further metabolism of the asparagine synthesized by this pathway in plants is confirmation of our earlier work (page 56 , and Lees, Farnden and Elliott, 1968a; 1968b) in which we were unable to demonstrate any in vivo or in vitro hydrolysis of asparagine in seedlings. In the E. coli extracts, however, the asparagine synthesized by the cyanide pathway was found to be rapidly hydrolyzed to aspartic acid.

These studies on the cyanide pathway have certainly not determined its significance in either cyanide or β-cyano-alanine detoxification or asparagine biosynthesis, but this pathway does remain the only in vitro pathway of asparagine biosynthesis so far reported to occur in plants that has been independently confirmed.

D. SUMMARY

1. The *in vivo* incorporation of [^{14}C]-HCN into asparagine by excised wheat shoots has been demonstrated.

2. The synthesis of asparagine from [^{14}C]-KCN and cysteine via β-cyano-alanine has been demonstrated in crude wheat leaf and *E. coli* extracts. The label was incorporated exclusively into the nitrile group of β-cyano-alanine and the amide group of asparagine.

3. The properties of the crude wheat leaf β-cyano-alanine hydrolase and synthase were investigated.

4. The intracellular distribution of β-cyano-alanine synthase and hydrolase was determined in wheat.

5. β-Cyano-alanine synthase activity was also detected in lupin shoot and root extracts, but not in any of the mammalian extracts examined.

6. β-Cyano-alanine hydrolase activity was detected in extracts of lupin leaves, and guinea pig liver, and in guinea pig serum.

CHAPTER V

STUDIES ON THE ENZYMES INVOLVED IN THE BIOSYNTHESIS AND UTILIZATION OF ASPARAGINE IN E. COLI

STUDIES ON THE ENZYMES INVOLVED IN THE BIOSYNTHESIS AND UTILIZATION OF ASPARAGINE IN E. COLI

A. INTRODUCTION

The initial report by Dunnill and Fowden (1965) of the enzymic formation of β-cyano-alanine from cyanide and cysteine in E. coli extracts was confirmed in the work described in the previous chapter and the hydrolysis of β-cyano-alanine to asparagine was also demonstrated. A major problem is whether the cyanide pathway enzymes in E. coli are of physiological significance in the biosynthesis of asparagine.

To investigate this a study of these enzymes in E. coli and a biochemical-genetic study on the biosynthesis of asparagine in this organism was undertaken. It was hoped that this would be useful in understanding the role of the pathway in plants. For example it was possible that a previously unknown physiologically significant source of cyanide or a related one carbon compound would be revealed in E. coli studies. The genetic manipulation possible in E. coli clearly makes such a problem much more easily tackled in this organism than in plants.

The studies on the enzymes involved in the cyanide pathway in E. coli are reported in this chapter. In the following chapter a study of the biosynthesis of asparagine in E. coli from a genetical approach is reported.

B. A STUDY OF THE E. COLI β-CYANO-ALANINE SYNTHASE, β-CYANO-HYDROLASE AND ASPARAGINASE

1. Demonstration of these activities in E. coli

The strains of E. coli used were from the collection of Dr. J.B. Egan and will be referred to by their collection number, e.g., E. coli E547. The original source of each strain is given on page 29.

(a) Demonstration of β-cyano-alanine synthase in E. coli

It was decided to survey a series of E. coli strains, conveniently available, for β-cyano-alanine synthase activity. Crude extracts of five strains of E. coli were assayed using cysteine and labelled cyanide as substrates (as described on p.41) protein concentrations of 30 mg/ml and incubation times of up to 3 hours were used. Under these conditions only E. coli E596 of those strains tested was found to have β-cyano-alanine synthase activity (Table V.1).

Exhaustive unsuccessful attempts were made to demonstrate β-cyano-alanine synthase activity in extracts of E. coli E547, E551, E563 and E586 grown both in complete medium and minimal medium. In these tests in addition to the standard assay procedure, cysteine was replaced by serine or O-acetyl-serine and pyridoxal phosphate and ATP were included in the assays. All tests were negative.

Dunnill and Fowden (1965) have suggested that the synthesis of β-cyano-alanine from cyanide and cysteine observed

TABLE V.1: β-CYANO-ALANINE SYNTHASE ACTIVITY IN *E. COLI* EXTRACTS

E. coli strain	β-cyano-alanine synthase activity[a]
E547	0.0
E551	0.0
E563	0.0
E586	0.0
E596	7.4

[a] 10^{-4} x cpm incorporated into β-cyano-alanine, asparagine and aspartic acid/mg protein/7 min.

The assay procedure and preparation of the *E. coli* extracts are described on pages 39 and 41. The specific activity of the [^{14}C]-potassium cyanide was 45.2 μC/μmole. All *E. coli* strains were grown in the minimal medium described on p. 30 .

in E. coli extracts may be catalyzed by cysteine synthase. To examine this, E. coli E563 was grown in minimal medium with reduced glutathione as the sole sulphur source. Under these conditions complete derepression of the cysteine biosynthetic enzymes occurs (Wheldrake, 1968). Extracts of these cells were made and assayed for β-cyano-alanine synthase activity. None was detected.

Attempts were also made to induce β-cyano-alanine synthase by growing E. coli E563 and E551 in both liquid and solid media supplemented with cyanide at concentrations varying from 10^{-6}M to 10^{-3}M. At 10^{-3}M, cyanide was found to completely inhibit the growth of cells in both minimal and complete media (Table V.2). At lower concentrations (10^{-5}M and 10^{-4}M) partial inhibition of growth was observed with more being seen in minimal than in complete medium. However, no β-cyano-alanine synthase activity was detectable in the standard assay in extracts prepared from E. coli E563 and E551 cells grown in either complete or minimal media supplemented with cyanide.

(b) Demonstration of β-cyano-alanine hydrolase activity in E. coli extracts

Crude extracts of the various E. coli strains grown in both complete and minimal media were assayed for hydrolase activity (Table V.3) using the assay described on p. 42 . Lauinger and Ressler (1970) have very recently reported a Km of 12mM for the E. coli β-cyano-alanine hydrolase. Due to

TABLE V.2: THE EFFECT OF CYANIDE ON E. COLI GENERATION TIMES

KCN concentration	complete medium		minimal medium	
	generation time (min)	% inhibition of growth	generation time (min)	% inhibition of growth
0	52	0	125	0
10^{-6}M	70	25	123	0
10^{-5}M	68	24	215	42
10^{-4}M	98	47	357	65
10^{-3}M	∞	100	∞	100

Cultures of E. coli E563 grown overnight with shaking at 37° in the minimal medium and complete medium supplemented with the potassium cyanide concentrations indicated were diluted (1:10) with fresh medium and incubated at 37° with shaking. Growth was determined by measuring the optical density at 600 mμ. Details of the growth media are given on p. 30 .

the expense and difficulty in preparing the [^{14}C]-β-cyano-alanine, the concentration of the [^{14}C]-β-cyano-alanine used in these assays was only 0.89mM. Therefore, assuming Michaelis-Menten kinetics, the initial velocities at a given enzyme concentration used in the calculation of the specific activities in Table V.3 would be only 6.9% of the Vmax.

In E. coli E551, E563 and E586, the β-cyano-alanine hydrolase specific activity was greater in extracts from cells grown in complete medium than from those grown in minimal medium (Table V.3). In these crude E. coli extracts the labelled asparagine, produced from the hydrolysis of the [^{14}C]-β-cyano-alanine was rapidly hydrolyzed to aspartic acid (80-90% of the product was aspartic acid). During the incubation of [^{14}C]-β-cyano-alanine in these crude extracts, a decrease in the total label in β-cyano-alanine plus asparagine and aspartic acid was also detected. It was assumed that this was due to the further metabolism of aspartic acid and was corrected for using an appropriate control.

Although it was not possible to detect any β-cyano-analine hydrolase activity in extracts of E. coli E596 using the standard assay conditions (Table V.3), the existence of the enzyme in this strain is shown by the fact that in the β-cyano-alanine synthase assay, where the specific activity of the cyanide is 1000 times that of the β-cyano-alanine used in the hydrolase assay, the conversion of the β-cyano-alanine

TABLE V.3: β-CYANO-ALANINE HYDROLASE ACTIVITY IN E. COLI EXTRACTS

E. coli strain	β-cyano-alanine hydrolase activity[a]		
	cells grown in		ratio complete/minimal
	complete medium	minimal medium	
E547[b]	0.84	-	-
E551	11.8	2.2	5.4
E563	10.6	3.8	2.8
E586	44.6	7.0	6.4
E596	0.0	0.0	-

[a] 10^4 x μmoles asparagine plus aspartic acid formed from β-cyano-alanine/mg protein/min.

[b] The growth requirements of this strain (see p. 29) were such that it was found necessary to further enrich the complete medium with 0.3% w/v yeast extract for rapid growth to occur.

The extracts were prepared as described on p. 39. The method of assay was modified to that described on p.42 in that [^{14}C]-β-cyano-alanine (0.178 μmoles; 14,000 cpm) was used in each assay and the total incubation volume was reduced to 0.2 ml. These assays had a final protein concentration of 5 - 15 mg protein/ml.

synthesized to asparagine and aspartic acid was detected.

(c) Demonstration of asparaginase activity in E. coli extracts

Crude extracts of the E. coli strains grown in minimal and complete media were assayed for asparaginase activity (Table V.4) as described on page 43.

As was found with the β-cyano-alanine hydrolase, a two to five fold increase in the specific activity of the asparaginase was found in E. coli E551, E563 and E586 cells grown in complete medium as compared with those grown in minimal medium (Table V.4). At the lower protein concentrations used compared with those used in the β-cyano-alanine hydrolase assays (Tables V3 and 4) no further metabolism of the aspartic acid formed from asparagine occurred.

(d) Summary of the preliminary studies on the E. coli β-cyano-alanine synthase, β-cyano-alanine hydrolase and asparaginase activities

The distribution of the cyanide pathway and asparaginase enzymes of the E. coli strains examined are qualitatively summarized in Table V.5. β-cyano-alanine hydrolase and asparaginase activities were detected in extracts of all the E. coli strains examined, but β-cyano-alanine synthase activity was only demonstrated in the E. coli E596 extract.

TABLE V.4: ASPARAGINASE ACTIVITY IN E. COLI EXTRACTS

E. coli strain	Asparaginase activity[a]		
	Cells grown in		Ratio $\frac{\text{complete}}{\text{minimal}}$
	complete medium	minimal medium	
E547	9.7	8.3	1.2
E551	14.0	4.1	3.4
E563	20.2	4.4	4.6
E586	15.7	6.4	2.5
E596	5.5	3.9	1.4

[a] 10^2 x μmoles aspartic acid released from [^{14}C]-asparagine/mg protein/min.

The assay procedure and method of preparation of extracts are described on pages 39 and 43. These assays had a final protein concentration of 0.025 - 0.15 mg protein/ml.

TABLE V.5: SUMMARY OF THE DISTRIBUTION OF THE CYANIDE PATHWAY AND ASPARAGINASE ENZYMES OF *E. COLI*

E. coli strain	β-cyano-alanine synthase	β-cyano-alanine hydrolase	Asparaginase
E547	-	+	+
E551	-	+	+
E563	-	+	+
E586	-	+	+
E596	+	+[a]	+

[a]Not detectable under normal assay conditions: see p. 84.

The conditions of assay and quantitative determinations have been presented in Tables V.1, 3 and 4.

2. Investigation of a possible relationship between β-cyano-alanine hydrolase and asparaginases

E. coli produces two distinct asparaginases (Schwartz, Reeves and Broome, 1966; Campbell, *et al.*, 1967; Cedar and Schwartz, 1967). These will be referred to as EC I and EC II in this text (Campbell *et al.*, 1967). It was decided to purify these asparaginases with the aim of finding out whether the β-cyano-alanine hydrolase activity of *E. coli* was distinct from the two asparaginases.

(a) Ammonium sulphate fractionation of the EC I and EC II asparaginases and β-cyano-alanine hydrolase

A modification of the ammonium sulphate fractionation procedure (see p.39 for details) described by Campbell *et al.* (1967) was used. The two ammonium sulphate fractions (AS I and AS II containing the EC I and EC II asparaginases respectively were assayed for both asparaginase and β-cyano-alanine hydrolase activities (Table V, 6 and 7). As described on p.84 at the substrate level used in the hydrolase assays, the activities reported in Table V.7 would be 6.9% of those at saturating substrate concentrations.

Asparaginase activity was found in both the AS I and AS II fractions of all strains examined (Table V.6). The ratios of the activities found in these fractions varied considerably with the strain used (Table V.6). β-cyano-

TABLE V.6: THE LEVELS OF ASPARAGINASE ACTIVITY FOUND IN THE *E. COLI* AS I AND AS II FRACTIONS

E. coli strain	Asparagine activity[a]				Ratio of the asparaginase activities in the AS II fraction / AS I fraction	
	Fraction AS I containing the EC I asparaginase		Fraction AS II containing the EC II asparaginase			
	Cells grown in		Cells grown in		Cells grown in	
	complete medium	minimal medium	complete medium	minimal medium	complete medium	minimal medium
E547	12.4	13.7	1.8	1.0	0.15	0.08
E551	7.3	4.9	32.4	4.6	4.4	0.94
E563	9.0	5.8	41.2	0.7	4.6	0.12
E586	9.5	3.0	202.6	12.6	21.3	4.2
E596	7.5	6.4	1.9	0.8	0.25	0.13

[a] 10^2 x µmoles aspartic acid formed/mg protein/min.

The assay procedure and method of preparation of extracts are described on pp. 39 and 43.

For other details see text.

TABLE V.7: THE LEVELS OF β-CYANO-ALANINE HYDROLASE ACTIVITY FOUND IN THE E. COLI AS I AND AS II FRACTIONS

E. coli strain	β-cyano-alanine hydrolase activity[a]			
	Fraction AS I Cells grown in		Fraction AS II Cells grown in	
	complete medium	minimal medium	complete medium	minimal medium
E547	0	-	2.2[b]	-
E551	0	0	40.1	3.3
E563	0	0	42.1	0
E586	0	0	233.0	10.3
E596	0	0	0	0

[a] 10^4 x μmoles asparagine plus aspartic acid formed/mg protein/min.

[b] This medium was supplemented with 0.3% w/v yeast extract as discussed on p. 85 .

The extracts were prepared as described on p. 39 . The method of assay was modified to thatdescribed on p.42 in that [^{14}C]-β-cyano-alanine (0.178 μmoles, 14,000 cpm) was used in each assay and the total assay volume was reduced to 0.2 ml.

alanine hydrolase activity was only found in the AS II fractions of *E. coli* E547, E551, E563 and E586 (Table V.7). Some β-cyano-alanine hydrolase activity was detected erratically in AS I preparations (less than 1%) and was considered to be of no significance. Campbell *et al.* (1967) found that fraction AS I contained 100% of the EC I asparaginase and less than 5% of the EC II asparaginase, whereas fraction AS II contained only EC II asparaginase.

A dramatic and parallel increase in the specific activities of both the EC II asparaginase and β-cyano-alanine hydrolase was found when *E. coli* strains E551, E563 and E586 were grown in complete medium as compared with those from cells grown in minimal medium (Tables V.6 and 7). It appears that both the EC II asparaginase and β-cyano-alanine hydrolase are induced (without necessarily implying specific substrate induction) by growth in an enriched medium. A slight increase in the specific activity of the EC I asparaginase was also detected in the cells grown in the enriched medium (Table V.6).

Asparagine did not cause a major induction of either the EC I or the EC II asparaginase (Table V.8), the 37% increase in EC I being not considered as a major effect. Cedar and Schwartz (1968) have likewise shown that asparagine does not induce the EC II asparaginase.

TABLE V.8: THE EFFECT OF ASPARAGINE IN THE GROWTH MEDIUM ON THE LEVEL OF THE E. COLI E563 ASPARAGINASES

Growth medium[b]	Crude Extract		Fraction AS I		Fraction AS II	
	Asparaginase activity[a]	% of Activity relative to medium (1)	Asparaginase activity	% of Activity relative to medium (1)	Asparaginase activity	% of Activity relative to medium (1)
(1) Minimal + NH_4Cl	1.38	(100)	3.62	(100)	1.83	(100)
(2) Minimal + $AspNH_2$	1.85	134	4.98	137	1.51	83
(3) Minimal + NH_4Cl + $AspNH_2$	1.89	137	3.55	98	1.28	70

[a] 10^2 x µmoles aspartic acid released from [^{14}C]-asparagine/mg protein/min.

[b] Ammonium chloride and asparagine were present at 1 mg/ml and 100 ug/ml respectively.

The assay procedure and method of preparation of extracts are described on pp. 39,43 Extracts were prepared simultaneously from bacteria grown under identical conditions to minimize random variations.

(b) DEAE-cellulose chromatography of E. coli E563 fraction AS II

Fraction AS II obtained from E. coli E563 grown in complete medium (see p.39 for details) was further fractionated by DEAE-cellulose chromatography. A sodium chloride gradient (0 to 0.35M) followed by 1.0M sodium chloride was used for elution. β-cyano-alanine hydrolase was found to co-chromatograph with the single asparaginase (EC II) peak (Figure V.1). Asparagine was hydrolyzed at a rate 8.7 times that of β-cyano alanine by the peak fraction. In this calculation a correction was made for the fact that the β-cyano-alanine hydrolase was not saturated with substrate in the assay, (p.84). A complete repeat of this fractionation gave an identical elution pattern for protein and for β-cyano-alanine hydrolase and asparaginase activities; in this the asparaginase:hydrolase ratio was 7.9, which agrees well with the first value.

(c) Sephadex G-100 chromatography of E. coli E563 fraction AS II

The same AS II fraction from E. coli E563 grown in complete medium was also fractionated by Sephadex G-100 chromatography. A single peak of β-cyano-alanine hydrolase and of asparaginase EC II activity was obtained using the assay procedures described previously for the DEAE-cellulose chromatography. The peak fractions were pooled and the protein

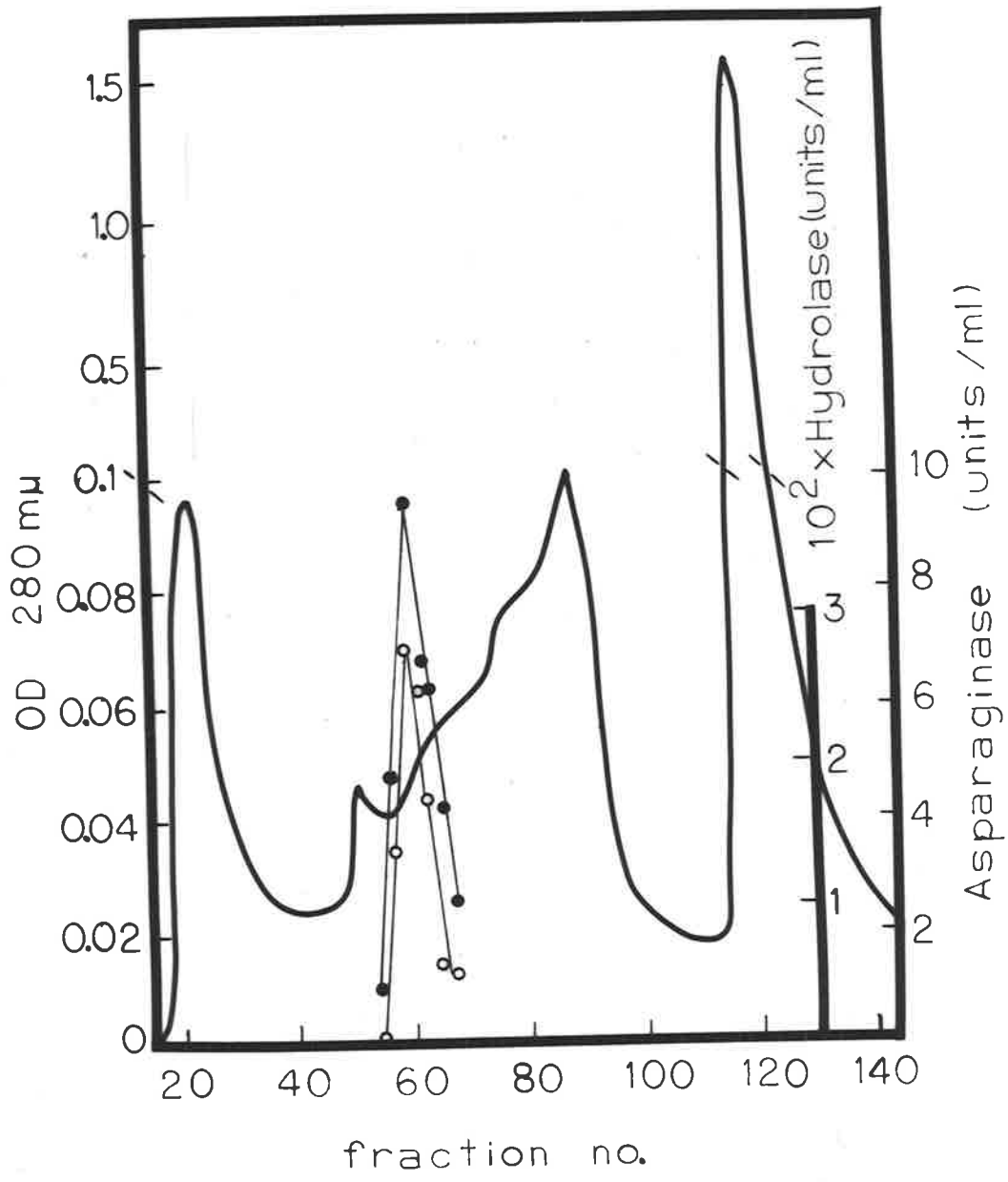
FIGURE V1. DEAE-CELLULOSE CHROMATOGRAPHY OF E. COLI E563 FRACTION AS II.

The DEAE-cellulose column (25 x 2.5 cm) was equilibrated with 0.02M sodium phosphate, pH 8.0. One ml of the AS II fraction (26.5 mg protein) in the same phosphate buffer was loaded onto the column and eluted with a continuous sodium chloride gradient (0 to 0.35M-sodium chloride in 0.02M-sodium phosphate, pH 8.0). At the completion of this (fraction 102) the column was further eluted with 1.0M-sodium chloride in 0.02M-sodium phosphate, pH 8.0. The optical densities of the fractions at 280mμ were determined. Fractions were assayed for asparaginase activity (p.43) and also for β-cyano-alanine hydrolase activity (as described on page 42 except that 0.064μmoles of [^{14}C]-β-cyano-alanine was used in each assay and the total assay volume was reduced to 0.2 ml). Activity is expressed as units/ml since protein concentrations were not detectable by the method of Lowry *et al*. One unit of activity is equal to that quantity of enzyme which will hydrolyze 1 μmole of substrate per hour in the standard assay.

○ ——— ○ β-cyano-alanine hydrolase activity

● ——— ● asparaginase activity

——— OD at 280 mμ.


1.5
1.0
0.5
0.1
0.08
0.06
0.04
0.02
0
OD 280 mμ
10^2 x Hydrolase (units/ml)
3
2
1
10
8
6
4
2
Asparaginase (units/ml)
20
40
60
80
100
120
140
fraction no.

precipitated with ammonium sulphate, redissolved in buffer and again fractionated on Sephadex G-100. Co-chromatography of the β-cyano-alanine hydrolase and asparaginase EC II activities was clearly demonstrated (Figure V.2). Asparagine was hydrolyzed at a rate 8.3 times that of β-cyano-alanine by the peak fraction. This value agrees with that obtained from the DEAE-cellulose chromatography (see p. 94). A molecular weight of approximately 60,000 was calculated for the β-cyano-alanine hydrolase and EC II asparaginase from this fractionation.

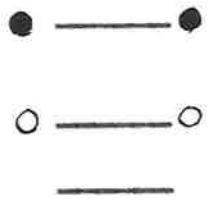
(d) Polyacrylamide gel-electrophoresis of the purified β-cyano-alanine hydrolase and EC II asparaginase

Aliquots of the peak fractions from the above DEAE-cellulose and Sephadex G-100 procedures were subjected to polyacrylamide gel-electrophoresis. The sample from DEAE-cellulose showed 1 major and 6 minor bands of protein (Figure V.3). The sample from Sephadex G-100 showed a major protein band running in the same position as that given by the DEAE-cellulose sample and four more minor bands, none of which corresponded exactly with the minor bands of the other sample (Figure V.3).

Attempts to directly demonstrate asparaginase and hydrolase activities in a single band (Figure V.3) were unsuccessful. Although asparaginase was found in the major

FIGURE V.2: CHROMATOGRAPHY OF E. COLI E563 FRACTION AS II ON SEPHADEX G-100

The Sephadex G100 column (45 x 1.5 cm) was equilibrated with 0.02M-sodium phosphate, pH 8.0. One ml of the AS II fraction (26.5 mg protein) was loaded onto the column in the phosphate buffer above and the column developed with the same buffer. The optical density of each fraction at 280 mμ was determined. Fractions were assayed for asparaginase and β-cyano-alanine hydrolase activity as described in the legend to the previous figure (Figure V.1). Activities are expressed as units/ml as defined in Figure V.1.



β-cyano-alanine hydrolase activity

asparaginase activity

OD at 280 mμ.

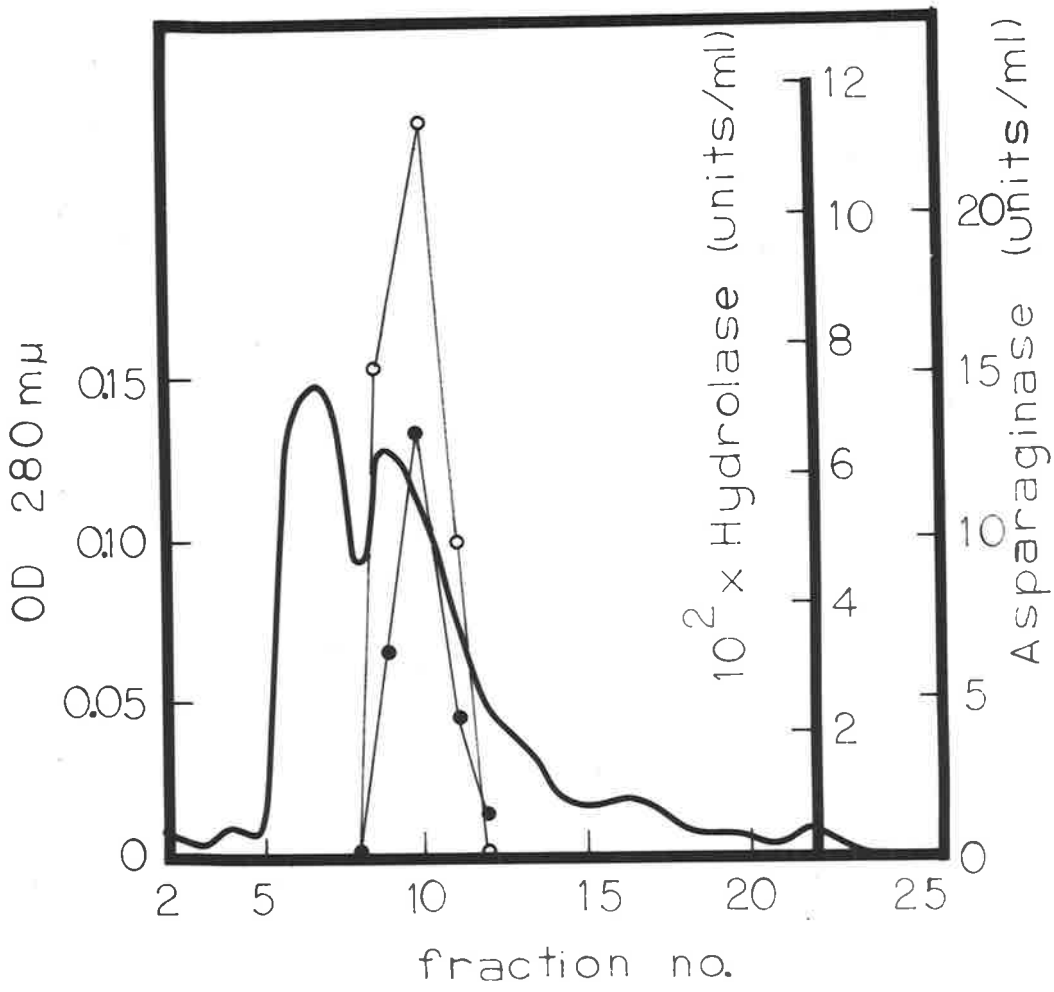

OD 280 mμ
0.15
0.10
0.05
0
2
5
10
15
20
25
fraction no.
10^2 x Hydrolase (units/ml)
12
10
8
6
4
2
Asparaginase (units/ml)
20
15
10
5
0

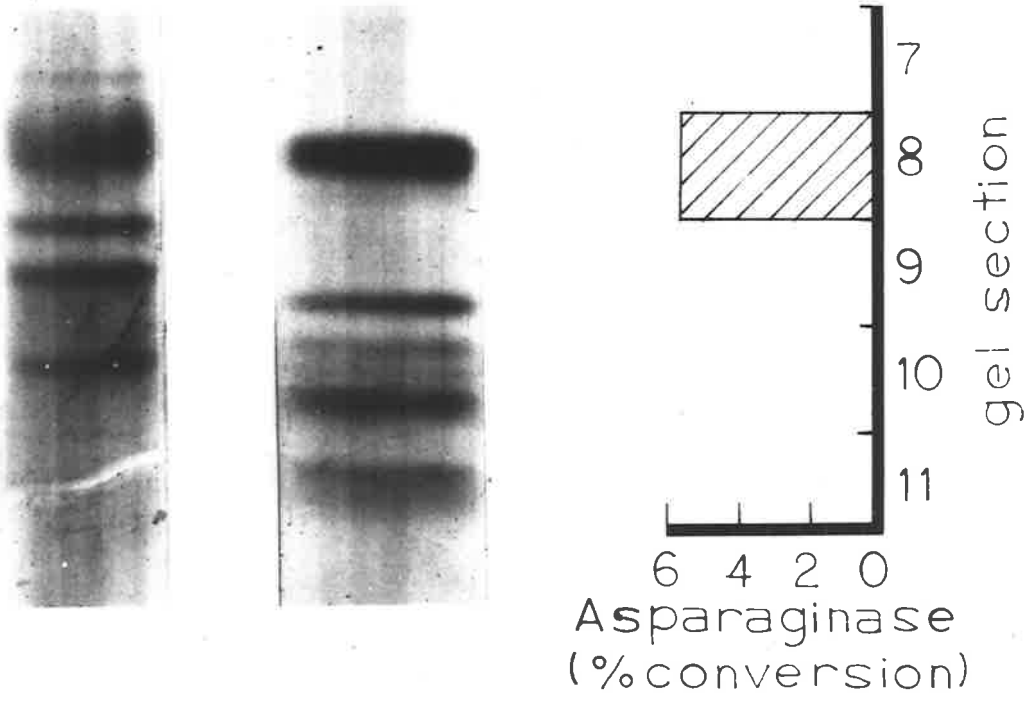
FIGURE V.3: POLYACRYLAMIDE GEL-ELECTROPHORESIS OF THE PURIFIED β-CYANO-ALANINE HYDROLASE AND EC II ASPARAGINASE

The fractions from the DEAE-cellulose and Sephadex G-100 chromatography containing the β-cyano-alanine hydrolase and asparaginase activities were pooled (vol. approx. 10 ml) and solid ammonium sulphate added to give a concentration of 4M. The precipitated protein was collected by centrifugation and redissolved in 1.0 ml of 0.05 M-Tris HCl buffer (8.0). Following dialysis against the same buffer a sample of this concentrated enzyme preparation was subjected to electrophoresis for 60 min. on 7% polyacrylamide separating gel at pH 9.5 as described on p. 47. The gel was longitudinally sliced and one of the halves stained with amido black for protein. For the determination of asparaginase and β-cyano-alanine hydrolase activity the other half of the gel was sliced into 0.1" sections. These were gently agitated in 0.05M-tris-HCl pH 7.8 buffer (0.5 ml) for 16 hours. Aliquots (0.2 ml) were incubated for 30 minutes at 37° with either [^{14}C]-β-cyano-alanine (0.064 μmoles; 14,000 cpm; 0.02 ml), or [^{14}C]-asparagine (0.2 μmoles; 0.025 μC; 0.01 ml).

Left. Gel showing the position of the protein bands from the Sephadex G-100 fractionated AS II sample.

Middle. Gel showing the position of the protein bands from the DEAE-cellulose fractionated AS II sample.

Right. Asparaginase activity found in the fractions of the middle gel.


7
8
9
10
11
gel section
6 4 2 0
Asparaginase
(% conversion)

band it was less than 5% of that loaded on the gel; at this level the failure to detect β-cyano-alanine hydrolase activity in the same band has no significance.

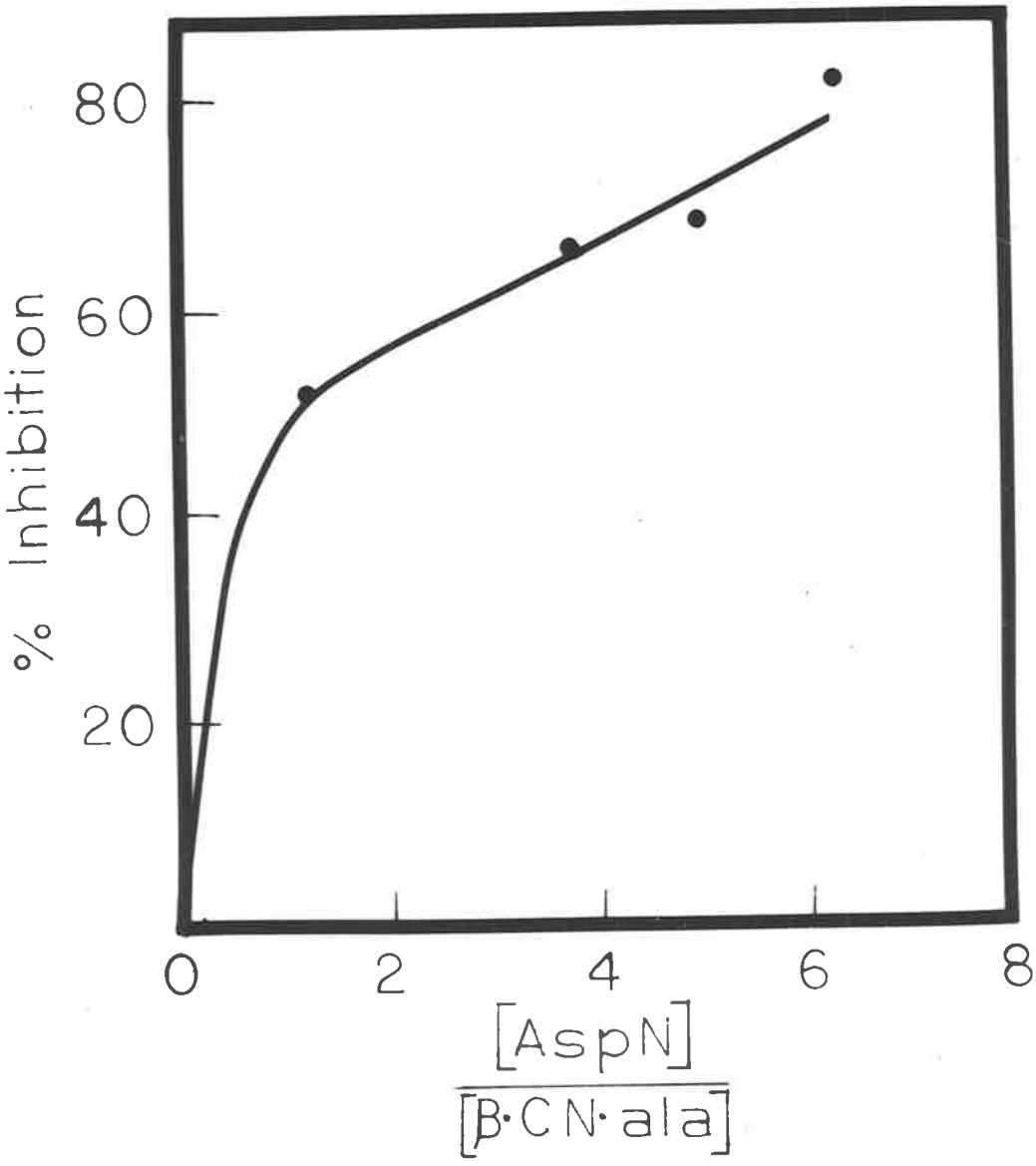
3. The apparent inhibition of β-cyano-alanine hydrolase by asparagine

The purification studies reported above indicated that the hydrolysis of β-cyano-alanine to asparagine in E. coli may be catalyzed by the EC II asparaginase. To examine this point further the effect of asparagine on the hydrolysis of β-cyano-alanine in E. coli extracts was studied.

The hydrolysis of [^{14}C]-β-cyano-alanine to asparagine and aspartic acid was inhibited by the addition of asparagine to the assay system (Figure V.4). But, since the EC II fraction rapidly hydrolyzes asparagine it is possible that the inhibition was due to aspartic acid. This was not investigated but Lauinger and Ressler (1970) have recently and independently confirmed that the hydrolysis of β-cyano-alanine is catalyzed by a partially purified preparation of the EC II asparaginase; furthermore this hydrolysis is inhibited 50% by aspartic acid when present at 20% of the concentration of β-cyanoalanine.

FIGURE V.4: THE APPARENT INHIBITION OF β-CYANO-ALANINE HYDROLASE BY ASPARAGINE

The hydrolysis of [^{14}C]-β-cyano-alanine (0.32mM) by an E. coli E563 AS II fraction (11.6 mg protein/ml) was determined in the presence of varying concentrations of asparagine (0 to 2.0 mM). The assay procedure is described on p. 42 . A 30 minute incubation period was used. The E. coli E563 AS II fraction was prepared from cells grown in a complete medium as described on pp. 30,39.


80
60
40
20
% Inhibition
0
2
4
6
8
[AspN]
[β·CN·ala]

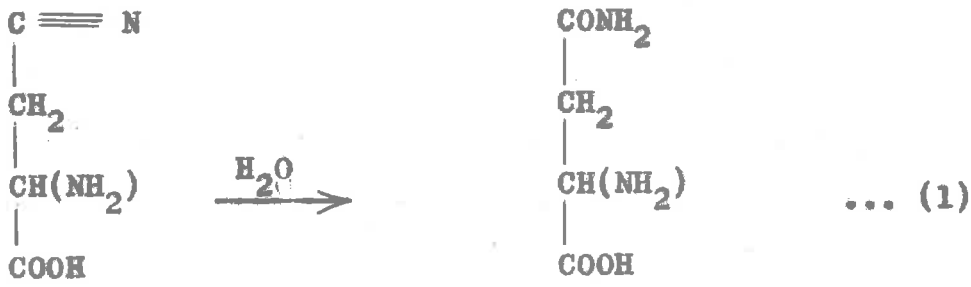
C. DISCUSSION

The demonstration of the enzymic formation of β-cyano-alanine from cyanide and cysteine in E. coli E596 confirms the existence of a β-cyano-alanine synthase in this organism as reported by Dunnill and Fowden (1965). However, it was not possible to detect synthase activity in all of the strains of E. coli examined. Despite the reservation that negative results of this type are necessarily inconclusive the physiological significance of β-cyano-alanine synthase in asparagine biosynthesis in E. coli must be questioned since those strains lacking the enzyme are not asparagine auxotrophs. Furthermore, none of the strains of E. coli examined were found to have both detectable β-cyano-alanine synthase and hydrolase activity in the standard assays.

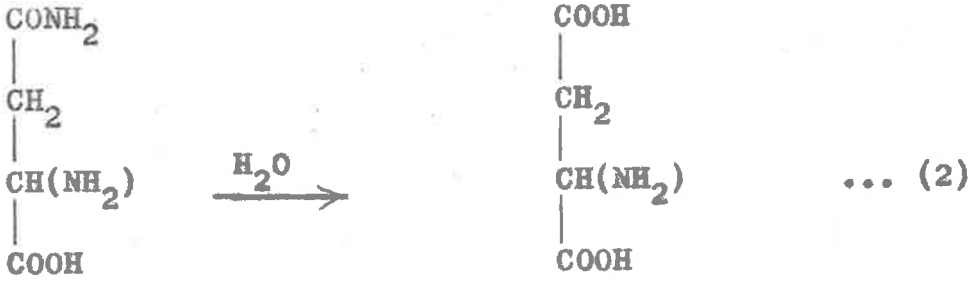
The cyanide toxicity studies show that 10^{-3}M cyanide completely inhibited growth. At 10^{-5}M, growth was inhibited 24% in a complete medium and 42% in a minimal medium. Therefore with a Km of 1.3×10^{-2}M reported for cyanide (Dunnill and Fowden, 1965) the rate of β-cyano-alanine synthesis (and of asparagine synthesis) by this pathway could only be a minute percentage of the maximum possible. (This of course is assuming that no metabolic compartmentation factors are present.)

The hydrolysis of β-cyano-alanine (Equation V.1) has

been proposed to be mediated by the action of an asparaginase (Equation V.2), (Giza, Ratzkin and Ressler, 1963). The work in this chapter suggests that the hydrolysis of β-cyano-alanine in E. coli extracts is catalyzed by the EC II asparaginase. Final proof of this can only be the isolation of a pure protein with both activities. Although this was not achieved, the fact that the two activities co-chromatographed on DEAE-cellulose and Sephadex G-100 strongly suggests that the EC II asparaginase hydrolyzes both asparagine and β-cyano-alanine. A preliminary account of this work has been published (Farnden and Egan, 1969). The parallel induction of these two activities also supports this conclusion. The induction of the EC II asparaginase during growth on an enriched medium has been recently independently confirmed (Cedar and Schwartz, 1968). The relative variation in the levels of the EC I and EC II asparaginases in various E. coli strains has also been recently and independently confirmed by a study of the enzymes in 28 strains of E. coli by Bilimoria (1969). A "highly purified crystalline" commercially available preparation of EC II asparaginase has been shown in the recent work of Jackson, Cooney and Handschumacher (1969) and Lauinger and Ressler (1970) to hydrolyze β-cyano-alanine as well as asparagine. EC II Asparaginase has further been shown to hydrolyze L-glutamine and D-asparagine



β-cyano-alanine asparagine ... (1)



asparagine aspartic acid ... (2)

(Campbell and Mashburn, 1969; Miller and Balis, 1969) and the decomposition of an asparagine analogue, diazo-4-oxo-L-norvaline (DONV), (Jackson, Cooney and Handschumacher, 1969; Cooney and Handschumacher, 1970), at the same site of the enzyme.

The present studies on the cyanide pathway enzymes

have raised doubts as to their significance in the biosynthesis of asparagine in E. coli mainly because of the following considerations. The E. coli strains that apparently lacked β-cyano-alanine synthase activity were not asparagine auxotrophs and no strain of E. coli examined was found to have abundant synthase and β-cyano-alanine hydrolase activity. It seems unlikely that a significant synthesis of asparagine could occur at non-toxic cyanide concentrations in view of the high Km reported for the cyanide ion. Finally from the purification and induction studies on the EC II asparaginase and β-cyano-alanine hydrolase it is likely that the EC II asparaginase hydrolyzed β-cyano-alanine but more slowly than asparagine. It must finally be stressed nevertheless that these studies have not provided conclusive evidence against the cyanide pathway being physiologically significant in asparagine biosynthesis in E. coli.

D. SUMMARY

1. β-cyano-alanine synthase, β-cyano-alanine hydrolase and asparaginase (EC I and EC II) were demonstrated in E. coli extracts.

2. E. coli β-cyano-alanine hydrolase and EC II asparaginase were induced by growing the cells in an enriched

medium. Asparagine alone did not induce the asparaginase activities.

3. β-cyano-alanine hydrolase activity was detected in the EC II asparaginase fraction isolated by ammonium sulphate fractionation.

4. β-cyano-alanine hydrolase and the EC II asparaginase were found to co-chromatograph on DEAE-cellulose and Sephadex G-100 chromatography.

5. The partially purified EC II asparaginase was found to hydrolyze β-cyano-alanine at a rate one eighth that of asparagine.

6. Asparagine was found to inhibit the hydrolysis of β-cyano-alanine but was rapidly hydrolyzed to aspartic acid during the reaction.

CHAPTER VI

A BIOCHEMICAL-GENETIC STUDY OF THE BIOSYNTHESIS OF ASPARAGINE BY THE CYANIDE PATHWAY ENZYMES OF E. COLI

A BIOCHEMICAL-GENETIC STUDY OF THE BIOSYNTHESIS OF ASPARAGINE BY THE CYANIDE PATHWAY ENZYMES OF E. COLI.

A. INTRODUCTION

A genetic study of the biosynthesis of asparagine in E. coli was undertaken with the aim of investigating the physiological significance of the cyanide pathway enzymes in the biosynthesis of asparagine.

The plan was to isolate an asparagine auxotroph of E. coli. It was reasoned that if the cyanide pathway of asparagine biosynthesis is physiologically significant then it might be expected that the asparagine requirement for such an auxotroph might be satisfied by a cyanide pathway intermediate (assuming that it was able to enter the cell). In addition it might be expected that one or more of the cyanide pathway enzymes would be absent (assuming that cyanide itself was not the missing metabolite). Alternatively, if the cyanide pathway is not involved in asparagine biosynthesis different findings would be expected.

Previously Giza, Ratzkin and Ressler (1963) reported that the asparagine requirement of a Neurospora crassa auxotroph was partially satisfied by β-cyanoalanine and proposed that β-cyanoalanine was hydrolyzed to asparagine by an asparaginase. No studies on the asparaginase or the cyanide pathway enzymes were, however, reported.

B. A GENETIC STUDY OF THE BIOSYNTHESIS OF ASPARAGINE FROM CYANIDE VIA β-CYANO-ALANINE

1. Isolation of an E. coli asparagine auxotroph

Wild type E. coli E596 cells were mutagenized by NNG treatment (see p. 45) which resulted in a 97.4% kill of the cells. The mutagenized culture was then incubated at 37° overnight, with shaking , in a minimal medium supplemented with low glucose (0.3% w/v, i.e. one third the normal level) and asparagine (100 μg/ml). Enrichment with respect to asparagine auxotrophs was carried out by penicillin treatment (see p. 45) which produced a further 99.8% kill.

Following the mutagenesis and penicillin enrichment approximately 5,000 cells were plated onto minimal plates supplemented with asparagine (100 μg/ml), (100 colonies per plate) and incubated for 24 hours at 37°. These plates were then replicated successively onto (a) minimal plus asparagine (100 μg/ml), (b) minimal, (c) minimal plus aspartic acid (100 μg/ml) and (d) tryptone broth plates, and incubated a further 24 hours at 37°. Minimal plus aspartic acid plates were included because in preliminary attempts to isolate asparagine auxotrophs, mutants were isolated that appeared to be asparagine auxotrophs but proved on further examination to be aspartic acid auxotrophs. They were presumably selected due to the hydrolysis of asparagine to aspartic acid during the prolonged incubation times at 37°.

Suspected asparagine auxotrophs (i.e. growth on (a) and (d) only) were further characterized as described in the following section.

2. Characterization of an E. coli asparagine auxtroph

(a) Growth studies in liquid and on solid media

Suspected asparagine auxotrophs (45 from the 5,000 colonies replicated) were streaked onto tryptone broth plates and incubated overnight. Cells from single colonies were inoculated successively onto selective agar plates as in the previous section. The sterile toothpick technique was used for inoculum transfers. An asparagine auxotroph isolated from a single colony showed itself by growth on plates (a) and (d) but not on plates (b) or (c). A single asparagine auxotroph was isolated in this way and will be designated as E. coli M36.

The growth of this asparagine auxotroph was compared with that of the wild type, E. coli E596 in liquid culture. Minimal media and minimal media supplemented with various levels of asparagine were inoculated with E. coli E596 and M36. The growth of the two organisms are shown in Figure VI.1. It can be seen that M36 shows a specific requirement for asparagine. Concentrations higher than 10 μg/ml are required for full growth of the mutant; at 100 μg/ml growth is the same as that of wild type.

(b) Susceptibility of the asparagine auxotroph to λ phage infection. As no genetic markers were

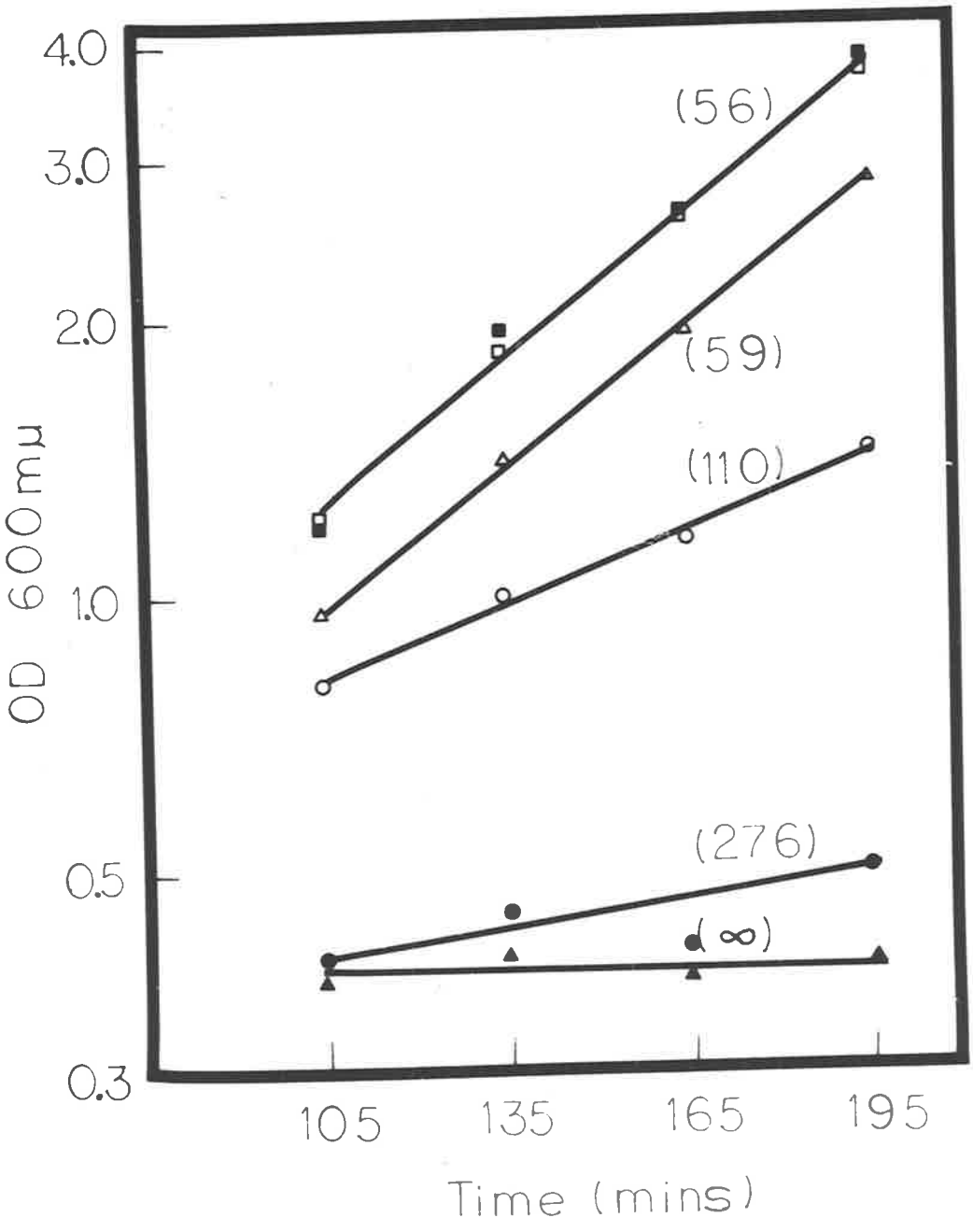

4.0
3.0
2.0
1.0
0.5
0.3
OD 600mμ
(56)
(59)
(110)
(276)
(∞)
105
135
165
195
Time (mins)

FIGURE VI.1. GROWTH CURVES OF E. COLI E596 AND M36 IN VARIOUS MINIMAL MEDIA

Overnight cultures of E. coli M36 and E596 grown in minimal medium (supplemented with asparagine for M36) were washed twice with minimal medium and inoculated into the media indicated below. Growth was followed by recording the optical density at 600 mμ. Generation times (min.) are indicated in the brackets in the figure.

(▲) M36 in minimal medium

(●) M36 in minimal medium + asparagine 1 μg/ml

(○) M36 in minimal medium + asparagine 10 μg/ml

(△) M36 in minimal medium + asparagine 100 μg/ml

(■) E596 in minimal medium

(□) E596 in minimal medium + asparagine 100 μg/ml.

present in the parent strain for confirmation that E. coli M36 was indeed an E. coli mutant and not a contaminant, the susceptibility of the asparagine auxotroph to λ phage infection was investigated.

E. coli M36 was shown to be susceptible to infection by this virus (see p.45 for details) prepared from the ultraviolet inducible E. coli E511 (C600, λ). This confirms that an E. coli asparagine auxotroph had indeed been isolated.

(c) Location of the genetic locus for the asparagine requirement of E. coli

The asparagine (asn) marker was mapped approximately by interrupted mating experiments using the Hfr-O-13 conjugation system (see p.46 for details). The asn marker of E. coli M36 was found to be transferred by the Hfr-AB2528 within the first two minutes of the formation of the mating mixture. This shows that the asn marker is located between the 72nd and 74th minute positions on the E. coli chromosome (Figure VI.2).

3. The biosynthesis of asparagine via the cyanide pathway in the E. coli asparagine auxotroph

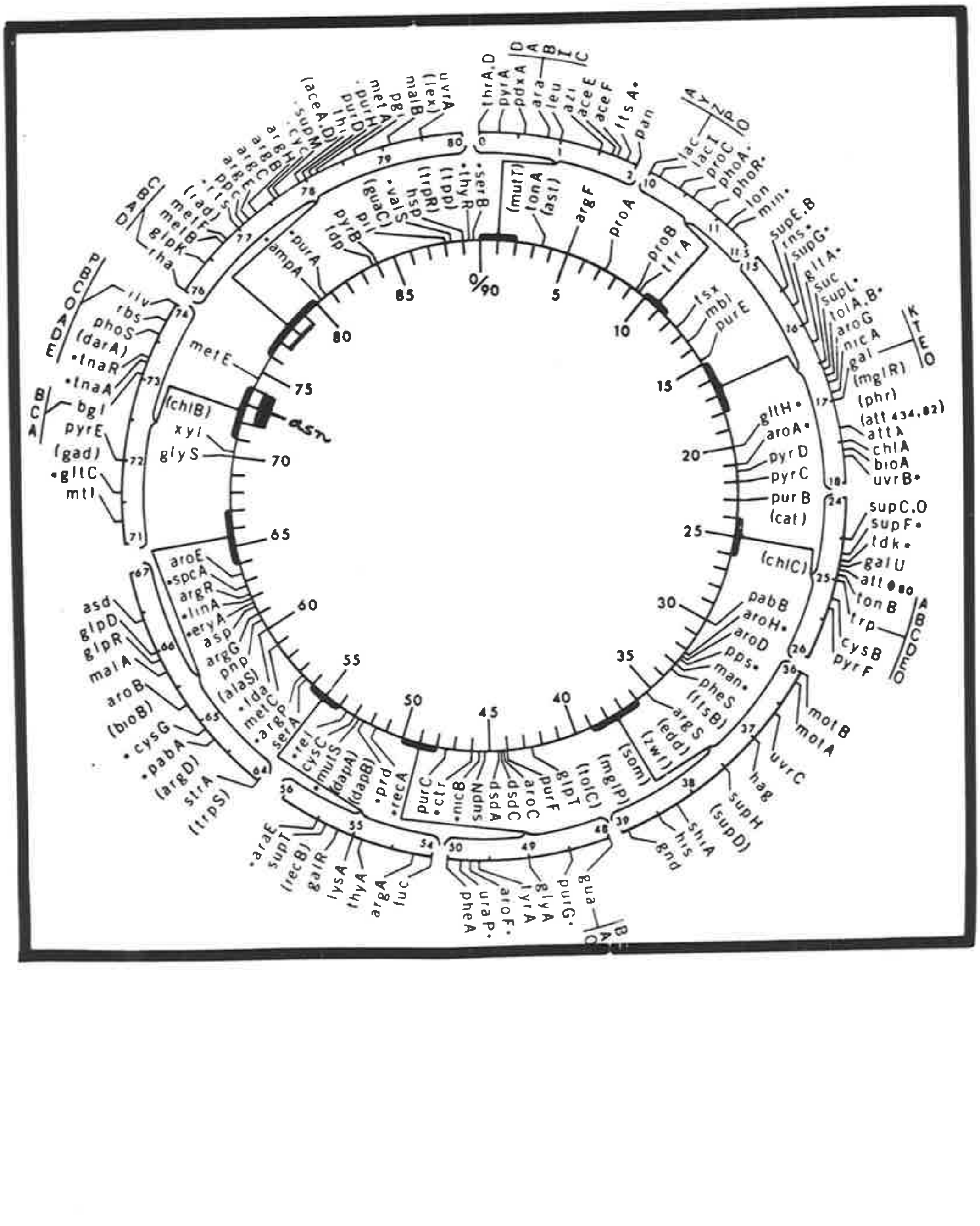
The following studies were carried out to establish whether the mutant E. coli M36 lacks an enzyme of the cyanide pathway.

(a) An attempt to demonstrate the in vivo utilization of cyanide in asparagine biosynthesis by E. coli E596 and M36

Late log-phase cultures of both E. coli E596 and M36 growth in minimal medium were inoculated into an equal

FIGURE VI.2: THE GENETIC MAP OF THE E. COLI CHROMOSOME SHOWING THE POSITION OF THE asn LOCUS

The asn locus was found by interrupted mating experiments to lie between the 72nd and 74th minute position on the E. coli chromosome as indicated on the map opposite. The genetic map reproduced here is from Taylor and Trotter (1967). The genetic symbols shown are defined by these authors.



0/90
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
argF
proA
proB
tsx
purE
gltH
aroA
pyrD
pyrC
purB
(cat)
(chlC)
pabB
aroH
aroD
supC,O
supF
tdk
galU
tonB
trp
cysB
pyrF
motB
motA
uvrC
hag
supH
(supD)
his
gnd
metE
(chlB)
asn
xyl
glyS
pyrE
(gad)
gltC
mtl
asd
glpD
glpR
malA
aroB
(bioB)
cysG
pabA
(argD)
strA
(trpS)
aroE
spcA
argR
asp
argG
pnp
(alaS)
serA
ampA
purA
tdp
pyrB
thrA,D
pyrA
pdxA
ara
leu
uvrA
(lex)
malB
metA
ilv
rbs
phoS
(darA)
tnaR
tnaA
bgl
glpK
rha

volume of the medium containing [^{14}C]-potassium cyanide. Samples were removed and the cells collected by millipore filtration and the filters rapidly washed with minimal medium. The uptake of [^{14}C]-potassium cyanide by these cells is shown in Figure VI.3. After an initial 'lag' period of 20 minutes an approximately linear uptake of label occurred. The [^{14}C]-potassium cyanide incorporated by the E. coli M36 was 80% of that incorporated by the E. coli E596 in minimal medium either with and without asparagine (Figure VI.3).

The protein from these labelled cells was isolated and counted as described on p. 47 . No incorporation of the isotope from [^{14}C]-potassium cyanide into protein was detected. It would have been possible to detect 0.05% of the isotope taken up by the E. coli cells in the isolated protein.

The soluble amino acids were also isolated from labelled cells (see p. 47) and separated by (a) paper chromatography in a phenol solvent and (b) paper electrophoresis at pH 5.5, (methods are described on p. 36). No detectable synthesis of β-cyano-alanine, asparagine or aspartic acid from the labelled cyanide was found . However, a 5% conversion of the isotope take up by the E. coli cells would have been necessary for detection.

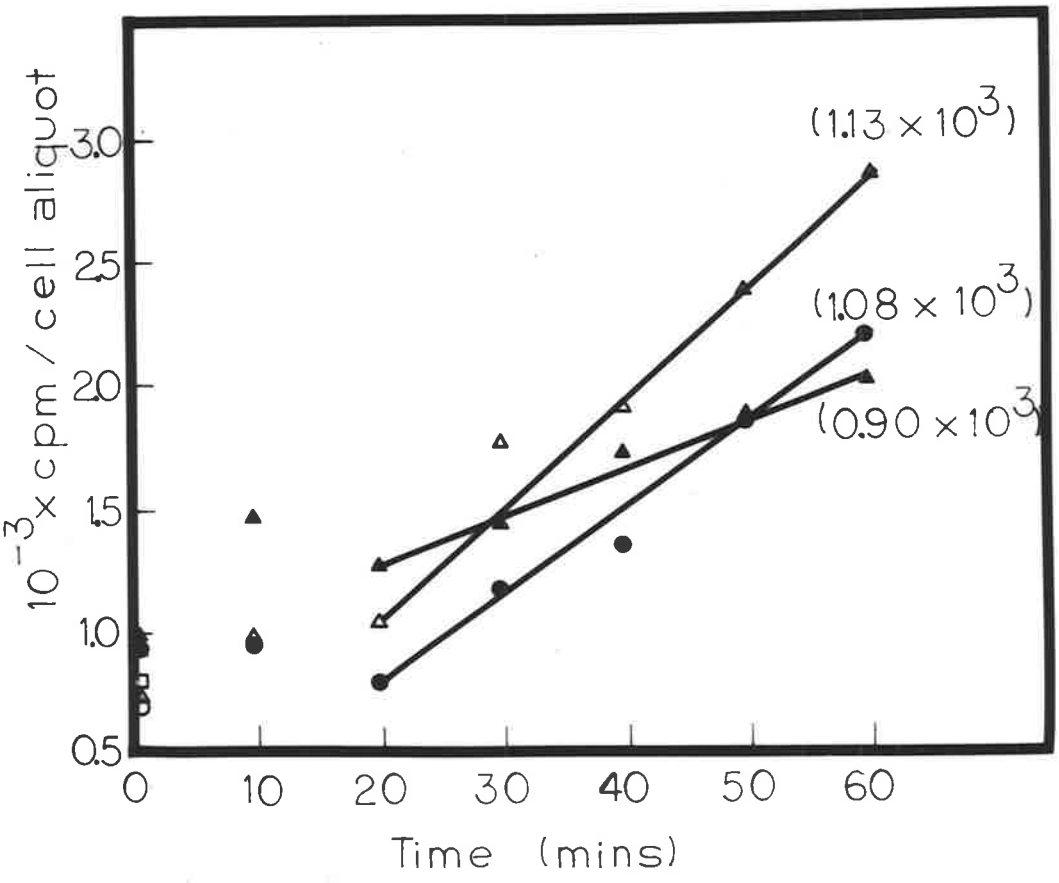
The studies (p. 83) on the inhibition of the growth of E. coli by cyanide suggest that the cyanide is penetrating the cells as does the continued accumulation of the isotope in Figure VI.3. Therefore, it seems likely that the cyanide

FIGURE VI.3: THE UPTAKE OF [^{14}C]-POTASSIUM CYANIDE BY E. COLI E596 AND M36 IN VIVO

Cultures of E. coli E596 were grown in minimal medium or in minimal medium plus asparagine (100 μg/ml). E. coli M36 was grown in the latter medium. The cultures reached optical densities at 600 mμ of 1.2, 1.6 and 1.0 respectively. These were diluted into an equal volume of medium containing [^{14}C]-potassium cyanide, (1.44×10^6 cpm, 45.2 μC/μmole) to give a total volume of 5 ml. Flasks were incubated at 37° with shaking. In addition, control flasks lacking cells or containing boiled cells were set up. Aliquots (0.18 ml) were removed and the cells collected on a millipore filter. The filter was washed with three successive 10 ml volumes of minimal medium. The filter was then dried and counted in a Scintillation Spectrometer.

The radioactivity incorporated per unit of cells in the 20 to 60 minute period are given in the brackets on the figure.

(□) Boiled cells control at zero time
(○) 'No cells' control at zero time
●——● E. coli E596 in minimal medium
△——△ E. coli E596 in minimal medium + asparagine (100 μg/ml)
▲——▲ E. coli M36 in minimal medium + asparagine (100 μg/ml).


10^{-3} x cpm / cell aliquot
3.0
2.5
2.0
1.5
1.0
0.5
0
10
20
30
40
50
60
Time (mins)
(1.13×10^3)
(1.08×10^3)
(0.90×10^3)

remains unchanged in the E. coli cells or is possibly complexed e.g. with aldehydes or imines giving cyanohydrins or aminonitrile respectively. However, the possibility that the cyanide is metabolized to either acidic or neutral compounds (mainly the organic acids and sugars respectively) is not ruled out.

Apparently neither the wild type E. coli E596 nor the asparagine auxotroph E. coli M36 can rapidly synthesize asparagine from exogenous cyanide (at least at a concentration of 3×10^{-6}M) in vivo.

(b) Attempts to satisfy the asparagine requirement of E. coli M36 with cyanide pathway intermediates

The results presented in the previous section suggest that asparagine is not synthesized significantly from exogenous cyanide (at least at a concentration of 3×10^{-6}M) via β-cyano-alanine. It was decided to examine this further by determining whether cyanide at a higher concentration (10^{-5}M and 10^{-4}M) could satisfy the asparagine requirement of the E. coli asparagine auxotroph. The results are shown in Table VI.1. Cyanide was unable to satisfy the requirement of E. coli M36 for asparagine at 10^{-4}M (Experiment 3) or at 10^{-5}M (Experiment 6). The situation is complicated by the toxicity of cyanide and the remaining experiments in Table VI.1 are controls for this. In these the effect of cyanide on growth in the presence of asparagine was tested. The conclusion to be drawn is that exogenous cyanide at 10^{-4}M will not replace asparagine; it should be noted that since 1 μg/ml

TABLE VI.1: ATTEMPT TO SATISFY THE ASPARAGINE REQUIREMENT OF *E. COLI* M36 WITH CYANIDE

Expt. No.	*E. coli* strain	Minimal medium supplemented with: potassium cyanide (M)	Minimal medium supplemented with: asparagine (μg/ml)	Cell density (OD 600mμ)	Growth of cells as percent of control
1	M36	0	10	0.059	(100)
2	M36	10^{-4}	10	0.041	69
3	M36	10^{-4}	0	0.006	0
4	M36	0	1	0.004	0
5	M36	10^{-5}	1	0.007	0
6	M36	10^{-5}	0	0.006	0
7	M36	0	0	0.006	0
8	E596	0	0	0.907	(100)
9	E596	10^{-4}	0	0.560	62
10	E596	10^{-5}	0	0.914	101

Washed E596 and M36 cells were used to inoculate the media indicated above. Cultures were incubated for 16 hours at 37° and the OD 600 mμ recorded as a measure of cell growth.

of asparagine (= 0.67 x 10^{-5}M) is insufficient for growth (Experiment 4) this experiment cannot be regarded as a particularly sensitive test. A slow conversion of cyanide to asparagine but insufficiently rapid to support growth is not ruled out. Nevertheless, it seems reasonable to conclude that the lesion in M36 is not due to a failure of the cell to produce cyanide.

As shown in Figure VI.4 the asparagine requirement of E. coli M36 could be partially replaced by β-cyano-alanine; at 100 μg/ml (0.88 mM) there was 38% of the growth found with 100 μg/ml, (0.67 mM) of asparagine. β-cyano-alanine was found to have no effect on the growth of the parent strain E. coli E596 nor had asparagine itself (Figure VI.4). The decreased growth observed with β-cyano-alanine as compared to asparagine is probably due to the low level of β-cyano-alanine hydrolase found in this strain (see p. 84).

(c) The demonstration of β-cyano-alanine synthase and β-cyano-alanine hydrolase activities in E. coli M36

β-cyano-alanine synthase activity was found in crude extracts of both E. coli E596 and M36 (Figure VI.5). Clearly therefore, the asparagine requirement of E. coli M36 is not due to the absence of this enzyme.

It was not possible to demonstrate β-cyano-alanine hydrolase activity in crude extracts of E. coli E596 or M36

FIGURE VI.4. THE PARTIAL ALLEVIATION OF THE ASPARAGINE REQUIREMENT OF E. COLI M36 WITH β-CYANO-ALANINE

Overnight cultures of E. coli M36 and E596 grown in minimal medium (supplemented with asparagine in the case of E. coli M36) were washed with minimal medium and inoculated into the media indicated below. Growth was determined by recording the optical density at 600 mμ. Generation times (min.) are indicated in the brackets in the figure.

(○) M36 in minimal medium

(●) M36 in minimal medium + β-cyano-alanine 100 μg/ml

(■) M36 in minimal medium + asparagine 100 μg/ml

(△) E596 in minimal medium

(▲) E596 in minimal medium + asparagine 100 μg/ml

(□) E596 in minimal medium + β-cyano-alanine 100 μg/ml.

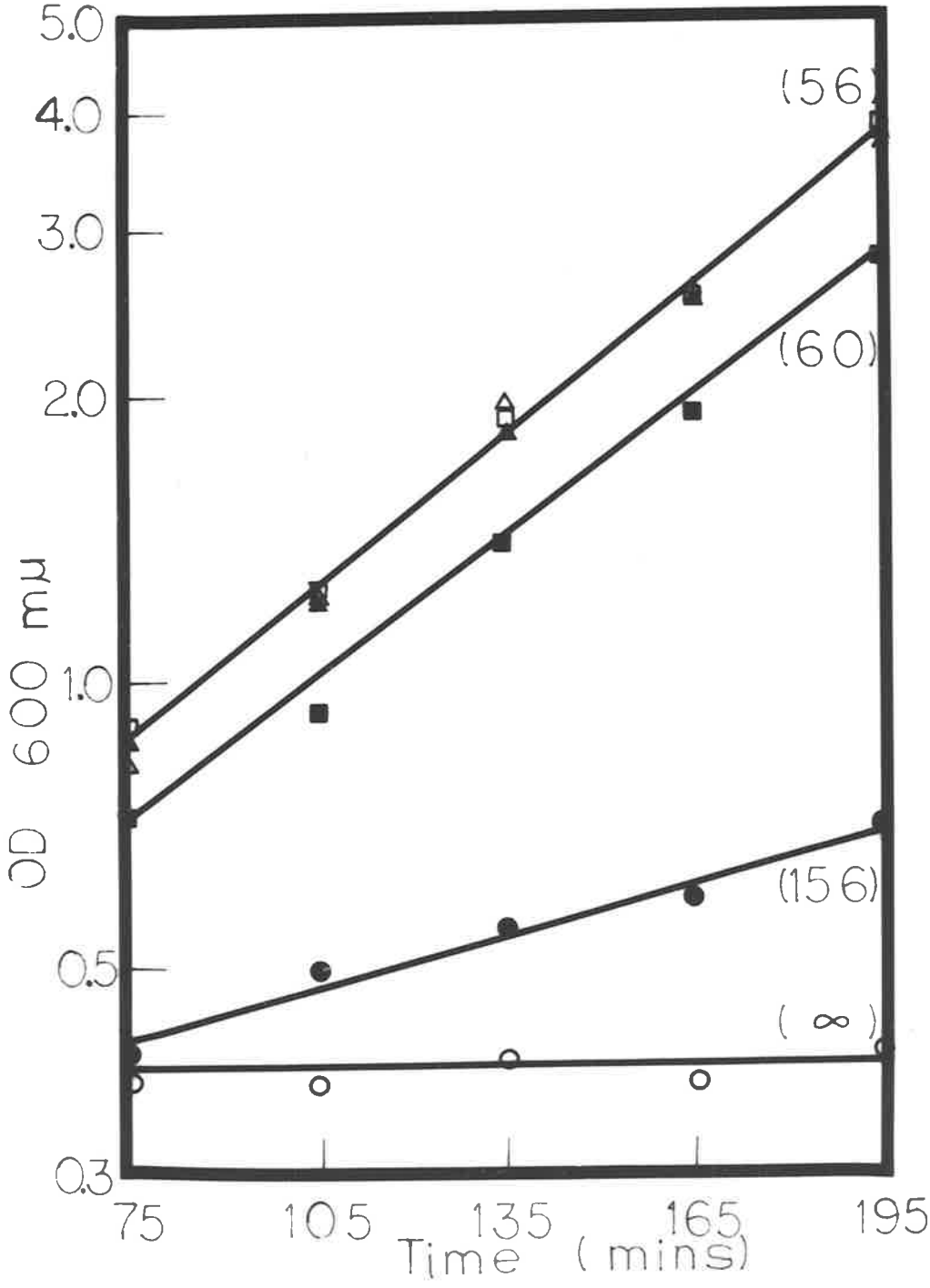

5.0
4.0
3.0
2.0
1.0
0.5
0.3
OD 600 mμ
(56)
(60)
(156)
(∞)
75
105
135
165
195
Time (mins)

FIGURE VI.5: β-CYANO-ALANINE SYNTHASE ACTIVITY IN *E. COLI* E596 and M36

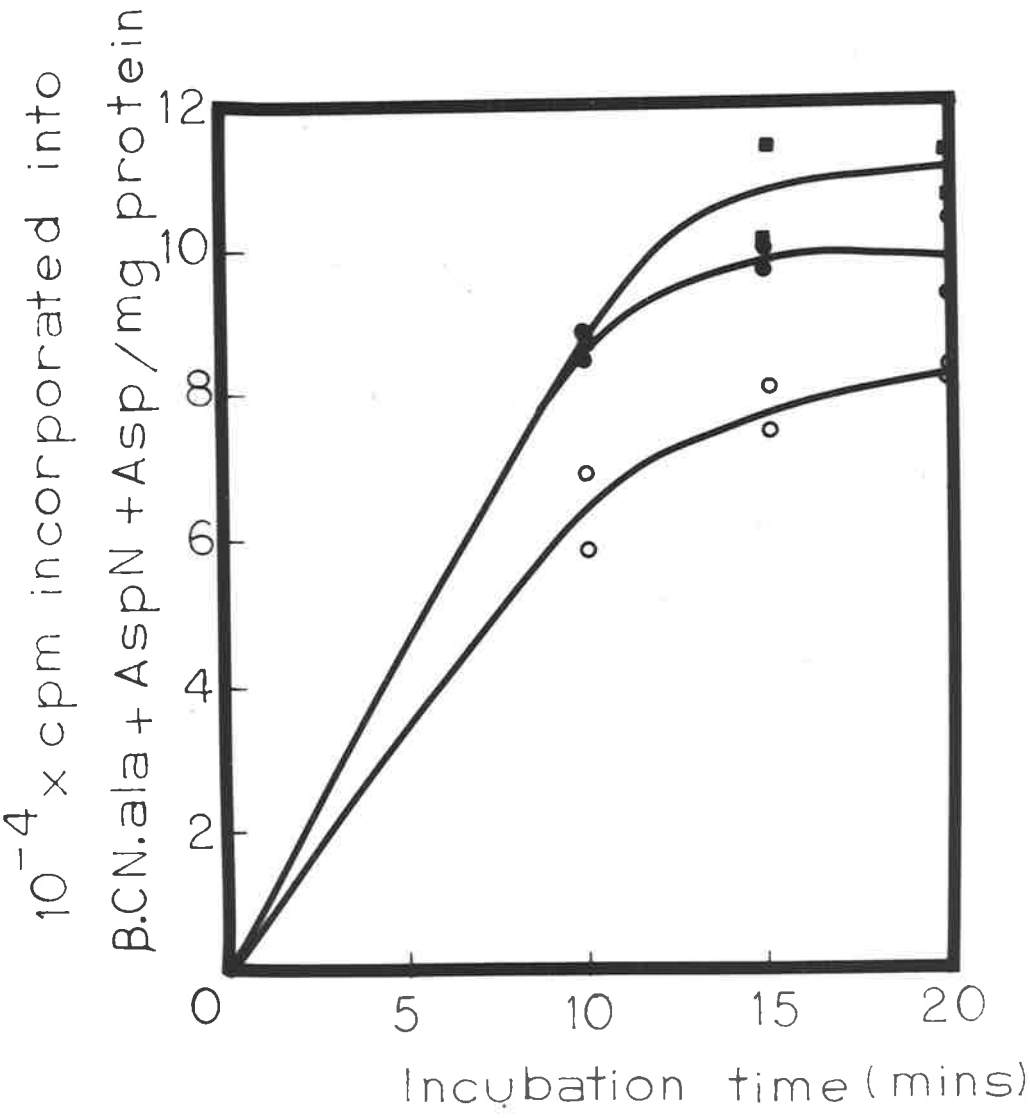
Crude extracts of *E. coli* E596 grown in minimal medium and *E. coli* E596 and M36 grown in minimal medium plus asparagine (100 μg/ml) were assayed for the synthesis of β-cyano-alanine from cysteine and [^{14}C]-potassium cyanide as described on p. 41. The labelling was determined in β-cyano-alanine, asparagine and aspartic acid at the completion of the incubation as described on p. 41.

The β-cyano-alanine synthase activity of;

● —● *E. coli* E596 grown in minimal medium

○ —○ *E. coli* E596 grown in minimal medium plus asparagine (100 μg/ml)

■ —■ *E. coli* M36 grown in minimal medium plus asparagine (100 μg/ml).


10^{-4} x cpm incorporated into
B.C.N.ala + AspN + Asp/mg protein
0
2
4
6
8
10
12
0
5
10
15
20
Incubation time (mins)

(see p. 84) under standard assay conditions, but the fact that virtually 100% of the label in the in vitro β-cyano-alanine synthase assay was found in aspartic acid and not in β-cyano-alanine or asparagine indicates the presence of a β-cyano-alanine hydrolase in the crude extracts of E. coli M36 and E596. The partial replacement of the asparagine requirement of M36 by β-cyano-alanine (Figure VI.4) is an additional indication of the existence of a β-cyano-alanine hydrolase in this mutant.

Asparaginase activity (presumably due to the high levels of ECI asparaginase found in this strain, see p.90) was also demonstrated in crude extracts of both E. coli E596 and M36 (Figure VI.6).

C. THE ASPARAGINE SYNTHETASE OF E. COLI

Following the studies described in the previous section of this chapter and Chapter V, a preliminary report of which was published (Farnden and Egan, 1969) Cedar and Schwartz (1969a,b) reported the demonstration of an asparagine synthetase in E. coli.

These authors demonstrated a synthesis of asparagine from aspartate and ammonia dependent on ATP and Mg^{++} in a mutant of E. coli K12 which produced only 5 to 10% of the EC 11 asparaginase of the wild type. The asparagine synthetase was also demonstrated by Cedar and Schwartz (1969a) in the

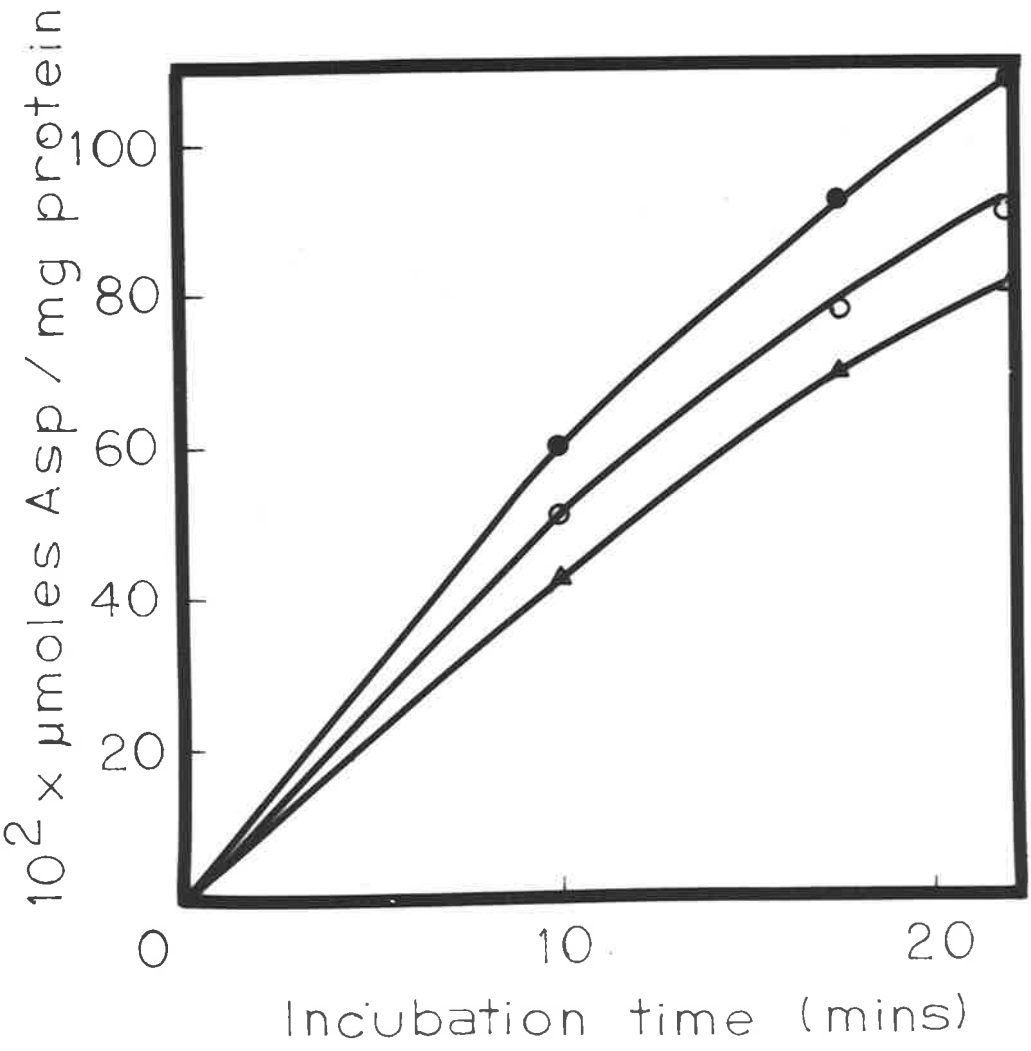
FIGURE VI.6: ASPARAGINASE ACTIVITY OF *E. COLI* M36 AND E596.

Crude extracts of *E. coli* E596 grown in minimal medium and *E. coli* E596 and M36 grown in minimal medium plus asparagine (100 µg/ml) were assayed for asparaginase activity as described on p. 43 . The asparaginase activity of;

●—● *E. coli* E596 grown in minimal medium

○—○ *E. coli* E596 grown in minimal medium plus asparagine (100 µg/ml)

△—△ *E. coli* M36 grown in minimal medium plus asparagine (100 µg/ml).


10^2 x µmoles Asp / mg protein
100
80
60
40
20
0
10
20
Incubation time (mins)

wild type E. coli using DONV to inhibit the EC 11 asparaginase.

Cedar and Schwartz (1969a) further demonstrated that this asparagine synthetase was not present in extracts of an asparagine requiring mutant. The genetic locus for the production of asparagine synthetase was found by these authors to lie between the 73rd and 74th minute of the E. coli chromosome.

It seemed of some importance therefore to confirm this, especially in view of the fact that the studies reported here gave no support for the belief that the cyanide pathway enzymes of E. coli are physiologically significant in asparagine biosynthesis. Furthermore, the E. coli asparagine auxotroph isolated in the present work mapped within the same region of the E. coli chromosome as that isolated by Cedar and Schwartz (1969a) (73rd - 74th and 72nd - 74th minute respectively).

1. In vitro biosynthesis of asparagine in E. coli

Initial unsuccessful attempts were made to demonstrate an asparagine synthetase in crude extracts of either E. coli E596 or M36. The extracts were prepared in 0.05M-tris-HCl, pH 8.0 from E. coli E596 cells grown in minimal medium and from E. coli E596 and M36 grown in minimal medium supplemented with asparagine. Using enzyme concentrations of 15, 1.5 and 0.15 mg protein/ml, attempts to demonstrate the synthesis of asparagine from [^{14}C]-aspartic acid,

ammonium and magnesium chlorides and ATP were unsuccessful. A time-dependent removal of [^{14}C]-aspartic acid was observed in these initial experiments (7% in 15 minutes) with a protein concentration of 15 mg/ml. Separation of the amino acids in a sample of the assay mixture revealed that a significant synthesis of alanine had occurred from the [^{14}C]-aspartic acid, but no labelling was detected in asparagine.

These crude extracts of E. coli E596 and M36 were known to contain asparaginase activity (see p. 87) and attempts were therefore made to inhibit this enzyme with carbobenzoxy-phenylalanine. This compound has been reported to be a potent competitive inhibitor of rat liver asparaginase (Mor and Lichtenstein, 1969). Carbobenzoxy-phenylalanine was found to inhibit the asparaginase activity of a crude E. coli E596 extract by 94% (Table VI.3).

A further attempt was therefore made to demonstrate the synthesis of asparagine in these crude extracts of E. coli using the procedure outlined above and including carbobenzoxy-phenylalanine in the incubation mixture. Again no synthesis of asparagine could be detected in any of the extracts examined. This could have been due to the rapid hydrolysis of any asparagine synthesized by the residual asparaginase activity (5%) still detected in the presence of carbobenzoxy-phenylalanine (Table VI.3). Unlabelled asparagine was therefore added to the incubation mixture to trap any asparagine synthesized from the [^{14}C]-aspartic acid. Under

TABLE VI.3: EFFECT OF CARBOBENZOXY-PHENYLALANINE ON THE ASPARAGINASE ACTIVITY OF E. COLI E596

Experiment	Asparagine mM	Cbz-phe mM	Ratio Cbz-phe / asparagine	Inhibition (%)
Control - no inhibitor	0.65	0	0	0
Inhibitor present	0.65	19.3	30	94

Crude E. coli E596 extract was prepared from cells grown in a minimal medium as described on p. 39 and contained 1.07 mg protein/ml. Asparaginase activity was determined as described on p. 43.

these conditions (i.e. in the presence of asparagine and carbobenzoxy-phenylalanine) asparagine synthesis was found to occur in a crude extract of E. coli E596 prepared from cells grown in a minimal medium, from [^{14}C]-aspartic acid, ammonium and magnesium chlorides and ATP (Figure VI.7). At the protein concentration used (1.5 mg/ml) no incorporation of label from the aspartic acid into alanine was observed. Using the same conditions that were necessary to demonstrate the synthesis of asparagine in the E. coli E596 extract no synthesis of asparagine could be detected in a crude extract of E. coli M36 (Figure VI, 7). In this case the cells were grown in a minimal medium supplemented with asparagine. It was possible therefore that the absence of the enzyme in this extract is due to repression of its synthesis by asparagine. However, this is not so, as a lower, but significant level of asparagine synthetase was found in an extract of E. coli E596 grown in a minimal medium supplemented with asparagine (Figure VI.7).

D. DISCUSSION

The results from the studies in this chapter support the concept that in E. coli the cyanide pathway enzymes are not physiologically significant in asparagine biosynthesis. The mutant E. coli M36 is most likely deficient in the same locus as the E. coli asparagine auxotroph isolated by Cedar and Schwartz (1969a), since both map at the same position on the E. coli chromosome. From this alone it might be deduced that

FIGURE VI.7. ASPARAGINE SYNTHETASE ACTIVITY OF E. COLI E596 AND M36.

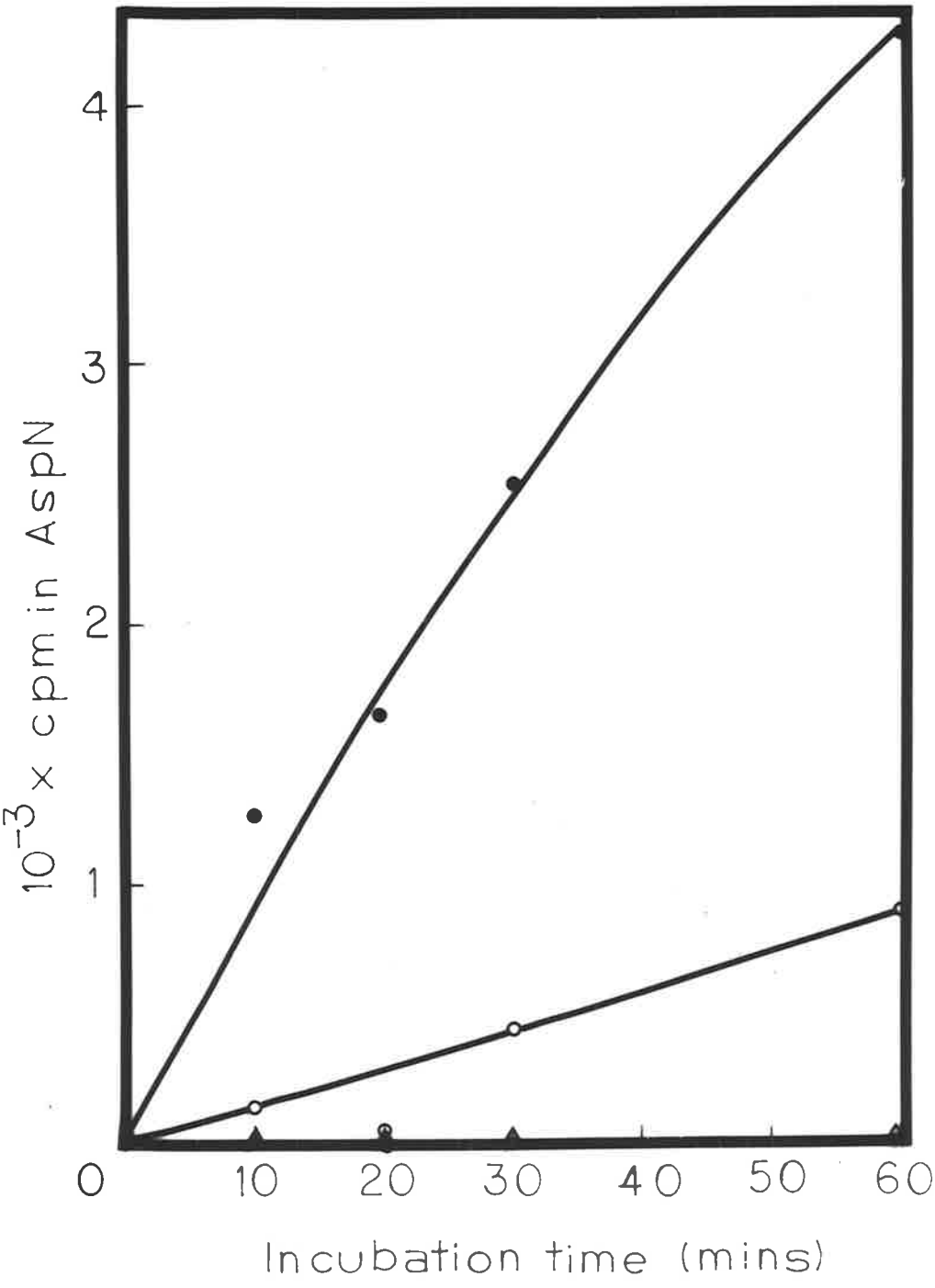
Crude extracts of E. coli E596 grown in minimal medium and E. coli E596 and M36 grown in minimal medium supplemented with asparagine (100 µg/ml) were prepared in 0.05M-tris-HCl, pH 8.0 as described on p. 39 . Asparagine synthetase activity was determined in the presence of carbobenzoxy-phenylalanine (20 mM) and asparagine (1 mM) as described on p. 43 .

The asparagine synthetase activity of;

●—● E. coli E596 grown in minimal medium

○—○ E. coli E596 grown in minimal medium supplemented with asparagine (100 µg/ml)

△—△ E. coli M36 grown in minimal medium supplemented with asparagine (100 µg/ml).


4
3
2
1
0
10
20
30
40
50
60
10^{-3} x cpm in AspN
Incubation time (mins)

E. coli M36 lacks an ATP-dependent asparagine synthetase; the *in vitro* studies reported here have confirmed this. Therefore, for the cyanide pathway to have a physiological role in asparagine biosynthesis it would be necessary to postulate that cells mutated in asparagine synthetase were also mutated in some enzyme function involved in the cyanide pathway, i.e. either in the production of cyanide, the synthesis of β-cyano-alanine or the hydrolysis of β-cyano-alanine to asparagine. This is most unlikely; the asparagine auxotroph isolated was not impaired in its ability to synthesize or hydrolyze β-cyano-alanine, and the asparagine requirement was partially alleviated by β-cyano-alanine. The asparagine requirement of this auxotroph could not be replaced by cyanide at the concentrations tested. The cyanide toxicity studies and the accumulation of the labelled cyanide by the *E. coli* cells indicate that exogenous cyanide penetrates the cells but both the wild type *E. coli* and the asparagine auxotroph were found not to significantly incorporate exogenous cyanide (present at a non-toxic level) into β-cyano-alanine, asparagine or aspartic acid. Although these considerations give no support to the cyanide pathway being physiologically significant in *E. coli* they are unfortunately not unequivocal. Nevertheless, if the cyanide pathway does operate, the mutant *E. coli* M36 must be blocked earlier than β-cyano-alanine synthase and cyanide must be produced at a rate greater than that achieved internally by supplying 10^{-4}M cyanide externally. It seems unlikely

that cells will reach a cyanide concentration greater than that which inhibits growth, but compartmentation possibilities have not been excluded. Finally for the cyanide pathway to be of significance one of the enzyme genes must map very close to asparagine synthetase.

Despite all of this it is interesting to note that a strain of Bacillus megaterium has been reported to incorporate labelled cyanide both in vivo and in vitro into β-cyano-alanine, asparagine and aspartic acid (Castric and Strobel, 1969). This strain of Bacillus megaterium was isolated by a cyanide enrichment technique from soil which had been cropped in flax (a cyanophoric plant) for 73 consecutive years. Furthermore, Brysk, Corpe and Hankes (1969), following their initial demonstration of the production of cyanide from the methyl group of glycine by non-proliferating cells of Chromobacterium violaceum (Michael, Hankes and Corpe, 1965) have now reported the in vivo incorporation of both exogenous cyanide and endogenous cyanide produced from glycine into β-cyano-alanine and asparagine by these cells. The relative physiological significance of the cyanide pathway as regards asparagine biosynthesis and cyanide detoxification in these cases has not as yet been determined.

The demonstrationhere of the in vitro biosynthesis of asparagine in E. coli from aspartate, ammonium and magnesium chlorides and ATP has confirmed the existence of an asparagine synthetase in E. coli first reported by Cedar and Schwartz

(1969a and 1969b). These authors have purified the enzyme some 370 fold and demonstrated that the synthesis of the amide resulted in the stoichiometric production of PPi and AMP. Glutamine could not replace ammonia as the amino donor. Studies on the mechanism reported by Cedar and Schwartz (1969b) support a two-step reaction mechanism involving an enzyme bound β-aspartyl-adenylate intermediate (Equation VI 1 and 2).

$$\text{Aspartate} + \text{ATP} + \text{E} \longrightarrow \text{E-aspartyl-AMP} + \text{PPi} \quad (1)$$

$$\text{E-aspartyl-AMP} + \text{NH}_3 \longrightarrow \text{Asparagine} + \text{AMP} \quad (2)$$

This reaction is identical to that proposed for the asparagine synthetases of Lactobacillus arabinosus (Ravel, Norton, Humphreys and Shive, 1962) and Streptococcus bovis (Burchall, Reichelt and Wolin, 1964).

The biosynthesis of asparagine by a partially purified preparation from the Novikoff hepatoma has recently been reported to proceed through the same β-aspartyl-adenylate intermediate but with glutamine as the preferred amino donor (Patterson and Orr, 1968). The synthesis of asparagine from aspartic acid in the presence of glutamine has also been demonstrated in chick embryo liver (Arfin, 1967) and in various guinea pig tissues (Holcenberg, 1969). A preliminary report by Al-Dawody and Varner (1961) indicated that asparagine was synthesized in yeast from aspartate and ammonia dependent on Mg^{++} and ATP by a reaction analogous to that of glutamine

synthesis which involved the intermediate formation of an enzyme bound β-aspartyl-phosphate resulting in the production of ADP and Pi. No detailed report, however, of this asparagine synthetase has as yet appeared. It would appear therefore that the biosynthesis of asparagine from aspartic acid by an asparagine synthetase in the vertebrates and micro-organisms is well documented. The biosynthesis of asparagine in plant tissues is, however, still not clearly resolved.

E. SUMMARY

1. An *E. coli* asparagine auxotroph has been isolated and characterized.

2. The genetic locus for the asparagine requirement of *E. coli* (asn) was mapped between the 72nd and 74th minute of the *E. coli* chromosome.

3. It was not possible to demonstrate the *in vivo* synthesis of asparagine from exogenous cyanide by either the asparagine auxotroph or the wild type *E. coli*.

4. The requirement for asparagine by the *E. coli* auxotroph could not be replaced with cyanide but was partially alleviated by β-cyano-alanine.

5. There was no significant difference in the levels of the cyanide pathway enzymes *in vitro* of the asparagine auxotroph and the wild type *E. coli*.

6. An asparagine synthetase was demonstrated *in vitro* in the wild type *E. coli*.

7. No asparagine synthetase activity could be detected in extracts of an *E. coli* asparagine auxotroph.

8. The level of asparagine synthetase found in the wild type *E. coli* was repressed by growing the cells on a minimal medium supplemented with asparagine.

CHAPTER VII

ATTEMPTS TO INVESTIGATE THE PHYSIOLOGICAL SIGNIFICANCE OF THE BIOSYNTHESIS OF ASPARAGINE BY THE CYANIDE PATHWAY IN PLANTS

ATTEMPTS TO INVESTIGATE THE PHYSIOLOGICAL SIGNIFICANCE OF THE BIOSYNTHESIS OF ASPARAGINE BY THE CYANIDE PATHWAY IN PLANTS

A. INTRODUCTION

The cyanide pathway remains the only route of asparagine biosynthesis reported to occur in plants that has been independently confirmed both *in vitro* and *in vivo* (see p. 75) As described in previous chapter *E. coli* was not found to rapidly incorporate exogenous cyanide (present at a non-toxic concentration) into β-cyano-alanine and asparagine (p. 107). But this situation in *E. coli* is in direct contrast to that found in plants, where a rapid *in vivo* incorporation of cyanide into β-cyano-alanine and asparagine occurs (p. 66). Km values of 5×10^{-6}M and 5×10^{-4}M for the cyanide ion have been reported for the β-cyano-alanine synthase of *Lotus tenuis* and *Lupinus angustifolius* (Floss, Hadwiger and Conn, 1965; Hendrickson and Conn, 1969), whereas a Km 1.3×10^{-2}M has been reported for the *E. coli* enzyme (Dunnill and Fowden, 1965). However, the significance of this rapid *in vivo* incorporation of cyanide into asparagine in plants has not been determined. With this problem in mind it was decided to return to the question of asparagine biosynthesis in plants.

Weissman (1959) and more recently, Siegel and Vogt (1968), Tromp and Ovaa (1969), and Hill-Cottingham and Cooper

(1970) have demonstrated that the application of a nitrogen fertilizer to plants increases the level of asparagine.

If a substantial increase in the synthesis of asparagine in wheat seedlings by ammonium sulphate feeding takes place then it might be used to determine whether normal metabolites are converted to cyanide (or to an intermediate on the cyanide pathway) by studying the incorporation of [^{14}C]-HCN into asparagine and β-cyano-alanine grown in the presence and absence of ammonium sulphate.

It was further considered possible that the major asparagine biosynthetic enzyme(s) would be induced following ammonium sulphate feeding if a large increase in the level of asparagine occurred. The occurrence of enzyme induction in plants in response to a change in a specific environmental factor is not unknown in the literature and has been recently reviewed by Filner, Wray and Varner (1969).

B. A STUDY OF THE BIOSYNTHESIS OF ASPARAGINE BY THE CYANIDE PATHWAY IN WHEAT SEEDLINGS GROWN IN THE PRESENCE AND ABSENCE OF AMMONIUM SULPHATE.

1. Demonstration of an increased level of asparagine following the administration of ammonium sulphate to wheat seedlings

Initially various concentrations of ammonium sulphate from 0.2% (w/v) to 10% (w/v) were supplied to wheat seedlings. It was found that 0.2% (w/v) ammonium sulphate stimulated shoot

growth but retarded that of roots. At higher levels of ammonium sulphate the growth of both the shoots and the roots was considerably retarded.

If the grains were presoaked in the 0.2% (w/v) ammonium sulphate prior to planting, germination was poor. On further investigation, however, it was found that 0.1% (w/v) ammonium sulphate did not effect either the germination of the wheat grain nor the growth of seedlings. This level was therefore routinely used for the administration of ammonium sulphate to seedlings as described on p. 32 .

Wheat grains were soaked in ammonium sulphate (0.1%, w/v) or water, and grown on vermiculite supplemented with either ammonium sulphate (0.1%, w/v), or on water alone. The soluble amino acids from the shoots of these two types of seedlings were isolated for comparison.

Initially the isolated amino acids were separated by electrophoresis at pH 2.2 and detected by ninhydrin as described on p. 36 . A far greater intensity of the characteristic brown asparagine spot was observed with samples isolated from the ammonium sulphate fed seedlings. No spot corresponding to β-cyano-alanine (grey-green on ninhydrin treatment) could be detected in either sample.

The quantitative changes in the concentration of asparagine and in some of the other soluble amino acids were determined. It can be seen that a dramatic increase in the level of asparagine and glutamine (3.5 and 5.7 fold respectively)

occurred with ammonium sulphate feeding (Table VII.1). However, the absolute increase in asparagine is ten times that of glutamine. The levels of the other amino acids examined (aspartic acid, glutamic acid, threonine, serine and proline) showed only small changes with ammonium sulphate feeding.

2. Incorporation of [^{14}C]-HCN into asparagine in wheat seedlings grown in the presence and absence of ammonium sulphate

It was expected that if the asparagine accumulated due to the administration of ammonium sulphate was in fact synthesized via the cyanide pathway, then the incorporation of [^{14}C]-HCN *in vivo* into asparagine might be reduced if there was a pool of endogenous cyanide significantly large as compared with that from the exogenous ion. The specific activity of the pathway intermediate, β-cyano-alanine would also be reduced.

In fact a 0.3 fold increase in the isotope incorporated into asparagine was found in the ammonium sulphate fed shoots compared to those fed with water alone (Figure VII.1). This experiment merely shows that there is no significantly increased endogenous cyanide pool due to the ammonium sulphate feeding or at least not one which rapidly equilibrates with exogenous cyanide. Such a negative result does not eliminate the presence of a small rapidly 'turning over' pool of

TABLE VII.1. CHANGES IN THE CONCENTRATION OF SOME OF THE SOLUBLE AMINO ACIDS IN WHEAT SHOOTS FOLLOWING AMMONIUM SULPHATE FEEDING

Amino acid	µmoles amino acid/gram wet weight		Ratio $\frac{(NH_4)_2SO_4}{H_2O}$	Change in amino acid concentration with $(NH_4)_2SO_4$ feeding[a]
	H_2O fed	$(NH_4)_2SO_4$ fed		
asp	1.98	2.99	1.5	+ 1.01
$aspNH_2$[b]	24.76	85.66	3.5	+60.90
glu	1.93	1.12	0.6	- 0.71
$gluNH_2$[b]	1.33	7.56	5.7	+ 6.23
thr	1.10	0.93	0.9	- 0.17
ser	3.24	1.87	0.6	- 1.37
pro	0.30	0.26	0.9	- 0.04

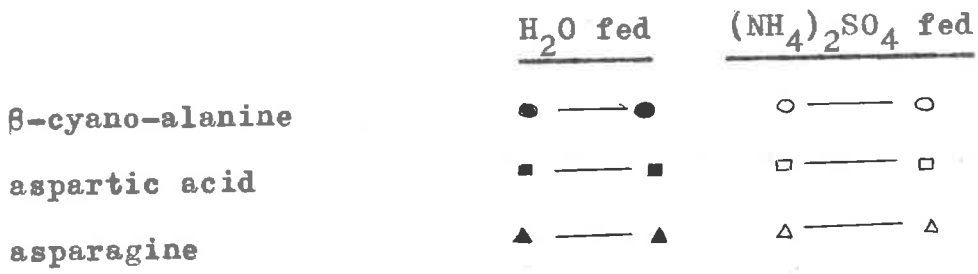
[a] µmoles amino acid/gram wet weight

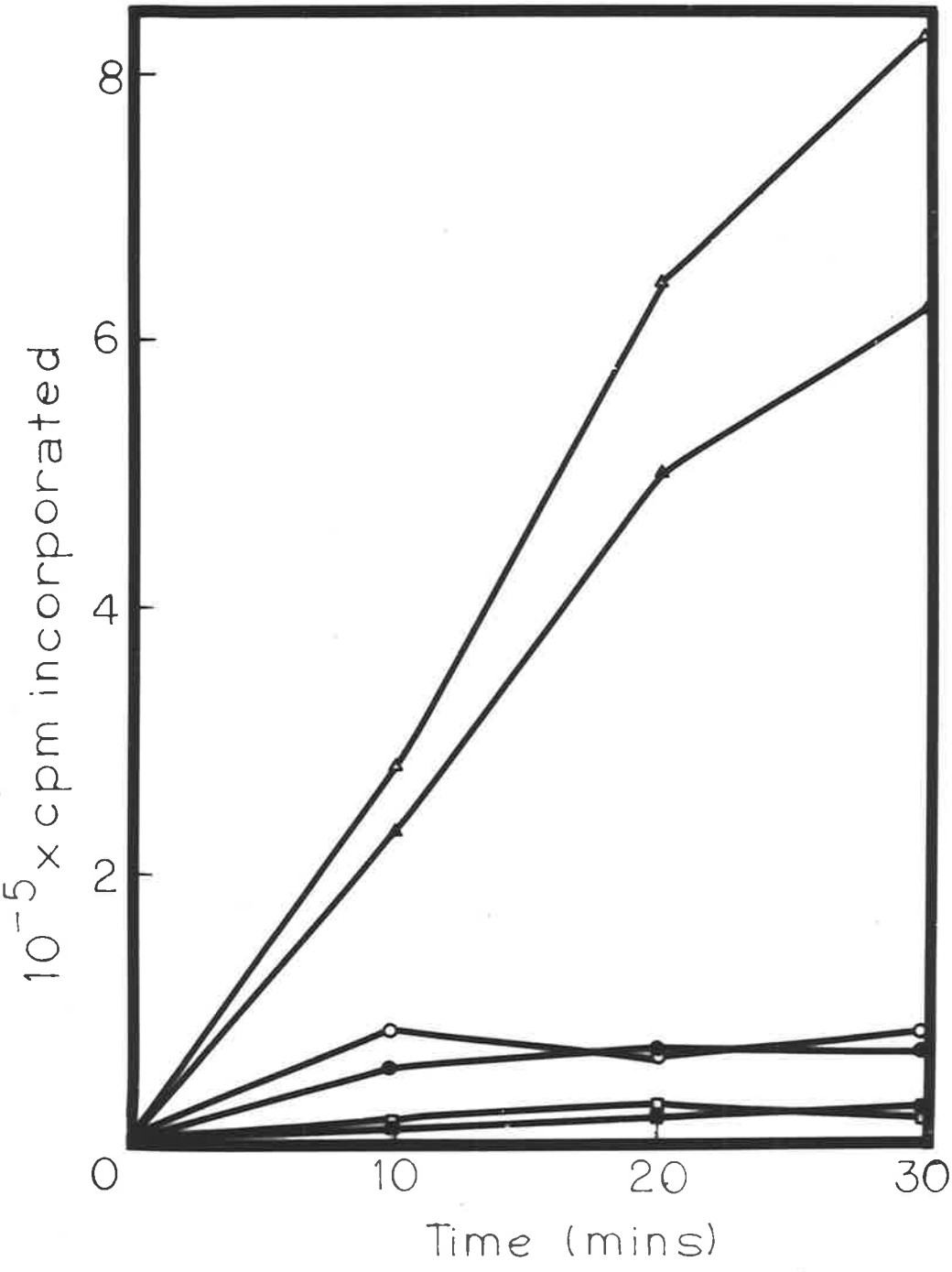
[b] determined as the increase in asp or glu on hydrolysis for 16 hours at 105° in 1N-HCl.

The grain was germinated and the ammonium sulphate 0.1% (w/v) administered as described on p. 32 . The soluble amino acids were isolated from the wheat shoots (8 day), and analyzed as described on p. 35. Taurine was used as an internal standard for the amino acid analysis which was aborted at proline.

FIGURE VII.1: THE INCORPORATION OF [^{14}C]-HCN INTO THE SOLUBLE AMINO ACID FRACTION OF AMMONIUM SULPHATE AND WATER FED EXCISED WHEAT SHOOTS

The administration of the [^{14}C]-HCN (83.33 μC; 1.5 μmoles; 0.034% v/v) in normal air to 5 excised wheat shoots from 8 day seedlings and the isolation of the soluble amino acids was as described on pp. 35,36 . The grain was germinated and the ammonium sulphate administered as described on p. 32 .




8
6
4
2
0
10
20
30
10^{-5} x cpm incorporated
Time (mins)

endogenous cyanide.

The label was found predominantly in asparagine (80%) indicating that a rapid hydrolysis of the β-cyano-alanine to asparagine occurred. Significant labelling was also found in aspartic acid and alanine (2 - 4%) and in the other amino acids examined, glutamine, serine, glycine and glutamic acid (0.03 - 1%), (Table VII.2).

An attempt was made to determine the specific activity of the β-cyano-alanine and asparagine labelled in the [^{14}C]-HCN incorporation experiment (Figure VII.1). These amino acids were purified by paper electrophoresis, hydrolyzed in 1-N-HCl for 4 hours at 110^{o} and the aspartic acid produced determined quantitatively on the Amino Acid Analyzer. The radioactivity was determined by liquid scintillation counting. Using this procedure, the specific activity of the asparagine labelled in the 10 minute sample of the water fed and ammonium sulphate fed shoots was 0.40 μC/μmole and 0.14 μC/μmole respectively. This 2.9 fold decrease in the specific activity of the asparagine in the ammonium sulphate fed plants substantiates the increased synthesis of asparagine demonstrated to occur with ammonium sulphate feeding (Table VII.1). In these determinations a correction was made for the aspartic acid eluted from the electrophoresis paper (interestingly proposed by Evered (1967) to have come from the asparagine in the original tree from which the paper was made). The aspartic acid correction amounted to 5% of the ammonium

TABLE VII.2: THE INCORPORATION OF $[^{14}C]$-HCN INTO THE SOLUBLE AMINO ACID FRACTION OF AMMONIUM SULPHATE AND WATER FED EXCISED WHEAT SHOOTS

	H_2O fed		$(NH_4)_2SO_4$ fed	
Amino acid	cpm incorporated x 10^{-4}	% of total incorporated	cpm incorporated x 10^{-4}	% of total incorporated
β-CN-ala	6.87	8.9	8.58	8.8
asp	3.11	4.0	2.30	2.3
$gluNH_2$	0.83	1.1	0.73	0.7
$aspNH_2$	62.51	81.0	83.30	84.3
ser	0.38	0.5	0.29	0.3
gly	0.74	1.0	0.50	0.5
glu	0.52	0.7	0.91	0.9
ala	2.18	2.8	2.16	2.2

Experimental details are given in Figure VII.1. The data above were obtained from the 30 min. time sample in Figure VII.1.

sulphate fed sample and 24% of the water fed sample. Without applying this correction a 2.3 fold decrease in the specific activity of the asparagine from the ammonium sulphate fed plants was calculated.

A similar method was used in an attempt to determine the specific activity of the β-cyano-alanine. However, the aspartic acid produced following the acid hydrolysis of the sample could not be detected in the amino acid analysis. The sensitivity of the method employed could have determined a level of β-cyano-alanine one-tenth that of the asparagine. It could be calculated therefore from this experiment that the specific activity of the β-cyano-alanine isolated from the wheat shoots grown in the presence and absence of ammonium sulphate following the incorporation of $[^{14}C]$-HCN was at least 4 μC/μmole and approaching that of the cyanide administered (55.7 μC/μmole).

3. A study of the levels of the cyanide pathway enzymes following the administration of ammonium sulphate to wheat seedlings

The levels of β-cyano-alanine synthase and β-cyano-alanine hydrolase were determined in crude leaf extracts of wheat seedlings grown with and without ammonium sulphate respectively.

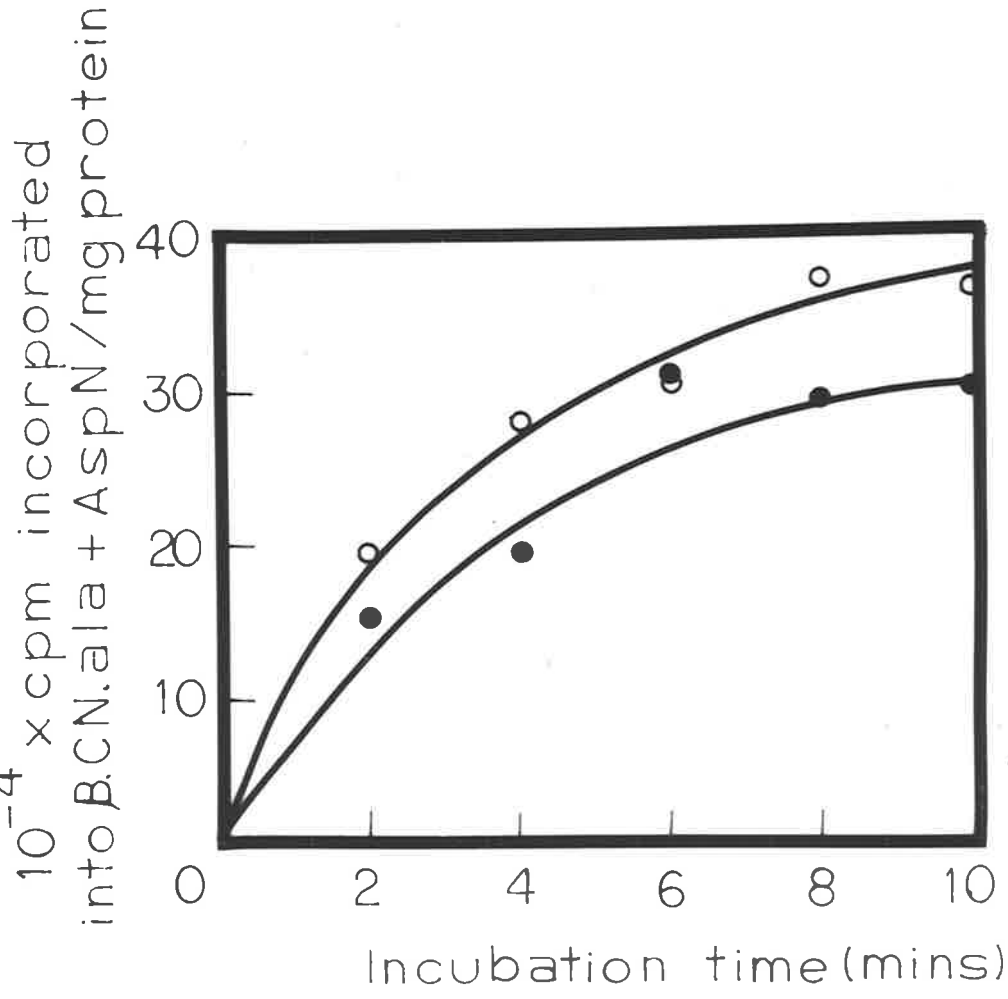
It can be seen in Figure VII.2 that a 0.3 fold increase in the specific activity of the β-cyano-alanine synthase has

FIGURE VII.2. TIME COURSE OF β-CYANO-ALANINE SYNTHESIS BY THE CRUDE WHEAT LEAF EXTRACTS OF AMMONIUM SULPHATE AND WATER FED SEEDLINGS

The growth of seedlings (8 day) and the preparation of the crude leaf extracts were as described on pp. 32,37 . The experimental details for the determination of β-cyano-alanine synthase activity are given on p. 42 .

O — O β-cyano-alanine synthase activity of the $(NH_4)_2SO_4$ fed plants.

● — ● β-cyano-alanine synthase activity of the H_2O fed plants.


10^{-4} x cpm incorporated into β.CN.ala + AspN/mg protein
0
10
20
30
40
2
4
6
8
10
Incubation time (mins)

been demonstrated following ammonium sulphate administration. It is not known whether this apparent enzyme induction was due in fact to de novo synthesis or enzyme activation.

This increase in the specific activity of the β-cyano-alanine synthase might be correlated with the corresponding 0.3 fold increase in the incorporation of cyanide into asparagine observed in vivo in the excised shoots of the ammonium sulphate.

There was no detectable change in the specific activity of the crude wheat leaf β-cyano-alanine hydrolase following ammonium sulphate feeding (Figure VII.3).

Further attempts to those described on p. 59 were made to detect an asparaginase in both the ammonium sulphate and water fed crude wheat leaf extracts; all were unsuccessful.

4. A study of the levels of glutamine synthetase following ammonium sulphate administration to wheat seedlings

The levels of glutamine synthetase in the crude wheat leaf extracts of both the ammonium sulphate and water fed seedlings were investigated with the aim of determining whether the increased synthesis of glutamine following the ammonium sulphate feeding (Table VII.1) was in fact due to the induction of glutamine synthetase.

No change in the level of this enzyme could be detected in the extract prepared from the ammonium sulphate fed seedlings

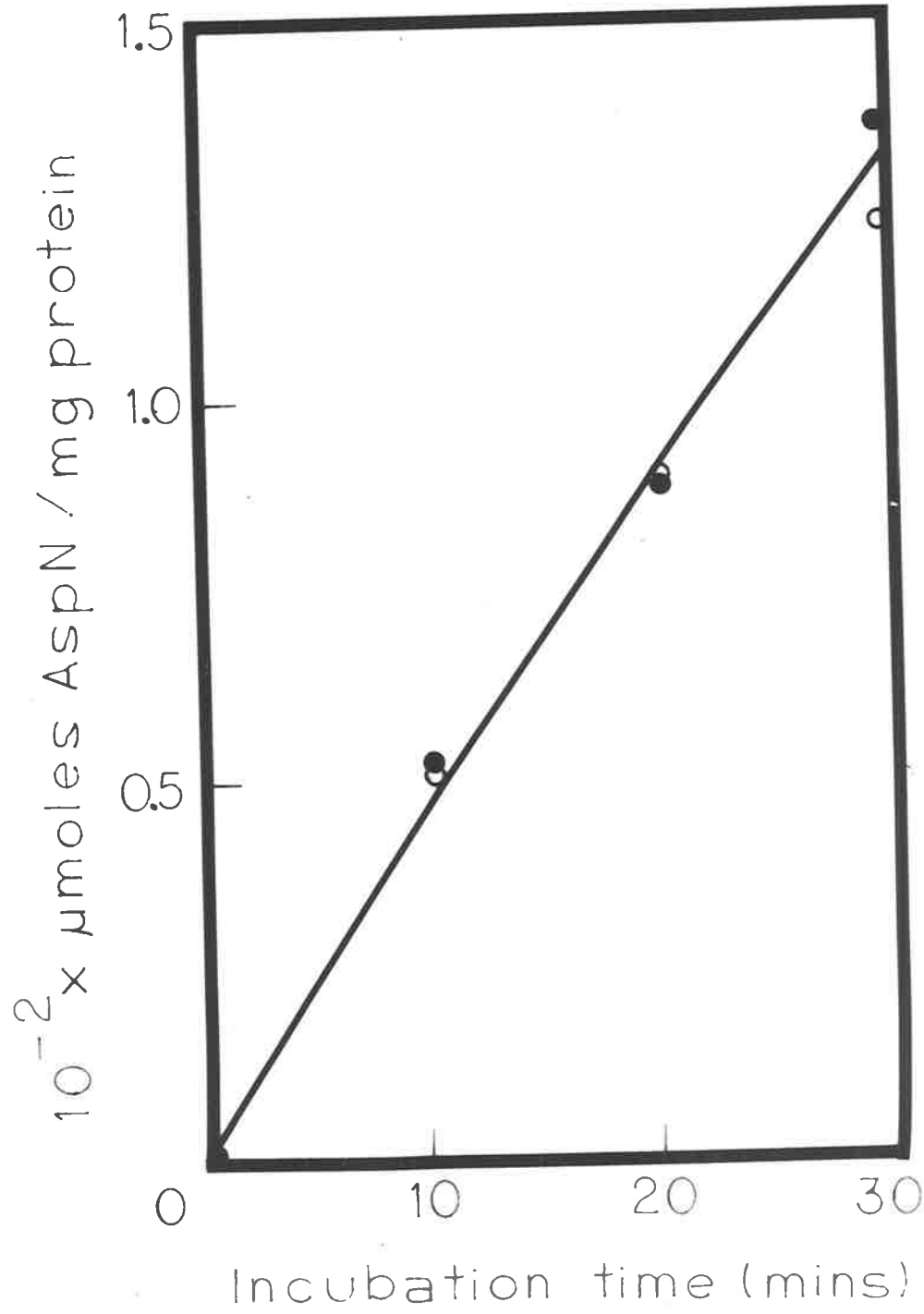

1.5
1.0
0.5
0
10
20
30
10^{-2} x µmoles AspN / mg protein
Incubation time (mins)

FIGURE VII.3. TIME COURSE OF β-CYANO-ALANINE HYDROLYSIS BY THE CRUDE WHEAT LEAF EXTRACTS OF AMMONIUM SULPHATE AND WATER FED SEEDLINGS

The growth of seedlings (8 day) and the preparation of the crude leaf extracts were as described on pp.32,37. The experimental details for the determination of β-cyano-alanine-hydrolase activity are given on p.42 except that [^{14}C]-β-cyano-alanine, (0.178 μmoles; 14,000 cpm) was used per assay in a total volume of 0.2 ml.

● — ● β-cyano-alanine hydrolase activity of the H_2O fed plants.

○ — ○ β-cyano-alanine hydrolase activity of the $(NH_4)_2SO_4$ fed plants.

as compared to that from control seedlings (Figure VII.4). The incorporation of label from the [^{14}C]-glutamic acid (1% in 30 min.) into aspartic acid was also found to occur in both these extracts.

Further attempts to those described on p. 54 were made to demonstrate an analogous asparagine synthetase in these extracts by replacing the glutamic acid in the assay described in Figure VII .4 with aspartic acid (0.5 μC; 106 μC/μmole). Again no conversion of the aspartic acid to asparagine could be detected in either the ammonium sulphate or water fed crude wheat leaf extracts. A significant conversion of the aspartate to alanine (0.3%), glutamine (0.5%) and glutamic acid (0.7%) was, however, demonstrated following a 30 min. incubation.

C. DISCUSSION

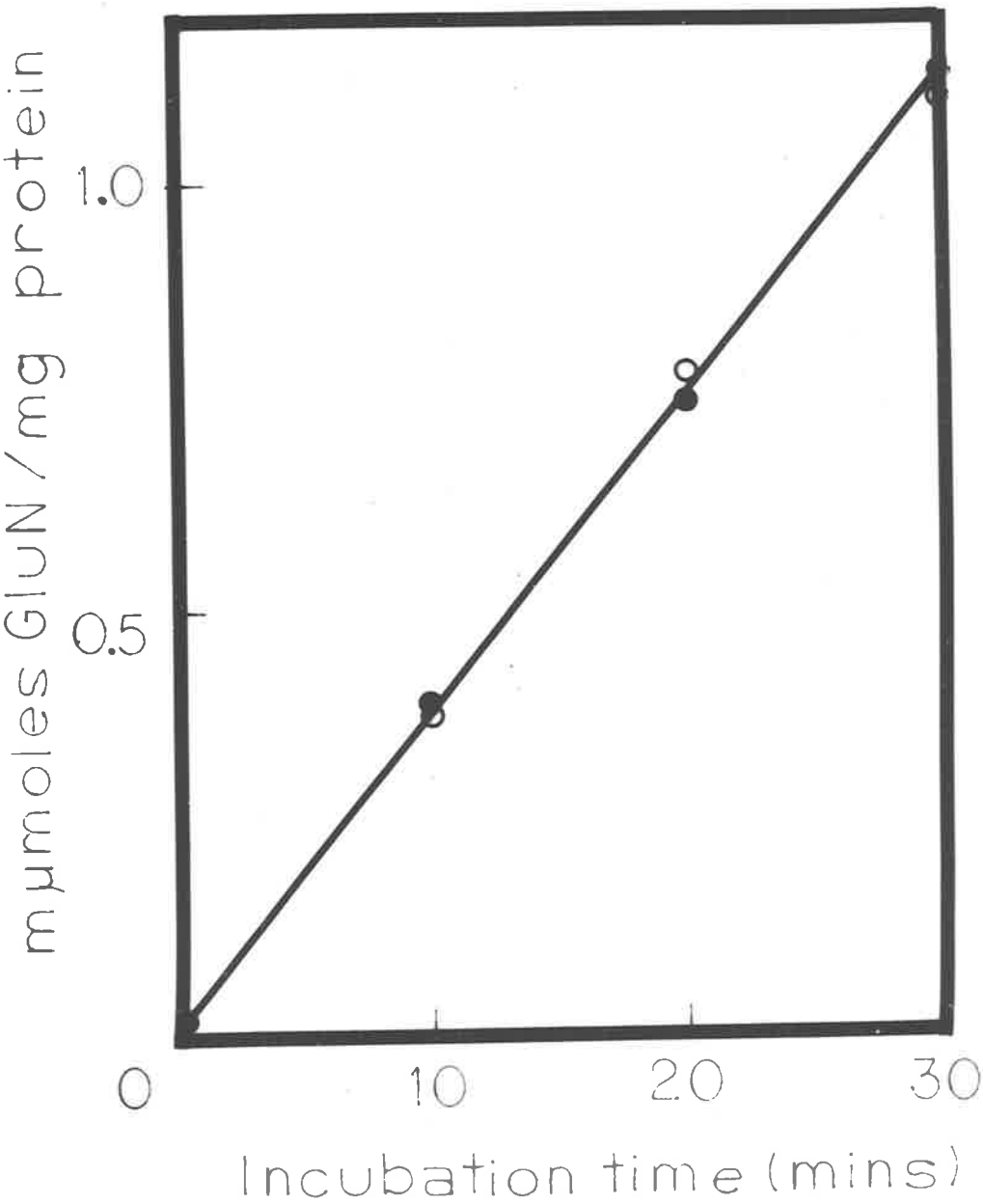
The [^{14}C]-HCN *in vivo* incorporation experiment reported here indicates that there is no significant increase accumulation of either cyanide or β-cyano-alanine associated with the increased synthesis of asparagine following ammonium sulphate feeding of the wheat seedlings. (This assumes the rapid equilibration of the exogenous [^{14}C]-HCN with such an endogenous cyanide pool.) Moreover, although it was not possible to quantitatively determine the β-cyano-alanine in these experiments, the results did indicate that the level of β-cyano-alanine in the wheat shoots was at the most,

FIGURE VII.4. TIME COURSE OF GLUTAMINE SYNTHESIS BY THE CRUDE WHEAT LEAF EXTRACTS OF AMMONIUM SULPHATE AND WATER FED SEEDLINGS.

The growth of seedlings (8 day) and the preparation of the crude wheat leaf extracts was as described on pp.32 and 37. The incubation mixture contained in a total volume of 0.25 ml, wheat leaf extract (0.16 ml, 1.2 mg protein) in 0.1M-KH_2PO_4 pH 7.1; Mg^{++} (12 mM); ATP (4 mM); NH_4Cl (24 mM); [^{14}C]-glutamic acid (1.0 μC, 4.885 mμmoles). After incubation at 30° for the times indicated the reaction was stopped by the addition of 2 volumes of ethanol and the labelling determined in glutamine and glutamic acid as described on p.36.

● — ● The glutamine synthetase activity of H_2O fed plants.

○ — ○ The glutamine synthetase activity of $(NH_4)_2SO_4$ fed plants.


mμmoles GluN / mg protein
1.0
0.5
0
10
20
30
Incubation time (mins)

one-tenth that of asparagine.

Unfortunately these negative results do not permit any conclusion to be made other than perhaps that if this pathway does have any physiological significance in asparagine biosynthesis, cyanide must be immediately converted to β-cyano-alanine which is subsequently rapidly hydrolyzed to asparagine.

In view of the toxicity of cyanide a rapid conversion of this ion to β-cyano-alanine is a likely situation to have evolved in any case. The low Km values of 5×10^{-6}M and 5×10^{-4}M reported for the cyanide ion in Lotus tenuis and Lupinus angustifolius respectively (Floss, Hadwiger, and Conn, 1965; Hendrickson and Conn, 1969) would facilitate such a rapid conversion in these plants. The Km for wheat β-cyano-alanine synthase is not known. However, in wheat, a Km of 2.2×10^{-3}M for β-cyano-alanine has been determined for β-cyano-alanine hydrolase (see p. 70). This Km would not favour the rapid hydrolysis of low levels of β-cyano-alanine to asparagine unless either metabolic compartmentation of the β-cyano-alanine pool existed or the levels of the enzyme were relatively high.

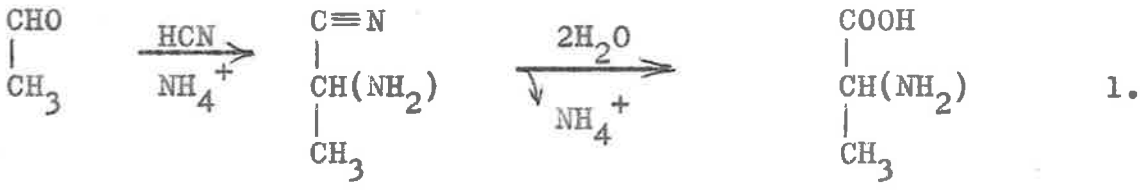
The rapid in vivo incorporation of $[^{14}C]$-KCN into asparagine without detectable accumulation of β-cyano-alanine has been observed in Lathyrus odoratus, Vicia villosa and Lathyrus sylvestris, (Ressler, Giza and Nigam, 1969).

These species have not been found to normally contain β-cyano-alanine. However, Vicia sativa seedlings which contain β-cyano-alanine and γ-glutamyl-β-cyano-alanine incorporate cyanide in vivo solely into these compounds (Ressler, Giza and Nigam, 1969). Since they are unable to hydrolyze β-cyano-alanine to asparagine they must synthesize the amide by another pathway. This suggests that the cyanide pathway in this species has no role in asparagine biosynthesis, but might be important in cyanide detoxification.

This is supported by the work of Nigam and Ressler (1964) who demonstrated the in vivo incorporation of serine into β-cyano-alanine in Vicia sativa seedlings only if potassium cyanide was added. However, during seed maturation in this plant Nigam and McConnell (1968) showed the in vivo incorporation of serine into β-cyano-alanine in the absence of an exogenous source of cyanide. This clearly establishes the existence of the pathway in vivo. Moreover, Abrol and Conn (1966) and Abrol, Conn and Stoker (1966) demonstrated the conversion of endogenously produced cyanide in vivo into asparagine. These authors found that the cyanide produced from the metabolism of cyanoglucosides was incorporated into asparagine, demonstrating that normal metabolites can be converted to cyanide which is then used in the synthesis of asparagine. The physiological significance of this synthesis of asparagine from endogenously produced cyanide obviously depends on how rapidly metabolites such as cyanoglucosides

are synthesized and subsequently converted to cyanide and β-cyano-alanine.

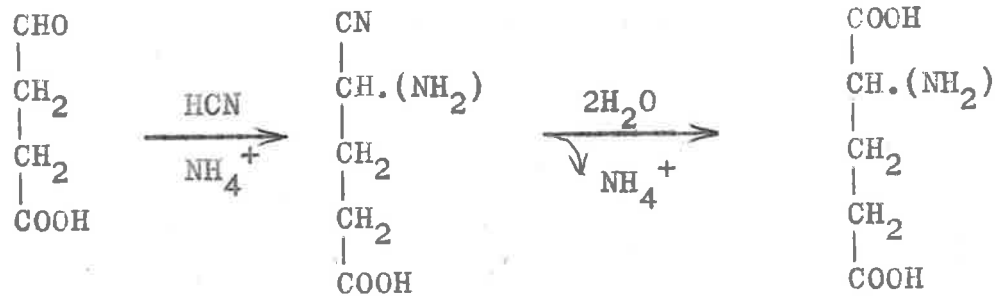
To date, the conversion of cyanide to asparagine via β-cyano-alanine is the only pathway of cyanide assimilation reported to occur in plants. However, in the in vivo [^{14}C]-HCN incorporation studies in wheat shoots reported in this chapter, significant labelling of aspartic acid, glutamine, serine, glycine, glutamic acid and alanine as well as of asparagine and β-cyano-alanine was observed. In view of the evidence reported in Chapter III which indicated that in wheat seedlings, asparagine is not further metabolized, it is likely that other pathways of cyanide assimilation may be operating. It would be of interest to know if the pathways of cyanide assimilation reported by Strobel (1966 and 1967) to occur in a cyanogenic fungus and that reported by Brysk and Ressler (1970) in cultures of Chromobacterium violaceum have a more widespread distribution. Strobel (1966) found that alanine is synthesized from cyanide, ammonia and acetaldehyde via α-aminopropionitrile in a cyanogenic psychrophilic basidiomycete (Equation VII.1).



1.

acetaldehyde α-aminopropionitrile alanine

Later it was found (Strobel, 1967) that an analogous synthesis of glutamate from cyanide, ammonia and succinic semi-aldehyde via γ-amino-γ-cyano-butyric acid occurred in the same fungus (Equation VII .2).



2.

succinic semialdehyde — γ-amino-γ-cyano-butyric acid — glutamic acid

γ-Cyano-α-amino-butyric acid has also been recently reported to accumulate in cultures of Chromobacterium violaceum when labelled cyanide was administered (Brysk and Ressler, 1970). The hydrolysis of this compound would also give glutamic acid but evidence of such a hydrolysis has not been presented; the pathway leading to the synthesis of this compound has not been elucidated but a preliminary in vivo study has suggested that γ-cyano-α-amino-butyric acid could be synthesized in this bacterium by the condensation of cyanide with aspartic acid or some derivative (Brysk and Ressler, 1970).

As stated at the beginning of this chapter it was considered possible that if the β-cyano-alanine pathway was

physiologically significant in asparagine biosynthesis, then the levels of the enzymes of this pathway might be induced on ammonium sulphate feeding. The work reported here has demonstrated that no such induction occurs. A negative result of this kind does not permit conclusions to be drawn. This is especially so in view of the fact that it was not possible to demonstrate that the increased synthesis of glutamine also observed with ammonium sulphate feeding was due to an increase in the level of glutamine synthetase.

An alternative possibility which must be considered is that the cyanide pathway enzymes of plants are of no physiological significance in either cyanide detoxification (which seems unlikely in view of the evidence discussed above) or asparagine biosynthesis. The synthesis and hydrolysis of β-cyano-alanine observed in plants could be merely catalyzed by enzymes catalyzing similar reactions as an accidental phenomenon virtually and without physiological 'purpose' or significance. Such reactions are not unknown. Thus pyruvate kinase will phosphorylate fluoride to give fluorophosphate (Tietz and Ochoa, 1958) and citrate synthase will form fluorocitrate from fluoroacetate (Ochoa, 1954).

β-Cyano-alanine hydrolysis has for example been proposed to be catalyzed by an asparaginase in E. coli (see p. 99). This is not the case, however, in crude wheat leaf extracts observed here for it has not been possible to demonstrate any asparaginase activity in these extracts.

(Such activity is only found in these tissues after 12 days, Lees and Blakeney, 1970; Lees, 1970.)

Although it is thus very unlikely that the hydrolysis of β-cyano-alanine observed in wheat seedling extracts is catalyzed by an asparaginase, it could be catalyzed by a nitrilase of low specificity. Such an enzyme occurs in a wide range of plant tissues, it hydrolyzes 21 different aryl and aliphatic nitriles (Mahadevan and Thimann, 1964; Thimann and Mahadevan, 1964) by the general reaction shown in Equation VII .3.

$$RCN + 2H_2O \longrightarrow RCOOH + NH_3 \qquad 3.$$

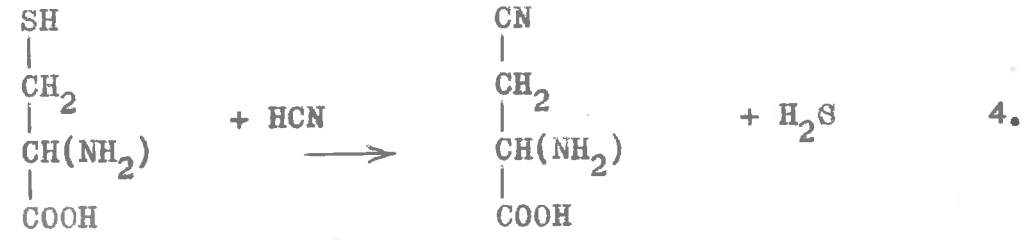
Intermediate formation of the amide was not observed, as would be the case if this enzyme hydrolyzed β-cyano-alanine to asparagine.

Nitrilase activity has been discovered by Strobel (1966, 1967) in cell-free extracts of an unidentified psychrophilic basidiomycete; α-amino-propionitrile is hydrolyzed to alanine and γ-amino-γ-cyanobutyric acid to glutamic acid.

A nitrilase has also been reported in an unidentified pseudomonad isolated from soil by enrichment with ricinine (Robinson and Hook, 1964). This enzyme hydrolyzes ricinine (the toxic alkaloid of Ricinus communis, Waller and Henderson, 1961), producing both the corresponding amide and acid (Hook

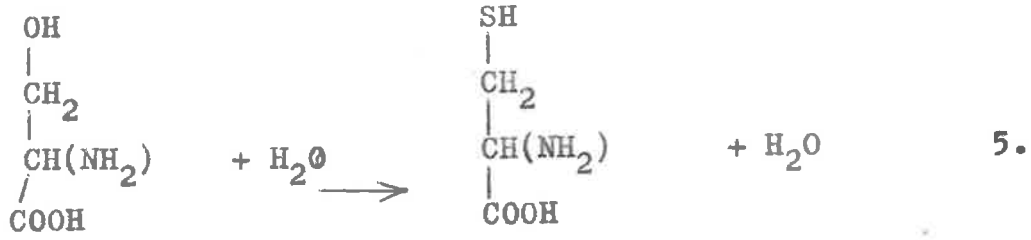
and Robinson, 1964). These authors claim to have demonstrated that extracts of the bacteria contained an enzyme which hydrolyzed β-cyano-alanine which was distinct from the ricicine nitrilase. No experimental details were given. Thus, there is no direct evidence for or against β-cyano-alanine hydrolysis in wheat being the non-specific action of a nitrilase.

It has been proposed that the synthesis of β-cyano-alanine is likewise due to the action of an enzyme not specifically concerned with this synthesis. Dunnill and Fowden (1965) proposed that the synthesis of β-cyano-alanine from cysteine and cyanide (Equation VII .4) is catalyzed by cysteine synthase (serine sulphydrase) which catalyzes the synthesis of cysteine from serine and sulphide (Equation VII .5).



cysteine β-cyano-alanine

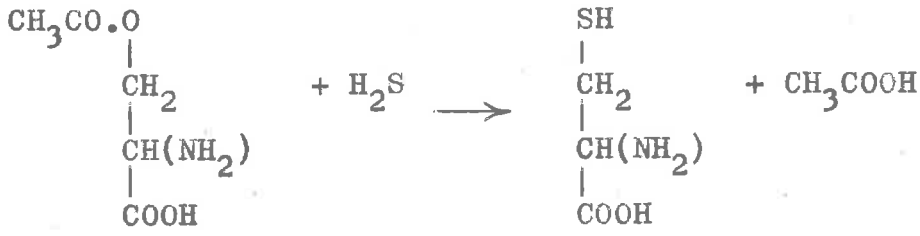
4.



serine cysteine

5.

Recent studies (Giovanelli and Mudd, 1967; Thompson and Moore, 1968) have shown that O-acetyl-serine rather than serine is the preferred substrate for the enzyme cysteine synthase (O-acetyl-serine sulfhydrase) in higher plants (Equation VII .6).



O-acetyl serine cysteine 6.

Hendrickson and Conn (1969) purified the β-cyano-alanine synthase of Lupinus angustifolius and separated it from O-acetyl-serine sulphydrase (β-cyano-alanine synthase is located in the mitochondria whereas O-acetyl-serine sulphydrase is a soluble enzyme). The purified β-cyano-alanine synthase catalyzed the formation of β-cyano-alanine from cyanide and either cysteine or O-acetyl-serine but not serine. With O-acetyl serine as substrate the rate of β-cyano-alanine synthesis was one-twentieth that observed with cysteine. The purified β-cyano-alanine synthase also catalyzed the synthesis of cysteine from O-acetyl-serine and sulphide at one-twentyfifth the rate of β-cyano-alanine synthesis (Hendrickson and Conn, 1969). The O-acetyl-serine sulphydrase catalyzed the synthesis

of β-cyano-alanine from cyanide and O-acetyl-serine at a rate one-tenth that of cysteine synthesis. These studies by Hendrickson and Conn (1969) suggest that the β-cyano-alanine synthase of Lupinus angustifolius is distinct from the cysteine biosynthetic enzymes. However, the physiological role of this enzyme in Lupinus angustifolius is not known since no source of cyanide is known in this plant (Hendrickson and Conn, 1969); the significance of this enzyme in either asparagine biosynthesis or cyanide detoxification is therefore doubtful. Conn and Butler (1969) have speculated that it may be a metabolic activity acquired early in evolution and retained by species that no longer have a need for such a process.

The results presented in this chapter and those from other workers discussed above have clearly established the existence of the cyanide pathway of asparagine biosynthesis both in vivo and in vitro in plants. No definitive evidence has been presented here which suggests that this pathway is physiologically significant in asparagine biosynthesis but the results are compatible with the pathway being a cyanide detoxification mechanism. It would appear that before significance can be attributed to this pathway in asparagine biosynthesis, evidence of a physiologically significant source of cyanide must be forthcoming.

D. SUMMARY

1. An increased level of both the soluble asparagine and glutamine (3.5 and 5.7 fold respectively) was found in the wheat shoots of seedlings fed with 0.1% w/v ammonium sulphate.

2. No increase in the pool of cyanide pathway intermediates could be detected in wheat shoots following ammonium sulphate feeding.

3. A slight increase (0.3 fold) in the specific activity of the crude wheat leaf β-cyano-alanine synthase of the ammonium sulphate fed seedlings as compared to the water fed seedlings was observed.

4. No detectable change in the specific activity of the crude wheat leaf β-cyano-alanine hydrolase or glutamine synthetase was found with ammonium sulphate feeding.

5. Neither the synthesis of asparagine from aspartic acid, ammonia, Mg^{++} and ATP, nor the hydrolysis of asparagine to aspartic acid could be demonstrated in crude wheat leaf extracts of either the ammonium sulphate or water fed seedlings.

CHAPTER VIII

FINAL DISCUSSION

FINAL DISCUSSION

Many of the points that are raised here have been summarized and discussed earlier in the thesis, but are recapitulated here to permit an overall discussion of the problem.

The work reported here on the *in vivo* synthesis and utilization of asparagine in seedlings is significant in that it perhaps shows some new light on nitrogen metabolism in seedlings. It suggests that asparagine biosynthesis is a response to ammonia accumulation whether from exogenous or endogenous sources. It seems to weaken the idea that it is a specific compound designed to translocate in the young seedlings carbon or nitrogen from the cotyledons or endosperm to the growing tip - although the recent work of Lees and Blakeney (1970) shows that in older plants the asparagine produced is re-utilized. This observation that asparagine appears to be a terminal metabolite in young seedlings has been recently confirmed by Ting and Tschoche (1970) and Maxwell and Bidwell (1970).

The unsuccessful attempts reported here and those of others (Elliott, 1951; Meister, 1962 and 1965b; Ting and Tschoche, 1970) to confirm the *in vitro* synthesis of asparagine from aspartate, as originally proposed by Webster and Varner (1955) and more recently by Nair (1969) has made the existence of such a conversion in plant tissues (Equation VIII.1)

somewhat questionable.

$$\text{aspartate} + NH_3 + ATP \xrightarrow{Mg^{++}} \text{asparagine} + ADP + P_i \quad .. 1.$$

This is extremely perplexing in view of the fact that aspartate has been demonstrated here and by others (Al-Dawody, Varner and Webster, 1960; Oaks and Johnson, 1970) to be readily converted to asparagine by plants in vivo. Furthermore, Al-Dawody and Varner (1961) had also claimed in a preliminary report that asparagine synthesis in yeast occurred by an identical mechanism. Recently Rognes (1970) has reported his inability to reproduce this work.

It would have been difficult to predict that with the modern biochemical methods available the synthesis of asparagine in plants would have remained an unsettled problem for so long. An asparagine synthetase catalyzing the reaction shown in Equation VIII.2 has been purified from the micro-organisms Lactobacillus arabinosus (Ravell, Norton, Humphreys and Shive; 1962), Streptococcus bovis (Burchall, Reichelt and Wolin, 1964) and Escherichia coli (Cedar and Schwartz, 1969a and b).

$$\text{aspartate} + NH_3 + ATP \xrightarrow{Mg^{++}} \text{asparagine} + AMP + PP_i \quad .. 2.$$

The amide nitrogen atom of glutamine rather than ammonia (Equation VIII.3) has been shown to be utilized primarily by the mammalian asparagine synthetases (Arfin, 1967; Patterson and Orr, 1968; Holcenberg, 1969).

$$\text{aspartate} + \text{glutamine} + \text{ATP} \longrightarrow \text{asparagine} + \text{glutamate} + \text{AMP} + PP_i \quad .. 3.$$

But when the experimental work reported here was concluded it had to be admitted with some regret that the massive synthesis of asparagine in plants was one of the few areas of amino acid metabolism where the pathway was still obscure. While it is dangerous in this perplexing field to make any predictions it seemed at the time of writing that the answer lay in one of three directions.

The first of these predictions is that there may be a totally novel and at present unsuspected route of synthesis occurring and sufficiently novel that no-one has added to an *in vitro* system whatever necessary (and perhaps unstable) component is needed.

Secondly it may be that the cyanide pathway, which occurs with such impressive efficiency from exogenous cyanide in plants is the answer. The study done in *E. coli* here strongly suggests that the cyanide pathway enzymes are not so involved in normal asparagine production in this organism. However, this does not exclude the plant pathway from being physiologically significant in asparagine biosynthesis. Bacteria were not found to readily convert exogenous cyanide to asparagine even though the cyanide pathway enzymes were demonstrated in this organism *in vitro*. Moreover, it is only in plants that massive accumulations of the amide occurs and

the pathway of synthesis here may be totally different. In fact the asparagine synthetase found to occur in E. coli may be an enzyme developed for efficient synthesis of the relatively minute amounts needed in this organism while the cyanide pathway is more 'suitable' for massive asparagine accumulation.

The third possibility is that an ATP-dependent asparagine synthetase exists but has not been assayed for under optimal conditions, or for some reason it is either very unstable, or very sensitive to some commonly occurring substance in plants which inhibits it. These possibilities are serious ones and ones that can be investigated only at random by trying new conditions and new methods of enzyme preparations. Such research is time-consuming and means nothing unless successful. Two recent reports (Streeter, 1970; and Rognes, 1970) which appeared in the literature during the final preparation of this thesis have added support to this possibility. These authors have presented evidence of a glutamine-dependent asparagine synthetase in plant extracts. Rognes (1970) states that 'no too far reaching conclusions should be made at this stage' but certainly the hypothesis that asparagine synthesis in higher plants occurs by mechanism similar to that found in animal cells (Equation VIII.3) is an exciting one and one well worth pursuing.

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