AMINOACID METABOLISM IN PLANTS:

THE BIOSYNTHESIS OF β-CYANOALANINE AND ASPARAGINE IN LUPINS

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Massey University.

Michael Lever

1970

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

My greatest debt is to Dr. G.W. Butler, of the D.S.I.R., who suggested this project, and who has provided valued criticism and guidance at all stages, as well as arranging laboratory facilities during the initial work.

The award of a University Grants Committee Post Graduate Scholarship made possible the initiation of this work. Its continuation when I moved to Lincoln College was facilitated by a grant from the Lincoln College Research Fund. I thank Professor B.H. Howard for arranging this, and for his continued help and interest.

Professor R.D. Batt, of Massey University, largely negotiated the arrangements under which this work has been carried out, and I am especially grateful for his making his department available for the completion of the last experiments described here. Of Dr. Butler's colleagues in the D.S.I.R. I would like to especially thank Dr. P.J. Peterson for his helpful criticism. Dr. E.E. Conn, during his stay in Palmerston North, also contributed useful criticism.

At Lincoln, the co-operation of Drs. Wilkinson and Simpson of the W.R.O., and of Dr. Allison of the D.S.I.R., greatly increased the laboratory facilities available to me.

I apologise to all those who helped, but are not mentioned here. One more, however, must be mentioned: my wife. But for Rhona this work may never have been completed.

1. SUMMARY

β-Cyanoalanine synthase in etiolated seedlings of Lupinus angustifolius is found mainly in the mitochondrial fraction of the cotyledons and stems. In seedlings developing at 25 °C in the dark it reaches a maximal concentration after 5 days, at the same time as asparagine accumulation is most rapid and other physiological changes occur. However, maximal ability to assimilate HCN gas to asparagine develops after 3 to 4 days, before asparagine accumulation begins.

A partial purification of lupin β-cyanoalanine synthase is described and its distinction from cysteine synthase confirmed.

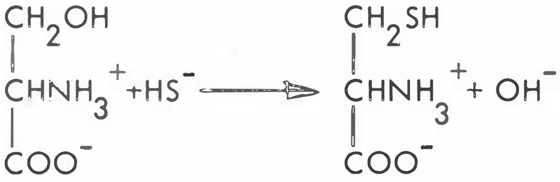
Carbon-14 labelled substrates were supplied to etiolated seedlings of lupin in order to identify precursors of asparagine. Four carbon acids related to the TCA cycle were readily converted to asparagine *in vivo* but not *in vitro*; the carbon skeleton of aspartate is retained in asparagine. Comparison of the distribution of label in the carbon skeletons of aspartate and asparagine from plants supplied carboxyl labelled fumarate shows a separation of the aspartate pool for asparagine biosynthesis from most cell aspartate. Metabolites that could be expected to give rise to cyanide and β-cyanoalanine are relatively ineffective as asparagine precursors.

Thus the β-cyanoalanine pathway is not of major importance in asparagine biosynthesis in lupins.

II. INTRODUCTION

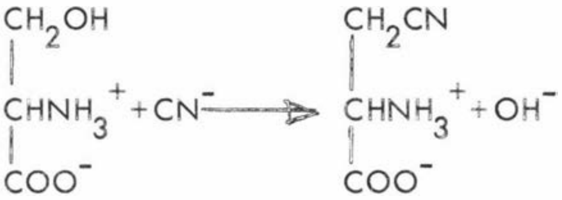
When Blumenthal-Goldschmidt et al. (1963) and Tschiersch (1963) observed that $H^{14}CN$ supplied to higher plants was converted into asparagine by way of β- cyanoalanine, serine providing the other three carbon atoms, the possibility suggested itself that the initial reaction of this pathway was catalysed by a relatively unspecific cysteine synthase, possibly the same as that described by Bruggemann <u>et al</u>. (1962)

(i) cysteine synthase



$$\begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CHNH_3^+}\\ |\\ \mathrm{COO^-}\end{array} + \mathrm{HS^-} \longrightarrow \begin{array}{l}\mathrm{CH_2SH}\\ |\\ \mathrm{CHNH_3^+}\\ |\\ \mathrm{COO^-}\end{array} + \mathrm{OH^-}$$

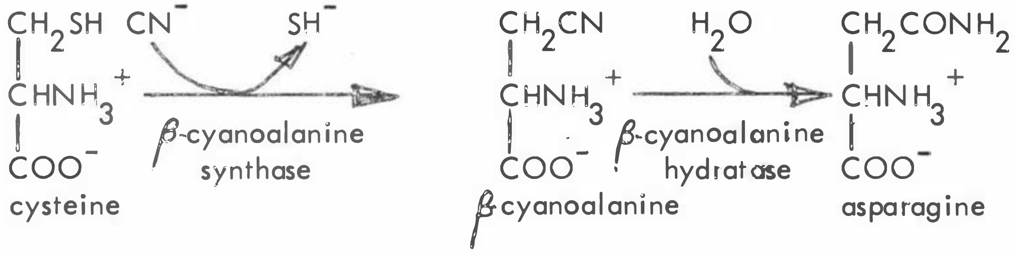
(ii) β-cyanoalanine synthase



$$\begin{array}{l}\mathrm{CH_2OH}\\ |\\ \mathrm{CHNH_3^+}\\ |\\ \mathrm{COO^-}\end{array} + \mathrm{CN^-} \longrightarrow \begin{array}{l}\mathrm{CH_2CN}\\ |\\ \mathrm{CHNH_3^+}\\ |\\ \mathrm{COO^-}\end{array} + \mathrm{OH^-}$$

Alternatively, these observations may be a clue to a solution of the vexed question of asparagine biosynthesis in plants. Described here is work intended to evaluate the metabolic role of these processes.

During the course of this work much relevant information has been published. Floss <u>et.al</u>. (1965) studied cell-free systems containing β-cyanoalanine synthase, and found that cysteine could replace serine as a substrate. Hendrickson and Conn (1969) have recently described a partial purification of β-cyanoalanine synthase from lupins, and they corroborate the evidence described here that the lupin enzyme will utilise cysteine as substrate but not serine. Meanwhile, doubt has been thrown on the significance of the enzyme described by Bruggemann et al., since the metabolically important substrate for cysteine synthase in higher plants now appears to be O-acetyl serine (Giovanelli and Mudd, 1967; Thompson and Moore, 1968). Hendrickson and Conn found that lupin β-cyanoalanine synthase will utilise O-acetyl serine as substrate at 5% of the rate that it will utilise cysteine. In addition an O-acetyl serine dependent cysteine synthase was found in their plant material, localised in a different subcellular fraction. Fowden and Bell (1965) have investigated the distribution of β-cyanoalanine hydratase, which converts β-cyanoalanine to asparagine in legume species, and found that it is absent in those that accumulate β-cyanoalanine and γ-glutamyl-β-cyanoalanine. Thus it is established that a wide variety of higher plants possess two enzymes, β-cyanoalanine synthase and β-cyanoalanine hydratase, that catalyse what will be called in this thesis the "β-cyanoalanine pathway".



This information, in conjunction with the earlier results described here, makes it most improbable that the enzymes of the β-cyanoalanine pathway are involved in cysteine biosynthesis in plants. The emphasis in this work was thus directed more towards elucidating the relationship between cyanide assimilation and asparagine biosynthesis in etiolated Lupinus angustifolius seedlings; these plants accumulate up to 30% of their dry weight as asparagine, and also readily assimilate cyanide. The development of β-cyanoalanine synthase activity was studied as a function of plant development, and compared with the development of the ability to assimilate exogenous cyanide and with the accumulation of asparagine. Radio-active substrates were supplied to intact plants to study which were the preferred substrates for asparagine biosynthesis, and to look for possible precursors of cyanide; attempts were made to locate the asparagine synthetase described by Webster and Varner (1955 a & b) but not found by other workers (Meister, 1962; Lees et al., 1968). The results suggest that the major pathway of asparagine synthesis is from four carbon dicarboxylic acids, and not from cyanide; but an isolation of an asparagine synthetase was not achieved.

III. METHODS AND RESULTS

1. GENERAL MATERIALS & METHODS

Seeds of blue lupin (*Lupinus angustifolius* L.) were obtained from Yates (N.Z.) Ltd. They were soaked in 0.2% "Zephiran" for 5 minutes, rinsed, and stood in tap water for 2 hours. They were then germinated on vermiculite, either in darkness at 25°C or in a controlled environment cabinet (12 hr. light period, 2000 f.c., 24°C).

Acetone powder preparations were made from 12 day old seedlings by blending the plant material with about 5 times its volume of acetone at -16°C, and rapidly filtering on a Buchner funnel. The filter cake was rehomogenised with the same volume of cold acetone and filtered, the washed cake being finally rinsed with cold "Analar" acetone and dried under reduced pressure.

Freeze dried plant powder preparations were made from 6 day old seedlings by first freezing the plant material on metal trays at -16°C or, preferably, by dropping it into liquid nitrogen, and then freeze drying. The dried plants were ground at 2°C in an electric coffee grinder set up in a cold laboratory: the ground material was then sieved through a 30 mesh sieve and the powder stored in a vacuum desiccator over calcium chloride.

Protein determinations were carried out by the colorimetric method of Lowry *et al.* (1951), using a modified Folin & Ciocalteau reagent. In each case the optical density at 750 nm was determined in a Bausch & Lomb "Spectronic 20" spectrophotometer, and the protein concentration estimated from a standard curve prepared using bovine serum albumen.

Unless otherwise noted, radioactive materials were obtained from the Radiochemical Centre, Amersham, England. Plant tissues that had been supplied with radioactive material were usually killed and extracted with boiling 80% aqueous alcohol, followed by water. Variations are described in each relevant section.

The separation methods used were high voltage paper electrophoresis and descending paper chromatography. Unless otherwise stated, electrophoresis was carried out on Whatman 3MM paper with a pyridine acetate buffer pH 5.3 made by diluting 25 ml pyridine and 10 ml glacial acetic acid to 2.5 litres with distilled water. The potential was from 3 to 5 KV, depending on the instrument used. Chromatography was carried out either on Whatman No 1 or 3MM paper. The solvent systems used are identified in the text by the code given in table III.1.1.

TABLE III.1.1

Chromatography Solvents

Solvent	Proportions	Code
n.butanol:acetic acid: water	12:3:5 by volume	BAW
n.butanol:ethanol:water	2:2:1 by volume	BEW
n.butanol:pyridine:water	1:1:1 by volume	BPW 1:1:1
n.butanol:pyridine:water	6:4:3 by volume	BPW 6:4:3
phenol:water	4:1 by weight	ØOH
n.propanol:water	7:3 by volume	PropW

Radioactivity was located on electropherograms and chromatograms either by autoradiography or with a chromatogram scanner. The chromatogram scanners used were a Nuclear Chicago "Actigraph" (Mark II) and a Packard instrument.

2. COMPARISON OF CYANIDE AND SULPHIDE ASSIMILATION

Materials & Methods

Seeds of blue lupin were germinated as described above.

Seeds of onion (Pukekohe: *Allium cepa*) were soaked in 0.1% "Zephiran" solution for 5 min, rinsed, and stood in tap water for 30 min. They were then germinated on vermiculite in the controlled environment cabinet.

Seeds of *Pinus radiata* were laid on a wad of water saturated filter paper and kept at 2°C for 14 days: at the end of this time they were germinated on vermiculite in the controlled environment cabinet.

Iodoacetamide and S-acetamidocysteine were prepared by the method described by Schiff (1964).

In vivo experiments:

Seedlings were removed from the vermiculite, washed with tap water, and exposed to either $H^{14}CN$ gas or $H_2{}^{35}S$ gas in the apparatus illustrated in figure III.2.1. The $K^{14}CN$ and $Na_2{}^{35}S$ used were 0.44 and 6.9 mCi per mole respectively at the start of this work, the latter decreasing substantially during the course of it. This was allowed for in experimental design and interpretation. Later work used cyanide of higher specific activity.

The radioactive gas was released by tipping in acid, and 0.5 to 4 hr after this, the complete stopper unit was removed in a fume cupboard and 50 ml boiling 80% ethanol added to kill the plant material. The flask was then boiled briefly on a hotplate to expel residual HCN or H_2S. The plant material and aqueous ethanol were homogenised in an MSE high speed homogeniser for 5 min, then filtered on a Buchner using Whatman No. 541 filter paper. The filter cake was washed and rehomogenised with 80% ethanol, filtered again, and this cake then homogenised with water. The filtrates were combined and evaporated to dryness in a rotary evaporator at 35°C, and the resulting material redissolved in 2 ml 10% aqueous isopropanol. Radioactive components were separated by high voltage electrophoresis and chromatography on Whatman No. 1 paper (solvents preferred: ØOH separates aspartate from β-cyanoalanine and asparagine; BPW 1:1:1 separates β-cyanoalanine from aspartate and asparagine).

Two modifications of this procedure were used to identify the labelled sulphur-containing aminoacids in the plant extracts from sulphide-feeding experiments. In the first, portions of the extracts prepared as described above were oxidised by evaporating them to dryness in a vacuum desicator with 30% hydrogen peroxide; this converted cysteine and cystine to cysteic acid, and cysteic acid was separated from sulphate and other sulphur compounds by paper chromatography with BPW 1:1:1, and by electrophoresis. In the second modification, after removing the stopper unit in the fume cupboard, crushed dry ice was added and allowed to evaporate to expel residual H_2S. Then 50 ml iodoacetamide (0.25%) in 80% ethanol was added at -16° to the still frozen plant material. The flask was stoppered and stored for at least 24 hr at -16°C. The plant material and solution were then homogenised and the soluble components extracted as described before, using in addition chromatography solvents BAW and PropW. In the second modification, cystine is not affected, but cysteine is converted to S-acetamidocysteine.

The insoluble residues left after filtration were digested with "Pronase" (A proteinase of broad specificity supplied by Calbiochem, Los Angeles, USA, and derived from Streptomyces griseus: Nomoto et al., 1960). Pronase was dissolved in 0.01M phosphate buffer at pH 7.2, with 50 µg chloramphenicol per ml added to prevent bacterial growth (Peterson & Butler, 1962). Each sample was incubated for two days at 35°C with 2 ml of this preparation, at the end of which time about 1 ml of hot ethanol was added and the suspension centrifuged. The supernatant was kept and the pellet resuspended in 50% ethanol, and then re-centrifuged. The combined supernatants were evaporated to dryness in a vacuum desicator, and the dry material redissolved in 0.5 ml 10% aqueous isopropanol as described before.

Control experiments were carried out on lupin and onion plant material that had been autoclaved for 20 min at 120°C. The plant material was autoclaved in conical flasks; the stopper units were added and the rest of the experimental procedure carried out exactly as before.

Tentative identifications of the substances separated were confirmed by co-chromatography with an authentic sample, using two dimensional chromatography and autoradiography. In the case of sulphate, co-chromatography was carried out with carrier free ^{35}S-sulphate and the chromatograms scanned. On scanning, only peaks due to sulphate were found, and the areas under these were found to be the sum of the areas under the sulphate peaks in parallel chromatograms of carrier free sulphate and suspected sulphate.

In vitro experiments:

These were carried out on acetone powder preparations of 12 day old lupins. Acetone powder (0.5 gm) was shaken with 20 ml potassium phosphate buffer (0.1 M, pH 7.3) for 1 hr at 0°C. At the end of this time the extract was centrifuged in a "Servall" refrigerated centrifuge (10,000 g for 20 min). The supernatant was dialysed against 5 litres of the same buffer, at 2°C.

Table III.2.1 summarises the relevant experiments carried out using this material: further work on this system is described in the next section.

At the end of the incubation period 1 ml 10% trichloroacetic acid was added to each mixture; the precipitated protein was removed by centrifugation at 2,000 g for 15 min. The aminoacids in the supernatants were absorbed on to "Zeokarb 225" columns. After washing with water, the aminoacids were eluted with 2% ammonia and the eluates evaporated to dryness: the dry material was redissolved in 1 ml 10% aqueous isopropanol. Portions (50 µl) were separated by chromatography on Whatman No. 1 paper, using the solvents BPW 1:1:1 and BEW; radioactive aminoacids were located by scanning.

TABLE III.2.1

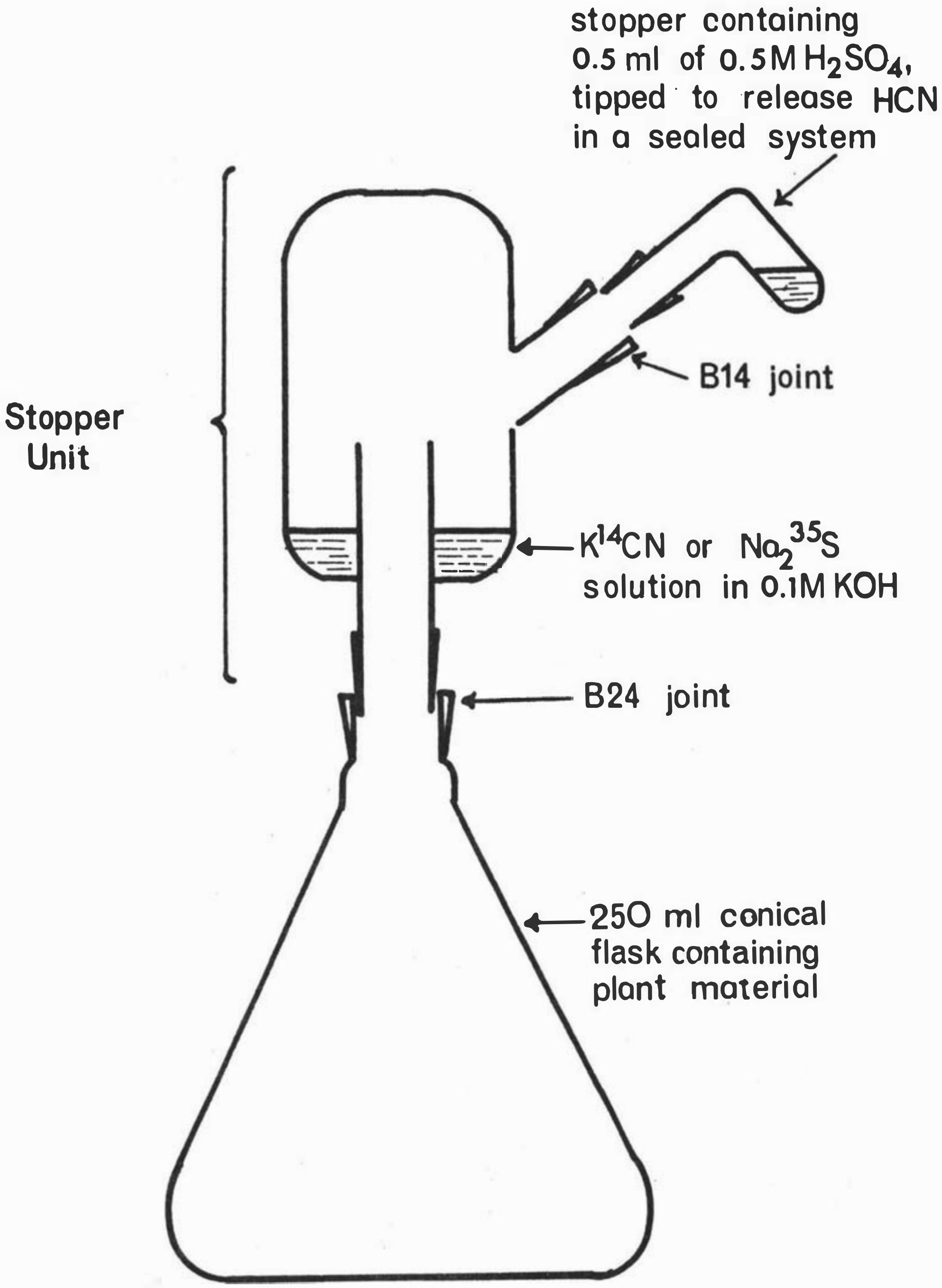
Assays for Substitution Reactions Catalysed by Lupin Acetone Powders

Aminoacid Substrate	Radioactive Substrate	PALP added	Extract Boiled
L cysteine	5μCi $K^{14}CN$	Yes	No
L cysteine	6μCi $Na_2{}^{35}S$	Yes	No
L cysteine	"	No	No
L cysteine	"	Yes	Yes
L serine	"	Yes	No
L cyanoalanine	"	Yes	No

In a total volume of 2.0 ml phosphate buffer (0.1M, pH 7.3) were mixed aminoacids (5 μmole), radioactive substrate, 1.0 ml fresh or boiled extract of acetone powder, and in some cases 0.2 μmole pyridoxal phosphate (PALP). $K^{14}CN$, 36.8 mCi/m mole; $Na_2{}^{35}S$, 6.0 mCi/m mole.

FIGURE 111.2.1

All Glass Apparatus for Supplying Radioactive Gas



Results

Lupins

When lupin plants were supplied with $H^{14}CN$ gas, the major labelled products were β-cyanoalanine and asparagine, with lesser amounts of label also appearing in aspartate and some unidentified compounds. This pattern was observed in plants 2 days and older, and no difference could be observed between dark grown plants and light grown plants. With younger plants and shorter time intervals the proportion of label in β-cyanoalanine increased. Quantitative data on cyanide assimilation is discussed in section III.4. In the control experiment with autoclaved lupin plants label appeared in many different compounds, but none in β-cyanoalanine, asparagine, or aspartate.

When lupin plants were supplied $H_2{}^{35}S$ gas the major labelled product was always sulphate, which accounted for nearly all of the label: aminoacid peaks were always at least an order of magnitude smaller. Plants grown in the light for 12 days or more incorporated some label into free cysteine or cystine, which could be detected as cysteic acid. Attempts to identify S-acetamidocysteine were inconclusive because of low activity (c.f. onions, below): thus lupins probably contain little free cysteine, and the cysteic acid is probably derived mainly from cystine. Most ^{35}S in aminoacids was found in the products of digestion with "Pronase", although in no case was it very substantial. Most effective incorporation was observed in 12 day old light grown plants. When these were cut up, and the separate organs exposed to $H_2{}^{35}S$, label was most pronounced in the aminoacids obtained by digesting the insoluble portion of the green stem and leaf sections.

Thus exogenous H_2S supplied to lupins appear to be more rapidly oxidised to sulphate than it equilbrated with any sulphide pool involved in aminoacid biosynthesis.

With autoclaved lupin plants, some label was incorporated into material that failed to move from the origin on chromatography and electrophoresis. A very slight amount of activity was observed in the position of sulphate, but no activity corresponding to sulphur amino acids was observed.

Onions

Onion seedlings were selected as examples of monocotylodons because onions are known to synthesise a variety of sulphur compounds related to cysteine (Virtanen, 1965). Onion seedlings incorporated $H^{14}CN$ into β-cyanoalanine and asparagine as soon as germination commenced (after 24 hours): but, as with lupins, label from $H_2{}^{35}S$ was not incorporated into sulphur amino acids in the first two weeks of development, although much labelled sulphate was produced. However, older onion seedlings incorporated label into cyst(e)ine, (identifiable both as cysteic acid and as S-acetamidocysteine), occurring both in the soluble fraction and in digests of the insoluble residues. The identification of S-acetamidocysteine demonstrates that onions, in contrast to lupins, readily incorporate exogenous sulphide into a substantial pool of free cysteine.

Pines

Pine seedlings were investigated as examples of gymnosperms. Two week old pine seedlings incorporated label into β-cyanoalanine and asparagine, at a very slow rate compared with lupin and onion seedlings. Incorporation was greater with 4-week old plants, but still about five times less than for other plants used. Label from $H_2{}^{35}S$ was not detected in material identifiable as cysteic acid or as S-acetamidocysteine. However, since the amount of labelled sulphate produced in these experiments was much less than that detected in the corresponding experiments with lupins and onions, it is possible that the length of the experiments was not sufficient for detectable label to accumulate.

Cell Free Systems

In the experiments summarised in table III.2.1 (p. 20), cysteine and cyanide were extensively converted to β-cyanoalanine. Quantitative data for this reaction are given in the following section (III.3). A labelled product, identified chromatographically as cysteic acid, was detected when cyanide was replaced by sulphide. The radioactivity in this was only about 5% (estimated from peak size on chromatogram scans) of that in β-cyanoalanine in the parallel experiment. Pyridoxal phosphate did not affect the incorporation of sulphide; in parallel experiments (next section), it did not affect the incorporation of cyanide. Recently, Hendrickson and Conn (1969) have shown that a partially purified β-cyanoalanine synthase preparation catalyses the exchange of the sulphydryl group of cysteine with inorganic sulphide; thus the labelling of cysteic acid is more likely to be catalysed by this enzyme than by pyridoxal phosphate dependent cysteine synthase.

Label was not incorporated into identified aminoacids in other incubation mixtures (table III.2.1). Thus β-cyanoalanine formation does not appear to be a readily reversible process; serine is not an effective substrate for β-cyanoalanine biosynthesis in lupins, and a cysteine synthase (serine dependent) does not appear to be present. Floss *et al.* (1965) found serine to serve as a substrate for β-cyanoalanine in crude extracts from *Lotus tenuis*; Hendrickson and Conn (1969) have confirmed that the serine is not a substrate for the purified lupin enzyme (see also III.3 and III.5 here), although O-acetylserine is a substrate. These last authors have also shown there to be an O-acetylserine dependent cysteine synthase in lupin seedlings. O-Acetylserine was not tried in the work described here, as this work was completed before its role in bacteria was published.

3. ASSAY METHODS FOR β-CYANOALANINE SYNTHASE

Methods

Crude extracts of acetone powders were prepared by shaking 1 gm powder with 40 ml potassium phosphate buffer (0.1 M, pH 7.4) or tris - HC1 buffer (0.2M, pH 8.4) for 1 hr at 2°C. Some extracts were dialysed for 5 hr against 5 litres of solution, using a rocking dialyser (Barbour and Gledhill, 1962).

β-Cyanoalanine synthase was assayed by three methods. The first was essentially that of Floss et al. (1965). To prepare in incubation mixture, 5 μmoles cysteine and 0.2 μmoles pyridoxal phosphate in 0.5 ml water were added to 1 ml buffered protein solution. The reaction was started with 5 μCi $K^{14}CN$ (36.8 mCi/mmole), incubated in stoppered tubes at 30°C for 1 hr and stopped with addition of 1 ml 10% trichloracetic acid and 2 μmoles β-cyanoalanine in 1 ml water. The precipitated protein was removed by centrifugation at 2,000 g for 15 min, and the amino acids in the supernatants were absorbed on a "Zeocarb 225" resin column. After washing with water, the aminoacids were eluted with 2% aqueous ammonia and the eluates evaporated to dryness in a rotary evaporator. The dry material was redissolved in 1 ml. of 10% aqueous isopropanol and 5 μl portions separated by chromatography on Whatman No. 1 paper, using ØOH as solvent. The radioactive areas on the chromatograms were located with the "Actigraph" chromatogram scanner and the areas under the peaks estimated as a measure of enzyme activity; this method was standardised by eluting one chromatogram and determining the activity in a "Packard" liquid scintillation spectrometer, using the solution described by Bray (1960).

Trials were carried out using different enzyme preparations, and both phosphate and tris buffers.

The other two assay systems depend on the assumption that HS^- is a product of the reaction (Dunnill & Fowden, 1965, Floss et al., 1965). In the second assay, production of $H_2{}^{35}S$ from L-cysteine-^{35}S was determined in Conway microdiffusion units. The outer compartment contained enzyme preparation in 0.1 M potassium phosphate buffer pH 7.3, 0.1 ml (5 μmoles) potassium cyanide solution, and 0.2 ml L-cysteine -^{35}S solution (made by mixing 30 μmoles fresh carrier L-cysteine in 3 ml with 8 μmoles L-cystine-^{35}S in 0.8 ml a few minutes before the start of the assays), in a total volume of 1.8 ml. The units were sealed and incubated at 30°C. The reaction was stopped by adding 1.0 ml 0.1 M NaOH to each centre well and 200 μl of concentrated sulphuric acid to each outer compartment. The units were immediately resealed and left overnight for diffusion to proceed to completion. Spurious results were obtained if the sodium hydroxide were put in place before the enzyme reaction had been allowed to take place: these can be attributed to the alkali absorbing the cyanide substrate, which at pH 7.3 is present mainly as unionised HCN. Portions (0.1 ml) of the alkali from the centre wells were counted in Bray's (1960) solution with a "Packard" liquid scintillation counter. The cysteine-^{35}S substrate solution was also counted under similar conditions, to calculate the enzyme activity in molar units.

The third and preferred method also depended on determining the production of sulphide, this time colorimetrically as methylene blue.

It was suggested by the assay method used by Siegel (1965) for sulphite reductase.

Fresh solutions of potassium cyanide (50 μmoles/ml in 0.05 M tris-HCl buffer pH 8.4), cysteine hydrochloride (10 μmoles/ml in 0.05 M tris-HCl buffer pH 8.4) were made up daily. 0.5 ml of the cysteine solution, a portion of the extract being assayed, and buffer to make a volume of 2.9 ml were placed in a Thunberg tube at 30°C. Cyanide solution (0.1 ml) was added, the tube stoppered, shaken and maintained at 30°C. At the end of the incubation, a mixture of 0.5 ml 0.03 M $FeCl_3$ in 1.2 M HCl and 0.5 ml of 0.02 M p-amino-N, N-dimethylaniline in 7.2 M HCl was tipped in from the stopper. After 20 min or more the precipitated matter was centrifuged at 2,000 g for 10 min and the supernatants were read at 650 nm in a Bausch and Lomb "Spectronic 20" spectrophotometer, against a blank made by carrying out the procedure without protein. The method was calibrated with solutions of sodium sulphide, standardised iodometrically with sodium arsenite (Vogel, 1951), by mixing these with the other components of the assay mixture after the incubation.

Results

Experiments using Floss Assay

The Floss assay was used to investigate the substrate requirements (table III.3.1). No activity could be detected with serine as substrate, though activity with cysteine as substrate corresponded to 10 nmole β-cyanoalanine per hr per mg protein. Dialysis of crude acetone powder extracts was necessary to remove substances that interfered with the isolation of β-cyanoalanine. Linearity of the assay with time was studied, and the data in table III.3.2 shows that the reaction is approximately linear for about three hours.

Tests on the stability of the enzyme were carried out. It was found that storing crude extracts of acetone powders in 0.05 M phosphate buffer (pH 7.3) at 0°C for 24 hours, and that freezing and thawing these same crude extracts, did not significantly diminish their activity. However, extracts made up in a cysteine phosphate buffer (0.05 M phosphate 0.025 M L-cysteine; pH 7.3) lost approximately two thirds of their activity in 24 hours at 0°C.

The effects of EDTA and glycerol on the stability of the enzyme were also determined. Portions of a crude extract from an acetone powder were mixed with equal volumes of either water, 0.01M EDTA solution, or glycerol. These mixtures were then incubated at 30°C for 0, 2 and 5 hours before the cysteine and cyanide substrates were added to start the usual assay. The results obtained are shown in table III.3.3. Glycerol proved markedly inhibitory; the hint of a possible slight stimulation by EDTA is confirmed in section III.5 (table III.5.4).

TABLE III.3.1

Substrate Requirements for Floss Assay

Substrates	Experiment 1	Experiment 2
Serine (μ moles)	..	5
Cysteine (μ moles)	5	..
Cyanide (μ moles)	0.13	0.13
β-cyanoalanine formed (nmole/hr)	27	0

TABLE III.3.2

Linearity of Floss Assay

Incubation Time min	Area Under β-CNala Peak cm^2	Area per Time $mm^2 min^{-1}$
5	0.4	8
20	1.5	8
60	4.6	8
180	13.0	7

TABLE III.3.3

Stability of β-Cyanoalanine Synthase (pH 7.3)

Reagent Added	Time of Preincubation Hrs.	Relative Activity %
Water	0	83
Water	2	95
Water	5	71
EDTA (0.01 M)	0	100
EDTA (0.01 M)	2	87
EDTA (0.01 M)	5	79
Glycerol	0	58
Glycerol	2	52
Glycerol	5	10

Equal volumes of crude extract of acetone powder and reagent were mixed at given time before assay.

A series of control experiments was also conducted in which radioactive cyanide was incubated under the usual experimental condition with no enzyme extract, or with a boiled enzyme extract. Though a variety of products were obtained with cysteine, pyridoxal phosphate and metal ions, only one such unidentified artefact appeared in the eluates of the ion exchange columns, and this was separated from β-cyanoalanine in the chromatography step.

Experiments Using ^{35}S-cysteine Assay and Microdiffusion

The second assay, using ^{35}S-cysteine in microdiffusion units, was tested for linearity with respect to the concentration of enzyme, with the result shown in figure III.3.1. Boiled controls and assays without pyridoxal phosphate or cyanide were also carried out, with results summarised in table III.3.4.

The time course of the reaction was examined both in the presence and in the absence of crude enzyme (table III.3.5).

A small, but not negligible, reaction takes place when cyanide is added to the incubation mixture in the absence of enzyme (tables III.3.4 & III.3.5; figure III.3.1). This appears to depend only on the cyanide and ^{35}S-cysteine, being approximately the same in all experiments, and not increasing with time. This is consistent with a rapid reaction between cyanide and a radiochemical impurity in the ^{35}S-cysteine.

A test of enzyme stability at 30°C in the presence of cyanide was also made. Portions from the incubations for 2 hr and 17 hr were assayed for activity after the start of the incubation, as shown in table III.3.6.

Cyanide has little effect on the stability of the enzyme, in contrast to the results obtained with cysteine, which markedly decreases the stability at the same pH (7.3).

TABLE III.3.4

Controls for ^{35}S-Cysteine Assay

Experiment	Activity (Total cpm x 10^{-3})
Complete Mixture	22.2
—pyridoxal phosphate	21.7
—cyanide	0.1
Boiled Extract	1.2

The complete mixture consisted of 1.0 ml crude extract of acetone powder, 5 μmoles cyanide, 2 μmoles ^{35}S-L cysteine and 0.2 μmoles pyridoxal phosphate in a total volume of 2.0 ml phosphate buffer. Activities in crude extracts were 70-75 nmoles hr^{-1} ml^{-1} by this assay, assuming that the ^{35}S-cystine equilibrated with the cysteine in the time allowed.

TABLE III.3.5

Linearity of ^{35}S-Cysteine Assay with Time

	Activity (cpm x 10^{-3})		
	15 min	30 min	60 min
Total Activity (cpm x 10^{-3}) with enzyme	3.6	5.2	10.0
Total Activity without enzyme	1.3	1.6	1.5
Difference Between Above	2.3	3.6	8.5
Boiled Enzyme	...	...	0.9
Without Cyanide	...	...	0.2

TABLE III.3.6

Stability of β-Cyanoalanine Synthase with Cyanide

	Activity (cpm product/hr)	
	2 hr Expt	17 hr Expt
Protein alone incubated	5.2×10^3	2.6×10^3
Cyanide + protein incubated	5.0×10^3	2.1×10^3

TABLE III.3.7

Stability of β-Cyanoalanine Synthase (pH 8.4)

Time Before Assay at Temp.	Activity (Absorbance x 10^3)	
(hr)	0°C	30°C
2.5	170	164
5	170	166
24	168	154

Portions (0.2 ml) assayed by colorimetric assay after storage at 0°C or 30°C for various times.

Colorimetric Assay

The third assay used, the colorimetric assay, is the method of choice. It is sensitive, simple, and requires much more readily available materials and apparatus since the product (methylene blue) absorbs in the visible region of the spectrum. The amount of reaction with time was studied in a medium buffered at pH 7.3, with the results summarised in figure III.3.2. It will be seen that the reaction is linear for at least 30 minutes, and that a small "blank" reaction also occurs (cf ^{35}S-cysteine assay); the latter does not change with time.

Figure III.3.3 shows that enzyme activity increases rapidly with increasing pH. This agrees with observations by Floss et al. (1965), which indicate that cyanide ion is the substrate: the pK_a of HCN is 9.1 at 25°C, so little cyanide ion would be present at neutral pH. By using a variety of buffers, Hendrickson and Conn (1969) confirmed the results given here, and extended the pH range to about pH 11; above pH 9.5, enzyme activity falls.

To achieve greater sensitivity, the linearity with enzyme concentration was checked at pH 8.4. Figure III.3.4 shows the relationship between enzyme concentration and activity; the response is linear in the range used in later experiments. Figure III.3.5 shows the time course; once more there is a small non-enzymic reaction. The enzymic reaction is linear for at least 5 min. This was the time interval chosen in further work.

The time taken for maximum colour development was investigated. After the p-amino-N, N-dimethylaniline was added, the absorbance was 192 at 10 min, 200 at 20 min and 201 at 60 min. Thus the reaction is complete within 10 to 20 min, and 30 min was routinely allowed in experimental work using this assay.

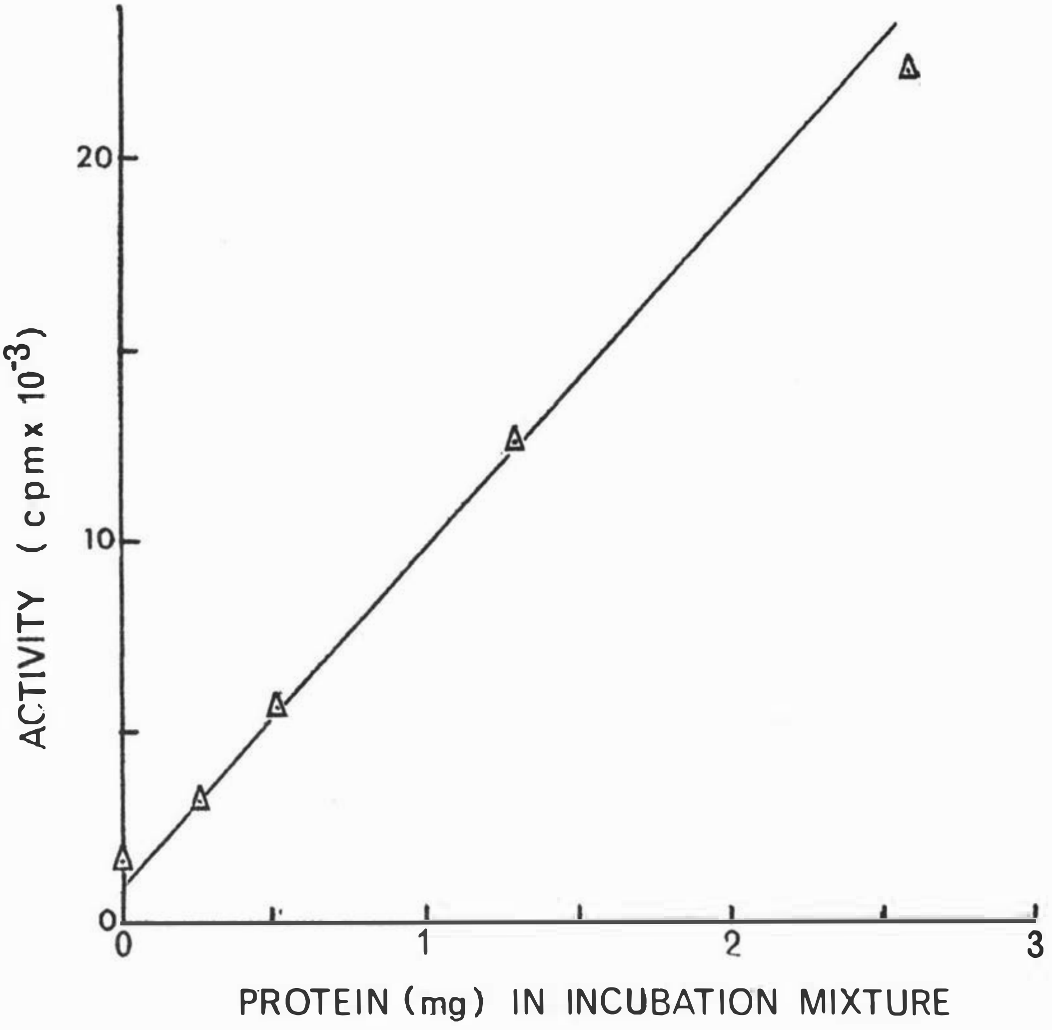
Some further tests on the stability of the enzyme were carried out (table III.3.7). It will be seen that β-cyanoalanine synthase is stable at pH 8.4, losing only 10% of its activity in 24 hrs at 30°C: this is greater stability than shown at pH 7.3 (table III.3.6). Samples of the same preparation were heated for 5 min in water baths at various temperatures, chilled in ice, and then 0.2 ml portions were assayed, with the results shown in figure III.3.6. The stability of the enzyme decreases sharply above 50°C.

When the colorimetric assay was standardised with a sulphide solution, it was found that absorbance was proportional to sulphide concentration, and that one absorbance unit was equivalent to 0.53 μmole sulphide.

Extracts of acetone powders contained 2.5 to 3.5 mg/ml protein. Extracts of lyophilised plant material contained 6 to 10 fold more protein, with correspondingly more enzyme activity (c.f. section III.5).

FIGURE III.3.1

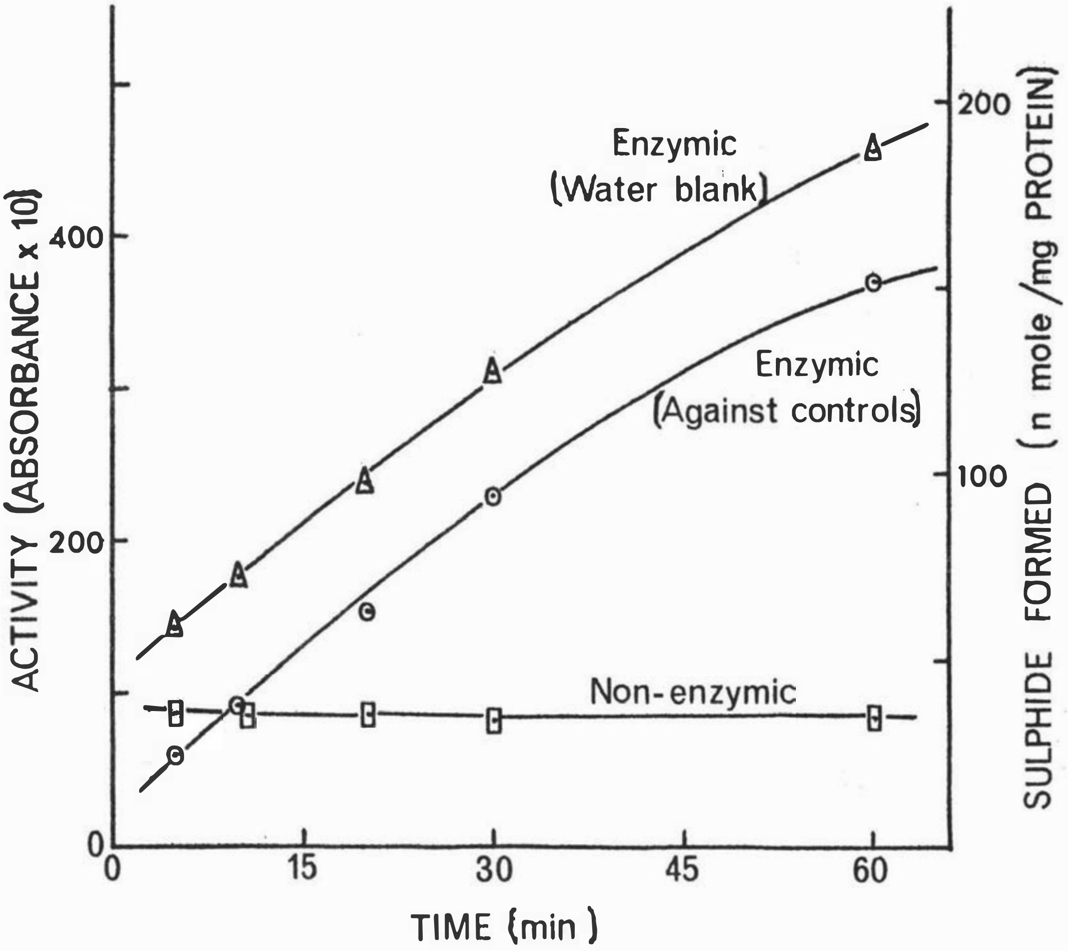
Linearity of ^{35}S-Cysteine Assay with Enzyme



Time interval 1 hr, pH 7.3. Assuming complete equilibration of ^{35}S-cystine with cysteine, activity corresponds to 33 nmole/hr/mg protein.

FIGURE III.3.2.

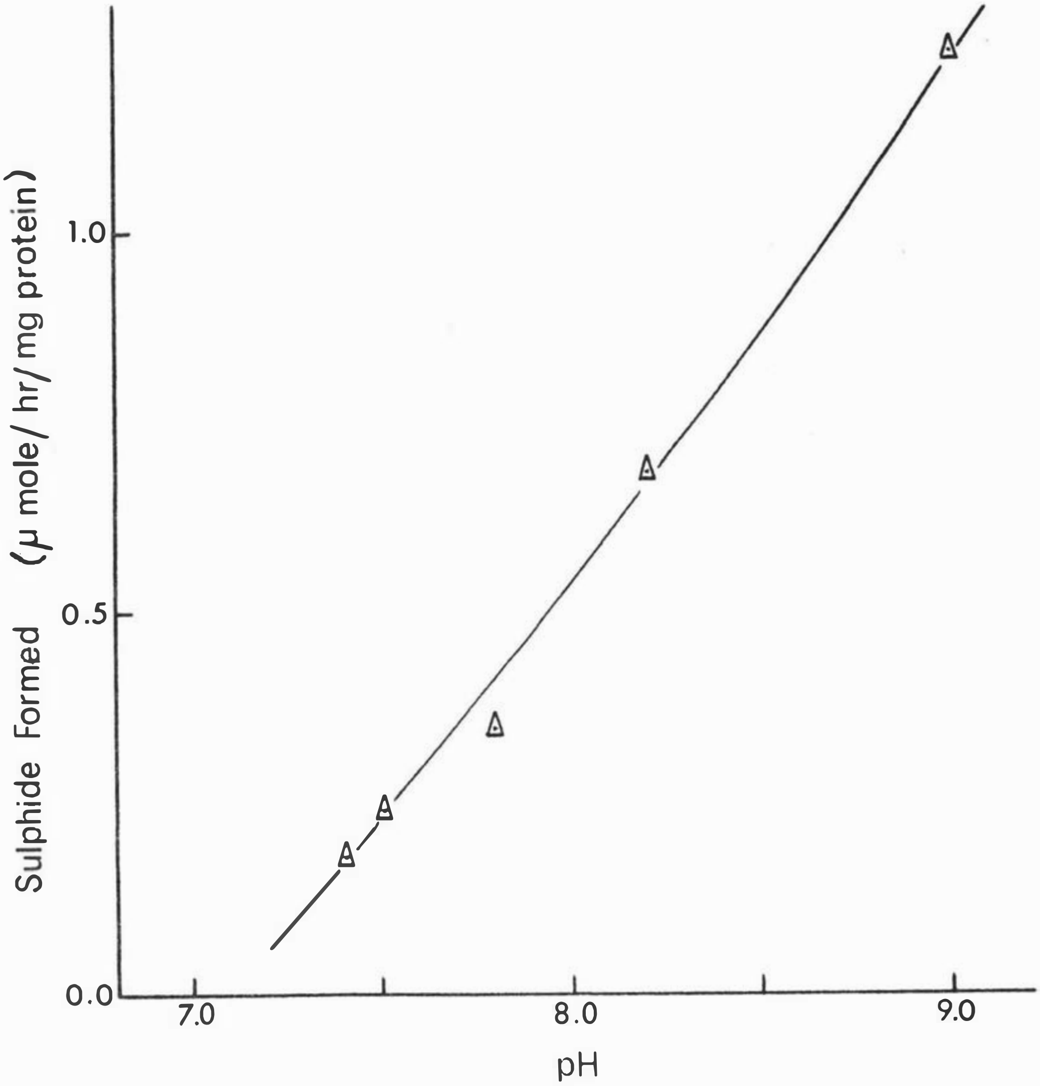
Linearity of Colorimetric Assay with Time (pH 7.3)



Activity measured at 650 nm with a Bausch and Lomb "Spectronic 20" spectrophotometer. Assays at pH 7.3 in tris-HC1 buffer. Cyanide and cysteine (5 µmole each) and 1.3 mg protein in 3 ml total volume.

FIGURE III.3.3.

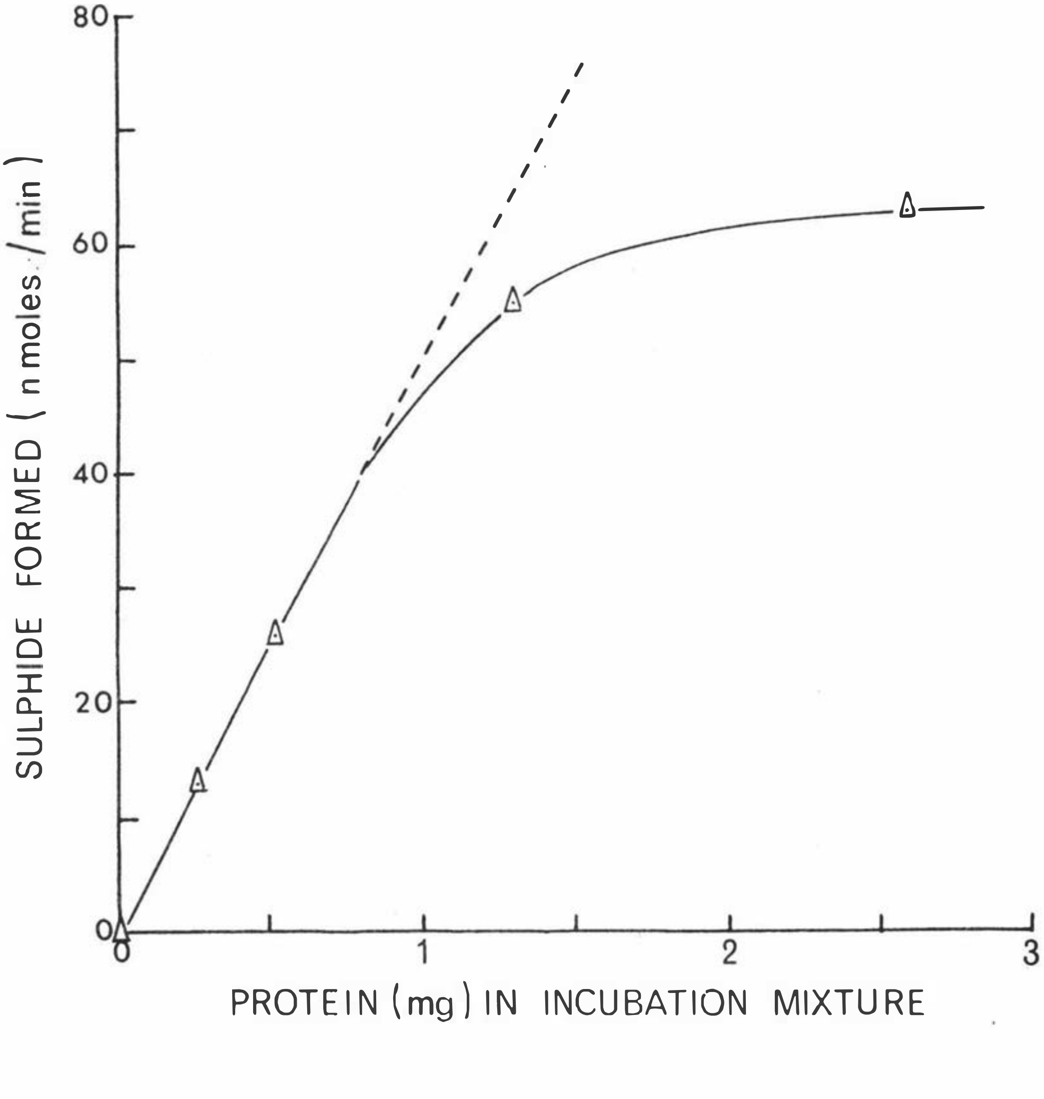
Effect of pH on β-Cyanoalanine Synthase Activity.



Protein (1.3 mg) incubated with substrate for 1 hr. Upper part of curve beyond linear response of assay.

FIGURE III.3.4

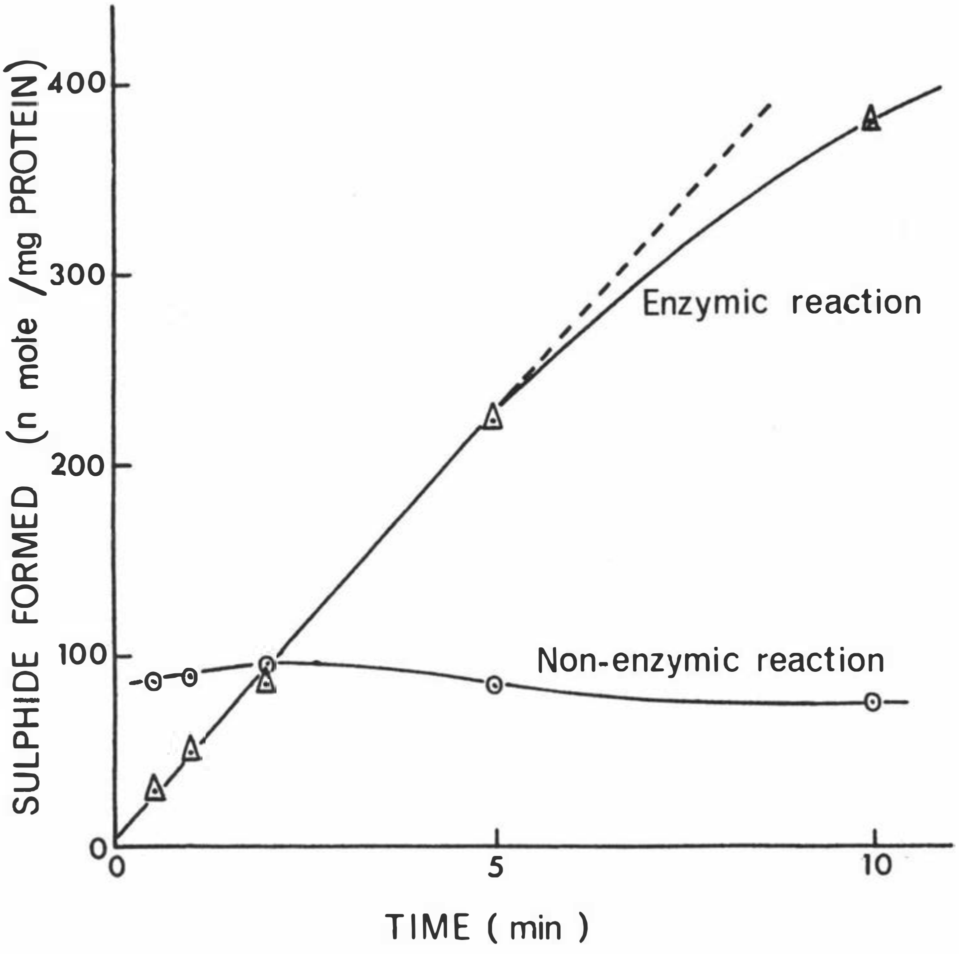
Relationship Between Colorimetric Assay and Enzyme Concentration (pH 8.4)



Time interval 30 min, pH 8.4. In later work (time interval 5 min), absorbance was measured in a range where the response is linear.

FIGURE III.3.5

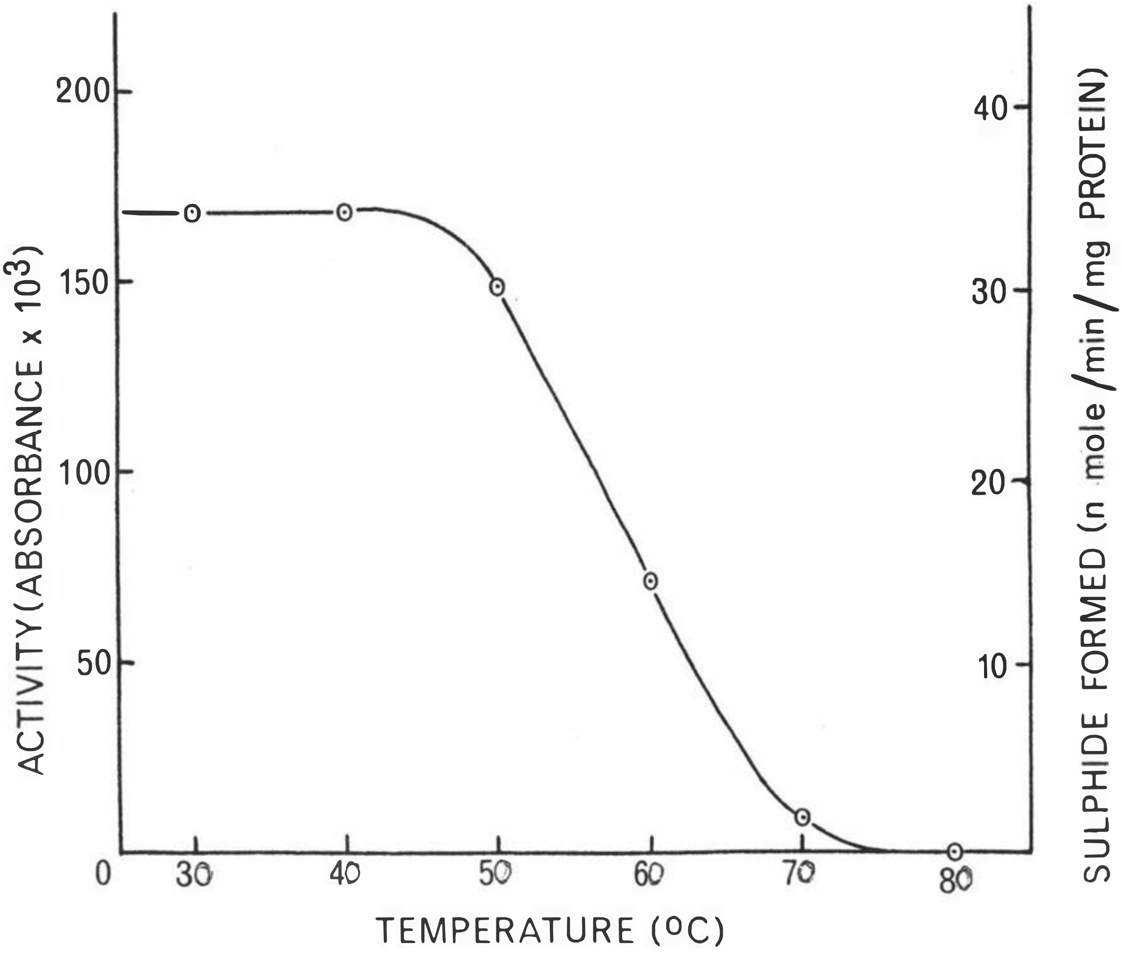
Time Course of Colorimetric Assay (pH 8.4)



Enzymic reaction data obtained with protein (0.5 mg) and substrates incubated at 30°C in a tris-HCl buffered medium (pH 8.4). Absorbance read against blank reaction (without protein) for each time interval. These blanks read against water, are plotted as "non-enzymic reaction".

FIGURE III.3.6

Effect of Heat on β-Cyanoalanine Synthase



4. DISTRIBUTION AND DEVELOPMENT OF THE CYANIDE ASSIMILATION SYSTEM

Methods

Plant material was prepared for β-cyanoalanine synthase assay and asparagine assay by one of two procedures. In the first, it was rinsed with water to remove the vermiculite, weighed, dropped into liquid nitrogen, and then freeze dried. The freeze dried material was ground in a mortar to a homogeneous powder. Portions of this were ground with the extracting solution in a Potter homogeniser, and the suspension clarified by centrifugation.

In the second, the vermiculite was washed off with water, and a few grams wet weight of plant material was homogenised with 50 ml tris-HC1 buffer (0.05 M, pH 8.4) in an MSE high speed homogeniser for 5 min. The homogenate was strained through two layers of boiled muslin and portions of the filtrate assayed.

Asparagine Assay:

Approximately 10 mg portions of the freeze dried powders were used for the asparagine assays. Each was ground in a glass Potter homogeniser with 5 ml glass distilled water, and the suspension clarified by centrifugation. Portions (1 ml) of the supernatant from each were mixed with 1 ml of a 0.5 mg per ml solution of asparaginase (Worthington Biochemical Corporation ASPC) dissolved in 0.05 M tris-HC1 buffer, pH 8.4: the mixture was incubated for 1 hour at 35°C, after which 0.5 ml 10% trichloracetic acid solution was added. The solution was again clarified by centrifugation and the supernatant assayed colorimetrically for ammonia with a modified Nessler's reagent. Separate blanks for each sample were put through the same treatment without the asparaginase step: other Nessler chromogens were present, and these absorbances were subtracted.

The modified Nessler's reagent used was based on that of Williams (1964). HgI_2 (5.5 gm) and 4.125 gm KI were dissolved in about 25 ml glass distilled water. 14.4 gm NaOH were dissolved in about 40 ml glass distilled water, and the solution cooled. Gum Arabic (0.2 gm) was dissolved in 5 ml hot glass distilled water, and the solution cooled. The gum arabic was added to the K_2HgI_4 solution and this solution chilled while the cold sodium hydroxide solution was slowly stirred in. Finally 0.5 ml 1 M potassium sodium tartrate was added and the total volume made up to 100 ml with glass distilled water. This reagent was found to be stable for some months at 2°C, when kept in the dark.

The colorimetric assay was carried out by diluting 0.5 ml of the supernatants with 5 ml glass distilled water. Nessler's reagent (0.5 ml) was added to each, and then rapidly mixed on a vortex mixer. After 20 to 25 minutes, the absorbance at 480 nm was read in a "Unicam" SP 600 spectrophotometer. A series of asparagine and another of ammonium chloride standards were used for comparison: these gave almost identical standard curves.

β-Cyanoalanine synthase Assay:

Portions (20 mg) of freeze-dried powders were extracted by homogenising them with 2 ml 0.05M tris-HCl buffer, pH 8.4, in a glass Potter homogeniser held in an ice bath. The suspensions obtained were centrifuged at 10,000 g for 20 min in a refrigerated centrifuge and the supernatants assayed for β-cyanoalanine synthase activity by the colorimetric procedure described in the preceding section. In the alternative method, crude homogenates were filtered through muslin, and 0.5 to 2 ml portions of the filtrates were assayed directly by the colorimetric procedure.

Changes in β-Cyanoalanine Synthase and Asparagine During Development

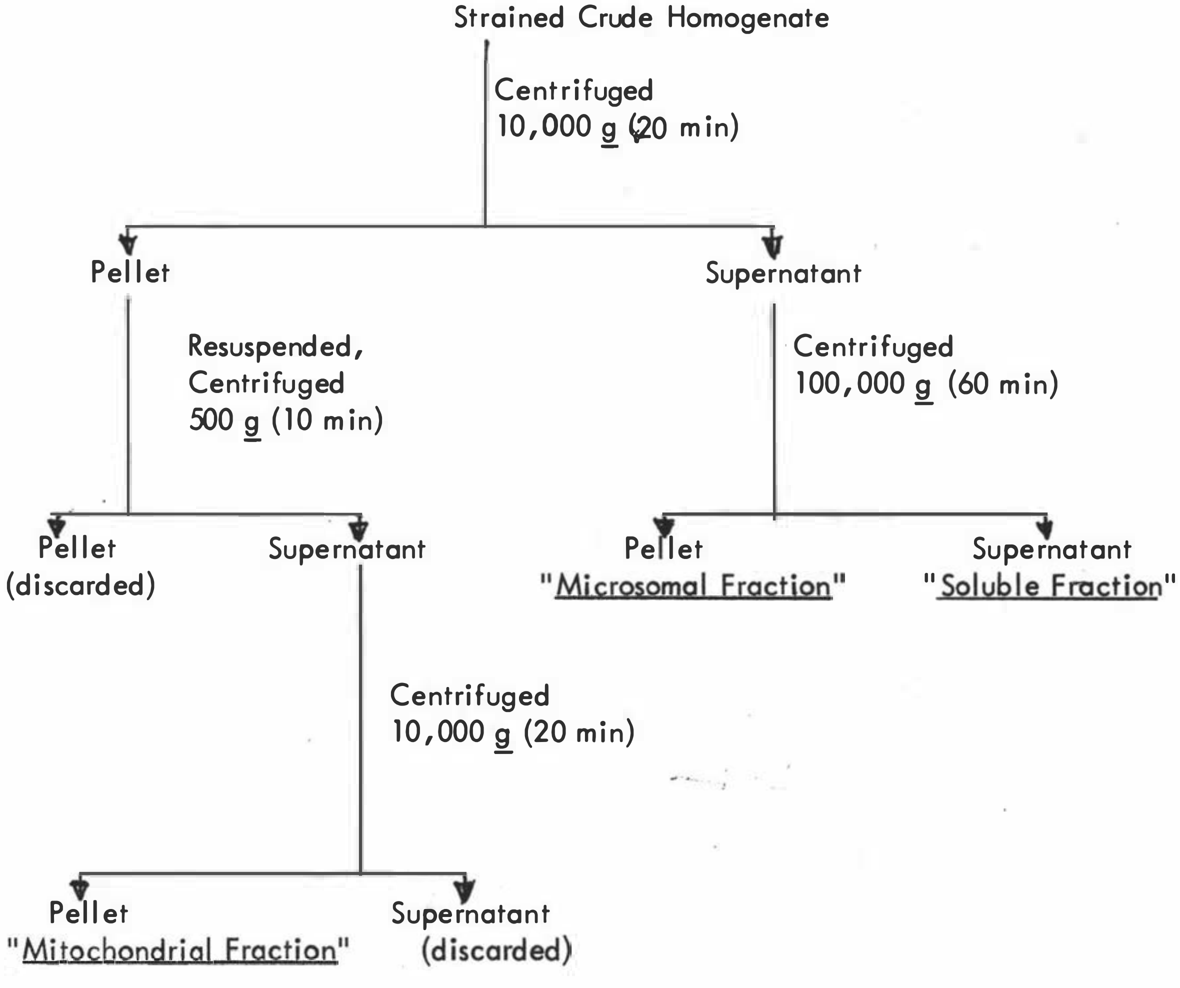
The first procedure used was to plant a batch of seeds and sample seedlings at different ages. Problems of unconsious selection of atypically vigorous plants, and of preparing fresh reagents each day, were minimised by freeze drying large samples of seedlings.

The alternative procedure was to plant seedlings at intervals and assay them all together. With small batches, the failure of a few seedlings to develop normally introduces large errors in this approach: the first procedure is therefore preferred.

Distribution of β-Cyanoalanine Synthase within the Plant

The distribution of the enzyme between different plant organs was investigated. Fresh and freeze-dried organs were homogenised, and the filtrates of these assayed.

To investigate the subcellular distribution of the synthase, the cotyledons, stems, and roots of 40 eight day old seedlings were separated and ground in a cold pestle and mortar with cold sucrose-phosphate buffer (0.4 M sucrose in 0.1 M potassium phosphate, pH 7.1; 1 ml buffer per gm fresh weight plant material). The homogenates were strained through boiled muslin and the filtrate treated as outlined in the scheme:



All centrifugation steps were carried out at 0°C, and all pellets resuspended in sucrose-phosphate buffer. The "mitochondrial" and "microsomal" fraction pellets were washed with buffer by resuspending and recentrifuging; the washed pellets were resuspended for assay in 25 ml (mitochondrial fractions) or 5ml (microsomal fractions) of buffer.

Portions (0.5 ml) of the mitochondrial, microsomal, and soluble fractions from each part of the plant were separately assayed for β-cyanoalanine synthase activity by the colorimetric method described in the previous section.

Changes in Ability to Assimilate HCN:

Samples of three plants from the batches of seedlings used in the development studies above were taken at various ages, and exposed to $H^{14}CN$ in the apparatus illustrated in figure III.2.1. The $K^{14}CN$, was in some cases diluted with carrier KCN so that the 10 μCi released into the gas space was contained in either 0.30 μmole cyanide or in 1.84 μmole. Two hours after the acid was tipped to release the cyanide, the complete stopper unit was removed in a fume cupboard and 50 ml boiling 80% ethanol poured in to kill the plants. These were then boiled briefly on a hotplate to expel residual HCN. The plant material and aqueous ethanol were homogenised in an MSE high speed homogeniser for 5 min, then centrifuged at 2000 g for 10 min. The supernatant was kept and the pellet washed by resuspending, rehomogenising and recentrifuging in 80% ethanol and then again in water. The combined supernatants were evaporated to dryness in a Buchi rotary evaporator at 35°C, and the resulting material redissolved in 5 ml 10% aueous isopropanol. Asparagine and β-cyanoalanine were separated from 20 μl portions of the resulting solutions by descending paper chromatography using the solvent BPW 6:4:3. The sections of the chromatograms containing the aminoacids were cut out as a standard sized strip and the ^{14}C in them counted by liquid scintillation counting: each paper strip was placed in a vial with 15 ml 0.4% PPO and 0.02% POPOP in toluene, and this was counted in a Beckman liquid scintillation spectrometer. The counting efficiency of this system is approximately 50%.

The identity of the labelled compounds on the chromatograms was confirmed by 2-dimensional co-chromatography with authentic amino acids in pairs of the solvents listed in table III.1.1.

Results

Development of β-Cyanoalanine Synthase and Asparagine

Lupin seeds contain little or no asparagine or β-cyanoalanine synthase activity. During the first 5 days after germination in the dark, β-cyanoalanine synthase activity increases but asparagine is not accumulated (table III.4.1); but, as soon as the plumule starts to grow at about day 5, the asparagine content increases rapidly. This apparent maximum rate of asparagine biosynthesis conincides with maximum activity of β-cyanoalanine synthase (figure III.4.1). Asparagine content is maximal after 10 to 12 days, at which time β-cyanoalanine synthase activity is declining rapidly and the dry weight of the plant also begins a rapid decline (table III.4.1) suggestive of exhaustion of reserves: most plants died in the fourth week. Figure III.4.1 summarised the results of assays of freeze dried powders prepared from 12 seedlings of each age. Also included is a study of the development of β-cyanoalanine synthase, carried out on homogenates of seedlings grown under the same conditions from the same batch of seed.

In the asparagine assays, the Nessler chromogens that were subtacted from the total colour also varied with age. Until the age of 5 days they exceeded the asparagine content: calculated as "μmoles NH_4^+" per plant they increased from 19 μmole at day 2 to a sharp peak of 119 μmole at day 5 and declined to 9 μmole at day 12. There was a small increase after this day; the plants were now grossly etiolated, although tissue death and decay was not apparent.

A comparison was made of the development of β-cyanoalanine synthase activity in light and dark grown plants, which were grown from a different batch of seeds from those used in the preceding experiments. The results, presented in figure III.4.2, show that light grown and dark grown plants have maximal β-cyanoalanine synthase activity at the same age. In view of the variations inherent in the method (the alternative one, p.46), the apparently slower decline of β-cyanoalanine synthase activity in the light grown plants may not be significant. It will be noted that plants from this seed source yielded much more enzyme activity, although the pattern of development is similar.

TABLE III.4.1

Development of Asparagine and β-Cyanoalanine in Etiolated Lupin Seedlings

Age of Plant (days)	Dry wt. Plant (mg/plant)	Asparagine (μmole/plant)	β-CNala. synthase (nmole/min/plant)
0	135	0	0
1	130	1	38
2	126	1	114
3	117	1	162
5	119	38	244
8	118	185	162
12	104	213	119
16	87	152	64

For each age, 12 seedlings were freeze dried and extracted together.

Distribution Between Plant Organs

As illustrated in table III.4.2, β-cyanoalanine synthase activity was found mainly in the cotyledons and stems; the ratio of activity in the cotyledons to that in the stems decreases during development, being nearly equal when the plants are about two weeks old. Asparagine accumulates proportionally more in the stems. The distribution of synthase is similar in plants grown in the light and in the dark.

Subcellular Distribution

In all tissues of 8 day old etiolated lupin seedlings, β-cyanoalanine synthase activity is located in the mitochondrial fraction. This is shown in table III.4.3. The distribution of protein differs from that of the synthase, and the cotyledon mitochondrial fraction has a high specific activity of β-cyanoalanine synthase (over 300 nmoles/min/mg protein); this is much greater than any other lupin fraction, and during the purification is only bettered after two purification steps (section III.5). Hendrickson and Conn (1969) used the mitochondrial location of β-cyanoalanine synthase to obtain a more active starting material than was used in the work described in the next section.

Changes in Ability to Assimilate Gaseous HCN

Dark grown seedlings of different ages were supplied with 10 μCi $H^{14}CN$ in 0.30 μmoles and in 1.84 μmoles, for 2 hours. These plants were sampled along with those used to obtain the data in table III.4.1 (p. 49).

TABLE III.4.2

Distribution of β-Cyanoalanine Synthase Between Plant Organs

	Age Plant (days)	Cotyledons	Stems	Roots
A. In low activity batch, dark grown.				
Asparagine	5	31	35	8
β-Cyanoalanine synthase	5	237	82	42
β-Cyanoalanine synthase	16	39	40	1
B. In high activity batch, dark grown.				
Wet weight (gm/plant)	12	0.35	1.26	0.45
Dry weight (mg/plant)	12	38	65	47
Protein content (mg/plant)	12	12	13	6
β-Cyanoalanine synthase	12	317	267	58
C. In high activity batch, light grown.				
Wet weight (gm/plant)	12	0.47	0.76	0.58
Dry weight (mg/plant)	12	28	65	78
β-Cyanoalanine synthase	12	426	276	56

Distribution of β-cyanoalanine synthase activity between organs of lupin seedlings derived from two different batches of seed. Units of β-cyanoalanine synthase, nmoles/min/plant.

TABLE III.4.3

Subcellular Distribution of β-Cyanoalanine Synthase

A. Synthase Activity (nmoles/min/plant)	Mitochondrial	Microsomal	Soluble
Cotyledons	69	0	1
Stems	22	1	3
Roots	6	0	1
B. Protein Content (mg/plant)			
Cotyledons	0.2	0.0	17.6
Stems	0.3	0.2	2.2
Roots	0.3	0.1	1.0

TABLE III.4.4

Effect of Changing HCN Concentration on the Assimilation of $H^{14}CN$

HCN level	1/HCN level	Act. Asn + β-CNala	% Incorp.	1/HCN Assimilated
(µmole)	(µmole^{-1})	(cpm)	%	(µmole^{-1})
0.30	3.33	8920	20	16.6
0.77	1.30	7520	18	7.7
1.84	0.54	6080	14	4.0
15.4	0.07	1030	2	2.8
156.	0.01	140	0.2	2.0

Eight day seedlings supplied 10 µCi $H^{14}CN$ in total HCN in column 1. In column 3 is activity in aminoacid products after 2 hr, determined in 1/250 total extract.

The percentage incorporation into asparagine and β-cyanoalanine is shown in figure III.4.3. It will be seen that maximal ability to assimilate HCN develops after 3 to 4 days, before significant asparagine accumulation has taken place and before maximum β-cyanoalanine synthase activity developed (figure III.4.1, p. 49). When 0.30 μmole HCN is supplied, the proportion of label appearing in β-cyanoalanine decreases from 21% at 3 days to 10% at 5 days, subsequently increasing slightly. Thus when β-cyanoalanine synthase activity is maximal, the smallest proportion of label from administered $H^{14}CN$ appears in β-cyanoalanine. Thus β-cyanoalanine hydratase activity is not rate limiting and may increase at about this stage in development.

Eight day old seedlings from the same batch were investigated with a wider range of cyanide levels. The results obtained are summarised in table III.4.4. As all samples were supplied with HCN for the same length of time, the mass of HCN assimilated by the β-cyanoalanine pathway (calculated from the activity observed in asparagine and β-cyanoalanine) is a measure of the rate of assimilation, provided this remains approximately constant over the two hour time interval. It will be seen from table III.4.4 that at low HCN levels, the percentage incorporation tends to an approximately constant value of about 20%, i.e. total cyanide assimilation is proportional to cyanide level. Unless there is further metabolism of asparagine to aspartate, and thence to the TCA cycle, depletion of substrate did not introduce a large error into these measures of average rates. Alternative pathways of cyanide assimilation are probably insignificant (section III.2; figure III.4.5), and little aspartate was labelled in these experiments.

The data in figure III.4.4 was also plotted in the manner of Lineweaver and Burke: the result is figure III.4.4. The system appears to conform to Michaelis kinetics, and the apparent maximum velocity is about 0.5 μmoles HCN assimilated by three plants in two hours. This incorporation is less than 1% of an integration of β-cyanoalanine synthase activity over the two hour period (table III.4.1).

Altough only a very approximate estimate, this difference of more than two magnitudes gives substance to the belief that β-cyanoalanine synthase is not rate limiting in cyanide assimilation. The rate limiting step may be one involved in absorption and transport of HCN to the mitochondrial site of conversion to asparagine. As the data of figure III. 4.5 shows, HCN incorporation into β-cyanoalanine and asparagine varies with age of plants, but reaches maximum activity earlier than the synthase.

Preliminary experiments were carried out with seedlings grown from the batch of seeds used to obtain the data in figure III.4.2. As this data shows, these develop high β-cyanoalanine synthase activity. They were supplied $H^{14}CN$ for only 0.5 hours. In this work, in contrast to that described before, up to 16% of the activity appeared in aspartate. No consistent differences were observed between light grown and dark grown plants. Detached cotyledons, stems, and roots of 12 day old seedlings were incubated with $H^{14}CN$, and it was found (figure III.4.5) that roots were the most effective in cyanide assimilation, accounting for 44% of the assimilation in dark grown plants and 57% in light grown plants; this contrasts with 10% and 7%, respectively, of the synthase activity in these organs (table III.4.2). These differences suggest that the rate of cyanide assimilation may be related to surface area, and are consistent with the hypothesis that the rate limiting step is cyanide absorption and not conversion to β-cyanoalanine.

The appearance of label in aspartate suggests that there could be a significant flux of ^{14}C from cyanide through the C-4 of aspartate to carbon dioxide. Although it seems unlikely that this could be large enough to invalidate the tentative interpretation proposed here, the possibility should not be discounted.

FIGURE III.4.1

Development of Asparagine and β-Cyanoalanine Synthase Activity in Lupin Seedlings

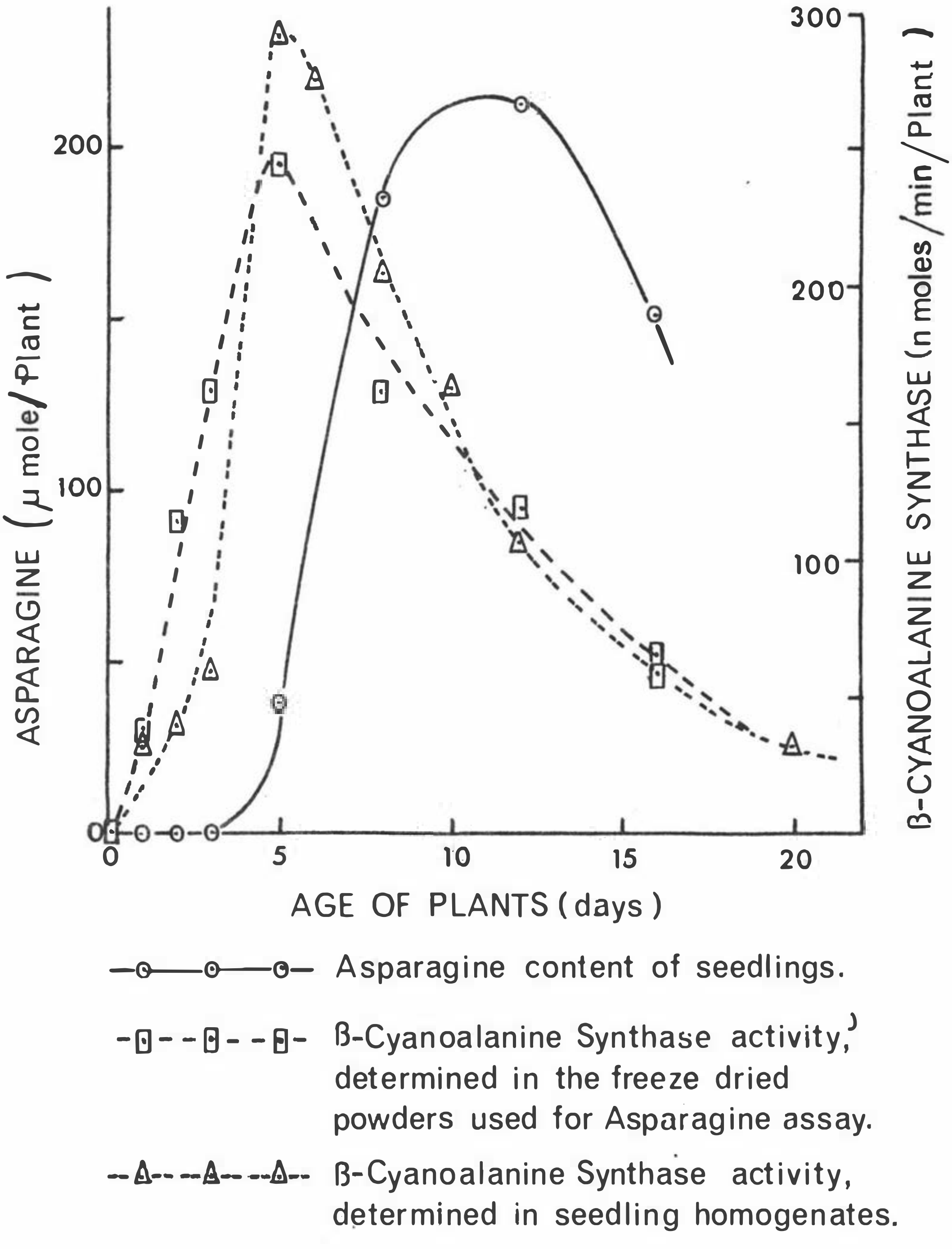
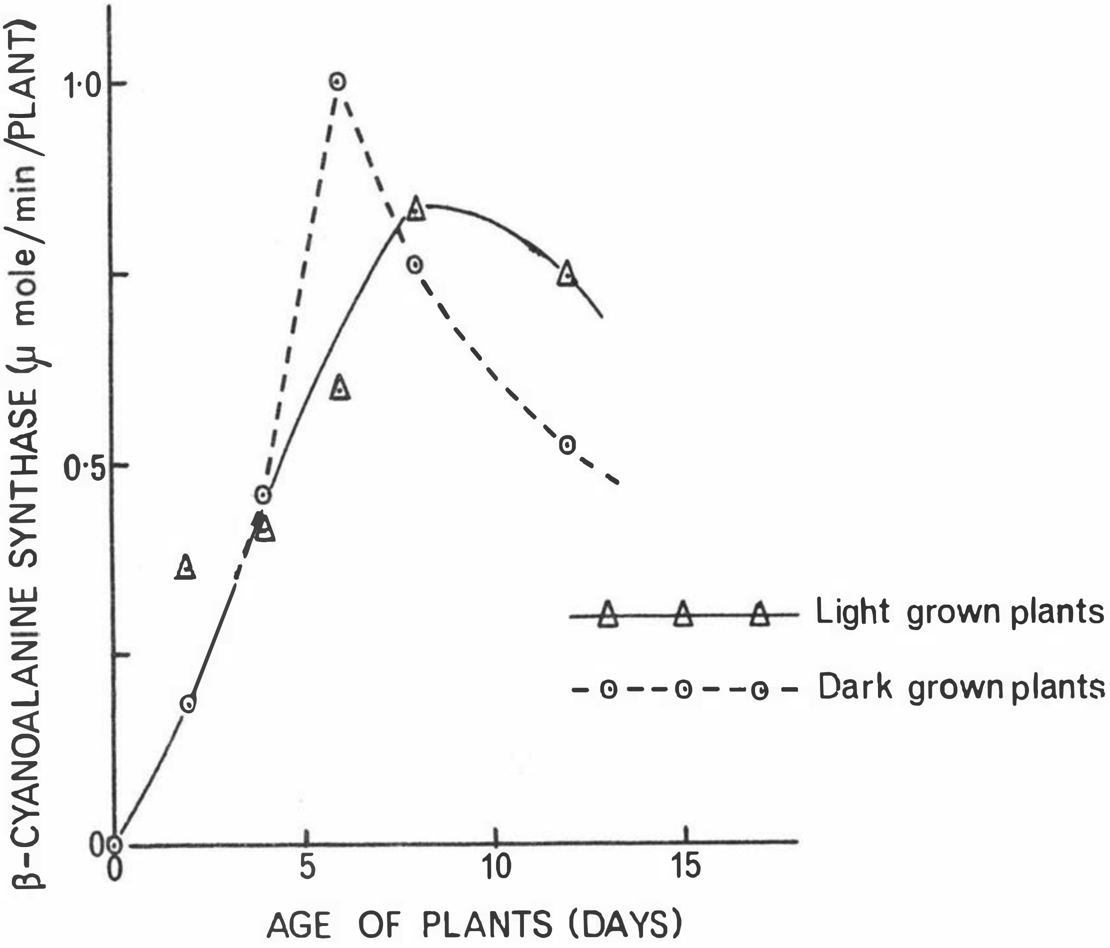


FIGURE III.4.2.

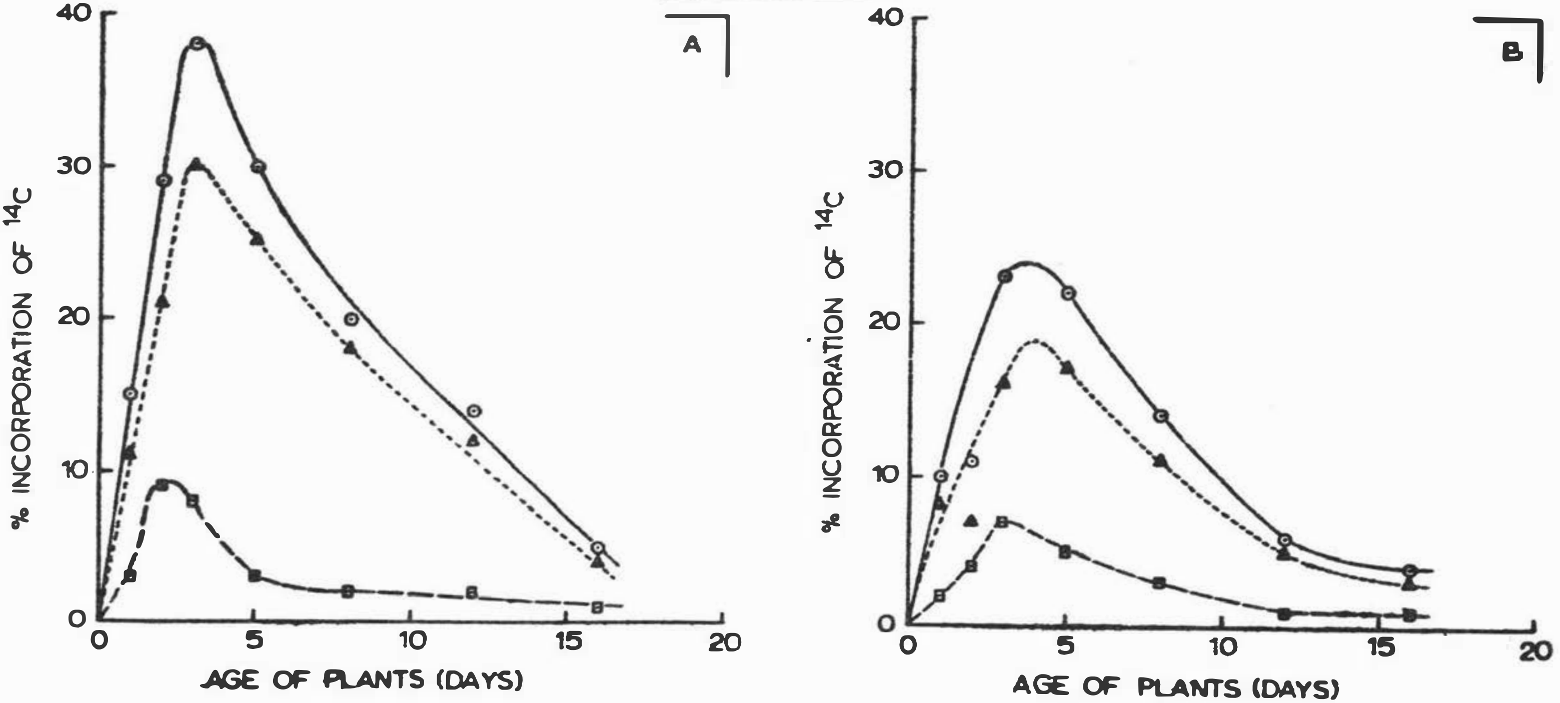
β-Cyanoalanine Synthase in Light and Dark Grown Seedlings



β-Cyanoalanine synthase activity determined in homogenates of light grown and dark grown seedlings from the same batch of seed.

FIGURE III.4. 3.

Cyanide Assimilation



% Incorporation of carbon^{-14} from $H^{14}CN$ into asparagine and β-cyanoalanine:(A) when 0.30 μmole HCN supplied; (B) when 1.84 μmole HCN supplied.

⊙—⊙—⊙ Total β-cyanoalanine + asparagine

▲--▲--▲- In asparagine

⊟—⊟—⊟ - In β-cyanoalanine

FIGURE III.4.4.

Lineweaver-Burke Treatment of HCN Assimilation Data

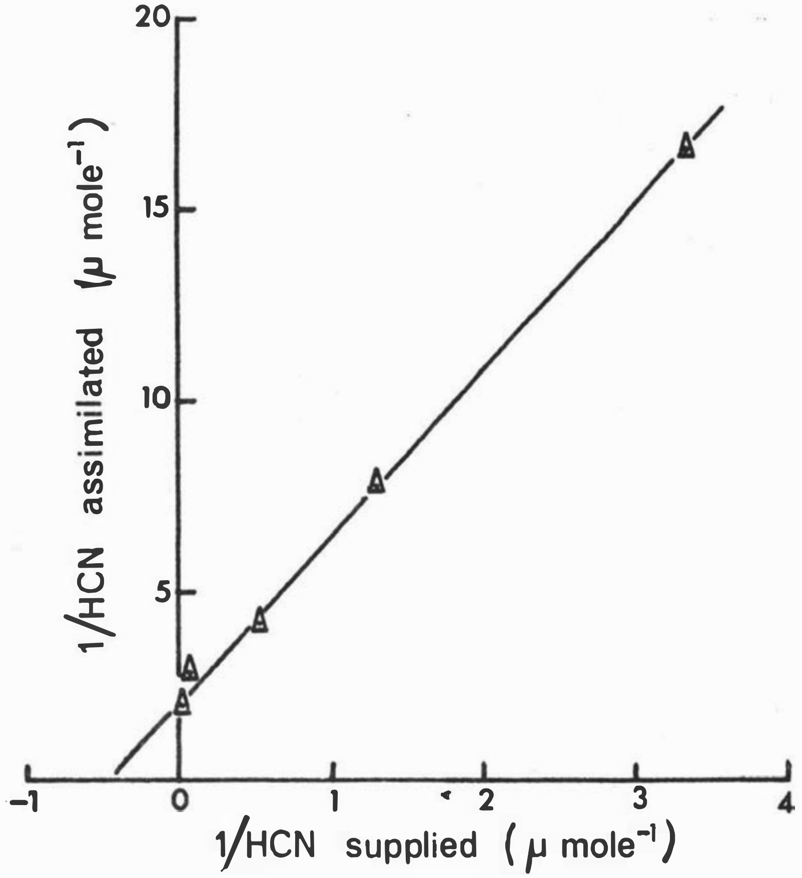
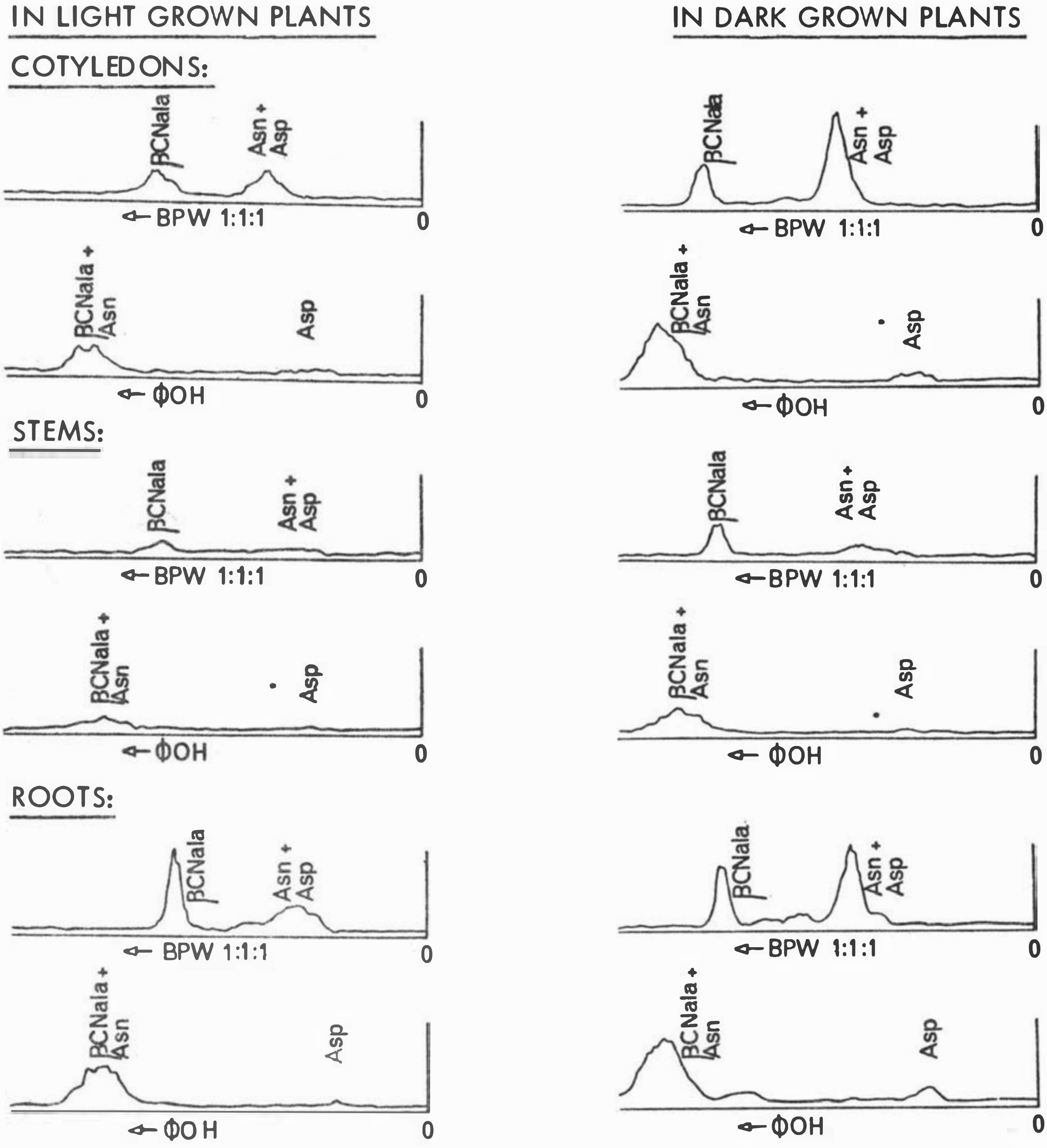


FIGURE III.4.5

Chromatograms of Cyanide Assimilation Products



IN LIGHT GROWN PLANTS

IN DARK GROWN PLANTS

COTYLEDONS:

STEMS:

ROOTS:

A set of traces of chromatogram scans showing the radioactive products isolated from cotyledons, stems and roots of 12 day old lupin seedlings. In each case detached organs from 4 plants were supplied. 20 µCi ^{14}C in 0.54 µmole HCN, for 0.5 hr.

5. PARTIAL PURIFICATION OF β-CYANOALANINE SYNTHASE

Some experiments were carried out on the purification of β-cyanoalanine synthase from acetone powders and from freeze dried powders (prepared as described in section III.1) of lupin seedlings. Freeze dried powders proved the more convenient source.

As described in the preceding section, the activity of synthase that could be extracted from these powders varied with age and with different batches of lupin seed. Because high activity seed became unavailable, and because it was known that Hendrickson had begun a similar purification programme (Hendrickson and Conn, 1969), the work was not pursued far.

The purification procedures investigated include acetone precipitation, ammonium sulphate precipitation, and gel filtration chromatography. These were carried out at 2° -3°C except where otherwise stated. Centrifugation steps were carried out in a "Servall" refrigerated centrifuge.

Some of the substrate and cofactor requirements were examined in more detail than described in section III.3, using partly purified synthase. The results of this work are described at the end of this section.

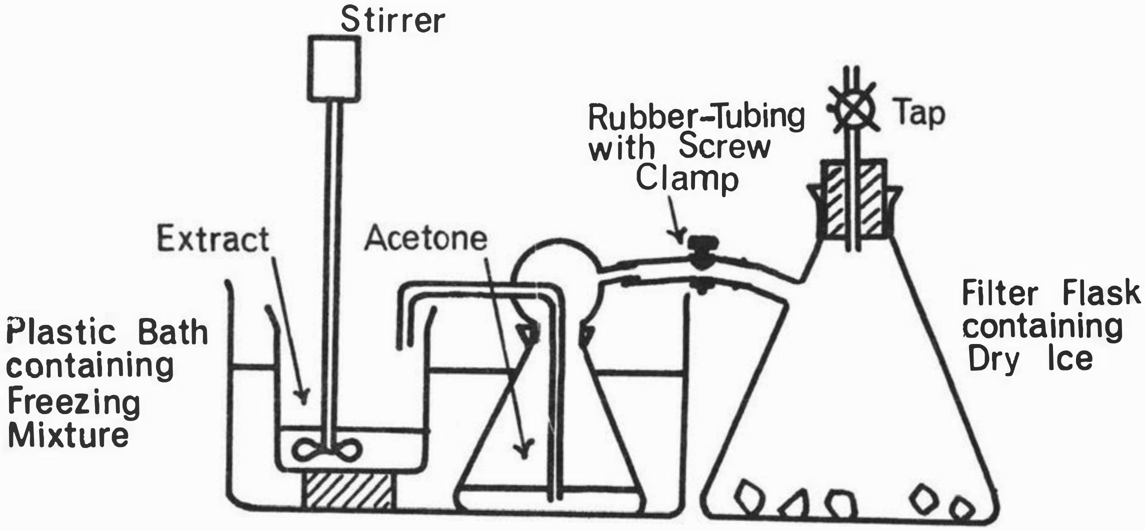
(i) Acetone Fractionation

Methods

Extracts of acetone powders were prepared by shaking 1 gm powder with 50 ml tris-HCl buffer (0.1M, pH 8.4) at 0°C. The extract was then clarified by centrifugation for 20 min at 10,000 rpm. In the first three trials 10 or 20 ml of such an extract was taken in a 50 ml centrifuge tube, which was then immersed in an acetone-ice mixture at -15 to -20°C. Appropriate

FIGURE III.5.1

Acetone Fractionation Apparatus



volumes of acetone at -15°C were added drop-wise from a graduated pipette while the mixture was agitated by hand. After each addition of acetone the mixture was stood for 5 min at -15°C before being centrifuged at 15,000 rpm for 4 minutes at -15°C. The precipitates were drained at -15°C and redissolved in tris-HC1 buffer (0.05 M, pH 8.4); portions of the fractions so obtained were assayed by the colorimetric assay described in section III 3. In the third trial the fractions were all flushed with a stream of nitrogen to rapidly remove the acetone.

The fourth trial was an attempt to carry out acetone fractionation on a preparative scale. Figure III.5.1 is a diagram of the apparatus employed.

Results

The results of the first three experiments are summarised in table III.5.1. This shows that the optimum fraction was found to be the 55% to 67% acetone fraction. The recovery of protein between 0 and 75% acetone was better than 80%, and enzyme recoveries of better than 40% were achieved.

In the first trial, 15% of the initial activity was recorded in the most active fraction (60-57%), with a purification of more than 2 fold; and in the second trial, taking a smaller fraction (60 - 64%) a 17% recovery of initial activity was obtained, with a purification of 4.6 fold. When only two fractions were taken (0-55% acetone and 55-67% acetone), and the precipitates flushed with nitrogen to rapidly remove acetone, 43% of the initial activity was recorded in the second fraction, with a purification factor of 4.8. However, when an attempt was made to scale this up 20-fold (using the apparatus shown in figure

TABLE III.5.1

Acetone Fractionation Experiments

Acetone Fraction %	Protein in Fraction mg	Activity in Fraction nmole $\min^{-1}$	Specific Activ. nmole $\min^{-1} mg^{-1}$
(a) Crude Extract	26	790	30
0-5	0.6	15	24
5-13	0.8	12	16
13-23	0.8	8	10
23-31	2.5	10	4
31-37	2.5	7	3
37-47	4.3	8	2
47-55	5.3	15	3
55-60	2.7	31	12
60-67	1.8	116	64
67-75	1.7	5	3
(b) Crude Extract	27	896	33
0-50	18	143	8
50-55	1.0	18	18
55-60	0.4	37	92
60-64	1.0	151	151
64-75	1.4	69	50
(c) Crude Extract	62	1760	28
0-55	32	372	12
55-67	5.6	753	134

Three experiments in acetone fractionation of β-cyanoalanine synthase; (a), (b) and (c) above are the initial crude extracts, and the figures below each show the activity in acetone fractions of each.

III.5.1), the recovery of activity dropped to 17%, with a purification of 4.6 fold.

(ii) Ammonium Sulphate Fractionation

Methods

Extracts of acetone powders were prepared by extracting 50 gm powder with 2 litres tris-HCl buffer (0.1M, pH 8.4) by stirring for an hour in a polythene bucket at 2°C. After centrifugation at 12,000 rpm for 10 min, the supernatant was dialysed overnight against 10 litres distilled water. The dialysed extract was frozen in a dry ice-alcohol bath, and then freeze dried. The product was redissolved in water, using one tenth of the initial volume, and dialysed against 5 litres tris-HCl buffer (0.05 M, pH 8.4).

Freeze dried powders (200 gm) were prepared by a similar method with 2 litres buffer: this ratio could not be achieved with acetone powders because of a tendency to form a gel. The protein concentration in extracts of freeze dried powders was higher than in those obtained from acetone powders: specific activities were also 1.5 to 2 times greater than found in extracts of acetone powders that had been concentrated by lyophilisation.

A saturated solution of ammonium sulphate (pH adjusted to 8.4 with ammonia) was added dropwise to portions of the extract. After 15 min the suspension was centrifuged at 12000 rpm for 10 min. The precipitates were redissolved in tris-HCl buffer (0.05 M, pH 8.4) and portions assayed for β-cyanoalanine synthase activity.

Results

Initial experiments with ammonium sulphate fractionation were carried out on a low specific activity crude extract (8 nmole/min/mg protein) that had been extracted from acetone powder by 0.1M tris, instead of tris buffer as usual. Table III.5.2 summarises the first two experiments, and also shows typical results from the later scaled-up preparative experiments; the last case shown illustrates the preparation of ammonium sulphate fractionated material for further purification (see below).

It will be seen from table III.5.2 that enzyme activity precipitated mainly between 47% and 60% saturation with ammonium sulphate, with 70% of the initial activity being recorded in this range and with a purification factor of better than 10 for the low specific activity crude extract. The crude extracts with the more typical specific activity of 26 nmole/min/mg protein, were purified 5.7 fold.

Table III.5.3 shows the results of a fractionation carried out on an extract of a freeze dried powder. Although the 45% to 60% saturated fraction has a similar specific activity to that obtained from extracts of acetone powders, the purification factor is less because of the higher initial specific activity.

(iii) Gel Filtration

Methods

"Sephadex G 200" was swelled in tris-HCl buffer (0.02 M, pH 8.4) and packed into columns of various dimensions, the bottoms of which were supported by sintered polystyrene discs. The gel was then washed with tris-HCl buffer (0.01 M, pH 8.4). The enzyme preparations used had already been partially purified by ammonium sulphate fractionation, and contained 20 to 40 mg per ml protein in 0.05 M or more concentrated tris-HC1 buffer. The preparation was layered on to the surface of the gel through a capillary, forming a layer below 0.01 M tris-HC1 buffer (pH 8.4). The protein was eluted with 0.01 M tris-HC1 buffer, collecting fractions with LKB apparatus.

The fractions collected were assayed for β-cyanoalanine synthase activity, and the most active fractions pooled. These were then frozen in a dry ice-alcohol bath, and freeze dried. The residues were redissolved in water. Further purification was achieved if this material was applied again to a smaller "Sephadex" column.

Results

The first trial of gel filtration was carried out on the material obtained in the "1st scaled up experiment" listed in table III.5.2, a 47% to 60% ammonium sulphate fraction of an acetone powder extract. The fractions obtained varied in volume from about 5 ml to 10 ml, as they were collected for constant time. Each was assayed for protein content and enzyme activity. Figure III.5.2 is a graphical representation of the results. In this experiment, the purification was 1.95 fold and the yield in fractions 4 and 5 estimated to be less than 25%. It will be seen that the peak of synthase activity occurs slightly after the main protein peak, and well before the second protein peak. This separation was improved in subsequent experiments in which protein concentration was monitored with an LKB "Uvicord" and recorder.

TABLE III. 5.2

Initial Experiments with Ammonium Sulphate Fractionation

$(NH_4)_2SO_4$ Frac. %	Protein in Fraction mg	Activity in Frac. nmole min^{-1}	Specific Activity nmole $min^{-1}mg^{-1}$
First experiment:			
Crude Extract	230	1846	8
0-33	38	110	3
33-50	28	695	25
50-67	18	1062	59
67-80	9	17	2
Second Experiment:			
Crude Extract	230	1846	8
0-33	36	68	2
33-41	18	136	8
41-47	9	186	21
47-55	10	665	67
55-60	7	650	93
60-67	11	140	13
1st Scaled up Experiment:			
Crude Extract	180	13,100	7
47-60	82	6900	84
2nd Scaled up Experiment:			
Crude Extract	750	19500	26
45-60	39	5760	148

"Crude extracts" are extracts of acetone powders that had been concentrated by lyophilisation.

TABLE III.5.3

Ammonium Sulphate Fractionation of Extract of Freeze Dried Powder.

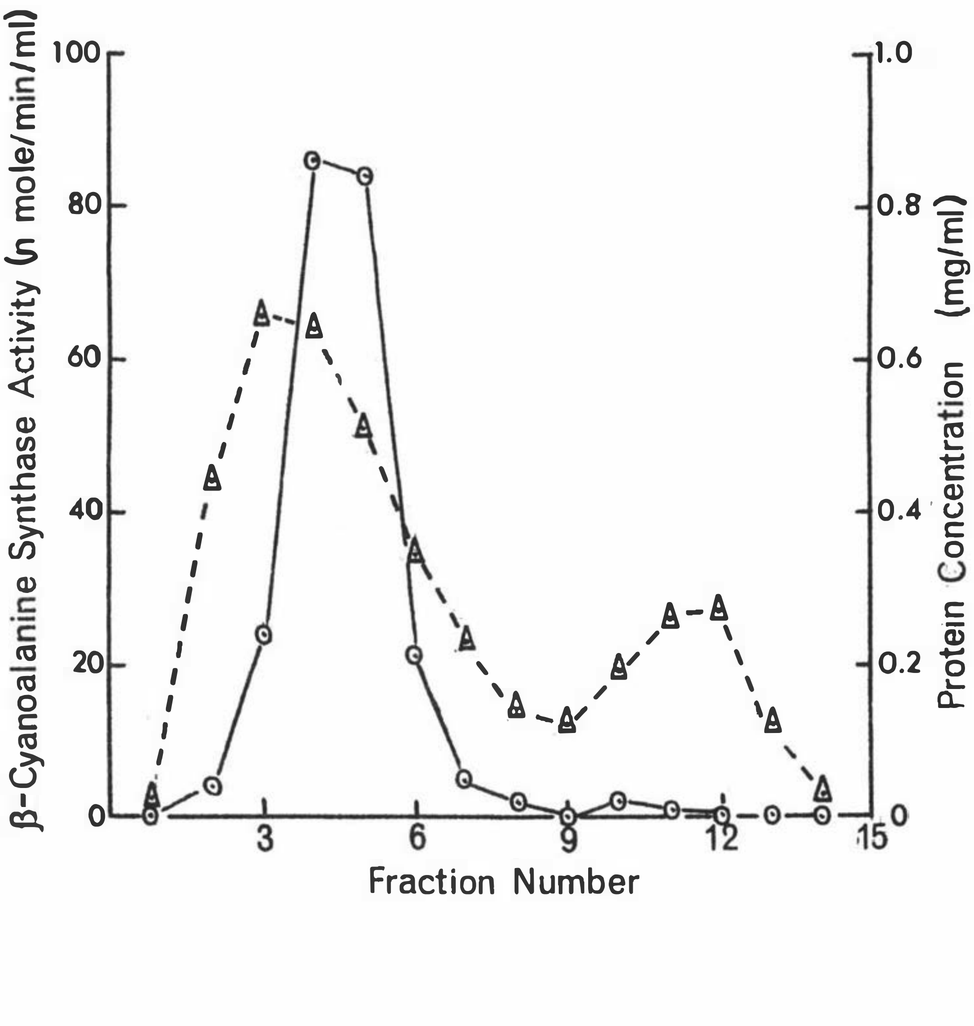
$(NH_4)_2SO_4$ Frac. %	Protein in Frac. mg	Activity in Frac. nmole/min	Specific Activity nmole/min/mg
Original Extract	140	7000	50
0-29	42	300	7
29-37	12	230	19
37-46	11	540	49
46-52	10	1350	135
52-61	15	2660	177
61-71	24	420	17

Complete records of recoveries at each step were not kept for these preliminary experiments on further purification. The highest specific activity material obtained had a specific activity of 6400 nmole/min/mg protein, which was a 250 fold increase on that of the crude extract used; this purification involved ammonium sulphate fractionation and two cycles through "Sephadex G200" columns. When this material was examined by disc electrophoresis in a polyacrylamide gel it was found to still contain at least five distinct protein components. Using a similar assay, Hendrickson and Conn (1969) purified β-cyanoalanine synthase from lupin mitochodria, and obtained, after preparative gel electrophoresis, a preparation with a specific activity of 33,800 nmole/min/mg protein. The earlier steps in their purification procedure involved ammonium sulphate precipitation, acetone precipitation, and chromatography on "Sephadex G100"; the acetone precipitation yields were improved over those described here by adding the acetone at -65°C.

Purification with DEAE cellulose and CM cellulose was also attempted, but a reproducible binding of the bulk of the enzyme to the ion exchange materiol was not obtained. Some protein could be removed from partly purified preparations by passing through a DEAE cellulose column, under such conditions that β-cyanoalanine synthase passes through unaffected; this was not used in the work described here.

FIGURE III.5.2

Chromatography of β-Cyanoalanine Synthase on "Sephadex G200"



(iv) Cofactors and Substrates

Methods

The 250-fold purified β-cyanoalanine synthase preparation was used for further examination of cofactor requirements and of substrate specificity. The substrates, cofactors, and possible inhibitors listed in table III.5.4 were added in solution in tris-HC1 buffer (0.05M, pH 8.4), replacing normal substrate or buffer in the usual colorimetric assay.

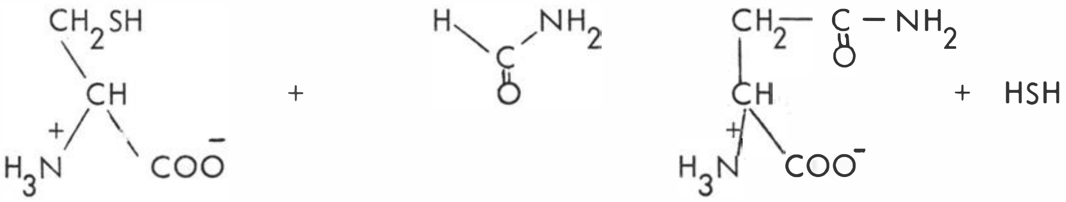
Results

The results obtained are summarised in table III.5.4. As with the crude enzyme preparation studied in section III.3., no evidence was obtained for a requirement of pyridoxal phosphate ("PALP"). The slight stimulation observed on adding this is paralled by adding EDTA, and thus can be explained by the removal of inhibitory trace metals. Isoniazid, similarly, has only a slight effect, as would be expected since the enzyme is not pyridoxal phosphate dependent. Hendrickson and Conn (1969) also failed to demonstrate a pyridoxal phosphate dependence of β-cyanoalanine synthase, although they suggested that an absorbance at 405 nm, which seemed to be associated with the enzyme, could be due to bound pyridoxal phosphate. The relative instability of the enzyme in the presence of cysteine (III.3, above) supports this view, and further evidence adduced by Hendrickson and Conn (1969) is the sensitivity of enzyme activity to inhibition by borohydride.

Glutathione proved to be neither an effective inhibitor nor an effective substrate for β-cyanoalanine synthase. The results show that it does not significantly complete with cysteine when both are supplied in equimolar concentrations.

Serine does not suppress sulphide production, and thus is not an effective substrate for the synthase, confirming the results with crude preparations (section III.3). Hendrickson and Conn showed that, while serine is not a substrate, O-acetyl serine is, although the rate in this case is only 5.4% of that with cysteine.

Formamide is not an effective substrate, so the reaction



does not take place to a significant extent.

TABLE III. 5. 4

Substrates and Cofactors for β-Cyanoalanine Synthase

Substrates	Additive	Activity (nmole/min)
cyanide , cysteine	...	41
cyanide , cysteine	PALP (0.3 μmole)	44
cyanide , cysteine	isoniazid (1 μmole)	49
cyanide , cysteine	serine (5 μmole)	42
cyanide , cysteine	glutathione (5 μmole)	39
cyanide , cysteine	EDTA (2 μmole)	49
cyanide , glutathione	...	2
formamide, cysteine	...	0

The effect of altering substrates and adding various substances to the colorimetric assay for β-cyanoalanine synthase. 5 μmoles of all substrates added.

6. ASPARTATE, FUMARATE & GLYCINE AS ASPARAGINE PRECURSORS.

Methods

Fumaric acid 1,4 (^{14}C), specific activity 12.0 mCi per mmole, and glycine 2 (^{14}C), specific activity 21.8 mCi per mmole, were obtained from the Radiochemical Centre; L aspartic acid 4(^{14}C), specific activity 19.5 mCi per mmole, was obtained from Calbiochem, Los Angeles, U.S.A.

Dark grown seedlings (5 days old) had their roots cut off with a razor blade, and each shoot was allowed to take up through the severed hypocotyl 50 μl of a solution containing 5 μCi of one of the radioactive substrates. For each substrate duplicate experiments were carried out for each of the time intervals 0.1 hr, 0.3 hr, 1.0 hr, and 3.0 hr.

At the end of the experiment, the plant material was killed by dropping it into boiling 80% ethanol and boiling briefly. It was kept at 2°C for 2 to 4 days, and then the aqueous ethanol decanted off. The plant material was transferred to a motor driven Potter homogeniser; about 5 ml of 80% ethanol was added and the material homogenised to an even consistency. This was then centrifuged and the supernatant added to the decanted aqueous ethanol. The pellet was rehomogenised with 80% ethanol, recentrifuged, and the supernatant was added to the rest of the extracted solution.

The pellet was further extracted twice with distilled water. The combined supernatants were evaporated to dryness at 35°C in a Buchi rotary evaporator; the final pellet was kept for digestion with "Pronase". The soluble extracted material after evaporation was redissolved in 1 ml 10% aqueous isopropanol, and 200 μl portions were separated by high voltage electrophoresis. The radioactive areas on the electropherograms were located by autoradiography and the neutral amino acids and aspartate areas eluted. These were then separated by chromatography on Whatman 3MM paper, using PropW as solvent system. The radioactivity was again located by autoradiography and the bands corresponding to aspartate and asparagine eluted.

The insoluble pellets were digested with "Pronase", as described in section III.2. About 1 ml ethanol was added to the digest, and the suspension centrifuged. The supernatant was collected and the pellet resuspended in 50% ethanol, and then recentrifuged. The combined supernatants were evaporated to dryness in a vacuum desiccator over concentrated sulphuric acid. The dry material was redissolved in 200 μl of pyridine acetate buffer pH 5.3 and separated by electrophoresis and chromatography as described above.

The radioactivity in the original extracts, and in the isolated asparagine and aspartate samples from each, was determined by liquid scintillation counting. Portions (50 μl) were applied to 2 cm x 6 cm rectangles of Whatman No 1 paper and then dried and placed in a vial with 15 ml of 0.4% PPO and 0.02% POPOP in sulphur free toluene. This was then counted in a Beckman liquid scintillation spectrometer with a counting efficiency of about 50%.

Sufficient asparagine and aspartate for the determination of the distribution of label were isolated similarly but using a larger scale; Whatman 3MM paper was used for the chromatography, first with BPW 6:4:3 as solvent system, followed by PropW. The isolated amino-acid fractions were eluted and stored frozen in 1.0 or 0.5 ml distilled water.

The distribution of label in the isolated aspartate and asparagine was determined by a modification of the decarboxylation procedure of Chappelle and Luck (1957). This was carried out on aspartate, asparagine, and on asparagine samples that had been hydrolysed to aspartate at 120°C, with equal volumes of concentrated hydrochloric acid in sealed tubes. In this procedure both C1 and C4 of aspartate are removed as CO_2, but only C1 of asparagine. From the difference between hydrolysed and unhydrolysed asparagine samples, the label in C4 was estimated.

After trial experiments (see below) the procedures adopted were as follows.

Into the main compartment of a two-armed Warburg flask was pipetted 3.0 ml 10% succinimide in sodium acetate buffer pH 4.7, and the sample (50 μl to 250 μl). Tetramethylammonium hydroxide (0.1 ml 25%, approx. 2.7 M) or sodium hydroxide (0.1 ml 5M) was placed in the centre well. Into one side arm was pipetted 0.5 ml 10% KI, and into the other 0.5 ml decarboxylation reagent. The latter was made by grinding N-bromosuccinimide in a pestle and mortar with 10% succinimide in sodium acetate buffer, pH 4.7, until a thin paste was obtained.

The flasks were shaken in a water bath at 30°C, and the reaction allowed to proceed for three hours after the decarboxylation reagent had been tipped in from the side arm.

The activity in the carbon dioxide collected in the centre well was counted with a "Packard" liquid scintillation spectrometer equipped with automatic external standardisation. When the carbon dioxide was collected in tetramethylammonium hydroxide, the entire contents of the centre well were transferred to a scintillation vial. When sodium hydroxide was used, this was first diluted to 5 ml in a volumetric flask, and 0.2 ml to 0.5 ml portions of this counted. The preferred scintillation solvent was a modification of that of Jeffay & Alvarez (1961). One litre contained 550 ml sulphur free toluene, 350 ml redistilled 2-methoxyethanol, 55 ml ethanolamine, 50 ml "Triton x 100" and 6 gm PPO.

The trial experiments that led to the adoption of this method were carried out on samples of uniformly labelled (^{14}C) - lysine and (^{14}C) - serine, to which carrier amino acid had been added.

Paper chromatograms were run of samples of each amino acid solution, using as solvent system BPW 6:4:3 on Whatman No. 1 paper. These chromatograms were scanned with a Packard chromatogram scanner; the lysine was found to contain only one significant radioactive impurity, which amounted to less than 1% of the total activity. The serine (which had been stored for over a year) contained substantial radioactive impurities; about 10% of the activity did not move from the origin.

For decarboxylation, 1 μmole of amino acid, containing 2 to 3 x 10^5 dpm (^{14} C), was used. Various methods of absorbing and counting the carbon dioxide were tried: the following is a summary of the results obtained.

1. The carbon dioxide was absorbed by 0.2 ml M sodium hydroxide soaked into a strip of Whatman No. 1 paper, 2 cm x 6 cm. This was then dried and counted in a vial as described above (p.78). When this was done only 60% to 70% of the expected activity was detected: the reason for this

was not investigated. It is suggested that the increased opacity of the paper that had been in contact with sodium hydroxide would account for the disparity, as the observed activity was stable.

2. The carbon dioxide was absorbed by 0.1 ml 5 M sodium hydroxide. This was diluted to 5 ml with water, and 0.5 ml portions of the solution so obtained were counted in the solvent described by Bray (1960). The vials were recounted 3 hr and 18 hr after the first counting; the results (expressed as % theoretical) are given in table III.6.1.

These suggested that activity was being lost from the vials; as no precipitate was apparent and activity was not recovered by agitating the vial contents (cf. below), it is presumed that the carbon dioxide excapes into, or is exchanged with, the atmosphere.

When the 5 M sodium hydroxide in the Warburg vessels was replaced with 1 M "Hyamine" hydroxide in methanol (0.1 ml) plus 0.1 ml water, and the entire contents of the centre well counted, the results summarised in table III.6.2 were obtained. These indicate that hyamine is no better than sodium hydroxide for holding carbon dioxide in Bray's solution, and appears to be less efficient as a trapping agent. Trials of a method in which carbon dioxide was trapped in sodium hydroxide and trasferred by microdiffusion (overnight) into hyamine hydroxide confirmed this.

TABLE III.6.1

Loss of $^{14}CO_2$ from Sodium Carbonate in Bray's Solution

	All Figures % Theoretical		
Amino acid	First count	Second count	Third count
Lysine	94	97	45
Lysine	96	73	2
Serine	78	76	18
Serine	79	78	9

The second count was made 3 hr and the third count 18 hr after the first count. $^{14}CO_2$ decarboxylation product of lysine (^{14}C) or serine (^{14}C)

TABLE III.6.2

Loss of $^{14}CO_2$ from Hyamine Carbonate in Bray's Solution

	All Figures % Theoretical		
Amino acid	First count	Second count	Third count
Lysine	83	81	40
Lysine	86	88	16
Serine	70	55	4
Serine	36	17	3

The second count was made 3 hr and the third count 18 hr after the first count.

3. The decarboxylations and carbon dioxide trapping were carried out as above, in 5M sodium hydroxide, and diluted samples of this were counted in various trial scintillation solvents.

First a modified Bray's solution, containing 10% 1 M hyamine hydroxide in methanol instead of 10% methanol (i.e. 0.1 M hyamine hydroxide in Bray's solution). Results obtained are given in table III.6.3. In view of the known contamination of the serine, these results were considered to be satisfactory.

At the same time a solvent system consisting of toluene 67%, 1 M hyamine hydroxide in methanol 33%, PPO 0.6% POPOP 0.03% was made up. This gave satisfactory results also.

The difficulty with these two solvent systems was severe quenching. The modified Bray's solution gave counting efficiencies below 30%. A scintillation solvent consisting of 60% toluene, 30% methanol, 10% 1 M hyamine hydroxide in methanol, 0.6% PPO and 0.4% POPOP was also satisfactory, giving counting efficiencies of 44-47%; this was initially used.

Attempts to use 1 M NaOH instead of 5 M NaOH to absorb the carbon dioxide showed that this did not give quantitative absorption.

4. This method was not suitable for the study of the distribution of label in some samples that contained a total activity of less than a few thousand counts per minute. In this case the transfer of the total contents of the centre well (0.1 ml of 5 M NaOH) to the last scintillation solvent described above was attempted. Loss of counts was again observed on repeated counting, but

TABLE III.6.3

Retention of Carbonate in a Modified Bray's Solution

Amino acid	All Figures % Theoretical First count	Second count	Third count
Lysine	102	108	103
Lysine	103	104	104
Serine	90	93	86
Serine	88	90	83

Bray's solution containing 0.1 M hyamine hydroxide was used. The second count was made 5 hr, and the third 18 hr, after the first.

this time most of the activity could be restored by shaking the vial (cf. above) indicating that sodium hydroxide and carbonate had separated from the solution as a precipitate. A visible precipitate could be seen. Adding 0.4 ml water, (making the aqueous sodium hydroxide content 0.5 ml 1 M solution) did not prevent this occurring; thus the limit of solubility is between 0.5 ml 0.1 M NaOH and 0.5 ml 1 M NaOH.

The solvent system of Jeffay & Alvarez (1961) was also tried; this was better but still unsatisfactory. It was noted that this quenched less than the solvent containing hyamine hydroxide, and quenching could be further decreased by adding "Triton X 100". The final mixture selected had the composition 55% toluene, 35% 2-methoxyethanol, 5.5% ethanolamine, 5% "Triton X 100" and 0.6% PPO. The counting efficiency in this, using the same gain settings, was 53-57%. In this mixture, not more than 25% of the initial activity was lost in 18 hours; but as extended overnight counting series were contemplated this was still considered unsatisfactory.

Other carbon dioxide absorbants were considered. Hyamine hydroxide was ruled out already, and potassium hydroxide was not considered because its natural radioactivity would be expected to interfere with samples of low activity. Instead was tried a 25% (c. 2.7 M) aqueous solution of tetramethylammonium hydroxide; 0.1 ml of this was used to replace the sodium hydroxide in the centre wells of the Warburg vessels. This did not increase the quenching in the last scintillation solvent described above. In duplicate trial decarboxylations of lysine the recovery of (^{14}C) was 100% of theoretical in both samples, and this remained unchanged after 18 hours. This base was then adopted as the preferred trapping agent.

It was noted that the apparent degree of quenching, and hence counting efficiency, fluctuated significantly in all methods. Thus it proved necessary to calibrate the automatic external standard (using cyclohexane -^{14}C) and determine the counting efficiency in each case; this was used to convert all cpm determinations to disintegrations per minute.

Results

The total disintegrations per minute in the soluble matter extracted from the plant material, and in aspartate and asparagine, are given in table III.6.4. Aspartate and fumarate are both effectivly converted to asparagine at a rate more than twenty fold faster than that of glycine. During chromatography, most activity from glycine-fed plants appeared to be in unchanged glycine.

When the solid residues were investigated, it was found that these were extensively labelled only in the case of the glycine-fed plants. In the case of fumarate and aspartate-fed plants, radioactivity was not detected in experiments of less than one hour, and was substantial only in the three-hour experiments. In all cases most activity was found in substances that moved with the neutral aminoacids on electrophoresis.

Thus glycine, a precursor of cyanide in Chromobacterium violaceum (Michaels et al., 1965) is not effective as a precursor of asparagine in lupins. The four carbon dicarboxylic acids, aspartate and fumarate, are more effective despite a relatively more rapid loss of activity from the plant; these substances are known to be rapidly metabolised, probably by reactions related to the TCA cycle, which involve decarboxylations (Naylor et al., 1958, for aspartate metabolism; Titus and Splittstoesser, 1969, for fumarate metabolism).

Table III.6.5 summarises the results of the degradation studies. Two plants were separately put through each treatment, and each of these duplicates was checked by repetition of the decarboxylations. The aspartate samples from fumarate fed plants gave unexpected results, and its identity was rechecked by chromatography in BAW and ØOH. Label from L-aspartate-

4-(^{14}C) and from fumarate-1,4-(^{14}C) appeared in C1 and C4 of asparagine in approximately equal amounts; little appeared in C2 and C3 of asparagine. Thus the four carbon skeleton is retained, but with extensive equilibration between C1 and C4 of aspartate.

However, asparagine from aspartate-fed plants consistently had more label in the amide group, whereas samples from fumarate-fed plants consistently had more label in the carboxyl group, suggesting that aspartate is probably not incorporated via fumarate. The randomisation observed when aspartate is supplied can be explained if exogenous aspartate equilibrates more rapidly with a pool of dicarboxylic acids than it does with the aspartate pool involved in asparagine biosynthesis. This aspartate pool is separate from most aspartate, as the fumarate-feeding experiments show. The label from fumarate enters most aspartate only after extensive metabolism, but the degradation of asparagine shows that it enters asparagine more directly, probably via a separate, smaller, pool of asparagine.

Glycine is not incorporated into asparagine via cyanide, as this would lead to a preponderance of label in C4, the converse of what is observed. The data obtained is consistent with glycine being metabolised via the TCA or a modified glyoxylate cycle.

In experiments where precursors were administered for less than an hour, and in all glycine experiments, the total activity in the isolated aspartate and asparagine was usually less than 10^4 dpm. In these cases the main uncertainty in the results should be the random errors inherent in radioisotope counting. The standard deviations of these results were calculated, considering only

TABLE III.6.4

Incorporation of ^{14}C into Aspartate and Asparagine

Compound Adminstered (5µCi)	Time (hr)	Activity (dpm x 10^{-6})			Incorporation Into Asparagine (%)
		Tot. Soluble	Aspartate	Asparagine	
L-Aspartate-4-^{14}C	0.1	1.4	0.7	0.01	0.1
	0.3	3.4	1.6	0.03	0.3
	1.0	5.4	1.9	0.2	1.8
	3.0	5.0	0.6	0.9	8.1
Fumarate-1,4-^{14}C	0.1	2.2	0.04	0.01	0.1
	0.3	2.4	0.1	0.02	0.2
	1.0	5.8	0.1	0.1	0.9
	3.0	5.2	0.2	0.7	6.3
Glycine-2-^{14}C	0.1	2.6	<0.01	<0.01	<0.1
	0.3	4.0	<0.01	<0.01	<0.1
	1.0	7.8	0.01	0.01	0.1
	3.0	6.4	0.01	0.03	0.3

TABLE III.6.5

Distribution of ^{14}C in Aspartate and Asparagine

Compound Administered	Time (hr)	Expt. No.	Aspartate (C1 + C4)		Asparagine	
				C1 + C4	C1	C4
Aspartate	0.1	1	0.96	0.86	0.34	0.53
	0.1	2	0.93	0.88	0.44	0.44
	0.3	1	0.98	0.98	...	...
	0.3	2	0.93	0.89	...	...
	1.0	1	0.89	0.96	0.42	0.54
	1.0	2	0.93	0.96	0.41	0.55
	3.0	1	0.91	0.90	0.43	0.47
	3.0	2	0.99	0.95	0.42	0.53
Fumarate	0.1	1	0.67	0.87	0.45	0.42
	0.1	2	0.75	0.93	0.49	0.44
	0.3	1	0.45	0.94	0.55	0.39
	0.3	2	0.81	0.92	0.57	0.35
	1.0	1	0.30	0.97	0.50	0.47
	1.0	2	0.77	0.90	0.46	0.44
	3.0	1	0.16	0.64	0.36	0.28
	3.0	2	0.27	0.87	0.43	0.43
Glycine	1.0	1	0.29	0.17	0.14	0.03
	1.0	2	0.21	0.13	0.13	0.00
	3.0	1	0.19	0.32	0.15	0.17
	3.0	2	0.22	0.33	0.17	0.16

Each horizontal line gives the data derived from one plant. Figures are portions of total radioactivity in the different carbon atoms of the aspartate and asparagine samples.

counting errors, and the seven sets of data with standard deviations greater than ± 0.02 are listed in table III.6.6. Thus with both aspartate and fumarate-fed plants there is a significant difference in the activity in C1 and C4 of asparagine, but the data does not detect any change in pattern with administration time.

TABLE III.6.6

Standard Deviations of Results

Radioactive Substrate	Time	Aminoacid	Portions of Label ± Standard Deviation*		
	(hr)	Isolated	C1+C4	C1	C4
Fumarate	0.1(1)	Asn	0.87±0.02	0.45±0.02	0.42±0.04
Fumarate	0.1(2)	Asn	0.93±0.05	0.49±0.03	0.44±0.06
Fumarate	0.3(1)	Asn	0.94±0.03	0.55±0.02	0.39±0.04
Fumarate	0.3(2)	Asn	0.92±0.03	0.57±0.02	0.35±0.03
Aspartate	0.1(1)	Asn	0.86±0.03	0.34±0.03	0.53±0.04
Aspartate	0.1(2)	Asn	0.88±0.08	0.44±0.05	0.44±0.10
Glycine	3.0(2)	Asp	0.22±0.05	...	...

*Determined from radioisotope counting errors only.

7. OTHER POSSIBLE PRECURSORS OF CYANIDE OR ASPARAGINE

Seedlings and shoots were used in this series of experiments, which were preliminary to those described in the previous section (III.6). In most cases it was assumed that asparagine is only slowly metabolised: as the results in sections III.4 and III.6 suggest, this appears to be true in as far as there is a large pool of asparagine that turns over slowly (c.f. Steward & Bidwell, 1966). Thus if a labelled metabolite is converted into asparagine, trace amounts of label supplied to the plant over a prolonged period should appear in asparagine and the label should accumulate. When significant labelling was observed in a long term experiment, short term experiments were carried out.

Etiolated seedlings of various ages were used. Three methods were used to supply these with labelled metabolites. The first was to supply the possible precursor through the roots. The seedling was washed with distilled water, and the roots dipped into 2 ml solution containing the labelled precursor. The second was to inject the plant with the labelled precursor, using a Hamilton syringe, in evenly spaced portions over the surface of the plant. The third was to remove the roots with a sharp blade, and to allow the severed hypocotyl to imbibe the precursor in 100 µl of solution.

No attempt was made to maintain strictly aseptic conditions during these preliminary experiments. However, all glassware was washed with dilute "Zephiran" before use, and rinsed with freshly boiled distilled water. During the incubation period all plants were covered by a large beaker that had been similarly washed.

The plant material was killed with boiling 80% ethanol (1 to 3 seedlings with 50 ml) and extracted by homogenising with further aqueous ethanol and with water. The insoluble matter was removed by centrifugation and the combined supernatants were evaporated to dryness at 35-40°C in a rotary evaporator. After evaporation, the soluble matter was redissolved in 2 ml 10% isopropanol (for chromatography) or pyridine acetate buffer (for electrophoresis). Portions of this solution were analysed.

Insoluble residues were digested with "Pronase" as described in section III.2, and the aminoacids separated from the digest by the same method as used there.

The possible precursors supplied were:

(i)	L Arginine (ureido ^{14}C), 15.7 mCi/mmole	p.95
(ii)	Glycine-2-(^{14}C), 31.7 mCi/mmole	p.96
(iii)	L Serine (uniformly labelled), 7.4 mCi/mmole & 87 mCi/mmole	p.97
(iv)	Potassium cyanate-(^{14}C), 7.0 mCi/mmole	p.98
(v)	Sodium pyruvate-1-(^{14}C), 9.3 mCi/mmole	p.98
(vi)	L Valine (107 mCi/mmole), L Isoleucine (174 mCi/mmole) & L Tyrosine (238 mCi/mmole) (all uniformly labelled)	p.98
(vii)	DL Aspartic acid-4-(^{14}C), 3.2 mCi/mmole	p.99

(i) Arginine

Seedlings, initially 6 days old, were allowed to take up 10 μCi L-arginine-ureido ^{14}C through the roots over a period of 6 days, and seedlings, initially 12 days old, were allowed to take up 10 μCi arginine through the roots over a period of 3 days. The asparagine was separated by electrophoresis followed by dhromatography of the neutral aminoacids in BPW 1:1:1; the label was detected with the "Actigraph" chromatogram scanner. Most of the label remained in the arginine; a small but significant incorporation into asparagine was observed with the 6 day plants after 6 days. The identity of this asparagine was confirmed by elution, followed by co-chromatography with authentic asparagine on a two dimensional chromatogram using ØOH in the first direction and BAW in the second: the brown ninhydrin positive area and the spot on an autoradiograph coincided.

Seedlings 2 days old, 6 days old, and 12 days old were injected with 10 μCi arginine, and after 3 hours killed and extracted. The asparagine was separated as before. Again, only the asparagine from the 6 day old plants contained detectable label. Sufficient of this for a study of the distribution of label between the carbon atoms was isolated by large scale electrophoresis followed by chromatography on Whatman 3 mm paper with BPW 1:1:1. The asparagine was degraded with N-bromosuccinimide, as described in section III.6. The CO_2 was collected in 0.1 ml 5M NaOH and this was diluted to 5 ml; 0.5 ml portions were then counted in Bray's solution. Results obtained with uniformly labelled serine decarboylated demonstrated at the same time that this procedure was giving almost quantitative results, probably because (unlike the later work described in III.6) new scintillation vials were used, with sealing inserts in the screw caps.

The amide group of asparagine was found to contain only 14% of the total activity (table III.7.1). This result is not consistent with the hypothesis that the ureido group of arginine is converted to cyanide, which is then converted to asparagine via the β-cyanoalanine pathway.

(ii) Glycine

A seedling initially 6 days old was allowed to take up 10 μCi glycine 2-^{14}C through the roots for 6 days. The asparagine was separated by chromatography in BEW and BPW 1:1:1 and the radioactivity located with the "Actigraph" scanner. Although most of the radioactivity remained in the glycine, considerable activity was found in the asparagine. Sufficient of this to study the distribution of label was purified by chromatography in BEW; the asparagine was eluted and further purified by chromatographing it again first in BAW and finally in BPW 1:1:1.

The asparagine obtained was degraded in the same manner as that derived from arginine (table III.7.1), and proved to have about 22% of the total label in each of C1 and C4 (c.f. results in III.6, table III.6.5 , p.90).

Three hour experiments on 5 day old plants are described in section III.6. A 15 day old seedling injected with 5 μCi glycine and left for 6 hours did not incorporate detectable radioactivity into asparagine. This is consistent with data in section III.4 (fig. III.4.1) which shows asparagine synthesis to be most rapid in plants 4-6 days old.

(iii) Serine

A seedling initially 12 days old was injected with 5 µCi of L-serine -U-^{14}C (7.4 mCi/mmole) and left for 2 days. The asparagine was separated by chromatography in propW, BPW 1:1:1, and BAW, and the radioactivity detected with an "Actigraph" chromatogram scanner. Extensive metabolism of the serine occurred, and asparagine was one of the labelled products.

A 5 day old seedling was allowed to imbibe 5 µCi of serine (87 mCi/mmole) through the severed hypocotyl for three hours. The asparagine was separated by chromatography in BPW 6:4:3, BAW, and ØOH and the radioactivity detected with a Packard chromatogram scanner. A small but significant conversion to asparagine was observed, although only 4% of that observed in a parallel experiment with 10 µCi DL-aspartate (table III.7.1). The identity of the asparagine was checked by cochromatography (see below, vi). Sufficient for degradation was isolated by chromatography on Whatman 3 MM paper in BPW 6:4:3 followed by elution and chromatography in BAW. The degradation was carried out as described for arginine; the solution of $^{14}CO_3^{2-}$ in alkali was then freeze dried and sent to another laboratory, where the CO_2 was transferred by microdiffusion into a solution of "Hyamine 10X" hydroxide and counted in this form. As will be seen from the results in section III.6 (p. 78) this is likely to underestimate the label a little. However, approximately equal activity was found in C1 and C4 (table III.7.1), and taking into account the probable counting errors in these low activity samples, the observed distribution of label is consistent with even labelling.

(iv) Cyanate

A seedling initially 6 days old was allowed to take up 5 µCi potassium cyanate $-^{14}C$ through the roots over a period of 6 days. The asparagine was separated by chromatography in propW, BPW 1:1:1 and BAW. The seedling contained little radioactivity, and no significant label was detected in asparagine.

(v) Pyruvate

Two seedlings 11 days old at the start were used: one was allowed to take up 10 µCi sodium pyruvate $-1-^{14}C$ through the roots for 3 days and the second was injected with 5 µCi pyruvate and left for 24 hours. The asparagine was separated by chromatography in propW, BEW, BAW, and BPW 1:1:1, and the radioactivity located with the "Actigraph" scanner. Very little activity was recovered from these plants, with no significant labelling of asparagine.

(vi) Valine, Isoleucine and Tyrosine

Three 5 day old seedlings were each allowed to imbibe 5 µCi of one of these three uniformly labelled L-aminoacids through a severed hypocotyl, and left for three hours. The asparagine was separated by chromatography in BPW 6:4:3, BAW, and ØOH. A considerable number of peaks were observed when these chromatograms were scanned with the "Packard" scanner, and peaks near where asparagine would be expected were eluted and their identity checked by cochromatography with authentic asparagine using two cimensional systems. These systems consisted of the two solvents from BPW, BAW and ØOH that were not used in the initial separation. The radioactivity was detected on these by autoradiography and the position of this compared with the brown ninhydrin spot of asparagine. Only asparagine from the isoleucine fed plant contained significant radioactivity; the conversion was poor even in this case, and was less than in the parallel serine experiment (iii. above) and much less than that when aspartate was supplied (vii. below). Degradation was not attempted.

(vii) DL Aspartate

A 5 day old seedling was allowed to imbibe 10 μCi of DL aspartate -4-^{14}C through the severed hypocotyl, and left for three hours. The asparagine was separated and its identity checked as described in the preceding paragraph (vi). Extensive metabolism of the aspartate had occurred, but the largest peaks on the chromatogram scans corresponded to asparagine; this was significantly larger than that corresponding to aspartate. DL Aspartate was clearly a much more effective precursor of asparagine than was the serine, valine, tyrosine, and isoleucine supplied in parallel experiments.

Sufficient asparagine for investigation of the distribution of label was isolated by electrophoresis followed by chromatography in BPW 6:4:3. The $^{14}CO_2$ samples were collected in alkali, freeze dried and counted as described for the parallel serine experiment. The results obtained suggest that C1 and C4 were equally labelled, with about 38% of the total label in each. As the counting method used would underestimate carboxyl label (section III.6, table III.6.2), this result is in consistent with those described in section III.6. The significance of this experiment lies in its allowing a comparison with serine, isoleucine, tyrosine, and valine as precursors. Under the same conditions, label from L-serine-U-^{14}C was incorporated into asparagine at less than one tenth the rate, and label from the other aminoacids even less effectively.

TABLE III.7.1

Distribution of Label in Asparagine Samples

Labelled Precursor	Time	Activity Decarboxylated (dpm x 10^{-3})	Portions of Activity in carbons		
			C1+C4	C1	C4
L-Arginine-ureido-^{14}C	6 days	1.2	0.40	0.26	0.14
Glycine -2-^{14}C	6 days	8.3	0.43	0.21	0.22
L-Serine-U-^{14}C	3 hrs	0.95	0.48	0.26	0.22
DL Aspartate-4-^{14}C	3 hrs	23.4	0.76	0.37	0.39

Counts were carried out on 1/10 of the CO_2 collected. In the first two cases, $^{14}CO_2$ was counted as sodium carbonate; in the other two, as hyamine carbonate. The latter were done for the author, and may be underestimated because of the method used. The activity in portions of asparagine decarboxylated are comparable between serine and aspartate only.

8. INVESTIGATIONS WITH CELL FREE SYSTEMS

Cell free preparations from dark grown lupins were investigated for the ability to catalyse four types of reaction that could be relevant to this study.

(i) Reduction of Carbamoyl Phosphate (or Cyanate) to Cyanide (p. 103)

Carbamoyl phosphate is, in solution, in equilibrium with cyanate and phosphate (Allen & Jones 1964). Thus a possible metabolic pathway for cyanide for asparagine biosynthesis was considered involving reduction of cyanate or carbamoyl phosphate to cyanide, and lupin preparations were investigated for enzymes that could catalyse either

$$O{=}C(O\text{Ⓟ})(NH_2) + NAD(P)H \longrightarrow {}^{-}C{\equiv}N + P_i + H_2O + NADP^+$$

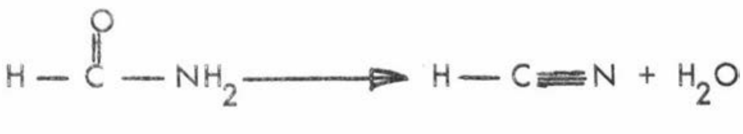
or

$$CNO^- + NAD(P)H + H^+ \longrightarrow CN^- + NAD(P)^+ + H_2O$$

In section IV.2 are given the reasons for suspecting a possible link between carbamoyl phosphate metabolism and asparagine biosynthesis.

(ii) Dehydration of Formamide (p. 104)

Formamide could conceivably arise from folate derivatives, and an enzyme that catalyses the reaction



$$H{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}NH_2 \longrightarrow H{-}C{\equiv}N + H_2O$$

was looked for. The data in table III.5.4 (p. 76) shows that formamide is not, itself, an effective substrate for β-cyanoalanine synthase.

(iii) β-Cyanoalanine Hydratase (p. 106)

There is radioisotopic evidence for the probable existence of this enzyme, as has been noted already. Fowden and Bell (1965) have shown that the reaction can take place in cell free systems of a number of plants. Preliminary experiments were carried out to assess a possible colorimetric assay that would enable some of the properties of this enzyme to be determined.

(iv) Asparagine Synthetase (p. 108)

The evidence for the occurrence in plants of this enzyme is discussed in section IV.3 (p. 125). It is a well established entity in animals and micro organisms.

(i) Carbamoyl Phosphate (or Cyanate) Reductase

Dilithium carbamoyl phosphate was prepared by the method of Spector et al. (1955) as modified by Davis (1962), and its purity assayed by the method of Fiske and SubbaRow as described by Leloir and Cardini (1955). The preparation used contained 65% carbamoyl phosphate.

Acetone powders were extracted with tris-HCl buffer (0.2M, pH 7.4) and centrifuged. Some of the supernatants were dialysed. Another extract was prepared by homogenising 25 gm tissue with 25 ml tris HCl buffer (0.1M, pH 8.4) and straining the homogenate through muslin. Substrate (potassium cyanate or carbamoyl phosphate) and extract were mixed in a silica spectrophotometer cell, and the reaction started by adding NADH or NADPH. Absorbance at 340 nm was followed in a Beckman DU or DK2 spectrophotometer. With KCNO as substrate, low activities were absorbed in all experiments: a little acetaldehyde was added at the end to check that some active enzyme and coenzyme were present. The results of this experiment (table III.8.1) provide no evidence for a nicotinamide coenzyme linked reduction of cyanate or of carbamoyl phosphate. The slight loss of absorbance observed occurred at least as fast in the absence of the suspected substrate as in its presence. That other enzyme activities had survived the preparation of these extracts was demonstrated by the addition of acetaldehyde: high alcohol dehydrogenase activities were observed. This also confirms the effectiveness of the coenzyme preparations. In the later experiments, high endogenous activity provided the same controls.

(ii) Formamide Dehydration

Lupin seedlings (5 day old) were homogenised with tris HCl buffer (0.1 M, pH 8.4), 1 ml buffer being used for each gm wet weight of plant material. The homogenate was strained through muslin and the filtrate used. Assays were carried out in 3.0 ml total volume buffer (0.05 M tris HCl, pH 8.4), containing of 2.0 ml filtrate, 0.5 ml buffer containing 5 μmole ATP, and 0.5 ml of buffer containing 10 μmoles formamide. The mixtures were incubated in stoppered tubes for 1 hr at 30°C, and then portions assayed for cyanide by the method of Aldridge (1945). The results obtained are summarised in table III.8.1. In all cases where filtrate had been included high absorbancies were found. As this also occurred in the boiled controls, in which a large portion of the cyanide would be expected to have been lost by volatilisation, it was suspected that the colour was to be attributed to interfering substances other than cyanide. This was confirmed by microdiffusion. Portions of the assay mixture were placed in Conway units with alkali in the centre well; the alkali was assayed for cyanide. Less than 0.1 μmole cyanide was present.

TABLE III.8.1

Assays for Reduction of Cyanate and Carbamoyl Phosphate

Extract	Substrate	Coenzyme	$-\frac{d}{dt}$ (Absorbance) x 10^3		
			With Substrate	Without Substrate	$+CH_3CHO$
A	KCNO	NADH	3	3	330
A	KCNO	NADPH	1	5	30
AD1	KCNO	NADH	4	4	...
AD1	KCNO	NADPH	0	0	...
AD2	CAP	NADH	13	16	...
AD2	CAP	NADPH	9	10	...
H	CAP	NADH	56	62	...
H	CAP	NADPH	74	84	...

Abbreviations: A; acetone powder extract. AD; dialysed acetone powder extract. H; crude homogenate. CAP; carbamoyl phosphate. All in volume 3ml; 5 μmoles substrate used in each case, and 0.3 μmole coenzyme. All assays in tris HC1 buffer; the first four at pH 7.4, the last four at pH 8.4.

Thus no evidence was found in this experiment to suggest that lupin seedlings can dehydrate formamide to cyanide.

(iii) β-Cyanoalanine Hydratase

The first attempt to measure the activity of this enzyme in a lupin system was carried out on an homogenate of 10 day old etiolated seedlings, prepared as described in (ii) above. The extract was dialysed against 1 litre of tris HC1 buffer (0.02 M, pH 8.4) before use.

With 1.0 ml homogenate were mixed 5 µmoles of β-cyanoalanine in buffer, and in some case 5 µmole ATP. The total volume was made up to 2.0 ml with buffer in all cases. The mixtures were incubated at 30°C for one hour, then placed in a boiling water bath for 5 min. After cooling, 1.0 ml of a solution of asparaginase was added, and the procedure of the asparagine assay of section III.4 (p.45) was followed. The results obtained are summarised in table III.8.2.

Apparent activity in the complete mixture is 64% greater than that in the boiled control, suggesting that some enzyme was detected. This difference corresponds to 0.35 µmole asparagine being formed in one hour. ATP appears to be inhibitory. However, interpretation is vitiated by the high absorbancies found in the controls, and these are eliminated if β-cyanoalanine is excluded. The purity of the β-cyanoalanine was checked chromatographically, and found not to contain significant asparagine. It was suspected that the asparaginase preparation was contaminated with a β-cyanoalanine hydratase; but recent work by Lauinger and Ressler (1970) suggests a more likely explanation. They found that asparaginases from guinea pig and E. coli slowly hydrolyse

TABLE III.8.2

Assay for Dehydration of Formamide

Experiment	Absorbance at 530nm
Complete mixture	0.35
- ATP	0.37
- Formamide	0.33
- Homogenate	0.05
Boiled Homogenate in Complete Mixture	0.42

The complete mixture contained, in 3 ml., 2.0 ml filtered homogenate from 5 day old lupins, 10 μmole formamide, and 5 umole ATP.

TABLE III.8.3

Assay for β-Cyanoalanine Hydratase

Experiment	Absorbance at 480 nm
Complete mixture	0.18
- ATP	0.24
- β-cyanoalanine	0.01
Boiled Homogenate in complete mixture	0.11

The complete mixture contained, in 2.0 ml, 1.0 ml dialysed homogenate 5 μmole -cyanoalanine, and 5 μmole of ATP.

β-cyanoalanine to aspartate; if the same is true for the preparation used here the high blanks are readily understood.

Because of the high asparagine content of the plant material used (section III.4, p. 51), inefficient dialysis will also cause high controls. Thus this method of assay is not satisfactory.

The assay for β-cyanoalanine hydratase was also carried out on extracts of freeze-dried powders; in these cases there were no differences between the experiments and the controls.

(iv) Asparagine Synthetase

Four attempts were made to detect an asparagine synthetase activity. In each case aspartate-4-^{14}C (either DL or L) was supplied to a crude homogenate and the radioactivity in asparagine investigated. Table III.8.4 gives a summary of these experiments, which were all negative. Asparagine was isolated by electrophoresis and chromatography (as described in section III.6); in the case of experiment 4 (using sucrose-buffer) a preliminary isolation of aminoacids was carried out using IR 120 ion exchange resin (as described in section III.3).

Thus no direct evidence was obtained for the presence of an asparagine synthetase, in agreement with the work of Lees et al. (1968) and in contrast with reports by Webster and Varner (1955 a & b) and Al Dawody (1961). The significance of this will be discussed later. Time did not permit two technical improvements of these assays; first, the use of "Sephadex G25" to speed up the separation of protein and asparagine (relative to dialysis), and second, the use of an ATP generating system to compensate for any ATP-ase present.

TABLE III.8.4

Attempts to Detect Asparagine Synthetase

	Experiment Number			
	1	2	3	4
Seedling Age (Day)	10	6	5	5
Homogenate in	Buffer	Buffer	Buffer	Sucrose/Buffer
Dialyed?	Yes	Yes	No	Yes & No
Substrates:				
DL Aspartate-^{14}C (μCi/ml)	0.8	0.8	-	-
L Aspartate -^{14}C (μCi/ml)	-	-	1.3	1.0
Fumarate-^{14}C (μCi/ml)	-	-	1.3	-
NH_4^+ (mM)	2	2	1.3	0.4
Glutamine (mM)	2	2	1.3	0.4
Carbamoyl Phosphate (mM)	-	2	-	-
Cofactors:				
ATP	2	2	1.3	1.0
Pyridoxal phosphate	-	-	0.3	0.2
Time of Incubation at 30°C	3hr	3hr	1.5hr	3hr

Concentrations of substrates and cofactors expressed in μCi/ml for radioactive one, remainder mmolar. Buffer tris HC1 (0.05M in assay medium, pH 8.4) with 0.2M sucrose added in experiment 4. Each experiment consisted on incubations of different combinations of the components listed.

IV DISCUSSION

1. RELATIONSHIPS BETWEEN CYANIDE AND SULPHIDE METABOLISM

In the introduction to this thesis a close connection between cyanide and sulphide assimilation was concluded to be unlikely. Here, the evidence for this will be considered more fully: the nature of the serine dependent and O-acetyl serine dependent cysteine synthases will be considered and compared with β-cyanoalanine synthase, and the data in section III.2 discussed.

(i) Serine Dependent Cysteine Synthase

This enzyme was described by Schlossmann and Lynen (1957) under the name "serine sulphhydrase". Schlossmann *et al.* (1962) purified it 50 fold from yeast. Similar enzymes have been found in *Escherichia coli* (Pasternak *et al.*, 1965) and *Neurospora* (Leinweber and Monty, 1965). Bruggemann *et al.* (1962) claimed to have detected this enzyme in spinach (very low activity), in a wide range of microorganisms and in rat and chicken tissues: indeed, they reported that chicken liver homogenates were as active as crude yeast extracts. This enzyme appears to be stimulated by ATP and pyridoxal phosphate. A cysteine synthase found in chicken embryo tissue by Fromageot and co-workers (Sentenac *et al.*, 1963; Fromageot and Sentenac, 1964) may be closely allied; it requires serine O-phosphate as substrate and will not utilise serine. These enzymes display a lack of specificity: Nakamura and Sato (1963) showed that a fungal enzyme would catalyse the exchange of thiosulphate groups as well as the sulphide and hydroxyl groups of cysteine and serine, and in the presence of Fromageot's enzyme sulphite, sulphide and phosphate groups are interchangeable. This lack of specificity may extend to cyanide.

Dunnill and Fowden (1965) observed ATP and pyriodoxal phosphate stimulation of cyanide assimilation into β-cyanoalanine in cell free preparations from E. coli; serine and cysteine could both serve as the other substrate. A similar enzyme activity has been described from Bacillus megaterium by Castric and Strobel (1969), and there is no reason to suspect that it is not more widespread. This activity can quite plausibly be attributed to a non-specific cysteine synthase.

Except for low activities reported by Bruggemann et al., using a rather non-specific assay, a serine dependent cysteine synthase has not been reported from higher plants. It has been reported from animals that are not known to be able to either synthesise β-cyanoalanine from cyanide or cysteine from serine. In section III.5, confirmed by Hendrickson and Conn, lupin β-cyanoalanine synthase was shown not to use serine as substrate, and it is stimulated neither by ATP nor by pyridoxal phosphate. Data in section III.2 (Table III.2.1, p.20) illustrates that acetone powders of lupin contain high levels of β-cyanoalanine synthase but no significant serine dependent cysteine synthase activity: these results have been confirmed by Hendrickson and Conn (1969).

Thus the formation of β-cyanoalanine in lupins cannot be attributed to a non-specific serine dependent cysteine synthase.

(ii) O-Acetyl Serine Dependent Cysteine Synthase

Later work has shown that the important enzyme in cysteine biosynthesis requires O-acetyl serine as its substrate: this applies in E. coli (Kredich and Tompkins, 1966), and higher plants (Giovanelli and Mudd, 1967; Thompson and Moore, 1968). An interesting difference between this cysteine synthase and the one discussed above is that pyridoxal phosphate does not stimulate its reaction, nor is ATP required. In these respects the O-acetyl serine dependent enzyme is similar to β-cyanoalanine synthase (sections III.3 and III.5). However, Hendrickson and Conn (1969) have demonstrated that the two enzyme activities are distinct in lupins, β-cyanoalanine synthase activity occurring in the mitochondria and cysteine synthase in the soluble portion of the cell.

(iii) β-Cyanoalanine Synthase

Sections III.2 to III.5 of this thesis, and Hendrickson and Conn (1969) contain much information about lupin β-cyanoalanine synthase. The only known aminoacid substrates are cysteine and O-acetylserine; the only known other substrates are cyanide and sulphide. The formation of β-cyanoalanine is not effectively reversible. β-Cyanoalanine synthase is a mitochondrial enzyme which can be easily extracted and is reasonably stable below 50°C in neutral or slightly alkaline solution. The stability is decreased by cysteine but not by cyanide. It may be a relatively hydrophobic protein since it is soluble in 60% acetone and does not bind readily to ion exchange materials (section III.5).

No cofactors have been identified, but there is some circumstantial evidence that the enzyme may contain bound pyridoxal phosphate (Hendrickson and Conn, 1969). This is an example of the intriguing similarity between this enzyme and the O-acetyl serine dependent cysteine synthase. Hendrickson and Conn also showed resemblances in substrate and cofactor requirements, molecular size, and absorption spectra, while they were establishing characteristic differences.

(iv) Comparative Assimilation of Cyanide and Sulphide

Exogenous cyanide is converted to asparagine whereas sulphide is rapidly oxidised to sulphate (section III.2). As β-cyanoalanine synthase can catalyse the exchange of the sulphhydryl group of cysteine with sulphide, the total lack of label in cyst(e)ine in young plants supplied ^{35}S-Hydrogen sulphide suggests that lupins can discriminate between sulphide and cyanide in such a way that whereas cyanide reaches the (presumably mitochondrial) site of the β-cyanoalanine pathway enzymes, sulphide does not. A preliminary experiment with $^{35}SO_4^{\ 2-}$ suggests that etiolated lupin seedlings can synthesise cysteine, as expected. If this is confirmed, failure to incorporate sulphide means that exogenous sulphide is not able to equilibrate with the pool of sulphide involved in cysteine biosynthesis, and this would be further evidence of barriers to intracellular sulphide movement.

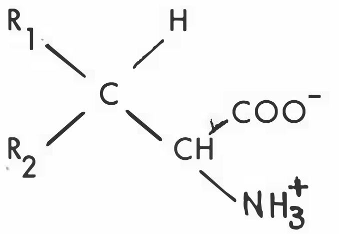
Thus, on the basis of arguments from several directions, the hypothesis that cyanide assimilation merely reflects a lack of specificity in sulphide assimilatory processes must be regarded as untenable.

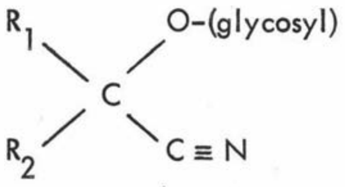
2. BIOGENESIS OF CYANIDE

The biosynthesis of cyanide is achieved by a remarkable variety of organisms, among which are representatives of the bacteria, fungi, and higher plants. Two biological origins have been identified: first from cyanogenic glycosides (recently reviewed by Conn and Butler, 1969) in plants and in some fungi (Stevens and Strobel, 1968), and second, from glycine in Chromobacterium violaceum (Michaels et al., 1965) and possibly some other bacteria (Wissing, 1968) and fungi (Ward and Thorn, 1966). The pathways involved in the metabolism of these precursors are discussed below, and the possibility of similar pathways operating in lupins considered. Then considered are the possibilities of cyanide biosynthesis starting from intermediates of the urea cycle or from tetrahydrofolate-linked one carbon units.

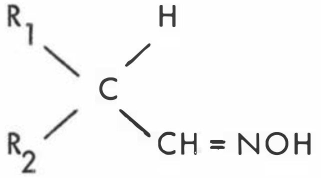
(i) Cyanogenic Glycosides

The term "cyanogenic glycoside" has been used for each of a number of naturally occurring glycosides that have been isolated, and which, on hydrolysis, give free cyanide. They are found widely distributed in many plant taxa, but even though Conn (1969) could cite approximately 1000 species, this is still only a small proportion of known species. In all cases where the structure is known, the aglycone is a cyanhydrin. Conn and Butler (1969) have lucidly reviewed these substances, and this review contains an account of the recent experimental work that they carried out with Tapper to elucidate the biosynthetic pathways involved. The cyanhydrin aglycones are structurally related to aminoacids, and in those plants that have been investigated there is a direct conversion of the aminoacid into the cyanogenic glycoside, with the retention of the α-amino nitrogen and the α-carbon as the nitrile group.

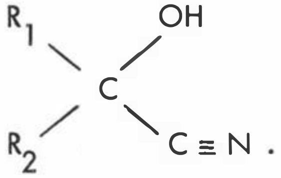




The pathway has not been completely elucidated, but most likely it involves the oxime derivatives.



and cyanhydrins



Aminoacids known to give rise to cyanogenic glycosides are phenylalanine, tyrosine, valine and isoleucine; the last three of these were shown in section III.7 (p. 98) not to be effective precursors of asparagine in the blue lupin. Unpublished experiments referred to by Blumenthal *et. al.* (1968) led to the same conclusion. Were cyanide being synthesised by lupins from a cyanogenic glycoside, label would be expected to be found in the amide group of asparagine, as observed by Abrol and Conn (Abrol and Conn, 1966; Abrol *et al.*, 1966) in cyanogenic *Lotus* and *Nandina* plants. In view of the rapid rate at which 5 day old lupin plants are synthesising asparagine, these negative results are evidence against the most common known cyanogenic glycosides being involved in asparagine biogenesis in lupins.

(ii) Glycine

The bacterium Chromobacterium violaceum (Michaels and Corpe, 1965; Michaels et al., 1965) and possibly the snow mold fungus described by Ward and Thorn (1966) convert glycine into hydrogen cyanide, the cyanide carbon being derived from the methylene carbon of the glycine. The conversion occurs without much dilution of the label, but the mechanism is not known: Conn and Butler (1969) observed that there is no reason why an oxime pathway should not operate in this case also. This is consistent with the observation of Brysk et al. (1969) that the α-amino nitrogen is retained. The studies described in sections III.6 and III.7 (pp.87 & 96) suggest that glycine gives rise to asparagine in lupins very slowly; the pathways involved seem to be related to the tricarboxylic acid cycle or glyoxylate cycle, rather than by conversion to cyanide followed by entry into the β-cyanoalanine pathway. Thus lupins that are rapidly accumulating asparagine appear not to synthesise cyanide from glycine. The possibility that cyanide is being synthesised from glycine in a separate metabolic pool cannot be ruled out: but as will be discussed in section IV.3, this is not the most likely explanation.

(iii) Urea Cycle Intermediates

It was observed by Schulze and Steiger as long ago as 1886 that asparagine accumulation is accompanied by arginine accumulation, and Reifer and Buraczewska (1958) noted that adding arginine to the medium in which pea shoots were growing stimulated intense asparagine production. As later discussed (section IV.3), the amide nitrogen of glutamine may give rise directly to the amide group of asparagine. Now the nitrogen of carbamoyl phosphate comes from glutamine in most tissues of higher organisms, including higher plants. Kleczkowski (1965) showed (with extracts of green pea acetone powders) that glutamine is 10 to 20 times more effective a precursor for citrulline biosynthesis than is ionic ammonium; were cyanide to be derived from urea cycle intermediates we could explain the apparent role of glutamine in asparagine biosynthesis. Animal systems which are not competent to catalyse reactions of the β-cyanoalanine pathway show a similar preference for glutamine;this has been differently explained, as will be seen later.

The possibility was considered that either the guanido group of arginine gave rise to cyanide, or that cyanide and arginine shared a common precursor such as carbamoyl phosphate: the cyanide could then be converted to asparagine. The involvement of arginine seems to be ruled out by the experiments described here.

Carbamoyl phosphate is too unstable in solution to supply directly: however, in solution it is in equilibrium with cyanate and phosphate (Allen and Jones, 1964). From the hypothesis being discussed here, one would predict that were the ^{14}C-cyanate supplied over a long period to plants rapidly synthesising asparagine, then label would appear predominatly in the amide carbon of asparagine. Insufficient incorporation was observed to check this prediction, possibly because of poor absorption of the cyanate. This and the attempts to demonstrate enzyme systems capable of reducing cyanate or carbamoyl phosphate are in themselves inconclusive, but the results do not favour the hypothesis that there is a close connection between the urea cycle and asparagine biosynthesis.

(iv) Folate Derivatives

Another possible source of cyanide that could enter the β-cyanoalanine pathway is the so called "one carbon pool" associated with tetrahydrofolate. In plants it would seem that the most important source of this pool would be the carbon 3 of serine. Cossins (1964) and Cossins and Sinha (1965) have investigated the metabolism of formate and methanol in plants; as might be expected label rapidly appears in serine and lesser amounts in other aminoacids. Very little label was detected in asparagine. Experimental work described in this thesis strenghtens the view that folate derivatives are not involved in asparagine biosynthesis, since serine is not an effective precursor of asparagine (Section III.7). Were C4 of asparagine derived from C3 of serine, and the other three carbons of asparagine derived more directly from serine also, one would expect that when U-(^{14}C)- serine is supplied to plants rapidly synthesising asparagine, then asparagine would be rapidly and asymmetrically labelled. This is not the case (III.7, p. 97).

Formamide could be plausibly derived from the one carbon pool. It could also be derived from carbamoyl phosphate. No evidence was found to suggest that formamide could be dehydrated to cyanide (section III.8) or that it could serve as a substrate for β -cyanoalanine synthase (section III.5, table III.5.4).

3. PATHWAYS OF ASPARAGINE BIOSYNTHESIS

In the introduction it was suggested that the β-cyanoalanine pathway might result from a lack of specificity in cysteine synthase, or that it might be important pathway in asparagine biosynthesis. The first suggestion can now be eliminated (section IV. 1); in this section the more likely pathways of asparagine biosynthesis will be discussed. Three plausible precursors of asparagine are known: β-cyanoalanine, β-oxosuccinamate, and aspartate; and the evidence for each of these as an asparagine a precursor will be considered.

(i) β-Cyanoalanine

The only known pathway of β-cyanoalanine biosynthesis is from cyanide and cysteine or serine. The β-cyanoalanine pathway can operate in cyanogenic plants, as discussed in the preceding section. These, however, are only a large minority of all plants; even in these there is no evidence that this is a major route of asparagine biosynthesis.

Labelled β-cyanoalanine is probably formed by all plants when $H^{14}CN$ is supplied to them, but in only a few does it accumulate free or as a γ-glutamyl peptide; in particular, this happens in some legumes, members of the genus *Vicia* (Bell, 1966). These provide useful material for studies of β-cyanoalanine biosynthesis, as they do not further convert this aminoacid into asparagine (Fowden and Bell, 1965). Some of these species (e.g. *V. angustifolia*) are known to synthesise vicianin, a cyanogenic glycoside derived from pheylalanine. When phenylalanine-2-(^{14}C) is supplied to these, label appears in β-cyanoalanine with a trace in asparagine (Tschiersch, 1966). However, when phenylalanine is supplied to V. sativa, another β-cyanoalanine accumulator, no significant label appears in β-cyanoalanine or asparagine (unpublished data quoted by Blumenthal et al., 1968; Ressler et al. 1969).

There is some confusion in the literature on the taxonomic status of these plants. Conn (1969) writes "Closely related if not identical species may differ greatly in this single property of cyanogenicity. Thus, common vetch (Vicia sativa L.) does not contain a cyanogenic glucoside whereas Vicia angustifolia L., which many botanists consider as a variety of V. sativa, is the plant in which the cyanogen vicianin occurs". Although Conn's V. sativa does not contain a cyanogen, Ressler et al. (1969) quote older literature to the effect that V. sativa seeds do contain a cyanogen. It is not clear whether this could in fact refer to V. angustifolia; however, Ressler et al. distinguished between V. sativa and V. angustifolia and detected low levels (100 ngm/gm seed). of HCN in their V. sativa seeds. The negative results of feeding phenylalanine, tyrosine, isoleucine and valine (quoted by Blumenthal et al., 1968) suggest that seedlings of this plant are not synthesising known cyanogenic glycosides.

As Ressler et al. (1969) observed, Vicia sativa seedlings synthesise β-cyanoalanine and γ-glutamyl-β-cyanoalanine when grown under sterile conditions. Four possible mechanism for this are (1) the plants can synthesis β-cyanoalanine by an unknown pathway; (2) the plants can synthesise cyanide by an unknown pathway; (3) the plants only synthesise cyanogens at a particular stage in their growth cycle, e.g. in the senescent stage or in maturing seeds;

(4) exogenous cyanide is required for β-cyanoalanine formation.

The first possibility seems unlikely; only asparagine is a likely precursor, and no evidence has been reported of this being converted to β-cyanoalanine. The enzyme that catalyses the reverse reaction is actually absent in the very species that accumulate the β-cyanoalanine derivatives (Fowden and Bell, 1965). The second possibility is also unlikely in view of the conclusions reached in the previous section. The third possibility deserves rather more consideration; it would be important to know the source of the cyanide detected in the seed material. This may have been synthesised at a late stage in the life cycle. The fourth possibility is related to the "cyanide cycle" proposed by Allen and Strobel (1966) and Strobel (1967). This is a proposed cycling of HCN between soil microorganisms and plants. Results in section III.4 (p. 52) suggest that seedlings may be able to assimilate the low levels of HCN such as could occur in their environment.

Thus present knowledge of β-cyanoalanine metabolism in Vicia does not lead to unequivocal solutions to problems about the role of the β-cyanoalanine pathway.

The accumulation of β-cyanoalanine in species not known to be cyanogenic suggests that the plants have access to an unknown source of cyanide. As we have seen, this argument is not conclusive. These plants can presumably all synthesise asparagine, which is a protein aminoacid. In those species lacking β-cyanoalanine hydratase, this would argue against the β-cyanoalanine pathway being important in asparagine biosynthesis.

In the related genus *Lathyus* there are no known cyanogenic species. These legumes do not accumulate β-cyanoalanine, but some species (Bell, 1966) accumulate β-amino propionitrile, which could be produced by the decarboxylation of β-cyanoalanine, as its γ-glutamyl peptide. Tschiersch (1964) showed that this became labelled in the nitrile group when $H^{14}CN$ was supplied to *L. odoratus* seedlings, which have no known source of endogenous cyanide. Label also appeared in asparagine. The biosynthesis of β-aminopropionitrile by *L. odoratus,* therefore, poses a similar problem to β-cyanoalanine synthesis in *Vicia.* In this case it is possible that, at certain stages in the life-cycle, asparagine is converted to β-cyanoalanine and then decarboxylated, or cyanide is synthesised by some unknown pathway: however, exogenous cyanide is an alternative possible source of the nitrile group.

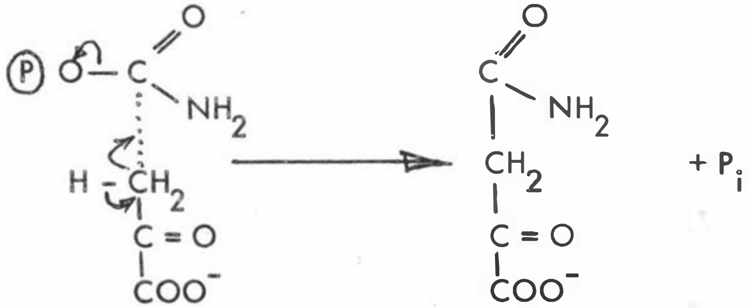
This would agree with the situation in lupins. Lupin seedlings develop the enzymes of the β-cyanoalanine pathway early (figures III.4.1 to III.4.3), but this does not appear to be related to asparagine biosynthesis. There are substantial levels of β-cyanoalanine synthase in younger seedlings that are synthesising negligible asparagine (figure III.4.1) and plants of this age have a high capacity to metabolise cyanide (figure III.4.3). The coincidence of maximal β-cyanoalanine synthase and maximal rate of asparagine accumulation at day 5 (figure III.4.1) are probably only two of many biochemical changes associated with morphological changes that occur as the plumule commences development. Attempts to demonstrate a source of endogenous cyanide in lupins have failed. As will be discussed below, the data in section III.6

suggests that a major pathway of asparagine biosynthesis in lupins is from aspartate. So it seems reasonable to suppose that although lupins possess the enzymes of the β-cyanoalanine pathway, these are not of major importance in asparagine biosynthesis. This pathway may be of secondary importance in enabling the plant to utilise HCN from its environment.

(ii) β-Oxosuccinamate

Animals (Meister and Fraser, 1954; Lerman and Mardashev, 1960) and plants (Suzuki, 1959; Guitton, 1963) possess asparagine aminotransferases; Cincerova (1969) has recently studied the specificity of the wheat enzyme and shown that glutamate is the preferred second amino acid. The α-ketoacid corresponding to asparagine is the β-amide of oxaloacetic acid, or β-oxosuccinamic acid.

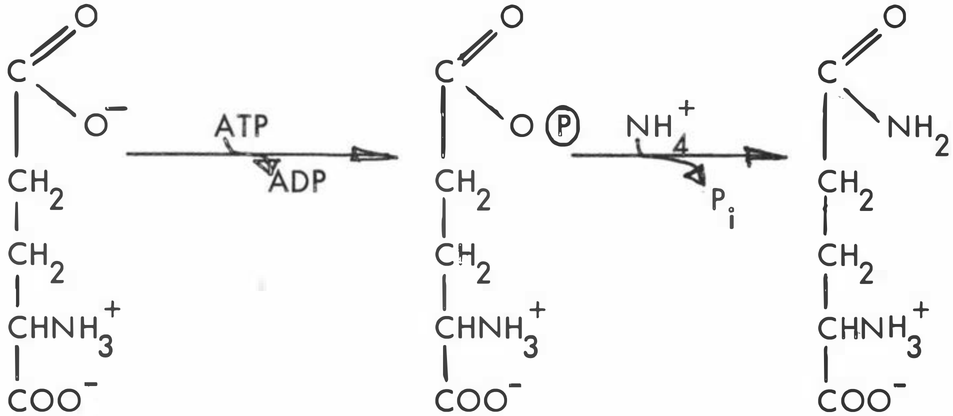
As yet, asparagine is the only known source of β-oxosuccinamate in living tissues. Another pathway considered involves the carbamoylation of pyruvate; one can envisage a plausible mechanism for the reaction between carbamoyl phosphate and pyruvate:



No enzyme catalysing this reaction has been described from any source. The negative experiments with ^{14}C cyanate and pyruvate (section III.7) are inconclusive because the plant may not have absorbed the precursors effectively; and so although this pathway is unlikely it deserves some further consideration.

(iii) Aspartate

The biosynthesis of glutamine from glutamate is a well known reaction. The enzyme involved (glutamine synthetase) has been purified from a number of sources and its reaction mechanism investigated. It catalyses an ATP-linked amidation of glutamate, with an enzyme bound γ-glutamyl phosphate as an intermediate:

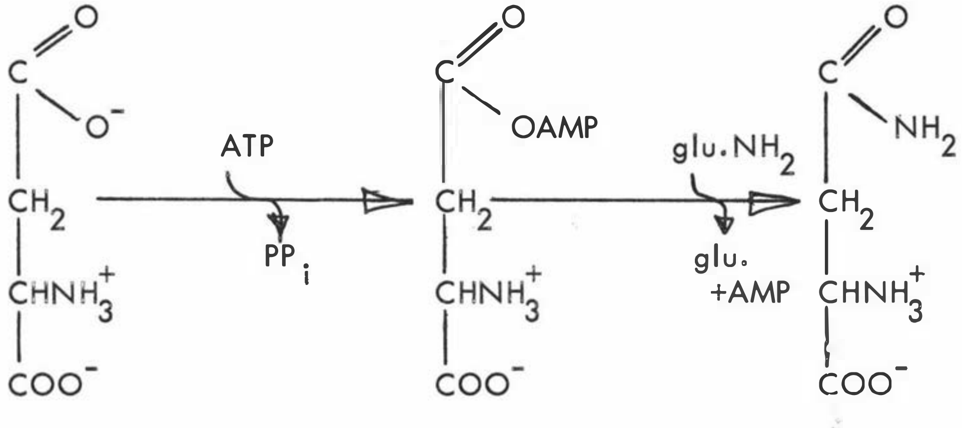


A parallel reaction with aspartate would give asparagine, and such an asparagine synthetase was described by Webster and Varner (1955 a & b) from wheat and lupin tissues. Al Dawody (1961) described a purification of an asparagine synthetase from yeast that is virtually identical to mammalian glutamine synthetase except, of course, for the substrate and product. He also claimed to have dtected asparagine synthetase in cell free extracts of pig heart and liver, dry peas, and pea and lupin seedlings. Al Dawody, Varner and Webster (1960) described asparagine biosynthesis from aspartate in tissue sections of lentil seedlings, in which the distribution of ^{14}C in isolated asparagine appeared to be identical to than in the DL-aspartate-4 ^{14}C supplied.

This work has not been confirmed. No details of the partial purification of asparagine synthetase have been published since the initial abstract

(Webster and Varner, 1955a), and of the other work by the same authors Meister (1962) says, "Although the wheat germ system was reported to catalyse the incorporation of ^{14}C aspartate into asparagine in the presence of magnesium ions, ATP, and ammonia, the incorporation was extremely low and net synthesis of asparagine was not observed. Attempts to carry out this and similar reactions in the author's laboratory have not been successful." Fowden (1967) also noted the weakness of the evidence for asparagine synthetase in higher plants. Lees *et al.* (1968) could not detect any asparagine synthetase activity in extracts of wheat and lupin tissue. On the other hand, Oaks (1967) described significant but low conversions of ^{14}C aspartate into asparagine in homogenates of wheat plants, and Nair (1969) has described an enzyme from γ-irradiated potatoes with properties very like those of Webster and Varner's enzyme. The attempts to demonstrate an asparagine synthetase described in section III.8 (p.**108**) confirm the results of Lees *et al.* rather than Webster and Varner; the data in sections III.6 and III.7 shows the distribution of label in asparagine synthesised from C4 labelled aspartate by lupins to be different from that described by Al Dawody *et al.* (1960).

Since the report of Al Dawody (1960), a number of workers (Patterson and Orr, 1967, 1968; Holcenberg and Pease, 1968; Holcenberg, 1969; Prager and Bachynsky, 1968 b) have described asparagine synthetases from a variety of mammalian sources. Although these reports differ in detail (section IV.4, below) they agree that mammalian asparagine synthetase *in vitro* will utilise ammonium ion or glutamine as the amide group nitrogen donor, with a preference for the latter; the reaction mechanism of the enzyme studied by Patterson and Orr (1968) involves enzyme bound β-aspartyl adenylate



The preference for glutamine explains the conversion in animal tissues of glutamine amide nitrogen to asparagine amide nitrogen, as observed by Levintow (1957), and Levintow *et al.* (1957).

Thus the enzyme described in animal tissues is similar to the bacterial asparagine synthetases described in Lactobacillus arabinosus (Ravel *et al.*, 1962), *Streptococcus bovis* (Burchall *et al.*, 1964), and *Escherichia coli* (Cedar and Schwartz, 1969 a & b). These also involve a β-aspartyl adenylate intermediate, although they are specific for ammonium ion as nitrogen donor.

Now asparagine is a ubiquitous protein aminoacid; we could, then, expect its biosynthesis in plants to resemble the process in other eukaryotes such as higher animals. There are indications that this is so. Lerman and Mardashev (1960) quote work by Bauerova and Shorm (1959) which demonstrates a transfer in rape seedlings of glutamine amide nitrogen to asparagine, similar to the process in animal tissues. In a review Kretovitch (1965) quotes work by his own group which demonstrates that nitrogen is assimilated into glutamine more directly than into asparagine, and among the materials mentioned are lupin and vetch shoots.

In section III.6 it was demonstrated that fumarate and aspartate are effective precursors of asparagine. Comparisons with glycine (table III.6.4 p. 89), and with serine and other substances (section III.7) show that these dicarboxylic acids are more effective than possible precursors of β-cyanoalanine pathway intermediates. The data in table III.6.5 (p. 90) show that the carbon skeleton of the four carbon dicarboxylic acids is retained in the asparagine. Since asparagine from the plants fed 1,4 ^{14}C-fumarate had significantly lower portions of label in C-4 than that produced from 4-^{14}C- aspartate, the carbon skeleton of asparagine is probably derived directly from aspartate. Comparison of the degradation data of the aspartate and asparagine isolated from the same plants (table III.6.5 , p. 90) shows that a discrete pool of aspartate is involved in asparagine biosynthesis.

Thus the data in section III.6 provide evidence that lupin seedlings possess an asparagine synthetase, even though this has not been demonstrated in a cell free system. There are also indications that glutamine is the preferred amide nitrogen donor, which parallels the observations made in animal systems. The reprt of Nair (1969) on an asparagine synthetase from potato must, however, be considered. This enzyme produces ADP and inorganic phophate as products, and uses ammonium ion as substrate: glutamine was not tried. Meister (1962) offered an alternative explanation for some similar data of Webster and Varner (1955 b). The enzyme being studied may be aspartate kinase, which produces β-aspartyl phosphate; this is a reactive species and could undergo non-enzymic reactions leading to β-aspartyl hydroxamate, or asparagine. Nair (1969) has documented his enzyme more convincingly than the earlier workers, but does not seem to have eliminated this possibility; he also used high concentrations of reactants in his assays (e.g. 30mM NH_4^+). If his work is confirmed, it would mean that the asparagine synthetase from at least some higher plants are more akin to glutamine synthetase than to animal and bacterial asparagine synthetases.

The view that aspartate is important as an asparagine precursor is not agreed with by Ressler et al. (1969). They studied the specific activity of asparagine isolated from plants after supplying various radioactive precursors for periods of up to eight days. They found that cyanide and β-cyanoalanine appeared more effective than aspartate. However, in view of the extensive and varied metabolism of aspartate observed even in one hour, (section III.6), one would expect only a small portion of the radioactivity from exogenous aspartate to appear in asparagine after a period of days, whereas cyanide and β-cyanoalanine would be almost exclusively converted to asparagine. Further, whereas the exogenous aspartate will be rapidly diluted by newly synthesised aspartate, exogenous cyanide and β-cyanoalanine will retain their initial specific activity unless there are other unknown pathways to these intermediates. In view of what has been said before, this last possibility is unlikely.

4. GENERAL DISCUSSION OF ASPARAGINE METABOLISM

In the previous section it was concluded that the principal biosynthetic route to asparagine in higher plants is probably from aspartate. Here, mammalian asparagine biosynthesis will be discussed more fully, for the difficulties encountered in isolating asparagine synthetases in animal tissues could provide a valuable guide for further investigation of the plant systems. This is used to develop a model of lupin asparagine metabolism.

Interest in mammalian asparagine biosynthesis was kindled by the discovery of the anti-leukemic activity of asparaginase. Adamson and Fabro (1968) have reviewed developments from the early observations of the beneficial effects of guinea pig serum, which is remarkable for its high asparaginase activity. Patterson and Orr (1967) provided evidence of a correlation between asparaginase susceptibility and low asparagine synthetase activity in many tumors; these tumors depend on serum asparagine for growth. This correlation has since been observed in several mammals, including man (Prager et al., 1969).

(i) Mammalian Asparagine Synthetases

Partial purifications of asparagine synthetase have been reported by Patterson and Orr (1968) from the Novikoff hepatoma, and by Holcenberg (1969) from guinea pig liver. The latter enzyme is reported to require a high salt concentration for activity, and the cation must be NH_4^+ or K^+ and the anion SO_4^{2-}. Other common ions are ineffective, or inhibitory. Glutamine is preferred to ammonia even in the presence of ammonium sulphate. The Novikoff hepatoma enzyme does not require high salt concentrations, and neither does the enzyme described from normal and malignant mouse tissues, and from guinea pig tissue, by Prager and Bachynsky (1968 b). In their experiments, asparagine synthetase was found to be microsomal in normal liver, although the supernatant fraction from malignant tissue contained a substantial portion of the activity. Asparaginase was present in the supernatant in all cases. Prager and Bachynsky homogenised their tissues with a sucrose medium containing calcium chloride; Hocenberg, who purified his enzyme from the 105,000 x g supernatant, and Patterson and Orr, who purified theirs from a 20,000 x g supernatant, used a similar medium but with magnesium chloride instead of calcium chloride. This may account for the different results. If mammalian asparagine synthetase is membrane bound the association is loose.

(ii) Organisation and Control of Asparagine Synthetase

Asparagine biosynthesis in animal cells appears to be separate from other metabolic processes. For example, asparaginase is present in the same cells, a possible explanation for earlier failures to detect asparagine biosynthesis. Lerman and Mardashev (1960) reported extensive dilution of the specific activity of aspartate during conversion to asparagine, and suggested that either there is an intermediate between aspartate and asparagine, or there are alternative pathways of asparagine biosynthesis, one of which does not involve aspartate. Puszkin *et al.* (1970) studied the biosynthesis in human platelet of aspartate and asparagine from labelled glucose and acetate, with similar results; they concluded that the aspartate pool in asparagine biosynthesis is distinct from the main aspartate pool. This explanation seems the more likely, and agrees with the situation in lupins (section III.6).

Thus asparagine biosynthesis in mammals is organised into a separate pool. It is probably subject to control. Both bacterial and mammalian asparagine synthetases are inhibited by asparagine. Holcenberg (1969) observed a strong inhibition of guinea pig enzyme with glutamine as substrate but only weak inhibition with ammonium. Patterson and Orr (1968), using glutamine as substrate, observed strong inhibition of the Novikoff hepatoma enzyme that did not obey the classical knetics of competitive or non competitive inhibition; as ammonium and glutamine appear to act at the same site, some kind of allosteric control would seem more likely than simple end product inhibition.

Patterson and Orr (1969) found that the level of asparagine synthase in rat tissues is closely related to asparagine requirement, and is greatly increased under conditions where active tissue growth is involved, whether this be normal regeneration or a tumor. Increases are also observed in animals injected with asparaginase and with animals fed asparagine deficient diets: the increase was found to be just sufficient to bring the asparagine pool size up to the normal controls. This suggests an efficient control mechanism. Prager and Bachynsky (1968 a) found a doubling time of 4 hours for asparagine synthetase activity after asparaginase treatment of asparaginase resistant mouse lymphomas, which they interpreted in terms of a derepression mechanism. This might also explain how, in cultures of asparagine-requiring Jensen sarcoma cells, variants can appear with the ability to synthesise asparagine: these consistently appear even in cultures derived from a single cell. However, Patterson et al. (1969) believe these to be spontaneous mutants.

Thus mammalian asparagine synthetases are under metabolic control, but little is known of the mechanism of this process as yet.

(iii) Asparagine Synthesis in Chicken Liver

One recent study of asparagine biosynthesis has been made in a non-mammalian animal system. Arfin (1967) investigated homogenates of chick embryo livers, and found that in these glutamine was required for asparagine biosynthesis, and another unidentified factor was also required. Asparagine synthesis would only occur in the presence of cell supernatant and intact mitochondria; the supernatant factor was protein in nature and possibly corresponds to mammalian asparagine synthetase. The mitochondria were required to supply energy, but ATP generating wystems could not replace them: neither could mitochondria from hatched chicks although the supernatants were still active. A range of nucleoside triphosphates were all ineffective or inhibitory. No furhter work on this has been published yet.

(iv) Application to Plant Systems

If plant asparagine synthetases are similar to the enzymes from other eukaryotes, difficulties in detecting it in cell free systems would be anticipated. The high asparagine content of plant cells may be strongly inhibitory, and complete removal may be difficult if the enzyme is unstable (section III.8). High salt concentrations or other unexpected cofactors may be required for activity. To detect the E. coli enzyme, Cedar and Schwartz (1969 a) cultured a strain low in asparaginase, and added the asparaginase inhibitor 5 -diazo-4-oxo-L-norvaline as well. The latter may prove useful in plant systems, for the conversion of $H^{14}CN$ to labelled aspartate (section III.4, p. 56) suggests that lupins do have the ability to convert some asparagine (that derived from β-cyanoalanine) to aspartate, although the enzyme responsible appears to be kept separate from the relatively inert pool of accumulated.

The changes in asparagine content during development (figure III.4.1, p. 58) imply some control of its biosynthesis. Some attempts to isolate asparagine synthetase from plants may have failed because asparagine synthesis was very slow at the stage of development chosen. Lupins (5 days old) would appear to be particularly favorable in this respect. The asparagine synthetase activity described by Nair (1969) was increased up to seven-fold by γ-irradiation, possibly by some derangement of control mechanism.

(v) A model of Asparagine Metabolism

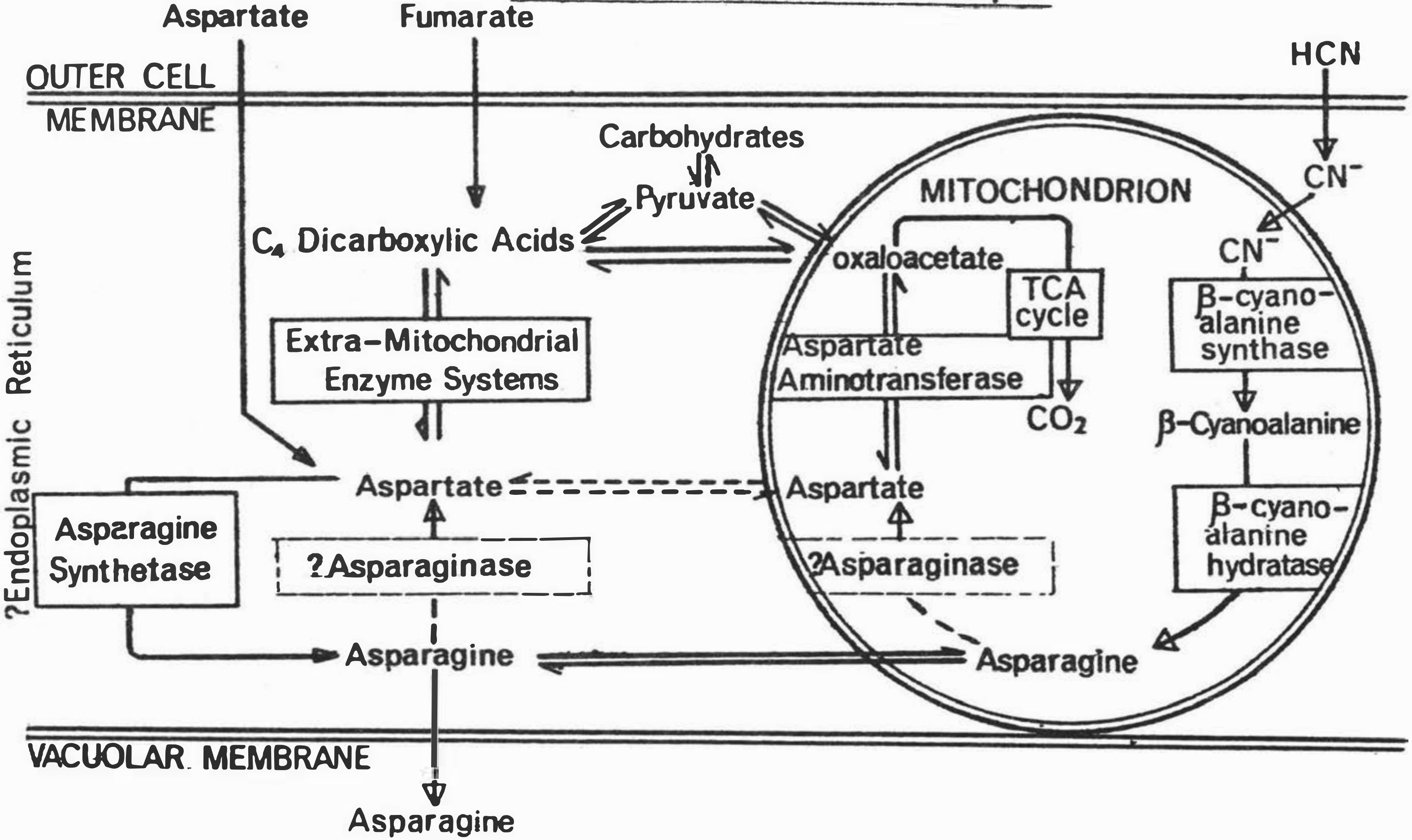
The data presented in this thesis can be explained by the model illustrated in figure IV.4.1. This is constructed by anticipating a resemblance between animal and plant asparagine biosynthesis. The model incorporates the interpretation of the data in section III.6 advanced before; aspartate synthesis is supposed to be most active in the mitochondrion. This can explain the distribution of label in aspartate after supplying carboxyl labelled fumarate (section III.6); carboxyl groups of fumarate that enter plant mitochondrial pathways are rapidly lost as carbonate (Titus and Splittstoesser, 1969), and only label that enters the 4-C dicarboxylic acids less directly (e.g. via carbohydrate) is retained. The label observed in isolated aspartate is predominantly that of mitochondrial aspartate, which is in equilibrium with the mitochondrial 4-C acids.

The significant preponderance of label in asparagine C-4 when C-4 labelled aspartate is supplied is evidence for aspartate being the direct precursor of asparagine; the similar predominance in C-1 when carboxyl labelled fumarate is supplied can similarly be interpreted as an effect of dilution of C-4 through the equilibration of C_4 dicarboxylic acids and pyruvate.

Thus present knowledge can be integrated into a consistent model. This leaves the β-cyanoalanine pathway as a separate mitochondrial pathway which converts exogenous HCN to asparagine, and does not appear to play an important role in asparagine biosynthesis. Whether it has a nutritional role in a cyanide microcycle (Allen and Stobel, 1966; Strobel, 1967), or is a detoxication pathway (Conn and Butler, 1969) for a powerful respiratory poison, will be more difficult to assess; but the mitochondrial location of β-cyanoalanine synthase, close to the site of respiratory activity, favours the latter suggestion.

FIGURE IV.4.1.

Model of Asparagine Metabolism in Lupins



V. REFERENCES

ABROL, Y.P., & CONN, E.E. (1966) Phytochemistry 5 237-242

ABROL, Y.P., CONN, E.E., & STOKER, J.R. (1966) Phytochemistry 5 1021-1027

ADAMSON, R.H., & FABRO, S. (1968) Cancer Chemother. Rep. Part 1 52 617-626

AL DAWODY, A.M.H. (1961) PhD thesis, Ohio State University

AL DAWODY, A.M.H., VARNER, J.E., & WEBSTER, G.C. (1960) Ohio J. Sci. 60 327-331

ALDRIDGE, W.N. (1945) Analyst 70 474-475

ALLEN, C.M, & JONES, M.E. (1964) Biochemistry 3 1238-1247

ALLEN, J. & STROBEL, G.A. (1966) Canad. J. Microbiol. 12 414-416

ARFIN, S.M. (1967) Biochim. Biophys. Acta 136 233-244

BARBOUR, R. & GLEDHILL, R.F. (1962) Laboratory Practice 11 619-620

BELL, E.A. (1966) in T. SWAIN (Editor) "Comparative Phytochemistry" Academic Press, London. p. 195-209

BLUMENTHAL, S.G., HENDRICKSON, H.R., ABROL, Y.P., & CONN, E.E. (1968) J. Biol. Chem. 243 5302-5307

BLUMENTHAL-GOLDSCHMIDT, S., BUTLER, G.W., & CONN, E.E. (1963) Nature 197 718-719

BRAY, G.A. (1960) Anal. Biochem. 1 279-285

BRUGGEMANN, J., SCHLOSSMANN, K., MERKENSHCLAGER, M., & WALDSCHMIDT, M. (1962) Biochem. Z. 335 392-399

BRYSK, M.M., LAUINGER, C. & RESSLER, C. (1969) Biochim. Biophys. Acta 184 583-588

BURCHALL, J.J., REICHELT, E.C., & WOLIN, M.J. (1964) J. Biol. Chem. 239 1794-1798

CASTRIC, P.A., & STROBEL, G.A. (1969) *J. Biol. Chem.* *244* 4089-4094

CEDAR, H., & SCHWARTZ, J.H. (1969)a *J. Biol. Chem.* *244* 4112-4121

CEDAR, H., & SCHWARTZ, J.H. (1969)b *J. Biol. Chem.* *244* 4122-4127

CHAPPELLE, E.W., & LUCK, J.M. (1957) *J. Biol. Chem.* *229* 171-179

CINCEROVA, A. (1969) *Biol. Plant.* *11* 139-148

CONN, E.E. (1969) *J. Agr. Food Chem.* *17* 519-526

CONN, E.E., & BUTLER, G.W. (1969) in J.B. HARBORNE & T. SWAIN (Editors) "*Perspectives in Phytochemistry*" Academic Press N.Y. p.47-74

COSSINS, E.A. (1964) *Canad. J. Biochem.* *42* 1793-1802

COSSINS, E.A., & SINHA, S.K. (1965) *Canad. J. Biochem.* *43* 685-598

DAVIS, R.H. (1962) *Arch. Biochem. Biophys.* *97* 185-191

DUNNILL, P.M., & FOWDEN, L. (1965) *Nature* *208* 1206-1207

FLOSS, H.G., HADWIGER, L., & CONN, E.E. (1965) *Nature* *208* 1207-1208

FOWDEN, L. (1967) *Ann. Rev. Plant Physiol.* *18* 85-106

FOWDEN, L., & BELL, E.A. (1965) *Nature* *206* 110-112

FROMAGEOT, P., & SENTENAC, A. (1964) *J. Biochem.* (Tokyo) *55* 659-668

GIOVANELLI, J., & MUDD, S.H. (1967) *Biochem. Biophys. Res. Comm.* *27* 150-156

GUITTON, Y. (1963) *Comptes Rendus* *257* 506-507

HENDRICKSON, H.R., & CONN, E.E. (1969) *J. Biol. Chem.* *244* 2632-2640

HOLCENBERG, J.S. (1969) *Biochim. Biophys. Acta* *185* 228-238

HOLCENBERG, J.S., & PEASE, J. (1968) *Biochim. Biophys. Acta* *158* 500-502

JEFFAY, H., & ALVAREZ, J. (1961) *Anal. Chem.* *33* 612-615

KLECZKOWSKI, K. (1965) *Acta Biochim. Polon.* *22* 243-249

KREDICH, N.M., & TOMPKINS, G.M. (1966) *J. Biol. Chem.* *241* 4955-4965

KRETOVITCH, V.L. (1965) Ann. Rev. Plant Physiol. 16 141-154

LAUINGER, C., & RESSLER, C. (1970) Biochem. Biophys. Acta 198 316-323

LEES, E.M., FARNDEN, K.J.F., & ELLIOTT, W.H. (1968) Arch. Biochem. Biophys. 126 539-546

LEINWEBER, F.J., & MONTY, K.J. (1965) J. Biol Chem. 240 782-787

LELOIR, L.F., & CARDINI, C.E. (1955) in S.P. COLOWICK & N.O. KAPLAN (Editors) "Methods in Enzymology" Academic Press, N.Y. Vol.III. p. 840-850

LERMAN, M.I., & MARDASHEV, S.R. (1960) Biokhimiya 25 946-953

LEVINTOW, L. (1957) Science 126 611-612

LEVINTOW, L.; EAGLE, H., & PIEZ, K.A. (1957) J. Biol. Chem. 227 929-941

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., & RANDALL, R.J. (1951) J. Biol. Chem. 193 265-275

MEISTER, A. (1962) in P.D. BOYER, H. LARDY, & K. MYRBACK (Editors) "The Enzymes" Academic Press, N.Y. Volume 6 p. 247-266

MEISTER, A., & FRASER, P. (1954) J. Biol. Chem. 210 37-43

MICHAELS, R., & CORPE, W.A. (1965) J. Bacteriol. 89 106-112

MICHAELS, R., HANKES, L.V., & CORPE, W.A. (1965) Arch. Biochem. Biophys. 111 121-125

NAIR, P.M. (1969) Arch. Biochem. Biophys. 133 208-215

NAKAMURA, T., & SATO, R. (1963) Nature 198 1198

NAYLOR, A.W., RABSON, R., & TOLBERT, N.E. (1958) Physiol. Plant. 11. 537-547

NOMOTO, M., NARAHASHI, Y. & MURUKAMI, M. (1960) J. Biochem. (Tokyo) 48 593-602 & 906-918

OAKS, A. (1967) Biochim. Biophys. Acta 141 436-439

PASTERNAK, C.A., ELLIS, R.J., JONES-MORTIMER, M.C., & CRICHTON, C.E. (1965) Biochem. J. 96 270-275

PATTERSON, M.K., MAXWELL. M.D., & CONWAY, E. (1969) *Cancer Res.* *29* 296-300

PATTERSON, M.K., & ORR, G.R. (1967) *Biochem. Biophys. Res. Comm.* *26* 228-233

PATTERSON, M.K., & ORR, G.R. (1968) *J. Biol. Chem.* *243* 376-380

PATTERSON, M.K., & ORR, G.R. (1969) *Cancer Res.* *29* 1179-1183

PETERSON, P.J., & BUTLER, G.W. (1962) *Aust. J. Biol. Sci.* *15* 126-146

PRAGER, M.D., & BACHYNSKY, N. (1968)a *Biochem. Biophys. Res Comm.* *31* 43-47

PRAGER, M.D., & BACHYNSKY, N. (1968)b *Arch. Biochem. Biophys.* *127* 645-654

PRAGER, M.D., PETERS, P.C., JANES, J.O., & DERR, I. (1969) *Nature* *221* 1064-1065

PUSZKIN, E., ALEDORT, L., & PUSZKIN, S. (1970) *J. Lab. Clin. Med.* *75* 234-243

RAVEL, J.M., NORTON, S.J., HUMPHREYS, J.S., & SHIVE, W. (1962) *J. Biol. Chem.* *237* 2845-2849

REIFER, I., & BURACZEWSKA, L. (1958) *Acta Biochim. Polon.* *6* 361-371

RESSLER, C., GIZA, Y.H., & NIGAM, S.N. (1969) *J. Amer. Chem. Soc.* *91* 2766-2775

SCHIFF, J.A. (1964) *Plant Physiol.* *39* 176-179

SCHLOSSMANN, K., BRUGGEMANN, J., & LYNEN, F. (1962) *Biochem. Z.* *336* 258-273

SCHLOSSMANN, K., & LYNEN, F. (1957) *Biochem. Z.* *328* 591-594

SCHULZE, E., & STEIGER, E. (1886) *Ber. dtsch. Chem. Ges.* *19* 1177-1180

SENTENAC, A., CHAPEVILLE, F., & FROMAGEOT, P. (1963) *Biochim. Biophys. Acta* *67* 672-673

SIEGEL., L.M. (1965) *Anal. Biochem.* *11* 126-132

SPECTOR, L., JONES, M.E. & LIPMANN, F. (1955) in S.P. COLOWICK & N.O. KAPLAN (Editors) "Methods in Enzymology" Academic Press, N.Y. Vol. III p. 653-655

STEVENS, D.L., & STROBEL,. G.A. (1968) J. Bacteriol. 95 1094-1102

STEWARD, F.C., & BIDWELL, R.G.S. (1966) J. Exper. Bot. 17 726-741

STROBEL, G.A., (1964) Canad. J. Biochem. 42 1637-1639

STROBEL, G.A. (1967) Soil Sci. 103 299-302

SUZUKI, Y. (1959) Phyton 12 27-29

THOMPSON, J.F., & MOORE, D.P. (1968) Biochem. Biophys. Res. Comm. 31 281-286

TITUS, J.S., & SPLITTSTOESSER, W.E. (1969) Phytochemistry 8 2133-2138

TSCHIERSCH, B. (1963) Flora 153 115-121

TSCHIERSCH, B. (1964) Phytochemistry 3 365-367

TSCHIERSCH, B. (1966) Flora, Abl. A 157 43-50

VIRTANEN, A.I. (1965) Phytochemistry 4 207-228

VOGEL, A.I. (1951) "A Text-book of Quantitative Inorganic Analysis" Longmans, London. 2nd. Edition p. 355

WARD, E.W.B., & THORN, G.D. (1966) Canad. J. Botany 44 95-104

WEBSTER, G.W., & VARNER, J.E. (1955)a. Federation Proc. 14 301

WEBSTER, G.W., & VARNER, J.E. (1955)b. J. Biol. Chem. 215 91-99

WILLIAMS, P.C. (1964) Analyst 89 276-281

WISSING, F. (1968) Physiol. Plant. 21 589-593