ASPARAGINE SYNTHESIS IN *ZEA MAYS*

THE EFFECT OF CYCLOHEXIMIDE, CYCLOHEXIMIDE ANALOGUES AND AZASERINE ON ASPARAGINE SYNTHESIS IN CORN ROOT-TIPS

By

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ABSTRACT

The experiments in this thesis were undertaken to: 1) compare the effects of cycloheximide and azaserine on asparagine synthesis in root-tip sections of corn; 2) study the effect of protein synthesis on asparagine synthesis in root-tip sections by the use of analogues of cycloheximide; and 3) study the properties of asparagine synthetase extracted from corn roots.

When [2-^{14}C]-acetate is fed to excised root-tip sections of corn pre-incubated in the presence of cycloheximide, protein synthesis is inhibited. The effect is almost immediate. Within the amide fraction, the levels of glutamine formed in these sections rises over the 3 hour pre-incubation period. Asparagine synthesis gradually declines over the same period. In similar experiments performed with azaserine in the pre-incubation media, protein synthesis was not markedly inhibited. Glutamine levels were immediately increased over the 3 hour period. The effect on asparagine synthesis was also rapid. In contrast to the situation with cycloheximide, the effect of azaserine on amide synthesis is constant over a 3 hour period.

Two analogues of cycloheximide - cycloheximide acetate and streptovitacin A - were found to produce effects similar to that of cycloheximide. These analogues were found to inhibit both protein synthesis and asparagine synthesis after a 3 hour exposure period. Six other analogues did not show marked inhibitory effects on either protein synthesis or asparagine synthesis.

Asparagine synthetase activity was found in extracts from corn seedling tissues. However, assays for asparagine synthetase revealed that the activity was low and that other aspartate utilizing enzymes were probably active in the extracts.

From the results of this investigation and those of earlier published results a model has been proposed in order to explain the regulation of asparagine synthesis in corn roots.

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TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd)

TABLE OF CONTENTS (cont'd)

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Plant seedlings prior to the initiation of their photosynthetic capacities, depend principally on seed storage material for both energy and basic metabolic intermediates. Amino acids derived from the hydrolysis of protein reserves and sugars derived from the hydrolysis of lipid and carbohydrate material are transported to actively growing areas and used in cellular biosynthesis. In corn, the conversion of sugar and lipid metabolites to amino acids and then to protein are limited by the supply of amino acids from the endosperms (2). The interconversion of amino acids may also occur depending on the particular requirements of the growing tissue and the physiological condition of the seedling.

A. The Amides

Of interest in nitrogen and carbon metabolism are two amino acids, glutamine and asparagine. These amino acids, in addition to their α-amino nitrogen, also have a reduced nitrogen moiety incorporated into their structure as an amide group. These molecules are

considered economical structures for the storage, transport and subsequent utilization of reduced nitrogen in cells owing to the fact that they have relatively high nitrogen to carbon ratios.

Asparagine has been considered a "dead-end metabolite" used only in protein synthesis. Evidence to support this conclusion has been the fact that under conditions of plentiful nitrogen supply asparagine is not extensively degraded in wheat seedlings (3) or in cotton roots (4). Yet, other evidence indicates that it is utilized. Asparagine can support growth in cultures of *Lemna minor* (5) and has been shown to be metabolized slowly in flowering *Pisum arvense* (6). In *Pisum arvense*, forty-eight hours after feeding [^{14}C]-asparagine, 60% of the radioactivity remaining in the soluble fraction was asparagine. The remaining 40% was found in many compounds including homoserine, proline, glutamate and serine. In the insoluble fraction about 50% of the label appeared as aspartyl residue. The remainder of the label found in the protein fraction comprised glutamate, lysine, threonine, arginine and proline. Vickery also indicates that there is turnover of asparagine in tobacco leaves but that pathways leading to asparagine predominate after 48 hours (7). In *Lupinus albus*, asparagine is the major nitrogen source translocated to the fruit and seed. At these sites, asparagine supplies greatly exceeded the

requirements of protein synthesis for the amino acid. Asparaginase, the enzyme that hydrolyzes asparagine to aspartate and ammonia, is sufficiently active *in vitro* to more than account for the estimated rates of asparagine utilization *in vivo*. In addition, the metabolic fate of the carbon skeleton and amide-nitrogen of asparagine was followed using doubly labeled [(U)-[^{14}C]-[^{15}N](amide)]-asparagine. It was found that essentially all the label was translocated in the form of asparagine. Interestingly, the amide group of asparagine entering the young seeds was traced to free ammonia, glutamine and alanine in the soluble endosperm while the carbon of asparagine appeared principally in non-amino compounds. Later, when storage protein synthesis began in the cotyledons, the carbon and nitrogen of asparagine was found to be distributed to a variety of protein amino acids (8). Thus, at least in this system asparagine is extensively metabolized before incorporation into protein.

In root-tip sections of corn excised from the rest of the seedling, asparagine synthesis increases for at least six hours under conditions of plentiful nitrogen supply (9). However, if the root-tip section remains intact, asparagine is not synthesized. In this situation, asparagine transported from the mature root and scutellum supplements the deficient synthesis in the growing area. In the root-tip, asparagine is

not extensively metabolized. Thus, in corn, asparagine supplied from regions of the seedling outside the root-tip supports the synthesis of protein in the growing area (10).

Storage is not the only fate for asparagine. It has been shown in several plants to be involved in transport. In many cases, it appears that asparagine functions to relay reduced nitrogen from sites of asparagine synthesis to meristematic growth areas. This is supported by the following examples. Atmospheric nitrogen fixed in root nodules of legumes by *Rhizobium* enters the transport system as asparagine. Pate found that asparagine in the stem exudate of *Pisum arvense* increased coincidentally with the start of nitrogen fixation in the root nodules when the root nodules began to decay, the levels of asparagine also declined (11). Streeter found that asparagine comprised up to 60% of the total amino nitrogen found in the stem exudate of soybean (12). Actively growing shoot areas are thus supplied with soluble nutrients from older tissues like roots and primary leaves.

In their review of asparagine metabolism in plants, Lea and Fowden suggested that "asparagine was stored somewhat 'distant' from the subcellular centre for active metabolism" (13). Yet, to the contrary, as the preceding examples have illustrated, this does not appear to be the case. Rather, asparagine metabolism

appears to be closely regulated at specific regions in the growing plant and, perhaps, its degradation is not a normal characteristic of most plant cells.

The levels of each amide commonly vary according to the growth conditions of the plant. The trends are particularly pronounced in leaf tissue. Poor nutritional conditions imposed by starvation in barley leaves (14) and under reduced photosynthesis as shown in bean leaves (15) give rise to a three-step transition in the physiology as reflected in the amides and ammonia levels. Initially, glutamine accumulates and then declines. Subsequently, there is a rise in the asparagine levels. Free ammonia levels rise after the decline in asparagine content is observed. This indicates that a general situation promoting catabolism continues under abberant conditions. When carbon limitations increase in their severity, protein amino acids are used as sources of energy liberating free ammonia. As a compensatory measure reduced nitrogen is thought to be transferred to the amides to prevent toxification and to conserve carbon. In this regard, a four carbon molecule, asparagine, is the last significant repository for the binding of reduced nitrogen.

The composition of soluble nitrogen in the amide amino acids undergoes diurnal variation in mint leaves grown under short days. Glutamine is most prevalent in the light whereas asparagine accumulates in the dark (16).

Investigations with chlorotic mutants of barley seedlings have also shown a relationship between photosynthetic capacity and asparagine formation. In mutants Xan-b^{15} and Xan-b^{18} chlorophyll content was increased at least two-fold when 2% sucrose was added to the basal medium. Analysis of the soluble amino acids indicated that asparagine levels were markedly decreased in these mutants in the presence of sucrose. Mutants that could not increase their chlorophyll content in the presence of sucrose had comparatively higher asparagine levels (17). Oaks has shown that the sugars, glucose and sucrose, inhibit asparagine formation from acetate in corn root-tips (9). She proposed that glucose or some derivative could inhibit the conversion of aspartate to asparagine. ATP availability, for instance, may be altered in corn root-tip sections which might account for this inhibition of asparagine formation. The effect of glucose in this system and light in the barley seedlings and mint leaves could possibly act through a common mechanism.

Interestingly, the glucose inhibition of asparagine synthesis is not found in mature root sections of corn (18). Other comparisons employing inhibitors also indicate that the regulation of asparagine synthesis changes during maturation of root cells. These examples will be discussed in the following section of the Introduction.

Glutamine serves as a protein amino acid and donates nitrogen in various biosynthetic pathways: amino acids, nucleic acids and specialized cell wall molecules (Table I). The glutamine synthetase reaction is thought to be a primary port of entry of ammonia into nitrogen metabolism (Figure 1). Glutamate, a substrate for glutamine formation is generated by the glutamate synthetase reaction: glutamine + α-ketoglutarate → 2 glutamate. When sufficient glutamate is available, ammonia is rapidly incorporated to form glutamine. The reaction is driven by ATP which is hydrolyzed to give ADP and inorganic phosphate. Glutamine synthetase has a high affinity for ammonia as shown for example by the low Km of about 10^{-5} M in pea (20). It has also been located in plastids and in leaf chloroplasts where carbon is fixed and adequate energy sources provided (21). In rice roots, this enzyme is also activated by the presence of α-ketoglutarate indicating regulation by carbon precursors. In the *Escherichia coli* enzyme deadenylation is promoted by α-ketoglutarate and is thought to hasten the reaction process by increasing the release of phosphate during the reaction. In addition, glutamine synthetase in *E. coli* is regulated by each of the end-products by a phenomenon called concerted feed-back inhibition (19). In mammalian and plant systems this type of regulation has not been demonstrated. If glutamine synthesis were

Table I

Molecules Requiring Glutamine

in their Biosynthesis

Glutamine contributes its amide-nitrogen group in the production of the following compounds:

i) Protein amino acids:

- -glutamate
- -tryptophan
- -histidine
- -asparagine

ii) Carbamyl phosphate

iii) α-ketoglutarate

iv) Nucleic acids:

- -CTP
- -AMP
- -GMP

v) Complex polysaccharides - Glucosamine-6'-phosphate

vi) Oxidative metabolism - Nicotinamide adenosine diphosphate

Figure 1: Glutamine can be considered the primary port of entry of reduced nitrogen into metabolism. Glutamate, the carbon substrate for glutamine formation is formed by the glutamate synthetase reaction: glutamine + α-ketoglutarate → 2 glutamate. Ammonia ion is used as the source of the amide nitrogen for glutamine by the enzyme glutamine synthetase when glutamate is available. Energy in the form of ATP drives this reaction. ADP and inorganic phosphate are the products. The amide nitrogen of glutamine is used in a variety of biosynthetic reactions. In one of these, asparagine synthetase transfers the amide group of glutamine onto aspartate. This reaction requires ATP hydrolysis to AMP and pyrophosphate to provide energy for the transfer. Glutamine is converted back to glutamate in this reaction which can provide the required substrate for the incorporation of more free ammonia by the glutamine synthetase reaction.

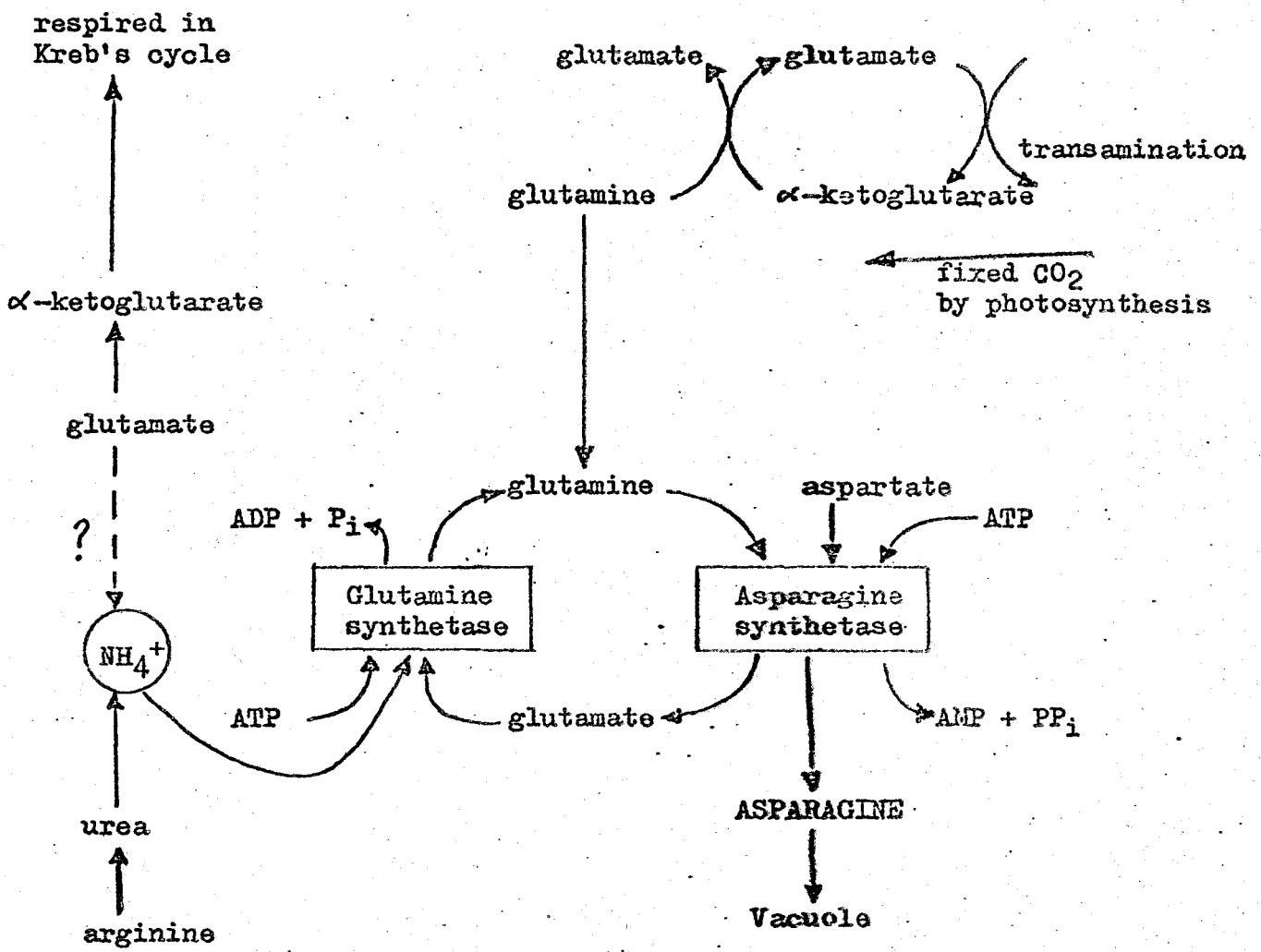


Figure 1: Glutamine Metabolism in Plants.

(Modified after Lea and Fowden, 1975.)

relatively unregulated, high glutamine concentrations could apparently upset cell metabolism in plants. An active transfer of nitrogen to asparagine could then provide a means of storing the excess reduced nitrogen in periods of high ammonia production. Formation of asparagine would liberate glutamate which in turn would then be available to accept an additional ammonium ion (22). In the final stages of catabolism when the reduced nitrogen present exceeds carbon available to act in nitrogen storage, ammonia would accumulate.

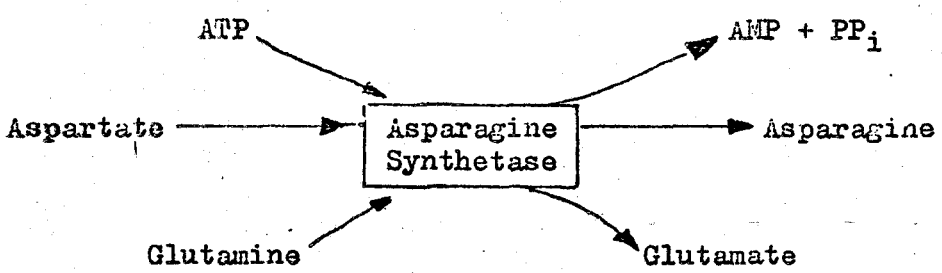
The understanding of asparagine formation and catabolism is not as complete as that for glutamine owing to difficulties in characterizing the enzyme required in asparagine biosynthesis. Several lines of evidence indicate that asparagine formation requires glutamine and is affected by metabolic intermediates. An alternate pathway for asparagine synthesis has been found in cyanogenic plants like sorghum. In this system, cyanide and β-cyanoalanine are condensed prior to the formation of asparagine as shown in Figure 2 (23). Certain lines of evidence indicate that this pathway is not a major route for the synthesis of asparagine in all plants. For example, the enzyme β-cyanoalanine synthetase has been shown to be located in mitochondria (24). Furthermore, in corn, Oaks has shown that there are limitations in the endogenous supply of cyanide and in the conversion of serine to

Figure 2: Aspartate Pathway.

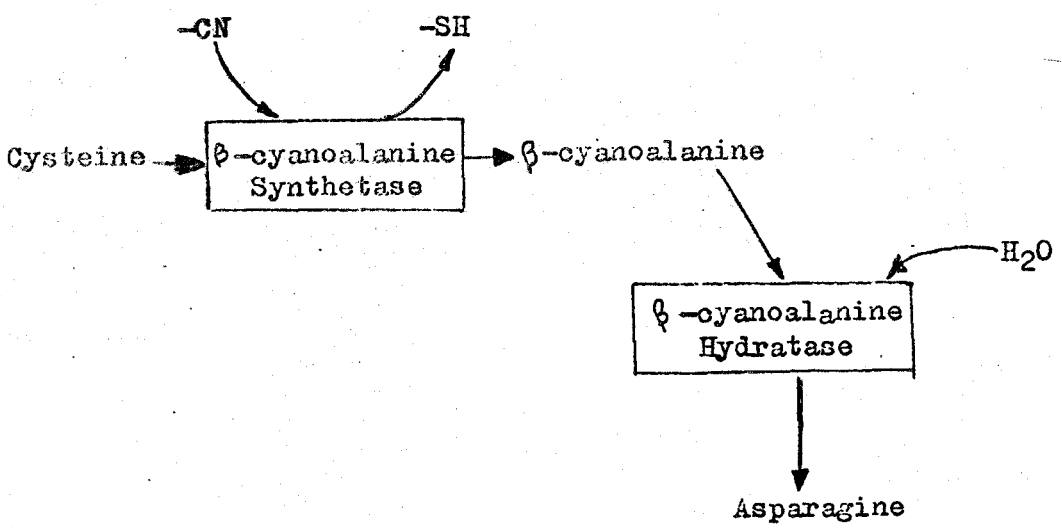
Glutamine donates its amide nitrogen to aspartate in the aspartate pathway. Energy in the form of ATP is required by asparagine synthetase to transfer the amide group and thereby form asparagine. Glutamate and AMP + pyrophosphate are the other reaction products.

Figure 2: Cyanide Pathway.

Cysteine is condensed with cyanide by the enzyme β-cyanoalanine synthetase. The reaction product β-cyanoalanine is then hydrolyzed by β-cyanoalanine hydratase to form asparagine.



Aspartate Pathway



Cyanide Pathway

cysteine which is necessary for the production of β-cyanoalanine (25). These findings suggest that the cyanide pathway is potentially present in most plants but that it probably functions to block the toxic effects of cyanide produced by bacterial sources (24).

Asparagine synthetases, catalyzing the formation of asparagine, were first extracted from bacterial systems (26,27,28). The reaction required L-aspartate, ammonium ion and ATP as substrates. Divalent metal ions, either Mg^{2+} or Mn^{2+}, were also necessary for activity. Asparagine, AMP and inorganic pyrophosphate are the products formed by these synthetases. End-product inhibition and repression of enzyme formation by asparagine have also been demonstrated in the bacterial system.

The asparagine synthetases found in plants and animals differ from the bacterial enzyme in that the amide nitrogen of glutamine rather than ammonia is the nitrogen source. The requirement of an amidotransferase reaction in these reactions is revealed by the low Km's for glutamine and the low affinity shown by the enzyme for ammonia (22,24,29).

The evolution of the change in substrate preference may parallel that found for tryptophan synthesis in *E. coli*. In mutant strains when the glutamine amidotransferase (GAT) function of anthranilate synthetase (anthranilate synthetase component II) is

deleted, there is synthesis of tryptophan using the ammonia-dependent anthranilate synthetase component I. Results suggested that the glutamine amidotransferase function may have evolved by addition of the GAT function to an existing aminase (30).

Glutamine-dependent asparagine synthetases have been isolated from the soluble fraction of extracts of lupin and soybean cotyledons. The *in vitro* enzyme preparation is unstable unless protected by high levels of thiols and glycerol (24,26,29). Substrate additions also seem to aid the maintenance of enzyme activity (29). The enzyme requirements have been established by several means. Substrate analogue studies, which indicate the substituent groups required in binding, and use of inhibitors of glutamine amide nitrogen transfer indicate that glutamine binds at the active site of asparagine synthetase (22). Lower Km's for glutamine than for ammonia also indicate a reaction mechanism preference for the amide group of glutamine. Rognes has concluded that the lupin asparagine synthetase appears to be unique with respect to molecular weight and regulatory properties when compared to similar enzymes from mammalian tumor cells (29). *In vitro* end-product inhibition by 2 mM asparagine has been demonstrated. A 50% inhibition by five millimolar α-ketoglutarate has also been observed with plant enzyme (22).

In vivo experiments also suggest that glutamine is directly involved in asparagine synthesis in plants. In corn, Oaks and Johnson found that azaserine, an inhibitor of glutamine requiring enzymes, inhibited asparagine formation in corn root-tips (1). Kanamori and Matsumoto found that glutamine additions to rice roots enhance the conversion of [^{14}C]-aspartate to [^{14}C]-asparagine. Ammonia was inactive as a donor of amide nitrogen for asparagine (31). Previously, they had suggested that aspartate was converted to asparagine in rice seedlings cultured for several days in an ammonia media (65). Apparently, the ammonium treatment alters the metabolism so that asparagine is a principal product. Their results suggest that the seedlings utilize ammonia in the production of glutamine and that the amide nitrogen of glutamine is transferred to aspartate to form asparagine.

B. Effect of Cycloheximide on Amide Metabolism

Cycloheximide, a naturally occurring glutarimide antibiotic, has been widely used to inhibit protein synthesis in eukaryotic systems. It has been demonstrated, using *in vitro* protein synthesizing systems from reticulocytes, that cycloheximide affects both initiation and extension of peptide synthesis by acting on the donor

site of the ribosomes (32). It is often employed in eukaryotic systems designed to determine the presence or absence of requirements for *de novo* synthesis of protein (33,34,35). In addition, amino acid biosynthesis and regulation have been examined under conditions of protein synthesis inhibition by cycloheximide. The basic assumption in these investigations was that amino acids should accumulate when not utilized for protein synthesis except under conditions where end-product inhibition is in effect and blocks their synthesis. Fletcher and Beevers found that the synthesis of several amino acids was reduced after cycloheximide addition in Paul's scarlet rose supporting this concept (36). Exceptions are also found, however, in leucine and proline biosynthesis in this system. Similar experiments performed with corn roots show that certain amino acids whose synthesis are sensitive to end-product control actually increase in amounts when low concentrations of cycloheximide are administered (37).

There are observations that suggest that cycloheximide is not entirely specific in its action. Changes in general metabolism, enzyme reactions and on membrane stability in cells have been found when this antibiotic is used (Table II). In particular, there are several instances from different systems which suggest that cycloheximide affects the utilization of the amide nitrogen of glutamine. According to this hypothesis,

Table II

Examples of Effects Produced by Cycloheximide

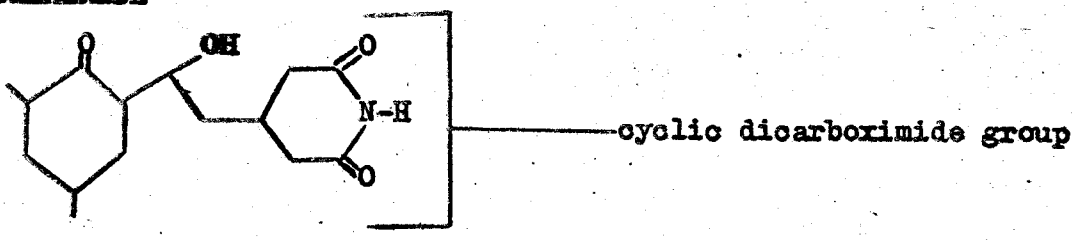
Cycloheximide, in addition to its general effect of inhibiting protein synthesis in most eukaryotic systems, affects other aspects of metabolism and physiology. Listed below are a few examples where cycloheximide is known to have either direct or indirect effects on:

		References
i)	RNA synthesis	38,39
ii)	Triglyceride metabolism	40
iii)	Lipid metabolism	41,42
iv)	Alkaloid metabolism	43
v)	Pyrimidine metabolism	44
vi)	Amino acid metabolism	45,46
vii)	Membranes	47
viii)	Transpiration, ion uptake; respiration and oxygen uptake	49,50,51
ix)	Enzyme activity:	
	-ornithine decarboxylase	52
	-tyrosine hydroxylase	53
	-diamine oxidase	54
	-ribonuclease	55

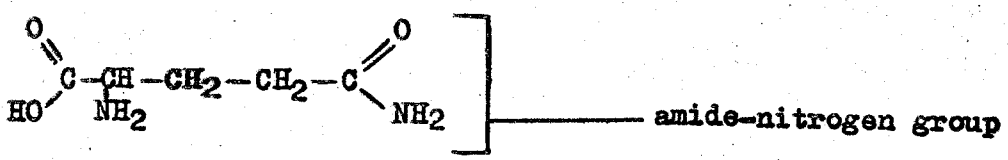
the cyclic dicarboximide group of cycloheximide (Figure 3) could be similar to the amide nitrogen of glutamine. Ross found in cocklebur leaf discs that cycloheximide inhibits the glutamine requiring conversion of UTP to CTP thus affecting pyrimidine nucleotide metabolism (44). In yeast, Widuczynski and Stoppani have observed an accumulation of [^{14}C]-glutamine from [^{14}C]-glutamate in the presence of the antibiotic. Cycloheximide inhibition of glutamine amide nitrogen transfer was suggested to account for this increase in glutamine (56).

In corn seedlings, Oaks and Johnson observed alterations in glutamine and asparagine formation in root-tip sections treated with this antibiotic (1). According to Chibnall and Vickery, asparagine should accumulate under conditions where protein turnover is high (57,7). Yet when this hypothesis was tested directly in root-tip sections with cycloheximide, a potent protein synthesis inhibitor in this system, asparagine synthesis was inhibited. Glutamine levels increased with this treatment. The situation was further examined by addition of 10^{-4} M azaserine, a known glutamine analogue and irreversible inhibitor of glutamine-dependent enzyme reactions (58). This antibiotic caused a similar inhibition of root-tip asparagine formation and also increased glutamine levels. There was no marked effect on protein synthesis. Mature sections of the corn root show characteristics different

CYCLOHEXIMIDE



GLUTAMINE



AZASERINE

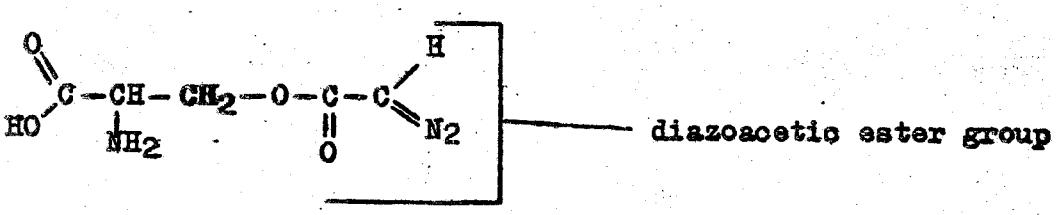


Figure 3: Structural Comparison of Cycloheximide, Glutamine and Azaserine.

The cyclic dicarboximide group of cycloheximide and the diazoacetic ester group of azaserine show similarities in molecular structure to the amide-nitrogen group of glutamine.

from the root-tip sections upon treatment with either antibiotic. Even though cycloheximide retains its effect on inhibiting protein synthesis in the mature section, it has, if anything, a stimulatory effect on asparagine formation. Azaserine, likewise, does not inhibit asparagine formation in mature root sections. To account for this, Oaks and Johnson suggested cycloheximide might be acting in the root-tip sections much like the known glutamine analogue azaserine. It was proposed that cycloheximide inhibited the utilization of glutamine by asparagine synthetase and thereby reduced asparagine formation. Further, when the root cells mature the asparagine synthetase may be altered in such a way that it no longer recognizes cycloheximide or azaserine (1).

Some similarities are apparent in the structure of the glutamine amide substituent group and the cyclic dicarboximide ring structure of cycloheximide. The diazoacetic ester group of azaserine which is involved in the irreversible inhibition of glutamine-dependent reactions also has structural characteristics similar to the amide substituent group (Figure 3).

The dicarboximide ring structure of cycloheximide is also considered important in the mode of action of cycloheximide and that of structurally similar molecules or analogues. Studies using analogues of cycloheximide in *Saccharomyces pastorianus* suggest that glutarimide

antibiotics, like cycloheximide, require a three point attachment to the substrate (59). The mode of action of these analogues is, however, variable if comparisons are made between systems (Table III). Differential effects on *in vivo* protein synthesis and RNA synthesis occur in *Achlya* (38) and on fungitoxicity in *S. pastorianus* when analogues are administered. Thus, it appears that minor changes in the structure of cycloheximide can produce significantly different effects in biological systems.

A number of other naturally-occurring and synthetic dicarboximides have been found to inhibit biological growth. For example, cis-4-cyclohexene-1, 2-dicarboximide, a molecule believed to inhibit germination in sugar beet fruit and dormant wheat seed, was found to inhibit lettuce seed germination. Captan, an N-thio substituted dicarboximide has been used as an agricultural fungicide (60).

This summary indicates that there is sufficient evidence in the literature to give support to Oaks and Johnson's suggestion that cycloheximide acts as a glutamine analogue in its inhibition of asparagine synthesis in corn root-tips. Many alternatives are possible however. For example, from a general survey (Table II) of the literature on cycloheximide effects, most effects may be attributed to direct action by the antibiotic on protein synthesis or membrane integrity.

Table III

Summary of Effects of Some Analogues of Cycloheximide

in *Achyla bisexualis* and *Saccharomyces pastorianus*

	Achyla bisexualis effect on *in vivo*:		*Saccharomyces pastorianus* % effectiveness compared to ED_{50} [cycloheximide] in:	
	protein synthesis	RNA synthesis	protein synthesis (*in vitro*)	Fungitoxicity
Cycloheximide	inhibited	inhibited	100	100
Analogues				
Anhydrocycloheximide	no effect	increased	no data	very low
Cycloheximide acetate	inhibited	increased	very low	very low
Cycloheximide oxime	inhibited	inhibited	very low	0.2
Cycloheximide semicarbizone	no effect	no effect	very low	2
Isocycloheximide	no effect	no effect	14	13
Streptovitacin A	inhibited	no effect	240	0.2

Interaction of the effects on both sites may also explain the difficulty that investigators have had in explaining the results of experiments using cycloheximide. The following work was carried out to clarify the situation in corn roots and to give further information on the mechanism of asparagine regulation.

The approach of this investigation was to:

1) compare the effects of cycloheximide and azaserine on asparagine synthesis in root-tip sections of corn;
2) use analogues of cycloheximide to study the effect on protein synthesis and asparagine synthesis in root-tip sections;
3) to study the properties of asparagine synthetase extracted from corn roots to determine:
   a) whether azaserine and cycloheximide inhibit the enzyme activity from root-tip sections, and
   b) whether the effect is still present in enzyme extracted from the mature section.

MATERIALS AND METHODS

A. Materials

Corn seed (W64A X 182E) was a gift from the Warwick Seed Company, Blenheim, Ontario, and soybean seed (Glycine max (L.) Merr.) was a gift from J.G. Streeter (24). The analogues of cycloheximide were obtained from three sources: cycloheximide oxime, cycloheximide acetate, cycloheximide semicarbizone, cycloheximide oxime acetate from G. Kangars, Agricultural Division, The Upjohn Company, Kalamazoo, Michigan; isocycloheximide, anhydrocycloheximide, streptovitacin A from D.H. Griffin (38); and cis-4-cyclohexene-1,2-dicarboximide from N.E. Tolbert (60).

Other materials were obtained from the following sources:Dowex resins, Bio-Rad Laboratories, Richmond, California; azaserine, Calbiochem, Los Angeles, California; agar, Difco Laboratories, Detroit, Michigan; 2-mercapto-ethanol, L-glutamine, 1,2-napthoquinone-4-sulfonate, Eastman Kodak Company, Rochester, New York; inorganic chemicals, Fisher Scientific, Fairlawn, New Jersey; Triton X-100, J.T. Baker Chemical Company, Phillipsberg, New Jersey; potassium penicillin G, Mann Research

Laboratories, New York, N.Y.; [2-^{14}C]-acetate (1.5 mCi/mM), [UL-^{14}C]-aspartate (>180 mCi/mM), omnifluor, New England Nuclear, Boston, Massachusetts; sephadex resins, Pharmacia, Uppsala, Sweden; adenosine-5'-triphosphate, P-L Laboratories Inc., Milwaukee, Wisconsin; and L-aspartate, L-asparagine, bovine serum albumin, cycloheximide, tricine buffer, streptomycin sulfate from Sigma Chemical Company, St. Louis, Missouri.

B. Germination Conditions

Corn seed used in the [2-^{14}C]-acetate feeding experiments was surface sterilized with 20% Javel (a commercial bleach containing approximately 5% sodium hypochlorite) for 20 minutes, soaked in 0.01 N HCl for 10 minutes and then rinsed well with sterile distilled water (6-8 times) according to a modified procedure introduced by Abdul-Baki (61).

Seed used in the investigation of asparagine synthetase activity in corn was not surface sterilized prior to planting.

The seed was then grown in the dark for 60 to 70 hours at 26^{o}C on sterilized petri plates containing 0.9% agar made up in 1/10 Hoagland's salts with 10 mM ammonium sulfate.

Soybean seed (Glycine max (L.) Merr). was surface sterilized in a 0.05% Javel solution for 15 minutes. The seed was then rinsed and germinated in moist vermiculite and sand at 27°C (24). Cotyledons used in the asparagine synthetase assay were taken from eight day old seedlings.

1. Experimental design

a. Acetate-feeding experiments:

For the [2-^{14}C]-acetate feeding experiments, roots 3.5 to 4.5 cm long were chosen and the 5 mm root-tip sections were used. The sections were placed in sterile 1/10 Hoagland's salts solution with 10 mM $(NH_4)_2SO_4$ plus appropriate additives. With the exception of azaserine and cis-4-cyclohexene-1,2-dicarboximide which were easily solubilized in distilled water, the analogues of cycloheximide were solubilized in distilled water heated to about 70°C for five minutes. All additive solutions were then passed through Millipore filters. To prevent bacterial growth, 10 µg/ml penicillin G and 325 µg/ml streptomycin sulfate were added to the above salt solution. The sections (40 per sample) were pre-incubated for 3 hours in the appropriate medium and then transferred to fresh sterile medium which contained [2-^{14}C]-acetate (5 µCi in 3 ml solution) in addition to the compounds

of the pre-incubation medium. The experimental time was routinely two hours. Thus, the standard exposure of the root sections to azaserine, cycloheximide and analogues of cycloheximide was a total of five hours. At the end of this experiment, samples of the medium were plated on Difco-bactonutrient agar. Samples were discarded if bacterial contamination was detected. The root-tip samples were killed by transferring them to 80% ethanol at the end of the experimental time.

2. Extraction procedures

The extraction procedure for the acetate feeding experiments are those described by Oaks and Johnson (9). The root sections were rinsed with water and extracted with 80% ethanol (W/V) with a hand homogenizer. Separation of the alcohol insoluble and soluble fractions were accomplished by repeated extractions of the homogenized root sections with 80% ethanol. The alcohol insoluble residue was hydrolyzed for twelve hours in 6 N HCl at 121^{o}C and 15 pounds pressure. The soluble samples were evaporated to dryness at 50^{o}C under reduced pressure. For quantitative determination of the radioactivity after each step, aliquots of samples dissolved in known amounts of distilled water were routinely placed on filter paper discs for counting. The amino acids of the soluble and insoluble fractions were separated by

use of ion exchange resins (Dowex 50 [H^+]: 1 x 6 cm). Elution of these columns with 60 ml of water removed sugars from the insoluble and the organic acids and sugars from the soluble fraction respectively. Amino acids were then eluted from the Dowex 50 [H^+] columns with 60 ml of 2 N NH_4OH. Each fraction was taken to dryness under vacuum at 45 to 50°C.

Glutamate and aspartate were removed from the amino acid fraction by passing this fraction over a Dowex-1-acetate column (1 x 6 cm). Neutral and basic amino acids plus amides were eluted by 50 ml of distilled water. The glutamate and aspartate fractions were then removed from this anion-exchange resin by 50 ml of 6 N acetate. The water eluates were hydrolyzed in 2 N HCl at 100°C for four hours to convert glutamine and asparagine to glutamate and aspartate, respectively. The hydrolyzed fractions were then passed over another Dowex-1-acetate column. Neutral and basic amino acids were eluted by 50 ml of water and the glutamate plus aspartate fraction then eluted by 6 N acetate. The glutamate plus aspartate fractions from the Dowex-1-acetate runs were separated using descending chromatography. Cold glutamate and aspartate were chromatographed concurrently. The solvent used was n-butanol:acetic acid and water (3:1:1 v/v). Each chromatograph was run for 14 hours, dried and run again for 14 hours.

The aspartate and glutamate spots were located under UV by fluorescence after application of 0.002%, 1,2-napthaquinone-4-sulfonate. The areas were cut out, placed in scintillation vials with 5 ml of omnifluor (POP-POPOP) in toluene and counted.

The routine method for quantitation of radioactivity in each fraction was as follows: The dried radioactivity compounds were re-solubilized in 2 ml of distilled water. Ten or 20 µl samples were taken from this solution and applied to small discs of chromatographic paper for counting using the above scintillation fluid. Cpm obtained from the scintillation counter were then corrected to obtain the cpm in the total fraction. For instance, if 20 µl was counted from a 2.0 ml total volume then the cpm obtained would be multiplied by 100 to give the total cpm in the fraction. Corrections for background radioactivity were subtracted from this total.

For the chromatographic separation of glutamate and aspartate radioactivity 0.1 ml from a 2.0 ml solution was initially applied to the chromatographic paper. The cpm data were then multiplied by 20 to give the total cpm in each fraction.

C. Extraction and Assay Method Used to Test for Asparagine Synthetase

The method used for *in vitro* examination of

asparagine synthetase activity is essentially that employed by Streeter (24).

Excised plant material (root, shoot, scutellum of corn or cotyledons from soybean) were chilled on ice and subsequent enzyme preparation steps carried out in ice or at 3°C. The material was macerated with a mortar and pestle or by Omnimix in 0.10 M phosphate buffer pH 7.0, containing 1 mM of 2-mercaptoethanol and 1 mM $MgCl_2$. The slurry was filtered through four layers of cheese cloth and centrifuged at 27,000 g for fifteen minutes. In some initial experiments, the supernatant was filtered through a 3 x 20 column of Sephadex G-25 (coarse). Later experiments routinely employed a 3 x 20 column of Sephadex G-100 (fine) for filtering of the crude extract since it gave better separation of large protein molecules from smaller substrate molecules. The column was equilibrated with 10 mM phosphate buffer pH 7.3, containing 1 mM 2-mercaptoethanol and 1 mM $MgCl_2$ prior to application of the protein. Protein was eluted from the column using the same buffer.

The standard reaction mixture contained the following:100 mM Tricine buffer pH 7.75; 10 mM $MgCl_2$; 10 mM glutamine or 50 mM NH_4Cl;2 mM aspartate; [UL-^{14}C]-aspartate (approximately 10^6 cpm, 0.5 µCi/sample); 5 mM ATP and protein extract in a total volume of 1.0 ml. Azaserine (1 mM), 20 mM asparagine and 10 mM cycloheximide

were added to the buffer when tested. Cpm in products formed were measured by the amount of radioactivity from [UL-^{14}C]-aspartate not retained by Dowex-1-acetate columns. Columns 5 mm in diameter and 50 mm in length were made up in Pasteur pipettes. Column effluent was collected directly in scintillation vials. Reaction mixtures were applied directly to the columns and three 0.5 ml portions of water were used to rinse reaction tubes and columns. Effluent was mixed with 15 ml of scintillation cocktail made from toluene and omnifluor (2:1). These samples were then counted in a Beckman liquid scintillation counter.

Identification of asparagine formed in the assays was accomplished by the use of descending chromatography. Three solvent systems - phenol solvent, phenol:ammonia (62) and butanol:acetic acid:water (3:1:1 v/v) were used to separate the reaction products. The relative separation of asparagine, aspartate and alanine after two runs of about 13 hours each is shown in Figure 4. Radioactive areas in the chromatographs were detected using a Nuclear Chicago Actigraph II strip counter and their positions compared to cold amino acids run concurrently in the same solvent system.

Mild acid hydrolysis of asparagine produces aspartate and ammonia. For the purpose of allowing further identification of asparagine formed in the assays,

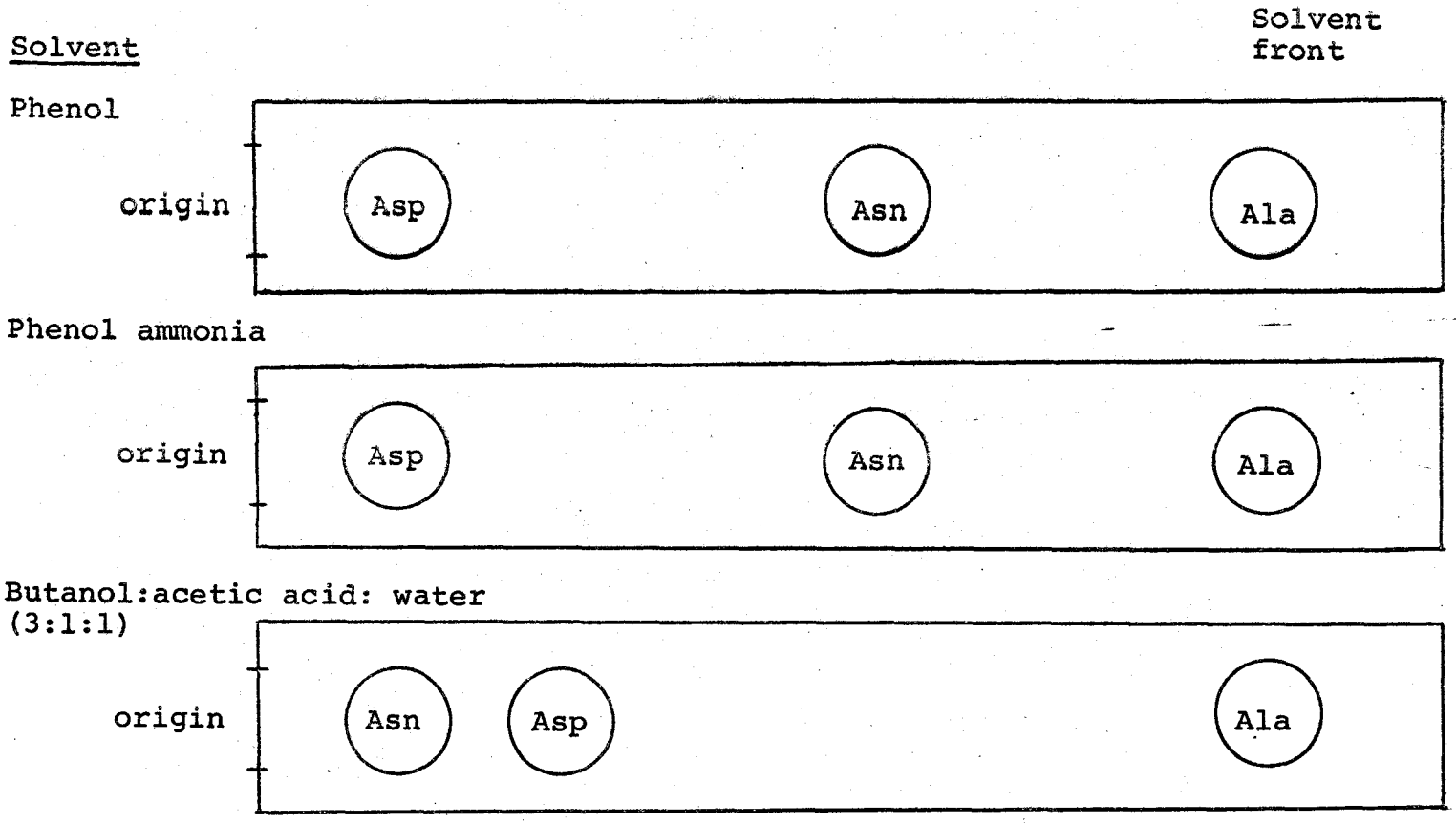


Figure 4: Relative Distribution of Standard Amino Acids After Chromatography

The amino acids were separated using descending chromatography in the solvents listed on the left. Chromatographs were run for about 13 hours, dried and run again in the same solvent for another 13 hours.

Abbreviations:

Asp = aspartate
Asn = asparagine
Ala = alanine

the reaction products or chromatographic areas suspected to contain asparagine were routinely hydrolyzed in 2 N HCl at 100°C for 4 hours. This hydrolysate was then dried and chromatographed using phenol solvent. After this treatment, any radioactivity appearing in the aspartate location was considered to be derived from asparagine.

RESULTS

A. [2-^{14}C]-Acetate-Feeding Experiments

1. Experimental design:

The experimental design for the routine experimental procedure has been outlined in Materials and Methods. Samples of 40 root-tip sections were pre-incubated for three hours with or without appropriate additive. The samples were then transferred to fresh media and [2-^{14}C]-acetate added. The samples were then incubated at 26^{o}C for two hours. After the two hour experimental period, the root-tip sections were killed in 80% ethanol. The standard exposure of the root-tip sections to the additives was therefore a total of five hours.

2. Concentration of cycloheximide used in [2-^{14}C]-acetate-feeding experiments:

Oaks and Johnson previously had determined the effect of cycloheximide concentration on protein synthesis, asparagine formation and glutamine formation. A cycloheximide concentration of 1 µg/ml (3.6×10^{-6} M)

produced an 80% inhibition of protein and asparagine synthesis when present three hours prior to [2-^{14}C]-acetate feeding. The cycloheximide effect on the amides appeared to be maximal at this concentration. Protein synthesis, however, could be further inhibited to 90% at a concentration of 5 µg/ml (1.8×10^{-5} M).

The cycloheximide concentration used in the following [2-^{14}C]-acetate feeding experiments was maintained at 1 µg/ml in order to: (1) significantly reduce asparagine synthesis; (2) to prevent possible additional effects on general metabolism due to greater cycloheximide concentrations.

3. Comparison of control and cycloheximide treated samples:

In root-tip sections, cycloheximide produces noticeable changes in the distribution of [^{14}C]-labelled compounds derived from [2-^{14}C]-acetate. Variability was, however, a common occurrence amongst similar experiments run on different days. The general distributions are noted by mean ± standard error for each fraction in Table IV. The statistical significance of the changes produced with cycloheximide in the cpm for each fraction was tested using a one-way analysis of variance programme. Data from 18 control and 6 cycloheximide treated experiments were analyzed by this experiment. The F-ratio which is the ratio of the two mean squares

Table IV

The Metabolism of Acetate [2-^{14}C]-Acetate: Comparison of Control and Cycloheximide Treated Samples

CPM x 10^{-3}

Mean ± Standard Error

Fraction	Control (18)	Cycloheximide (6)	F-ratio
CO_2	61.81 ± 7.92	102.27 ± 17.92	5.65*
Insoluble:			
Total	194.57 ± 11.58	108.33 ± 22.15	13.53**
AA	128.74 ± 8.64	48.47 ± 7.81	25.88**
Soluble:			
Total	2286.35 ± 146.42	2081.78 ± 152.30	0.57
OAS	518.29 ± 29.35	373.38 ± 48.17	6.24**
AA	1670.23 ± 112.82	1609.93 ± 116.28	0.001
NB & Amides	359.90 ± 23.10	653.49 ± 86.37	22.16**
NB	55.35 ± 5.38	105.03 ± 21.52	10.75**
Glutamate	859.12 ± 91.29	603.44 ± 86.92	2.33
Aspartate	47.29 ± 5.27	36.88 ± 9.94	0.94
Glutamine	181.90 ± 16.48	312.06 ± 95.51	4.70**
Asparagine	53.42 ± 3.69	15.96 ± 2.30	37.77**
Average % Recovery	78	70	

* values significant at p = .05 level
** values significant at p = .01 level

See Table VI for abbreviations (page 50).

The experimental design was similar to that described in Materials and Methods.

for the control and treated samples is also shown for each fraction. F-ratios greater than the critical F-ratio 4.30 are considered to show statistically significant differences between control and treated samples at the p = 0.5 level ($F_{.95}(1,22 = 4.30)$. F -ratios greater than 7.95 show statistically significant differences at the p = .01 level ($F_{.99}(1,22) = 7.95$).

From the analysis of variance comparison it appears that cycloheximide treatment results in statistically significant increases in [^{14}C]-glutamine, [^{14}C]-neutral and basic amino acids and $^{14}CO_2$ respired. Significant decreases are found in the [^{14}C]-insoluble fraction (protein), [^{14}C]-organic acids and sugars and in [^{14}C]-asparagine. The [^{14}C]-label in the total soluble fraction, total amino acid fractions, glutamate and aspartate are not significantly different from the controls.

Data from cycloheximide treated samples published by Oaks and Johnson are similar in the following aspects: the decrease in the insoluble amino acid fraction, the increase in the neutral and basic fraction, the increase in glutamine and the decrease in asparagine. However, the increase in respired $^{14}CO_2$ and the decrease in the organic acids and sugars fraction were not noted in their experiments.

B. Time of Addition of Antibiotics

1. Experimental design

The standard experiment described in Materials and Methods was modified for the timing experiments with cycloheximide and azaserine. Excised sections were placed in the pre-incubation salt solution for three hours. At appropriate intervals prior to [2-^{14}C]-acetate feeding the compound being tested was also added to the pre-incubation media. This compound was present later during the routine two-hour [2-^{14}C]-acetate feeding period. Time intervals stated in the data thus refer to time of exposure to the compound prior to [2-^{14}C]-acetate feeding.

The data from the acetate feeding experiments can be represented in several ways. For example, in Figures 5 to 12, the cpm data were given as percent control and percent soluble fraction in graphs A and B, respectively. Direct reference to graphs A and B of each figure is not always given in the text, but both graphs are present to facilitate comparison of the data.

2. Effect of time of addition of cycloheximide

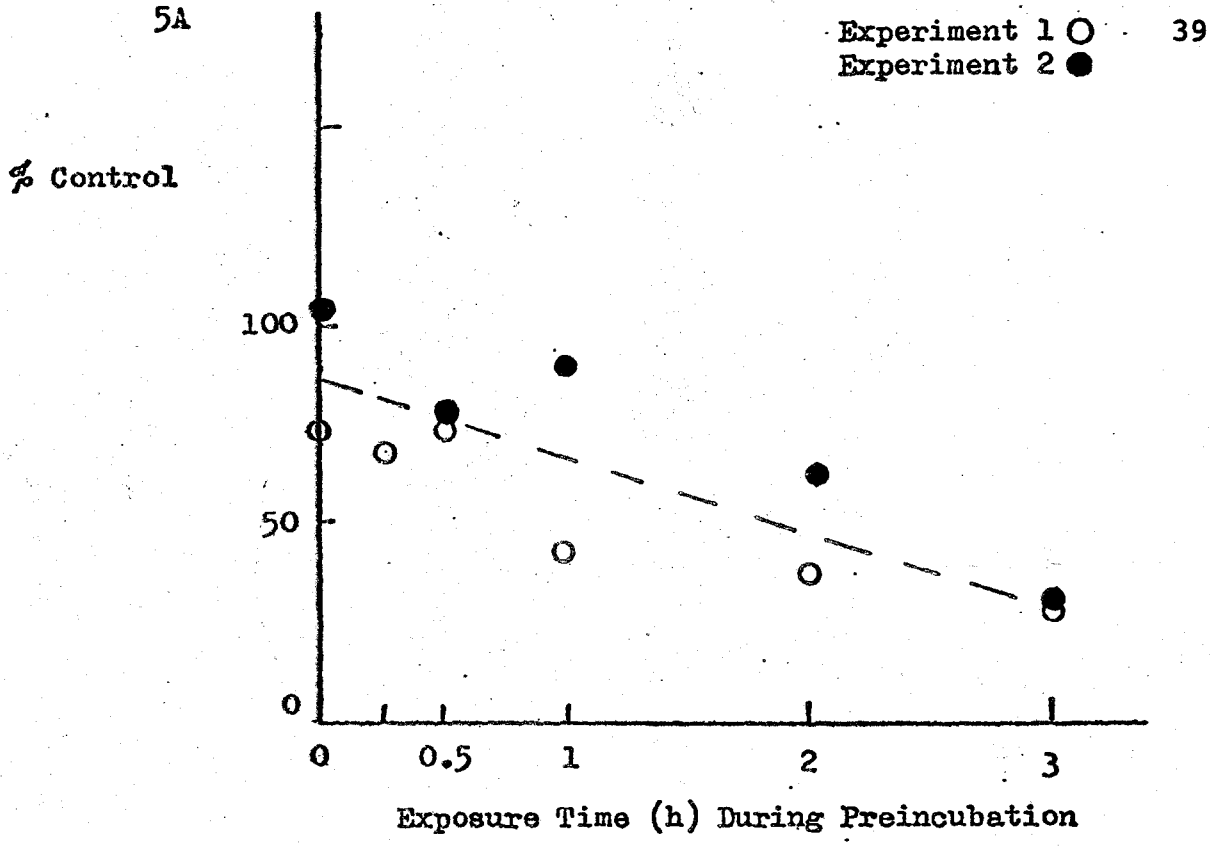
a. Asparagine formation

The data indicate that inhibition of asparagine

formation by cycloheximide increases with increasing time of exposure to the antibiotic. Asparagine formed from acetate when expressed as a percent of the total soluble fractions shows an immediate decrease at time zero (Figure 5B). This decline continues, with slight variability, from about 2% to slightly less than 1% with longer exposures to cycloheximide. For pre-incubation times of three hours, the data indicate that asparagine formation is about one-third that noted in the control situation. The trend noted above is also observed when the data for asparagine formation are expressed as a percentage of the control (Figure 5A). In the first experiment, an immediate decrease in asparagine is observed. However, in the second experiment, the immediate decrease to 80% of the control is not found. Instead, a slight increase of 5% above the control value is found at 0 hours. Variability in the decline in asparagine formation is revealed at one hour exposure to cycloheximide. However, by three hours exposure, both experiments indicate that asparagine formation has been inhibited to about one-third that of the control.

Figures 6A and 6B normalize the data with respect to the controls for both experiments. This procedure indicates that the pattern of decrease in asparagine is similar in both experiments. This gradual decline in *in vivo* formation of asparagine is the same

5A



5B

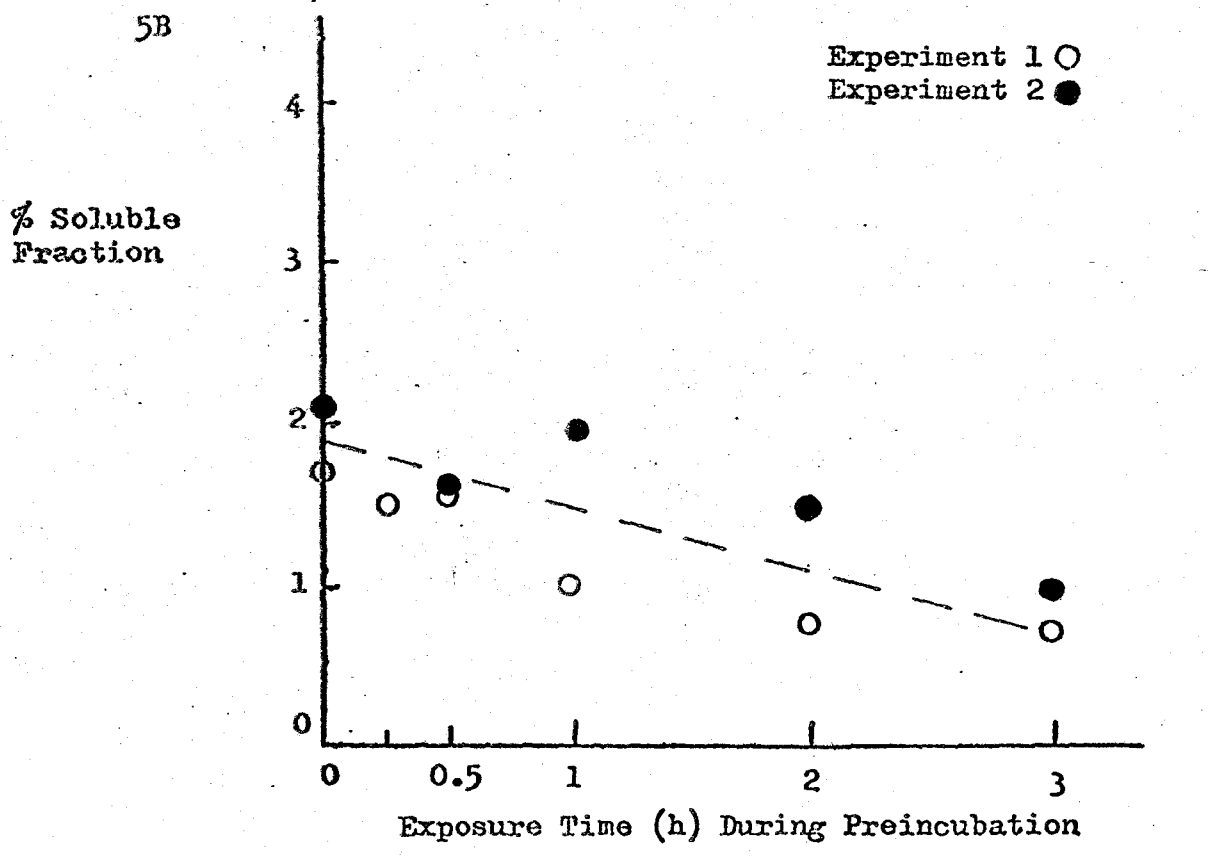
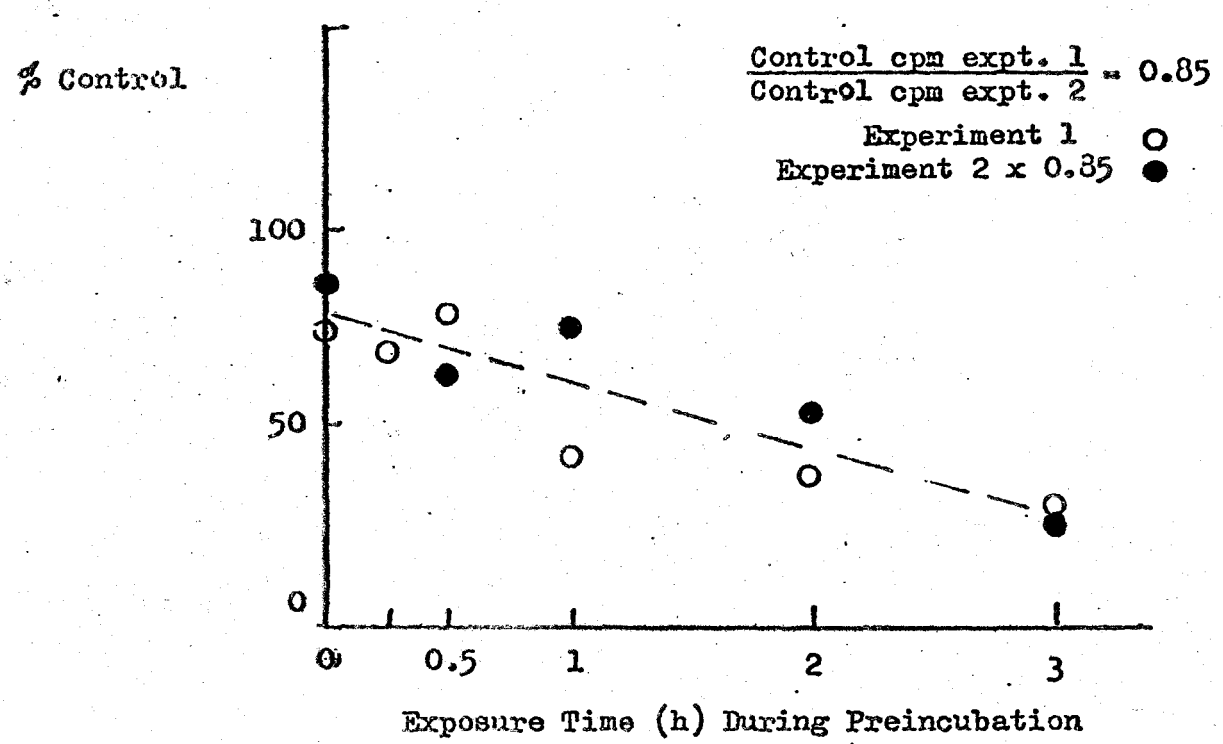


Figure 5: The Effect of Time of Addition of Cycloheximide on Asparagine Synthesis: Experiments 1 and 2.

6A



6B

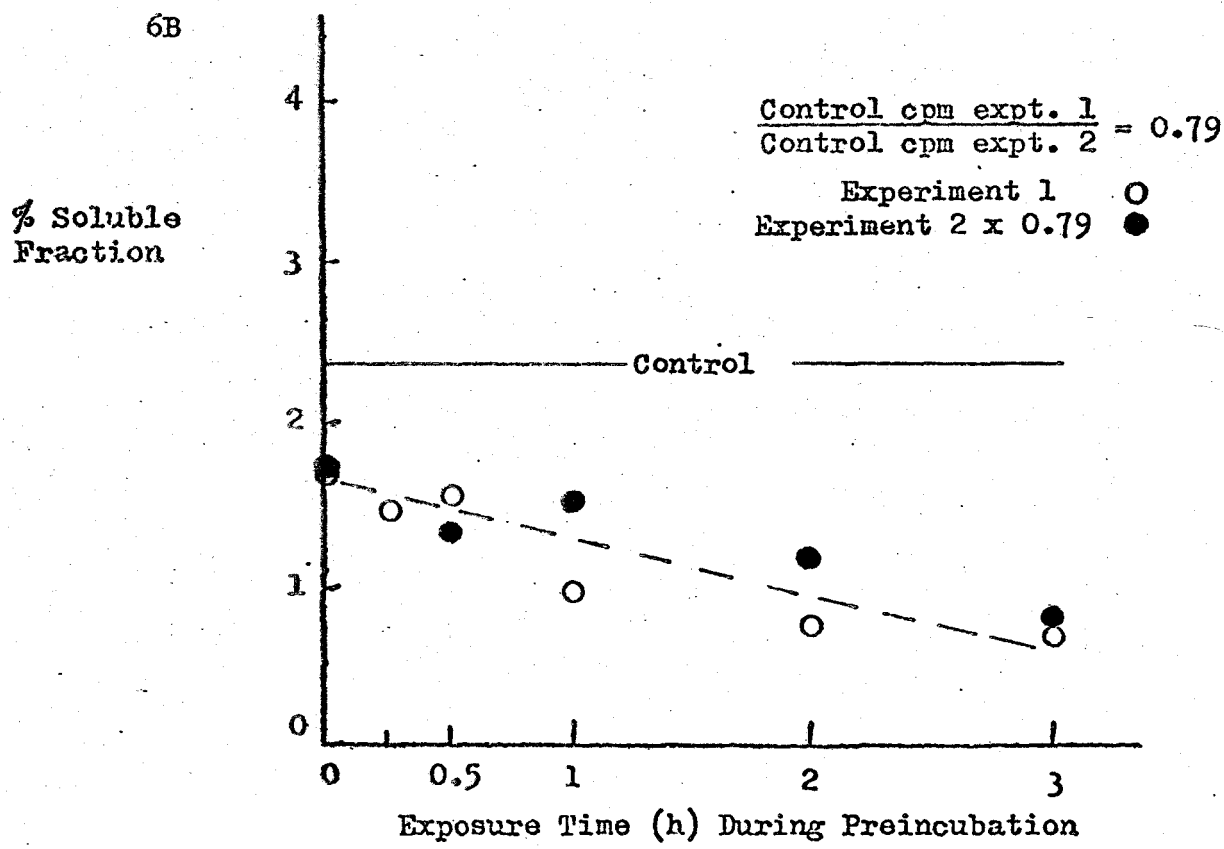


Figure 6: The Effect of Time of Addition of Cycloheximide on Asparagine Synthesis.

(Data from Figure 5 normalized with respect to control data.)

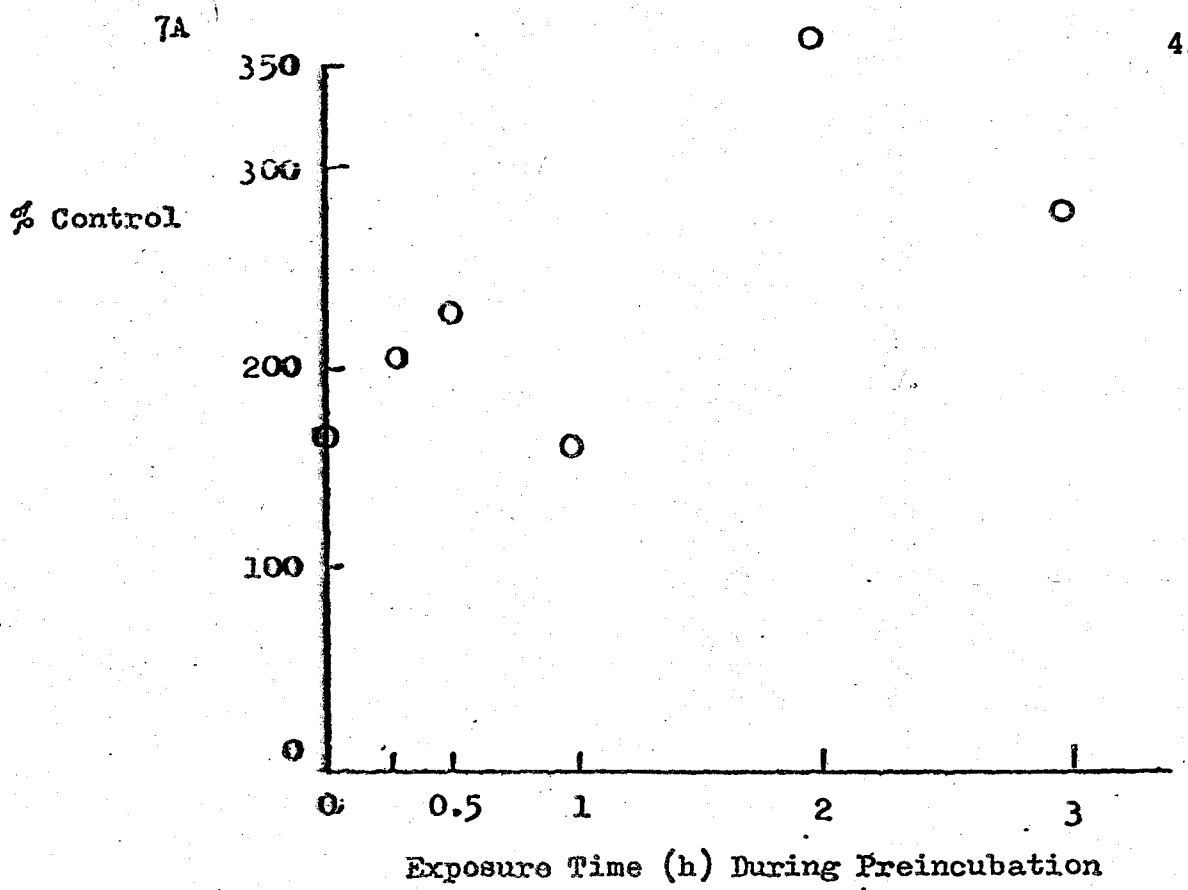
for both experiments whether expressed as a percent of the control (Figure 6A) or as a percent of the total soluble fraction (Figure 6B).

b. Glutamine formation:

The data indicate that a general increase in glutamine occurs with increasing exposure to cycloheximide. This increase appears to be immediate. In both experiments, at 0 hours, glutamine was about 5% above the percent soluble fraction value found in the controls. In experiment number 1 (Figure 7B) glutamine increases from 12% of the soluble fraction at 0 hours to 19% at three hours pre-incubation exposure. Similarly, in experiment number 2, glutamine increases from 16% at 0 hours to about 32% of the soluble fraction at three hours (Figure 8B). As shown in Figure 9B when the control values are normalized similar patterns in the glutamine increase with time of exposure.

When the same data is expressed as a percent of the control, the data indicates that glutamine increases immediately to about 190% at 0 hours and increases to at least 270% at three hours (Figure 10A). A discernable pattern is not readily apparent using the percent control data for glutamine. However, the general trend is consistent with that increase found in the percent soluble fraction data (Figure 10B).

7A



7B

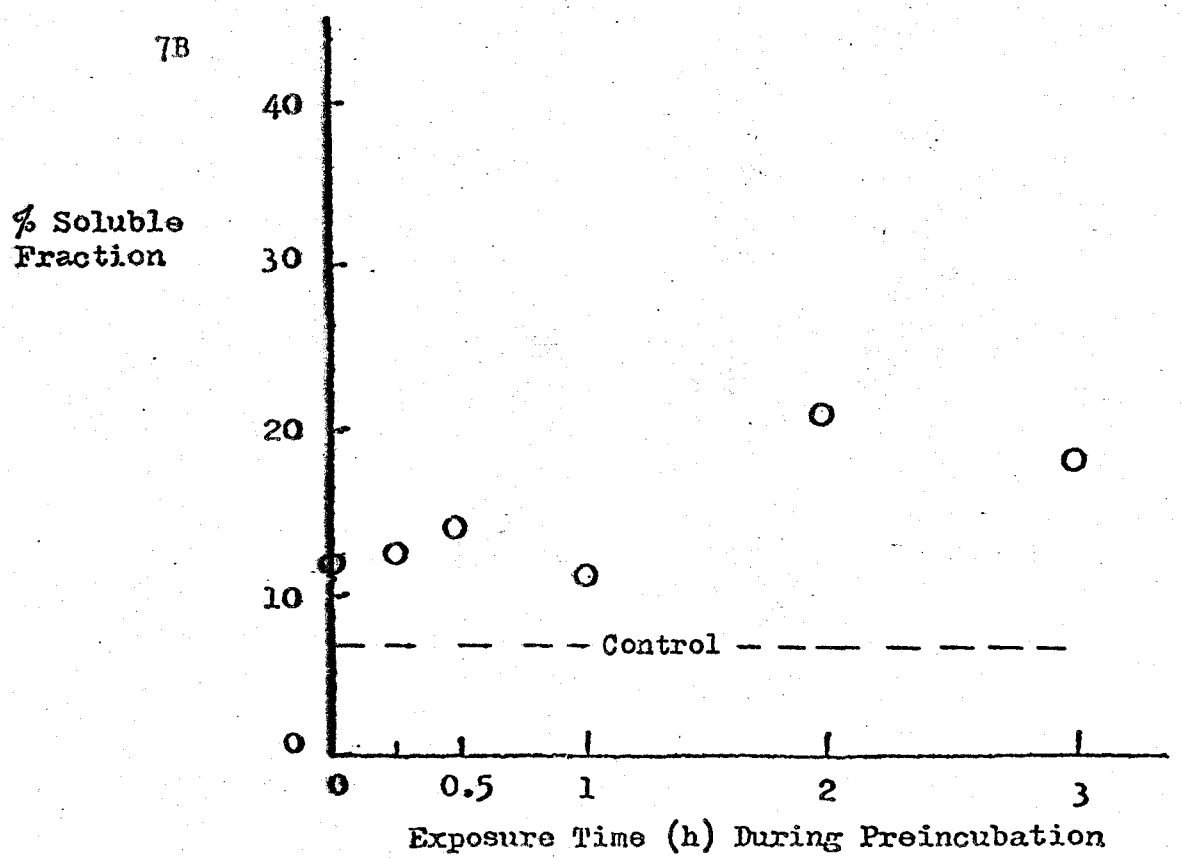
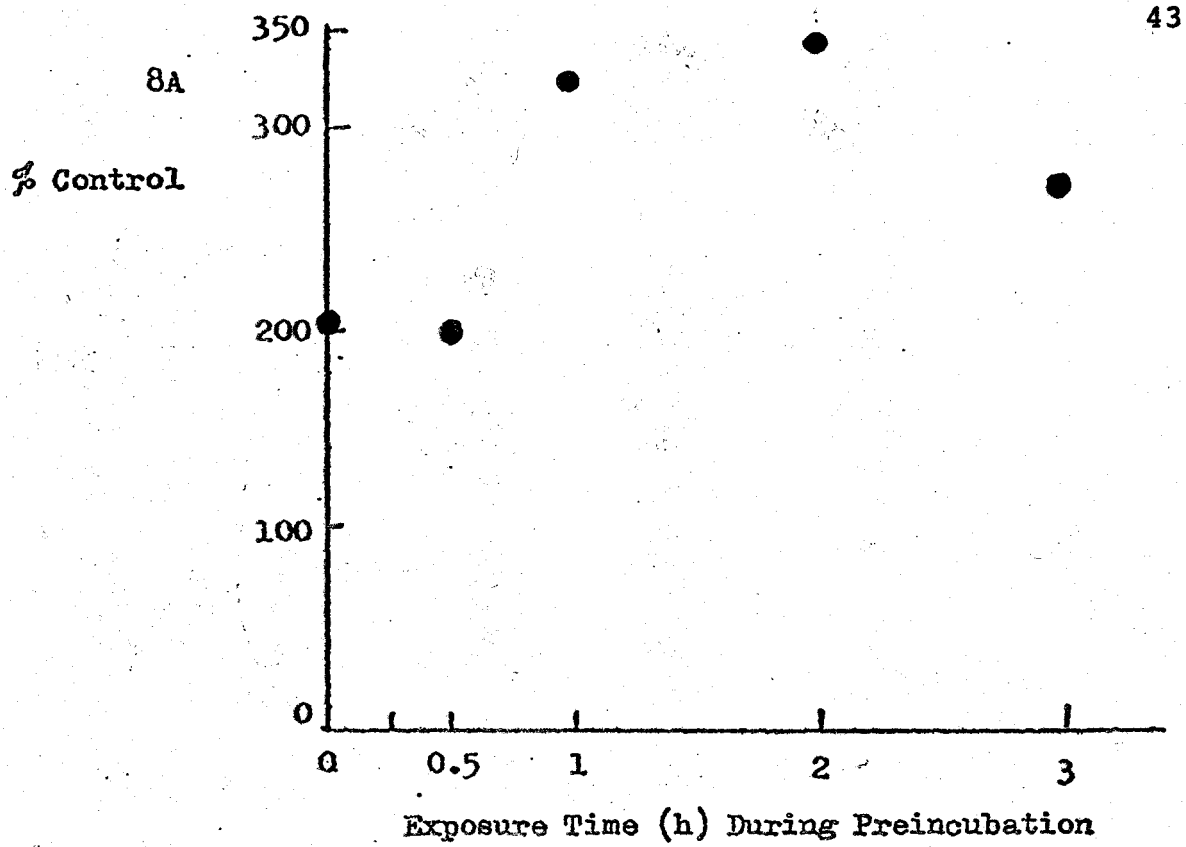


Figure 7: The Effect of Time of Addition of Cycloheximide on Glutamine Synthesis: Experiment 1.



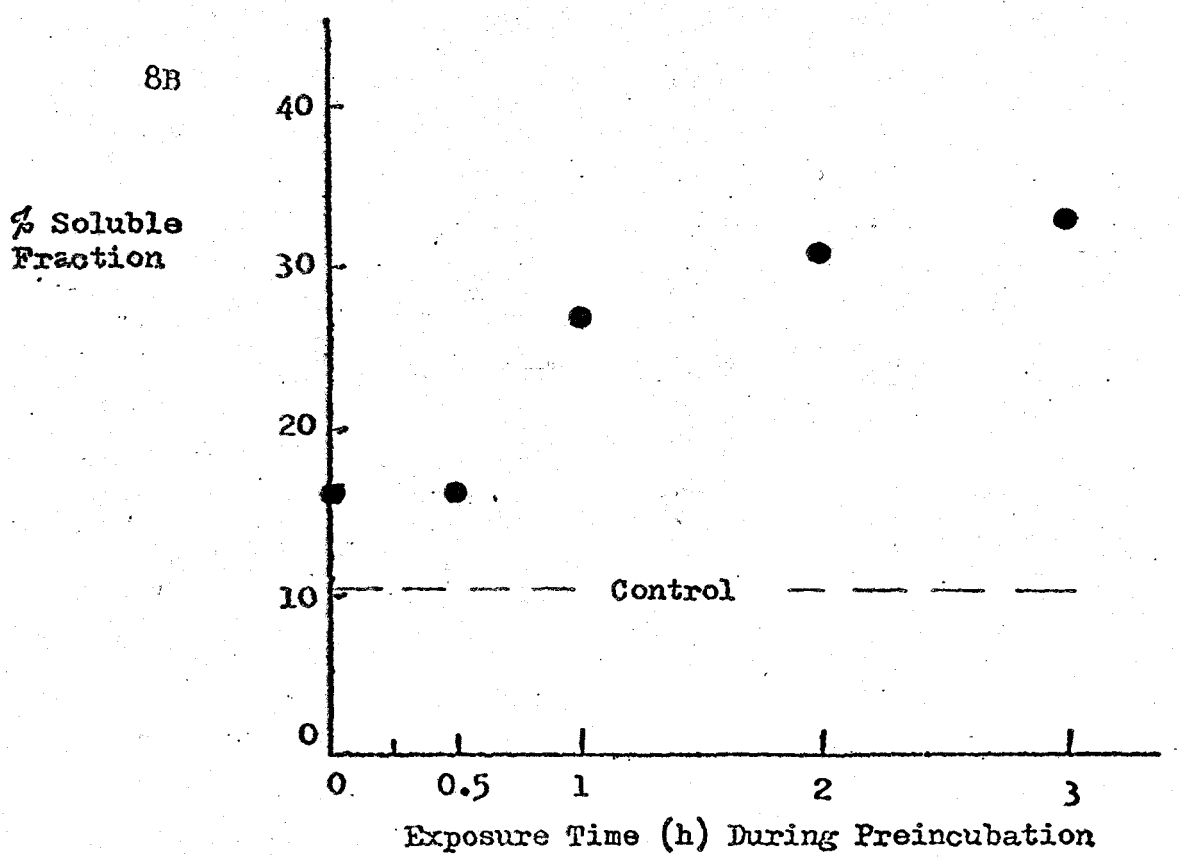
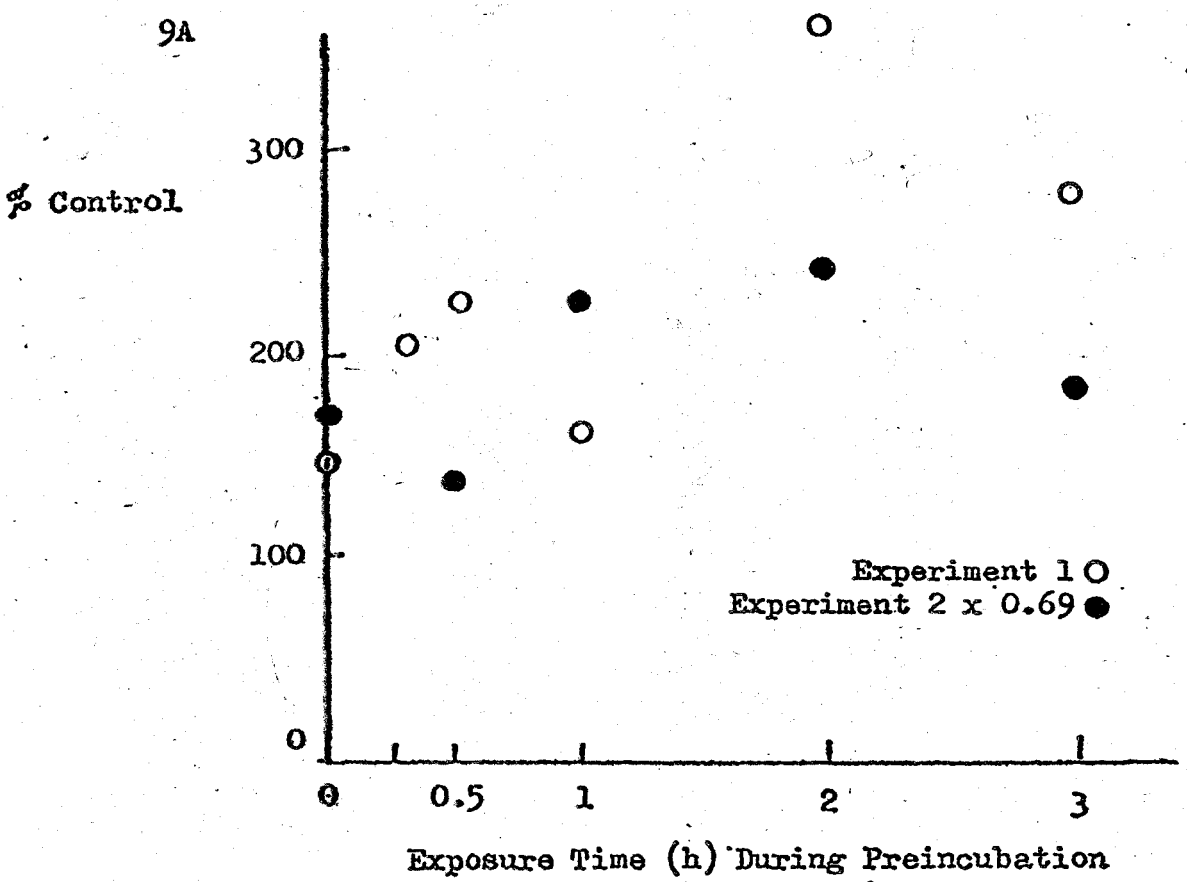


Figure 8: The Effect of Time of Addition of Cycloheximide on Glutamine Synthesis: Experiment 2.

9A



9B

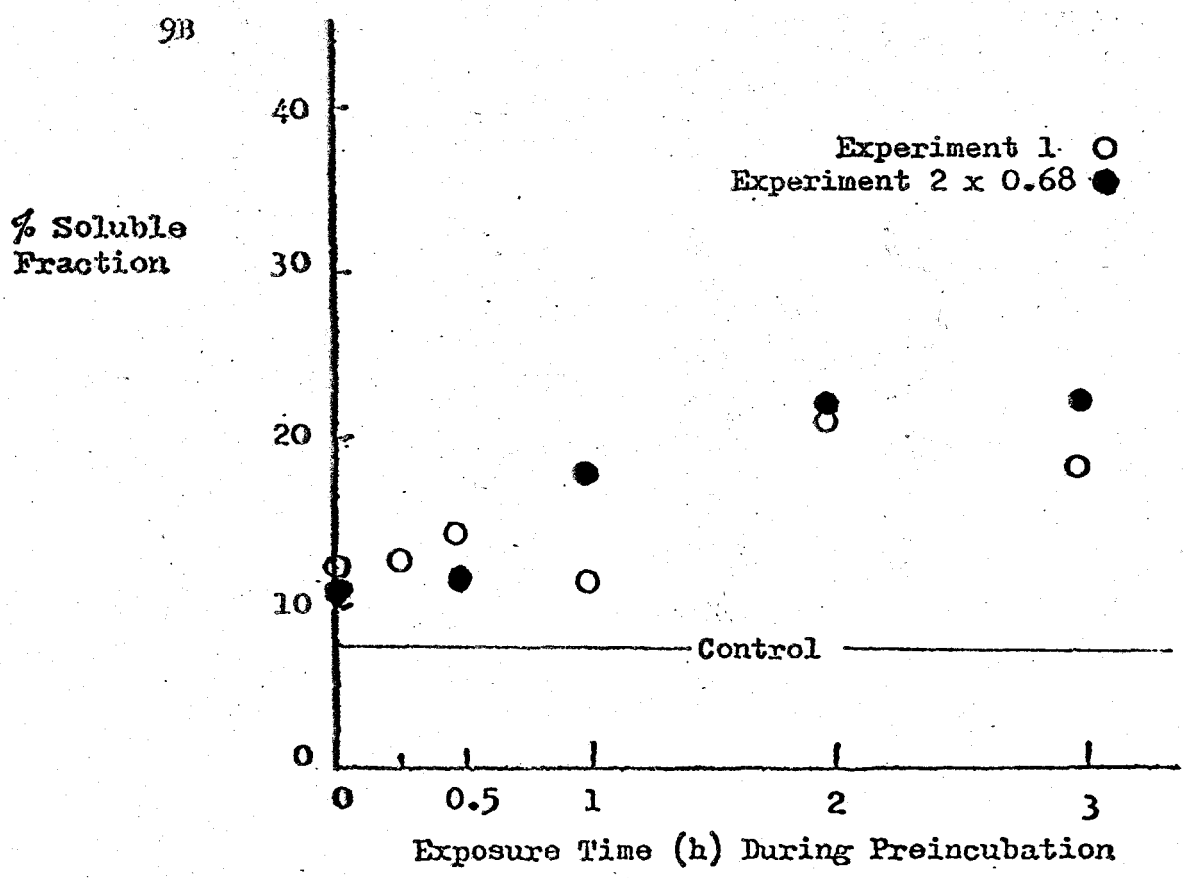
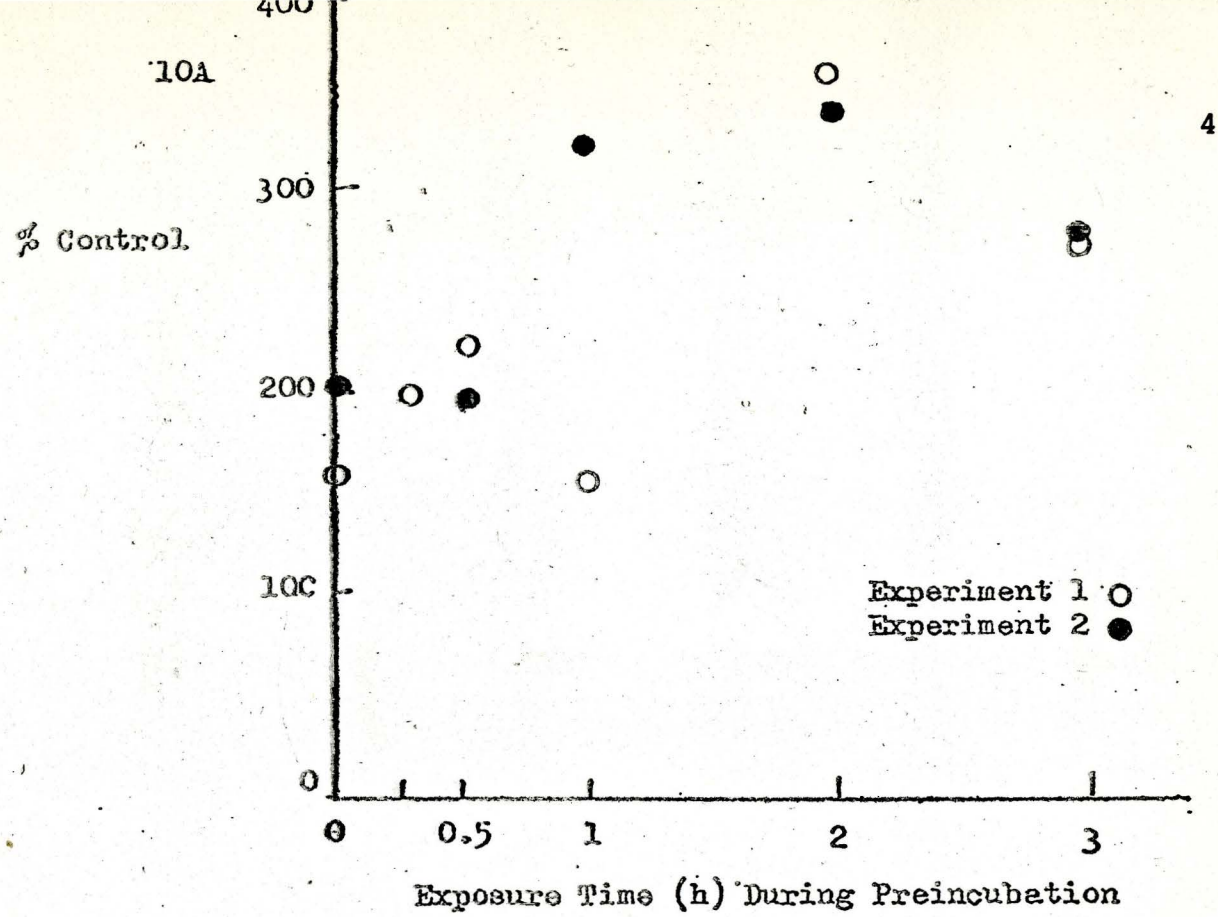


Figure 9: The Effect of Time of Addition of Cycloheximide on Glutamine Synthesis.

(Data from Figures 7 and 8 normalized with respect to controls.)



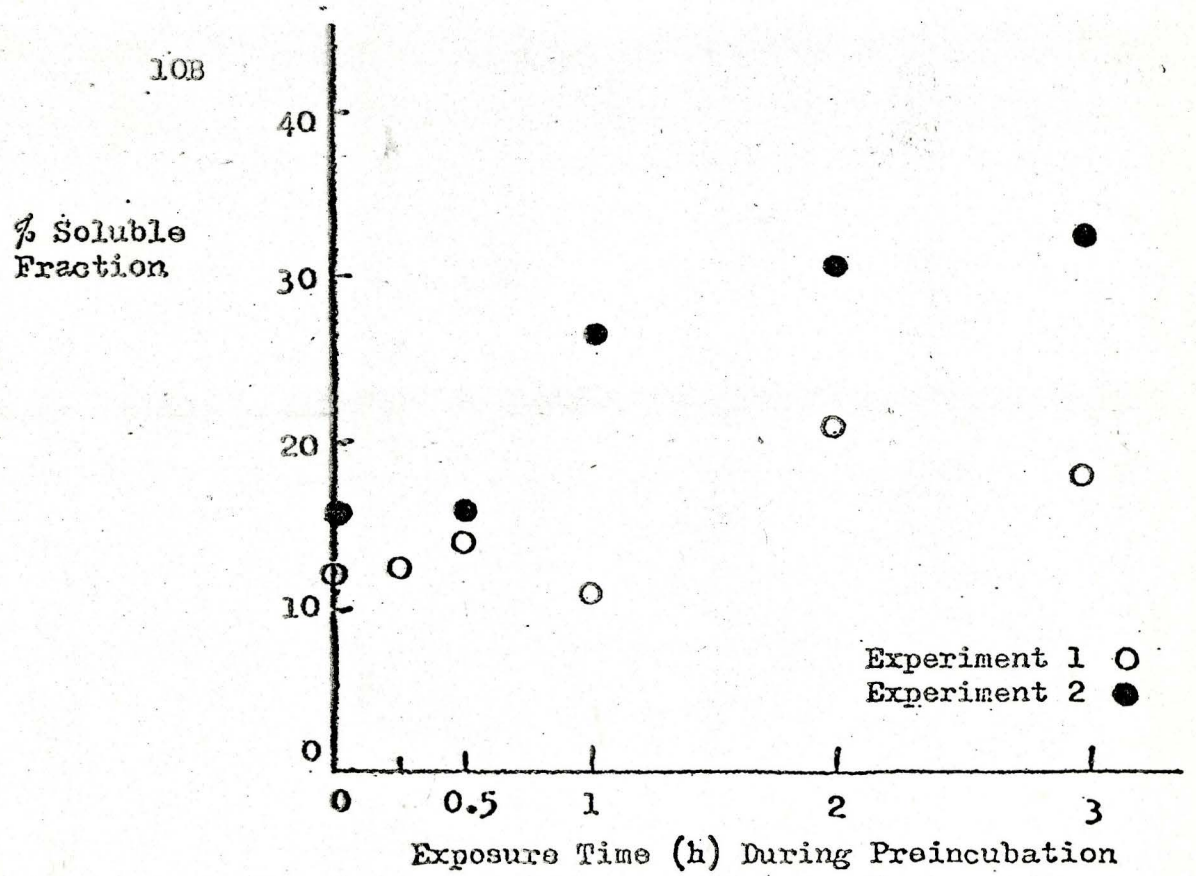


Figure 10: The Effect of Time of Addition of Cycloheximide on Glutamine Synthesis: Experiments 1 and 2.

c. Protein synthesis

The data for the amino acids recovered from protein in both experiments indicate that protein synthesis was inhibited to about 35% of the control values (Table V). This inhibition is found immediately upon addition of cycloheximide to the root-tip samples.

The remaining components of the soluble fraction were examined relative to the mean cpm of the controls as shown in Table V. The variability in the controls is approximated by the percent range value. The percent range is the percent which the mean deviates from the most extreme control value.

The distribution of acetate carbon into the fractions is similar to that found in the comparison of the control and three-hour cycloheximide treated samples. Essentially, the fractions which did not show statistically significant differences in the initial comparison also did not appear to be markedly different from control throughout the intervals used here. This is seen in the percent mean control values of the total soluble fraction, the total amino acid fraction, the glutamate fraction and the aspartate fraction which are in general within the range of values seen in the controls for these fractions. The organic acids and sugars fraction is less than the mean control value at three hours pre-treatment with

Table V

Effect of Time of Addition of Cycloheximide

		Mean cpm ($\times 10^{-3}$) = 100%	Range in control (%)	Data expressed as % mean control 0h	0.25h	0.5h	1h	2h	3h
Insoluble amino acids	1.	81.4	± 16	-	33.7	43.6	33.2	46.7	43.0
	2.	114.2	± 3	51.3	-	53.8	40.0	33.8	41.6
	average	97.8	± 20	51.3	33.7	48.7	36.6	40.3	42.3
Total soluble fraction	1.	2320.3	± 6	104.6	113.1	116.8	104.2	125.1	114.4
	2.	2270.0	± 27	138.6	-	132.1	133.5	122.9	90.8
	average	2295.2	± 28	121.6	113.1	124.6	118.8	124.0	102.6
Organic acids and sugars	1.	576.8	± 4	92.0	89.7	81.7	72.5	99.1	85.2
	2.	536.7	± 28	101.5	-	99.0	102.0	109.9	70.8
	average	556.8	± 31	96.8	89.7	90.4	87.3	104.5	78.0
Amino acids	1.	1660.8	± 11	115.6	114.7	126.5	108.8	128.3	121.1
	2.	1647.6	± 28	148.2	-	146.7	145.3	125.8	97.0
	average	1654.2	± 29	131.9	114.7	136.6	127.1	127.1	109.1
Neutral and basic amino acids	1.	34.2	± 14	178.4	181.5	230.4	558.8	262.6	274.3
	2.	33.1	± 18	576.1	-	79.2	154.1	235.6	123.9
	average	33.7	20	377.3	181.5	154.1	356.9	249.1	199.1
Glutamate	1.	941.7	± 5	96.6	74.8	117.3	93.1	97.4	89.9
	2.	938.8	±32	135.9	-	135.0	110.9	54.5	54.2
	average	940.3	±33	116.3	74.8	126.5	102.0	76.0	72.1
Aspartate	1.	82.3	± 2	75.7	43.2	100.0	71.4	73.6	82.5
	2.	62.2	±19	106.9	-	127.7	125.9	137.5	77.1
	average	72.3	±30	91.3	43.2	113.8	98.7	105.6	79.8
Glutamine	1.	173.2	±10	170.1	205.5	226.9	173.5	368.0	288.8
	2.	249.5	±34	203.8	-	199.2	328.9	352.0	271.5
	average	211.4	±34	187.0	205.5	213.1	251.2	360.0	280.2
Asparagine	1.	55.4	± 1	76.4	72.0	81.2	45.3	41.5	32.0
	2.	65.4	± 7	105.0	-	75.8	90.5	66.1	32.6
	average	60.4	±11	90.8	72.0	78.7	67.9	53.8	32.3

Root tip sections were pre-incubated in a salts media for 3 hours. At appropriate intervals during the pre-incubation, cycloheximide (1 µg/ml) was added. This was followed by a 2 hour experimental time with cycloheximide and [2-^{14}C]-acetate (5 µCi in 3 ml).

In the table, the time intervals refer to the period of exposure to the antibiotic prior to the experimental time.

cycloheximide. However, the effects at shorter pre-treatment times are quite variable and overlap into the control value range. Nevertheless, the neutral and basic amino acid fraction does increase well above the mean control value. This increase is in agreement with the statistical comparison. With the exception of only one value at 1/2 hour pre-treatment, all the percent mean control values are above the control neutral and basic amino acid levels. The increase when averaged between the experiments is greater than 50% above the controls. The neutral and basic amino acid fraction thus increases immediately after addition of cycloheximide to the samples.

3. Effect of Time of Addition of Azaserine

a. Experimental design

The standard assay was modified as previously stated for the cycloheximide timing experiments. Excised root-tip sections were placed in the pre-incubation salt solution for three hours. At appropriate time intervals prior to [2-^{14}C]-acetate addition, the azaserine (final concentration = 10^{-4} M) was also added to the pre-incubation media. The azaserine was present later during the routine two hour labelling period. Time intervals stated in the data tables thus refer to the duration of exposure to the azaserine prior to [2-^{14}C]-acetate feeding.

b. General description of results

Azaserine affects the distribution of acetate carbon into the other components of the soluble fraction as shown in Table VI. Overall, the effects produced are fairly constant after 1/2 hour pre-treatment with azaserine. The total soluble fraction decreases to about 85% of the mean control after 1/2 hour. This decrease originates within the amino acid fraction which is about 75% of the control. Organic acids and sugars increase by about 30% but since they are not the major component of the soluble fraction, this increase is not reflected in the total soluble fraction. Within the amino acid fractions, the neutral and basic amino acids decrease by about 30%, glutamate levels remain fairly constant and aspartate levels in general increase by 30% when azaserine is added to the root-tip samples. Marked changes in the distribution of ^{14}C-label are noted within the amide fraction. Glutamine formation is increased and asparagine formation is decreased in the presence of azaserine.

The data from the insoluble fraction indicates that there does not appear to be a major decline in protein synthesis with azaserine present in the pre-incubation media. After 1/2 hour, the percent mean control values of the total insoluble fraction decline by about 20%. The protein amino acids although consistently decreased below the mean control value, are

Table VI

Effect of Time of Addition of Azaserine

	Mean cpm ($\times 10^{-3}$) = 100%	Range in control (%)	Data expressed as % mean control				
			0h	0.5h	1h	2h	3h
Insoluble fraction total	245.3	±14	39.0	82.6	81.9	82.4	80.7
AA	139.4	±21	54.9	90.2	80.5	96.0	95.3
Total soluble fraction	2936.6	± 4	95.6	81.2	82.6	85.9	85.4
OAS	498.4	± 3	182.0	137.5	141.3	135.5	130.8
AA	2183.0	± 2	78.1	64.8	64.9	72.7	68.8
NB	75.1	±12	99.1	61.1	65.4	69.6	63.6
Glu	504.9	±21	118.9	111.4	98.2	80.9	98.8
Asp	50.7	± 6	131.6	170.8	132.9	133.9	73.8
Gln	258.0	± 5	186.4	199.2	192.6	195.0	187.6
Asn	57.6	± 8	45.5	60.6	41.0	47.0	54.3

Experimental design is described in Table V.
Azaserine (10^{-4} M) was added to the pre-incubation salts media at the above times prior to the experimental period.

Abbreviations:

OAS = organic salts and sugars

AA = amino acids

NB = neutral and basic amino acids

Glu = glutamate

Asp = aspartate

Gln = glutamine

Asn = asparagine

not greatly reduced. In the presence of azaserine, protein synthesis is reduced about 10% after 1/2 hour.

4. Effect of Azaserine on:

a. Asparagine formation

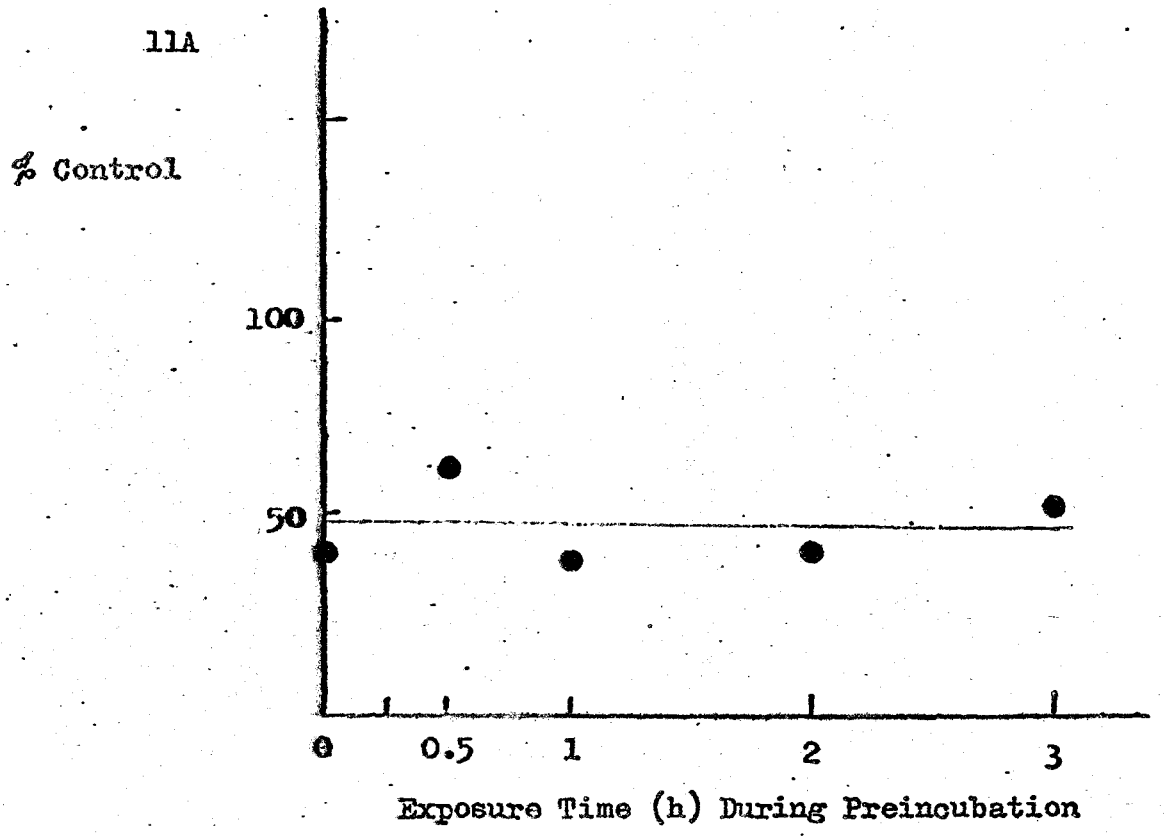
The effect of azaserine on asparagine synthesis is shown in Figures 10A and 11B. When the cpm in asparagine is expressed as a percentage of the control (11A) the data indicates that asparagine formation is inhibited immediately at 0 hours pre-incubation. This inhibition remains constant with increasing exposure to the glutamine analogue. Asparagine formation is inhibited by 10^{-4} M azaserine to about 50% of the control throughout the three hour pre-incubation period.

More scatter is noted in the data when asparagine formed is expressed as a percent of the soluble fraction (Figure 11B). There is a general consistency in the percent control and percent soluble fraction comparison in that both indicate a decrease in asparagine formed of about 50% that of the controls.

b. Glutamine formation

Azaserine has an immediate effect on the glutamine formed in root-tip sections. When expressed as a percent of the control the data indicates that glutamine has

11A



11B

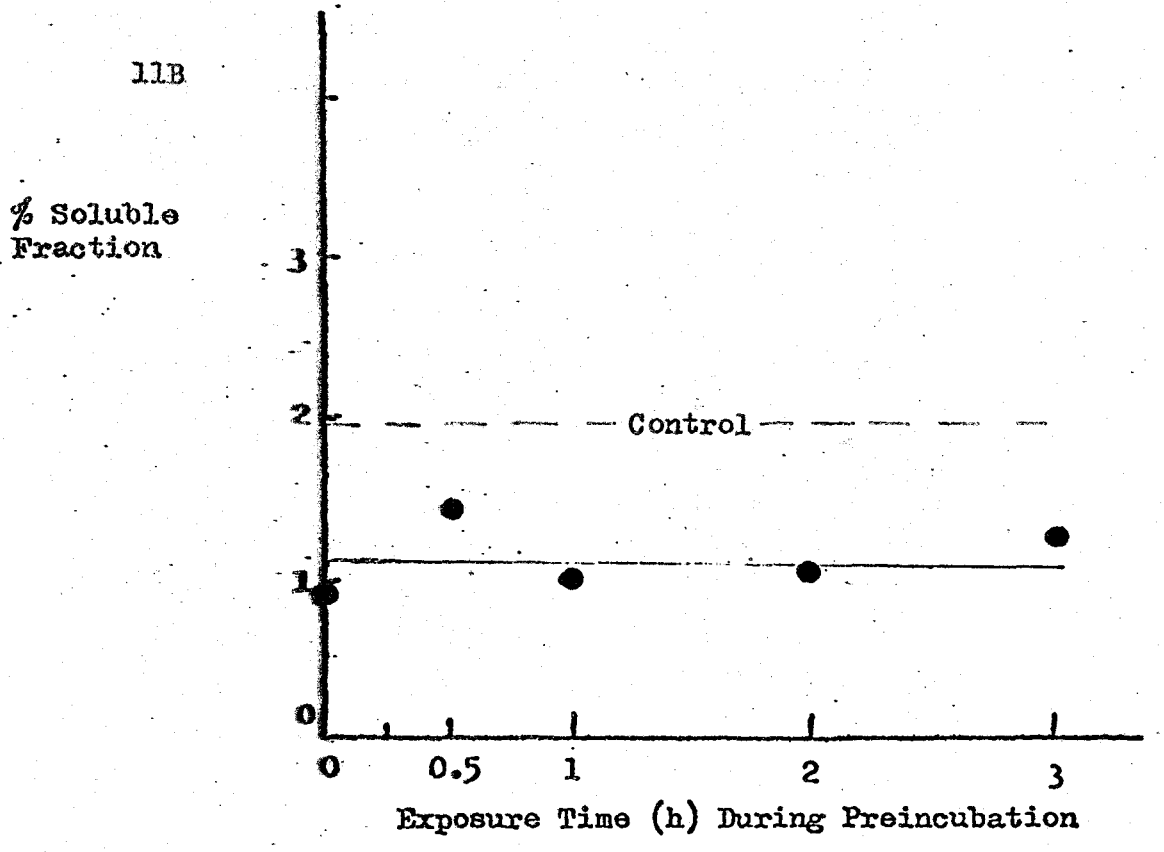
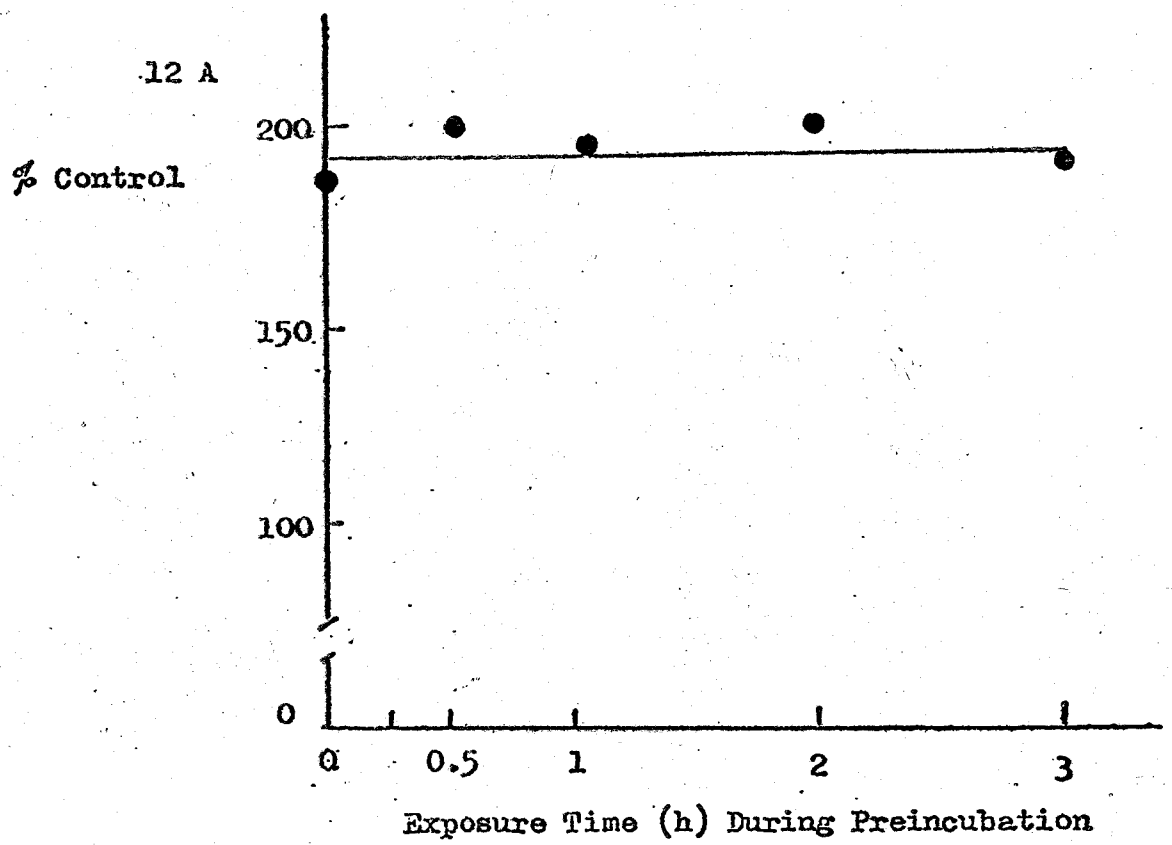


Figure 11: The Effect of Time of Addition of Azaserine on Asparagine Synthesis.

12 A



12B

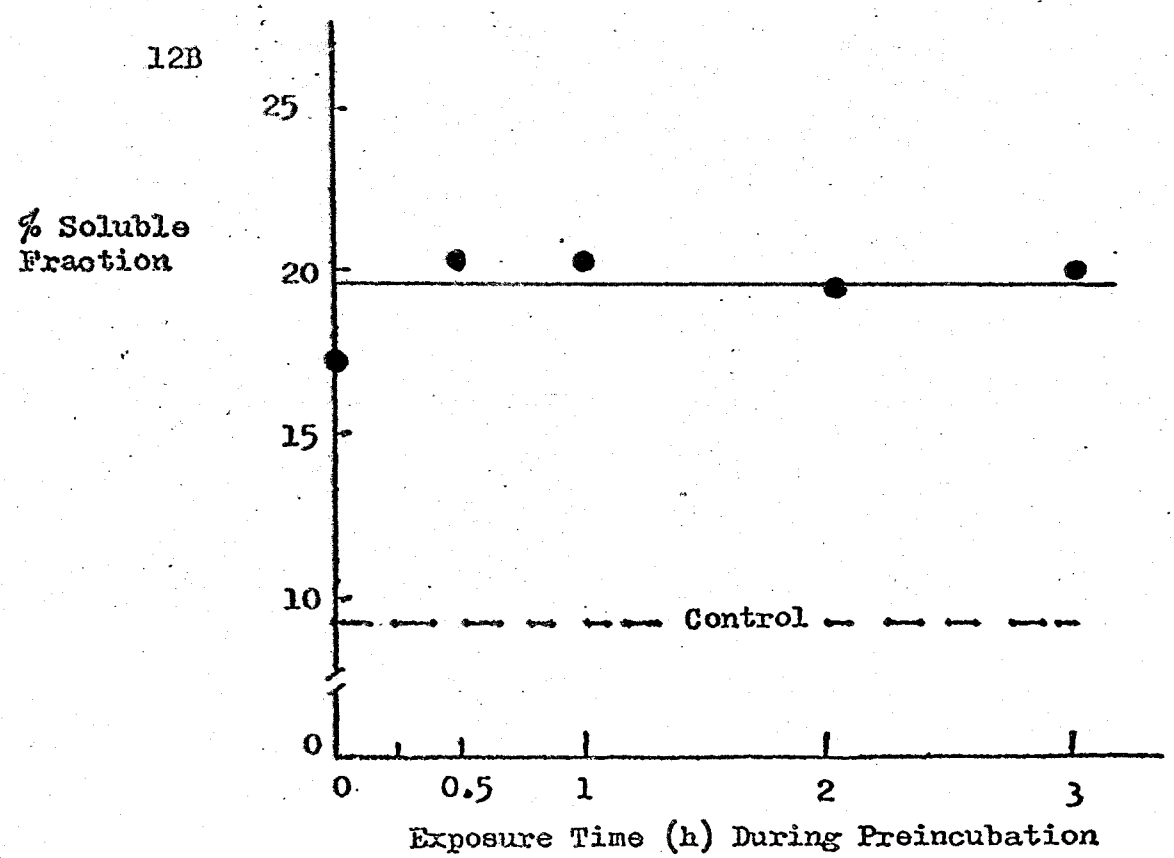


Figure 12: The Effect of Time of Addition of Azaserine on Glutamine Synthesis.

doubled immediately and consistently during the 0 to three hour pre-incubation period (Figure 12A). The glutamine formed represents about 190% that of the control.

The glutamine formed in the presence of azaserine also represents about 20% of the total soluble fraction (Figure 12B). This is an 11% increase above the control values which are about 9% of the soluble fraction.

c. Protein synthesis

Azaserine does not appear to significantly affect the incorporation of amino acids into protein as shown in Table VI. With the exception of the 0 hour data, which may be accounted for by control variability, protein synthesis is about 90% that of the control from one-half hour to three hours pre-incubation with azaserine.

5. General Comparison of Timing Experiments

Azaserine produces similar effects on the amide amino acids in that the effects appear to be immediate and constant for all pre-incubation times. Asparagine declines by about one-half while glutamine levels increase. The effects of cylcoheximide on asparagine and glutamine formation are similar in that both effects appear to gradually increase with increasing exposure to cycloheximide. The trend for glutamine is to

gradually increase concomitant with a gradual decrease in asparagine formation. It is possible that protein synthesis inhibition by cycloheximide may affect the general use of glutamine in the cells of the corn root-tip and as such may account for the scatter in the data.

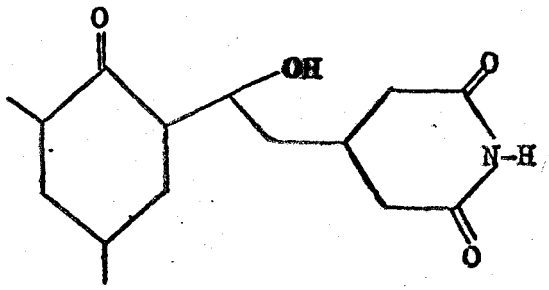
C. Analogue Studies

The analogues of cycloheximide were tested using the standard method. Excised root-tip sections were pre-incubated in the salt solution with 1.8×10^{-5} M (final concentration) of the appropriate analogue for three hours. The sections were then transferred to fresh media which contained the analogue and [2-^{14}C]-acetate in addition to the additives of the pre-incubation medium. The experimental time was routinely two hours. Thus, the samples were exposed to the analogues for a total of five hours.

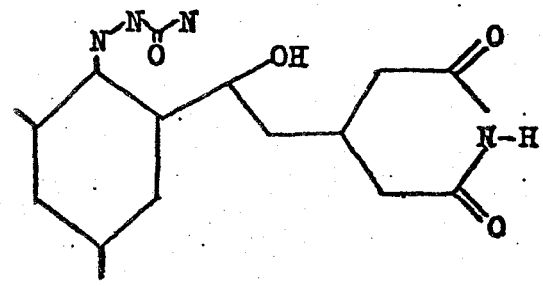
1. Analogue concentration

The analogues of cycloheximide shown in Figure 13 were maintained at concentrations equal to five times that used in the cycloheximide treated samples. Concentrations of this level were chosen in order to see clear inhibitions if that should be the case. Should

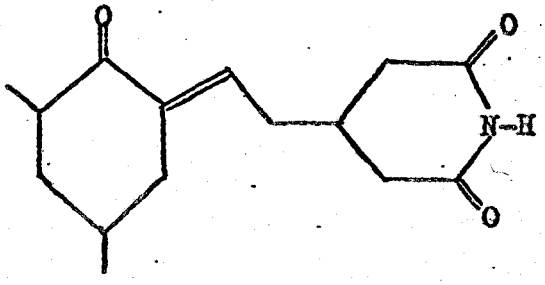
Cycloheximide



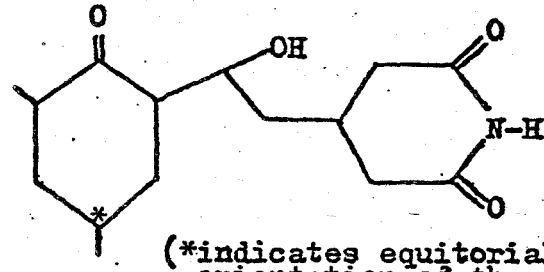
Cycloheximide Semicarbizone



Anhydrocycloheximide

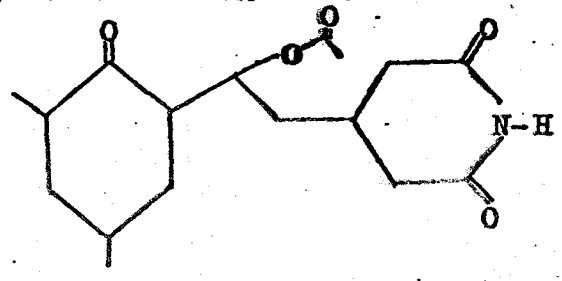


Isocycloheximide

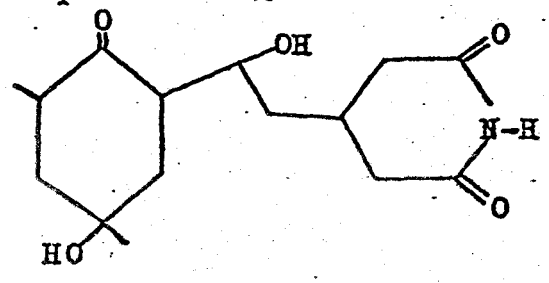


(*indicates equitorial orientation of the methyl group)

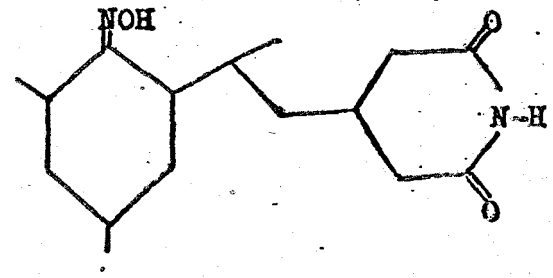
Cycloheximide Acetate



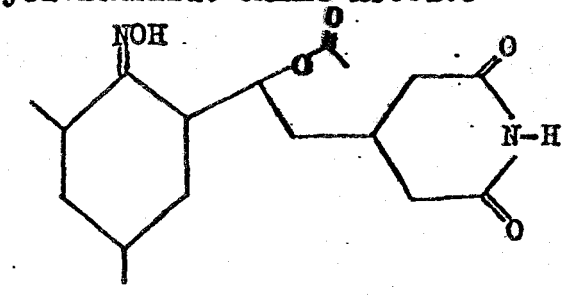
Streptovitacin A



Cycloheximide Oxime



Cycloheximide Oxime Acetate



Chdm (cis-4-Cyclohexene-1,2-dicarboximide)

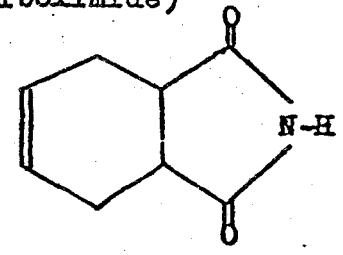


Figure 13: The Chemical Structure of Cycloheximide and the Analogues of Cycloheximide.

fractions were variable during 0 to three hours exposure. This did not appear to deviate greatly from that shown in the controls for this fraction. Similarly, the effect on respiration is not clearly distinguishable from the controls in the timing experiments. Only the three criteria mentioned above and the immediate effect of increasing the neutral and basic fraction show clear trends in the timing experiments. Because of this finding, only the three criteria are considered in detail in the analogue experiments.

3. Results of analogue experiments

The data from the investigation of asparagine synthesis in root-tip sections of corn using analogues of cycloheximide are summarized in Table VII and Table VIII. The following is a general description of the effects of the analogues which have been separated into two groups according to their effects on asparagine synthesis: Group I analogues of cycloheximide are those analogues without a significant inhibitory effect on asparagine synthesis. Group II analogues are those exhibiting an inhibitory effect on asparagine synthesis.

The Group I analogues (Table VII) comprise anhydrocycloheximide, cycloheximide oxime, cycloheximide oxime acetate, cycloheximide semicarbizone, isocycloheximide and chdm (cis-4-cyclohexene-1,2-dicarboximide).

The Group II analogues are cycloheximide acetate and streptovitacin A. The results for these two analogues are presented in Table VIII. This group produces effects similar to that found for the cycloheximide effect in corn root-tips; an inhibition of protein and asparagine synthesis and enhanced glutamine levels.

The data for cycloheximide treated root-tip samples has been presented in both tables to facilitate comparison of the analogue data.

Group I analogues as shown in Table VII have no appreciable effect on asparagine synthesis, glutamine synthesis or protein synthesis. In this group, mean percent control values range from 89.0 to 102.6% for asparagine; 83.4 to 128.3% for glutamine; and 89.9 to 112.1% for protein synthesis. Mean percent control values for cycloheximide show clear differences from the Group I analogues. With cycloheximide present, asparagine is reduced to 37.7%, glutamine is increased to 200.4%, and protein synthesis reduced to 45.2%. Similarly, the mean percent soluble fraction data also reflects that these analogues do not greatly change the distribution of acetate carbon in these fractions. Group I analogues produce mean percent soluble fraction values ranging from 1.9 to 2.6% for asparagine, and 5.7 to 9.5% for glutamine. Cycloheximide reduces the mean percent soluble fraction of asparagine to 0.8% and increases glutamine to 14.6%.

Table VII

Effect of Group I Analogues of Cycloheximide on Protein, Asparagine and Glutamine Synthesis

Mean ± SE

	CH	Anhydro-CH	CH oxime	CH oxime acetate	CH semicarbizone	Iso-CH	Chdm
Protein synthesis							
cpm x 10^{-3}	48.5±7.8	137.9±1.9	171.3±30.7	167.5±19.6	129.7±13.9	140.0±1.6	157.1±31.9
% control	45.2±6.5	109.7±10.6	100.2±12.1	94.9±10.7	89.8± 8.4	96.3±3.8	112.1±11.6
Asparagine							
cpm x 10^{-3}	15.9±2.3	52.8±13.4	46.2±13.1	54.1±13.5	56.2± 8.3	57.6±12.9	62.6±24.9
% control	37.7±4.6	102.7±23.9	90.9± 3.4	93.6±12.5	88.8± 6.7	89.1± 7.5	107.9± 7.6
% soluble fraction	0.8±0.1	1.9± 0.5	1.9± 0.4	2.2± 0.1	2.5± 0.2	2.4± 0.3	2.6± 0.3
Glutamine							
cpm x 10^{-3}	312.1±95.5	149.4±46.7	199.8±48.5	154.1±41.5	179.6±27.2	164.1±18.8	159.5±37.5
% control	200.4±26.3	128.3±15.6	123.4± 6.6	125.6±39.1	96.8±10.4	83.4± 7.4	86.3±12.0
% soluble fraction	14.6± 4.2	5.9± 1.6	9.5± 0.4	6.2± 1.7	7.6± 0.8	7.0± 0.2	6.8± 0.6

Chdm = cis-4-cyclohexene-1,2-dicarboximide.

The experimental design is described in the Materials and Methods. Root-tip sections were pre-incubated with the analogues in a salts media for 3 hours. This was followed by a 2 hour experimental time with the analogue and [2-^{14}C]-acetate (5 µCi in 3 ml). The cycloheximide concentration was 1 µg/ml or 3.6 x 10^{-6} M and the analogue concentrations were 1.8 x 10^{-5} M.

$$\% \text{ control} = \frac{\text{cpm treated}}{\text{cpm control}} \times 100\%$$

$$\% \text{ soluble fraction} = \frac{\text{cpm treated fraction}}{\text{cpm total soluble fraction (treated)}} \times 100\%.$$

These data indicate that cycloheximide treatment produces effects which differ greatly from the Group I analogues. The distribution of label from [2-^{14}C]-acetate into asparagine and glutamine fraction indicates that the Group I analogues do not appear to change the distribution from that found in the controls.

The Group II analogues (Table VIII) are similar to cycloheximide using the three criteria. Asparagine synthesis is reduced to a mean value of 45.0% of the control for cycloheximide acetate and 42.1% for streptovitacin A. The mean percent soluble fraction values are 0.9% and 0.8%, respectively. Mean percent control values for glutamine increase to 485.5% for cycloheximide acetate and 279.1% for streptovitacin A. These glutamine values represent mean percent soluble fraction values of 29.9% for cycloheximide acetate and 18.7% for streptovitacin A. Protein synthesis is also reduced by both analogues in this group. Cycloheximide acetate reduces protein synthesis to a mean percent control value of 39.8% whereas streptovitacin A reduces protein synthesis to 57.6% of the control.

As shown in Table IX, there are differences between the Group I and the Group II analogues in other components of the soluble fraction. On the average, the Group II analogues caused decreases in the organic acids and sugars fraction and increases in the neutral and basic

Table VIII

Effect of Group II Analogues on Protein, Asparagine and Glutamine Synthesis

Mean ± SE

	Cycloheximide	Cycloheximide acetate	Streptovitacin A
Protein synthesis			
cpm x 10^{-3}	48.47 ± 7.81	46.27 ± 5.17	103.15 ± 2.49
% control	45.21 ± 6.48	39.76 ± 9.21	57.63 ± 11.98
Asparagine			
cpm x 10^{-3}	15.96 ± 2.30	17.90 ± 3.33	22.89 ± 1.82
% control	37.70 ± 4.62	45.00 ± 4.89	42.17 ± 10.18
% soluble fraction	0.80 ± 0.09	0.85 ± 0.16	0.75 ± 0.05
Glutamine			
cpm x 10^{-3}	312.06 ± 95.51	631.53 ± 104.4	567.1 ± 31.95
% control	200.36 ± 26.33	485.53 ± 38.38	279.10 ± 3.87
% soluble fraction	14.59 ± 4.22	29.87 ± 4.72	18.74 ± 1.24

Experimental design is described in Table VII.

Table IX

Effect of Analogues of Cycloheximide in Root-tip Sections of Corn

	Average Percent Control								
	Group I Analogues							Group II Analogues	
	CH	Anhydro-CH	CH oxime	CH oxime acetate	CH semi-carbizone	Iso-CH	Chdm	CH acetate	Strep. A
Total soluble fraction	102.6	107.8	120.1	99.6	99.0	102.9	111.2	114.0	122.2
OAS	78.0	109.7	126.1	108.9	102.1	119.0	111.0	87.4	95.5
AA	109.1	95.3	123.6	90.4	99.4	93.8	111.6	126.2	126.1
NB	199.1	139.7	107.5	94.4	97.5	106.6	128.4	203.9	187.3
Glu	72.1	97.3	107.4	89.9	102.2	87.2	123.1	74.2	98.3
Asp	79.8	191.7	77.8	90.8	100.0	109.9	90.8	158.4	63.0
Gln	200.4	128.3	123.4	125.6	96.8	83.4	86.3	495.5	279.1
Asn	37.7	102.7	90.9	93.6	88.8	89.1	107.9	45.0	42.2

The experimental design is described in Table VII

Abbreviations:

CH = cycloheximide

Chdm = cis-4-cyclohexene-1,2-dicarboximide

OAS = organic acids and sugars

AA = amino acids

NB = neutral and basic amino acids

Glu = glutamate

Asp = aspartate

Gln = glutamine

asn = asparagine

amino acid fraction when compared to the Group I analogues. These trends shown by the Group II analogues are also found in the same soluble fraction components after cycloheximide treatment. The analysis of the neutral and basic fraction performed by Oaks and Johnson was not carried out (37).

D. Results of the *In Vitro* Assay for Asparagine Synthetase

Preliminary experiments with corn extracts suggested that asparagine was not the product of the assay when extraction and assay conditions were similar to those described by Streeter (24). Accordingly, experiments using soybean were undertaken to examine asparagine synthetase from a known source.

The results in Table X where the enzyme preparation was passed over a Sephadex G-100 (fine) column show that the activity of asparagine synthetase from soybean cotyledons was significantly inhibited by the omission of either of the substrates, glutamine or ATP, and was inhibited by the absence of $MgCl_2$. These results are essentially similar to those reported by Streeter.

Similar enzyme extracts passed over Sephadex G-25 (coarse) did not have the expected appropriate substrate requirements. Inadequate separation of small substrate molecules from the larger protein molecules was probably

Table X

Assay of Asparagine Synthetase from Soybean Cotyledons

cpm x 10^{-3}/h/1.5 mg protein

	Streeter's data	Sephadex G-100		Sephadex G-25	
	% complete	cpm above boiled enzyme*	% complete	cpm above boiled enzyme*	% complete
Complete	20.3=100%	10.7	100	36.8	100
-glutamine	7	0.3	3	13.0	35
+ 50 mM NH_4Cl	31	2.4	22	13.7	37
-ATP	8	1.2	11	9.4	26
-$MgCl_2$	3	0.9	8	5.3	14
+ 10 mM cycloheximide	-	9.3	87	37.7	102
+ 20 mM asparagine	-	7.2	67	36.9	100

* boiled enzyme cpm = 3.9.

10^{-3} M azaserine produced a precipitate in the reaction mixture. Hence, the results obtained with this antibiotic are unreliable.

the reason for this descrepancy.

The G-25 filtered soybean extract was found to have more activity than that found by Streeter. The G-100 filtered soybean extract had about one-half the activity of the Streeter preparation and the omission of the substrates had similar inhibitory effects on the enzyme activity when compared to Streeter's data. In particular, marked inhibition (97%) was found when glutamine was omitted from the assay mixture. Fifty millimolar NH_4Cl was found to restore about 22% of the complete activity.

Chromatographic analysis of the G-100 reaction product indicated that the compound produced, behaved as expected for asparagine. After separation with the solvents: phenol solvent, phenol:ammonia and butanol: acetic acid: water, the radioactivity was found in areas similar to that shown by asparagine (Figure 14). After mild acid hydrolysis (2 N HCl at 100^{o}C for 4 hours), the assay product was also found to shift its location to that of aspartate (Figure 14). From these tests, it is concluded that the product formed in the assay of the G-100 filtered extract was asparagine.

Examination of the soybean asparagine synthetase activity after addition of cycloheximide or asparagine to the assay mixture indicated that neither compound had any affect on the G-25 filtered extract (Table X). Slight

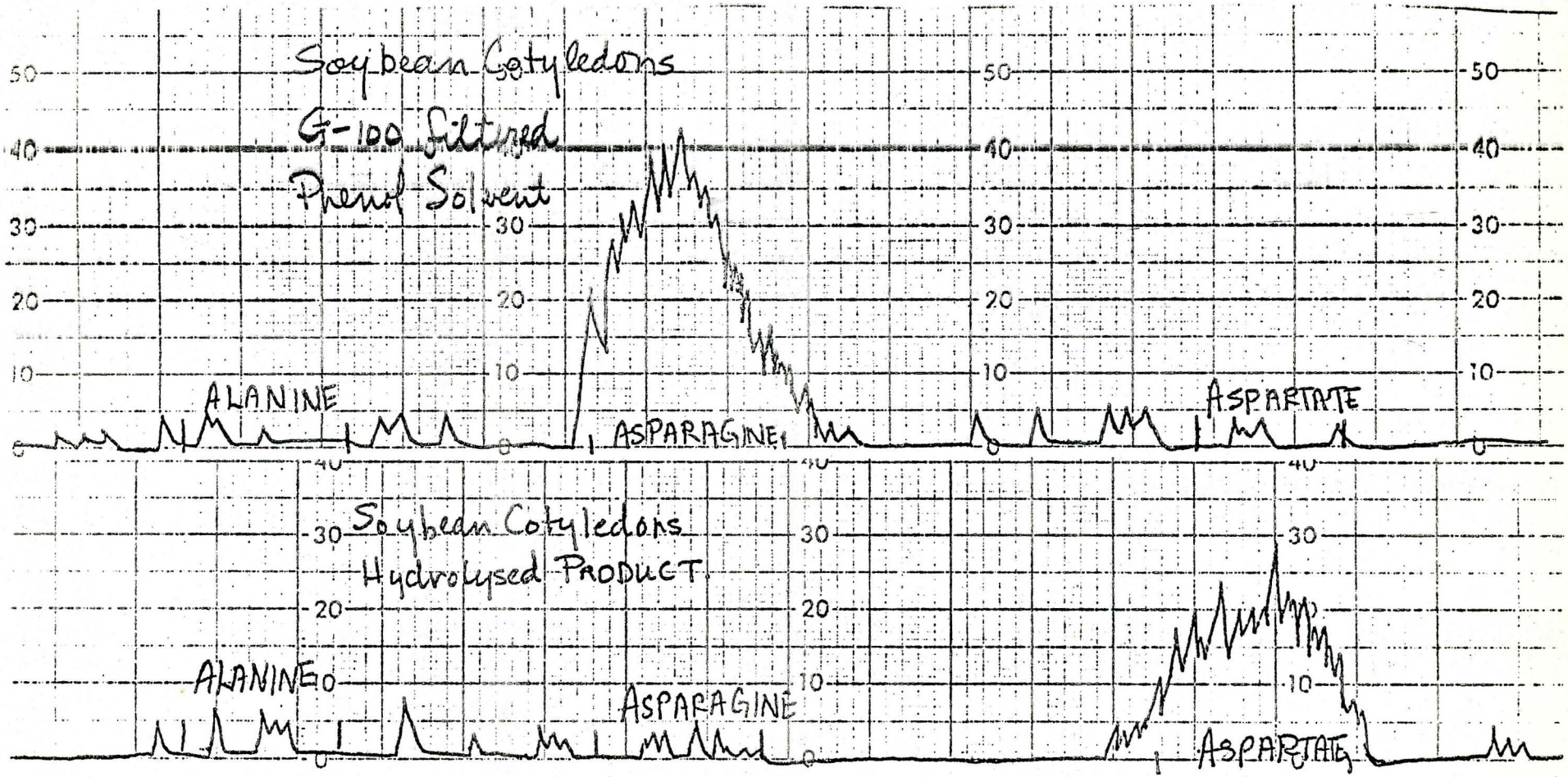


Figure 14: Strip Counter Tracings of the Product and Hydrolysed Product from the asparagine Synthetase Assay of Sephadex G-100 filtered extracts of Soybean Cotyledons.

In the top tracing, the radioactive product formed in the soybean asparagine synthetase assay was found to chromatograph at the asparagine position. After mild acid hydrolysis, this radioactive product chromatographed at the aspartate position indicating that the original product was asparagine. Using the butanol:acetic acid water solvent the radioactive product also moved with asparagine.

Table XI

Assay for Asparagine Synthetase in Corn Seedling Tissues

CPM x 10^{-3} above boiled enzyme

	Scutellum	Root	Shoot	5 mm root-tips pre-incubated in Hoagland's + 10 mM $(NH_4)_2SO$	
				0h	6h
Complete	11.3	3.4	4.7	8.9	9.5
-Glutamine	9.0	7.1	5.4	6.6	20.6
+ 50 mM NH_4Cl	6.9	7.5	5.2	7.7	8.5
-ATP	4.7	9.0	7.5	7.1	5.4
-$MgCl_2$	8.1	7.4	7.4	6.6	5.3
+ 10 mM cycloheximide	15.9	9.8	7.4	7.2	7.3
+ 10^{-4} azaserine	16.5	10.2	7.1	7.1	7.3
+ 20 mM asparagine	14.7	9.3	6.4	9.5	6.6
Boiled enzyme	2.3	1.4	1.6	1.6	1.6

The standard reaction mixture containing [UL-^{14}C]-aspartate (approximately 10^6 cpm) was incubated at 30°C for 60 minutes. Enzyme activity was determined by measuring the conversion of [UL-^{14}C]-aspartate as indicated by the amount of radioactivity which was not retained by Dowex-1-acetate columns.

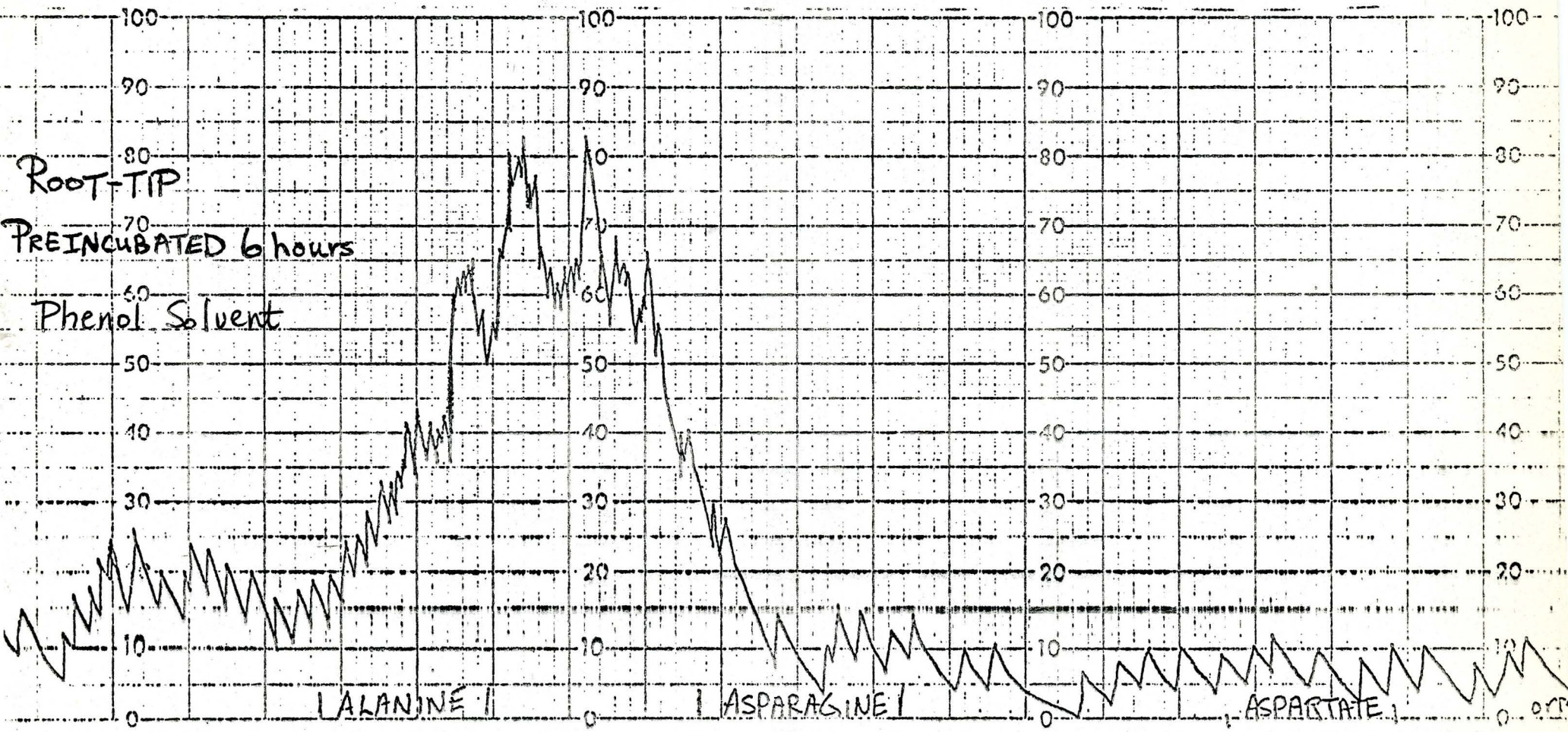


Figure 15: Strip Counter Tracing of the Assay Products formed in the Asparagine Synthetase Assay of Root-tip Sections of Corn Pre-incubated for 6 Hours.

The radioactive products formed in the assay of root-tip extracts were found to chromatograph over a broad region. Most of the radioactivity was located in positions between alanine and asparagine.

reductions in activity were found with cycloheximide in the G-100 treated extracts. High asparagine concentrations (20 mM) were found to reduce the activity by about one-third.

Assays for asparagine synthetase activity in corn seedling tissues were routinely carried out using Streeter's assay method modified by the use of Sephadex G-100 to filter the crude extracts. As shown in Table the cpm data obtained from the assay of scutellum, root and shoot extracts were much lower than similar reactions with soybean cotyledons. Pre-incubation of root-tip sections for 6 hours in 1/10 Hoagland's salts + 10 mM $(NH_4)_2SO_4$ did not significantly increase the cpm in the complete mixture. In contrast to the results with soybean, consistent inhibition of activity was not observed when the substrates were omitted.

Chromatographic analysis of the assay products from the corn seedling tissues was carried out using phenol solvent separation. As shown in Figure 15, the radioactivity from the assay of the 6 hour pre-incubated root-tips was principally located in an area between alanine and asparagine. The spread of the radioactive area indicates that several products perhaps including alanine and β-alanine were formed during the assay. Assays of extracts from shoots and scutellum were found to have more radioactivity in the area expected for

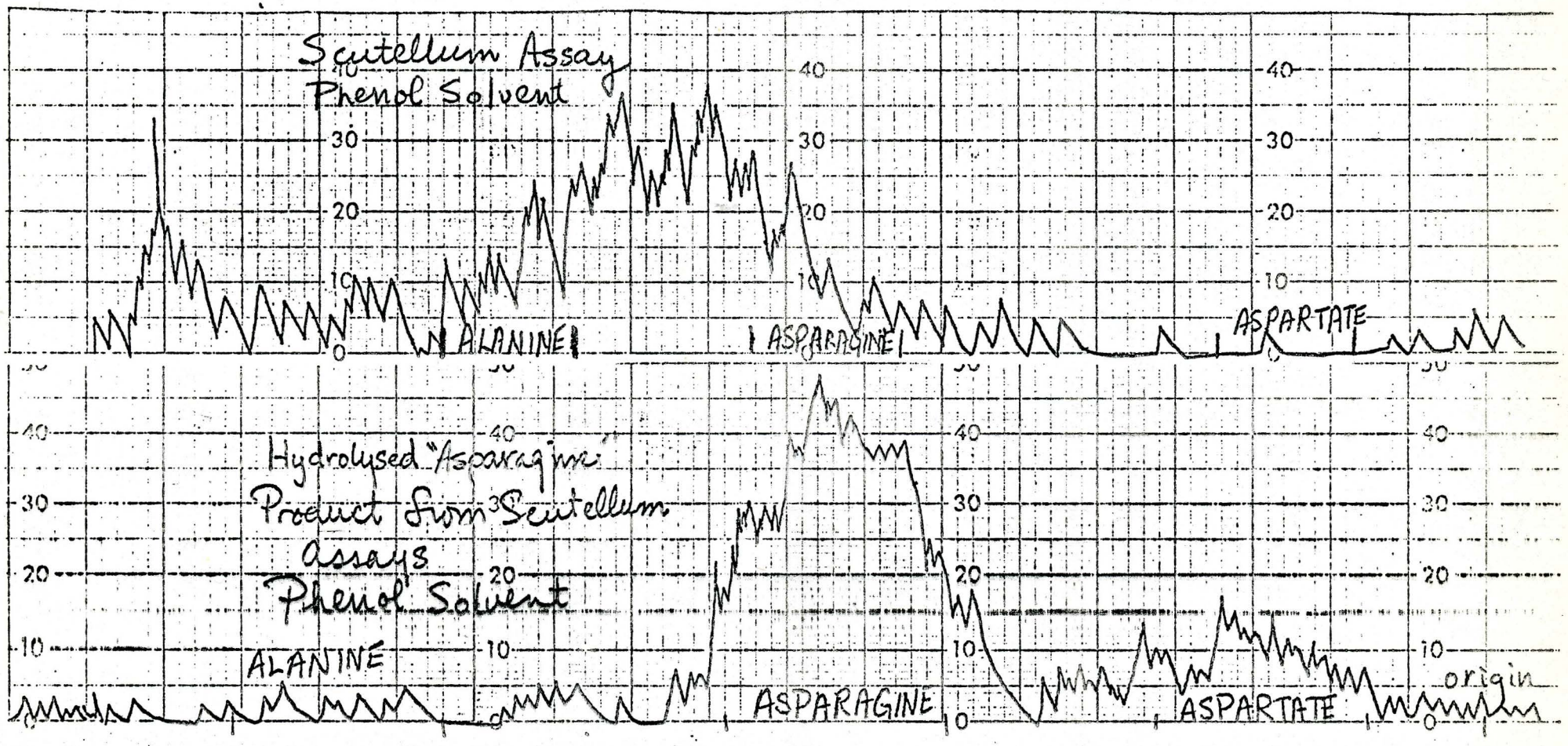


Figure 16: Strip Counter Tracings of the Assay Products Formed in the Asparagine Synthetase Assay of Extracts from Corn Scutella.

In the top tracing, the radioactive products formed in the assay for asparagine synthetase in scutella extracts were found to chromatograph over a broad region. Some of the radioactivity was located at the asparagine position. After mild acid hydrolysis, the radioactive products found in the asparagine position formed two peaks. A small amount of the hydrolyzed products were found to chromatograph at the aspartate position.

asparagine (Figure 16). Closer examination of this area was undertaken by eluting the radioactivity from these areas and subjecting the eluate to mild acid hydrolysis. Chromatographic analysis of this hydrolysate (Figure 16) indicated that the major portion of this fraction still chromatographed in the asparagine location. Some of the activity, however, was found in the aspartate position as expected after mild hydrolysis of asparagine.

By this analysis, asparagine was shown to represent only a minor component of the total radioactivity of the assays. It also appears that extracts derived from corn tissue contain many active enzymes capable of utilizing [^{14}C]-aspartate when assayed by Streeter's method. The additional activity of the other enzymes probably accounts for the confusing data obtained in the substrate omission assays.

DISCUSSION

In aged leaves (7) and in senescing cotyledons (24) increases in asparagine synthesis appear to be coincident with a reduction in the rate of protein synthesis. A similar situation occurs in corn seedling roots. Intact root-tips of corn do not synthesize asparagine from acetate carbon in detectable amounts. Under these conditions, other seedling tissues like the scutellum or mature regions of the root synthesize asparagine. This asparagine is transported to the growing root-tip and hence can supply the asparagine required for protein synthesis (10). Initially, after excision from the mature root tissue, root-tip sections show low levels of asparagine synthesis. However, upon aging in a salts medium,asparagine formation increases with time for at least six hours (9). After excision, there is no longer a measurable increase in protein in the root-tip which suggests that turnover of protein in this section increases when sources of nutrients are cut off.

Comparisons made between root-tip and mature sections of the corn root indicate that the regulation of asparagine is modified during the development of root cells. Various treatments can result in decreased

asparagine synthesis in the root-tip sections. Glucose, an important metabolite transported to the root-tip sections, has been shown to reduce the incorporation of acetate carbon into asparagine (9). A three hour pre-treatment with 1 μg/ml cycloheximide, a potent protein synthesis inhibitor in corn roots, inhibits asparagine formation in the root-tips. Similar exposure of these sections to 10^{-4} M azaserine, a glutamine analogue, also reduces asparagine synthesis (1). In excised mature root sections additions of glucose (18), cycloheximide or azaserine do not inhibit asparagine formation (1). Cycloheximide does enter the cells since protein synthesis is inhibited. These changes in asparagine metabolism noted between meristematic and differentiated regions probably reflect alterations in the regulatory properties of the cells.

Examination of the response to time of addition of cycloheximide shows that the effects on amide synthesis occur at different times in the root-tip samples. Protein synthesis is inhibited within minutes of addition of the antibiotic. Glutamine levels rise gradually with increased exposure and the inhibition of asparagine formation increases with increasing time of exposure to cycloheximide.

Azaserine, an irreversible inhibitor of glutamine dependent reactions, exhibits different effects from those produced by cycloheximide. While protein synthesis is not markedly inhibited by azaserine - even after

long exposure, the effects on the formation of the amide amino acids are rapid. Glutamine formation is increased immediately and remains fairly constant over the three hour interval. Asparagine synthesis is reduced immediately by azaserine additions.

The time of addition studies performed with these two antibiotics indicate that their mode of action on asparagine synthesis is clearly different. Azaserine probably acts to inhibit the transfer of glutamine amide-nitrogen to aspartate by asparagine synthetase as expected for this inhibitor. The effects produced by cycloheximide, on the other hand, are not those expected for a direct effect on the enzyme. Rather, the effect on asparagine synthesis appears to be generated after cycloheximide inhibits protein synthesis. This indicates that inhibition of protein synthesis probably blocks formation of the enzyme or some critical component of the enzyme involved in asparagine synthesis. This hypothesis is supported in part by the studies carried out using the analogues of cycloheximide. Small modifications in the structure of cycloheximide did not distinctly separate the cycloheximide effect on protein synthesis from its effect on asparagine synthesis. Cycloheximide acetate and streptovitacin A, Group II analogues, did, however, produce similar effects to those exhibited by cycloheximide.

In particular, these analogues inhibited both protein synthesis and asparagine synthesis following a three hour pre-treatment. The remaining six analogues from Group I produced effects which were not markedly different from the controls. In general, the findings from the cycloheximide and analogue studies indicate that those molecules which inhibit protein synthesis in corn roots also produce reductions in asparagine synthesis.

Two of the Group I analogues of cycloheximide tested, cycloheximide oxime and chdm (cis-4-cyclohexene-1,2-dicarboximide) have been shown to inhibit protein synthesis in other systems (38,60) as have the Group II analogues (32,38,59). The differences in the response shown by the corn root system may reflect variations in the mechanism of protein synthesis between these systems.

The definitive experiment to firmly disclose whether cycloheximide can function as a glutamine analogue and inhibit asparagine synthetase directly requires an active enzyme preparation from the corn seedling. Extracts from corn seedling tissues had low amounts of active asparagine synthetase. Other enzymes in the preparation utilized aspartate and thus made quantitative evaluation of the possible direct effects of the antibiotics on asparagine synthesis impossible. In assays of extracts derived from soybean cotyledons, there was essentially

no effect produced by cycloheximide. Even though this finding would tend to support indirect inhibition of asparagine synthesis by cycloheximide, differences in the responses of the asparagine synthetases obtained from young and mature cells could still occur. In order to examine this possibility further, a more suitable method for assaying asparagine synthetase from these regions is required.

The *in vivo* part of this investigation gives information which can be used in comparing the changes in asparagine regulation shown between root-tip and mature root sections (1). The azaserine study indicates that glutamine is probably the amide-nitrogen donor for asparagine synthesis in root-tips. The cycloheximide analogues studied indicate that some component involved in asparagine synthesis, *i.e.*, asparagine synthetase, requires continual protein synthesis to retain normal activity in root-tip sections. This situation may be similar to that shown for ornithine decarboxylase, a rate-limiting enzyme in the pathway for the synthesis of polyamines, putrescine, spermidine and spermine which are important for cell proliferation. This enzyme has been shown to undergo a reversible modification with cycloheximide *in vivo*. A seven-fold increase in the Km for the substrate pyridoxal-5-phosphate occurs over a 100 minute exposure to cycloheximide. Modification of

the enzyme apparently follows inhibition of protein synthesis which prevents synthesis of a small protein factor that must be continually synthesized to maintain the optimal activity of the enzyme (52). The cycloheximide effect in the root-tip sections of corn could be explained by a similar mechanism. In this case, an unstable enzyme subunit, glutamine amidotransferase (GAT), would require continual synthesis for optimal asparagine synthetase activity. Under the condition of reduced protein synthesis, the apparent Km for glutamine would rise gradually as the relative number of GAT subunits declined through inactivation by enzymic degradation. This in turn, would rate-limit asparagine synthesis at the amide-nitrogen transfer reaction (Figure 17). With this model, the decline in asparagine synthesis observed in root-tip sections after cycloheximide additions would be parallelled at the level of the enzyme by a decline in the availability of the GAT subunit and not by substrate limitations. The site of action of azaserine in its reduction of asparagine synthesis would be on the catalytic site of the GAT subunit. Its time of action, however, would be dependent only on its ability to penetrate to the catalytic site of this subunit.

Evidence from mature sections of the corn root indicates that neither cycloheximide nor azaserine produce

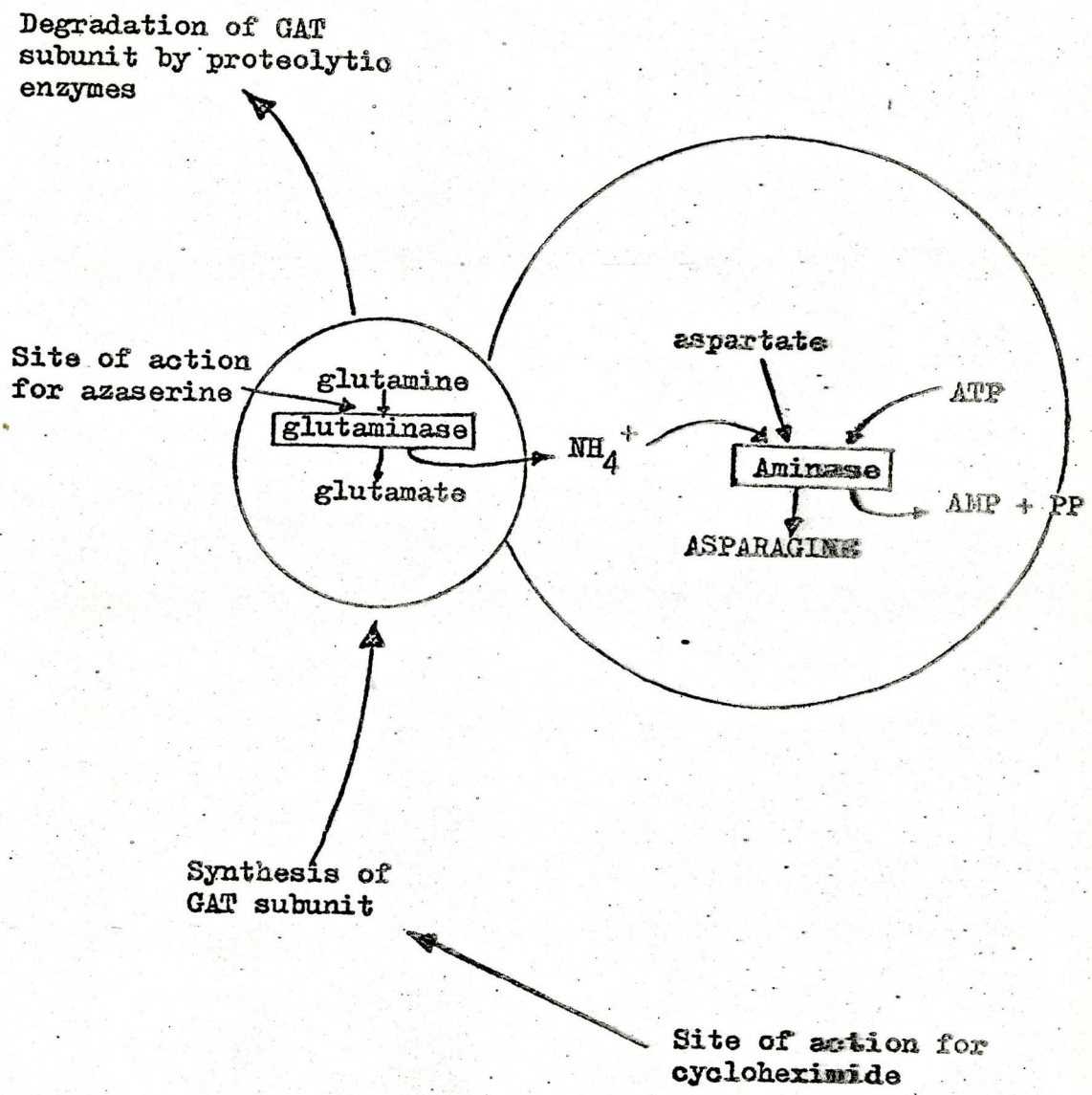


Figure 17: Proposed Model for Regulation of Asparagine Synthetase in Root-tip Sections of Corn.

reductions in asparagine formation (1). These results could be explained by two hypotheses compatible with a method of asparagine regulation by means of a GAT subunit (Figure 18). In the first hypothesis, the asparagine synthetase of the mature root is considered to be regulated less stringently by protein synthesis. Inhibition of protein synthesis by cycloheximide would not affect a GAT subunit which is more stable and thereby less affected by active turnover. Furthermore, the mature root enzyme could be stabilized by being associated inside an organelle of the root cells. The lack of inhibition of asparagine synthesis by azaserine could be explained as resulting from permeability restrictions at the membrane of the organelle. In the second hypothesis, asparagine formation in the mature section would be considered to be no longer glutamine dependent owing to the loss of the GAT function during development. Cycloheximide and azaserine inhibition of asparagine synthesis could not occur since the site on which both eventually act would not be present in the mature root cells. This second alternative thus predicts that free ammonia would be the amide-nitrogen donor in the older root tissues.

Oaks (63) and Rognes (29) suggest that ATP levels within cells regulate asparagine formation. The modulation by ATP may work at the level of the asparagine synthetase enzyme much like that shown by aspartate kinase

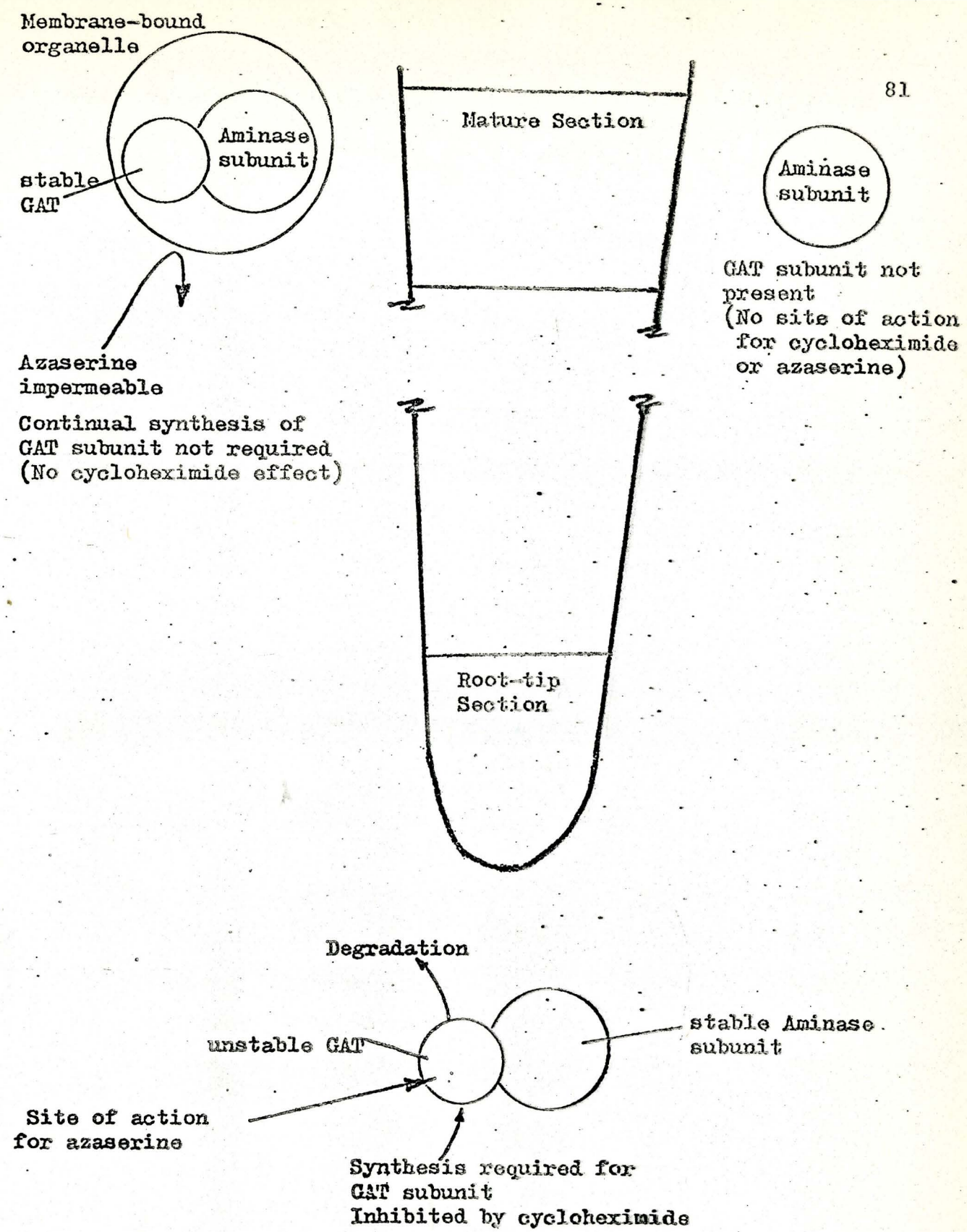


Figure 18: Possible Explanations for the Loss of the Cycloheximide and Azaserine Effects in Mature Root Sections.

(64). For example, with aspartate kinase, ADP and AMP probably inhibited the activity through a Mg-complex acting on the enzyme. By this method, this pathway is probably regulated by the overall energy level in the system. The similarity of asparagine synthetase to this enzyme is supported by the observations that conditions which could affect the energy levels of the cells also influence the channelling of nitrogen and carbon into asparagine relative to other protein amino acids of the aspartate family (29).

The NH_4^+ to C balance within the cells, for instance, may determine which general metabolic state is favoured. Relatively large amounts of carbon, e.g. glucose, for carbohydrate synthesis in addition to sufficient free ammonia for amino acid and protein synthesis should promote conditions for cell growth and proliferation. Should ammonia levels rise without adequate carbon reserves then the pathways leading towards growth would become unbalanced and/or competitive leading to a general inhibition of these pathways perhaps through substrate limitations. The strategy of the cell may be to reduce the high ammonia levels and to generate carbon skeletons in order to return to a balanced situation where growth is promoted. Channeling reduced nitrogen into asparagine would efficiently assist this strategy. ATP not utilized by other pathways would be available for use in asparagine

formation. Exogenous glucose application or conditions where photosynthate is available would reverse the carbon depletion in the cells. This, in turn, would promote transaminase reactions which would compete for glutamine amide-nitrogen and α-ketoacid derived carbon as suggested by Lea and Fowden (13). Furthermore, with the physiological balance again set in favour of growth, ATP energy would be no longer available for asparagine synthesis. An inhibition of asparagine synthetase activity would thereby result from the re-routing of nitrogen and carbon and the energy demands of those pathways required in growth.

Certain modifications in the extraction and assay techniques for asparagine synthetase from the lupin system may prove useful in characterizing this enzyme in the corn system. The primary hindrance in the study of this enzyme in other systems was its instability *in vitro*. Rognes has shown that $MgCl_2$ and the addition of the reaction substrates glutamine, aspartate and ATP provide a considerably more stable conformation for the enzyme. In particular, the presence of Mg ATP aids the formation of a dioligomer conformation in the enzyme which may be important in its regulatory properties. Sulphydryl protection by relatively high concentrations of mercapto-ethanol and addition of glycerol also increase stability as does addition of KCN to the extraction buffer (29).

In order to remove the activity of other enzymes in their extracts, Lea and Fowden added a specific inhibitor of aspartate amino transferase, aminoxyacetate, to their reaction mixture (22).

The proposed model for the regulation of asparagine synthetase in corn root-tips could be examined in a number of ways. For example, *in vivo*, inhibition of RNA synthesis by actinomycin D would establish whether the RNA(s) involved in asparagine synthetase synthesis is stable or require continual synthesis with active enzyme preparations. The Km for glutamine of asparagine synthetase could be examined with time of exposure to cycloheximide in a manner similar to that shown for ornithine decarboxylase (52). If the model were correct the requirement for glutamine would decline with increasing time of exposure to cycloheximide. Further purification of the asparagine synthetase would be required to establish the number of subunits in the enzyme. Perhaps, then additional experiments involving [H^3]-and [^{14}C]-amino acids incorporation into the subunits could give information about the relative rates of turnover of the subunits after cycloheximide treatment.

The situation in the mature sections of the corn root could be examined in a similar manner. Certain observations would be expected assuming that the model in correct. For instance, if the GAT subunit were no

longer present in the mature section then the enzyme activity should be seen with NH_4^+ and not with glutamine as the nitrogen donor. If the enzyme retained the GAT subunit in the mature section then closer examination of the stability of GAT unit would be required. This could be achieved by the Km studies on glutamine preference with exposure to cycloheximide. In this case, no increase in the Km for glutamine would be expected with increasing cycloheximide treatment if the GAT subunit were stable.

SUMMARY

Protein synthesis is required to maintain normal levels of asparagine synthesis in corn root-tips. Cycloheximide has been shown to inhibit protein synthesis rapidly and inhibit asparagine formation gradually with increasing time of exposure. The inhibitory effect on asparagine synthesis produced by cycloheximide could not be separated from the inhibitory effect on protein synthesis using analogues of cycloheximide. With azaserine, which does not markedly inhibit protein synthesis, the effect on asparagine synthesis is constant over a three hour period. The differences in the effects on asparagine formation produced by these two antibiotics indicates that their mechanisms of action are not similar in corn root-tip sections.

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