CHAPTER 1

CHAPTER 1

INTRODUCTION

The lucerne grass, synonymously termed as alfalfa is referred to as 'Lasan Ghas' in the local language, (Botanical name; *Medicago sativa*) is used as a fodder for herbivorous animals namely cattle, sheep, horses and pigs. It is especially fed to the horses as it is known to increase the strength of these animals. Lucerne grass or alfalfa is highly valued as fodder due to the presence of high content of protein, calcium, and free amino acids. It also has a very high digestibility coefficient (74 %).

It is known that the herb contains the unusual free amino acids in large quantities as compared to the other grasses. Unusual amino acids are known to act as precursors of naturally occuring antibiotics, toxins and other proteins. In animals they are known to function as hormones and neurotransmitters.

In lucerne grass it is felt that these unusual amino acids may play a role in increasing the value of the fodder by way of giving extra strength to the animals. The present investigation proposes studies on metabolism of these amino acids particularly those containing guanidine groups.

The morphological aspect of the lucerne grass plant is given below :

1.1 CLASSIFICATION AND DESCRIPTION OF ALFALFA

Division	-	Spermatophyta
Class	-	Angiospermae
Sub-class	-	Dicotyledonae
Group	-	Calyciflorae
Order	-	Rosales
Family	-	Leguminosae
Sub-family	-	Papilionaceae
Species & Genus	-	Medicago sativa

The plant is herbaceous, perennial, 0.6 - 1.6 m in height having leaves pinnately trifoliate with slightly dentate margin and two stipules adnate to the petiole. The plant bears flower smaller in size having yellow, white or purple colour.

The flower is zygomorphic, hermaphrodite, borne on dense slender and short auxillary racemes. The calyx is gamosepalous, five-teethed and sub-equal. The corolla is five petalled, the petals are having imbricate nature. The fruit of the plant is a legume which is spirally coiled with kidney shaped greenish yellow seeds(2).

Lucerne grass has a very high content of protein and hence its use as a fodder is advantageous. Table I shows the comparison between leucerne grass and other fodders that are commonly used.(3)

TABLE I

NUTRITIVE VALUE OF LUCERNE & OTHER CULTIVATED INDIAN FODDERS

(Percent dry basis)

FODDER	PROTEIN	SOL.ASH	DIGESTIVE PROTEIN	TOTAL DIGESTIVE NUTRIENTS	NUTRITIVE RATIO
LUCERENE	19.90	11.73 CaO, 2.8	15.92	57.79	2.6
JOWAR YOUNG STAGE	8.91	4.2 CaO, 0.51	4.2	56.07	12.4
OATS	14.63	10.81 CaO, 0.67	10.50	66.70	5.4
MAIZE	6.74	4.99 CaO, 2.7	4.14	58.28	15.5
BAJRA JUST BEFORE FLOWERING	16.25	10.90 CaO, 1.06	-	-	-

1.2 CHEMICAL COMPOSITION OF LEUCERNE GRASS

The plant as a whole is enriched with several essential nutrients such as proteins, vitamins, lipids, minerals, etc.

PROTEINS :

The biological value of protein in the plant is 60.5% while the digestibility coefficient is 74%. Owing to the high content of protein lucerne grass can help to correct the protein deficiency of the herbivorous animals.

The content of essential amino acids from the proteins of the plant is as follows; (values expressed in mg / 100 gms) (3)

Arginine	-	3.5	Histidine	-	1.5
Lysine	-	4.2	Tryptophan	-	1.5
Phenyl alanine	-	4.1	Methionine	-	1.3
Threonine	-	5.0	Leucine	-	7.9
Isoleucine	-	4.3	Valine	-	4.9

VITAMINS :

The fresh plant is enriched with vitamin C, vitamin A and vitamin E (4). The other vitamins which include pantothenic acid, biotin, folic acid, pyridoxin, choline, vitamin B_{12}, niacin, thiamine, β - carotene and α - tocopherol have been reported.

The sun-cured field grown plant contains vitamin D_2 (48 mg/g) and vitamin D_3 (0.63 mg/g) while laboratory grown plant when artificially irradiated contains vitamin D_2 at a concentration of 80 mg/g and vitamin D_3 at 1.0 mg/g (5). The plant loses more than 80% of its vitamin C on drying either in direct sunlight or under a fan at 25°C (6).

The vitamin B_{12} content of the fresh plant is 50 - 62 p.p.b. which is reduced to 12.45 p.p.b. as observed in dehydrated plant hay. At least 85% of vitamin B_{12} activity is due to factor other than B_{12} (7). The riboflavin (15.9 v/g), pantothenic acid (33.7 v/g), niacin (40.4 v/g) and folic acid (3.5 v/g), were not lost during dehydration of the plant.

The vitamin B_2 content is high, about 1190 v/100g of dry matter of the plant (8).

PIGMENTS :

The pigments xanthophylls (including lutein, violaxanthin, zeaxanthin and neoxanthin) β - carotene and chlorophyll makes up the major concentration. The tricin has been isolated and charecterized from lucerne plant which is an inhibitor of smooth muscle movement (9).

ACIDS :

The acids reported in the plant are citrate, malate, malonate and oxalate. Smaller amounts of succinate, fumarate, shikimic acid and quinic acids are also present (9).

MINERALS :

The minerals in the form of oxides are found to be present in the plant. The average value on the dry basis are given, as follows :

Potassium (K_2O)	-	4.11	Sodium (Na_2O)	-	0.35
Magnesium (MgO)	-	0.44	Calcium (CaO)	-	2.80

LIPIDS :

The glycerides found in the plant are composed of saturated fatty acids (19.9%), oleic acid (31.0%), linoleic acid (16.9%) and linolenic acid (32.2%) while the phosphatides are composed of lecithin (0.08%), saturated fatty acids (13.3%), oleic acid (36.8%), linoleic acid (14.7%) and linolenic acid (35.2%) (9).

ENZYMES :

The enzymes which were isolated and charecterized from the plant include lipase, peroxidase, coagulase, emulsin, invertase and pectinase (9). The enzyme ribulose biphosphate carboxylase was purified from the leaves(10) whereas NADP $^{+}$- isocitrate dehydrogenase was purified from the cytosol of the plant (n).

MISCELLANEOUS CONSTITUENTS :

The toxic saponin (0.5 - 2%) and an alkaloid - 1 - stachydrine (0.14%), meristone and alfalfone were found to be present in the seeds (9). Two more triterpene glucosides namely caulosaponin B and glycoside medicoside C (12) were isolated from the roots of the plant.

The phytolexins are the antibiotics produced after fungal wilt disease in the leucerne plant. The phytolexins vesitol, sativan and medicarpin have been derived from the leucerne plant which are derived from the condensation of a cinnamyl Co A and three acetyl Co A (13).

WAX :

The waxes myricyl stearate, nonacosane, hentriacontane, n - triacontanol and n - octacosanol are found to be present in the leucerne grass plant (9).

The chemical composition of leucerne and its products is discussed in Table II. The differences in the protein, fibre and fat composition is evident with respect to different climatic conditions.(9)

TABLE II

CHEMICAL COMPOSITION OF LUCERNE & IT'S PRODUCTS

	FIBRE	PROTEIN %	FAT %	N-FREE EXTRACT %	ASH %	TOTAL DIGESTIVE NUTRIENT %	NUTRITIVE RATIO
GREEN FEED							
Punjab	29.51	19.90	1.81	34.68	14.10	57.79	2.6
Bihar	21.54	22.71	2.87	38.39	14.49	58.47	2.2
Bangalore	30.13	20.24	2.32	36.62	10.69	60.17	2.7
HAY (on dry basis)							
Bangalore	29.41	21.26	1.41	35.18	12.74	55.90	2.4
DEHYDRATED							
Meal	24.0	17.7	2.5	38.4	10.1	54.4	3.4
Silage Wilted	11.4	6.3	1.4	13.9	3.2	21.5	4.0
Straw	40.6	9.2	1.5	34.6	6.8	42.6	8.1

1.3 CHEMICAL COMPOSITION OF ALFALFA PRODUCTS

a) **Hay** :

The plants cut at the pre-bloom stage are used for green leafy hay. The loss of leaves is avoided during drying. It is mixed with bhusa to reduce the loss of leaves during storage. About eighty to ninety percent carotene is lost during curing.

Vitamins :

The vitamin content in alfalfa hay is as follows : (values expressed per 100g)

Vitamin A	-	3.013 i.u.	Thiamine	-	3.84 mg
Pantothenic acid	-	1.78 mg	Vitamin E (as α- tocopherol)	=	2.60 mg
Biotin	-	0.018 mg	Vitamin D (D_1+D_2)	-	199.5 i.u.
Riboflavin	-	1.37 mg			

Due to high content of nutritive value the alfalfa hay serves as nutritive roughage for all classes of livestock, particularly dairy cows, breeding stock and horses. Alfalfa hay should be fed to dairy cows after milking as otherwise it is liable to change the flavour of milk (9).

b) <u>Silage</u> :

The good silage is obtained by ensuing wilted cutting of alfalfa, fermentation of which is assisted by the addition of molasses. The mixture of alfalfa and grass also make good silage.

c) <u>Alfalfa meal</u> :

The alfalfa meal is obtained by grinding its dehydrated cuttings or hay. The standard meal is graded on the basis of protein content viz; 20%, 17%, 15%, 13%, etc. It is used in the poultry, dairy cows and horses to supply vitamin A and vitamin K because it has rich content of vitamin C, vitamin A, vitamin K alongwith β - carotene, xanthophyll and chlorophyll.

The byproducts of alfalfa meal phytol and sterols are useful for manufacture of sex hormone (9). A comparison of the carotene content of the lucerne products is shown in table III (9). The content of carotene is highest in dehydrated leaf meal and is lost during curing.

TABLE III

CAROTENE CONTENT OF LUCERNE PRODUCT

	CAROTENE MG|100 G	VITAMIN A ACTIVITY I.U.|100 G
GREEN FORAGE	6.2	10,398
HAY (Average value)	1.8	3,013
MEAL (dehydrated) 17 % Protein	5.3	8,813
LEAF MEAL (dehydrated)	13.9	23,111
SILAGE	3.3	5,548

Due to high content of protein, (32.7%) and calcium (1.84%), the lucerne leaf powder is used as an excellent supplement to poor rice diet.

It is a good source of pectinase, the enzyme used for clarification of fruit juice. The sugars extracted from the lucerne have been fermented to alcohol and used for human consumption. The processed lucerne leaves concentrate which involve the removal of odour, fibre and taste charecteristic of lucerne, is also used for human consumption. It contains high content of protein 44.2%; carotene 110 mg/100g; and ascorbic acid 51.6 mg / 100g. It also contains calcium, phosphorous and thiamine, after processing.

Using as a cover crop, lucerne increases the yield of successive crops. The gram negative bacteria can not grow on aqueous as well as ether extract of lucerne plant. The use of lucerne as pasturage, is also suggested. The honey obtained contains sucrose (4.42%), dextrin (0.34%), and invert sugar (76.90%).

The seeds of lucerne shows the presence of various bases, alkaloids including stachydrine, 1 - homostachydrine, proteins and fats (9).

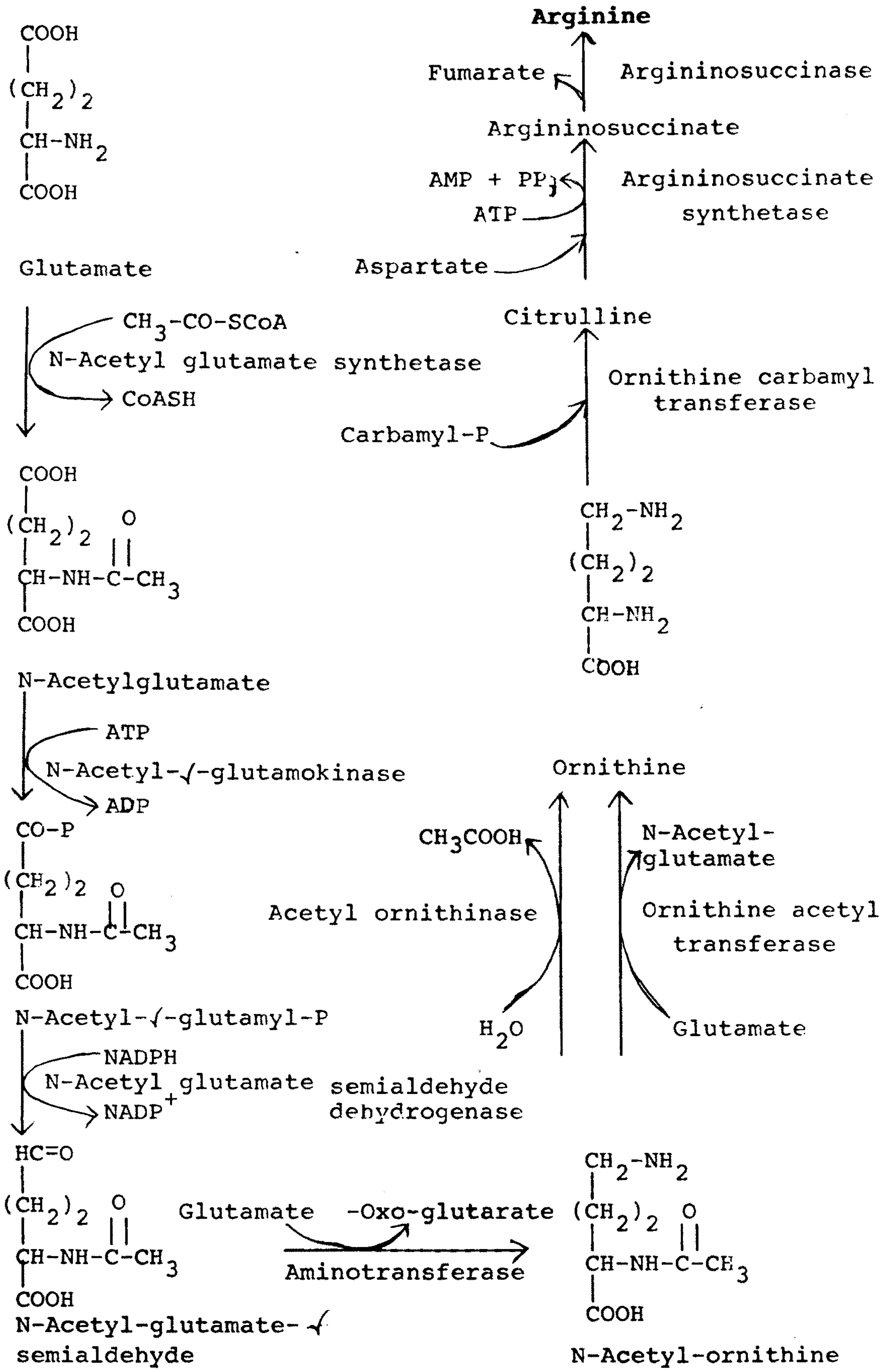
1.4 METABOLISM OF GUANIDINE COMPOUNDS

The extract of lucerne grass showed high amounts of guanidine positive material. Our attempts have been made to charecterize this product and to understand its physiological and nutririve value. L - arginine is the natural basic amino acid containing guanidino group. The metabolism of this amino acid is clearly understood.

Arginine serves as an intermediate of the urea synthesis. As the pathway of arginine synthesis and urea synthesis is the same, the first step in arginine synthesis is formation of ornithine from N - acetyl glutamine which is obtained by acetyl transfer from acetyl - SCoA to glutamate. This reaction is important in controlling pathway by feedback inhibition where enzyme acetyl glutamate synthetase is inhibited by arginine.

The sequence of the reactions involved in the synthesis of arginine are shown in the figure I (14).

FIGURE I ARGININE BIO-SYNTHESIS



1.4.1 The Synthesis of Urea :

The formation of carbamyl phosphate is the first step in the urea synthesis using ammonia as the nitrogen donor. The enzyme catalyzing this reaction is carbamyl phosphate synthetase. In the mitochondrion, carbamyl phosphate reacts with ornithine to form citrulline using the enzyme ornithine carbamyl transferase.

The product citrulline and aspartate react with each other in the cytosol to form arginosuccinic acid, the reaction is catalysed by arginosuccinate synthetase. The arginosuccinate is then cleaved by the enzyme arginosuccinase to arginine and fumarate. An enzyme arginase also cleaves arginine to ornithine and urea. Thus ornithine ie re-formed and the cycle continues to form arginine repeatedly.

The synthesis of urea is the first cyclic metabolic pathway to be described. It was elucidated by Krebs and Henseleit in 1932. A diagramatic representation of the urea cycle is depicted in figure II (15).

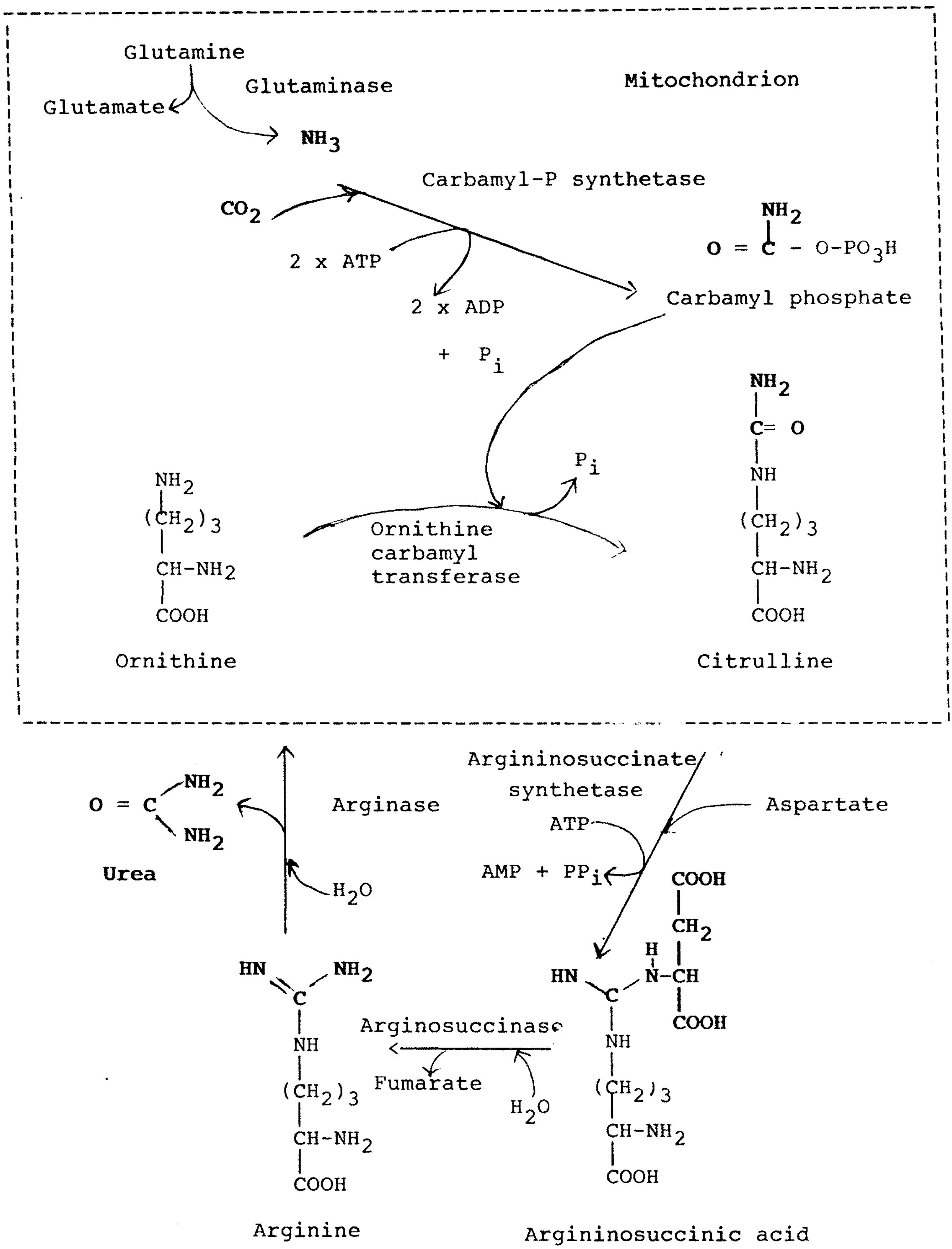


FIGURE II SYNTHESIS OF UREA

1.4.2 Arginine Phosphate :

The muscle phosphogen in invertebrates is in the form of arginine phosphate. It phosphorylates the adenosine diphosphate to adenosine triphosphate during heavy exertion and gets itself rephosphorylated when there is sufficient ATP production.

The muscle phosphogen in the vertebrates and marine annelids is creatine phosphate and in the earthworm, it is a guanidine phosphate (16).

1.4.3 Biosynthesis of Creatine and Creatinine :

In mammals the creatine is synthesized from arginine. During this pathway the guanidinoacetic acid is formed by the reaction between arginine and glycine. The enzyme catalyzing this reaction is glycine guanidino transferase, which also cleaves arginine to ornithine and urea. The reaction is dependent on S - adenosyl methionine.

In mammals the creatine cyclizes to form creatinine by the dehydration, which is otherwise excreted through the urine. The pathway is shown in Figure III.

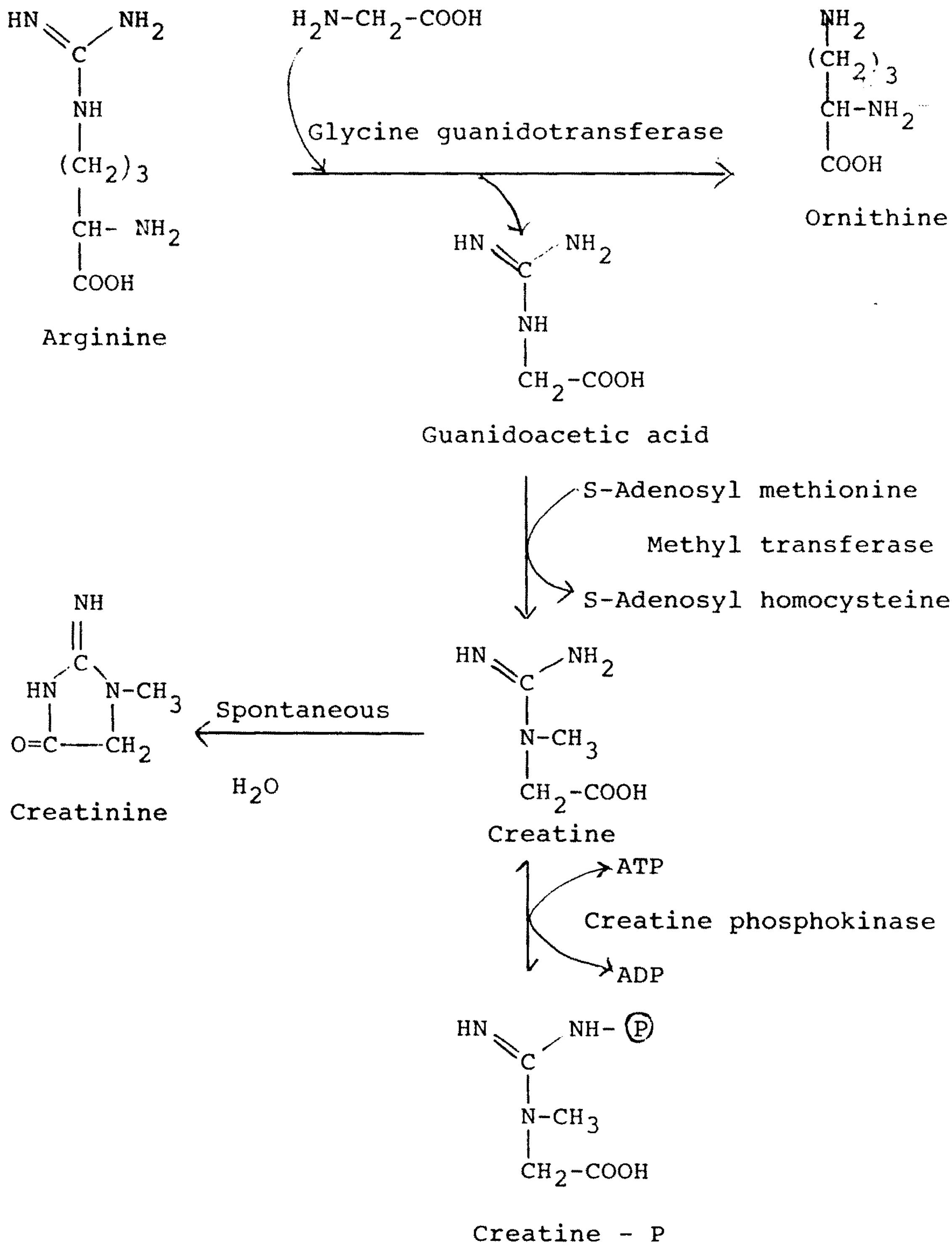


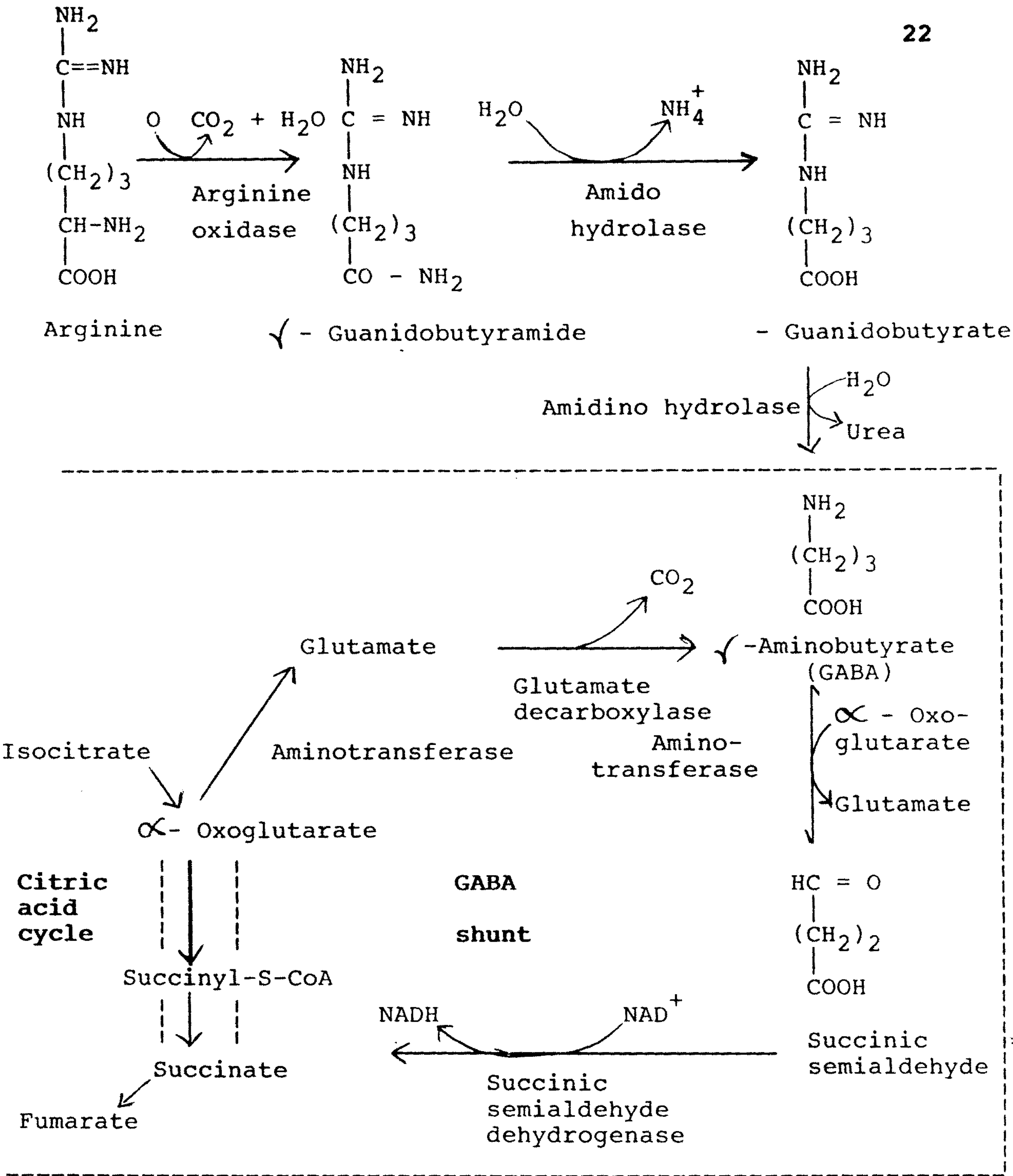
FIGURE III SYNTHESIS OF CREATINE & CREATININE

1.4.4 <u>Arginine Catabolism</u> :

Arginine catabolism in most organism follows through the enzymatic hydrolysis to ornithine and urea using enzyme arginase. The transfer of δ - amino group ornithine to suitable acceptor gives rise to glutamate - γ - semialdehyde which further oxidizes to glutamate.

While in some bacteria, agmatine, a decarboxylation product of arginine is formed which hydrolyses to urea and diamine. The agmatine acts as precursor of diamine putrescine.

The organism which are deficient in arginase activity, catabolize arginine by the enzyme arginine oxidase which oxidatively decarboxylate arginine to γ- guanidobutyramide (fig. IV). The enzyme is specific for arginine, homoarginine and concavanin. The further hydrolysis of γ- guanidobutyramide by amido hydrolase gives rise γ- aminobutyrate and ammonia. The γ - aminobutyric acid (GABA) undergoes transamination to succinic semialdehyde and successive dehydrogenation to succinate which is an intermediate of the citric acid cycle.



<u>FIGURE</u> IV THE PATHWAY OF γ- GUANIDINOBUTYRAMIDE

The γ - aminobutyrate is an important neurotransmitter formed also by decarboxylation of glutamic acid using an enzyme glutamate dehydrogenase (15).

The insects have an active enzyme arginase. During the development of silk moth, arginase develops at the time of emergence of the winged imago, in flight muscle cytoplasm. The flight muscle mitochondrial membrane is impermeable to glutamate but permeable to proline. Figure V represents the scheme of mitochondrial substrate uptake showing that proline enters the mytochondrion where it can be converted to α - oxoglutarate by the action of enzyme proline dehydrogenase to form Δ' - pyrroline - 5 - carboxylate which is furtheroxidised to glutamate.

In the cytosol, Δ' - pyrroline - 5 - carboxylate reduces to form proline. Alongwith acting as a source of carbon for citrate cycle, this mechanism reoxidizes cytoplasmic NADH and transfers it to the mitochondrion. The cytoplasmic Δ' - pyrroline - 5 - carboxylate is mainly formed from arginine.

Figure V represents the role of arginase in insect flight muscle.

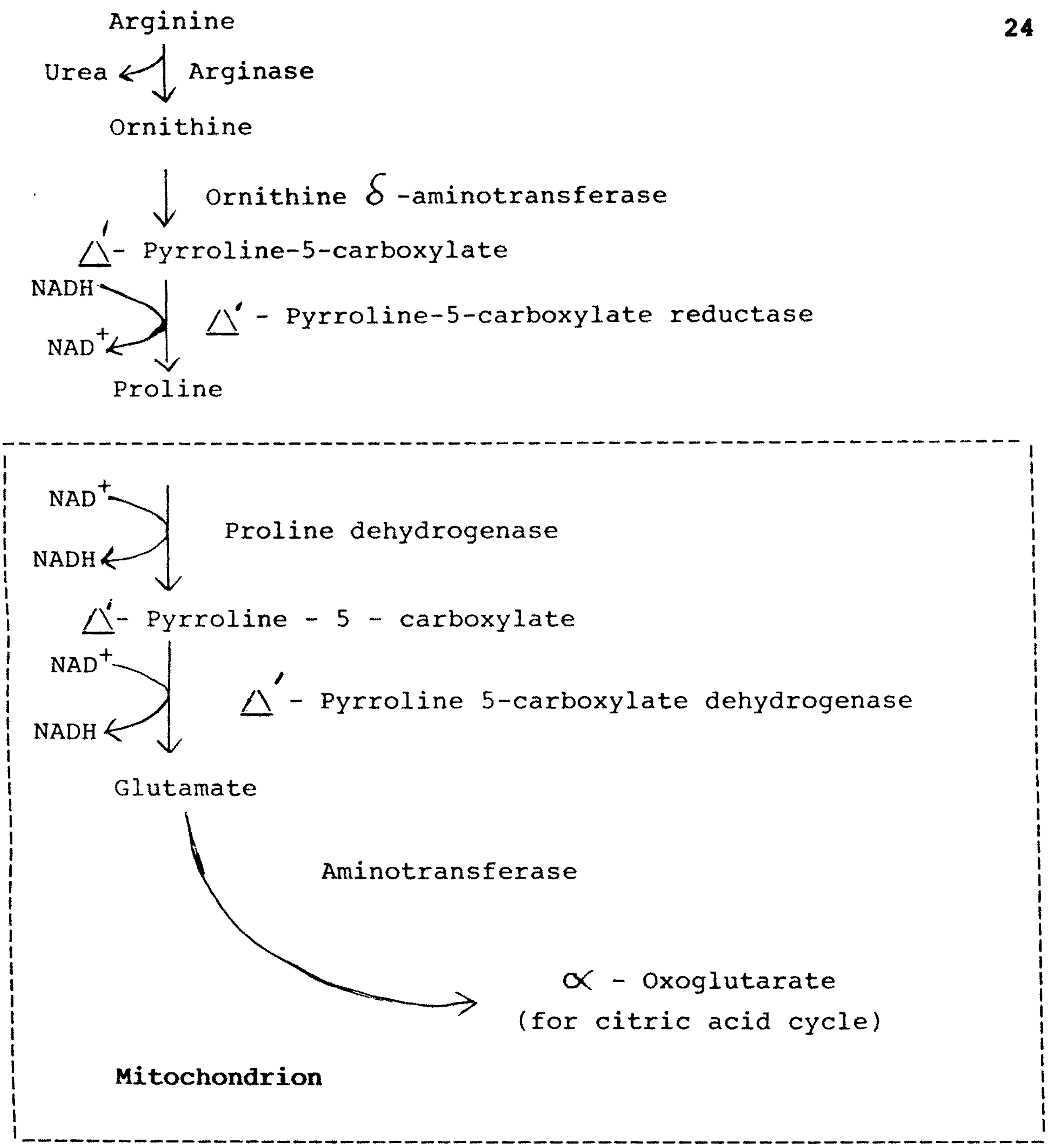


FIGURE V ROLE OF ARGINASE IN INSECT FLIGHT MUSCLE

Arginine accumulates when the protein synthesis is arrested (for example, as in certain storage organs such as tulip bulbs) or as a result of certain mineral deficiency (for example, sulfur).

It is found that most of guanidine derivatives in nature are ultimately derived from arginine by transamination. A number of guanidine derivatives occuring in higher plants and fungi are formed from oxidative degradation of arginine and subsequent metabolic reactions. L - arginine is also an important precursor of mono, di and polyamines biosynthesis. Thus L - arginine is a key metabolite in guanidine metabolism.

1.4.5 Biosynthesis of Diamines and Polyamines :

Polyamines are widely distributed in plants. Its concentration is very high in proliferating tissues.

a) Putrescine : In the bacteria, formation of putrescine takes place from l - ornithine by the action of the enzyme l - ornithine decarboxylase, or from arginine via agmatine (16). Figure VI represents the scheme of biosynthesis of putrescine.

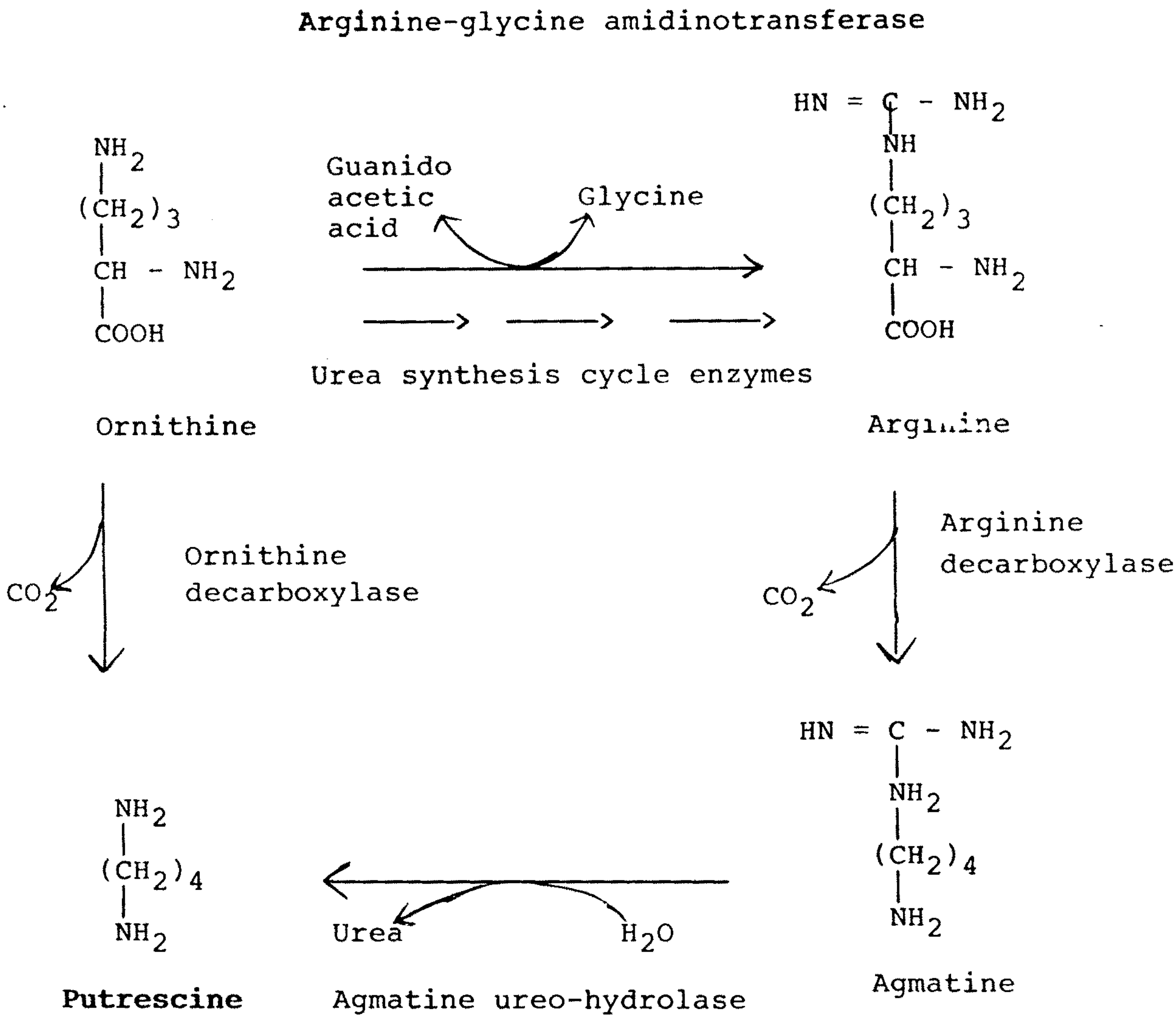


FIGURE VI BIOSYNTHESIS OF PUTRESCINE

In the micro-organisms putrescine and urea are formed by the action of amidinohydrolase on agmatine. In maize, agmatine degradation results in N - carbamoyl putrescine which on subsequent degradation yields putrescine (17). In the growing cells of sugar cane, the end product of arginine catabolism was found to be N - carbamoyl putrescine (18).

b) Spermine and Spermidine : The biosynthesis of spermine and spermidine takes place from putrescine and S - adenosyl - 1 - methionine.

S - adenosylmethionine is decarboxylated by S - adenosyl - 1 - methionine decarboxylase yielding decarboxy - S - adenosylmethinine in yeast and fungi. In higher plants spermine and spermidine are not reported while in pea plants simple carbamoyl derivatives of these are reported.

A schematic representation of spermine and spermidine is shown in figure VII.

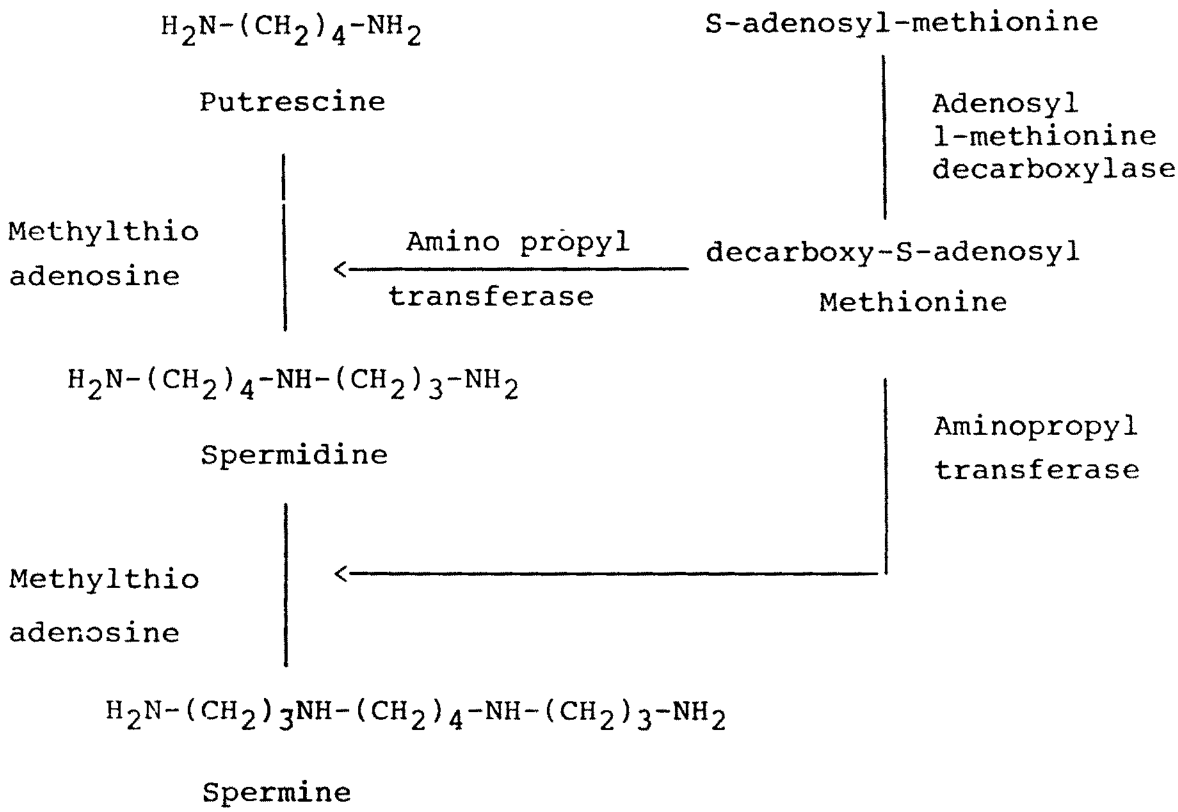


FIGURE VII BIOSYNTHESIS OF SPERMIDINE & SPERMINE

according to Tabor and Tabor (19), the membrane adsorption of polyamines by bacteria may be connected with osmotic shock resistance.

Ames and Dubin (29) showed the high polyamine levels of bacteriophages. Most of mammalian tissues show the presence of polyamines. Human blood, for example contains high content of spermidine (90.96 mg/l blood) and spermine (1.3 mg/l blood).

1.4.6 <u>Polyamine Catabolism</u> :

The exchange of an amide group of glutamine with variety of polyamines and lysine is due to the action of an enzyme, amidotransferase. Bacterial polyamine oxidase attack on substrate in two ways as follows :

1) In spermidine degradation, the butyl side chain of central imino group is attacked first forming 1, 3 - diaminopropane and γ - aminobutyraldehyde.

$$\underset{\text{Spermidine}}{H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH_2} \longrightarrow \underset{\text{1,3- diamino propane}}{H_2N\text{-}(CH_2)_3\text{-}NH_2} + \underset{\gamma\text{ - amino butyraldehyde}}{OCH\text{-}(CH_2)_3\text{-}NH_2}$$

Δ' - pyrroline is then formed by the spontaneous dehydration and cyclisation of γ - aminobutyric acid, which is converted to γ - aminobutyrate and then to succinate, the citric acid cycle intermediate.

2) While in Mycobacterium and Pseudomonas species, polyamine oxidase is attacked to the propylamine side of central imino group of spermidine. The products formed by this reaction are putrescine and aminopropinaldehyde, the latter may be catabolised by the way of β - alanine.

$$H_2N\text{-}(CH_2)_3\text{-}NH\text{-}(CH_2)_4\text{-}NH_2 \longrightarrow H_2N\text{-}(CH_2)_2\text{-}CHO + H_2N\text{-}(CH_2)_4\text{-}NH_2$$

Spermidine — Amino - Propinaldehyde — Putrescine

Spermine, spermidine and acrolein are oxidised by cupro protein, in mammalian serum. Spermidine on oxidation is converted into putrescine and acrolein.

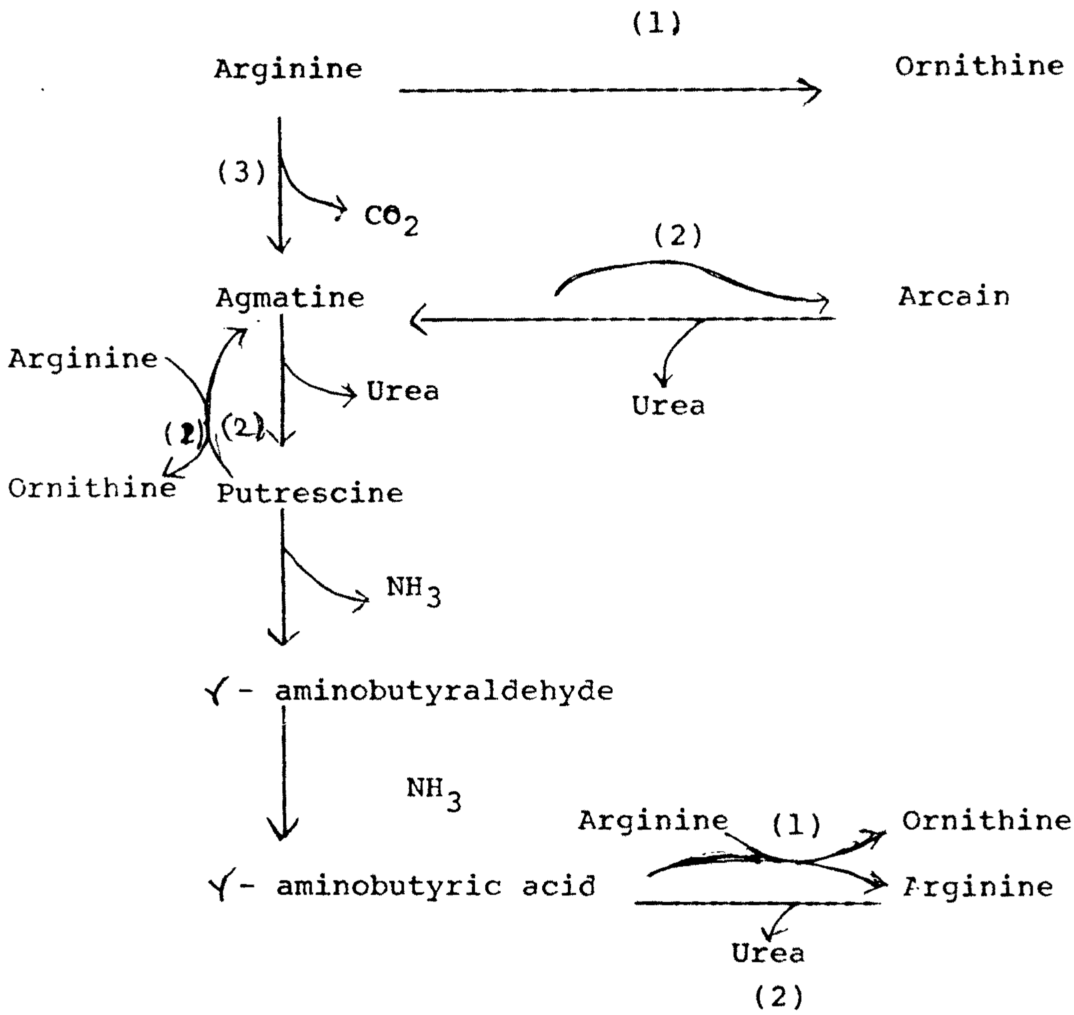
1.4.7 Catabolism of Arginine in Fungi :

In fungi the putrescine and urea are formed by the decomposition of arginine to form Lα oxo- guanidinovaleric acid which on spontaneous decomposition by hydrogen peroxide gives rise to γ - guanidinobutyric acid (GABA) (20).

In the fruit bodies of *Panus tigrinus*, l - arginine is first decarboxylated to form agmatine, which yields putrescine and urea by the action of urea hydrolase (21,22). Both putrescine and agmatine can function as an amide group acceptors, giving rise to agmatine and arcaine (23).

In some fungi and yeast such as *Candida guillermondii* and *Candida sicezevisiae*, the urea formed from arginine, is further metabolised to allophanic acid and allophanate.

Another pathway leads via transamination of arginine to guanidinobutyrate and γ- aminobutyrate (24). γ- guanidinobutyrate and urea as seen in figure VIII. Urea is then decomposed by urease. Thus arginase is by-passed (25). Diamine oxidase transforms putrescine to γ- aminobutyraldehyde and then to aminobutyric acid. L - arginine carboxylase is inhibited by agmatine, γ - guanidinobutyrate and γ- aminobutyrate (25, 26).



(1) Amidino transferases

(2) Amidino (urea) hydrolases

(3) L - arginine carboxylase

<u>FIGURE</u> VIII ALTERNATE PATHWAY OF ARGININE METABOLISM

1.4.8 Guanidine Metabolism in Plants :

The guanidines are recorded in many higher plants (27) and crops (28, 29). The formation of many guanidines is caused by transamidation from arginine (30), eg. canaline, canavanine.

Some guanidines may have widespread distribution while others have rare or infrequent distribution throught the plant and in different fungi.

γ - guanidinobutyric acid was found in the plant products such as cocoa and chocolate (31), and in potato, clover, wheat and rye(31). Creatine arises by the methylation of guanidinoacetic acid. Creatinine,the anhydride of creatine is also found in cocoa and chocolate (32).

The arginine derivatives like octopine and nopaline have been found in the crown gall tumours and not in the normal plant tissues, by the induction with infection of Agrobacterium tumefacians, Nicotiana tabacum (33, 34). Some amount of octopine is recorded in the root tips, and hypocotyls of pea seedling, normal tissues of tobacco and sunflowers (35).

The homooctopine is reported in the crown gall tissues (36). Octapinic acid, nopalinic acid and lysopine are derived from ornithine and lysine respectively. Octopine dehydrogenase converts octopine to pyruvate and arginine. Indospicine (2 - amino - 6 - aminohexonic acid) from Indigofera spicata, which possess hepatotoxic activity, is an antimetabolite of 1 - arginine increases arginine incorporation into protein (37).

Several guanidine alkaloid like terpenoid guanidines are reported in the plants, for example Galegine (38), 4 - hydoxygalegine which might be transamidation products of dimethylallylamine and 1 - arginine. In the Galega offcinalis seedlings, galegine is synthesized from guanidinoacetic acid (38, 39). The crown gall tissues of Canavalia ensiformis contains various canavanine metabolites (40).

Sphaerophysin, dimethylallylguanidine compound which occurs in Sphaerophysa salsula Pall . (fabaceae) may be derived from agmatine and mevalonic acid or acetate. (41)

$$CH_3-\underset{\substack{|\\ CH_3}}{C}=CH-CH_2-NH-(CH_2)_4-NH-\underset{\substack{\|\\ NH}}{C}-NH_2$$

Structure of sphaerophysin

The oxidation of arginine gives rise to γ - hydroxyarginine (42), δ - guanidinovaleric acid, γ - hydroxyhomoargine (43, 44) were identified in the leguminous genera Vicia and Lathyrus.

The fruit bodies of puff balls, Lycoperdon species, phlox plant seeds (45) show the prescence of keto analog of l - arginine, α - oxo - δ - guanidinovaleric acid which is the oxidative deamination product of l - arginine formed by the action of l - amino acid oxidase (46) and argininc acid (i.e. α - hydroxy - δ - guanidinovaleric acid) might be its reduction product formed by dehydrogenase action. α - oxo - δ - guanidinovaleric acid is also found in <u>Neurospora crassa</u> (47)

In the genus Lathyrus, lysine is converted into homoarginine and is cyclized to form lathyrine (48, 49, 50). Carbamoylagmatine, arcaine, coumaroylagmatine, arise from agmatine (51). L - homoarginine is formed by transamidination with lysine as amidino acceptor. The l - citrulline may give gigartinine on amidination. In the mushroom agmatine is formed either from l - arginine by carboxylation or from putrescine by amidino group transfer (52). The guanidines are summerised in the table IV.

1.5 AMINO ACIDS OCCURING IN PROTEIN :

There are twenty common amino acids which are incorporated into protein, following their initial activation by twenty specific aminoacyl - t-RNA synthetase enzymes. These amino acids are listed below.

Cystine is normally encountered into plant protein while cell wall of higher plants contains trans - 4 - hydroxy proline (75).

TABLE V

AMINO ACID	**AMINO ACID**	**AMINO ACID**
Alkyl :	Imino acid :	Sulphur :
Glycine	Proline	Cysteine
Alanine	Hydroxy :	Methionine
Valine	Serine	Aromatic :
Leucine	Threonine	Phenyl alanine
Isoleucine	Basic :	Tyrosine
	Lysine	Tryptophan
	Arginine	
Acidic :	Histidine	Amide
Aspartic acid		Glutamine
Glutamic acid		Aspargine

1.6 UNUSUAL NONPROTEIN AMINO ACIDS

In addition to the twenty or so universally distributed protein amino acids at this time, over 400 other amino acids have been obtained from natural sources (51), 240 of which are found in various plants. Prokaryotic organisms and fungi also produce nonprotein amino acids. About 50 nonprotein amino acids are produced by animals.

Nearly all amino acids which are unusual, nonprotein in nature occur in free form, but occasionally are also found to be attached to a carbohydrate moiety and many other are found as glutamyl linked peptide. Some of these compounds bear some structural analogy to protein amino acid counterpart and most of nonprotein amino acids are hydroxylated aromatic or heterocyclic in structure. Heterocyclic nonprotein amino acids may contain oxygen, nitrogen and sulphur in addition to carbon in the ring. The phenyl group is associated mostly with alanine or glycine in case of aromatic unusual amino acid, while heterocyclic compounds are often β - substituted alanines in which pyrimidine, pyrone, pyridine, pyrazole, thiazole, or isoxazoline structures are evident. Many are constructed from azetidine,pyrrolidine, and piperidine units.

1.6.1 Amides, Substituted amides & Dicarboxylic acids :

An extensive range of the amides and substituted amides are found in plants, for example, substituted aspargines are same products of Cucurbitaceae. N^4 - methylaspargine, 5a from seeds of Corallocarpus epigaeus (52), N^4 - ethyl aspargine, 5b and N^4 - hydroxyethyl aspargine, 5c were isolated and charecterised from Ecballium elaterium (53) and Bryonia Diocia (54).

In the onion and garlic, many γ- glutamyl peptides of S - substituted cysteine are found (53) while in cucurbita seeds γ- glutamyl β - pyrazoll -ylalanine is found (56).

The isomers of γ- methylene glutamic acid (57) and an cis & trans form of α- (2 - carboxycyclopropyl) glycine, and 3 - carboxy - D - phenylglycine, 3 - carboxy - phenyl alanine, D - (3 - carboxy - 4 - hydroxy phenyl) glycine have been reported in the family Resedaceae (58).

γ - hydroxyglutamic acid and γ- hydroxyglutamine from Phlox decussata (59); γ- methyleneglutamine from Peanut plants (60); β and γ substituted dihydroxy glutamic acids from different plants (61); γ- ethelideneglutamic acid from seeds of Tulipa (62) and from legume Tetrapleura tetraptera (63) were isolated.

1.6.2 Basic Unusual Amino Acids :

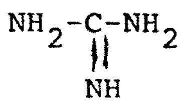
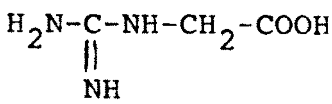
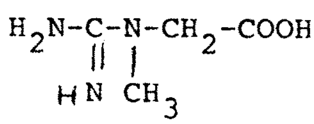
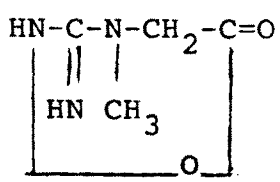
The linear aliphatic, substituted compound of three to six carbon atoms are found to occur in the plants. The α , γ - Diaminobutyric acid and β - oxalyldiaminopropionate are the neurotoxins which produce the symptoms of lathyrine in animals, especially in man (64). Amidine is also toxic and causes liver lesions in the sheep grazing on that legume (65). β - cyanoalanine, γ - glutamyl - β -cyanoalanine present in the seedcoat of Vicia species (66).

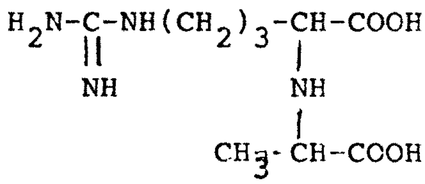
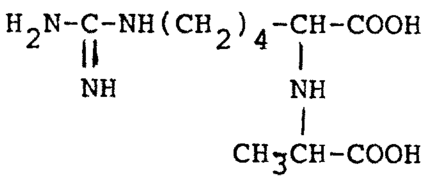
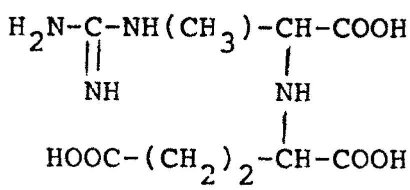
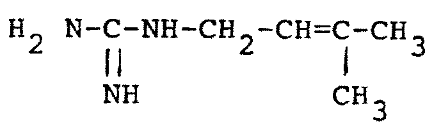
The Laminaria species show the prescence of laminine, while the Saccharomyces cerevaceae and other fungi show the prescence of saccharopine (67).

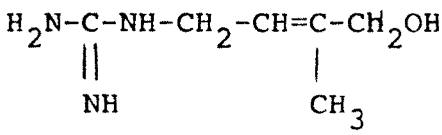
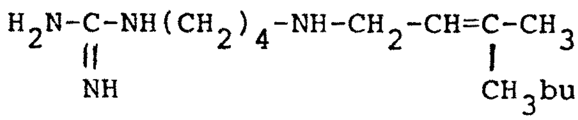
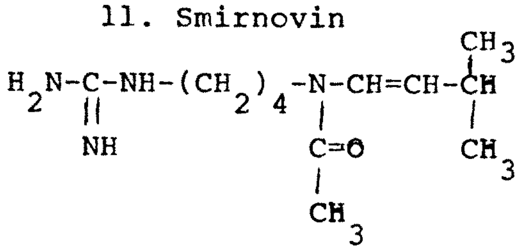
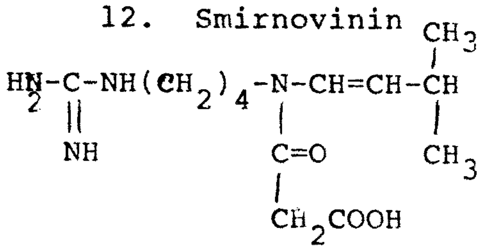
Lysopine, N α- substituted lysine from crown gall tumours and N α- acetylornithine from bacteria are other lysine substituted products (68). The canavanine (69), lathyrine (70) are other basic unusual amino acids of legume family. The basic guanidino - substituted amino acids are reported in the table IV

TABLE IV

GUANIDINO COMPOUNDS IN HIGHER PLANTS AND FUNGI

COMPOUND	OCCURANCE
1. Guanidine 	Seedling of *Vicia sativa*, *Pisum sativum*, Rice roots, fungi other higher plants, presumably involved in arginine catabolism of sugar cane culture cells.
2. Guanidino acetic acid (Glycosamine) 	Precursor of galegine in *Galega officinalis*
3. Methyl guanidine acetic acid (Creatine) 	Potato, cow - pea, clover, wheat, rye.
4. Creatinine (anhydride of creatine) 	Lupine, wheat, Coacoa beans, etc.

COMPOUND	OCCURANCE
5. D-Octopine (N-C -(1-carboxyethyl)-l-arginine) 	Crown gall tissue of <u>Helianthus</u> <u>tuberosus</u>.
6. Homooctopine (N-C -(1-carboxyethyl)-L-arginine) 	Crown gall tissue of Scorzonera species
7. Nopaline [N_2-(1,3-dicarboxy propyl)-L-arginine) 	Crown gall tissues of several plants
8. Galegine (3-methyl but-2-enyl guanidine) 	Seeds of <u>Galega</u> <u>officinalis</u>, Metabolism in Galega, suggested in transamidination of isopentyl amine with arginine in shoots, leaves &fruits of Galega.

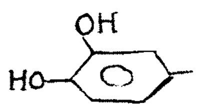
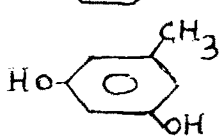
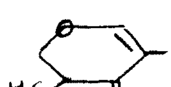
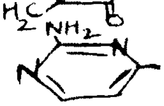
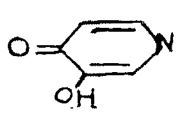
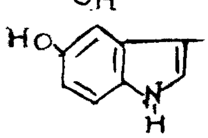
COMPOUND	OCCURANCE
9. 4-hydroxy galegine 	*Galega officinalis*.
10. Sphaerophysin 	*Sphaerophysa salsula*, *Smirnovia turkestana* (involved in metabu0olism of sphaerophysa seedlings in transamidination with arginine.
11. Smirnovin 	*Smirnovia turkestana*, *Eremosparton aphyllum* *Astragalus tibetanicus*
12. Smirnovinin 	Eremosparton species *Smirnovia turkestana*.

1.6.3 Cyclic Unusual Amino Acids :

Alicyclic compounds mainly contain cyclopropane ring, for example, 1 - aminocyclopropane - 1 - carboxylic acid occurs in fruit pears and cow berries (71). β - (methylenecyclopropyl) - β - methylalanine is present in the seeds of *Aesculus californica* (72).

Aromatic compounds contain m - carboxy substituted amino acids together with hydroxyphenylalanine and orcylalanine. These compounds are listed below :

TABLE VI

'R' IN AMINO ACID	NAME OF UNUSUAL AMINO ACID	PLANT ORIGIN
	3,4 Dihydroxy phenyl alanine	*Vicia faba*
	Orcylalanine	*Agrostemma githago*
	Stizolobic acid	*Stizolobium lassjo*
	Lathyrine	Lathyrus species
	Mimosine	Mimosa & Leucaena species
	5 - hydroxy tryptophan	*Griffonia simplicifolia*

1.6.4 Unusual Amino Acids containing Sulphur :

these compounds include S - substituted cysteines in the form of mainly thioesters found in the families liliaceae, cruciferae and leguminosae based on the structure,

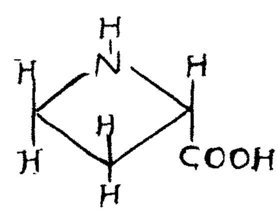
$$R.S: CH_2\text{-}CH(NH_2)COOH$$

where, R group may be methyl or substituted (73).

S - hydroxymethyl homocysteine has been isolated from algae (74) while ethionine (S - ethyl homocysteine) is reported in several bacteria (75).

1.6.5 Imino Acids :

The imino acids found in the plants include azetidine, pyrrolidine and piperidine rings, for example L - azetidine - 2 - carboxylic acid is found in <u>Convallaria</u> <u>majalis</u> (76) and many species of liliaceae(77)



Structure of L - azetidine - 2 - carboxylic acid

3 - aminopyrrolidene - 3 - carboxylic acid (cucurbitin) from cucurbita and Kainic, allokainic and domoic acid from seaweeds are well studied (78).

The free hydroxyproline isomers, for example Cis(allo) - 4 - hydroxy - 1 - proline, 7e and trans - 3 - hydroxy - 1 - proline are reported in the leaves of _Santalum album_ (79) and in the seeds of _Delonix regia_ (80). The fruits of the family Rosaceae, apples and pears show the presence of transmethyl - 1 - proline, 7d and 4 - hydroxymethylproline (81).

The pipecolic acid is mainly found in the seeds of the legumes, for example, trans - 5 - hydroxy - 1 - pipecolic acid, 8c and trans - 4 - hydroxy - 1 - pipecolic acid, 8c are found to be present in acacia species (82).

1.6.6 Hydroxyl Amino Acids :

Most of the nonprotein amino acids are hydroxylated, for example, the δ - hydroxy derivative of lysine from alfalfa (83), O - oxalylhomoserine from lathyrus species (84), γ - hydroxy derivative of valine from _Kalanchoe diagremontiana_ (85) and hydroxy derivative of leucine along with lysine and valine, from Deutzia gracilis (86), are isolated.

1.6.7 Unsaturated Amino Acids :

The free amino acids obtained from the seeds of Aesculus californica (87), include the structure as following

$$R-CH_2-CH=\underset{\displaystyle \backslash CH_3}{C}-CH_2-CH(NH_2)-COOH$$

Where R = H and R = OH

many fungi contain 3 - methylene - 2 - amino-pentenoic acid (88).

The same unusual amino acids found distributed in many species, give probability of these species being evolved from a common ancestral form in which the genome controlling the biosynthesis of that particular amino first arose. The prescence of the same uncommon amino acid, in taxanomically different species or groups, proposes the presence of the independent metabolic pathways leading to this amino acid.

When more than one unusual amino acid is found in same species, the unusual amino acids may be related to each other as a precursor and derivative on a common biosynthetic pathway controlled by unrelated genomes. When two or more unusual amino acids that are biochemically different, occur together in more than one species then these species may have two or more distinctive and unusual genome.

The higher plants have proved to be a source of unusual amino acids and many have been isolated from leguminoseae. The lucerne grass also belongs to the leguminoseae family.

1.7 PHYSIOLOGICAL IMPORTANCE OF UNUSUAL AMINO ACIDS

The unusual amino acids are nonprotein amino acids the function of which in plants are not fully understood. The possible role of unusual amino acids which are free in nature can be enumerated as follows due to their common biological properties observed (89).

a) A number of free amino acids are present in selected taxa of plants concerned with nitrogen storage in the seeds and vegetative storage organs.

b) The free unusual amino acids may act as metabolic precursors of proteins or naturally occuring antibiotics as the building stones of proteins due to their structural analogy to common amino acids.

The accumulation of soluble nitrogen in particular form might be the consequence of ammonia detoxification in these plants which are unable to excrete nitrogen waste compounds. Special detoxification products arise functioning in nitrogen storage and translocation so that limiting nitrogen can be reutilized in nitrogen consuming processes in relation to growth and development. This concept was based upon earlier finding that compounds such as aspargine, glutamine, urea (in fruit bodies of some fungi) can be accumulated in large amount.

The low molecular nitrogen rich compounds for example, glutamine, l - arginine, l - canavanine are the nitrogen translocating compounds in selected plant taxa, studies by means of N - compounds, dominant increasing number of soluble nitrogen compounds are detected by chromatography.

Large number of unusual amino acids found in the legumes, for example, canavanine, act as a toxin against larvae of Prodenia eridania. L - canavanine, a guanidoxy structural analog of l - arginine, is present in jack-bean seeds (Canavalia ensiformis) about 5% of dry weight. There is considerable evidence for the view that stored canavanine is consumed in seedling development functioning as soluble nitrogen storage (89). During seed germination stored canavanine is depleted suggesting its involvement in nitrogen with respective species. L - canavanine, being a structural analog of arginine by the replacement of δ - methylenene group by oxygen, it competes with uptake and metabolism of arginine in the cells.

The unusual amino acid might be formed by modification of general metabolic pathways by mutation and selection or by some mode of genetic enrichment by recombination, gene duplication, etc. leading to novel route to biosynthesis in close connection with morphological co-evolution (89). The antimetabolite properties of many nonprotein amino acids are well studied. The formation ability of these compounds may reflect subtle structural alterations of the plant genome in evolution providing modified enzymes leading to novel plant constituents. The production of new compound seems without severe consequences in basic metabolite patterns but may confer some selective advantage on the producer of analog.

The plant leaves, roots, rhizomes also shows high concentration of unusual amino acids, for example, leaves of *Lucaena leucocephala* contains 8% unusual amino acid (dry weight of mimosine).

A number of unusual amino acids are highly toxic for man and his domestic animals. For example, α - amino - B - oxalylaminopropionic acid from *Lathyrus sativa* which causes human lathyrism. Indospicine is known as teratogen and hepatotoxin in sheep. Mimosine can cause hair loss in cattle and the shedding of the fleece in the sheep (90, 91) These properties may confer the producer plants to protect against animal predators or pathogenic fungi.

Azetidine - 2 - carboxylate, a structural analog of l - proline in the rhizome of the liliaceous species inhibiting invertase and acid phosphatases in excised pea roots as well as nitrate reductases formation. The rate of uptake of proline for growth in the microorganism is also inhibited by azetidine - 2 - carboxylate (92).

The unusual nonprotein amino acids normally do not play role in deciding the structure and hence the biological activity of the proteins. Further they are unlikely to have any common function in plant metabolism.

Most of the compounds rich in nitrogen act as nitrogen storage of seeds. As in early phases of seedling growth, concentration of these compounds changes, mostly reduces, suggesting its use in vegetation or growth of the plant, for example canavanine, homoserine, α, γ - di - aminobutyric acid, etc.

While some amino acids show their presence after seed germination. The high concentrations of these amino acids in seedling are directly involved in nitrogen transport within the respective species, for example, γ - methylane glutamic acid and γ - methylene glutamine in peanut and homoserine in peas, etc.

The plant nonprotein unusual amino acids may play important role in the plant-insect, plant-mammal and plant-plant interrelationship, for examp] the insecticidal action of plant constituents a chemical weapon or defence agent against animals or other plants and insects. While the presence of some unusual amino acids may be acting as feeding attractant, for example, glucosinolates in the plant family, brassicaceae (93).

The allelochemical activity of some amino acids helps plants to minimise the efficiency of organisms causing plant disease and destruction.

The accumulation of unusual amino acid may be advantageous to species in which they are present. These may play important role in pharmacalogical or chemotaxonomical and phytogenetic significance. Thus the toxic, repellant, attracting activity of unusual amino acids is supposed to be the chemical signals of plants to communicate with other living beings.

1.8 RECORDED AMINO ACIDS FROM LUCERNE

An extensive study of the chemical composition of the lucerne plant was carried out by Vickery, Osborne, Wakeman and Levenworth (94). The study was done by using the protein free concentrated extract of lucerne and it was referred to as the alfalfa filtrate.

An amide aspargine was charecterized and isolated from alfalfa filtrate by Vickery. Small amount of tyrosine was also isolated. About 55% of amide was recovered. The method of protein analysis showed the prescence of alanine, valine, leucine, phenyl alanine and serine in addition to aspartic acid and asparagine. The content of these acids are recorded in the table VII (95).
121

The precipitate of basic lead acetate of alfalfa filtrate on hydrolysis contain appreciable amounts of adenine, arginine, lysine, stachydrine, aspartic acid and tyrosine. Table VIII represents these amounts. (96).
121

The nitrogen base fraction of alfalfa filtrate that contains quaternary bases has been shown to contain stachydrine, choline, trimethylamine and betaine, which occur as free compounds or as salt and not as components

TABLE VII

CHEMICAL COMPOSITION OF LUCERNE PLANT

SUBSTACES	AMOUNT	N	FRESH PLANT	DRY PLANT PLANT	'N'as'N' OF PLANT	'N'as'N' OF LUCERNE FILTRATE	SUBSTANCE / gm N of LUCERNE FILTRATE
	gm.	gm.	%	%	%	%	gm.
Asparagine	18.03	3.364	0.075	0.411	1.52	6.10	0.327
Aspartic Acid	6.196	0.6524	0.026	0.112	0.294	1.18	0.112
Tyrosine	0.117	0.009	0.0005	0.0027	0.004	0.016	0.0021
Phenylalanine	0.821	0.0698	0.0034	0.019	0.032	0.127	0.015
Serine	2.407	0.321	0.010	0.055	0.144	0.582	0.0436
Leucine	2.07	0.221	0.0086	0.472	0.095	0.401	0.0376
Valine	2.887	0.345	0.012	0.0658	0.155	0.625	0.0524
Alanine	1.163	0.183	0.0049	0.0265	0.082	0.332	0.021
TOTAL	33.69	5.165	0.140	0.739	2.326	9.326	

The yield of amino acids corresponds to 23.96 kg. of fresh plant

TABLE VIII

AMINO ACIDS & OTHER BASES OF LUCERENE

	SUBSTANCES gm.	NITROGEN gm.
Adenine	0.168	0.087
Arginine	0.053	0.017
Lysine	1.75	0.336
Aspartic Acid	7.091	0.729
Tyrosine	0.368	0.028
Stachydrine	1.409	0.137
TOTAL	10.839	1.334

of more complex substances. The stachydrine content was more as compared to other quaternery bases as shown in table IX (97).

1.7.1 Free Amino Acids of Lucerne :

The work of Leavenworth, et al. have reported the prescence of free bases and acidic amino acids along with some quaternery bases. The proteins of the alfalfa were precipitated by means of alcohol and subjected to successive hydrolysis of alfalfa filtrate which has established the prescence of measured amount of basic amino acids like arginine, lysine, the quaternery bases stachydrine,choline, adenine, aspargine and tyrosine as free in nature. Table X represents the content of free amino acids and other bases.

1.7.2 Unusual Amino Acids From Lucerne :

An unusual amino acid γ- aminobutyric acid was reported to be present in the lucerne leaves by Synge(95). While Wilding has isolated the hydroxy derivative of lysine, i.e. hydroxy lysine from the rot of the plant (83).

TABLE IX

ALKALOIDS & OTHER BASES FROM LUCERENE

(Quaternary Bases from Lucerene)

	FRESH PLANT %	DRY PLANT %	'N' as ' N ' JUICE %	SUBSTANCES per gm N IN JUICE gm.
Stachydrine	0.144	0.785	6.09	0.624
Choline	0.0115	0.063	0.58	0.050
Trymethylamine	0.0013	0.0069	0.13	0.0055
Betaine	0.00095	0.0052	0.049	0.0041

The betaine fraction of the alfalfa filterate corresponds to 23.96 Kg. of the fresh plant.

TABLE X

FREE AMINO ACIDS & OTHER BASES OF LUCERNE

	SUBSTANCES gm	NITROGEN gm
ARGININE	0.522	0.172
LYSINE	0.073	0.014
STACHYDRINE	3.768	0.371
CHOLINE	0.249	0.036
ADENINE BASE	0.290	0.146
CHLORIDES OF PURINE	0.048	0.014
ASPARAGINE	3.193	0.596
TYROSINE	0.224	0.0173
TOTAL	8.367	1.3663