Studies on the Biochemical Synthesis of Asparagine

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Within recent years the idea has become almost universally accepted that proteins consist in part of amino acids joined together by the "peptide" linkage,--CONH--. It is tacitly assumed, as there is at present no evidence to the contrary, that amino acids are the building blocks in protein synthesis, these acids being incorporated into the protein molecule by the formation of a peptide bond. The mechanism of the synthesis of this bond is a major unsolved problem in biochemistry.

The synthesis of protein from amino acids is an upgrade reaction in the sense that it is necessary to put energy into the system to create the pepteide linkage (from 2000 to 4000 calories per bond). Reactions of this kind are difficult to study under simple experimental conditions because energy-yielding reactions must take place simultaneously to supply the driving force for the synthesis, and the energy-yielding and energy-using reactions must be coupled. The fact that in many cases such coupled reactions require the intact cell structure precludes the use of relatively simple <u>in vitro</u> experiments as a tool in such studies. Other difficulties lie in the facts that even under <u>in vivo</u> conditions the net rate of protein synthesis is slow, and the mechanism is obscured by the whole complex of metabolic changes taking place in living tissue.

However, the peptide bond occurs naturally in cases other than in proteins; the three most common structures containing this grouping are hippuric acid (benzoyl glycine) found in the urine of horses and other animals, and the amides-asparagine

and glutamine, found in plant tissues. Strictly speaking these two last named compounds are not peptides, but they may be considered as peptide derivatives in which hydrogen has replaced the amino acid residue. From a thermodynamic viewpoint the synthesis of asparagine from aspartic acid and ammonia, and the synthesis of a peptide from two amino acids are similar in that both reactions require energy to drive them forward. Indeed the synthesis of the amide group in asparagine requires nearly the same amount of free energy (3460 cal) as does the formation of a peptide bond.

The facts that peptides and amides have the common properties of chemical configuration and a positive free energy requirement for synthesis suggested that a study of amide formation might throw some light on the mechanism of peptide synthesis and on the general problem of protein formation. The amide chosen for study was asparagine, $CONH_2$-CH_2-$CH(NH_2)$-COOH, partly because energy data are available for this compound, and partly because asparagine occurs in some plants as the only amide, whereas glutamine-forming plants usually contain asparagine. Since there is practically no definite information of the chemistry of asparagine formation, an investigation of the possible precursors of this amide was undertaken.

I. Survey of Earlier Work on the Asparagine Problem

1. General

The occurrence of asparagine in plants was first reported by Delaville in 1802 (1), who found the compound in young asparagus shoots and named it from this source. Sometime later the compound "Althain" from altheae sprouts was also indentified as asparagine (2). By the middle of the last century the amide was known to have a rather wide distribution in germinating plants and especially in etiolated seedlings. Thus Pasteur reported a yield of 6 gms. of the compound from 1 liter of the expressed juice of vicia (3), and considerably higher yields were obtained by various workers from other members of this genus as well as from vetch, beet, almond, potato, dahlia, and the legumes (4).

In general the compound is found in the seedlings of most higher plants to the extent of 1 to 3% of the dry weight, and is often associated with glutamine, the amide of glutamic acid. In the legumes especially, asparagine makes up a conspicuously high proportion of the dry weight of the young plant. In this connection may be cited the classical experiment of Schulze in 1876, an experiment which strikingly demonstrated the important position of asparagine in the nitrogen economy of plants and served as a basis for much subsequent work on the origin and role of the amide. Schulze found that ungerminated lupin seeds contained 45% protein and practically no asparagine; when the seeds were germinated 8 days in the dark the protein decreased to 8% of the original seed weight, while the asparagine increased to 25%, and constituted over 60% of the total nitrogen. After several weeks the amide disappeared

and finally only succinic and malic acids were found (5,6). In this and similar experiments Schulze showed that as much as 75% of the nitrogen arising from the decomposition of protein was accounted for as asparagine. Since the protein contained only 2-3% of free amide nitrogen and aspartic acid to the extent of about 10%, it was conclusively demonstrated that asparagine must have a secondary origin and does not arise as a primary split product of protein as Pfeffer and Borodin had proposed (7,8).

Work on the origin of the amide was further extended by Schulze. In experiments on Lupinus luteus he found that after 15 days germination protein decomposition ceased, but asparagine continued to accumulate. The increase in the asparagine nitrogen could be accounted for by a simultaneous decrease in amino acid nitrogen, and especially of the monoamino fraction (9). If aspartic acid were present and was being utilized as precursor, such a decrease in this fraction would be expected.

When the amide is formed from the products of protein breakdown oxygen is necessary; under anerobic conditions only the amino compounds accumulate, This was shown by Suzuki, who grew barley and soy beans under anerobic conditions and found an accumulation of amino acids but no asparagine (10), by Butkewitsch (11), who anesthetized lupin seedlings with toluene (which inhibits anabolic oxygen-requiring reactions, among others) and found an accumulation of ammonia but no asparagine, by Loew (12), who proposed the overall reaction $2NH_3 + O_6H_{12}O_6 + 3O_2 \rightarrow$ Asparagine $+ 2CO_2 + 5H_2O$, and by others (Palladin, Godlewski, Wassilieff).

Two futher observations of Schulze and his pupils should be mentioned to complete the evidence upon which this investigator's

final opinions were founded. Firstly, by analyzing separately different portions of seedlings, Schulze found that asparagine accumulated less in the leaves and plumules than in the petioles and stem, while the former structures contained the greatest amounts of protein. This was interpreted as evidence that protein synthesis occurred at the expense of the amide. Secondly, it was shown that an artificial supply of ammonia and carbohydrate via the external medium would bring about a direct primary synthesis of asparagine in the plant (13, 14, 16). When etiolated seedlings were illuminated the accumulated amide usually disappeared, presumably being utilized for protein synthesis; in those cases where asparagine increased after photosynthesis began, Schulze believed that the carbohydrate formed in response to illumination produced the amide at a greater rate than the latter was used in the formation of protein.

In the light of the findings briefly outlined above, Schulze's position with regard to the formation and role of asparagine may be summarized as follows: in the etiolated germinating seedling, protein breaks down to yield amino acids; these acids then undergo a secondary change and split off ammonia, from which asparagine is formed. Oxygen is necessary for the oxidative formation of ammonia and probably for the synthesis of asparagine. In the light (or when sugars are fed) asparagine is formed from ammonia and carbohydrate. The amide itself is a precursor of protein, hence it formation from amino acids as an initial step in the process, and its final utilization in situations where active protein synthesis is taking place. Schulze very cautiously made no mention of what he thought was the precursor of the carbon skeleton of asparagine, although he did report the detection of succinic acid in lupinus and cucurbita (pumpkin) and pointed out that the following

reaction might occur:

$$R\text{-}COONH_4 \rightarrow R\text{-}CONH_2 + H_2O$$

(ammonium succinate?)

The above views were held by most students of plant biochemistry in the first decade of this century and are based mainly on Schulze's careful experiments. A large part of the subsequent work on the origin of asparagine has only served to confirm these factual findings and but little has been added since. On the other hand new opinions soon developed regarding the role of the amide in plant metabolism.

Schulze's pioneering work was carried on by his pupil Prianischnikow, who made extensive use of the water culture technique in his studies. Prianischnikow first repeated Suzuki's work on ammonium absorption (16) by growing barley in solutions of ammonium chloride or sulfate. The plants yielded increased amounts of total nitrogen and asparagine (as Suzuki had found), and the medium became acid. Peas were also grown in ammonium solutions (which contained calcium to balance acidity) and showed a seven-fold increase in asparagine and a five fold increase in total nitrogen over water controls. In neither case did the ammonium content of the plants increase significantly (17). In the experiments on peas it was found that the increase in asparagine nitrogen was only slightly less than the increase in total nitrogen, from which it was inferred that the assimilated ammonium nitrogen had supplied both the amino and amide nitrogen for the synthesis of the asparagine molecule. The important generalization made from this experiment was that amide synthesis does not depend directly on aspartic acid arising from protein metabolism; indeed the asparagine may arise by a complex of completely independent reactions (Vickery,18

Another point of interest in these experiments is the relatively small increases in ammonia as compared to the large increases in asparagine nitrogen. This suggested to Prianischnikow that asparagine is an an ammonium "buffer" in that the toxic ammonia is synthesized into and stored as the innocuous amide, a role which he compared to that of urea in animal metabolism. This concept was a definite departure from Schulze's theory of the significance of asparagine.

Prianischnikow further noted that in general those seedlings accumulate asparagine that have considerable carbohydrate reserves, while seedlings with low reserves increase in ammonia during germination. Thus barley forms asparagine from ammonia derived from protein breakdown or from an external medium, the pea accumulates asparagine only when supplied with calcium salts, and the starving etiolated lupin accumulates only ammonia (17, 19). To substantiate this argument with regard to the importance of carbohydrate in asparagine synthesis, Prianischnikow cut off the cotyledons of barley to remove the carbohydrate reserves; the seedlings then accumulated ammonia as in the case of "lupin type" plants. In the reverse direction it was found that lupins, supplied with carbohydrate by photosynthesis, were able to absorb ammonia and convert it to asparagine, illustrating a reversion to the "barley type" of plant.

The early observations of Suzuki (16) that lupins were unable to absorb ammonia from an external medium and convert it to asparagine, and the work of Schulze and Prianischnikow as outlined above, suggested a new type of experiment to Smirnow, who had been working in Prianischnikow's laboratory on the utilization of ammonia by various plant groups. By the use of a sterile technique, this

investigator was able to grow Lupinus angustifolius on a medium containing ammonium salts and glucose (20). The following table shows the results of an experiment in which lupins were germinated in the dark for 13 days on media with and without glucose (values are in mg. nitrogen per 100 plants):

	Total N	Protein N	Ammonia N	Asparagine N
No glucose	904	194	63	467
Glucose added	934	262	34	446

It is seen that the glucose slightly increased the nitrogen uptake from the medium, and lowered the ammonium content of the tissues, but did not increase the asparagine content. Smirnow was unable to explain this last observation satisfactorily. In an experiment identical to this one but lasting 26 days, the results with regard to the amide were more decisive:

	Total N	Protein N	Ammonium N	Asparagine N
No glucose	1003	216	123	490
Glucose Added	1249	380	82	619

On the basis of these data, Smirnow concluded that carbohydrate supplied to the plant artificially could promote amide synthesis just as could carbohydrate arising by photosynthesis and that the latter compound was involved in some way in the sparing or synthesis of protein. Earlier workers had conducted similar experiments and arrived at the same conclusions (13, 14, 16), but Smirnow's investigations were more striking due to his elaborate precautions in preventing infection and thus in being able to carry the germination over a longer period. The analytical methods employed by Smirnow have recently been criticised by Burkhart (21).

Another approach to the study of the formation of asparagine has been made through investigations of the changes taking place in leafy shoots and detached leaves floating in water or in nutrient

media. Suzuki first showed (22) that during the night the protein of attached leaves decreased, but no simultaneous increase in amide nitrogen occurred, presumably because of translocation (bean, potato). Schulze and co-workers placed the shoots of various forest trees in water and noted an accumulation of asparagine in the leaves (23). The same author found that the glutamine content of beet leaves increased when these organs were detached and kept in a moist dark chamber (24). Avena Sativa (oat) and Vicia faba (bean) were shown by Butkewitsch to increase in asparagine in the dark; the rate of increase of asparagine nitrogen was approximated by the rate of decrease of protein nitrogen, hence it was inferred that the amide was being formed at the expense of protein (25).

The earlier work on changes occuring in leaves was given further attention by Chibnall in a series of papers beginning in 1924. Chibnall first showed that during the night the protein content of attached runner bean leaves decreased, and the nitrogenous products resulting from the protein breakdown were translocated out of the leaf (26). However, when the leaves were detached and placed with their petioles in water, the products of protein decomposition were found to accumulate. These products were mainly asparagine and amino acids (27). In an attempt to isolate the amide, Chibnall was able to obtain 48.8% of the "amide" nitrogen as crystalline asparagine. This writer pointed out the difficulties in effecting a quantitative separation of a compound such as asparagine from mixtures as complicated as tissue extracts. Chibnall's conclusions from his experiments are that leaf proteins are continuously decomposing, but during the day this process is masked by synthesis; the decomposition products are amino acids and asparagine, which are trans

located out of the leaf mainly as asparagine, and hence the amide plays the role of a translocation compound. He further emphasized the fact that Prianischnikow's analogy between urea and asparagine is misleading in that urea is a non-utilizable waste product whereas asparagine is readily utilized and in no sense has an excretory function.

Mothes has sought to extend Chibnall's investigations on the amide metabolism of leaves. This worker has confirmed the observation that during the night leaves decrease in total nitrogen (28). He computed his data on the basis of the loss of a given form of nitrogen in terms of the percent of that form originally present. On this basis amide and amino nitrogen decrease while protein nitrogen changes but little in the night (vicia faba, lupinus luteus) ; however, this mode of computation obscures the relative importance of the changes in the different constituents, and the data has been recast into the following tables;

Vicia Faba leaves. N as % of fresh weight.

	NH_3	2x amide	Amino	Protein
Evening value	.04	.11	.43	4.49
Loss during night	-.03 (gain)	.05	.22	.28

Lupinus luteus leaves. N as % of fresh weight.

	NH_3	2x amide	amino	Protein
Evening value	.03	.07	.18	6.05
Loss during night	.00	.03	.05	.25

It is seen that the amide nitrogen is relatively very low as compared to protein nitrogen and the absolute change in the former fraction is slight. In view of this relation it is difficult to draw any definite conclusions as to the origin and role of asparagine in etiolated leaves although Mothes' experiments have been widely cited.

Mothes also investigated the effects of carbohydrate on the nitrogen metabolism of leaves. In detached darkened leaves the protein decreased and the amino acids and amide increased at a greater rate than in illuminated controls, although there was considerable variation of behavior in this regard. In sugar feeding experiments conducted under sterile conditions ammonium nitrogen remained at a low level as compared with water culture controls, and in all cases carbohydrate exerted a protein sparing action. Under conditions of chloroform narcosis or anaerobiosis synthetic reactions were inhibited and ammonia accumulated as in the case of seedlings. In general these experiments only served to confirm the earlier work; more recent investigations of Mothes concerned with the immediate precursors of asparagine will be discussed in a later section.

In an investigation of the nitrogen metabolism of the germinating lupin Bonnet (28) has pointed out the fundamental difference in the nature of the nitrogen metabolism of plants and animals; in the latter the breakdown products are excreted unchanged or after secondary transformations, whereas in plants the degradation products either accumulate or undergo further changes which lead eventually to resynthesis. It is this economy in the utilization of nitrogen which obscures the exact origin and role of a compound such as asparagine.

Bonnet's data shows that in the lupin germination is reflected metabolically in proteolysis and accumulation of amino, amide, and "peptone" nitrogen. He believes asparagine plays the role of a protein precursor, and has suggested the following scheme as a general outline of the changes taking place:

$$\left.\begin{matrix}\text{Protein}\\ \text{Amino acids}\end{matrix}\right\} \longrightarrow \left.\begin{matrix}\text{Ammonia}\\ + \\ \text{Carbohydrate}\end{matrix}\right\} \longrightarrow \text{asparagine} \longrightarrow \text{protein}$$

McKie (29) has followed the nitrogen metabolism of germinating lupins over a 25 day period, but her methods of treating the material were such that secondary changes in the labile constituents probably occured. The results indicate an irregular hydrolysis of protein.

Vickery's studies which began in 1931 on amide metabolism in the tobacco plant have brought to light several new aspects of the problem. When tobacco plant leaves are placed in water or nutrient solution the usual protrolytic changes occur and protein disappears at about the same rate both in the light and dark for the first 75 hours (30). The darkened leaves accumulate asparagine and ammonia and continue to hydrolyze protein after this period. The illuminated leaves, however, form every little ammonia and only moderate amounts of asparagine, but relatively large quantities of glutamine arise. Nitrogen in the form of ammonia, asparagine, and glutamine can be accounted for by the decrease in protein and nitrate nitrogen.

Vickery explained the effect of illumination on glutamine synthesis as follows: in the dark the precursor of asparagine is already present in the tissues and is readily utilized. When the supply of precursor is exhausted (after about 100 hours) ammonia accumulates in large quantities. In the light photosynthetic reactions supply the precursor of glutamine, which is formed in greater amounts than asparagine. The abundance of glutamine precursor insures that very little free ammonium appears in the illuminated plants, and hence the role of the amides is to detoxify ammonia (a view Vickery has recently strongly repudiated).

While Vickery believed that the glutamine precursor was a "carbohydrate metabolite" he did not explain the fact the glucose supplied in his nutrient solutions was apparently unable to supply the precursor. Furthermore, his arguments suggest that the precursor

of glutamine has a considerably different origin than that of asparagine, although in later statements he expressed the belief that both amides arise from carbohydrate. It is certainly not clear why the asparagine precursor is so quickly exhausted in the dark unless photosynthesis is involved. The fact that glutamine arises both in the light and dark in rhubarb leaves (Vickery and Pucher, 31) further obscures the problem of the necessity of carbohydrate as a precursor of this amide.

Positive evidence as to the importance of carbohydrate has been adduced by Yemm in an investigation of chemical changes occuring in starving barley leaves (34). This worker found that the period of rapid asparagine synthesis corresponded with the period of steady low carbohydrate depletion; ammonia finally arose when the carbohydrate supply was exhausted. The usual implication of this type of experiment is that the sugars directly furnish a carbon skeleton for the amide. While this is an attractive hypothesis there is no positive evidence in it's support. Since carbohydrate is an important energy source in leaf metabolism, it seems not unreasonable that it's most important function in amide synthesis is to provide energy. Exhaustion of carbohydrate is characterized by a cessation of synthetic reactions (i.e. those reactions where ΔF is positive), and by the predominance of downgrade reactions which yield energy, hence when carbohydrate is available one may postulate a "continuing metabolism" much as Borsook and Kieghley have originally suggested for animals (35). This supply of energy promotes or "maintains" synthetic reactions such as those forming amino acids, asparagine and protein. Indirect evidence for such a continuing metabolism in plants is to be found in experiments of Vickery and Schoenheimer (35a), in which ammonium nitrogen containing N^{15} was

fed to buck-wheat plants; the amide and protein nitrogen was found to have undergone replacement with the isotope even more rapidly than corresponding replacements in animal tissues. In the case of protein nitrogen, a replacement of 6% was observed in 47 hours. These observations point to a continual dynamic breakdown and resynthesis of metabolites and tissue constituents, processes which require an external supply of energy.

Negative evidence as to the importance of carbohydrate in amide synthesis has been presented by Petrie and Wood (32,33), who have studied the metabolism of two grasses, Phalaris tuberosa and Lolium multiflorum, with the object of correlating changes in the nitrogenous constituents with carbohydrate and water content. Their statistical analysis of the data indicates that ammonium is the only constituent which bears a significant relation to asparagine, from which they conclude that the amide arises from ammonia and a non-nitrogenous precursor. Since no correlation between asparagine and carbohydrate was observed, these findings suggest that the conversion of the latter to asparagine is not a direct process.

In a comprehensive investigation of the nitrogen and carbohydrate metabolism of high-oil-reserve (pumpkin, peanut) and high-protein-reserve plants (lupin), Burkhart (36) has considerably extended Yemm's findings. The material Burkhart used was grown in a sugar-free physiological salt solution containing ammonium nitrogen and the plants were analyzed at various stages of germination. While the responses of the groups studied varied as to the time relations, the general metabolic pictures were the same. In the early stages of germination while carbohydrate was available, ammonium was still absorbed and utilized in growth and protein synthesis. In the intermediate period when carbohydrate was almost depleted ammonium was stil absorbed, but to a lesser degree. This stage was characterized by

protein breakdown and formation of amides. The third stage of germination was the period of complete carbohydrate starvation, characterized by no ammonium absorption, and by decomposition of both protein and asparagine and a corresponding accumulation of ammonium.

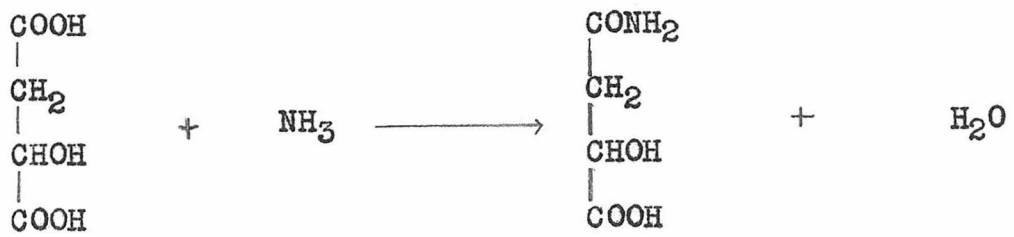
Burkhart has discussed the theories of the function of amide formation, and on the basis of his own observations concludes that so called "ammonium injury" may be the result of carbohydrate starvation rather than of ammonium excess. On this basis asparagine synthesis is not a detoxification mechanism but is the "resultant of certain (as yet unknown) carbohydrate-nitrogen relations". This latter is a fair statement of the present status of the problem.

The foregoing discussion is an attempt to present in review the most important papers dealing with the physiological aspects of the synthesis of asparagine. The justification for such a review lies in the fact that the whole problem is still on the borderline between plant physiology and biochemistry, and before an effective biochemical approach can be made, the pertinent facts relative to the strictly physiological precursors must be considered. The next section will deal with the work that has been done in an attempt to define the immediate precursors of the amide.

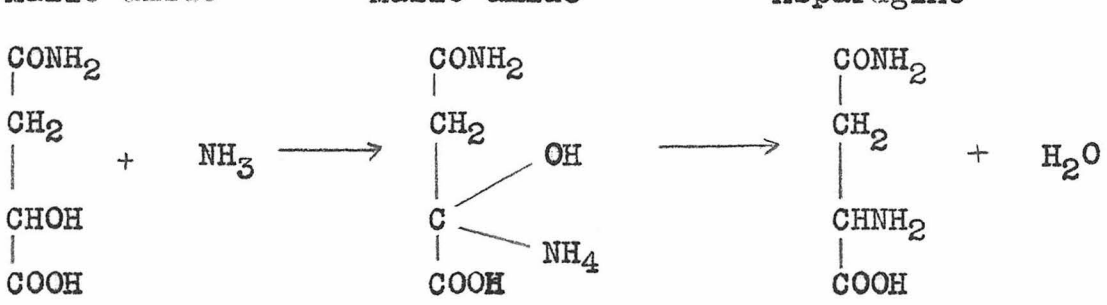
2. Immediate Precursors of Asparagine

In view of the structural relation of asparagine to the 4-carbon dicarboxylic acids, it is tempting to associate these latter compounds with the biological synthesis of the amide. The first suggestion of this kind was made by Muller in 1886 who pointed out the obvious relation of succinic and malic acids to the carbon skeleton of asparagine (Smirnow, 20; 37). Franzen and Keyssner (55) have critically reviewed the available literature on the occurence of malic acid in plants, which appears to have a fairly wide distribution. Schulze and more recently Ruhland and Wetzel (50) reported the detection of succinic acid in lupinus, pumpkin, and begonia.

Actual experimental work on the carbon skeleton precursors of asparagine seems to have been stimulated by the observations of Schulow in 1913 and of Petrow in 1917 on the nitrogen metabolism of maize. Petrow grew maize in a medium containing malic acid, whereas Schulow used asparagine in his substrate, but both reported the same results. It was found, upon subsequent analysis of the plants, that when the amide nitrogen value was doubled to account for the amino group of asparagine, and this value added to the ammonia nitrogen, the sum exceeded the non-protein-nitrogen (N.P.N.). These investigators attempted to explain this surprising result by assuming that the malic acid had first formed the amide, and had not yet completed the amination reaction (20, 38, 39), thus:



This postulate suggested the order and mechanism of asparagine synthesis from 4-carbon dicarboxylic acids such as malic acid:



Similar experiments on maize were carried out by Smirnow in 1923 (20), who used ammonium succinate, malate, and aspartate in his substrates. The following table shows the results of an experiment in which the plants had grown 13 days:

Mg. N per 100 plts.

Medium	Total N	Protein N	Asparagine N
Ammonium sulfate	253	168	33
Ammonium malate	287	158	55
Ammonium succinate	273	172	44
Seeds	211	201	--

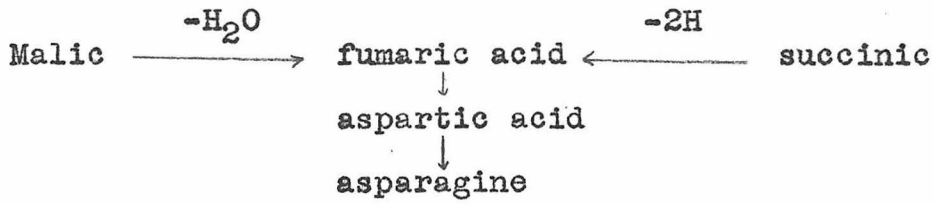
Ammonium aspartate was tested under slightly different but comparable conditions and produced considerably less asparagine than the malate or succinate.

Smirnow concluded from these experiments that succinic and malic acids brought about an "intensive" amide formation. It is to be noted however, that the decrease in protein nitrogen alone could account for more than 60% of the increase in asparagine nitrogen, and no data is given on changes in amino acid nitrogen. On this basis the author's conclusion seems rather poorly established. In general the most severe criticism of this type of experiment lies in the fact that there is so much more protein than asparagine present that it is quite possible that increases in amide content

can be ascribed to secondary synthesis following protein degradation. Smirnow failed to confirm the work of Schulow and Petrow, and indeed Smirnow stated that he believed amination would occur before amidization in asparagine synthesis.

The existence of an "asparaginase" involved in the deamidization of asparagine was demonstrated early in the century, (Kiesel (41), Butkewitsch (42), 1909, Kato (43), 1911),but received little attention until 1927. In that year Grover and Chibnall (40) showed that an enzyme preparation from barley would deamidize asparagine to aspartic acid and ammonia at an optimum pH of 7. Since this preparation would also split glycylglycine, they concluded that it was a non-specific peptidase and that the amide group of asparagine was of a peptide character. Geddes and Hunter (44) made somewhat more detailed investigations of an "asparaginase" from yeast and ascribed fairly specific properties to their enzyme. It had an optimum pH of 8 and showed some glutaminase activity, but was inactive toward a number of other amides. These observations, while not confirming the entity of an asparaginase, do indicate the existence of an enzyme that will hydrolyze the amide group of asparagine. This enzyme is fairly widely distributed in animals, moulds, bacteria, and higher plants.

In 1926 Quastel and Woolf (45) reported that resting B. coli were able to synthesize aspartic acid from fumaric acid and ammonia. To a lesser degree malic acid could also be so converted, but maleic acid was not acted upon. The work of Woolf (46), and of Virtanen and Tarnanen (47) has extended these observations, and has shown that malic, succinic, and fumaric acids can all serve as precursors of aspartic acid, which theoretically can be converted to asparagine by asparaginase:



This scheme has not been shown to occur as such in plants, but it did offer a starting point and a rationale for the experiments to be described which attempted to elucidate the mechanism of the synthesis. More recent developments concerning asparaginase will be discussed later.

Mothes attacked the asparagine problem by means of a new method, namely by "vacuum infiltration" of excised leaves (48). Briefly the technique consists in placing leaves in a nutrient medium and evacuating the container; upon readmitting air, the nutrient solution is forced into the intercellular spaces via the stomata, and thence into the cells where it enters the metabolic picture. The application of this method in a study of amide precursors has given results of sufficient importance to justify some discussion.

Mothes first ran an orienting experiment in which almost equal amounts of ammonium succinate, furmarate, malate, and asparaginate were infiltrated into leaves of Phaseolus multiflorus (bean). After 30 hours the leaves were analyzed for total and aspargine nitrogen; the asparagine nitrogen of the succinate treated leaves was 3 times as great, and the leaves treated by the other salts almost 4 times as great as the corresponding value for a water control. Unfortunately an ammonium sulfate control was omitted.

To determine whether the acids were furnishing the carbon skeleton for the amide, Mothes proceeded in an indirect manner; carbohydrate-rich and carbohydrate-poor (etiolated) leaves were infiltrated with ammonium malate, with the following results:

		Total N	Protein N	Asparagine N
CHO rich	water control	709	614	31
	amm. malate	865	635	124
CHO poor	Water control	713	565	61
	amm. malate	858	547	190

Since the carbohydrate-poor leaves synthesized even more amide than those well supplied, Mothes concluded that the infiltrated acid had served as a carbon skeleton. Similar results were obtained with tobacco.

These experiments are very striking in that the increase in amide nitrogen cannot be accounted for simply by a corresponding decrease in protein and amino nitrogen; however they are not absolutely conclusive because no controls containing only inorganic ammonium were run. Furthermore it is difficult to conceive of plant tissue so exhausted in carbohydrate that no carbon is available for synthesis, yet having sufficient metabolic energy reserves to synthesize amides and amino acids. Nevertheless this new method and it's initial results represent a distinct forward step in the solution of the problem, and must be placed in the list of experiments which indicate that the 4C-dicarboxylic acids are involved in asparagine synthesis.

In an extensive investigation dealing with several aspects of the origin of asparagine, Schwab (49) has repeated some of Mothes' infiltration experiments, and has produced evidence which tends to place the latter author's results in a different light. Using excised leaves of phaseolus, Schwab infiltrated ammonium succinate and acetate, and upon subsequent analysis found that the acetate treated leaves contained over one-half as much asparagine as the succinate controls. However the actual utilization of

ammonia was much less in the acetate treated leaves, and in neither case was protein or amino nitrogen changes followed. A similar experiment on Fittonia Verschaffetii (acanthaceae) was performed, and the increase in amide N was actually greater in the acetate treated leaves than in those infiltrated with succinate. Ammonium oxalate, bicarbonate, and sulfate likewise caused an accumulation of asparagine nitrogen of about the same magnitude as did succinate. In another series of experiments, ammonium bicarbonate, and sodium asparaginate and glutaminate (the amides) were infiltrated into leaves of Triticum sativ (wheat). The amounts of asparagine synthesized were almost identical in all three cases.

Schwab concluded from these investigations that asparagine synthesis occurs independently of the carbon skeleton associated with the ammoniumion, and that the only effect of the anion is to exert some control over the NH_3 concentration prevailing in the tissues. This latter in turn is one of the limiting factors affecting the intensity of amide synthesis. Mothes' results were also interpreted on this basis by Schwab.

There are several severe criticisms which can be adduced against the work just discussed. Firstly, the protein nitrogen, in those cases where it was determined, was from 6 to 13 times as great as asparagine nitrogen; in no instances were amino nitrogen changes followed. Secondly, in all experiments in which ammonium succinate or malate was infiltrated there was either no proteolysis (one experiment) or an actual protein synthesis. In all other experiments where protein changes were reported, hydrolysis occurred, usually of such magnitude as to account for the increase in amide nitrogen. This second criticism illustrates an important principle

to be taken into account in the interpretation of results of infiltration or feeding experiments; the synthesis of asparagine in which ammonia from an external source is utilized often occurs under conditions associated with no proteolysis or with actual protein synthesis. This suggests that the carbohydrate metabolism is affording sufficient energy to maintain or drive forward synthetic reactions. When proteolysis does occur, as in some of Schwab's experiments, an indequate carbohydrate metabolism may be prevailing, and the energy requirements for viability are being supplemented by protein breakdown (that is continuing metabolism is failing). It is possible that anions such as oxalate and acetate, forced into the metabolic machinery in high concentration by mechanical means, so disrupt carbohydrate metabolism that proteolysis and secondary synthesis of asparagine thus occurs. If this explanation of Schwab's results be accepted, his investigations indicate nothing more than the possibility that succinic and malic acids may be concerned in asparagine synthesis, as these compounds are known to have a physiological origin. It is the writer's opinion that Schwab's work has not been critically evaluated by Yemm (34) and by Vickery (30).

Modern Aspects of the Problem

The work of Smirnow, Mothes, and Schwab has clearly posed the asparagine problem in its modern aspect. What 4-carbon nonnitrogenous acid is converted to the amide, and what is the mechanism of the conversion? Succinic acid has been shown to occur normally in plants (Schulze; Ruhland and Wetzel, 50; Onslow). The three possible sources of this compound are from carbohydrate metabolism (respiration), from deamination of amino acids, and from the oxidation of arginine to yield guanidine and succinic acid (Kiesel,

51). The investigations of Ruhland and Wetzel have shown that both malic and succinic acids arise simultaneously with the formation of ammonia, and these workers conclude that these substances owe their origin to nitrogen rather than to carbohydrate metabolism. However, in animal tissues succinate may arise from carbohydrate via puruvate, acetate, oxalacetate, citrate, and α-ketoglutarate. The recent work of Braunstein and Kritzman (52) on the donation of amino groups to keto acids serves to explain the possible inter-relationships between carbohydrate and nitrogen metabolism, although this donation reaction has not yet been demonstrated in plant tissues.

Granting that succinic acid can be formed in the plant it remains to find a path for it's conversion to asparagine, with possibly aspartic acid a link in the chain. Thunberg in 1921 demonstrated the presence of succinic dehydrogenase in phaseolus (53). The action of this enzyme converts succinic to fumaric acid, which by the addition of ammonia could form aspartic acid, a reaction known to occur in various bacteria (page 18) and in liver (54). Malic acid also occurs in plants (55) and can be dehydrated enzymatically to fumaric and thence to aspartic acid. Virtanen and Tarnanen have stated that the enzyme occurs in higher plants (47), but later studies by Jacobson (54a) have not definitely confirmed this.

An alternative path for the formation of aspartic acid involves oxalacetic acid as an intermediate. In 1929 Robinson (56) suggested that oxalacetic might be converted to aspartic acid in plants. The oxalacetate could arise by the oxidation of malate catalyzed by the malico dehydrase found in seeds by Thunberg (57) and in heart muscle by Green (58). According to Green the reaction is greatly

inhibited by traces of the Keto-acid (the inhibition is due to the keto group), hence the formation of aspartic acid, by removing the reactive oxygen, would allow the reaction to continue. At the time Robinson made her suggestion concerning the intermediation of the oxalacetate, evidence for the conversion of keto acids to amino acids was based chiefly on experiments similar to those of Embden in which ammonium pyruvate was perfused through surviving liver and alanine was found in the perfusate. At present the Braunstein-Kritzmann transamination reaction offers a much more clearly defined mechanism for this conversion. According to recent studies by Cohen (59) the most active keto acids in the transamination reaction with glutamate are pyruvate and oxalacetate. The reaction with oxalacetate is as follows:

$$\text{glutamate} + \text{oxalacetate} \rightarrow \text{aspartate} + \text{ketoglutarate}$$

$$K = \frac{(\text{Ketoglut})\ \ (\text{asp})}{(\text{glut})\ \ (\text{oxal})}$$

From Cohen's data as well as thermal data compiled by Borsook K is of the order of magnitude of unity, showing that even at equilibrium a significant amount of aspartic acid would exist. It is possible that the formation of asparagine, by removing the aspartic acid, would shift the reaction to the right until the glutamate was used up, thus necessitating a mechanism for the regeneration of this carrier.

A possible scheme for the formation of the glutamate from Ketoglutaric acid and ammonia is to be found in the enzyme which catalyzes the reaction:

$$\text{glutamic} \longrightarrow \text{Ketoglutaric} + NH_3$$

This enzyme was demonstrated in kidney (Krebs, 60), in certain seeds (Anderson, 61), and recently in legume seedlings (62). Euler has studied the mechanism in some detail in liver and con-

cludes that two reactions are involved:

$$\text{Glutamic} + \text{Co} \xrightarrow{1} \text{imminoglutaric} + \text{CoH}_2$$

$$\text{iminoglutaric} + \text{H}_2\text{O} \xrightarrow{2} \text{ketoglutaric} + \text{NH}_3$$

Reaction 1 requires a specific protein and coenzyme I or II depending on the source of the enzyme; in higher plants coenzyme I is required. Reaction 2 takes place spontaneously. It thus seems enzymatically possible to convert oxalacetic acid, a carbohydrate degradation product, to aspartic acid by reaction with ammonia, ketoglutaric acting as a nitrogen carrier.

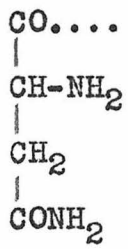
The possibility of direct reaction between oxalacetic acid and ammonia in plant tissue is still open, as the conversion of aspartate to the keto acid has been demonstrated in muscle (60), and presumably the reaction may reverse. Recently Kritzman has claimed to have found an enzyme in plants that will catalyze the transamination reaction with aspartic acid in a system free of the glutamic-ketoglutaric enzyme, and for which amino acids possibly act as nitrogen donators. (60a)

Virtanen, working in Finland, has claimed that nitrogen fixation in the pea occurs through the formation of the oxime of oxalacetic acid, which then forms aspartic acid (64). Hydroxylamine was suggested as a possible intermediate although there is at present no evidence on this point. Virtanen has also reported finding as much as 1 mg. of oxalacetic acid per gram of pea tissue, and states that even excised pea nodules will fix nitrogen if supplied with the acid. The reaction was postulated thusly:

$$N_2 \ (NH_2OH \quad ?) + \begin{matrix} COOH \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{matrix} \longrightarrow \begin{matrix} COOH \\ | \\ CH_2 \\ | \\ CNOH \\ | \\ COOH \end{matrix} \longrightarrow \begin{matrix} COOH \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{matrix}$$

Wyss and co-workers at Wisconsin have not been able to confirm Virtanen's observations, either with respect to the presence of the ketoacid in legumes or to the fixation of nitrogen by excised nodules (65). It was suggested that a difference in the environment of the two stations might account for the lack of agreement.

The early work on asparaginase has already been mentioned. In addition to the report of Geddes and Hunter, the only significant contribution to an elucidation of the properties of this enzyme has been made by Grassman and Mayr (66). Working with a highly purified preparation of yeast asparaginase, these investigators have showed that the enzyme is specific for l-B-asparagine. Slight activity was found against aspartic acid diamide, but the enzyme was inactive toward glutamine, hydroxy asparagine, asparagyl dipeptides, and the mono- and di-amides of succinic acid. The configurational requirements are satisfied by the following structure:



The enzyme is unstable at pH 5 or below, but is not inactivated by H_2S, pyrophosphate, or cyanide. The optimum pH is in the range 7.8-8.0.

On the basis of the work discussed in this section there appear to be two alternatives with regard to the synthesis of asparagine: it may be assumed either that the above enzyme systems can catalyze the reaction between aspartic acid and ammonia when coupled with energy yielding reactions, or that other enzymes, using unidentified substrates, are required for the synthesis.

II. The Effect of pH on Asparagine Synthesis
--Physiological Considerations--

Even among the early workers in plant physiology who were interested in the origin and role of amides, it was generally known that not all plants contained these compounds, and that some did have large amounts of preformed ammonia. Furthermore it has only been within the last twenty years that the importance of hydrogen ion concentration in biological systems has received its due recognition; it is not surprising therefore, that a clear statement concerning the relation of cellsap pH to amide content was made only as late as 1926. In that year Ruhland and Wetzel (67) published the results of investigations on begonia which showed that some of these plants contained large amounts of preformed ammonia but little or no amide. Associated with this ammonia were high concentrations of oxalic, succinic, and malic acids, which often occurred in sufficient excess to produce pH values of the cell sap as low as 1.3. Old plants or those which had grown on nitrate medium contained but little free acid, and yielded some amide. On the basis of these observations and the fact that approximately neutral cell-sap plants show much more amide than ammonia, Ruhland and Wetzel have proposed a classification in which such plants as begonia are to be called "acid" or "ammonium" types, and more neutral plants the "amide" type. In a later publication (67) rhubarb was also placed in the acid group, and arguments were presented to show that the acid and ammonia arise by deamination of amino acids.

Kultzscher has considerably extended the above work with an exhaustive investigation of the relation of pH of the cell sap to the ratio amide /NH_3 in a large variety of plants (68). It was

almost universally observed that the pH increase in going from one species to another was reflected in an increase in the amide /NH_3 ratio. Below pH 3 distribution was almost entirely in favor of NH_3, and above pH 5 well in favor of amide. These pH values may be taken as the upper and lower limits respectively of acid and amide plants. Between these limits the ratio showed the greatest change with pH and plants in this range were called "mixed types". Kultzscher has concluded from this evidence that an equilibrium exists between amide and NH_3 which depends upon pH. The direction of the pH effect suggests that it is the NH_4^+ ion which is involved in asparagine synthesis.

--Theoretical--

On the basis of physico-chemical data and theoretical considerations it is possible to calculate the distribution of the various reactants which may be involved in amide synthesis. For this purpose the following reaction will be assumed:

$$A^- + NH_4^+ \longrightarrow Asp.H_2O$$

A^- = Aspartate
$Asp.H_2O$ = Asparagine monohydrate

$$(1)\quad K = \frac{(Asp.H_2O)}{(A^-)\ (NH_4^+)}$$

Since the concentrations of A^- and NH_4^+ depend upon pH, this latter factor must also be considered. The relations are derived in the following manner:

$$A^+ \leftrightarrow A + H^+$$

$$(2)\quad K_1 = \frac{(H^+)\ (A)}{(A^+)}$$

$$A \leftrightarrow A^- + H^+$$

$$(3)\quad K_2 = \frac{(H^+)\ (A^-)}{(A)}$$

$$A^- \longleftrightarrow A^= + H^+$$

$$(4) \qquad K_3 = \frac{(H^+)\ (A^=)}{(A^-)}$$

$$(5) \quad \Sigma A = A^+ + A + A^- + A^=$$

ΣA = all forms of aspartate

$$NH_4OH \leftrightarrow NH_4^+ + OH^-$$

$$(6) \quad K_b = \frac{(NH_4)\ (OH)}{(NH_4OH)}$$

$$(7) \quad \Sigma NH_3 = (NH_4OH) + (NH_4^+)$$

From the simultaneous equations (2) to (5) it is possible to solve for (A^-) in terms of known or stipulated quantities:

$$(A^-) = (\Sigma A) \times \left\{ \frac{(H^+)\ K_1\ K_2}{(H^+) + K_1 \times (H^+) + (H^+)K_1K_2 + K_1K_2K_3} \right\} = (\Sigma A)\ f_1$$

Similarly from equations (5), (6), and (7):

$$(NH_4^+) = (\Sigma NH_3) \left\{ \frac{(H^+)\ K_b}{KW + (H^+) \cdot Kb} \right\} = (NH_3{}^{\Sigma}) \cdot f_2$$

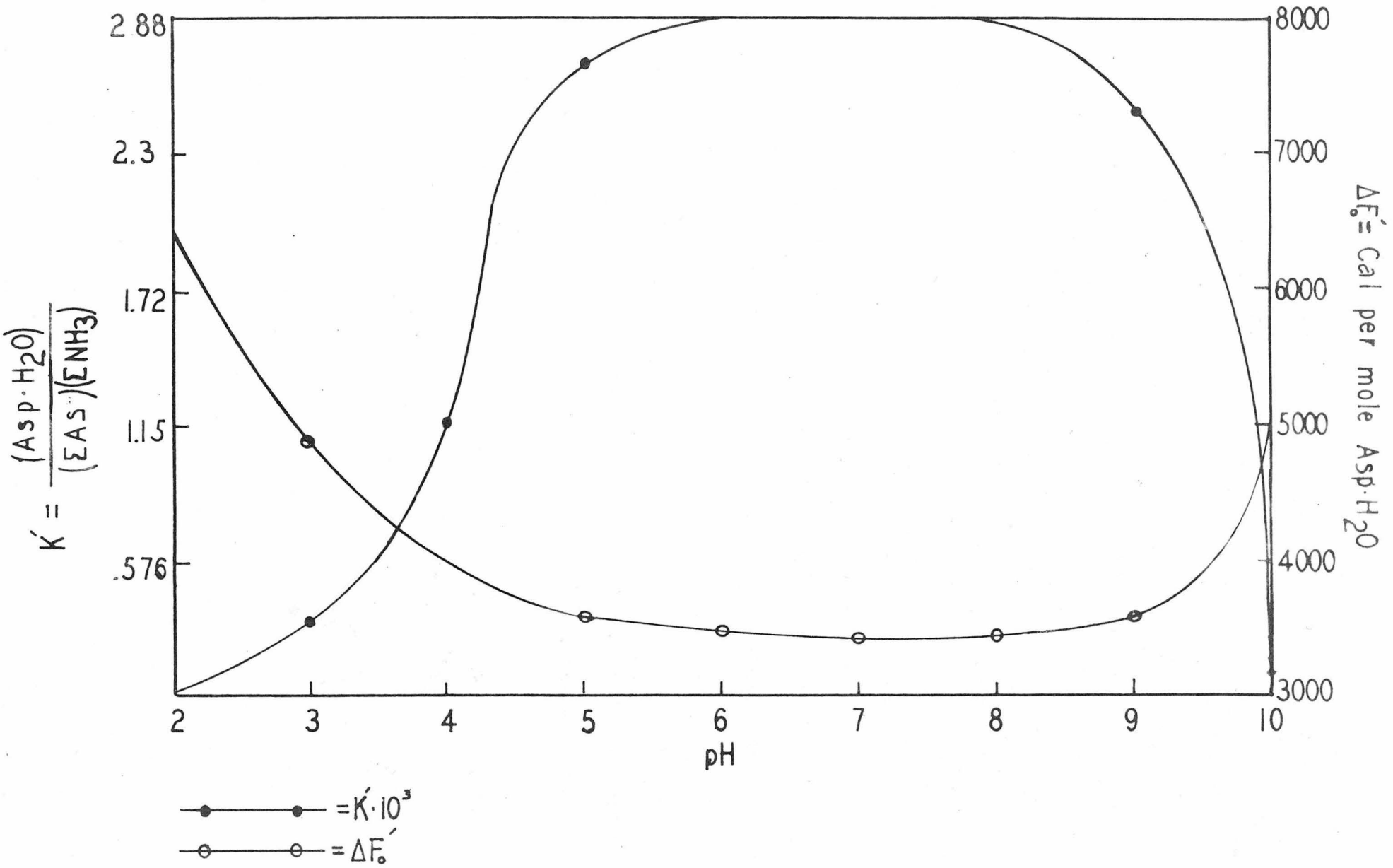
Substituting in equation (1)

$$Kf1f2 = \frac{(asp.H_2O}{(\Sigma A)\ (\Sigma NH_3)} = K'$$

From thse considerations it may be seen that K varies with the (H^+) and consequently with the pH. Using values for K_1, K_2 , and K_3 obtained from the titration data of Greenstein (69), values for K′ at various pH's have been calculated and plotted. The graph shows a theoretical basis for the physiological observations previously discussed. Above pH 5 the equilibrium shifts in favor of amide synthesis (amide type) and below pH 3 in favor of hydrolysis (ammonia type). The intermediate range indicates conditions in the mixed type plants.

The standard free energy of synthesis of the asparagine hydrate was calculated according to the relation

$$\Delta F.' = -1365 \log K - 1365 \log f_1f_2$$


2.88
2.3
1.72
1.15
.576
8000
7000
6000
5000
4000
3000
2
3
4
5
6
7
8
9
10
pH
K' = (Asp·H2O) / (ΣAs)(ΣNH3)
ΔF°' = Cal per mole Asp·H2O
= K'·10³
= ΔF°'

The value of K was obtained from the free energy data of Huffman, Ellis, and Fox (70), recalculated as values of the free energy of formation in aqueous solution of l-aspartate, NH_4^+, and asparagine monohydrate (Borsook and Huffman, 71)

The change in the free energy of synthesis of asparagine with pH is plotted on the same chart as that showing the variation of K' with pH. The curve indicates that the minimum energy requirement for synthesis is above pH 5.

The foregoing discussion is offered only to illustrate a possible physico-chemical basis for the relation of cell sap pH to the amide/ammonia ratio. Experimentally only very approximate values for this ratio can be determined, especially when the ratio differs considerably from unity. The true "thermodynamic" ratio, which is the value that applies in theoretical considerations, would be extremely difficult to determine. A few examples of the experimentally determined ratio taken from the analytical data of Kultzscher are shown below:

Plant	pH	amide/ammonia (leaf)
Medinilla mag.	1.92	only NH_3
	2.06	.04
oxalis dep.	1.53	only NH_3
	1.72	.58
rumex scu.	2.09	only NH_3
	2.47	.5
polygonum cusp.	3.8	.7
beta vulg.	6.36	1.9
phaseolus multif.	5.59	38
lupinus alb.	-	190

These values correspond with the theoretical curve only in a general way; in the pH region around 2 ammonia is very much in excess of amide, in the pH 5-6 region amide is definitely in excess, while in the intermediate region the ratio has intermediate values. In view of the lack of information on the activities of ammonia and amide in plants it seems futile to attempt to speculate as to whether a true equilibrium exists in the tissues between these constituents, especially since other factors in the reaction are unknown. However it may be pointed out that the data show that in the neutral pH region the reaction appears to be shifted far toward the side of amide synthesis.

III . Materials and Methods

Introduction

The work to be described here is a study of the synthesis of asparagine in plants. The only way open at present to attack this problem is by means of in vivo experiments, as in vitro coupled reactions involving the synthesis are at present unknown. Two general approaches immediately suggest themselves, namely the method of vacuum infiltration, and the simple feeding method, both of these being devices for introducing compounds into the metabolic mill without destroying the synthesizing apparatus of the plant.

Vacuum infiltration requires the use of leaves, which contain relatively large amounts of protein; the severe limitations of protein-rich tissue for studying synthetic reactions involving the structural units of protein itself have been pointed out, and this method was not considered for the reasons given.

Hitherto, feeding experiments have consisted in growing whole plants in synthetic nutrient media, or in placing excised structures such as leaves or stalks in such media, and analyzing the material after some hours or days. When germinating whole plants are used the energy requirements may be furnished by carbohydrate already present in the cotyledons, but protein reserves are also present and undergo hydrolysis during the early stages of growth. In excised leaves and stalks the whole metabolic picture is one of catabolism, with synthetic processes occurring only as side reactions. In order to avoid the difficulties inherent in such material, it was decided to use isolated structures which would contain minimal amounts of protein, and which actually could grow on carbohydrate and nitrogen supplied artificially.

Since Schulze's early work on the occurrence of asparagine in lupinus, this genus has become the classical material for studies on the origin of the amide. A preliminary investigation of the nitrogen metabolism of this plant during germination has been carried out in this laboratory, with the observation that hydrolysis of the protein of the cotyledon results in an accumulation of asparagine in the hypoctyl (72). It was not known whether the actual synthesis of the amide occurred in the cotyledon or stem, but on the assumption that the latter structure had the potentialities for carrying out this reaction, attempts were made to grow the excised hypocotyl (with root) on an artificial medium. When this was accomplished it was then possible to analyze the tissue, which will hereafter be called the embryo, before and after periods of culture on various media, and to ascribe the metabolic changes to synthetic reactions. The interpretation of such feeding experiments is based on the assumption that if the embryo is equipped to utilize the substances in the medium it will absorb them in its growth, and conversely failure to absorb suggests inability to utilize. If a compound is utilized in metabolic reactions then it may be suspected that it arises naturally in the plant. Final proof of the normal occurence of a suspected intermediate rests upon its isolation from the plant.

This method falls far short of being a true "tissue culture" as defined by White (73). The recent success attained in the potentially unlimited growth of roots (Bonner,74, White,75) offers a nearer approach to such a culture, and nitrogen metabolism studies of this material may be expected to yield fundamental information as to the processes carried out by these organs.

Technique of the Culturing of Excised Embryos

GERMINATION

The Hartwegii species of dark blue lupin was used throughout, as in former experiments. At the end of a 3 day germination period in a moist chamber the embryos are about 13 mm. long. This is a convenient length for "planting" and at this stage the root and hypocotyl tissue contain but little protein. These embryos were to be cultured under sterile conditions in agar medium contained in 125 ml. conical flasks, hence the most difficult problem was to sterilize the embryos and carry out the culturing under aseptic conditions. At first the seeds were germinated with no special precautions and the hypocotyls cut from the cotyledons at their junction with the latter. Attempts were then made to sterilize these excised embryos by treatment with various dilutions of formaldehyde, bromine water, phenol, mercuric chloride, and calcium hypochlorite. In all cases where the treatment was vigorous enough to actually sterilize the tissue, severe injury occurred as evidenced by discoloration and eventual death of the embryo.

It was necessary therefore to sterilize the seeds before germination while the hull was still intact, and to carry out all the subsequent operations under aseptic conditions. This was satisfactorily accomplished by the following method: About 200 seeds were treated with 95% alcohol for 2 minutes, then placed in .1% mercuric chloride for 20 minutes. Following this, the seeds were washed once with sterile redistilled water and set out on moist filter paper in Petri dishes. At the end of 3 days in the dark at 25 degrees the embryos were ready for culturing.

CULTURE METHODS

Using instruments dipped in alcohol and flamed, and working under a glass plate in a sterile room previously sprayed with a dilute Lysol solution, 5 germinated plants at a time were transferred to a sheet of sterile filter paper and the embryos cut from the cotyledons. The cotton plug was removed from a 125 ml. culture flask, five small punctures made in the agar medium with a pair of long thin forceps, and the excised embryos inserted into the agar. The plug was replaced, the instruments sterilized, and the operation repeated using 5 more plants and another filter paper and flask.

CULTURE MEDIA

Only after a satisfactory culture method had been worked out was it possible to proceed with the problem of developing an acceptable basic culture medium. At the outset it should be mentioned that in addition to agar as a substrate, paraffined wire netting suspended over the nutrient solution, and sand soaked in the nutrient was tried. In the case of the netting, such a large area of liquid was exposed that it was almost impossible to avoid infections after a few days. Sand had no advantages over agar and in addition was difficult to saturate to just the same degree in each of several flasks. Hence agar at a concentration of 1.5% was used in all experiments, and was quite satisfactory.

Attention was first given to the inorganic salt constitution of the medium. The mixtures of Kogl and Haagen-Smit (76), of White (77) and of Bonner and Addicott (78) were the only ones tested. These solutions were made up to 10 times the final concentration and diluted when used. Water redistilled from an all-Pyrex still was used in all solutions. The initial experiments consisted in culturing excised embryos in media containing these mixtures plus

20 mg.% $(NH_4)_2SO_4$, 100 mg.% vitamin B_1 hydrochloride, and 3% sucrose (this choice of sugar and concentration was confirmed as satisfactory in later experiments). At room temperature and in ordinary diffuse daylight the embryos attained their maximum growth in about 12 days, and at this period were removed and the length of the hypocotyl measured. The increased length was almost indentical for the three different media. It was finally decided to use White's medium because of the presence of certain trace elements (iodine and mangenese), although there was no evidence that these factors are necessary. Bonner's mixture was superior to that of White for the lupin variety Texensis.

Following is the salt composition of the medium used. (10x):

$Ca(NO_3)$. $4H_2O$	1050 mg. per liter
KNO_3	800
KCl	650
KH_2PO_4	125
KI	7.5
$MnCl_2$. $4H_2O$	57
$ZnSO_4$	15
H_3BO_3	16
Fe tartrate (separate solution)	25

It seemed not unlikely that the addition of small amounts of organic nitrogen would further promote the growth of these excised embryos, especially in view of the fact that in the early stages of development there were no secondary leaves to carry on photosynthetic reactions which might otherwise furnish certain growth factors or amino acids. This organic nitrogen was added as a mixture of amino acids. Because of the time required to work out the actual requirement and concentration of each acid, a mixture

of the following composition was prepared and diluted 1-100 upon use (this mixture was chosen from those amino acids recommended by Bonner and Addicott and by White for isolated roots):

dl- phenylalanine
l- tryptophane
dl- isoleucine
d- glutamic acid
l- asparagine
leucine
d- valine
d- arginine
l- histidine..HCL
d- lysine. 2HCL
glycine
d- alanine
dl- serine
l- cystine
d- ornithine
proline

Stock solution contained 31 mg.% of each amino acid.

As used in the final medium, the above mixture afforded a concentration of 5 mg. of total amino acids per 100 ml. of solution. These compounds were not added for the purpose of acting as mass nutrients but rather to supply the embryo with small amounts of indispensible amino acids. The substitution of this mixture for the inorganic nitrogen promoted about the same growth in length of hypocotyl as did the latter, but a somewhat more extensive development of secondary leaves was observed on the amino acids. To recapitulate, the medium finally adopted contained White's inorganic salts, 100 mg.%

thiamin chloride, 3% sucrose, the amino acid mixture, and 1.5% agar. Each cluture flask contained 30 ml. of the medium.

STERILIZATION OF MEDIA

The basic medium was conveniently sterilized by autoclaving for 20 minutes at 15 lbs. pressure. However when $(NH_4)_2SO_4$ was added at concentrations of 250 mg.% or higher, some decomposition of the sucrose took place. When this occurred the basic medium and the added substances were either autoclaved separately and mixed under sterile conditions in proportions to give the correct final concentration, or the final medium was steamed for 15 minutes on three successive days.

In several experiments extremely labile substances were used; these were sterilized by passing the concentrated solutions through 5 on 3 Jena sintered glass filters and then adding them to the basic medium. These instances will be discussed in more detail in later sections.

CULTURE CONDITIONS:

Experiments were conducted to ascertain any possible effects of etiolation on the nitrogen metabolism of the embryos. Analytical data on 12 day old embryos grown on the basic medium indicated that there was no great difference in the metabolic picture between etiolated and illuminated plants. However, illumination would bring out potentialities for leaf development, hence the plants were exposed to diffuse daylight plus light from a 100 watt bulb placed at a distance of 16 inches from the flasks. This lamp was automatically controlled to operate between 6 A.M. and 6 P.M.

The temperature of the culture room was maintained between 23 and 25 degrees C. by means of an electric heater.

Analytical Methods

EXTRACTION OF THE TISSUE

In every experiment 5 flasks containing 5 embryos each were set up. Occasionally extensive infections occurred compelling the rejection of a whole experiment, but usually it was possible to obtain from 18 to 22 sterile plants for analysis. After washing away adhering agar, the hypocotyl length was measured and the tissue placed in a heavy walled test tube fitted to receive a glass grinding rod. A small amount of acid-washed sand was then added and the material ground to a pulp. After centrifuging, the supernatant tissue fluid was decanted into a volumetric flask. One ml. of 4% Na_2SO_4 solution was then added to the pulp, the grinding and centrifuging repeated, and the supernatants combined. This was repeated until the supernatant above the pulp became clear; the combined extracts were then made to volume and mixed.

The method of extraction as outlined above presents several important advantages over the procedures generally followed in studies of this kind. The use of 4% Na_2SO_4 insures that globulins as well as albumins will be extracted, the former being soluble in dilute salt solutions, and constituting the bulk of most plant proteins. Since boiling, extracting at high temperatures, and drying were not involved there was no heat hydrolysis of such compounds as asparagine, glutamine, peptides, or protein. The relatively short time required for the process ($\frac{1}{2}$ hour) minimized changes due to enzymatic action, which was cut short by the addition of trichloracetic acid to precipitate the protein. The pH range of the extract was well out of that required for the action of asparaginase, a point of especial importance in these studies. In general it was possible to extract from 96 to 98% of the total plant nitrogen, and

by "total N" will be meant that amount determined in extracts prepared as above.

DETERMINATION OF NITROGEN FRACTIONS

The system of micro analysis developed by Borsook and Dubnoff (79) was used to analyze the tissue extract. The final determination in all cases except for amino N depends upon the micro-distillation of ammonia, and titration of the excess acid with .03n NaOH delivered from a micro burette, using a glass electrode to indicate the end point. The distillation vessels were turned on the lathe from Lucite, and consisted of an inner circular well to contain the excess of standard HCl, and an outer concentric well to contain the sample to be analyzed. The addition of alkali to the sample, quickly followed by covering the vessel with a glass plate, results in the distillation of ammonia, which quantitatively passes over into the acid in the central well in 2 hours when the vessels are placed in an air bath at 40°. The procedure for each fraction will be briefly outlined in the following paragraphs.

TOTAL AND PROTEIN NITROGEN

One-tenth ml. samples of the extract were delivered into micro digestion tubes, digestion mixture was added and the excess water driven off at 110° in an oven. The digestion was completed by placing the tubes on a metal rack and heating until the solution became colorless. The digest was made to volume and aliquots treated with 25% NaOH in distillation vessels. The ammonia driven off was determined as total N.

The original extract was made 2% in trichloracetic acid to precipitate proteins. A N determination was made on the resulting NPN extract and the difference between total N and NPN reported as protein N.

AMMONIA NITROGEN

The ammonia liberated from the NPN extract by the action of pH 10 borate buffer represents ammonia.

AMIDE NITROGEN

The most important and possibly the only amide occurring in lupins is asparagine (Schwab, 49). The carbamino group of this compound is hydrolyzed by treatment with 1n. H_2SO_4 in a boiling water bath for 3 hours (Pucher, 80). The ammonia thus liberated was determined by micro distillation, and after being corrected for preformed ammonia was reported as amide N.

AMINO NITROGEN

An aliquot of the NPN extract was carefully brought to pH 7. An equal volume of 1:1 formaldehyde was added, and after two minutes the solution was titrated to pH 8. Corrections were made for preformed ammonia and the formaldehyde blank. This pH range of titration is an arbitrary one but quantitatively determines the amino N of asparagine, which constitutes most of this fraction.

ERRORS

The largest analytical errors occurred in the determination of total N. It has been generally observed by many workers that plant proteins are difficult to digest; in addition to this source of trouble, lupins contain several alkaloids (lupanine, sparteine, anagyrin), certain resistant amino acids such as arginine, histidine, and tryptophane, and unknown nitrogenous compounds. Although total N and NPN determinations were carried out in triplicate, differences of 3-5% were often observed, and analytical errors of this magnitude must be accepted for total and protein N. The differences between duplicates in the analyses of the other fractions was less than 1%.

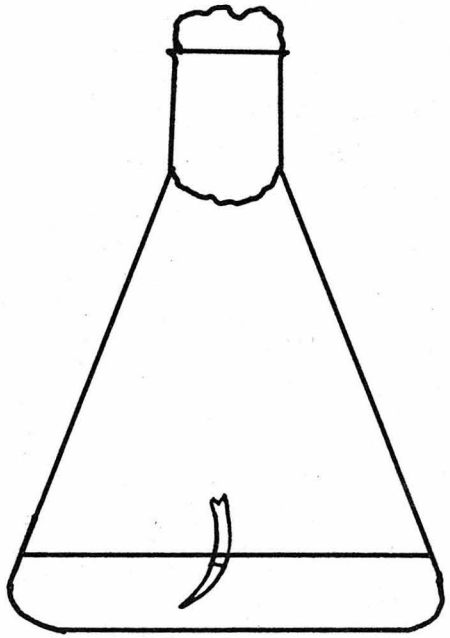
An average of 20 plants were taken for analysis. This relatively small number introduces a significant sampling error. Since there was not enough "spread" in the number of plants analyzed in each of several identical experiments, it was impossible to calculate directly the magnitude of this error. However, one experiment was repeated three different times, taking the same number of embryos for analysis. From the analytical data it was found that the standard deviation from the mean for total N was 2.4 mg. nitrogen per 50 plants. Since protein N was determined by the (relatively small) difference between two such values, the calculated standard deviation for this fraction was a high as 3.3 mg. nitrogen per 50 plants. Actually maximum differences for protein N were of the order of magnitude of 2 mg. The standard deviations for amide and amino N were 0.4 and 0.25 mg. nitrogen per 50 plants respectively. Mean values in the above considerations were, total N 27.5, protein N 6.6, amide N 8.3, and amine N 9.8 mg N per 50 embryos

In assessing the significance of the above data two points must be considered. First, the deviations represent composites of both the sampling and analytical errors. Second, the data are based on only three experiments. It is probable that if a larger number of identical experiments had been carried out the errors as given above would be somewhat smaller.

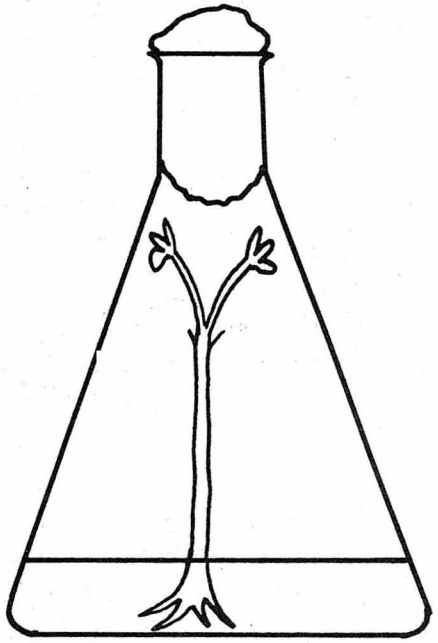
Scheme for the Culture of Excised Embryos

3 day old seedling

excised embryo



excised embryo in agar medium



12 day old embryo

IV. Four-carbon Dicarboxylic Acids as Asparagine Precursors

-Effect of Dicarboxylic Acids at Low Ammonium Concentration-

Until recently it has been the commonly accepted view that ammonia is toxic to plants; indeed some workers believe asparagine synthesis to be a mechanism for ammonia detoxication. It still remains to be shown whether these toxic effects are to be ascribed to the ammonium per se or to indirect metabolic causes. However, in the experiments reported here it was tacitly assumed that high ammonium concentrations were harmful, and $(NH_4)_2SO_4$ was fed at a low level.

Media were prepared containing the basic constituents plus 20 mg.% $(NH_4)_2SO_4$ and various 4-carbon dicarboxylic acids. These media were brought to pH 4.7 to correspond to that of the control, sterilized, and innoculated with 3 day old excised embryos. Following a 12 day culture period, the tissues were analyzed; the data are reported in terms of 50 embryos. Analytical data on unclutured 3 day embryos are also reported to show the order of magnitude of the synthetic processes which took place.

	1 3 day embryos	2 Control. Basic 20mg% $(NH_4)_2SO_4$	3 100 mg% Malate	4 500 mg% malate
Total N	8.1	21.6	24.4	22.5
Protein N	3.8	4.5	2.9	3.0
Amide N	.75	6.5	6.1	6.1
Amino N	2.6	8.1	8.7	9.6
Length (mm)	13	26	38	32

Experiments 1 and 2 show clearly that the increases in amide and in amino N are the result of synthetic reactions, as there is no significant change in protein N. It is also of interest to note the increase in the amide/amino ration from exp. 1 to 2. Comparison

of exps. 3 and 4 with the control indicates that malate has no ability to promote the synthesis of asparagine at this low ammonium level, although it appears to stimulate growth in the length of the hypocotyl.

The next group of substances tested consisted of maleate (cis-isomer, fumarate (trans isomer), and succinate. The experiments were carried out in the same manner as that with malate.

	5 100 mg.% maleate	6 500 mg.% maleate	7 500 mg.% fumarate	8 500 mg.% succinate
Total N	20.2	19.2	16.9	19.0
Protein N	4.2	6.2	4.1	5.3
Amide N	4.4	3.7	4.0	5.2
Amino N	7.1	5.8	6.2	7.4
Length (mm)	29	26	27	26

These results indicate that the above compounds were unable to stimulate N metabolism or promote growth when added to a basic medium very low in ammonium. A slight indication of protein synthesis is seen in experiments 6 and 8.

It was clear that in these experiments the limiting factor in asparagine synthesis was the available N rather than the carbon skeleton, which was adequately supplied by the carbohydrate in the medium. Accordingly the ammonium level of the basic medium was raised in subsequent experiments in order to supply more nitrogen and thus tax the synthesizing mechanism and the carbon skeleton supply.

-Effect of Dicarboxylic Acids at Moderate-

Ammonium Concentrations

When the basic medium was autoclaved in 250 mg.% $(NH_4)_2SO_4$ solution the sucrose was partly decomposed and the mixture became

brown. To avoid this difficulty the ammonium-free basic medium was autoclaved in 150 ml. flasks, and 2 ml. of a sterile $(NH_4)_2SO_4$-dicarboxylic acid solution added before the agar set. The strength of this added salt solution was adjusted to result in the final desired concentration in the medium. The results of feeding experiments using this basic medium are shown below:

	9 Control. 250 mg.% $(NH_4)_2SO_4$	10 250 mg.% malate	11 500 mg.% malate
Total N	22.2	25.0	14.8
Protein N	1.9	1.8	2.7
Amide N	7.3	6.8	3.9
Amino N	7.6	8.4	5.5
Length (mm)	31	24	27

The above data indicate that a high concentration of malate acting in conjunction with a moderate ammonia concentration inhibits the absorption and utilization of ammonia (exp. 11). Malate at a moderate concentration has no significant effect on amide synthesis. This observation is not in accord with Smirnow's, who reported an increase in asparagine content when malate was fed to maize seedlings (20). According to Vickery, malic acid occurring in tobacco leaves is not converted to asparagine but rather to citric acid (18). Franzen and Keysner cite two early references in which it is claimed that malic acid was found in lupins, but these reviewers state that the evidence was poor in both cases (55).

Although malate did not stimulate amide synthesis the possiblity still existed that the reaction: malate oxalacetate 2H did occur. To investigate this possibility an experiment was conducted as follows: hypocotyl tissue was ground with phosphate buffer, the mixture centrifuged, and both residue and supernatant tested for

dehydrogenase activity in presence of coenzyme by the Thunberg technique, using methylene blue as hydrogen acceptor and boiled yeast extract as a source of the coenzyme. No difference in color of the dye was observed after two hours between a control and a test with malate as added substrate. This experiment taken in conjunction with the analytical data argues against the existence of enzymatic mechanisms for the metabolism of malate.

Passing now to the utilization of succinate and fumarate at moderate ammonia concentrations, experiments were carried out with these substrates as indicated:

	12 500 mg.% succinate	13 500 mg.% fumarate	14 100 mg.% fumarate
Total N	21.3	18	31.4
Protein	4.4	2.3	3.7
Amide	5.5	5.6	7,9
Amino	6.6	6.6	9.4
Length	21	18	35

These data show that succinate and fumarate at concentrations of 500 mg.% were not effective as amide precursors and even caused slight inhibition of the synthesis. Succinic dehydrogenase was not demonstrable in hypocotyl extracts. Fumarate at 100 mg.% promoted a considerable increase in total N and a moderate increase in protein and amino N and in length of hypocotyl. The increase in amide over the control was insignificant. In view of the extensive evidence adduced by Schulze, Mothes, and Smirnow that under favorable conditions of carbohydrate nutrition asparagine is converted to protein, it seems justifiable, in interpreting these experiments, to consider the increases in both amide and protein N (and indirectly total N) as indices of asparagine synthesizing ability.

On this basis fumaric acid may be classed as a pos ible precursor of the amide, although the effect is not pronounced.

So far as the writer is aware there have been no published attempts to test maleic acid as an amide precursor, possibly because this compound has not been reported as arising under physiological conditions. In animal tissues (muscle) Thunberg, Szent-Gyorgyi and others have noted that this acid inhibits respiration. It is known to poison succinic dehydrogenase, possibly by combining with the free sulfhydryl groups of the enzyme (Morgan,81). It was surprising therefore to find that this compound stimulated markedly the N metabolism of the excised lupin embryo.

	15 500 mg.% maleate	9 control
Total N	37.2	22.2
Protein	3.2	1.9
Amide	9.4	7.3
Amino	11	7.6
Length	26	31

Compared with the control these embryos showed very little development of roots and secondary leaves, but the effect on N metabolism seems clear. There was no large accumulation of free ammonia. No explanation is offered for this observation.

V. Carbon Sources Other Than Sucrose as Substrates

Few attempts have been made to replace the usual carbohydrate sources employed in asparagine studies with simpler compounds. Glucose has been used most frequently and can supply the carbon skeleton for amide synthesis. Experiments 1 and 2 show that sucrose likewise is utilized, and other experiments indicated that it is slightly superior to glucose for work of this kind.

If amide synthesis could be shown to occur at the expense of intermediates involved in carbohydrate metabolism, a possible path for the conversion of sucrose or glucose to the amide might be revealed Conversely, if a carbohydrate source were found which would support growth but could not act as an asparagine precursor it would then be possible to clearly define the effects of added tes substances. To investigate these possiblities media were prepared containing 100 mg.% $(NH_4)_2SO_4$, and various carbohydrate intermediates instead of sucrose. The pH was adjusted to 5.8 to correspond with normal tissue juice and the sterilization carried out by steaming. The details of the procedure were as in preceeding experiments. The results are given in the following tables:

	16 Control No CHO	17 1.5% hexose- diphosphate	18 1% gluconate
Total N	No growth. Embryos not analyzed.	No growth	12.4
Protein			1.8
Amide			1.5
Amino			5.5
Length			18

	19 1% glycero-phosphate	20 1% glycerate	21 1% glycerol
Total	14.9	13.1	13.8
Protein	1.2	.9	2.2
Amide	3.8	3.1	3.4
Amino	5.9	5.2	5.3
Length	20	22	23

	22 1% lactate	23 1% pyruvate
Total N	12.7	8.9
Protein	1.1	1.5
Amide	3.1	2.1
Amino	4.6	2.9
Length	15	19

These data show that the N uptake in all cases was small compared with that when sucrose was used and the amide synthesis was insignificant. All thse embryos grew poorly and exhibited little or no development of roots or secondary leaves. The results with hexesediphosphate were especially disappointing as it might be expected that this compound would be more readily absorbed than hexese. The failure of pyruvate and lactate to act as amide precursors was reported by Mothes (48) and is confirmed by the data from experiments 22 and 23.

Glucose and fructose were compared with sucrose in the following experiments in which ammonium sulfate was 250 mg %.

	3% sucrose	3% glucose	3% fructose
Total N	26.9	24.5	21.1
Protein	4.0	2.6	3.5
Amide	6.9	7.1	6.0
Amino	6.9	8.5	6.7
Ammonia	3.0	3.5	2.7
Length	27	21	21

These experiments indicate only slight differences in the amide promoting properties of the above sugars and suggest that configurational differences in these compounds are not important in the problem of amide synthesis.

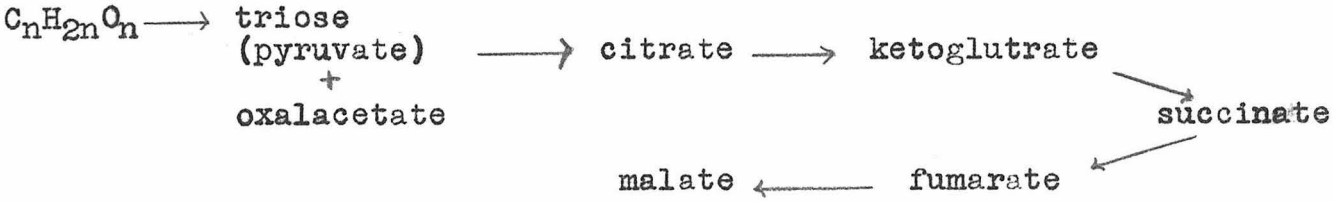
VI. Asparagine Synthesis in Response to the Ammonia Concentration of the Substrate

It was Schulze's view in 1898 that asparagine arises from ammonia derived from the breakdown of amino acids, and that the amide serves as an easily transportable source of nitrogen for protein synthesis in growing regions. His pupil Prianischnikow proposed a somewhat different concept of the role of the amide, namely that it was a mechanism for the detoxication of ammonia. The latter concept has become the classical explanation of the formation of the amide. It is true that ammonia fed to certain species via the nutrient medium or through vacuum infiltration is largely converted to the amide, but there is a growing body of evidence that this mechanism is not exclusively an ammonia detoxication. A priori there is no more reason to consider ammonia as toxic than there is to consider any other readily absorbed ion as toxic if fed at very high concentrations, and indeed the very fact that ammonia can be utilized by the plant argues against it's toxic nature. Foreign compounds substances known to be excreted (such as CO_2), and enzyme poisons, may justifiably be called toxic.

An excellent example of the non-toxic nature of ammonia is to be seen in the so-called "acid plants", some of which can tolerate as much as 37% of their total N as preformed ammonia. Certain species of oxalis, medinilla, and rumex have been found to contain large amounts of this compound and yet be entirely free of amides (page 30). These plants showed no symptoms of ammonia "poisoning", possibly because of an adequate carbohydrate metabolism, the disruption of which may well be the fundamental cause of the usual toxic symptoms observed when ammonia rises to abnormal levels.

This departure from the classical concept has been championed by Burkhart and by Vickery. The former writer stated that he "prefers to think of the pathological condition during which ammonium accumulates as caused by an unfavorable complex of conditions in which depletion of available carbohydrate plays a predominant role, and that the accumulation of ammonia is probably a resulting phenomena, but not a cause of the conditions" (36). Burkhart's data on the ammonium nutrition of seedlings indicate that amide synthesis from ammonia derived from the medium occurs only as long as carbohydrate is available. Depletion of carbohydrate results in the accumulation of free ammonia and the amides themselves are partly decomposed. In this respect amide synthesis is a reflection of carbohydrate metabolism and hence of respiratory activity and may be influenced by ammonia only when this ion becomes limiting or causes osmotic or physico-chemical disturbances.

Vickery (82) has developed a similar interpretation of asparagine and glutamine synthesis based on Chibnall's discussion (3) of the Krebs citric acid cycle as applied to plants. This cycle provides a mechanism whereby oxalacetic and ketoglutaric acids may arise from carbohydrates as outlined thus:



These keto acids then supposedly serve as carbon skeletons for the corresponding amides, and their supply thus controls the rate and extent of the synthesis. To illustrate the minor influence ammonia has on the conversion, Vickery grew tobacco plants in media containing various concentrations of ammonia, with the following results:

ammonia / total N (medium)	ammonia / NPN-NO3 (plants)	amide / NPN-NO3 (plants)
0	2.2	5.1
40	3.9	6.2
60	10.3	5.5
80	26.6	6.8

These figures clearly show that in tobacco the free ammonia may increase ten-fold and yet evoke no significant increase in amide formation.

The effect of increasing concentrations of ammonia on the asparagine synthesis in isolated lupin embryos has been similarly studied. Media were prepared containing the basic constituents plus ammonium sulfate at various concentrations. These solutions were adjusted to pH 7, sterilized by successive steamings, and innoculated with 3 day embryos. In 12 days the plants were analyzed by the methods previously described, and the results are shown in the following tables:

	24 Control.No ammonia	25 20 mg.% $(NH_4)_2SO4$	26 250 mg.% $(NH_4)_2SO4$	27 500 mg.% $(NH_4)_2SO4$
Total N	16.2	21.6	26.9	24.1
Protein	1.0	4.5	4	4.2
Amide	3.0	6.5	6.9	5.3
Amino	4.7	8.1	6.9	6.4
Ammonia	.07	.4	3.0	5.4
Length	25	29	30	

	28 800 mg.% $(NH_4)_2SO_4$	29 1000 mg.% $(NH_4)_2SO_4$	30 1500 mg.% $(NH_4)_2SO_4$
Total N	27	24	Toxic
Protein	4.4	3.6	
Amide	6.0	4.7	
Amino	---	5.5	
Ammonia	6.6	6.7	
Length	27	23	

These data amply confirm the principle that if ammonia is supplied over a minimal value, increasing concentrations cause no corresponding increases in the ammount of asparagine synthesized, even in an "amide" plant. Between values of ammonium sulfate concentration of 20 to 800 mg.% in the medium a twenty-fold increase in ammonia content of the tissues was produced. This ammonia increase had no significant effect on the amount of asparagine synthesized, which remained almost constant. At a concentration of 500 mg.% ammonium slufate caused toxic symptoms such as darkening of the roots and inhibition of secondary leaf formation. These signs of distribution became progressively stronger until there was complete inhibitions of growth at the 1500 mg.% level. However even at 800 mg.% there was some indication of protein synthesis, suggesting that carbohydrate metabolism had not completely failed.

Since in all cases there was an adequate supply of carbohydrate, mere lack of this substrate cannot explain the limited amide formation. Two possible reasons suggest themselves; either the increasing ammonia concentrations inhibited the uptake of sucrose and thus limited the primary carbon supply, or "metabolic activity" acted as the controlling factor by limiting the secondary supply of a proper carbon skeleton for the synthesis. In view of experiments to be

reported later it seems unwise to place the entire burden of synthesis on keto acids supplied by respiratory activity as Vickery has done. The apparent effect of respiration on the synthesis may be more important from the standpoint of energy supply than of carbon supply.

Irrespective of theories as to the role of carbohydrates in amide formation it seems clear that a simple detoxication explanation for this mechanism is an inadequate explanation of the facts presented above.

To determine whether carbon skeletons which might serve as asparagine precursors would exert any effect on N metabolism at high ammonium levels, fumaric and maleic acids were added to media containing 800 mg.% ammonium sulfate. At the same time the effect of etiolation was investigated. The data are shown on the following page.

Illuminated

	31 100mg.% fumarate 800 mg.% $(NH_4)_2SO_4$	32 100mg.% maleate 800mg.% $(NH_4)_2SO_4$
Total N	25.1	23.8
Protein	2.8	3.2
Amide	6.1	5.1
Amino	6.1	6.5
Ammonia	5.5	5.9
Length	19	18

Etiolated

	33 100mg.% fumarate 800mg.% $(NH_4)_2SO_4$	34 100mg.% maleate 800mg.% $(NH_4)_2SO_4$
Total N	27.6	27.5
Protein	3.9	4.6
Amide	6.9	7.1
Amino	8.4	8.1
Ammonia	5.6	6.2
Length	---	---

Experiments 31 and 32 as compared with 28 indicate that these acids are unable to stimulate amide formation when fed at high ammonium levels. Etiolation stimulates the general N metabolism slightly and as regards asparagine formation partly compensates for the inhibitory effect of the ammonia. It is difficult to explain these phenomena, and especially to account for the increased protein in the etiolated embryos.

VII. Organic Nitrogen-Containing Compounds as Asparagine Precursors

General

It is well known that plants growing in soil under natural conditions obtain orgainc nitrogen from decaying vegetable and animal matter. These compounds include amino acids, amides, purines and pyrimidines, urea, and other substances. Such materials contain their nitrogen in a highly reduced form, hence the large energy expenditures required in the reduction of nitrate is unnecessary; furthermore such organic N is frequently already built into carbon chains that may be utilized directly. For these resons it might be expected that certain compounds of this type would be excellent nitrogen sources.

The utilizability of a given material is a composite of the readiness with which it is absorbed and the availability of enzymatic apparatus for its conversion once it is in the tissues. Feeding experiments and subsequent analysis of the plant can only give information on the combined effect of these two factors, but as a working hypothesis it may be assumed (with reservations) that a compound that can be metabolized will be absorbed to some extent. On this basis it has been observed that creatin, nucleic acid, xanthine, guanidine, arginine, histidine, and asparagine are readily utilized by wheat. (84). Virtanen, in 1935, found that oats and barley grew well on substances supplied from legum nodules which grew on peas in the same container. Recently aspartic acid has been identified as an important synthetic product of nodule activity (64) and this acid supplied artificially has been found to act as a good nutrient for legumes and barley, as has also glutamic acid and asparagine (84a). Macht has grown lupins in various amino acids

and found definite growth responses to aspartic acid and leucine (85), and urea and arginine have been used successfully as sources of nitrogen for maize and beans (84b), (84c). However it seems to be a general observation that organic nitrogen is somewhat inferior to ammonia and nitrate as a mass source of nitrogen, but certain organic forms are frequently able to promote the absorption of the inorganic forms to a surprising degree; these effects are probably due to the hormone-like action of such compounds as adenine which stimulates leaf growth. Factors such as sterility, pH, aeration, and the presence of accessory salts in the medium are known to play an important part in the responses of plants to such nutrients.

Organic Nitrogen in Asparagine Synthesis

Many workers in the field of asparagine formation have proposed that aspartic acid may be the immediate precursor of this amide, the reaction consisting of the splitting out of water from the ammonium salt of the acid:

In spite of this obvious relation very few attempts have been made to use aspartic acid as a precursor in asparagine studies. Smirnow (20) fed ammonium aspartate and succinate to maize and obtained results as shown in the following protocol. The figures represent mg. N per 100 plants.

	Seeds	Ammonium Succinate	Ammonium aspartate
Total N	348	468	471
Protein	327	317	330
Ammonia	-	20	21
Asparagine	-	92	78

Smirnow concluded from these data that succinate was superior to aspartate, but he did not consider the possibility of an impaired metabolism as indicated by the slight hydrolysis of protein in the succinate experiment, whereas the aspartate fed plants maintained a positive protein balance.

Mothes, in infiltration experiments on bean leaves, observed that ammonium aspartate was slightly superior to the ammonium salts of malic, succinic, and fumaric acids as an amide precursor but he gave no special emphasis to this point. In tobacco leaves ammonium asparaginate was only half as effective as these N-free acids in this regard (48). Schwab has reported that sodium asparaginate and ammonium bicarbonate were equally effective as asparagine precursors in infiltrated wheat leaves. On the basis of the work discussed on page 21 and of the experiments of Mothes, Schwab concludes that asparagine arises from amino acids derived from the breakdown of protein. The apparent species differences in the responses to the substrates tested and the lack of proper controls in the investigations of these two workers makes it difficult to properly assess their results.

To test the effect of aspartic acid and asparagine on the amide synthesis in isolated lupin embryos, the following experiments were set up, the medium adjusted to pH 7, and techniques for culturing and analysis being identical with those already described. The concentrations of aspartate corresponded approximately with

those of fumarate in preceeding experiments, but asparaginate was used at one half this concentration because of the presence of two nitrogen atoms in its molecule.

	35 570 mg.% aspartate 20 mg.% $(NH_4)_2SO_4$	36 250 mg.% asparaginate No $(NH_4)_2SO_4$
Total N	19.6	41
Protein	3.0	9.3
Amide	5.1	10.3
Amino	8.1	13.8
Ammonia	.1	.1
Length	28	26

	37 570 mg.% aspartate 250 mg.% $(NH_4)_2SO_4$	38 250 mg.% asparaginate 250 mg.% $(NH_4)_2SO_4$
Total N	30.6	47
Protein	7.1	8.4
Amide	8.1	13.7
Amino	10.2	19.3
Ammonia	2.3	3.5
Length	22	30

	39 100 mg.% aspartate 250 mg.% $(NH_4)_2SO_4$	25 20 mg.% $(NH_4)_2SO_4$
Total N	28.7	21.6
Protein	2.1	4.5
Amide	9.5	6.5
Amino	12.1	8.1
Ammonia	1.8	.4
Length	31	26

26
250 mg.% $(NH_4)_2SO_4$

Total N	26.9
Protein	4
Amide	6.9
Amino	6.9
Ammonia	3.0
Length	27

Considering first experiment 35 and using experiment 25 as a control, it appears that aspartate acting with very small concentrations of ammonium is not readily absorbed and has no amide promoting properties. This may be interpreted as showing that aspartic acid is unable to furnish N for the carbon skeleton. Lack of ammonia caused no outward symptoms of metabolic inhibition, as the embryos grew well and developed secondary leaves. Passing now to experiments 37 and 39 and using experiment 26 as the control, slight but definite evidence of increased amide formation in the presence of aspartate is indicated by the analytical data. The smaller amide content in experiment 37 as compared to 39 may be explained by the higher protein content of the embryos grown in 570 mg.% aspartate. That asparagine can serve as a protein precursor is clearly indicated by experiment 36 where a conspicuously high protein content has arisen almost entirely from asparagine N. This latter observation appears to confirm the postulate of Schulze, which was based on the finding that etiolated seedlings, when exposed to light, increased in protein and decreased in asparagine content.

It might be argued that asparagine acts to increase protein content simply by being readily absorbed and hydrolyzed at the

amide group to yield ammonia, which could then be converted to protein via carbohydrate metabolites. If this were the case relatively large amounts of ammonia might be expected to arise in the tissues; actually the value for this fraction was very low in experiment 36 but high in experiment 38, suggesting that the available ammonia in the last experiment was not being readily utilized. These data taken together strongly indicate that asparagine N is directly utilized in preference to ammonia N in the synthesis of protein.

The low protein content in experiment 39 is difficult to explain, especially in view of the high amide value. It seems likely that a rather delicate balance exists between asparagine and protein, and unknown factors possibly prevented the utilization of asparagine to form protein in this experiment.

The next group of experiments were concerned with the properties of the mono- and di-amides of the 4-carbon dicarboxylic acids as precursors of asparagine. The work of Schulow and Petrow on the initial amidization of malic acid has already been discussed on page 16. Their hypothesis that the amidization precedes amination has never been experimentally tested, yet information on this point would be of fundamental importance.

Attempts were first made to prepare the monamide of fumaric acid by the method of Griess (86), in which asparagine is exhaustively methylated and the amino N removed by treatment of the resulting compound with concentrated NaOH, leaving a fumarate skeleton with the amide group on one carboxyl. The product obtained in several trials liberated free ammonia from basic solution indicating that the amide group had been hydrolyzed. The compound described in the original paper was probably the ammonium salt (personal communication from E.R. Buchman). An attempt was also made to prepare the monamide by mild hydrolysis of the cyclic

imide of fumaric acid, prepared by dry distillation of the ammonium salt. Hydrolysis even by warming decomposed the imide to yield the ammonium salt and not the amide.

Although succinic acid had not proved effective as an asparagine precursor, amides of this compound were obtainable and were tested. The monamide was prepared as the Ca salt by hydrolysis of succinimide in $Ca(OH)_2$ solution (87). The diamide was supplied by Mr. W. Baumgarten. These compounds were fed at pH 7 and at molal concentrations corresponding to those of aspartic acid in the preceeding experiments.

	40 88mg.% succinmonamide 250 mg.% $(NH_4)SO_4$	41 87mg.% succindiamide 250 mg% $(NH_4)_2SO_4$
Total N	25	23.3
Protein	2.8	2.9
Amide	6.4	6.0
Amino	9.0	7.3
Ammonia	1.7	2.5
	42 500 mg.% succinmonamide 250 mg.% $(NH_4)_2SO_4$	43 500mg.% succindiamide 250 mg.% $(NH_4)_2SO_4$
Total N	-	37.2
Protein	-	8.2
Amide	7.0	10.1
Amino	9.4	11.8
Ammonia	.97	3.4
Length	22	27

All of the above embryos appeared especially well developed. The data indicate that the two amides are not effective at low concentrations but that the diamide stimulates asparagine formation at the 500 mg.% level. It is to be noted however, that the ammonia content is high in the diamide experiment, suggesting that this compound may

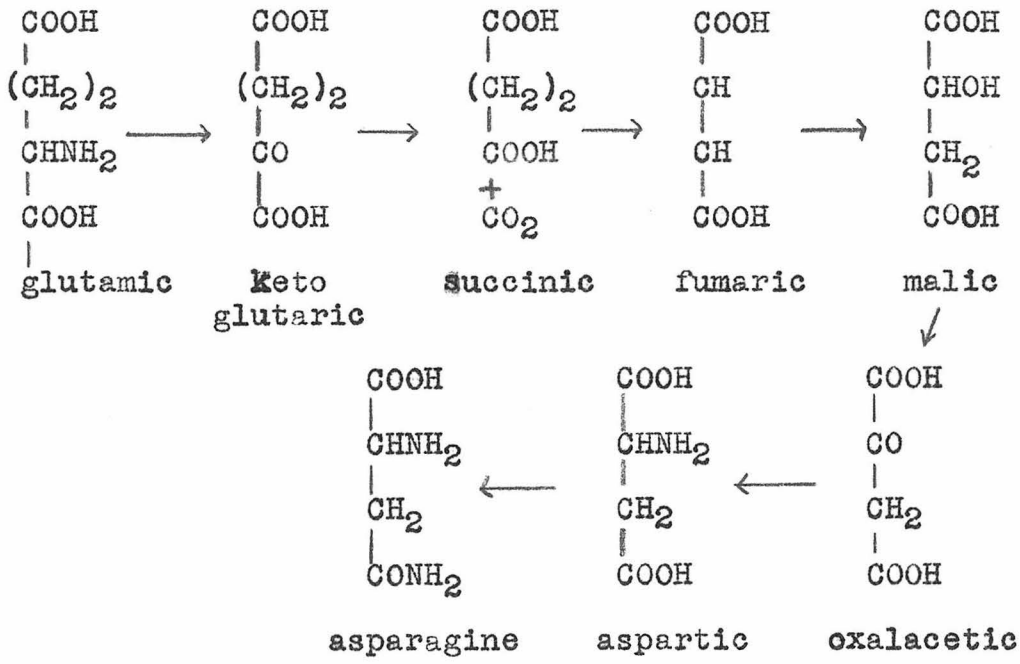
act by increasing ammonia uptake or by being hydrolyzed in the tissues to yield ammonia in metabolically active situations. Experiment 43 represents another illustration of that complex of conditions favorable for both asparagine and protein synthesis. Indirect evidence that the analytical data on amide content represents asparagine amide and not succin-amide is to be found in the fact that the ratio amide N/amino N has the same value as in preceeding experiments; had the succinamides been taken up unchanged this ratio would be greater than unity, whereas it was actually less.

The interpretation of the above group of experiments is obscured by the more effective utilization of the diamide than the monamide in asparagine and protein synthesis. Conclusions as to the mode of action of these compounds must await further investigations.

Attention was next directed to glutamic acid, the 5-carbon homologue of aspartic acid. The writer is aware of no experiment in which this compound has been tested as a precursor of glutamine or asparagine, although it's relation to the former amide is obvious. Schwab infiltrated ammonium bicarbonate and sodium asparaginate and glutaminate into wheat leaves and found four times as much asparagine as glutamine in all cases (this species normally contains both amides in varying ratios). The infiltrated amides stimulated protein synthesis more than did the ammonium bicarbonate (49). Chibnall infiltrated ketoglutaric acid into leaf tissue and demonstrated the simultaneous disappearance of the oxy-acid and the appearance of glutamine(83). The obvious experiment of infiltrating glutamic acid itself was apparently omitted.

The series of reactions whereby possible 4-carbon asparagine precursors could arise from glutamic acid has already been mentioned

in chapter I, part 2. Such a scheme is a follows:



This series of conversions appears unlikely in the lupin embryo because of the failure of succinic and malic acid to serve as precursors, unless it be assumed that these compounds were not absorbed or that there were other limiting factors. However the importance of glutamic acid in plant and animal tissue respiration justified an investigation of its effect on asparagine synthesis, and the compound was fed at a concentration corresponding to that of aspartic acid:

	44 630 mg.% glutamate no ammonia	45 630mg.% glutamate 250mg.% $(NH_4)_2SO_4$
Total N	17	43
Protein	1.7	6.2
Amide	4.3	13.3
Amino	6.1	16.0
Ammonia	.3	1.8
Glutamine amide	--	.5
Length	28	30

Experiment 45 shows a striking effect of glutamate on the synthesis of asparagine and on N metabolism in general. That the amide formed was not glutamine is indicated by the analytical data (88). Experiment 44 shows that glutamate alone is not readily absorbed or utilized as a N source. To obtain some evidence as to whether glutamate was acting in a transfer system (i.e. catalytically) the compound was fed at low concentrations:

	46 50 mg.% glutamate 250 mg.% $(NH_4)_2SO_4$	47 100 mg.% glutamate 250 mg.% $(NH_4)_2SO_4$
Total N	34	34.5
Protein	2.4	2.5
Amide	9.9	10.8
Amino	11.9	13.5
Ammonia	1	2.5
Length	40	25

These experiments show that even the very low concentration of 50 mg.% glutamate exerts some action, which increases with increasing concentrations of the amino acid. The possibility arose that this effect could be explained by the following reaction:

glutamate + oxalacetate $\longrightarrow$ ketoglutarate + aspartate $\rightarrow$ asparagine

and in which an increase in glutamate concentration would force the reaction to the right (oxalacetate presumably arises from carbohydrate). An experiment in which glutamate and oxalacetate were fed simultaneously in the absence of ammonia, and an experiment to see if oxalacetate would react directly with ammonia are shown below.

Preparation of oxalacetic acid: Ethyl oxalacetate (kindly supplied by Dr. E.R. Buchman) was hydrolyzed in the cold with concentrated HCl. The HCl was removed in vacuum, the white product remaining behind washed repeatedly with small

portions of cold HCl, and finally dired. The compound gave a yellow color with phenylhydrazine in basic $NaNO_2$ (65). Because of the instability of the keto acid, a neutral solution of the compound was sterilized by filtration through a 5 on 3 Jena filter. This solution was then added to an agar medium just before solidification; after mixing, the medium was chilled.

Results using these substrates follow:

	48 100 mg% oxalacetate 630 mg% glutamate	49 100 mg% oxalacetate 250 mg% $(NH_4)_2SO_4$
Total N	19.1	28.5
Protein	2.8	3.6
Amide	4.3	8.4
Amino	6.2	10.8
Ammoina	.1	1.3
Length	22	18

Experiment 48 indicates that oxalacetate and glutamate in the absence of free ammonium are unable to be taken up and undergo reaction in the tissues. Experiment 49 shows that exalacetate with ammonium can slightly stimulate amide synthesis. At a concentration of 500 mg% the keto acid was toxic to excised embryos.

Recapitulation of the foregoing investigations at this point shows:

1. That glutamate stimulates N uptake and asparagine synthesis only in presence of ammonia.
2. That glutamate and oxalacetate do not appear to react in the absence of ammonia. Perhaps ammonia is necessary to convert aspartate to asparagine and thus permit the reaction to proceed to the right.

Two possibilities as to the role of glutamate remain open, namely, that the acid is metabolized to yield a carbon skeleton for the

formation of asparagine, or that the acid undergoes a reaction which furnishes energy for the synthesis of amide and protein from ammonia and carbohydrate metabolites.

In vitro experiments showed that the addition of glutamate to a hypocotyl tissue extract containing boiled yeast juice (coenzyme I and II) accelerated the reduction of methylene blue even faster than did added glucose. The necessity of coenzyme suggested that the reaction might be an oxidative deamination as found by Euler in liver (63). This point was investigated in more detail by a quantitative in vitro experiment in which an actual increase in ammonia was observed when glutamate was incubated for 24 hours with hypocotyl tissue extract, boiled yeast juice, and methylene blue. The ammonia liberated from glutamate was 65% greater than that from a control experiment without the amino acid. The product of this postulated reaction, ketoglutaric acid, should then be unable to stimulate N metabolism if the action of glutamate depends soley on deamination. The following experiment shows that ketoglutarate is quite inactive.

	50 630 mg.% ketoglutarate 250 $(NH_4)_2SO_4$	51 99 mg.% glutarate 250 mg.% $(NH_4)_2SO_4$
Total N	18.5	14.3
Protein	2.9	1.1
Amide	5.5	2.1
Amino	6.2	3.4
Ammonia	1.9	2.8
Length	14	21

Glutaric acid, even at a low concentration, actually inhibited N metabolism showing that this compound plays no part in these considerations. The failure of the keto acid to be utilized indicates that glutamate is not acting primarily as a carbon skeleton

(if the logical assumption that the keto acid is a product of glutamate metabolism is made). That the action of glutamate is not simply to supply ammonia in some reactive form is brought out by the following data, in which glutamate and the appropriate ammonia acceptor, aspartic acid, were fed.

	52 315 mg.% glutamate 285 mg.% aspartate	37 570 mg.% aspartate 250 mg.% $(NH_4)_2SO_4$
Total N	14	30.6
Protein	2.6	7.1
Amide	3.1	8.1
Amino	5.0	10.2
Ammonia	.07	2.3

If glutamate were able to furnish aspartate with ammonia, amide synthesis over that of an aspartate plus ammonia control (exp.37) would be expected, but the two acids alone completely failed to stimulate N metabolism as the above experiment shows.

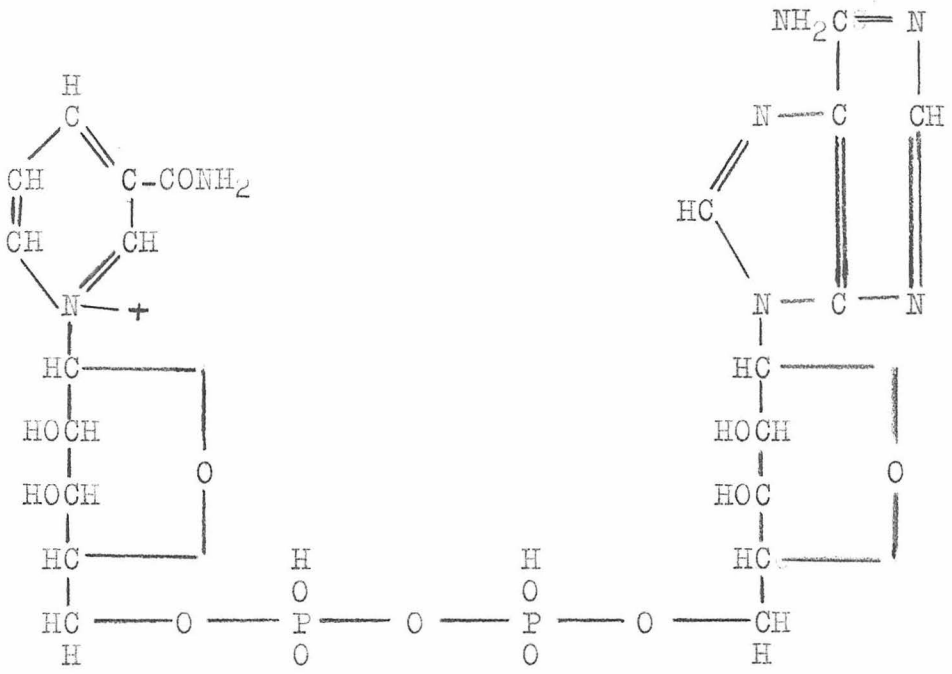
These experiments suggest that the reaction

$$\underset{\text{(coenzyme)}}{Co} + \text{glutamate} + H_2O \longrightarrow \text{ketoglutarate} + CoH + H + NH_4$$

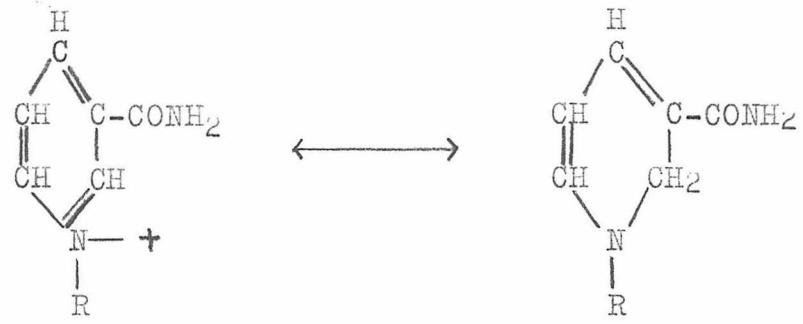
maybe of fundamental importance in asparagine synthesis, since neither the ammonia nor ketoglutaric acid formed appear to be the decisive factors. It seems likely therefore that the action of glutamate depends upon its property of reducing oxidized coenzyme. When significant amounts of the reduced coenzyme are available it may be possible to actually demonstrate an *in vitro* synthesis of the amide, using the appropriate precursors. In figure 3 is shown the structure of coenzyme I and the changes ocurring on reduction.

To investigate the possibility of glutamate playing a role in the conversion of ammonium aspartate to asparagine, an *in vitro* experiment was set up in which hypocotyl tissue extract, boiled yeast

Figure 3



Diphosphopyridine nucleotide
Coenzyme I



Oxidized form

Reduced form

juice, ammonium sulfate, and various substrates were incubated 15 hours at 38 degrees under anaerobic conditions. The following protocol shows the results:

Substrates	Amide N/ml.
Water control	5.8×10^{-3}
Glutamate	5.6
Aspartate	5.8
Glutamate+aspartate	6.7

These data indicate that aspartate and glutamate acting together in a suitable enzyme system yield amide N.

An indirect method of studying the action of glutamate consists in adding enzyme inhibiters to the medium and investigating the resulting changes in the metabolism of the embryos. The ideal inhibitor for this purpose would be one specific for glutamic dehydrogenase, but no such compound has yet been described, and relatively non-specific poisons must be emplyed. The action of such poisons has been reviewed by Cohen (89). Quastel and Wooldridge, using resting b. coli, have demonstrated that malonic acid inhibits glutamic dehydrogenase to the extent of about 30%. Urethane has been observed to inhibit succinic, lactic, and malic dehydrogenases, while iodoacetate appears to inhibit the synthesis of adenylpyrophosphate when redox systems furnish the energy. In the following experiments the action of these enzymes has been studied in a medium containing 630 mg.% glutamate and 250 mg.% $(NH_4)_2SO_4$.

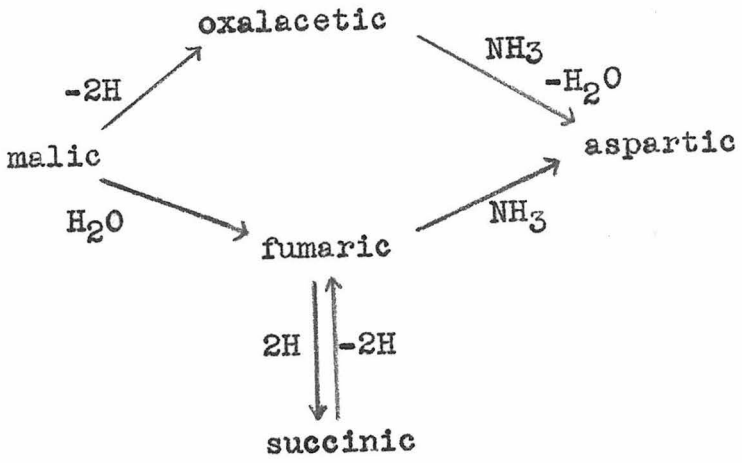
	53 200 mg.% malonate	54 500 mg.% urethane	55 100 mg.% iodeacetate
Total N	33.1	33.3	Toxic
Protein	3.8	4.0	
Amide	10.4	10.5	
Ammonia	1	1.3	

	53 (cont.)	54 (cont.)	55 (cont.)
	200 mg.% malonate	500 mg.% urethane	100 mg.% iodeacetate
Length	25	19	Toxic

A comparison of the formation of asparagine and protein in the above experiments with that of the control (exp.45) shows that the poisons have partially inhibited the action of the glutamate. The non-specific nature of these inhibitors in N and carbohydrate metabolism militates against drawing definite conclusions as to their effect on glutamate oxidation in embryos, but the data suggest that inhibition of glutamic dehydrogenase inhibits asparagine synthesis.

VIII. Discussion

The experiments described in the proceeding chapters were carried out to test the possibility of asparagine synthesis from compounds closely related to the amide or from carbohydrate metabolites. The 4-carbon dicarboxylic acids, to be converted to asparagine, would first have to be aminated to aspartic acid according to most logical structural considerations. This amination would occur in all probability either by the addition of ammonia to fumaric acid or by the reductive amination of exalacetic acid. The following scheme illustrates these conversions



The evidence for the existence of these enzyme systems in plants has already been discussed, but the point to be emphasized here is that the reductive amination of keto acids by trasamination with glutamate may require the intermediation of coenzyme to transfer the energy from the oxidative to the reductive compnent of the redox system:

$$Co + H_2O + \text{glutamic} \longrightarrow CoH + H^+ + NH_4^+ + \text{Ketoglutaric}$$

The reduced coenzyme formed in this reaction can then react with exalacetic acid to form aspartic acid:

$$H^+ + CoH + NH_4^+ + \text{oxalacetic} \rightarrow Co + H_2O + \text{aspartic}$$

In this way the coenzyme is reoxidized and can enter the cycle again and the net reaction may be represented thus

glutamic + oxalacetic → aspartic + ketoglutaric

There are known paths for the conversion of succinic, malic, and fumaric acids to exalacetic acid, which can then be aminated to aspartate, but none of the first three compounds were observed to promote either asparagine or amino N synthesis, suggesting that these conversions were not active under the conditions of the experiments. In view of the relatively high concentrations of substrate used it seems unlikely that failure of absorption can account for these results, and one may conclude that either enzyme systems for these reactions are non-existent or that some unknown factor or factors were limiting.

Maleic acid was better able to promote asparagine formation than any compound in this class, an observation for which there appears to be no obvious explanation. While this acid was tested primarily as a carbon skeleton it is possible that its action depends upon the inhibition of succinic dehydrogenase and a rerouting of the latter compound into some other channel leading to asparagine. This seems unlikely in light of the <u>in vitro</u> experiment which indicated the absence of succinic dehydrogenase in the first place. Burris has observed that maleic acid stimulates respiration in Rhizebium (90), but was unable to explain the phenomenon.

The experiments employing increasing concentrations of ammonia in the medium offer conclusive evidence that this component is limiting only when fed at very low levels. Much of the earlier literature purporting to show the effect of ammonia on amide synthesis can probably be explained upon the basis that the ammonia in the tissues were not yet limiting, and the writer is entirely in agreement with Vickery that the old 'detoxication' theory is not tenable.

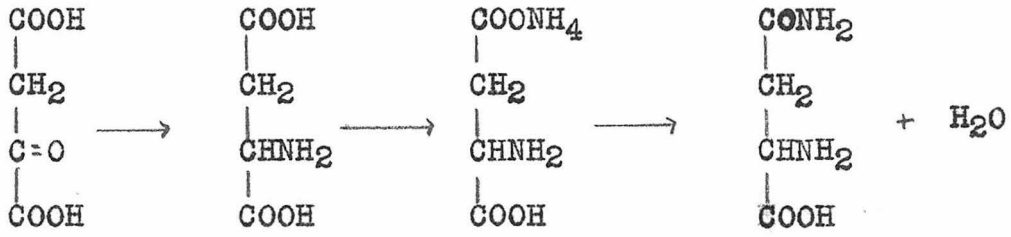
Aspartic acid, the most probable precursor of asparagine, showed some effect in this respect but was much less active than glutamate. Evidence has been presented that the possible products of glutamate metabolism (i.e. ketoglutarate or succinate) are not utilizable as asparagine precursors, and the assumption has been made that the energy liberated in the oxidation of glutamate is in some way especially available for amide synthesis. The fact that the glutamate oxidation occurs in vitro only in presence of coenzyme suggests that reduced coenzyme may act as an energy transferring intermediate in the synthesis. This energy factor may then have been the limiting element in all the other experiments. It may be argued that carbohydrate metabolism should be able to supply the necessary energy, and indeed the fact that asparagine synthesis normally occurs only when sugars are available supports this contention. The additive effect of glutamate over that when sugars are available might be explained by assuming that energy from glycolysis is distributed to numerous energy requiring reactions, only one of which synthesizes asparagine.

The metabolism (oxidation) of glutamate then makes available an additional supply of energy which can add to the normal quota delivered to the asparagine synthesizing mechanism. Energy from both glucose and glutamate metabolism has a final common path in amide formation.

Within the last 5 years the keto acids have been given a key position in possible schemes of asparagine synthesis, notably by Chibnall and Vickery. Reviewing this aspect of N metabolism in plants Krebs has stated: "Since the carbon skeletons of the amides, being normal intermediates in carbohydrate breakdown, are more abundantly available than any other analogous substances, it is easy

to understand why the cells choose to synthesize glutamine and asparagine for the binding of ammonia" (91). The intermediates referred to were ketoglutaric and oxalacetic acid. When the above statement appeared Vickery believed it disposed of the matter much too simply, but in a recent publication he has subscribed to essentially the same reasoning with some added details. There has been, however, a surprising reluctance on the part of the adherents of this scheme to even speculate on the details of the conversion of the keto acids to the amides.

If oxalacetic acid is a precursor of asparagine then it seems probable that the keto acid must pass first to aspartic acid, and thence to asparagine with the splitting out of water from the ammonium salt



There is no reasonable alternative to this course of reactions. On this basis aspartic acid should be a better precursor of asparagine than oxalacetic acid, and as a matter of fact the former acid yielded 13% more amide N than the latter compound when each was fed at the 100mg.% level.

Evidence has been presented in the preceeding chapter relative to the role of glutamate as a N carrier in the conversion of oxalacetate to aspartate. Whether glutamate and oxalacetate react directly in a "transamination" reaction or through the intermediation of a coenzyme cannot yet be stated with certainty, although the writer is inclined to belive a coenzyme may be involved. The most serious difficulties occur however when an attempt is made to

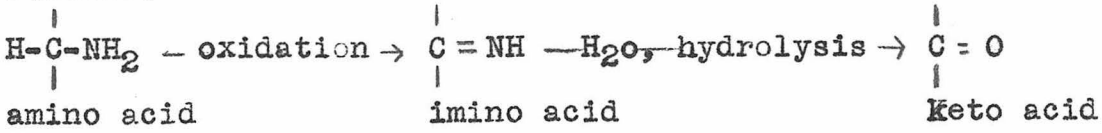
explain the conversion of the terminal carboxyl group of aspartic acid into an amide group. Whatever the mechanism may be with respect to the precursors in this last phase of amide synthesis, attention must first be directed to the details of the coupled reaction which yields the energy for the synthesis of the amide group. On the basis of known biochemical conversions the action of glutamate has been ascribed to its role in transamination with oxalacetic acid, but the possibility is still open that glutamate may be more importantly involved in supplying energy for the amidization of the terminal carboxyl group of the aspartic acid. No accumulation of amino N (aspartic acid) was observed when glutamate and oxalacetate were fed without ammonia, but glutamate with ammonia greatly stimulated asparagine formation; perhaps then the sequence

$$\text{oxalacetate} \xrightarrow{1} \text{aspartate} \xrightarrow{2} \text{asparagine}$$

is driven to the right by the continual removal of aspartate to form the amide, and when this reaction is impossible due to lack of ammonia, reaction 1 does not proceed to any significant extent. Thus reaction 2 is the limiting factor in our present knowledge of asparagine synthesis.

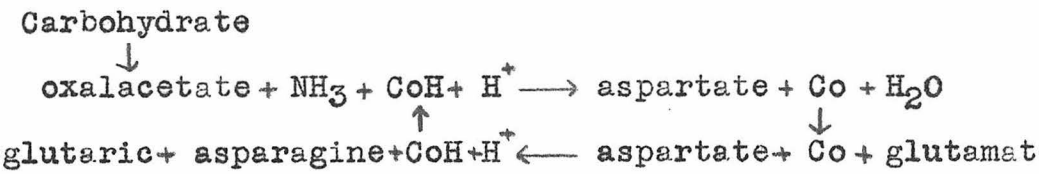
The in vitro experiment in which an increase in amide N was observed when glutamate and aspartate were incubated with enzymes from the embryo lends credence to the postulate that glutamate is involved in the amidization of aspartic acid. This amidization reaction involves the splitting out of water from the ammonium salt and no oxidation or reduction occurs in the overall process; this circumstance makes it impossible to state a mechanism involving only the electron change in oxidative deamination of glutamate and the removal of water from ammonium aspartate. However, oxidative deamination with the formation of the keto acid uses one water molecule

as follows:



It is possible that the reaction above requiring water is the one coupled with the amidization. The greatest difficulty in this postulation lies in the fact that the water might be expected to come more readily from the solution than from the ammonium salt, but irrespective of the mechanism proposed this same difficulty would exist. Assuming that this reaction occurs, one may then say that the energy from oxidative deamination is made available through the formation of imino acid which is highly reactive toward water and may bring about the amidization of the ammonium salt.

A scheme embodying these concepts is shown below:



It must be admitted that there is no positive evidence that coenzyme plays a role in the synthesis of asparagine in the plant, or that an enzyme mechanism is active in the removal of water from ammonium aspartate and the utilization of this water in the oxidative deamination of glutamic acid, but some mechanism of this general type must eventually be proposed to account for the energetics of asparagine formation.

The experiments described in the preceeding chapters have failed in their original purpose of discovering the precursors of asparagine, but the results with glutamic acid have thrown new light on the problem. They have shown that emphasis must be placed on investigations dealing with energy sources rather than carbon sources, and only when this aspect of the biological reaction has been solved

will it be possible to make an exhaustive study of precursors.

Summary

Methods are described for the in vitro cultivation of the excised lupin embryo on a synthetic medium.

The postulate that asparagine synthesis is not conditioned primarily by the presence of excess free ammonia and hence is not a detoxication mechanism has been experimentally confirmed in the lupin.

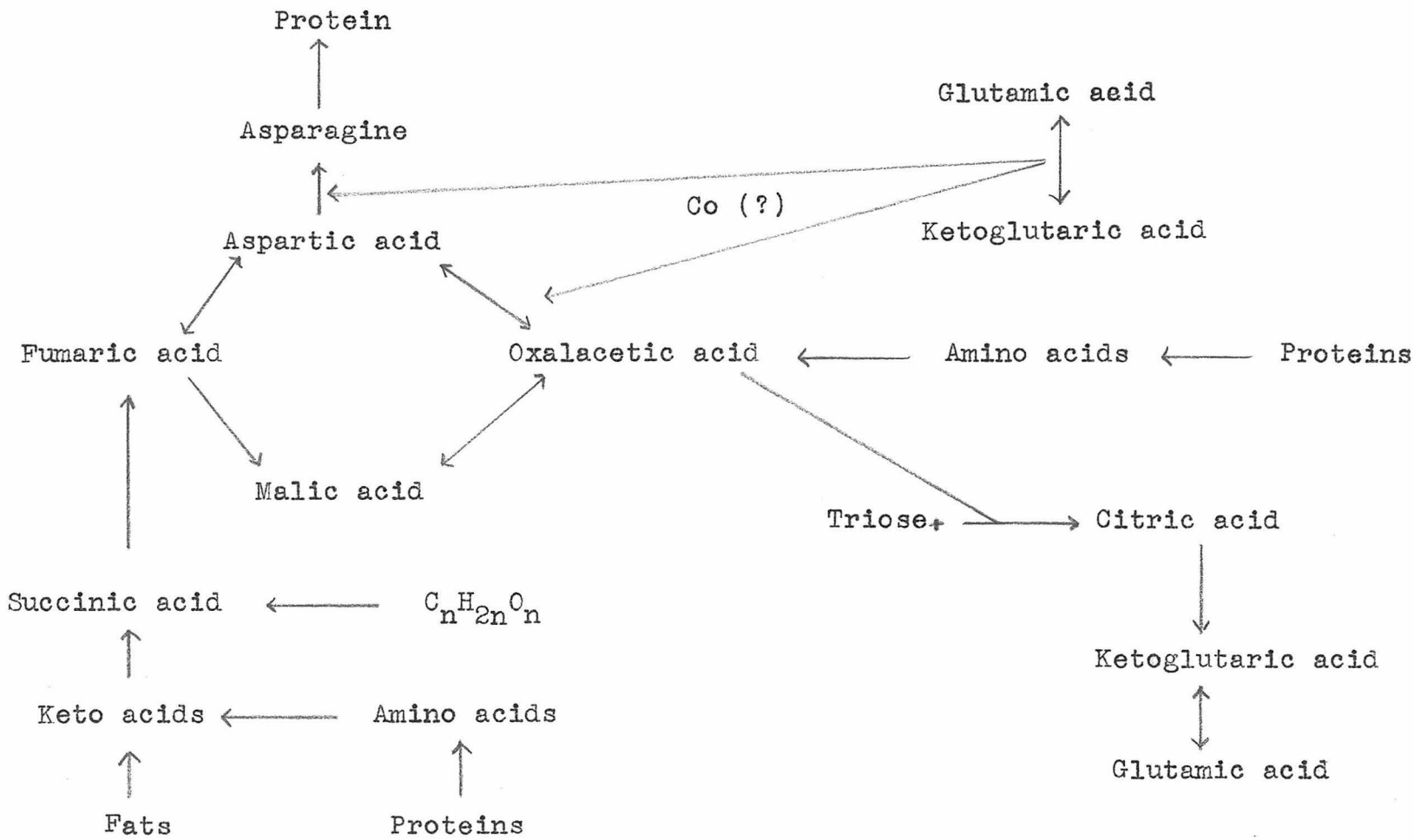
Isolated embryos, when supplied with adequate carbohydrate and nitrogen in the medium, are unable to absorb and convert succinic, malic, pyruvic, lactic, and glyceric acids, and glycerol and succinmonamide to asparagine.

Under similar conditions of carbohydrate and nitrogen supply fumaric, maleic, aspartic, oxalacetic, and glutamic acids, and succindiamide are effective in stimulating the synthesis of asparagine. Of these compounds glutamic adid is the most effective. Metabolic products of glutamic acid such as ketoglutaric, glutaric, and succinic acids are not effective. Combinations of glutamic acid with aspartic and oxalacetic acids in the absence of ammonia are likewise not effective in the stimulation of asparagine synthesis.

An extract of embryo tissue is able to oxidize glutamic acid in the presence of boiled yeast juice. Amide formation has been demonstrated in such an extract to which was added glutamic and aspartic acids and ammonia.

An attempt has been made to unify the above observations, and to explain the role of glutamate as an energy source in asparagine synthesis, with the energy transfer possibly taking place through the intermediation of a pyridine coenzyme.

The unsatisfactory status of the postulated conversion of aspartic acid to asparagine has been pointed out and a scheme proposed to explain the introduction of energy into the system.



A Unified Scheme of Nitrogen Metabolism

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NITROGEN METABOLISM OF THE PLANT EMBRYO

-LUPIN-

The analysis of growing plants at various stages of development, under controlled conditions, is a classical method in plant physiology. However, quite apart from the interest and significance this method has for the plant physiologist, it may in many cases be applied in more strictly biochemical studies, especially those relating to synthetic reactions. In the experiments reported here, germinating plants were analyzed at successive stages for nitrogenous constituents known to be concerned in protein metabolism, with the view toward seeking a quantitative relation between protein synthesis or degradation and the appearance or disappearance of the corresponding split products. The site of these processes and the effect of one physical condition, namely illumination, was also investigated.

In the early stages of growth, the germinating plant depends on the food reserves in the seed for its supply of nutrient material, and the reserve nitrogen is stored chiefly as protein. The present study deals with the lupin, which has a relatively high protein reserve as compared to the grasses, which are low in protein and high in carbohydrate, or the legumes, which contain both protein and carbohydrate. Using micro methods developed in this laboratory, Borsook and Dubnoff have studied the nitrogen metabolism of these two latter classes of plants, especially with regard to the synthesis of protein, and their results suggested the experiments reported here.

Attention should be called at the outset to the confusion in the literature resulting from the use of the terms "soluble" and "insoluble" nitrogen in analyses of plant tissues. Usually an aqueous extract of the tissue is taken for analysis, and the residual nitrogen is reported as insoluble nitrogen. Probably this insoluble fraction is mostly protein. By using dilute Na_2SO_4 in the extracting solution as explained below, practically all of the nitrogen becomes soluble, and the relations between the various fractions can be more clearly appreciated.

Morphologically the bulk of the lupin seed consists of two cotyledons, which unlike the pea, become the functional leaves during germination. By analysing separately the cotyledons, and the stem and root (which together will be called the embryo) at various stages of development, it is possible to construct a fairly complete nitrogen balance sheet for each of these parts and for the plant as a whole. In this way the general metabolic trends of the nitrogenous constituents may be followed.

EXPERIMENTAL

Culture of the Plants. Etiolated series.

The seeds used were the Hartwegii species of dark blue lupin, obtained from the Ferry-Morse Seed Company. In these experiments the seeds were grown under starving conditions on cotton saturated with an inorganic salt medium. The seeds (100-200) were sterilized by a 20 minute treatment with formaldehyde 1-250, thoroughly washed in sterile water and set out under aseptic conditions on sterile soaked cotton in the bottom of a deep petri dish. The solution used was of the following composition:

$K H_2 PO_4$	.272 gms.
$Ca Cl_2 2H_2O$	.179 "
$Mg SO_4 7H_2O$	.494 "
Ferric Tatrate	.008 "
Boric Acid	.006 "
$Mn Cl_2$	.008 "
Redistilled water	1 liter

Autoclaved 20 min. at 20 lbs. pressure.

The petri dish was covered with a large beaker and set away in the dark. The temperature varied between 23° and 25°.

Culture of the Plants: Illuminated Series.

Analysis of the plants grown in the dark indicated that the nitrogen free salt solution had no detectable effect on the metabolism of the plant in these early stages as compared with water controls. It was also evident that infection occurred mainly on seeds that failed to germinate. With this information, the following method of culturing was found satisfactory: the seeds were soaked three hours in redistilled water, and placed individually in vials containing cotton saturated with water. The vials were placed upright in a glass covered box, and the plants illuminated continuously by a 100 watt lamp placed at a distance of 15 inches from the vials. The temperature was maintained between 23° and 25°.

Extraction of the Tissue.

The plants were separated from the cotton, the hulls removed, and excess moisture taken up with filter paper. The cotyledons were cutt off at the junction of leaf stem and leaf, and embryo and cotyledons weighed and treated separately in all subsequent processes.

The tissue was placed in a small heavy walled test tube fitted to receive a glass grinding rod. A small amount of sand was added and the tissue ground to a fine pulp. One ml. of a 4% solution of $Na_2 SO_4$ (to dissolve globulins as well as albumins) was then added and the grinding continued 2 minutes more. The mixture was centrifuged and the supernatant transferred to a 10 ml. volumetric flask. This process was repeated until the supernatant was clear. The extract was made to volume and the nitrogen determinations carried out by the method of Borsook and Dubnoff (1). In this treatment, the insoluble N was never more than 5% of the total N. Water content was determined by the difference in weight of the tissue before and after drying at 100° for 10 hours. In addition to the nitrogen fractions shown in the following section, ammonia nitrogen was determined by micro distillation and peptide nitrogen by the use of a peptidase mixture described by Orcutt and Wilson (2).

RESULTS

The results are expressed graphically in figures 1 to 4, in which the various forms of nitrogen (as percentage of total plant nitrogen) are plotted against the time in days. The total nitrogen in 100 plants averaged 102 mg. The concentrations of amide and amino nitrogen in the water of the tissues are given in tables A to D.

Plants Grown in the Dark:

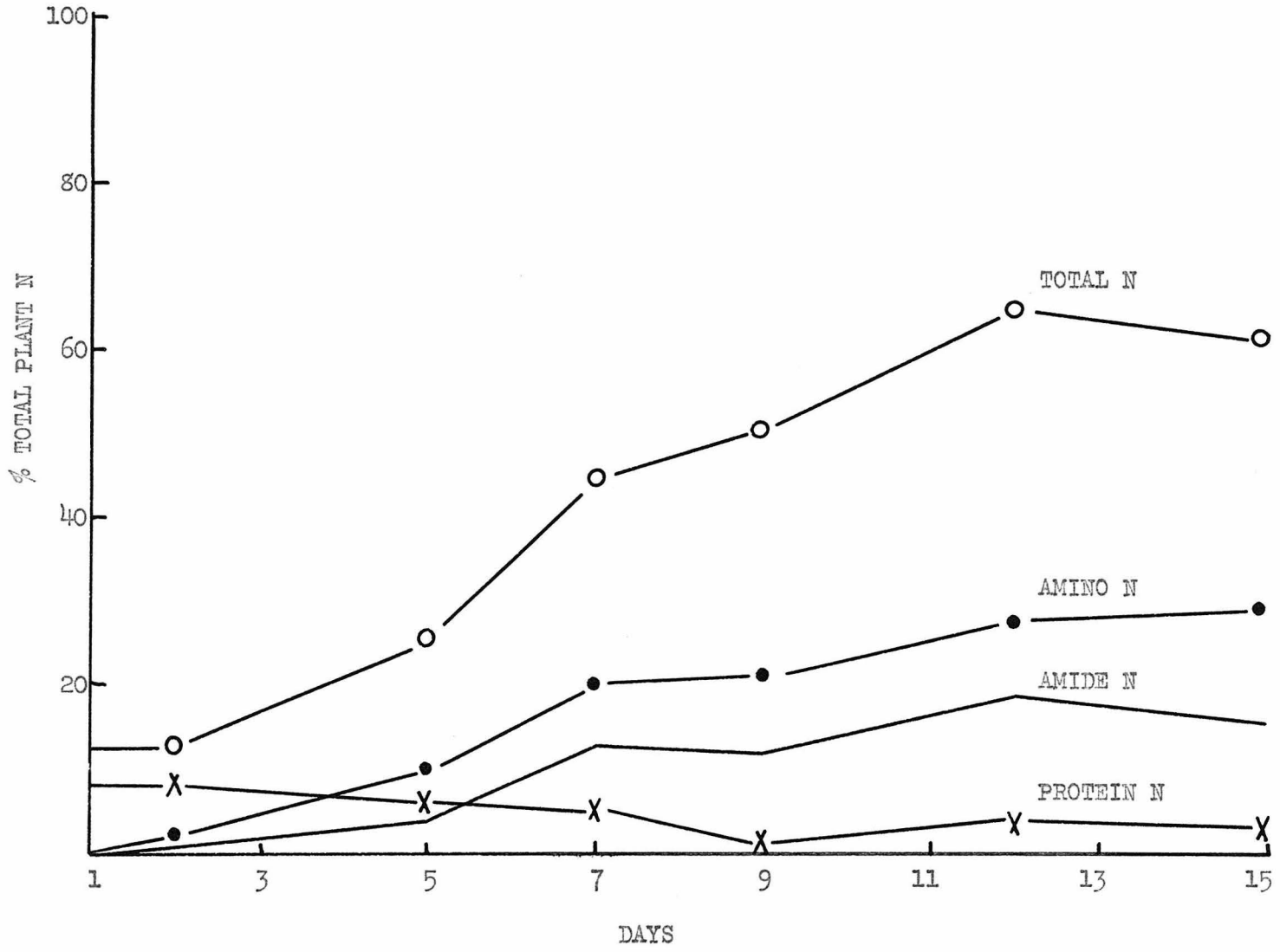
Examination of figures 1 and 2 shows that nitrogen is transferred from the cotyledons to the embryo for about the first 12 days. This increase in total embryo nitrogen is paralleled by rises in amino and amide nitrogen, which together account for this increase. However, the decrease in cotyledon nitrogen is paralleled closely by a decrease in protein content (fig. 2) in the same tissue. The protein nitrogen of the embryo, and the amino and amide nitrogen of the cotyledon show relatively little change during the period studied.

From the values given in table A, it is seen that the concentration of amino nitrogen, and hence of amino acids, remains fairly constant in the embryo, dropping to a somewhat lower value by the 15th day. Corresponding values for the amino nitrogen in the cotyledons are at a higher level. With the exception of the irregularity on the 2nd day, the amide concentration in the embryo remains constant up to the 12th day, after which it drops. The amide concentration in the cotyledons falls steadily throughout the period studied.

Plants Grown in the Light:

The data for the embryos are shown in figure 3. The increase in total nitrogen was less rapid than in the dark, and reached a constant value by the ninth day. At this time the plant had reached its maximum growth in height (4.6-4.8 cm). The amino and amide nitrogen values for the embryo increased throughout this period, while the protein content showed little change.

The nitrogen changes in the cotyledons are represented in figure 4. Total and protein nitrogen fell together until the 9th day; the protein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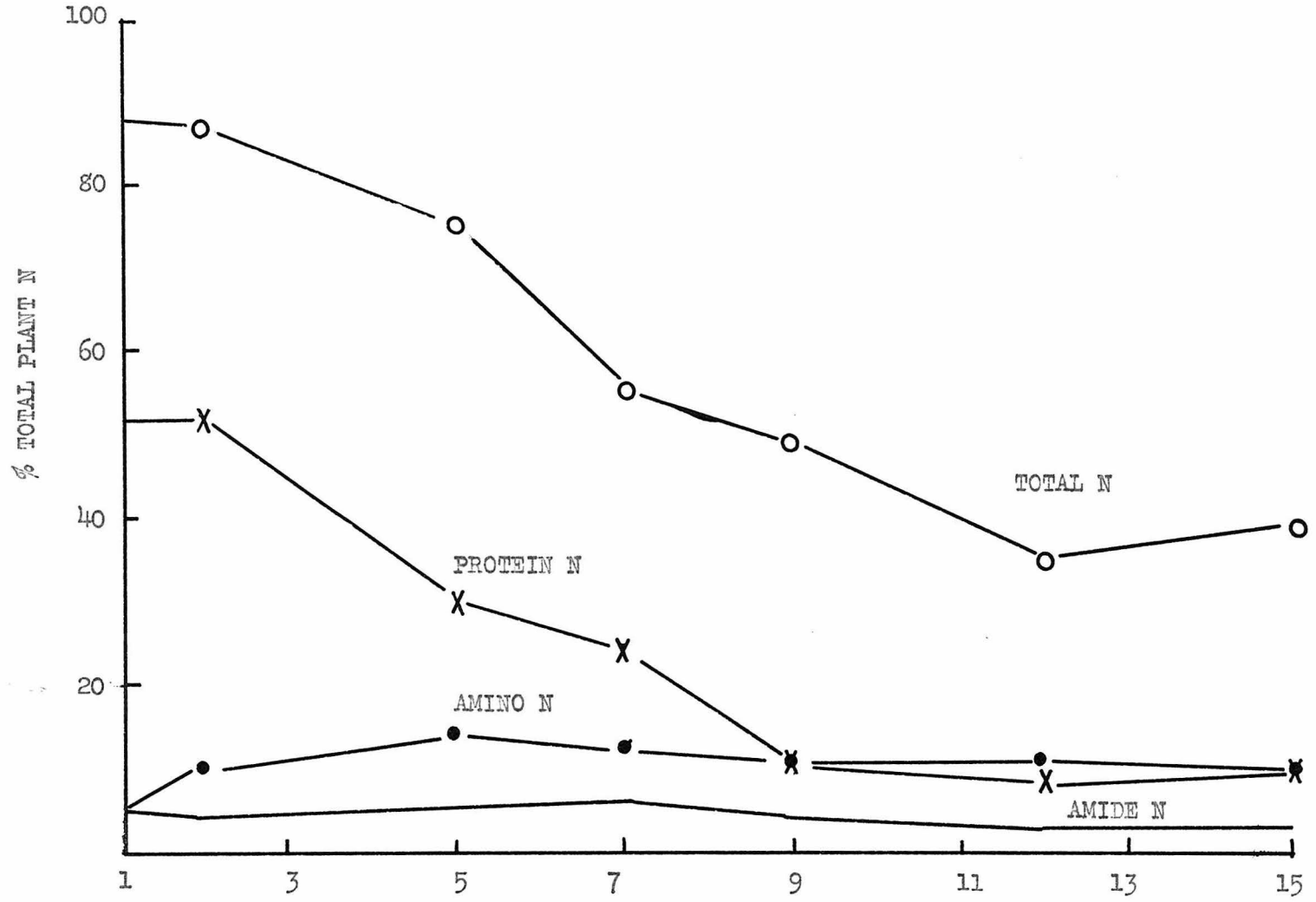
DAYS

FIGURE 1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DAYS

FIGURE 2

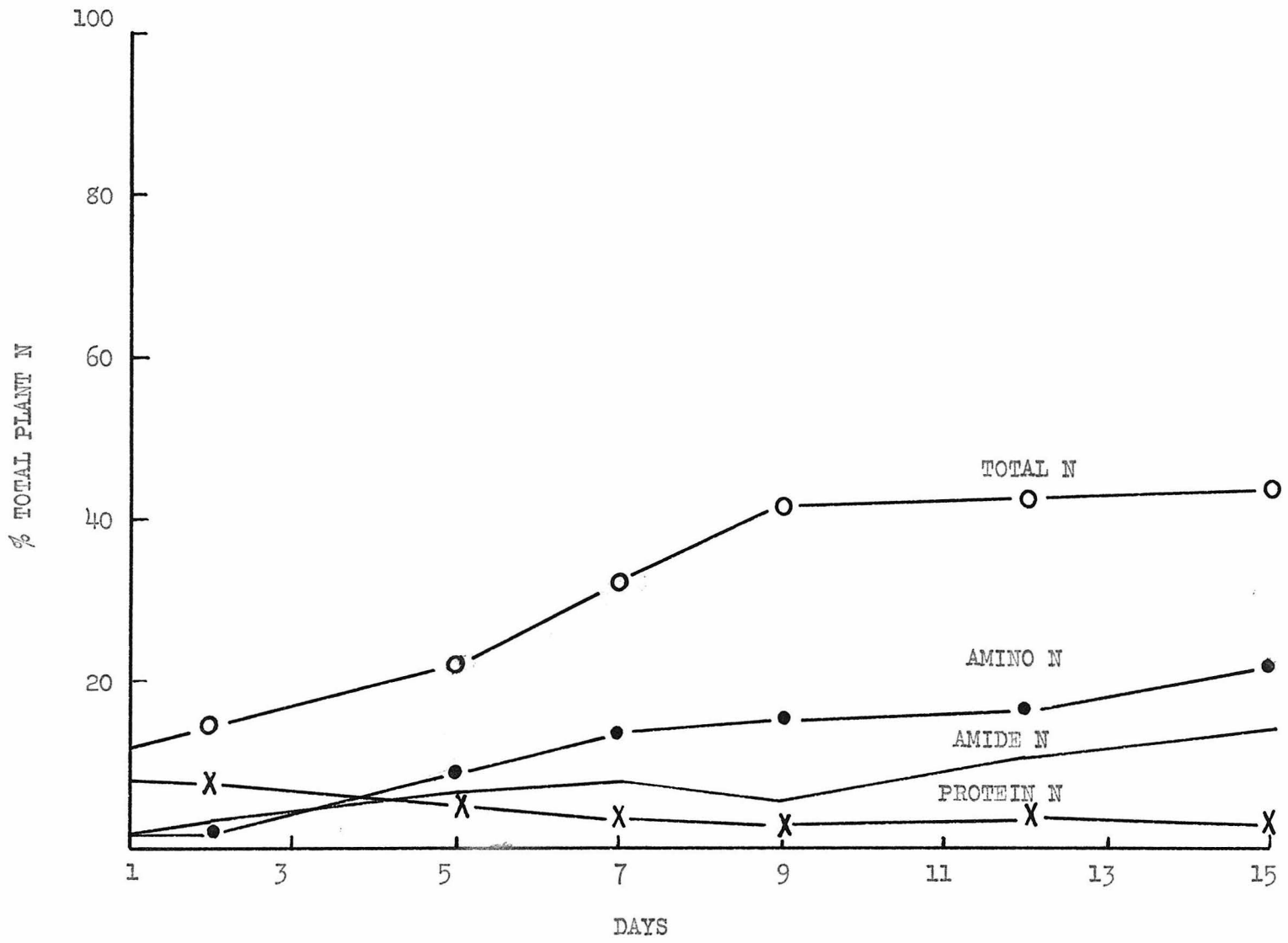
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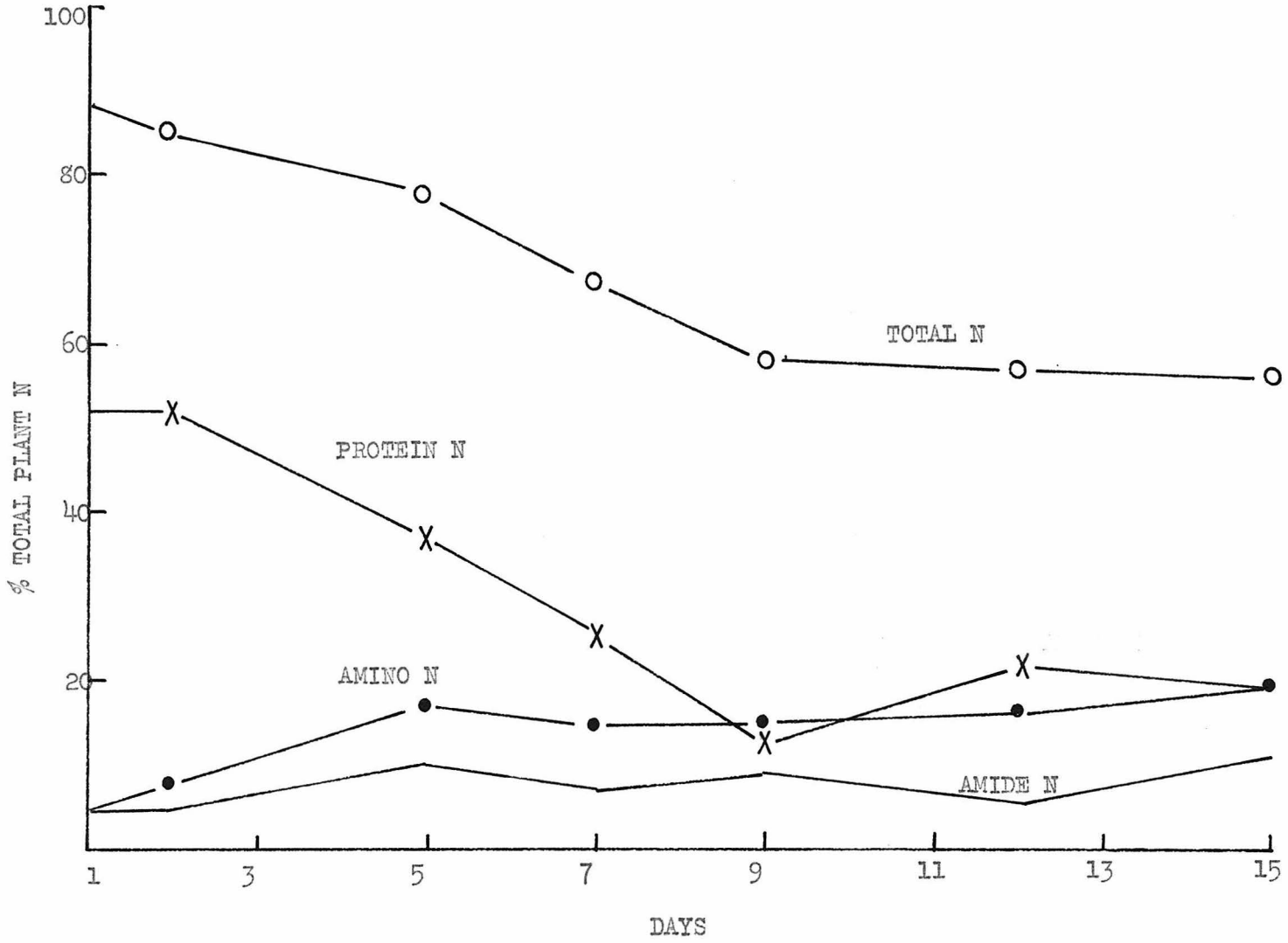
FIGURE 3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DAYS

FIGURE 4

Table A - Conc.of Amino and Amide N in Water of Embryo. Dark Series.

Time in Days	1	2	5	7	9	12	15
Molal conc. of amino N	.19	.17	.23	.14	.16	.16	.11
Molal conc. of amide N	.09	.045	.09	.09	.09	.11	.06

Table B - Conc.of Amino & Amide N in Water of Cotyledon. Dark Series.

Time in Days	1	2	5	7	9	12	15
Molal conc. of amino N	.18	.34	.35	.25	.27	.24	.19
Molal conc. of amide N	.16	.14	.13	.13	.09	.08	.06

Table C - Conc.of Amino & Amide N in Water of Embryo - Illuminated Series.

Time in Days	1	2	5	7	9	12	15
Molal conc. of amino N	.19	.16	.29	.14	.16	.16	.16
Molal conc. of amide N	.09	.21	.16	.09	.06	.1	.11

Table D - Conc.of Amino & Amide N in Water of Cotyledons - Illuminated Series.

Time in Days	1	2	5	7	9	12	15
Molal conc. of amino N	.18	.23	.34	.23	.17	.16	.18
Molal conc. of amide N	.16	.14	.19	.11	.10	.05	.10

then increased somewhat, possibly at the expense of amide nitrogen, which fell slightly at this time. After the 5th day the amino nitrogen content was constant.

Tables C and D give the data for the concentration of amino and amide nitrogen in the water of the tissues. For the embryo, the amino nitrogen concentration was fairly constant with the exception of the value on the 5th day. The other values were too irregular to be of much significance.

In all the experiments, ammonia nitrogen never exceeded 2% of the total, and in most cases was much less. The peptide nitrogen in the embryo constituted about 2% of the total nitrogen in both light and dark series. In the cotyledons of the dark series the peptide nitrogen constituted 10% of the total nitrogen for the first five days, then it dropped to less than 2% by the 9th day. In the illuminated series, the peptide nitrogen dropped to less than 2% of the total nitrogen by the 5th day.

DISCUSSION

Bonnet (3) in a study of the nitrogen metabolism of Lupinus luteus in the very early stages of germination, reported significant increases in amino and amide nitrogen when the embryo just appeared (which takes place between the 2nd and 3rd day under the conditions reported in this paper). He gave no data on the subsequent changes occurring in embryo and cotyledon.

McKie (4) studied the changes occurring in lupins grown in a soil-sand mixture up to 25 days. She reported a minimum value for protein nitrogen on the 18th day, and decreasing values for amino and amide nitrogen from the 8th day onward. The asparagine content rose to a maximum of 53% of the total nitrogen on the 18th day under her conditions of culture and analysis, and in all her experiments amino and amide nitrogen was very low as compared to protein and insoluble nitrogen. The position occupied by "insoluble nitrogen" in the whole process of nitrogen metabolism is very obscure, unless it is assumed to represent protein nitrogen.

By the method of extraction used in the experiments reported here, a relatively unchanged tissue extract was obtained, as rapid treatment of the fresh tissue

during the extraction and up to the point of precipitating the protein, obviated significant changes due to enzymic hydrolysis or coagulation of soluble protein.

A consideration of figures 1 and 2 (dark series) shows that the amino and amide nitrogen of the cotyledon changed but little after the 5th day, whereas the protein content continued to fall; concomittant with this protein decrease, the amide and amino N content of the embryo increased in close parallelism with each other, suggesting a quantitative relationship between these three constituents. It should be pointed out that after the 9th day the protein content of the cotyledon changed very little, but the amino acids of the embryo continued to increase.

With regard to the plants grown in the light, it appeared that illumination either promoted protein synthesis or inhibited its hydrolysis after the 8th day, as the protein content of the cotyledon was at a higher level in the illuminated series as compared with the etiolated plants. A similar phenomenon was found by Vickery et al (5) to occur in the excised tobacco leaf after 73 hours. From the 9th to the 15th day a reciprocal relation existed between amide and protein in the cotyledon. The "protein sparing" action of light might derive from the fact that the illuminated plant obtains its energy for growth by the oxidation of carbohydrate synthesized in the presence of light, whereas the etiolated plant hydrolyzes protein to supply some of the required energy, unless it has adequate carbohydrate reserves.

Associated with the increased protein content of the illuminated plants, were definite morphological differences; thus the etiolated plants had small yellow cotyledons and tall thin stems (8.5 cm. in 15 days) whereas the illuminated plants had large, green functioning leaves and short, sturdy stems (4.7 cm. in 15 days). These relations indicate that longitudinal growth and protein synthesis are not necessarily associated, and that even a reciprocal relation may hold.

Attention has already been called to the constancy of the values for amide and amino N concentration in the water of the tissues. Although these concen-

trations are irregular, they are of the same order of magnitude for each constituent throughout the period studied. This suggests that a dynamic relation exists between protein hydrolysis and the concentration of the soluble hydrolytic products.

SUMMARY

Data are presented on the values and distribution of the chief nitrogenous constituents of the germinating lupin.

Protein disappearing from the cotyledon was found to accumulate in the stem as amino acids and amides.

Plants grown in the dark were found to hydrolyze more protein and transport the products to the stem, than did plants grown in the light.

Longitudinal plant growth may take place to a marked extent with little or no change in the actual protein content of the elongating part.

The amino and amide nitrogen concentration in the water of the tissues remain at the same order of magnitude throughout the first 15 days of germination.

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