THE FORMATION OF ASPARAGINE IN ETIOLATED SEEDLINGS OF *Lupinus albus* L.

by A. N. Meiss

THE CONNECTICUT AGRICULTURAL
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CONTENTS

INTRODUCTION 5

HISTORICAL 5

BIOCHEMISTRY OF ASPARAGINE FORMATION 7

- Review of Recent Work 9
- Amide Accumulation in Relation to the Reserve Food Materials of the Seed 9
- Asparagine-Glutamine Relationships in Higher Plant Materials 12
- Changes in Nitrogenous Constituents During Seedling Growth 13
- Role of the Organic Acids 14

EXPERIMENTAL 16

- Part I. Germination and Growth of Etiolated Seedlings 16
  - Experimental Procedure 16
  - Results 19
- Part II. Analytical Investigation 22
  - Analytical Procedures 22
  - Results of the Analytical Work 28
    - Nitrogen Fractions 28
    - Lipids 32
    - Soluble and Insoluble Carbohydrates 35
    - Organic Acids 40
      - Identification of Organic Acids on Paper Strip Chromatograms 43
    - Moisture and Ash 48
    - Summary of Changes in Composition 48
    - Carbon Distribution 48

DISCUSSION AND INTERPRETATION OF THE EXPERIMENTAL RESULTS 53

- Relationship of Asparagine Accumulation and Growth 53
- Some Considerations as to the Source of the Asparagine Nitrogen 57
- Source of the Carbon of the Asparagine Synthesized, in Relation to the Sources of Respiratory Carbon Loss 59
- Interpretation of the Data on Organic Acids and Mineral Constituents 65

CONCLUSIONS 68

LITERATURE CITED 71

THE FORMATION OF ASPARAGINE IN ETIOLATED SEEDLINGS OF *Lupinus albus* L.[1,2]

Alfred N. Meiss

INTRODUCTION

During germination and early growth of seedlings of many legumes, the most striking chemical phenomenon is the accumulation of asparagine[3] as a principal constituent of the tissue. In the historical development of plant biochemistry, this phenomenon has played a central role, and has been the subject of a very large proportion of the classical studies of seedling metabolism. Although amide metabolism continues to be one of the major fields of interest in plant biochemistry, the general significance of asparagine accumulation in seedlings is by no means clear, even at the present time. This bulletin is the result of an effort to help clarify the situation through an intensive experimental study of a single plant species which is noteworthy for the accumulation of asparagine.

The experimental work reported herein was planned as an initial phase of a broader study of seedling metabolism, and is limited in scope to measurement of seedling growth and the chemical analysis of the dried tissues.

HISTORICAL

Comprehensive historical summaries of the occurrence of the common plant amides, asparagine and glutamine, and their roles in plant metabolism have been pubished by Vickery and coworkers (1937),[4] Chibnall (1939), Archibald (1945), and Prianishnikov (1945).[5] The reader is referred to these sources for a detailed history of the work on asparagine formation in seedlings.

Interest in asparagine dates from its discovery, in the juice of the asparagus plant, in 1806. The earlier work was rather sparse and fragmentary, but the problem of asparagine formation and its role in seedlings was the subject of continuous, and for the most part, noteworthy research from Boussingault's day, 1859, until the 1920's. During this period the field was led, at successive times, by three outstanding scientists: W. Pfeffer, German

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1 This bulletin is based on a dissertation presented to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy, 1950.

2 The work was carried out in the Biochemical Laboratory of The Connecticut Agricultural Experiment Station. Special acknowledgment is made to Dr. Hubert B. Vickery, head of the Department of Biochemistry, for suggesting the problem, and for his continuing interest and helpful advice throughout the course of the work. Grateful acknowledgment is also made to the Experiment Station for providing the facilities and financial support, to the Department of Analytical Chemistry for making the spectrographic analyses of the plant ash, and to Dr. C. A. Hargreaves, II, for his introductory chromatographic study of the organic acid fraction.

3 Asparagine, $NH_2OC\text{-}CH_2\text{-}CHNH_2\text{-}COOH$, is the *B*-amide of aspartic acid, or 2-amino-4-succinamic acid, according to the Geneva system of nomenclature.

4 References to literature cited are given by author and date.

5 Originally published in Russian only, Prianishnikov's book, "Nitrogen in the Life of Plants", became available in English translation in 1951.

botanist, Ernst Schulze, the Swiss protein chemist, and D. N. Prianishnikov, Russian plant physiologist. Each of these men summarized his results and views on the problem as the culmination of a long period of research.

The essence of Pfeffer's views, published in 1872, was that protein was converted to asparagine and sugar in the storage tissue of the seed, the asparagine was then translocated to the developing seedling axis, and there recombined with carbohydrate to form the protein of the new tissues. Ritthausen's hypothesis that amino acids, as such, were present in the structures of protein molecules, came too late (1872) to influence Pfeffer's conclusions, but was accepted immediately by Schulze, who used it to great advantage in developing his contributions to seedling metabolism. Schulze's principal conclusions, stated in a comprehensive paper in 1898, were: first, that the final products of protein decomposition in seed germination were amino acids; second, that while some of the asparagine and glutamine formed undoubtedly arose directly from protein decomposition, most of these substances must have been of secondary origin, through transformation of other amino acids; and finally, that the metabolic role of asparagine was to serve as a neutral, stable, and most readily available intermediate between the storage protein and the protein formed in the developing plant. Prianishnikov, who had been a pupil of Schulze, established definite correlations between rates of protein decomposition and asparagine accumulation, and found that asparagine was formed in seedlings by a definite synthetic reaction, dependent on the oxidative production of ammonia. His principal contribution was the discovery that the ability to assimilate ammonium ion from the substrate depends on the supply of available carbohydrate, and he finally demonstrated that even the lupines, given an external supply of carbohydrate along with ammonia, can synthesize asparagine without apparent participation of the storage protein in the process.

Knowledge of how asparagine formation and accumulation takes place in etiolated seedlings has not advanced significantly since Chibnall's discussion of the question in 1939. The dependence of secondary asparagine formation on aerobic respiratory activity is firmly established, and the production of ammonia by oxidative deamination of protein amino acids is generally accepted as the source of most of the nitrogen. The immediate non-nitrogenous precursor of the asparagine must, almost of necessity in the light of modern biochemistry, be oxalacetic acid, but the source, among the constituents of the storage tissue, of the oxalacetic acid (i.e., the carbon skeleton of the asparagine molecule) is not at all clear. Chibnall showed, by computations made with some of Schulze's data on *Lupinus luteus*, that non-nitrogenous reserve materials were not sufficient in amount to provide for both respiration and asparagine synthesis, and that if non-nitrogenous reserves furnished the asparagine carbon, protein must have been the principal respiratory substrate. In support of the view that the storage protein was the source of the entire asparagine molecule, he indicated that known reactions of the amino acids, applied to the lupine seed proteins, could produce a sufficient supply of precursors to account for the protein as the direct source of the carbon skeleton of all of the asparagine formed.

BIOCHEMISTRY OF ASPARAGINE FORMATION

In order to consider the process of amide formation in seedlings in the light of what is known of the biochemistry of asparagine and glutamine, the following brief summary is presented.

Chibnall (1939) constructed a hypothetical scheme to interpret the metabolic activities of asparagine and glutamine in leaves, basing it on the concept of the tricarboxylic acid respiratory cycle of Krebs as the point of convergence of the various pathways of metabolism involving proteins, carbohydrates, and lipids. The concept has received wide acceptance among biochemists and has been the basis of interpretation of most of the subsequent data on amide metabolism (cf. Vickery & Pucher, 1939; Archibald, 1945; Braunstein, 1947; Steward & Street, 1947; Yemm, 1949).

The metabolic activities and relationships leading to the formation of asparagine in etiolated seedlings are diagrammed in Figure 1. Many aspects of this scheme of utilization of the food reserves of seeds are entirely unknown and subject only to conjecture; these are indicated by the question marks on the figure. The most serious gap lies in our ignorance of the manner in which the pectic substances of seeds may enter into the seedling metabolism (cf. Bonner, 1936, 1946; Hirst, 1942, 1949; Hirst & Jones, 1946). Also, the metabolism of the lipids in higher plants is a practically untouched field, and any inferences regarding the manner of utilization of lipid reserves during seed germination must derive, for the most part, from studies with animal tissues.

Transamination and the Utilization of Amino Acids

At present, two enzyme systems, glutamic-aspartic and glutamic-alanine transaminases, are known to be highly active in the transamination reaction, and although transaminations involving other amino acids have been demonstrated, it has been contended that only in these two systems does the reaction proceed rapidly enough to be of major significance in metabolism (Braunstein, 1947; Green et al., 1945; Rautanen, 1948). In the breakdown of protein into its constituent amino acids, some asparagine arises by direct liberation from the seed protein,[1] and any aspartic acid liberated on hydrolysis is immediately available as a substrate for asparaginase. The utilization of the whole molecules of glutamine, glutamic acid, and alanine can take place readily through transamination (following deamidation in the case of glutamine), coupled with synthesis of oxalacetic acid from the resulting keto acids, via the organic acid cycle. It is assumed that any other amino acids which can provide ammonia and nitrogen-free residues for asparagine formation must do so by a scheme of metabolism which leads to the synthesis of aspartic acid, glutamic acid, or alanine directly, or involves oxidative deamination and the production of substrates which may enter the respiratory cycle (cf. Roine, 1947; Rautanen, 1948; Krebs, 1943, pp. 245-6; Braunstein, 1947, regarding the primary roles of glutamic and aspartic acids and alanine). Although little is known of the specific metabolism of the amino acids in higher plants, there is evidence that in animal tissues and micro-organisms all of the amino acids, with the exception of glycine and tryptophan, can be metabolized to yield products which can be shunted into amide synthesis (Braunstein, 1947).

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[1] cf. Archibald, 1945, pp. 169-171. Practically all of the aspartic acid of the lupine seed proteins is present in the form of the amide.

Figure 1. Biochemical pathways leading from food reserves of seeds to asparagine formation during germination and early growth of seedlings.

Recent studies with bacteria (Feldman & Gunsalus, 1950) and with a number of higher plants, including a lupine species (Stumpf, 1951), indicate that transamination may be a more general reaction of the amino acids. The transamination with α-ketoglutarate, as a general reaction of the amino acids, coupled with the glutamic→ oxalacetic transamination, would provide a much simplified picture of the pathway by which the bulk of the free α-amino nitrogen can be channeled into asparagine synthesis. A further point of considerable interest is that the transfer of the amino group from glutamic acid to oxalacetic acid, forming aspartic acid, is by far the predominating reaction among all amino group transfers that have been studied in plants (Leonard & Burris, 1947; Albaum & Cohen, 1943; Smith & Williams, 1949; Rautanen, 1946, 1948). The view that asparagine is formed in the stepwise sequence,

$$\text{oxalacetic acid} \xrightarrow{NH_3} \text{aspartic acid} \xrightarrow{NH_3} \text{asparagine},$$

is supported by the demonstration by Virtanen and Laine (1941) that asparagine and glutamine do not undergo transamination until hydrolysis of the amide group has taken place.

A possibility that is of particular interest in the present work is whether nearly all of the amino acids undergo oxidative deamination readily, but do not deliver forms of nitrogen other than α-amino and amide nitrogen into the metabolic pathway leading to amide formation. Such a situation would imply that the nitrogen of the imidazolyl group of histidine, the guanidino group of arginine,[1] the ϵ-amino group of lysine, and the indolyl group of tryptophan may be utilized more or less directly in the synthesis of other nitrogenous substances.

REVIEW OF RECENT WORK

Amide Accumulation in Relation to the Reserve Food Materials of the Seed

The relationship between the nature of the reserve food materials of seeds and the behavior of the etiolated seedlings with respect to the accumulation of asparagine and glutamine requires clarification. It was earlier thought that species having oil-bearing seeds were in general glutamine formers, and that species having highly proteinaceous seeds were predominately asparagine formers, while starchy seeds were associated with little or no accumulation of amides (Chibnall, 1939).

Data of Vickery and Pucher (1943) can be interpreted to support the view that it is the nature and relative availability of the respiratory substrates which determine whether, and to what extent, etiolated seedlings of a plant species will accumulate asparagine. The pertinent data are plotted in Figure 2. The nitrogen-free reserves of the squash seed consist mainly of fatty substances, with smaller amounts of starch and sugars. The reserves of the vetches are mainly starch, with very little lipid. The lupines, in addition to pectic substances and complex sugars (Schulze & Godet, 1909; Schulze,

[1] Renewed interest in the metabolism of arginine in seedlings has arisen from recent evidence that urea may be a third amide in the normal metabolism in some plant species (Damodaran & Venkatesan, 1948; Reifer & Melville, 1949). The work of Damodaran and Venkatesan suggests that a mechanism analogous to the Krebs ornithine cycle occurs in germinating seedlings of certain species of legumes.

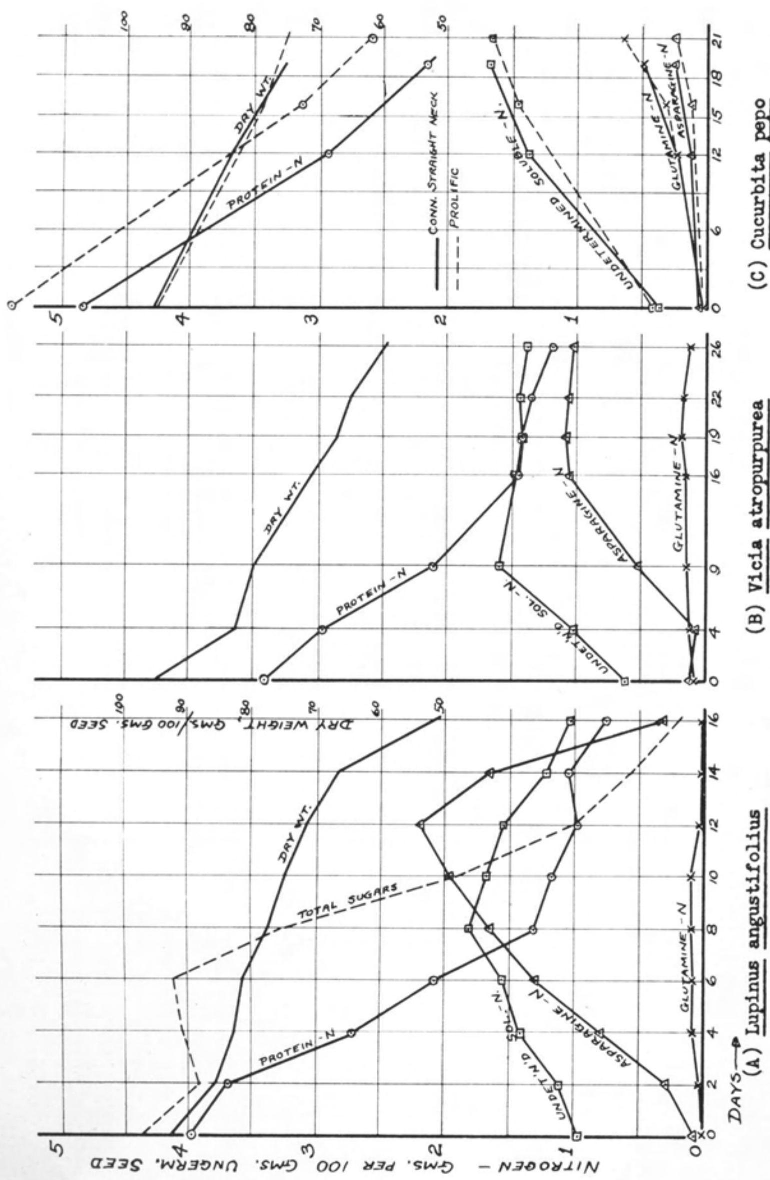

Figure 2. Formation of asparagine and glutamine in etiolated seedlings of species having different types of nitrogen-free reserve foods in the seed.

(From data of Vickery and Pucher, 1943)

1910a,b), contain lipids in the range from 6 to 10 per cent of the dry substance. From the figures it can be seen that the initial protein contents of the seeds were of the same order, but that the intensity of protein decomposition and asparagine accumulation increased with the change from lipids to starch to pectic substances as the principal nitrogen-free reserve of the seed. Over the period of asparagine accumulation, about 81 per cent of the seed protein of *L. angustifolius* (at 12 days) was decomposed in addition to that utilized for resynthesis of seedling protein, while the values for the other species were: *Vicia* (16 days), 58 per cent; and the *Cucurbita pepo* varieties, Connecticut Straight Neck and Prolific, 55 and 50 per cent, respectively. The species differences are even further accentuated if a comparison is made of the quantity of asparagine formed with the net increase in undetermined soluble nitrogen. Thus, in the lupine, the quantity of newly formed asparagine nitrogen amounts to 370 per cent of the increase in undetermined soluble nitrogen, while for the vetch and the cucurbits the values are 110 per cent and less than 20 per cent, respectively. The increase in undetermined soluble nitrogen represents, for the most part, amino acids which have accumulated from protein decomposition but have not been further metabolized. The data can also be interpreted to indicate that glutamine was an intermediate in the synthesis of asparagine in vetch and lupine, but that the asparagine and glutamine in squash arose directly from the hydrolysis of protein, there being no secondary formation.

The view that there was no significant secondary formation of either asparagine or glutamine in the squash seedlings is supported by new analytical values for aspartic and glutamic acids in hydrolyzates of seed globulins of *Cucumis sativus* (cucumber) and *Cucurbita maxima* (Hubbard squash). These values, from the work of Steward and Thompson[1] are, for *Cucumis sativus*, 9.3 grams of aspartic acid and 21.4 grams of glutamic acid per hundred grams of dry protein, and for *Cucurbita maxima*, 6.8 and 24.2 grams, respectively. Using these figures to compute the amounts of asparagine and glutamine which would arise from the quantity of protein which Vickery and Pucher found to be utilized, the following results are obtained:

Cucurbita pepo:	var. Conn. Straight Neck		var. Prolific	
	Found	Calculated[1]	Found	Calculated[1]
gms. protein decomposed	2.68	......	2.61	......
gms. asparagine formed	.20	.25 — .18	.22	.24 — .18
gms. glutamine formed	.49	.57 — .62	.64	.56 — .63

[1] The numbers on the left were calculated from the analytical values for *Cucumis sativus* seed globulin, those on the right from the *Cucurbita maxima* values.

The equilibrium levels of asparagine and glutamine involved in protein synthesis would be accounted for, more or less, by the quantities of the free amides in the ungerminated seed, and the resynthesis of protein in the growing seedlings is accounted for in the computation of the amount of protein

[1] Communicated to Dr. H. B. Vickery by Dr. F. C. Steward.

decomposed. Accordingly, it seems highly probable that no accumulation from secondary synthesis is represented in the asparagine and glutamine levels in the *Cucurbita* seedlings.

Besides rendering untenable the hypothesis that glutamine accumulation occurs as the result of secondary synthesis in etiolated seedlings grown from oil-bearing seed, these data suggest inquiry as to whether secondary accumulation of glutamine occurs at all in etiolated seedlings under conditions where no exogenous substrates or precursors are supplied.

Asparagine-Glutamine Relationships in Higher Plant Materials

Scrutiny of the results of recent researches leads one to certain conclusions as to the comparative functions of asparagine and glutamine in seedling metabolism. In particular, asparagine is not an *active* constituent of the tissues in the same sense that glutamine is (Yemm, 1949; Steward & Street, 1946). Whereas glutamine appears to be the key substance mediating the synthesis of amino acids and proteins, asparagine seems to have a largely passive role that of a stable product of amino acid oxidation, and concomitantly, a storage pool supplying ammonia and four-carbon dicarboxylic acids which are used in protein synthesis.

It is now suggested that the labile amide bond of glutamine may be the principal factor in a system for coupling the energy of respiration with peptide synthesis, but there is little or no evidence in support of a similar role for asparagine in the higher plants (cf. Yemm, 1949). In a recent study of glutamyl transphorase of pumpkin seedlings, partially purified preparations of this enzyme, which effects an exchange of isotopic ammonia with the amide group of glutamine, were inactive toward asparagine (Delwiche, Loomis, & Stumpf, 1951).

Rautanen's (1948) data on pea seedlings strongly suggest that glutamine and glutamic acid are primary products in the assimilation of ammonia, and may lie on the pathway by which asparagine is formed from ammonia and nitrogen-free substances, and possibly even when aspartic acid is utilized for asparagine production. Yemm (1949) has concluded, similarly, that glutamine plays a primary role, with respect to asparagine, in the normal metabolism of barley plants, with asparagine synthesis promoted by starvation conditions. It had been shown previously that in starving grass leaves, amino acids, glutamine, asparagine, and ammonia attained maxima in that order (Yemm, 1937; Wood et al., 1943).

Furthermore, a low and relatively stable glutamine level is generally associated with secondary asparagine accumulation in seedlings, as seen in the 1943 data of Vickey and Pucher, and in the results of Damodaran and coworkers (1946 with three legume species. Similarly, Kretovich and Eustigneeva (1949) reported that the infiltration of turnip, wheat, and lupine sprouts with salts of aspartic and glutamic acids raised the asparagine level, but did not affect the glutamine content.

Christiansen and Thimann (1950), in their studies on the metabolism of etiolated pea stem sections, found that, during growth for 24 hours, the glutamine content decreased to about one-third of the initial value, regardless

of treatment, but that the asparagine content was decidedly responsive to the conditions of growth. When growth was inhibited without enzyme poisoning, the asparagine content nearly doubled (Thimann et al., 1950), as occurred in the controls on water or auxin, but when respiratory inhibitors were used to suppress growth, only slight increases in the asparagine content were obtained. Their observations thus suggest widely different metabolic roles for the two amides.

Changes in Nitrogenous Constituents During Seedling Growth

A general pattern is observed in the metabolism of storage protein in etiolated seedlings of many legume species: protein decomposition usually reaches a level of maximum intensity within 24 to 48 hours after the seeds begin to take up water, and thereafter follows a logarithmic curve which approaches a minimum level asymptotically while the seedling tissues are still turgid but approaching a cessation of synthetic activity (McRary, 1940; Vickery & Pucher, 1943; Pucher, 1946, unpub.; Damodaran et al., 1946).

In the study of the amino acid changes taking place in germinating legume seedlings, no data have as yet been obtained to indicate the distribution of amino acids between the protein and non-protein fractions and between the cotyledons and seedling axis. Christiansen and Thimann (1950) demonstrated that isolated sections of etiolated pea stems, during growth in water, utilized nitrogen of free amino acids in the synthesis of approximately equivalent quantities of protein and asparagine. A chromatographic analysis of the free amino acids showed that this synthetic activity was associated with marked decreases in all of the 29 ninhydrin-reacting substances that were observed. Dunn and coworkers (1948) analyzed acid hydrolyzates of whole etiolated seedlings of *L. angustifolius*, harvested at intervals over a 15-day period. Estimations of 12 amino acids by microbiological methods showed a five-fold increase in aspartic acid (i.e., asparagine), together with marked decreases in all of the other amino acids except histidine, which showed no change, and methionine, which decreased from 0.20 mg. per seed to 0.15 mg. per seedling at 15 days. The major contributors to the increase of 20.2 mg. per seedling of aspartic acid were glutamic acid, 10.3 mg.; arginine, 2.8 mg., and the leucines, 4.0 mg. In a comparison of the partial composition of *L. angustifolius* seed protein with that of cotyledons and seedling axis tissue of 14-day etiolated seedlings, Lugg and Weller (1941) found that all of the methionine remained in the protein fractions, and postulated that the supply of methionine was limiting to the regeneration of protein in the seedling. While the demands for particular amino acids (which the seedling is unable to synthesize rapidly enough) required in the resynthesis of seedling protein might be an important condition controlling the extent of hydrolysis of seed protein, this should have no influence on the rate or extent of amino acid oxidation, nor, therefore, on secondary asparagine formation.

Urea does not accumulate in etiolated *L. albus* seedlings (Klein & Tauböck, 1932; Echevin & Brunel, 1937), notwithstanding a rapid disappearance of total arginine. While the secondary accumulation of amides is known to involve both asparagine and urea[1] in some legume species (Damodaran et al.,

[1] Secondary accumulation of urea is considered as that arising in excess of the amount which can be accounted for by the hydrolysis of the amidine group of the arginine which disappears.

1946; Klein & Tauböck, 1932), urea metabolism appears not to complicate the scheme of asparagine formation in *L. albus*, since it has never been found in more than a trace amount in this species.

Of the other non-protein nitrogenous constituents which have been studied in lupine seedlings, including betaine and creatinine (Tokarewa, 1926), purines, uric acid, allantoin, and allantoic acid (Echevin & Brunel, 1937), none were present in sufficient amounts to have any quantitative significance in asparagine accumulation. This is likewise apparent with the lupine alkaloids, which contain only about 1 to 2 per cent of the total nitrogen (Wallebroek, 1940).

Direct investigation of the accumulation of asparagine in relation to the utilization of specific amino acids, supplied as substrates after removal of the storage tissue of the seed (i.e., cotyledons), has not yet been undertaken with lupine seedlings. Such investigation is obviously one of the more urgent needs in the field of seedling metabolism at the present time.

Role of the Organic Acids

Ritthausen (1872) found oxalic and malic acids in small quantities, and several times as much citric acid, in the seed of *Lupinus luteus*. Malic acid was isolated in an amount equivalent to about 0.35 per cent of the seed weight.

Unpublished data of Pucher (1946) indicate that malic and citric acids make up only a small portion of the organic acid fraction in *L. angustifolius*, as shown in Figure 3. It should be noted that the maximum in the total acid curve occurs just prior to the time of maximum accumulation of asparagine. Citric acid declines rapidly at first, remains nearly constant during the period of rapid asparagine synthesis and then begins to fall off again. Malic acid increases at first, maintains a constant higher level during the period of rapid asparagine synthesis but drops rapidly with the cessation of amide accumulation. The quantities of acids involved were small: citric acid comprised 0.56 per cent of the weight of the ungerminated seed and malic acid, 0.11 per cent. The more or less constant level of each of those acids during the rapid synthesis of asparagine is considered to be the "dynamic equilibrium concentration", that is, the level attained as a resultant of the velocities of formation and decomposition of the acid as an intermediate in the chain of precursors of asparagine. Any excess over this concentration, as in the case of citric acid, is available as a respiratory substrate or as raw material for synthetic processes.

Such a "dynamic equilibrium concentration" would be affected by changes in the balance of the mineral cations and anions as well as by the velocities of the enzymic reactions by which the acid is formed and decomposed (cf. Turner & Hanley, 1949). A constant level of acidity during a period of active metabolism must mean, therefore, that there is no change in inorganic ion balance during that period or that there are compensating drifts produced by other factors affecting the concentration of organic acids. The former condition must have held in Pucher's experiments, since the culture medium was water alone.

In Rautanen's (1948) ammonia feeding experiments with cotyledonless pea seedlings, the formation of both glutamine and asparagine was accom-

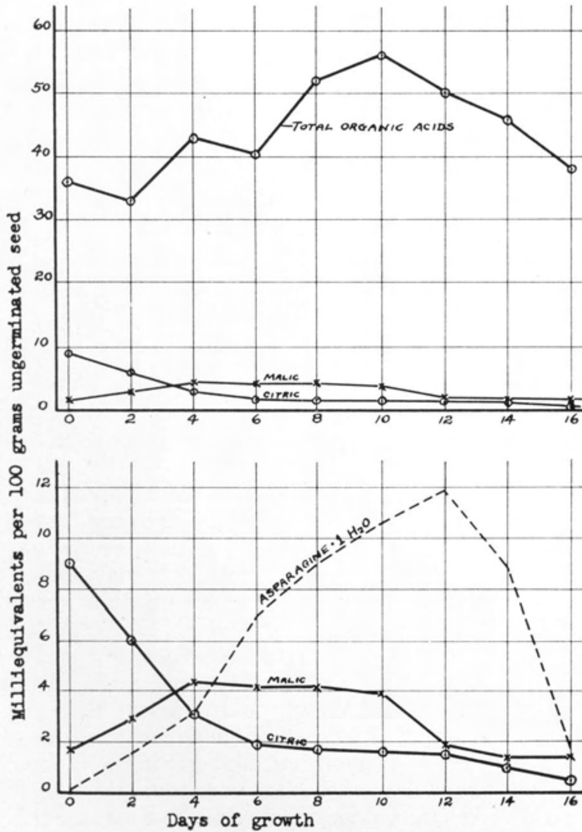

Figure 3. Organic acids of etiolated seedlings of *Lupinus angustifolius*.

(Unpublished data, Pucher, 1946)

(Asparagine monohydrate is in grams per 100 grams ungerminated seed, on the same scale as meq. of malic and citric acids.)

panied by a sharp decrease in the malic acid content of the tissue. When glutamic acid or aspartic acid was fed, malic acid increased, and did so to a much greater extent with glutamic acid than with aspartic. These observations provide concrete evidence that malic acid is an intermediate in the formation of the amides from ammonia and nitrogen-free substances.

EXPERIMENTAL

The general plan of the experimental work was, briefly, as follows. Seeds of the white lupine, *Lupinus albus* L., were germinated in a controlled environment and the seedlings were grown in darkness, supplied only with tap water in a fine spray, until physiological breakdown of the tissues commenced. Triplicate groups of seedlings were harvested at 3-day intervals throughout the period. These were separated into three fractions each, seed coats, cotyledons, and plant axes, and after the fundamental data on growth was obtained, were prepared as dry samples for analytical determinations. The experiment was limited in its scope to the growth measurements and a detailed analytical investigation involving nitrogenous and carbohydrate components of the tissue, lipid fraction, total organic acidity, and ash constituents.

Part I. Germination and Growth of Etiolated Seedlings

EXPERIMENTAL PROCEDURE

Selection of Plant Material

The choice of *Lupinus albus* for the experimental material was based on several desirable properties of this species. It is, of course, one of the species which is noteworthy for the accumulation of asparagine during germination and early growth of the seedling, previous studies having shown that asparagine accumulates to the extent of as much as one-fourth of the dry weight of the whole seedlings (Vickery, Pucher, & Deuber, 1942). Although *Lupinus luteus* has been used more often in the study of amide metabolism in seedlings, it was considered less suitable for the purpose of this work. *L. albus* seed[1] is more readily obtained, is larger and therefore easier to work with, and contains less protein, but more lipid and carbohydrate, than *luteus*. Because of the lower protein content and higher content of lipid and carbohydrate, *albus* is considered the better species for investigation of the probable source of the carbon skeleton of the asparagine. Individual seeds of *L. albus* usually weigh from four- to five-tenths of a gram, and the size and shape of the seed and seedling make it very simple to remove the seed coat and to separate the cotyledons from the plant axis rapidly with large numbers of seedlings.

Equipment Used for Growing Seedlings

A steel box, 27 inches high, 27 inches deep, and 40 inches long, with the front hinged to swing open, was used as a growth chamber. The cabinet is divided into upper and lower sections by four lengths of three-eighths inch pipe running lengthwise, and spaced evenly at a level 14 inches above the bottom. The upper and lower sections are each provided with a spraying device. In operation, these fill the entire cabinet with a dense, fine spray, which serves as a temperature regulator as well as water supply. The temperature was controlled by applying heat to the water pipe leading to the sprayers. By this means the temperature was held between the limits of 21 and 23°C.

[1] The seed used in this investigation came from the 1947 crop grown in the vicinity of Palermo, Sicily, and was obtained through Mr. Leonard Sclafani, 181 Mott St., New York, N. Y.

over the 26-day period in which the seedlings were grown. Each of the two sections accommodates four galvanized sheet iron trays, 13 by 19 inches, and three-quarters of an inch deep, with a drainage hole at the base of the rim at each end, so that only a thin film of water remains on the bottom of the tray. The seedlings were grown in quarter-inch mesh wire baskets, nine inches in diameter and seven inches deep, placed two on each tray. The bottoms of the baskets are square, with the projecting corners bent downward at right angles to form feet which elevate the bottoms of the baskets proper to one and one-half inches above the level of the tray. All metal parts of the equipment, with the exception of the sprayers, are coated with asphaltum paint, and a steam line is provided to sterilize the cabinet and contents before starting a lot of seedlings. The seedling cabinet is set up in a basement laboratory room, but no attempt was made to maintain total darkness. The brief periods of dim illumination incidental to caring for the plants could not result in significant photosynthetic fixation of atmospheric carbon.

Germination and Culture of the Seedlings

Triplicate sets of seedlings were grown for periods of 3, 6, 9, 12, 15, 18, 21, and 24 days from the start of germination. Since the cabinet could accommodate only 16 mesh baskets at one time, it was necessary to stagger the times of planting of the 24 sets of seedlings. The three sets of seedlings for each length of growth period were planted and harvested simultaneously. In order to obtain sufficient dry weight of plant axis tissue for all of the analyses, 300 seeds were planted in each of the sets of three- and six-day seedlings, and 200 in the nine-day sets. In each of the other sets, 100 seeds were germinated.

The seeds for each set were counted out (from a lot that had been carefully sorted for uniformity and perfection), weighed, and spread evenly over the bottom of the wire baskets. The quality of the seed stock was such that only a very few seeds were discarded in a careful culling to remove odd-sized ones, and those with cracked seed coats, insect injury, or other defects. Preliminary trials had shown that surface sterilization of the seed was unnecessary, and possibly detrimental. By virtue of having cleaned and steamed out the equipment thoroughly before beginning the germination of the seed, using clean and carefully selected seed, and culturing by means of the water spray technique, in which the surfaces of the seedlings and seeds were continually being washed with clean water, it was possible to grow seedlings until the beginning of physiological breakdown without fear of interference with the seedling metabolism by bacterial or mold growth.

At the start, the positions of the various sets of seedlings in the cabinet were determined by random selection. Thereafter, occasional rearrangements were made to help equalize the distribution of water to all of the baskets, since the water spray, while entirely filling the cabinet, was somewhat more dense in the immediate vicinity of the sprayers. After the first week, the water sprays were cut off from one to several hours during each day, the time depending upon the rapidity with which the temperature changed.

In every instance, 95 per cent or more of the seeds germinated and were showing one-half to two centimeters of growth of the hypocotyl within 24 hours.

Harvesting Procedure

On removing each set of seedlings from the growth chamber, the contents of the wire basket were transferred to a large tray and sorted carefully. Seed that failed to germinate and seedlings displaying abnormality in size or form, or which were contaminated with mold or bacterial growth, were discarded. A count was made of the number of good seedlings and sufficient measurements made to give an estimate of the lengths of hypocotyl and epicotyl, as growth indices. Then, as rapidly as possible, the plants were divided into portions comprising seed coats, cotyledons, and seedling axis. Snapping off the cotyledons resulted in a clean break at the junction of the tissue of cotyledons and hypocotyl. Fresh weights were obtained as soon as all free water appeared to be gone from the surfaces of the plant parts. Free water was removed from the seed coat and cotyledon fractions by blotting and rolling in towels, and from the axis portion by spreading the material out on a tray and allowing the surface water to evaporate, turning the mass over frequently. The axis portion was weighed as soon as droplets of water could no longer be detected on the hypocotyls.

As soon as the fresh weights were obtained, the various portions of plant tissue were spread out on individual porcelain enameled trays and placed in a circulating hot air drier at 78° to 80° C. until thoroughly dry. The drying periods ranged from five to eight hours in length. The dry tissues were transferred, while still warm, to tared sample bottles, capped tightly, and weighed. The equilibration of the samples with the atmosphere was deferred until the harvest of the entire 24-day series was complete.

For determining the composition of the ungerminated seed, three samples of 100 seeds were selected, weighed, the seed coats removed carefully, and the fresh weights of the naked seeds and seed coats obtained. The losses in weight incidental to seed coat removal were 0.70, 0.30, and 0.20 per cent of the initial total weight. Some of this weight loss may have been due to loss of water during the operation, but most is believed to represent the loss of fine fragments of the seed coats. The seed coats and seeds were then dried to constant weight at 80° C., and stored in tightly closed containers.

Equilibration of Samples

The rapidity with which oven dry plant tissues absorb atmospheric moisture renders the accurate weighing of analytical samples exceedingly difficult. On the other hand, tissues which have been equilibrated with air at room temperature and in the relative humidity range from about 50 to 70 per cent are much more stable, and under most conditions, permit the weighing of hundred-milligram samples with a reproducibility of ±0.5 per cent or less; the moisture content will remain practically constant over long periods of time if the samples are maintained in tightly closed containers except when actually removing material for weighing. Since a controlled condition room at 75° Fahrenheit and 50 per cent relative humidity was available, those conditions were used for the equilibration and to establish the "equilibrated dry weight" basis on which the analytical results are expressed. The term "dry weight" as used throughout this work refers to the weight after drying at 80° C. and equilibration (i.e., exposure of the tissues until constant weight was attained) in the standard atmosphere mentioned above.

RESULTS

The progress of seedling growth in terms of length of hypocotyl and epicotyl is shown in Figure 4. It is of interest to note that growth of the epicotyl proceeded very slowly until after the 15th day, when the hypocotyl had attained its maximum length, but that from the 15th to beyond the 21st day the epicotyl increased in length at a rate nearly equal (see Figure 5) to the maximum rate of elongation of the hypocotyl, attained during the period from the 6th to the 9th day. During the period of rapid elongation of the

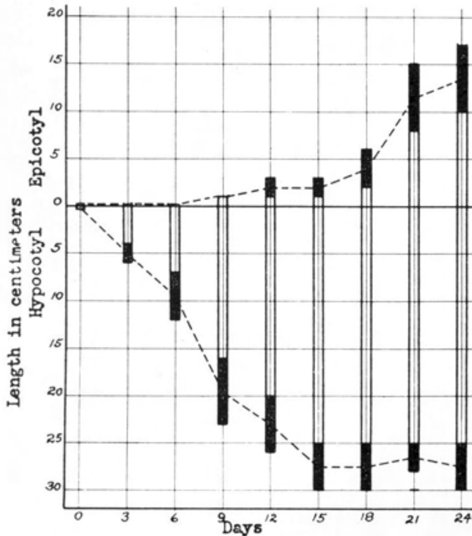

Figure 4. Seedling growth in terms of length of hypocotyl and epicotyl.

(The darkened sections indicate the range in length in each group of seedlings.)

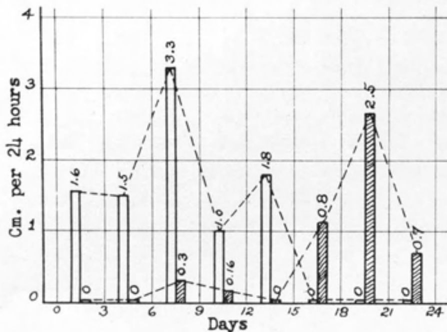

Figure 5. Linear growth rates of hypocotyl and epicotyl during successive three-day periods.

(Open bars, hypocotyl; shaded bars, epicotyl.)

epicotyl, there was a parallel increase in the size of the true leaves. The cotyledons increased in the maximum dimension from 1 to 1.5 cm. in the ungerminated seed to 2 to 2.5 cm. after three to four days of growth, and thereafter showed little increase in size. The cotyledons retained a uniform bright yellow color until after the 18th day. Some cotyledons in the 21-day groups had developed brown areas at the edges, and there was somewhat more of the brown discoloration in the 24-day cotyledons. The hypocotyls remained white and turgid until after the 21st day, when general physiological breakdown commenced. This was clearly indicated in the 24-day old seedlings, which had a general water-soaked appearance and were somewhat flaccid. Breakdown of the tissues was similarly evident in the leaves and roots at that stage.

The changes in fresh weight during germination and growth are shown in Figure 6, plotted from the data of Table 1. These and all subsequent data dealing with absolute quantities are computed on the basis of a weight of 100 grams of original seed material (on the equilibrated dry weight basis) with seed coats removed. Since the number of seedlings required to give this weight of material ranged only from 251 to 265[1] for the 24 groups, the

Table 1. Fresh and Dry Weights[1]

(All values are expressed as grams per 100 grams ungerminated seed, on equilibrated dry basis without seed coats.)

	Fresh Weights			Dry Weights (Equilibrated)			
	Seed coat	Cotyledons	Seedling axis	Seed coat	Cotyledons	Seedling axis	Cotyledons + axis
Seed	23	104	....	22.4	100.0	....	100.0
3 days	71	259	104	22.1	85.8	10.1	95.9
6 days	70	285	428[2]	22.1	63.8	28.5	92.3
9 days	72	288	845	22.1	41.2	48.6	89.8
12 days	57	263	1138	22.4	29.0	57.4	86.4
15 days	69	289	1229	22.0	26.1	58.1	84.2
18 days	61	280	1261	22.1	22.3	59.4	81.7
21 days	....	297	1280	....	20.1	58.1	78.2
24 days	....	286	1165	....	19.6	54.9	74.5

[1] The figures given in Table 1 are the mean values of the three sets of 100 or more seedlings harvested at each stage of growth. The agreement between replicates was such that only very minor variations in the plots of the data were obtained when the mean was compared with plots made from single replicates taken at random from among the sets of three for each point on the curves.
The values for the individual replicates are contained in the dissertation on which this bulletin is based. (Meiss, A. N., 1950; copies are on file in Yale University Library and Osborn Botanical Laboratory, New Haven, Conn.)

[2] 6-3P not included in the mean.

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[1] The factor used for this computation is based on the observed weights of the seed coats and the weight loss, to the equilibrated dry weight condition, as determined on the three ungerminated seed samples. Using this factor, the dry weight of each lot before germination was calculated, and from that weight, the number of seedlings required to correspond to 100 grams of dry seed substance. Average value, 257.7.

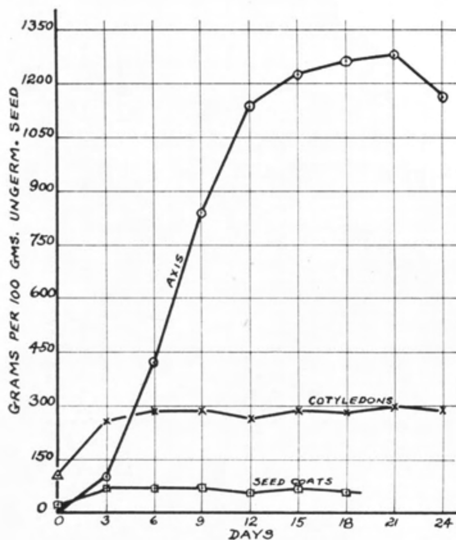

Figure 6. Changes in fresh weight during growth.

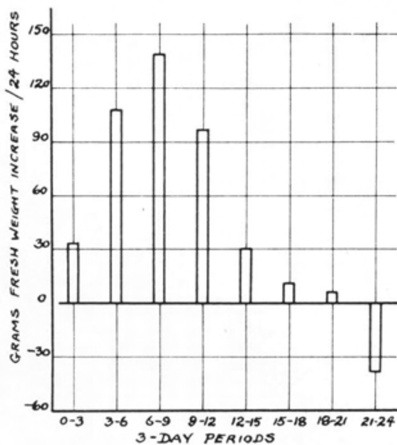

Figure 7. Rate of increase of fresh weight, seedling axis.

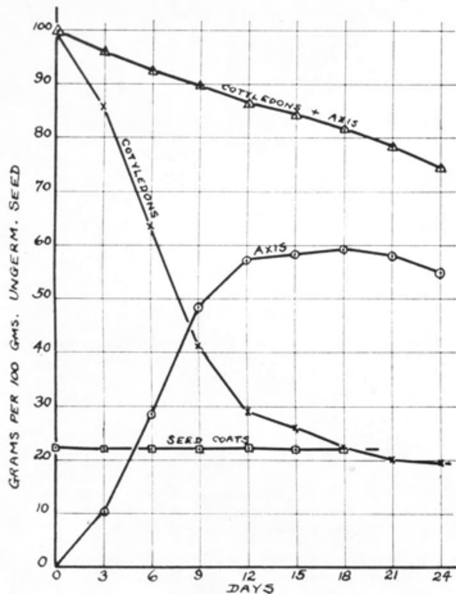

Figure 8. Changes in dry weight during growth.

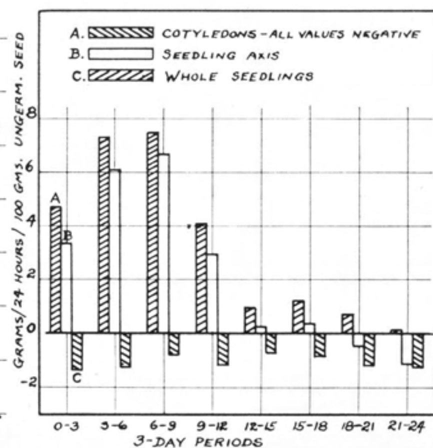

Figure 9. Rates of change of dry weight.

method of expression is satisfactory as a biological unit (i.e., absolute quantities per 258 seedlings) as well as a basis for determining the amount of change from equal initial weights of material involved in the metabolism. Throughout this work, it is assumed for convenience that all of the substance of the peeled seed is contained in the cotyledons, so that all plots of data on the seedling axis arbitrarily originate at zero. A few seeds were dissected and weighed. The results showed that the weight of the embryo is only 3 to 5 per cent of the weight of the peeled ungerminated seed.

It should be noted that the fresh weight of the cotyledons increased only until about the third day, remaining constant thereafter in a manner paralleling the increase in size. Similarly, the increase in fresh weight of the seedling axis corresponds closely with the linear growth of the hypocotyl. The decrease in fresh weight between the 21st and 24th day represents water lost after the onset of breakdown of the tissues. One of the three sets of 21-day seedlings also showed a sharp decrease from the 18-day values. The fresh weight increments per day for the successive 3-day intervals are shown in Figure 7.

The changes in dry weight are shown in Figure 8 (data in Table 1). The rates of change are indicated in Figure 9. These curves are characteristic of growth in the dark of seedlings provided with an abundant supply of stored food. After a short lag period, growth of the embryo proceeds at a rapid rate until the supply of stored food translocated from the cotyledons begins to diminish; then the leveling of the growth curve is followed by a diminution in dry weight after exhaustion of one or more substances essential for maintaining the integrity of the seedling tissues. The loss in weight of the cotyledons represents the substances utilized in the growth of the new seedling axis and the respiratory substrates consumed in supplying energy for the growth and metabolism. The over-all decrease in dry weight of the system approximates the quantity of material used up in respiration. The data for seed coats, falling on a horizontal straight line, conform with the assumption that they do not participate in the metabolic processes.

Part II. Analytical Investigation

ANALYTICAL PROCEDURES

The methods of analysis used in this work are for the most part those which have been used and modified over the years in the Biochemical Laboratory at The Connecticut Agricultural Experiment Station. However, in keeping with the experience in that laboratory, it has been necessary in the present instance to make some modifications in analytical procedures to adapt them to the material under study. Modifications in manipulative technique have been adopted when an increase in convenience or precision could be effected thereby. The accuracy of methods of analysis of plant tissues is usually established by study of the recovery of known constituents, alone or added to tissue extracts; changes in such factors as hydrolysis time, concentration of reagents, and so forth, have been made during this investigation when an improvement in accuracy resulted, as judged by the recovery criterion.

Preparation of samples: After they had attained moisture equilibrium, the samples were ground in a Wiley mill to pass a 40-mesh screen. Each sample was mixed thoroughly in the bottle before withdrawing material for an analytical determination.

Nitrogen Fractions

Total nitrogen was determined by a semi-micro Kjeldahl procedure developed by Pucher (Vickery et al., 1946, p. 11), but with the following modifications. Two ml. of a 2.5 per cent solution of anhydrous sodium selenate were used as the digestion catalyst instead of mercury, and no sodium thiosulfate was added to the sodium hydroxide solution used to neutralize the digestion mixture. The reduction with iron powder was not carried out, since tests with diphenylamine reagent established the absence of nitrate from all tissues. The distillation time was increased from six minutes to eight. Nitrogen in amounts as high as 10 milligrams was recovered quantitatively (99.7%) from ammonium sulfate by the distillation technique used, and in general an agreement within 1 per cent of the quantity determined was obtained in check analyses.

To determine *protein nitrogen*, small samples were extracted for 16 hours with a refluxing water-alcohol mixture,[1] and washed once with hot water; nitrogen was determined on the insoluble residue by the Kjeldahl procedure. The extraction was carried out on 100 or 200-milligram samples, each wrapped securely in a 2.5-inch square of fine mesh cotton cloth and secured with a steel paper clip, to make a flat packet about one inch square. Groups of 24 or more such samples were extracted at one time in a multiple extraction apparatus.[2] To wash with water, the tissue was transferred to a 25 by 200 mm. test tube with 20 ml. of water and heated for 10 minutes with occasional stirring on the steam bath. The tube was then centrifuged, the supernatant solution was decanted through a paper filter, and the washed tissue, including any particles caught on the paper, was transferred to a Kjeldahl flask with a minimum of water. The digestion reagents were added immediately and, after the excess water was boiled off, the total nitrogen procedure was followed. A slightly greater precision was obtained in the protein nitrogen determination than in the total nitrogen determination.

The *soluble nitrogen fractions* were determined on a water extract prepared as follows: One gram of dry tissue was steeped in 80 ml. of water at 80-85° C. for 10 minutes with continual stirring, cooled, made to a volume of 100 ml. and centrifuged. The extract was decanted from the solids, and aliquots were used for the various determinations. *Free ammonia* was determined by the method of Pucher, Vickery, and Leavenworth (1935) and *glutamine-amide* and *asparagine-amide nitrogen* by the methods of Vickery, Pucher, Clark, Chibnall, and Westall (1935). On the basis of recovery data, an increase in the severity of hydrolysis conditions was made for the tissues richest in asparagine by increasing the concentration of acid in the hydrolysis mixture from 1.0 N to 1.5 N. The determination of the ammonia collected in all of these procedures was done titrimetrically (i.e., the ammonia distillate was received in standardized 0.1 N HCl and the resulting solution back-titrated with standardized 0.02 N NaOH), rather than by the Nessler technique. *Free amino* and *total amino nitrogen* were determined according to the procedure of Vickery and Pucher (Vickery et al., 1946, p. 12), using the

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[1] A mixture containing 70% ethyl alcohol by volume was used. The vapor phase and hot condensate, which actually leached the sample, contained 88% alcohol by volume.

[2] cf. L. S. Nolan, Anal. Chem. 21: 1116, 1949.

manometric amino nitrogen method of Van Slyke (Peters and Van Slyke, 1932). The correction of the free amino nitrogen value for the amide nitrogen of glutamine was not applied in view of the minute quantities of glutamine amide nitrogen found; however, the glutamine amide nitrogen values were corrected by subtracting 2.6 per cent of the asparagine amide nitrogen values for the corresponding samples (Vickery, Pucher, Clark, Chibnall, and Westall, 1935).

Hydrolyzates of the *protein nitrogen fraction* of the ungerminated seed, and 6-day and 18-day cotyledons were analyzed for *amino* and *ammonia nitrogen*. The tissue remaining after the preparation of the water extract was recovered quantitatively, extracted with 70 per cent alcohol as in the protein nitrogen determination, and then hydrolyzed under reflux for 24 hours with 25 ml. of 10 *N* sulfuric acid. The insoluble humin of the hydrolyzate was recovered by filtration and humin-nitrogen determined by the Kjeldahl procedure. The clear hydrolyzate was diluted to 100 ml. and 10 ml. aliquots used for the determination of ammonia and amino nitrogen. The ammonia was collected in HCl and determined titrimetrically after distillation in the apparatus used in the free ammonia and amide nitrogen determinations. The residue was then acidified with glacial acetic acid, diluted to 50 ml. and amino nitrogen determined on 5 ml. aliquots by the Van Slyke method. Kjeldahl nitrogen determinations were run on the hydrolyzate and on the alcoholic extract.

Lipids

Determination of total lipids: A 500 milligram sample of tissue was placed in a glass-stoppered centrifuge tube (32 mm. diam. x 180 mm. long, including the taper) and extracted by shaking with three successive portions of petroleum ether. The first portion was allowed to stand in contact with the sample overnight. The rack holding the set of tubes was supported on an oscillating shaking machine in such a position that there was continual mixing of sample and solvent. The successive portions of extract were collected, after centrifuging, by decantation into an Erlenmeyer flask. After distilling off the petroleum ether, the residue was transferred to a tared weighing bottle with a small quantity of petroleum ether, evaporated free of solvent, dried for one-half hour at 105° C., cooled, and weighed. Results obtained by this method were in close agreement with those obtained by an overnight extraction with refluxing petroleum ether.

Soluble Carbohydrates

Preparation of soluble carbohydrate fractions: The dried residue from the lipid determination was extracted, in the same tube and using the same technique as above, with three successive portions of water at room temperature. The combined portions of extract were made up to 100 ml. and aliquots taken for the various determinations.

Sugar titration method: With the exception of the pentose determinations, all of the sugar analyses were based on the reduction of copper by clarified filtrates, using Pucher's modification of the Somogyi method (Pucher, Vickery, and Leavenworth, 1948). The clarification procedure involved treatment of the solution with neutral lead acetate, centrifugation to remove

precipitated organic lead salts, precipitation of excess lead with dibasic potassium phosphate, and finally, treatment with Lloyd's reagent for the absorption of alkaloids and other unknown interfering substances. All carbohydrate fractions except pentose were calculated in terms of glucose.

Free reducing sugar was determined by clarification and titration of an aliquot of the extract, without further treatment. In this and all other determinations, a complete blank was run parallel with each series of unknowns.

Total sugar was determined as the reducing value after treatment of the extract with invertase. 0.2 ml. of invertase solution (Pfanstiehl) was added to 5 ml. of extract, and the mixture allowed to stand for one hour at room temperature before the clarification and sugar estimation were carried out.

Unfermentable sugar was determined as the reducing value after treatment with yeast. The yeast cells from 5 ml. of a standard suspension (8 grams of dried baker's yeast suspended in 50 ml. of distilled water after thorough washing with four or more 300 ml. portions of water, and prepared on the day of use), collected by centrifugation, were added to a small Erlenmeyer flask with 10 or 12 ml. of extract, and the flask was then shaken by machine for one-half hour at room temperature. After removal of the yeast cells by centrifugation, an aliquot of the treated extract was clarified and the reducing value determined.

Total soluble carbohydrate was determined after dilute acid hydrolysis of the water extract. An aliquot, with sulfuric acid added to make a concentration of 0.7 normal, was heated in a boiling water bath for two hours. The acid was neutralized with barium hydroxide and the precipitated barium sulfate removed by the centrifugation during the usual clarification process.

Pentose was determined by an adaptation of the method of McRary and Slattery (1945), which is based on the formation of a deep green color when a pentose is heated in the presence of a strongly acid solution of orcinol. Since lead salts produce a strong interfering color, the clarification procedure was omitted. Glucose interference was eliminated by yeast treatment, following the procedure of the unfermentable sugar determination. An aliquot of the yeast-treated extract and the reagent were mixed in a small conical centrifuge tube and heated for 30 minutes in a boiling water bath to develop the color. After being cooled, the tube was centrifuged at high speed for 5 minutes. The optical density was then measured at 670 mμ in the Beckman model D spectrophotometer. After the appropriate blank reading was subtracted, the quantity of pentose in the aliquot was read from a standard curve based on a series of concentrations of pure arabinose treated with yeast in the same manner as the unknowns.

Insoluble Carbohydrates

Hydrolysis of residue: Twenty-five ml. of 0.7 *N* sulfuric acid were added to the dry tissue residue in the large centrifuge tube, which was then heated for 2½ hours, with occasional agitation, in a boiling water bath. The contents of the tube were then diluted to the 50 ml. mark with distilled water, and the hydrolyzate separated from the solids by centrifugation.

Total insoluble carbohydrate was determined by neutralizing an aliquot of the hydrolyzate and following the procedure for total soluble carbohydrate.

Unfermentable carbohydrate in the hydrolyzate was determined by the procedure for unfermentable sugars in the water extract, using an aliquot of the hydrolyzate which was first neutralized and separated from the precipitated barium sulfate.

Pentose was determined on neutralized, barium-free portions of the hydrolyzate.

Starch was shown by qualitative tests to be absent from all tissues studied. However, in preliminary work, analyses for starch were made on a number of tissue samples by the method of Pucher, Vickery, and Leavenworth (1948). Results were negative in every case.

Total Organic Acids

The preparation of the organic acid fraction and titration of total organic acids were done according to the methods of Pucher, Wakeman, and Vickery (1941). The only change in procedure was in the transfer of the acids from the ether phase to the alkaline aqueous solution. Instead of adding the water and alkali to the ether and evaporating the latter completely, the ether was evaporated to a volume of about 50 ml. and transferred to a separatory funnel, where the acids were extracted from the ether by shaking out into the dilute sodium hydroxide. The aqueous phase (about 12 cc.) was then drawn off into a 25 ml. volumetric flask, and was made to volume by washing the ether with successive 2 to 3 ml. portions of water and adding the washings to the flask. Titration of the ether residue established that only about 1 per cent of the total organic acids was retained. This change was made necessary by the large quantities of lipids present in the ether extract. If carried along with the aqueous phase, the fatty materials became deposited on the glass electrode in a thick layer during the titration.

Inorganic Constituents

Moisture content was determined as the weight loss in drying 500-milligram samples to constant weight at 110° C. The time required to reach constant weight with the various tissues was noted.

Ash was determined on the dried samples from the moisture determination, by heating, first over a low flame until completely charred, then in an electric muffle at 580±20° C. for two hours. The ash from one determination on each of a series of different tissue samples was subjected to detailed spectrographic analysis in the Department of Analytical Chemistry.

Alkalinity of ash titrations were performed with the ash from the rest of the determinations. After being weighed, the ash was digested with excess standardized hydrochloric acid solution, which was boiled to expel carbon dioxide, and then back-titrated with standardized sodium hydroxide solution. The results were expressed as milli-equivalents per gram of dry tissue. To calibrate the alkalinity titration method, sodium citrate (Mallinckrodt A. R. grade), in quantities roughly equivalent to the ash found in the tissues, was ashed and titrated as above. The experimental values for the alkalinity of the citrate ash ranged from 104 to 106 per cent of the theoretical values.

The pH of the dry tissue was measured with the Beckman model G pH meter. One hundred milligrams of tissue were mixed with 2.5 ml. of distilled water and the pH measurement made after the mixture had stood for one-half hour with occasional stirring.

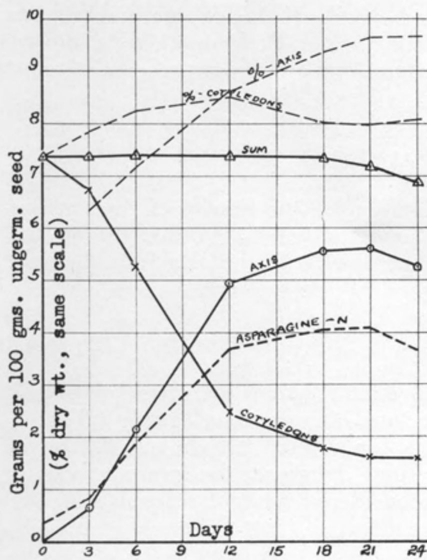


Figure 10. Total nitrogen.

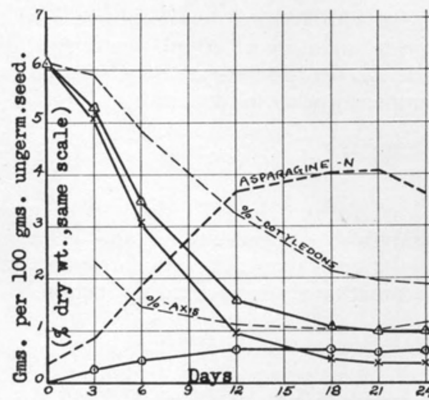


Figure 11. Protein nitrogen.

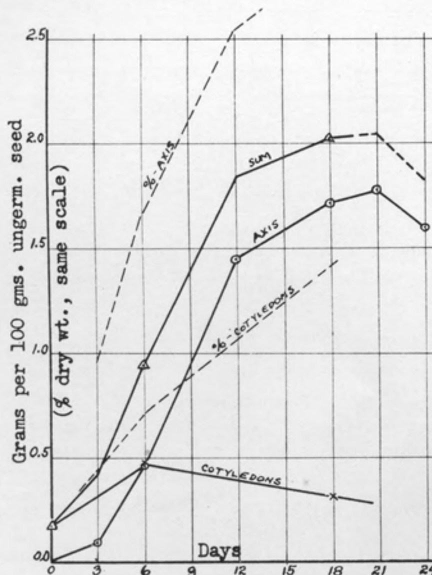


Figure 12. Asparagine amide nitrogen.

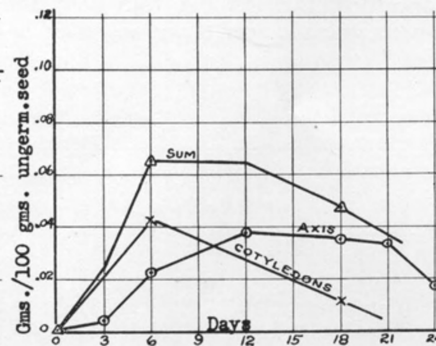


Fig. 13A. Glutamine amide nitrogen.

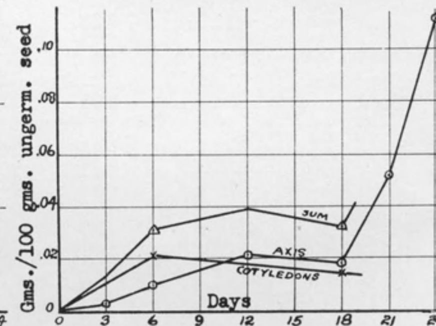


Fig. 13B. Free ammonia nitrogen.

Titratable acidity of the dry tissue was determined by potentiometric titration of hundred-milligram portions, each suspended in 20 ml. of distilled water. Two samples were used to establish the complete titration curve; one was titrated with 0.02 *N* sodium hydroxide to beyond pH 8.0 and the other with 0.02 *N* hydrochloric acid to beyond pH 2.6. Water blanks were run by titration of 20 ml. portions of distilled water. Preliminary runs showed that the titration curves of a hot water extract and a tissue suspension at room temperature were identical.

RESULTS OF THE ANALYTICAL WORK

This section is limited to a presentation of the results of the various analytical determinations and certain computations based directly on the analytical values. The interpretation and evaluation of the data form the subject matter of the succeeding section.

Nitrogen Fractions

The results of the analyses of the nitrogen fractions are given in Table 2 and in Figures 10 through 16. From the total nitrogen data (Table 2,B) it is seen that the system is a closed one, insofar as the nitrogen metabolism is concerned, through the 18th day, and that, therefore, the interconversions among the nitrogen fractions may be considered without reference to any

TABLE 2. NITROGEN FRACTIONS

(*A*). *As percentage of dry weight*[1]							
	Seed	3 days	6 days	12 days	18 days	21 days	24 days
TOTAL NITROGEN							
Seed coat	0.54	....	....	0.46	0.44	....	....
Cotyledons	7.39	7.84	8.24	8.50	8.02	7.99	8.10
Seedling axis	....	6.33	7.17	8.59	9.35	9.62	9.65
PROTEIN NITROGEN							
Cotyledons	6.11	5.88	4.82	3.25	2.14	1.96	1.90
Seedling axis	....	2.38	1.46	1.12	1.04	1.02	1.15
ASPARAGINE AMIDE NITROGEN							
Cotyledons	0.175	....	0.74	....	1.41	....	....
Seedling axis	....	0.95	1.65	2.54	2.89	3.06	2.90
FREE AMMONIA NITROGEN							
Cotyledons	nil	....	0.033	....	0.065	....	....
Seedling axis	....	0.028	0.035	0.037	0.030	0.090	0.205
GLUTAMINE AMIDE NITROGEN							
Cotyledons	nil	....	0.066	....	0.055	....	....
Seedling axis	....	0.050	0.078	0.067	0.060	0.059	0.030
FREE AMINO NITROGEN							
Cotyledons	0.213	....	1.13	....	2.36	....	....
Seedling axis	....	1.37	2.05	3.32	3.80	3.88	3.80
PEPTIDE NITROGEN							
Cotyledons	0.500	....	1.25	....	0.264	....	....
Seedling axis	....	0.405	0.519	0.260	0.100	0.121	....

[1] The figures given are the mean values for each of the three replicate groups. See note, Table 1.

gains from or losses to the environment. The similarity of the total nitrogen curves to the dry weight growth curves is immediately apparent.

During the entire period of growth, 94 per cent of the protein nitrogen originally present in the cotyledons disappeared. Of this, 85 per cent was gone by the 12th day and 92 per cent by the 18th day. Synthesis of protein in the seedling axis proceeded in a manner corresponding to the increase in dry weight, reaching a maximum value by the 12th day and remaining essentially constant thereafter.

To illustrate the magnitude of the asparagine synthesis, a curve representing the asparagine nitrogen of cotyledons plus axis has been superimposed

TABLE 2. NITROGEN FRACTIONS

(*B*). *Grams per* 100 *grams ungerminated seed*

	Seed	3 days	6 days	12 days	18 days	21 days	24 days
TOTAL NITROGEN							
Seed coat	0.12	....	....	0.10	0.10	....	....
Cotyledons	7.39	6.73	5.26	2.46	1.79	1.60	1.59
Seedling axis	....	0.64	2.16[1]	4.93	5.55	5.59	5.29
Sum, cotyledons + axis	7.39	7.37	7.42	7.39	7.34	7.19	6.88
PROTEIN NITROGEN							
Cotyledons	6.12	5.05	3.07	0.94	0.48	0.39	0.37
Seedling axis	....	0.24	0.42	0.64	0.62	0.59	0.58[2]
Sum, cotyledons + axis	6.12	5.29	3.49	1.58	1.10	0.98	0.95
ASPARAGINE AMIDE NITROGEN							
Cotyledons	0.175	....	0.47	....	0.31	....	....
Seedling axis	....	0.096	0.47	1.46	1.72	1.78	1.59
Sum, cotyledons + axis	0.175	0.42[3]	0.94	1.85[3]	2.03	2.05[3]	1.82[3]
FREE AMMONIA NITROGEN							
Cotyledons	nil	....	0.021	....	0.014	....	....
Seedling axis	....	0.003	0.010	0.021	0.018	0.052	0.112
Sum, cotyledons + axis	nil	....	0.031	....	0.032	....	....
GLUTAMINE AMIDE NITROGEN							
Cotyledons	nil	....	0.042	....	0.012	....	....
Seedling axis	....	0.004	0.023	0.038	0.035	0.033	0.017
Sum, cotyledons + axis	nil	....	0.065	0.065[3]	0.047	....	....
FREE AMINO NITROGEN							
Cotyledons	0.213	....	0.72	....	0.51	....	....
Seedling axis	....	0.139	0.58	1.91	2.25	2.26	2.08
Sum, cotyledons + axis	0.213	0.61[3]	1.30	2.52[3]	2.76	2.73[3]	2.50[3]
PEPTIDE NITROGEN							
Cotyledons	0.500	....	0.80	....	0.058	....	....
Seedling axis	....	0.041	0.149	0.150	0.060	0.071	....
Sum, cotyledons + axis	0.500	0.69[3]	0.95	0.58[3]	0.118	....	....

[1] 6-3P not included in mean.
[2] 24-3P not included in mean.
[3] Mean based in part on interpolated or estimated values.

on both the total nitrogen and the protein nitrogen curves. The data on asparagine amide nitrogen, which represents one-half of the total nitrogen of asparagine, are shown graphically in Figure 12. As with the increase in dry weight, asparagine arose very rapidly in the seedling axis between the 3rd and 12th days, but thereafter continued to increase at a relatively more rapid rate than the dry weight change, and reached a maximum on the 21st day, somewhat later than the dry weight. The dry weight of the axis on the 12th day was 97 per cent of the maximum, while the asparagine amide nitrogen content at the same time was only 82 per cent of the maximum. The increase in the seedling axis after the 12th day may have been due in part to translocation from the cotyledons. On the basis of 5.4 per cent as the aspartic acid (asparagine) content of seed meal of *L. luteus* (Heinrich, 1941), more than 90 per cent of the total asparagine formed, as shown in Figures 10-12, is of secondary origin.

The free ammonia and glutamine amide fractions were minute in quantity. In Figure 13 the data are plotted on a scale that is twelve times that of the asparagine amide plot in Figure 12. The striking increase in free ammonia from the 18th day onward is associated with the commencement of physiological breakdown of the tissues at that time.

A line representing the amino nitrogen of asparagine is shown on the free amino nitrogen graph, Figure 14. The area between that line and the curve for the total of free amino nitrogen in the seedlings represents the amino nitrogen of all free amino acids other than asparagine, and in addition, any free α-amino groups in the peptide fraction. As growth progressed, the proportion of free amino nitrogen from asparagine was greater in the axis tissue than in the cotyledons; the percentage figures at 18 days were 76 and 65, respectively.

The difference between total and free amino nitrogen constitutes the peptide nitrogen fraction (Figure 15). In the interest of brevity, the figures for total amino nitrogen are not presented, but the original data can be obtained by adding the corresponding values for free amino nitrogen and peptide nitrogen. A considerable increase in peptide nitrogen in the cotyledons occurred, as might be expected, during the period of rapid hydrolysis of protein, and as the protein nitrogen approached its minimum value, peptide nitrogen decreased along with it, both in the absolute sense and in the ratio of peptide to protein.

The results of the analysis of the insoluble nitrogen of the ungerminated seed and cotyledons are summarized in Table 3. These data have been used to compute the quantities of amino and ammonia nitrogen produced by decomposition of the storage protein as seedling growth progressed. These amino and ammonia nitrogen fractions are derived from the peptide and amide linkages, respectively, of the intact protein molecules. In the discussion of the present work, they are considered to be that part of the protein nitrogen that is readily available for amide synthesis. The computed values for "available nitrogen" of protein are given in Table 4. A small amount of the available nitrogen was lost in the analytical process through the formation of humin, but the total nitrogen of the humin is so small that the loss may be disregarded for practical purposes.

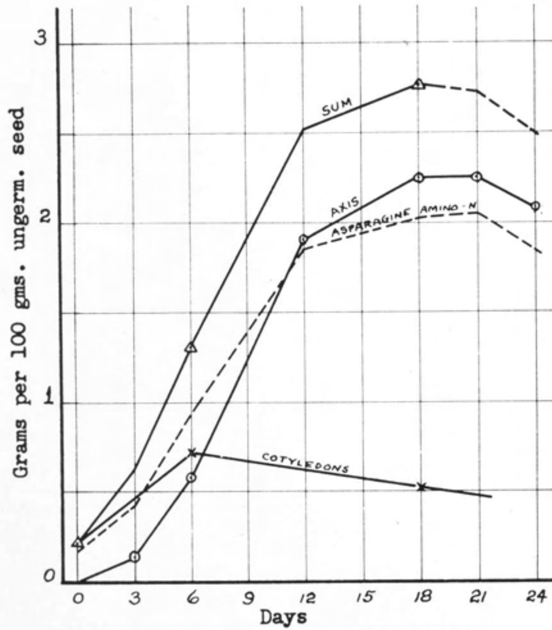


Figure 14. Free amino nitrogen.

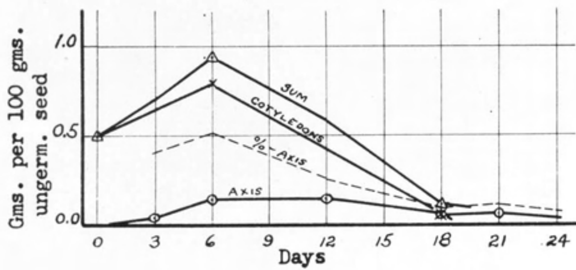


Figure 15. Peptide nitrogen.

(percentage line on same scale as grams per hundred grams ungerminated seed.)

TABLE 3. ANALYSIS OF PROTEIN NITROGEN FRACTIONS IN SEED AND COTYLEDONS

(Figures given are the average values for three seedling groups.)

	Seed	Cotyledons: 6 days	Cotyledons: 18 days
(1) Total N of "protein", grams per 100 g. ungerm. seed	5.015	1.842	0.446
Percentage distribution of the total N:			
(2) Humin nitrogen	1.6	2.8	4.6
(3) Amino N in hydrolyzate	63.1	63.6	65.6
(4) Ammonia N in hydrolyzate[1]	13.2	13.5	12.4
(5) "Available Nitrogen", (3) + (4)	76.3	77.1	78.0
(6) Undetermined N in hydrolyzate, by difference	22.1	20.1	17.4

[1] Presumably this arises almost entirely from amide groups of glutamine and asparagine residues in the intact protein molecule.

TABLE 4. "AVAILABLE NITROGEN" FROM PROTEIN

	Seed	Cotyledons, age in days: 3	6	12	18	21	24
Protein nitrogen (Table 2), gms.	6.12	5.05	3.07	0.943	0.478	0.394	0.373
Percentage of "available N"[1]	76.3	76.7	77.1	77.5	78.0	78.2	78.4
AVAILABLE N, grams per 100 grams ungerminated seed[2]	4.67	3.87	2.37	0.73	0.37	0.31	0.29

[1] From line (5), Table 3. Values for 3 and 12 days were obtained by interpolation, and for 21 and 24 days by projection.

[2] Protein nitrogen (first line) multiplied by percentage of available nitrogen (second line, above).

The distribution of the total nitrogen among the various fractions is shown in Table 5 and in Figure 16.

The detailed analysis of the fractions comprising lipids, carbohydrates, and total organic acids was carried out on samples S-1, cotyledons and seedling axis of 6-1, 12-1, and 18-2, and cotyledons of 21-1.[1]

Lipids

The results of the lipid determinations are shown in Table 6 and plotted in Figure 17. Eighty-seven per cent of the lipid disappeared from the cotyledons by the 12th day, and the utilization of the lipid fraction was still continuing, but at a much reduced rate, on the 21st day, when only 5 per cent remained of the lipid initially present. Approximately 10 per cent of the lipid

[1] Explanation of sample designations: The first number indicates the number of days of growth, and the second, the number of the replicate, 1, 2, or 3. S designates a sample of the ungerminated seed, and C and P the cotyledons and plant axis, respectively.

lost from the cotyledons was accountable for by lipid synthesis in the seedling axis, which continued until the 12th day. Samples of the lipid fraction were examined qualitatively in the infra-red spectrometer by S. G. Linsley, of the Department of Analytical Chemistry. Visual observation indicated the presence of two fractions, one a clear, pale yellow, viscous oil, freely soluble in petroleum ether, and the other a white solid material which required warming for 15 to 20 minutes to redissolve. The oil was characterized as a homogenous

TABLE 5. PERCENTAGE DISTRIBUTION OF THE TOTAL NITROGEN

A. Distribution in the Whole Seedlings

	Seed	3 days	6 days	12 days	18 days	21 days	24 days
Protein nitrogen	82.8	71.8	47.0	21.4	14.8	13.3	13.6
"Available N" of protein[1]	63.3	52.5	31.9	9.9	5.0	4.2	3.9
Peptide nitrogen	6.8	9.3	12.7	7.8	1.6	......	......
Total N of peptides[2]	8.8	12.1	16.5	10.1	2.1	......	......
Asparagine nitrogen	4.7	11.4	25.4	50.0	54.7	55.3	49.4
Glutamine nitrogen	0.0	0.6	1.8	1.8	1.3	1.0	0.5
Free ammonia nitrogen	0.0	0.2	0.4	0.5	0.4	0.9	1.7
Other amino nitrogen	0.5	1.7	2.6	6.4	7.6	7.0	7.8
Total N of amino acids[3]	0.6	2.2	3.4	8.3	9.9	9.1	10.1
Undetermined soluble N[4]	5.2	5.0	10.1	12.1	18.9	19.8[5]	20.1[5]
Nitrogen lost from system[6]	0.0	0.0	0.0	0.0	0.7	2.7	6.9

B. Distribution in Cotyledons and Seedling Axis as Percentage of the Total Nitrogen of the Whole Seedlings

	Cotyledons		Seedling axis					
	6 days	18 days	3 days	6 days	12 days	18 days	21 days	24 days
Protein nitrogen	41.4	6.5	3.3	5.6	8.7	8.3	8.0	8.6
Peptide nitrogen	10.7	0.8	0.6	2.0	2.0	0.8	1.0	0.7
Total N of peptides	13.9	1.0	0.8	2.6	2.6	1.0	1.3	0.9
Asparagine nitrogen	12.7	8.3	2.6	12.6	39.4	46.4	48.0	43.1
Glutamine nitrogen	1.1	0.3	0.1	0.7	1.0	1.0	0.9	0.5
Free ammonia nitrogen	0.3	0.2	0.0	0.1	0.3	0.2	0.7	1.5
Other amino nitrogen	2.0	2.3	0.4	0.7	4.2	5.3	4.6	5.2
Total N of amino acids	2.6	3.0	0.5	0.9	5.5	6.9	6.0	6.8
Undetermined soluble N	2.7	5.8	1.7	7.4	11.1	13.1	12.4	11.6
Total nitrogen as percentage of total nitrogen of whole seedlings	70.9	24.2	8.7	29.1	66.7	75.1	75.6	71.2

[1] Based on values in Table 4.

[2] 1.3 x peptide nitrogen.

[3] 1.3 x NH_2-N; includes free amino nitrogen other than that of asparagine and glutamine.

[4] Includes non-α-amino nitrogen (excluding amide N) of peptides and amino acids.

[5] Peptide nitrogen is included.

[6] For the computation of the nitrogen lost from the system, the initial total nitrogen value for the 18, 21, and 24-day seedlings was assumed to be 7.39 grams per 100 grams of ungerminated seed, that being the mean value for the seed, 3, 6, and 12-day seedlings.

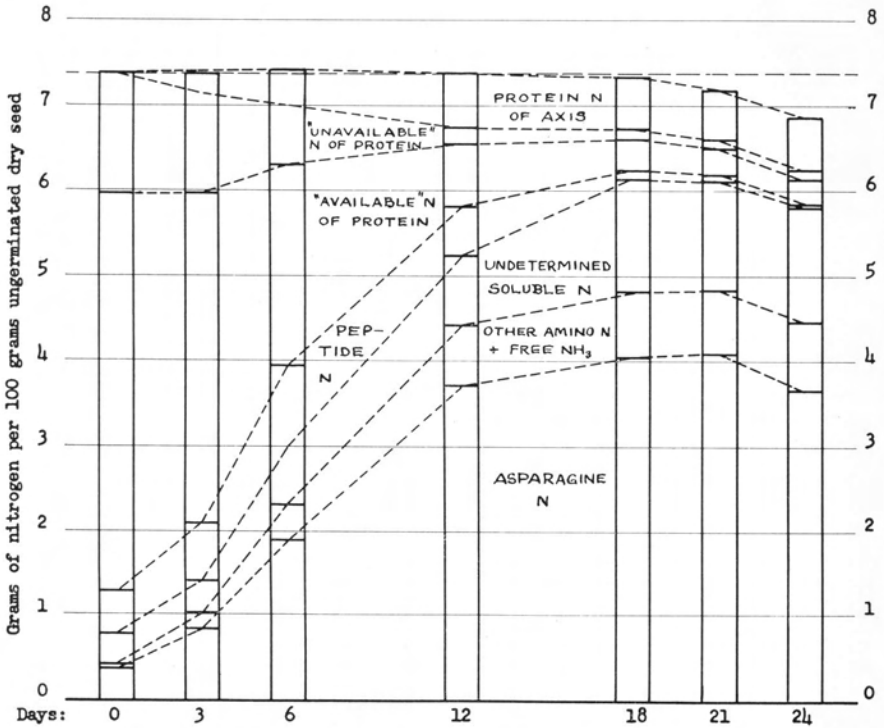


Figure 16. Distribution of the total nitrogen (whole seedlings).

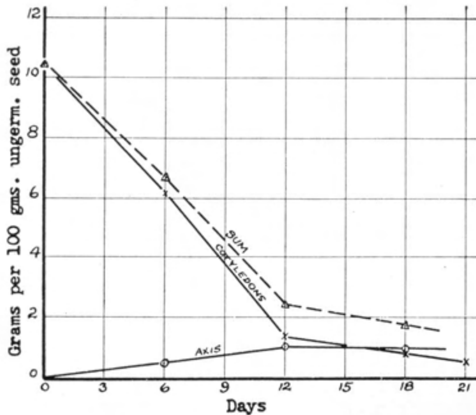


Figure 17. Lipids.

unsaturated ester, and the white solid was considered to be saturated free fatty acid. The lipid fraction in the ungerminated seed consisted mostly of the oil, while the solid portion became predominant in both the cotyledons and seedling axis as growth progressed.

Soluble and Insoluble Carbohydrates

The results of the analysis of carbohydrate fractions are given in Table 6. These data have been illustrated in Figures 18 and 19. From Figure 18A it is seen that all of the soluble fractions in the cotyledons approached a stable low limit by the 12th day, at which time 92 per cent of the total soluble reducing substances had disappeared from those tissues. However, translocation to the seedling axis proceeded, during the first six days, at a rate much greater than the rate of utilization, so that the change in total sugars (Figure 18) for the whole seedlings maintained a fairly constant rate throughout the period of growth and asparagine synthesis. As illustrated in Figure 18A, utilization of sugars in the seedling axis was nearly balanced by translocation from the cotyledons during the period from the 6th to the 12th day, but thereafter the accumulated reserve in the plant axis was rapidly depleted.

The net loss of reducing substances from the insoluble fraction was 62 per cent by the 6th day, but subsequently the loss was slight, only an additional 8 per cent having been lost by the 18th day, giving a total utilization of 70 per cent of the insoluble carbohydrate during the period in which asparagine was accumulating (Figure 19). The net loss of soluble carbohydrate over the same period was 77 per cent. However, since the supply of soluble carbohydrates was heavily augmented only during the first six days by soluble products derived from the insoluble carbohydrate fractions, the change in total carbohydrate (Figure 19A) should be used to give a truer picture of the rates at which the carbohydrates were metabolized. During the period from 0 to 6 days, the total carbohydrate was metabolized at an average rate of 1.62 grams per day per hundred grams original seed, while for the 6th to 12th and 12th to 18th-day periods, the respective rates were 0.61 and 0.60 grams.

From the data on unfermentable reducing substances (see Figures 18 and 18A), it may be inferred that during the first six days the main activity was the transfer of the bulk of this fraction from the cotyledons to the axis, followed by minor utilization during the period from 6 to 12 days, and a somewhat greater rate of disappearance thereafter, to give a total utilization of about 52 per cent during the 18-day period.

The analysis of the hydrolyzate of the insoluble fraction is shown in Figure 19. It should be noted that the curve for unfermentable reducing substances very nearly coincides with that for the total reduction, with both calculated as glucose.[1] Of the arabinose from the pentosan fraction of the un-

[1] The reality of the difference between the total insoluble carbohydrate and the unfermentable portion cannot be ascertained with the present data, in which all reducing values have been arbitrarily calculated as glucose. The reducing value of arabinose has been experimentally determined as 1.12 times that of glucose, but the corresponding relative values for galactose and galacturonic acid have not been determined for the present analytical method, nor is any estimate available of the relative proportions of these two substances in the pectin fraction. For Bertrand's copper-reduction method, the reducing values for galactose and galacturonic acid have been shown to be approximately 95% and 70%, respectively, of the glucose value. (Browne and Zerban, Phys. and Chem. Methods of Sugar Analysis, 3rd ed., New York: Wiley, 1941. Table 20, Appendix.) Therefore, the quantities of galactose and galacturonic acid as determined here may actually be 10% to 20% low.

TABLE 6. LIPID FRACTION AND CARBOHYDRATES

A. As Percentage of Dry Weight

	Seed	6-1C	12-1C	18-2C	21-1C	6-1P	12-1P	18-2P
Lipid Fraction	10.48	9.56	4.94	3.52	2.60	1.82	1.82	1.64
Soluble Carbohydrates								
Total soluble carbohydrates	10.50	5.85	3.66	3.63	2.73	....	......	2.69
Free reducing sugars	0.68	0.71	0.91	1.84	1.20	10.34	4.93	1.56
Total sugars (invertase)	8.48	5.06	2.57	2.38	1.27	11.89	5.90	1.99
Unfermentable sugars	2.46	0.81	1.04	1.22	0.85	6.95	3.32	1.55
Unfermentable after hydrolysis	3.90	1.54	1.30	......	......	2.55	......	......
Soluble pentose	0.52	0.52	0.60	0.39	0.47	0.70	0.40	0.40
Insoluble Carbohydrates[1]								
Total insoluble carbohydrates	12.84	5.12	7.76	7.40	6.86	5.27	3.92	3.82
Unfermentable insoluble carbohydrates	12.49	4.72	7.22	6.71	6.22	5.27	3.77	3.55
Insoluble pentose	4.20	......	6.32	5.25	5.12	2.75	2.38	2.62

[1] Analysis of dilute acid hydrolyzate.

Table 6. Lipid Fraction and Carbohydrates (*continued*)

B. As Grams per 100 Grams Ungerminated Seed

	Seed	6-1C	12-1C	18-2C	21-1C	6-1P	12-1P	18-2P	Sum, axis + cotyledons		
									6	12	18
Lipid fraction	10.50	6.20	1.39	0.81	0.54	0.53	1.04	0.96	6.73	2.43	1.77
Soluble Carbohydrates											
Total soluble carbohydrates	10.52	3.80	1.03	0.84	0.57	4.99[1]	4.52[1]	1.57	8.79[1]	5.55[1]	2.41
Free reducing sugars	0.68	0.46	0.26	0.42	0.25	3.02	2.81	0.91	3.48	3.07	1.33
Total sugars (invertase)	8.50	3.28	0.72	0.55	0.26	3.48	3.36	1.16	6.76	4.08	1.71
Unfermentable sugars	2.46	0.53	0.29	0.28	0.18	2.03	1.89	0.90	2.56	2.18	1.18
Unfermentable after hydrolysis	3.91	1.00	0.37	......	......	0.75	......	......	1.75	......	......
Soluble pentose	0.52	0.34	0.17	0.09	0.10	0.20	0.23	0.23	0.54	0.40	0.32
Insoluble Carbohydrates											
Total insoluble carbohydrates	12.87	3.32	2.19	1.70	1.43	1.54	2.23	2.23	4.86	4.42	3.93
Unfermentable insoluble carbohydrates	12.51	3.06	2.03	1.55	1.30	1.54	2.15	2.07	4.60	4.18	3.62
Insoluble pentose	4.21	2.40[1]	1.78	1.21	1.07	0.80	1.35	1.53	3.20[1]	3.13	2.74
Difference, unfermentable minus pentose	8.31	0.66[1]	0.25	0.34	0.23	0.74	0.80	0.54	1.40[1]	1.05	0.88

[1] Based on interpolated values.

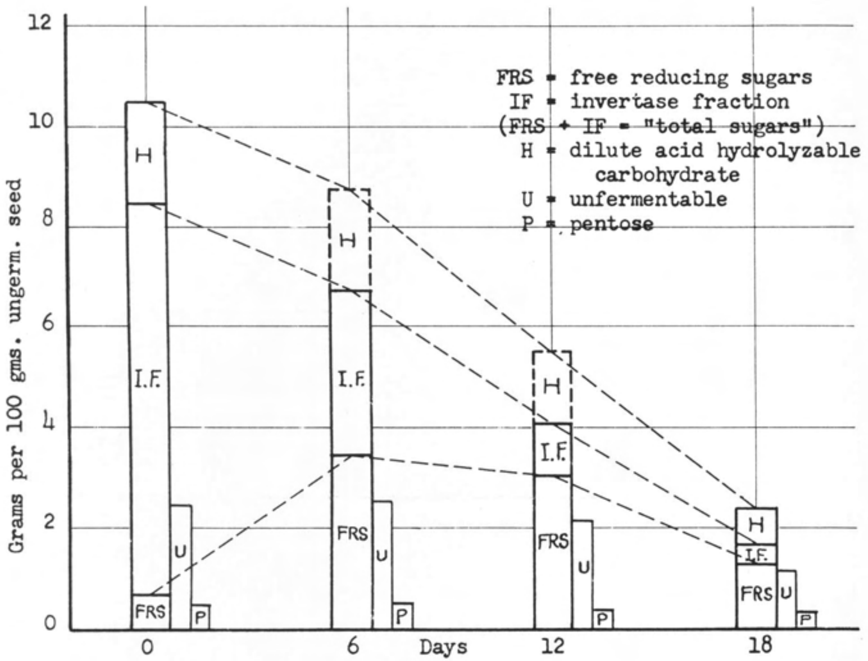


Figure 18. Soluble carbohydrates, whole seedlings.

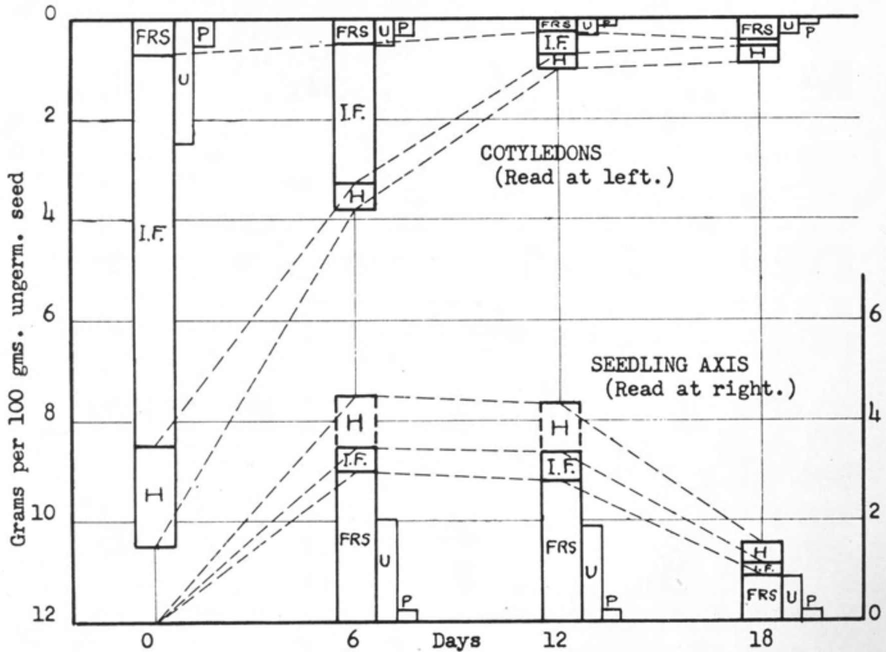


Figure 18A. Soluble carbohydrates in cotyledons and axis.

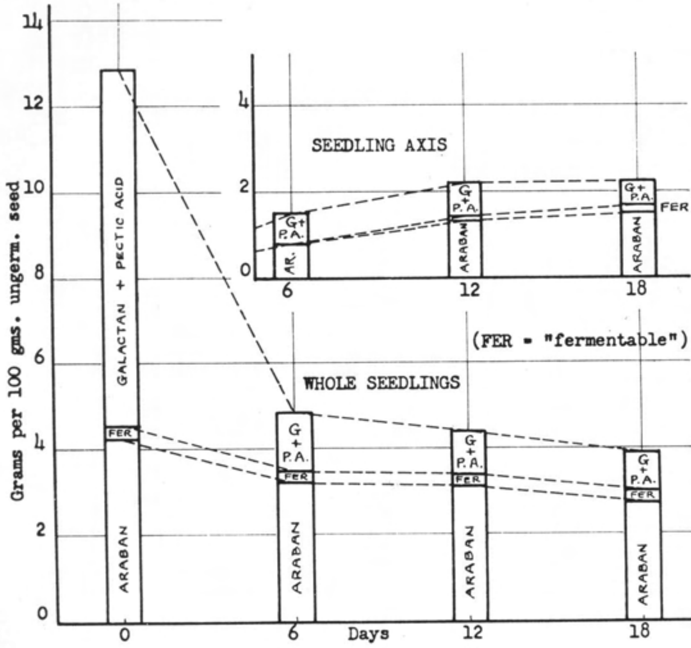


Figure 19. Insoluble carbohydrates.

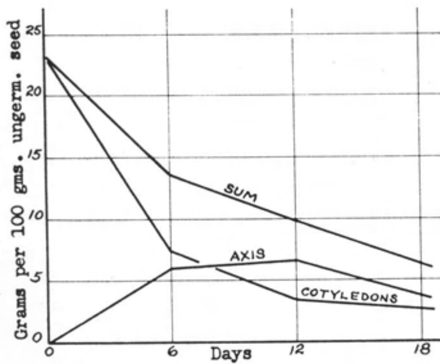


Figure 19A. Total carbohydrates.

germinated seed, 1.47 grams, or 35 per cent, disappeared, an additional 36 per cent is accounted for by resynthesis of pentosan in the seedling axis, and the rest remains, to comprise 71 per cent of the total insoluble carbohydrate of the cotyledons at 18 days, as against a value of 33 per cent in the ungerminated seed. The insoluble carbohydrate of the seedling axis at 18 days contained approximately 68 per cent pentosan. The galactan and pectic acid fractions (polymers of galactose and galacturonic acid, respectively), obtained by subtracting the pentosan value from the unfermentable reducing substance value, disappeared to the extent of nearly 90 per cent of the quantity present in the seed. Of the total quantity of insoluble carbohydrate which disappeared from the cotyledons and could not be accounted for in the pectic substance of the seedling axis, approximately 83 per cent came from the galactan and pectic acid fractions, which comprise but 65 per cent in the seed.

The quantities of pentose in the soluble fraction are small, and it is not known whether any of this existed in the free state, or whether it was present entirely in combination in nucleotides and polysaccharides. The treatment of the filtrate during the pentose determination, heating at 100° C. for 30 minutes in the presence of 9 *N* HCl, is sufficiently drastic to destroy such combinations and liberate the pentose. About 80 per cent of the pentose of the soluble fraction disappeared from the cotyledons during 18 days, half of this being accounted for in the soluble fraction in the seedling axis, where the pentose content remained stable from the sixth day onwards.

Organic Acids

Total organic acids are shown in Table 7 and are plotted in Figure 20, along with the data of Pucher from Figure 3, for comparison. The total acids, computed as malic acid, appear on that basis to make up over 6 per cent of the dry substance of the seed, but less than one-fifth disappeared during 18 days of growth. The organic acid fraction, therefore, does not seem to contribute any appreciable quantity of synthetic or respiratory substrates to the seedling metabolism. However, the common plant acids may make up only a small proportion of the total organic acidity as here determined.

Table 7. Total Organic Acids, Alkalinity of Ash, and Titratable Acidity

(All data computed as milliequivalents per 100 grams of ungerminated seed.)

	Seed-1	6-1C	12-1C	18-2C	21-1C	6-1P	12-1P	18-2P	21-1P
(1) Total organic acids	71	44	27	20	22	26	27	36	....
(2) Alkalinity of ash	23	18	14	11	....	13	21	21	....
(3) Titratable acidity,[1] to pH 8.0	25	21	16	13	....	26	47	40	36
(4) Titratable acidity,[1] to pH 2.6	80	77	49	42	....	36	80	83	89
(5) Titratable acidity, pH 2.6 to 8.0 (3+4)	105	98	65	55	....	62	127	123	125
(6) Total ash computed as K_2CO_3	38	27	17	15	....	16	34	31	....

[1] Direct titration of 100 mg. of dry tissue suspended 20 ml. of water at room temp.

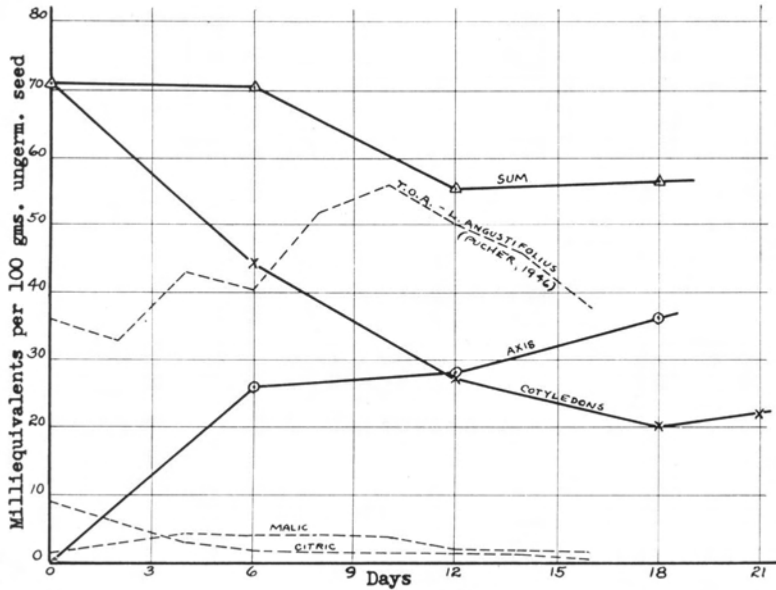


Figure 20. Total organic acids.

(Unpub. data of Pucher (1946)(broken lines) on whole seedlings of L. angustifolius are shown for comparison.)

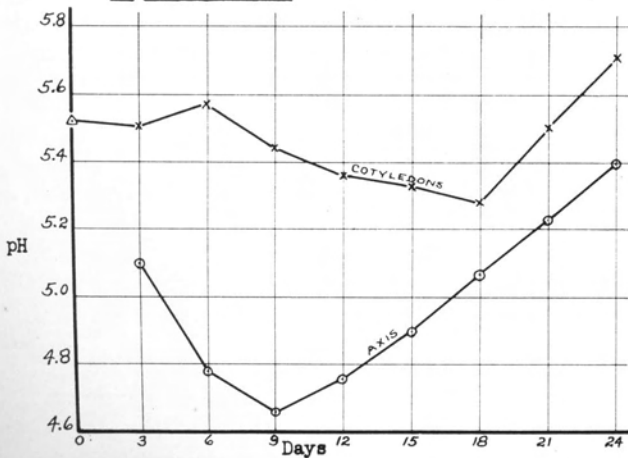


Figure 21. pH values of the dried tissues.

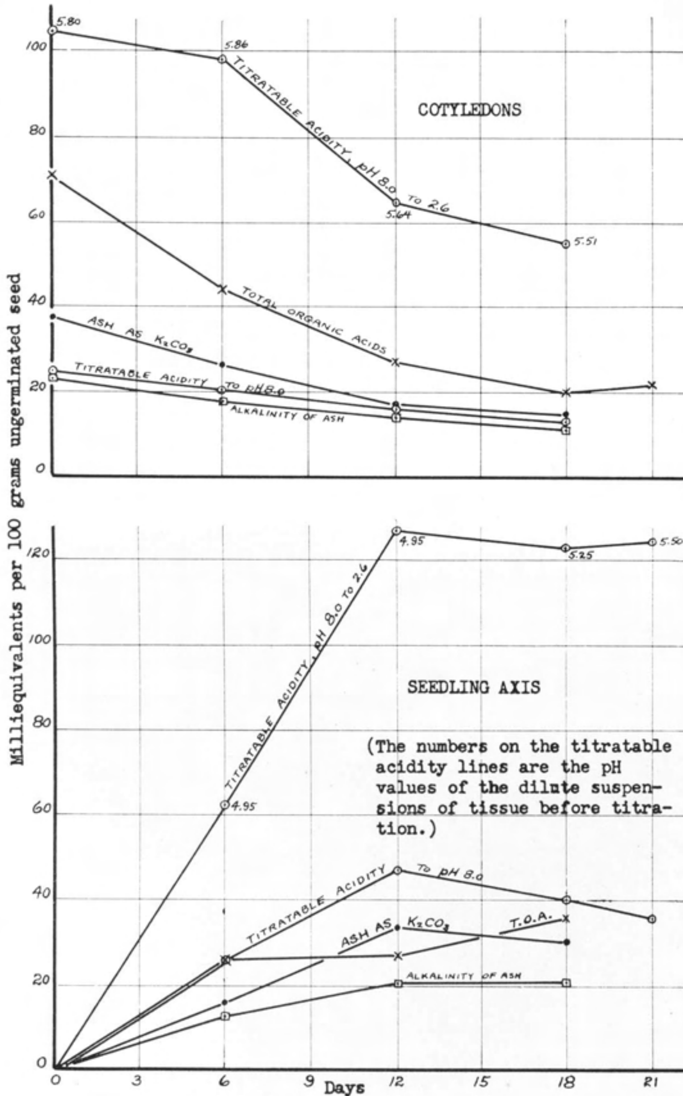


Figure 22. Comparison of total organic acids with alkalinity of ash, titratable acidity, and total ash as K_2CO_3.

In addition to the total organic acid values, Table 7 contains the data for the same tissues on alkalinity of ash, total ash computed as milliequivalents of potassium carbonate, and the data from the direct titration of tissue suspensions between the limits of pH 2.6 and 8.0. To facilitate comparisons, these acidic and basic components of the tissue have been plotted in Figure 22. The pH values of the dry tissue suspensions (100 mg. in 2.5 ml.) are plotted as Figure 21.

IDENTIFICATION OF ORGANIC ACIDS ON PAPER STRIP CHROMATOGRAMS[1]

The organic acid fractions from samples of ungerminated seed, cotyledons of 6, 12, and 18-day-old plants, and seedling axis tissue of 3, 6, 12, and 18-day-old plants were examined. The ether extracts were prepared in the usual way from dry tissues which had been given a preliminary extraction with petroleum ether. The ether solutions were dried over sodium sulfate, filtered, the sulfate washed exhaustively with ether, and the combined filtrate and washings evaporated to dryness. The residues were then dissolved and made to a volume of 25 milliliters in acetone. 0.1, 0.2, and 0.3 ml. portions of the acetone solution were applied to 5 by 37 cm. strips of Whatman No. 1 filter paper for chromatographic separation.

The sample solution was applied in a spot 3.5 cm. from the bottom of the paper, which was then suspended, without equilibration, in a closed glass cylinder containing the solvent mixture. The solvent mixture was prepared by equilibrating, with 10 per cent of its volume of water, a solution of 7 parts of ether and 3 parts of toluene, made four normal with formic acid. The upper, organic layer of this mixture constituted the moving solvent phase in the development of the chromatograms. The paper was dipped into the solvent to a depth of 0.5 cm., and the chromatogram developed by allowing the mobile phase to ascend for a period of 16 hours at $60 \pm 3°F$. At the end of the development period, the paper strip was steamed to remove the formic acid and then sprayed with an indicator solution containing two parts of bromphenol blue to one part of methyl yellow, in a total concentration of 0.04 per cent. The acids showed up as discrete yellow spots (with red centers in the more strongly acid spots) on a bright blue background. Mixtures of known acids in definite concentrations were run simultaneously with the unknowns on some of the strips.

As a result of the chromatographic analysis, it was evident that, in common with most other plant materials, citric and malic acids were the predominant organic acids. In addition to citric and malic, the only acids detected positively were phosphoric acid and one unknown organic acid. This chromatographic system permitted identification by direct comparison with a group of nine known acids, which separate to form discrete and well defined spots on a single strip of paper. These acids, listed in their order from the origin on the developed chromatogram, and with the characteristic R_F value for each, were: tartaric (0.10), citric (0.18), malic (0.27), isocitric lactone (0.35), glycolic (0.40), α-ketoglutaric (0.48), malonic (0.55), succinic (0.61), and fumaric (0.85). The unknown acid had an R_F value very close to that of malonic acid in this system. The presence of phosphoric acid in the

[1] The chromatographic work was carried out by Dr. C. A. Hargreaves. This contribution and his advice in the interpretation of the results are gratefully acknowledged.

organic acid fraction was suspected and a detailed study was made of extracts of a single tissue, the 6-day cotyledons. On the basis of phosphate estimations on the organic acid extract and the observation that roughly 50 per cent of any phosphoric acid present is neutralized in the total organic acid titration, it was shown that in this tissue a significant part of the acidity determined as total organic acid was actually phosphoric acid. When phosphoric acid was applied to a chromatogram, about one-third of it remained at the origin after development, while the rest migrated to form a spot which coincided with that of citric acid. Sulfuric acid remains entirely at the origin in this system, but sulfate analyses on a number of extracts have given such low results that it is highly probable that the spot remaining at the origin in the present instance represents part of the phosphoric acid, with no sulfuric present. In evaluating the results from the chromatograms of the unknowns, it is accordingly assumed that the citric acid spot consists of citric acid together with a quantity of phosphoric acid equivalent to about twice the amount represented by the spot remaining at the origin.

In the extract from a sample of ungerminated seed, only the spots for phosphoric and citric acids were detected; phosphoric, citric, and malic were found in the extract of cotyledon tissue of 6-day-old seedlings, and phosphoric, citric, malic, and the unknown acid were found in all other tissues examined. An approximation in terms of + signs of the relative concentrations of the acids observed in the different tissue samples is shown in the following tabulation:

	Seed	6-C	12-C	18-C	3-P	6-P	12-P	18-P
Unknown			+	+	2+	+	+	+
Malic		+	4+	5+	6+	6+	6+	4+
Citric	3+	4+	5+	5+	+	2+	2+	+
Phosphoric	2+	3+	4+	4+	6+	3+	3+	+

Estimations of the actual quantities of material shown on the paper are at best only very rough approximations. The tabulation above is based on a comparison of the strips on which 0.3 ml. of solution, representing the extract of six milligrams of dry tissue, was applied. For each acid, a single + sign represents the smallest quantity of substance observed; the order of magnitude of a single mark, in micrograms, for the various acids has been estimated as: unknown, 3 to 5; malic, 5 to 10; citric, 10 to 15; and phosphoric, 5. If one uses these data to compute figures for citric and malic acids in terms of milliequivalents per 100 grams of ungerminated seed, the range of values obtained is comparable to the analytical values for malic and citric acids obtained on *L. angustifolius* by Pucher (see Figure 20). For instance, the range of 8 to 12 meq. so computed for the ungerminated seed includes the analytical figure of 9 meq. obtained by Pucher, and the highest range computed for malic acid, 5 to 9 meq. for sample 12-P, includes his maximum

value for malic acid, 5 meq. However, little more than this can be said at present concerning the relative quantities of acids per 100 grams of ungerminated seed. In the cotyledons, considering the shrinkage in organic solids which occurred, it is likely that the absolute quantities of all four acids decreased over the 18-day period, notwithstanding the increases in concentration. On the other hand, the observations on the seedling axis over the period from 3 to 18 days indicate a probable large increase in the unknown acid, malic, and citric, but perhaps little over-all change in the total amount of phosphoric acid present in the organic acid fraction of the tissue.

TABLE 8. MOISTURE AND ASH

(*A*). *As percentage of dry weight*[1]

		Seed	3 days	6 days	12 days	18 days
MOISTURE:	Cotyledons	5.72	6.00	6.03	6.39	7.46
	Seedling axis	......	......	16.11	14.02	11.19
ASH:	Cotyledons	2.58	2.52	2.79	4.17	4.61
	Seedling axis	......	......	3.91	4.16	3.78

(*B*). *As grams per hundred grams*[1] *ungerminated seed*

		Seed	3 days	6 days	12 days	18 days
MOISTURE:	Cotyledons	5.72	5.15	3.84	1.85	1.67
	Seedling axis	......	......	4.59	8.04	6.65
ASH:	Cotyledons	2.58	2.19	1.78	1.21	1.03
	Seedling axis	......	......	1.11	2.39	2.24

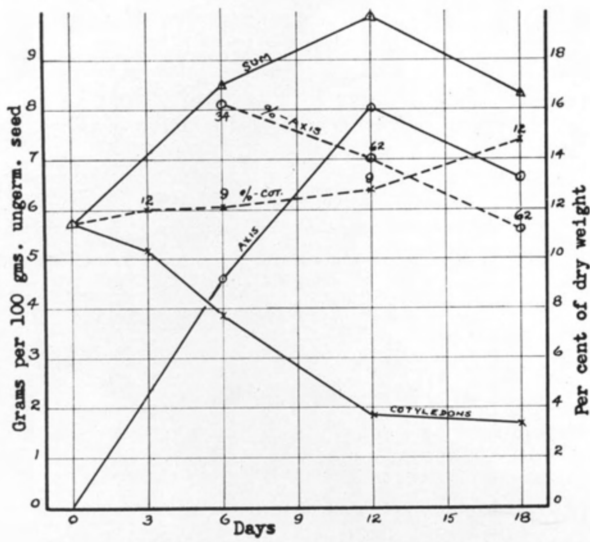
(*C*). *Constituents of the plant ash*[2]

	Seed	6-C	12-C	18-C	6-P	12-P	18-P
	Grams per 100 grams ungerminated seed						
Potassium	0.56	0.32	0.12	0.09	0.24	0.52	0.48
Calcium	0.22	0.16	0.13	0.12	0.07	0.16	0.22
Magnesium	0.12	0.08	0.06	0.06	0.04	0.08	0.09
Phosphorus	0.42	0.26	0.12	0.08	0.14	0.26	0.28
Manganese	0.40[3]	0.38[3]	0.22[3]	0.15	0.012	0.018	0.027
Iron	0.003	0.003	0.0028	0.003	0.0009	0.005	0.0065
Aluminum	0.001	0.0007	0.0003	0.0006	0.0003	0.0015	0.001
Zinc	0.01	0.007	0.005	0.006	0.003	0.019	0.024
Sodium	0.035	0.019	0.02	0.02	0.045	0.01	0.20
	Parts per million parts ungerminated seed						
Copper	14	16	10	10	7	24	26
Boron	6	13	9	8	17	4	9
Molybdenum				4.2		3.8	4.7

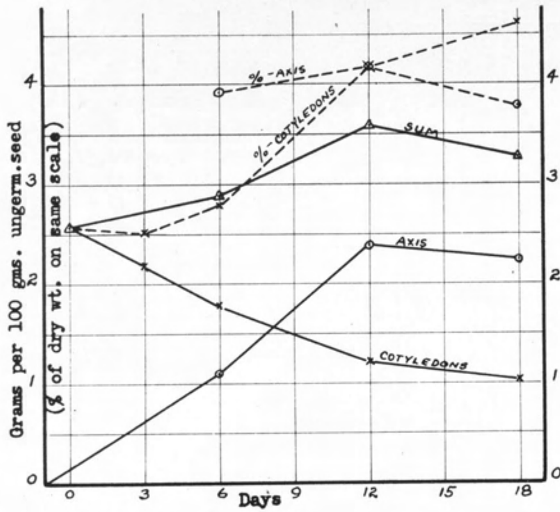
[1] Mean values for three replicates. See note, Table 1.

[2] Based on the analysis of duplicate samples of ash. See dissertation, Meiss, 1950, for more detailed data.

[3] By chemical analysis.



(A). Moisture content of the dried tissues.



(B). Ash.

Figure 23. Moisture and ash.

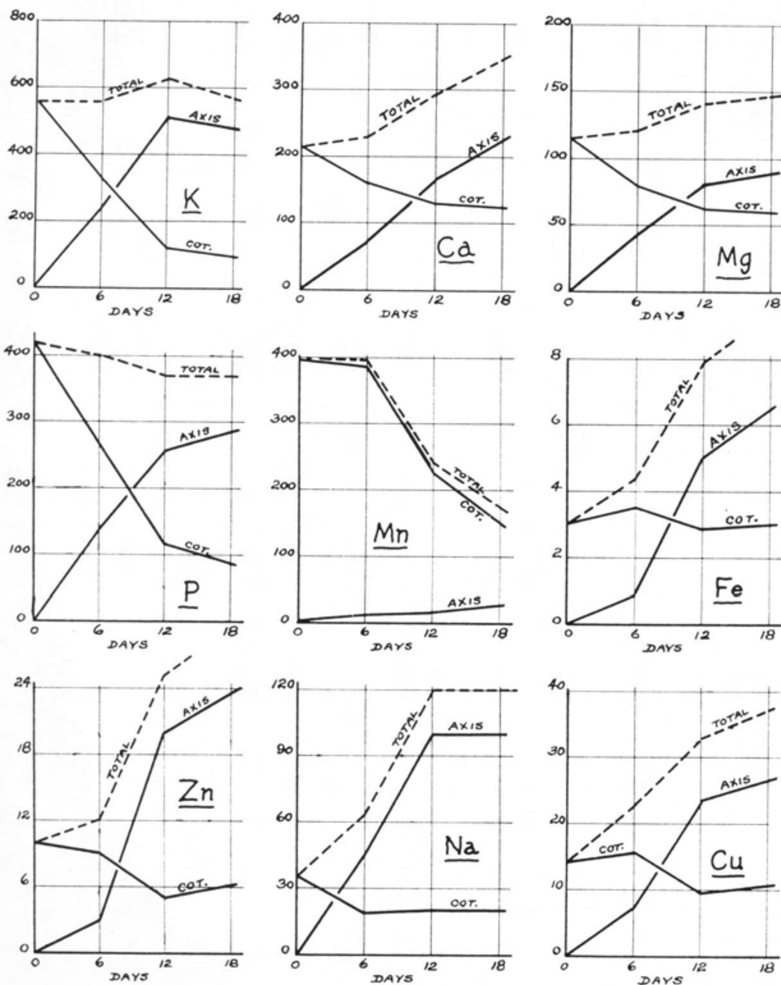


Figure 24. Distribution of certain constituents of the plant ash.

(See Table 8. All data are in milligrams per 100 grams of ungerminated seed except those for copper, which are in parts per million parts of ungerminated seed material.)

Moisture and Ash

Table 8 and Figure 23 show the moisture and ash values. The correspondence of the distribution of ash with the pattern of dry weight changes in the seedling growth should be noted. The increase in total ash during growth presumably represents accumulation from the tap water in which the seedlings were cultured.

The results of the spectrographic analysis of the ash for certain constituents are also shown in Table 8. The distribution of the various elements between cotyledons and plant axis during growth is shown in Figure 24.

Summary of Changes in Composition

Table 9 summarizes the composition of the ungerminated seed, and tissues representing 6, 12, and 18 days of growth. The quantities of protein were computed on the basis of 18.1 per cent as the nitrogen content of conglutin, the principal protein of *L. angustifolius* seeds (Osborne & Campbell, 1897). The quantities of free amino acids (excluding asparagine) and peptides were computed on the assumption that these substances contain approximately 10 per cent of α-amino nitrogen. The data of Table 3 were used as the basis for computing the total nitrogen of the peptide and free amino acid fractions, on the assumption that these fractions contained α-amino nitrogen and undetermined nitrogen (line 6 of Table 3) in the same proportions as the hydrolyzate of the protein nitrogen fraction, and that, further, they contained no amide nitrogen. A factor of 1.3 was used, therefore, to convert the amino nitrogen of the free amino acids and the peptide nitrogen to the respective total nitrogen figures. Then, to obtain the nitrogen of undetermined soluble nitrogenous compounds, the following quantities were subtracted from the total nitrogen of the tissue: (1) protein N, (2) 2 x asparagine amide N, (3) free ammonia N, (4) glutamine amide N x 0.8, (5) total N of free amino acids (except asparagine), and (6) total N of peptides. The weights of unknown soluble nitrogenous substances were then obtained by multiplying the remainder of the nitrogen by five.[1] In two instances, cotyledon tissues of 6- and 12-day-old plants, small negative values were obtained for the undetermined nitrogenous substances. Since these were associated with large values for peptides and moderately high values for free amino acids, the discrepancies probably result from unknown errors in determining the magnitude of those two fractions. Total carbohydrates have been arbitrarily computed in terms of glucose.

Carbon Distribution

The distribution of the total carbon among the various tissue fractions is shown in Table 10 and in Figure 25. The data were obtained by multiplying the quantities of the tissue constituents and fractions by the appropriate factors for the carbon content (see Vickery et al., 1946, pp. 71 ff.). Many of the carbon factors are arbitrary or only approximations, but since the errors introduced are systematic, the relative changes have considerable interpretative value. Protein carbon was computed from the value of Osborne and Campbell for conglutin, 51 per cent. Carbon of the free amino acid and peptide fractions was taken as 4.4 times the amino nitrogen, that being the estimated ratio of amino nitrogen to carbon in the protein hydrolyzate. Carbon

[1] See Vickery et al. (1946, pp. 65 ff.) for a detailed discussion of this and other factors used in computing the changes in composition.

TABLE 9. CHANGES IN TISSUE CONSTITUENTS

(Grams per 100 grams ungerminated seed)

	Seed-1	6-1 total	12-1 total	18-2 total	6-1C	12-1C	18-2C	6-1P	12-1P	18-2P	Net change (totals)
Protein (N x 5.55)	33.97	19.24	8.56	6.02	16.87	5.02	2.62	2.37	3.54	3.40	-27.95
Asparagine (asp.-amide N x 9.44)	2.04	8.98	(17.44)[1]	18.99	4.70	4.72	2.84	4.28	12.72	16.15	+16.95
Other amino acids[2] (amino N x 10)	0.40	3.60	(7.10)	7.00	2.50	(1.90)	2.00	1.30	5.20	5.00	+6.60
Peptides (N x 10)	4.37	10.05	(6.00)	0.91	8.49	(4.50)	0.50	1.56	1.50	0.41	-3.46
Other soluble N compounds (N x 5)	1.15	1.00	(1.85)	5.45	-0.75	-1.30	2.25	1.75	3.15	3.25	+4.30
Lipids	10.50	6.73	2.43	1.77	6.20	1.39	0.81	0.53	1.04	0.96	-8.73
Total soluble carbohydrates	10.52	8.79	5.55	2.41	3.80	1.03	0.84	(4.99)	(4.52)	1.57	-8.11
Total insoluble carbohydrates	12.87	4.86	4.42	3.93	3.32	2.19	1.70	1.54	2.23	2.23	-8.94
Total organic acids as malic acid	6.30	6.10	4.82	5.02	3.89	2.21	1.74	2.21	2.61	3.28	-1.28
Total organic substance determined	82.12	69.35	58.17	51.50	49.02	21.66	15.30	20.53	36.51	36.25	-30.62
Dry weight	100.20	94.12	85.09	79.65	64.88	28.18	21.31	29.24	56.91	58.34	-20.55
Moisture	5.85	8.73	9.52	8.76	3.90	1.79	1.78	4.83	7.73	6.98	+2.91
Ash	2.62	2.99	3.54	3.13	1.85	1.18	1.02	1.14	2.36	2.11	+0.51
Organic solids	91.73	82.40	72.03	67.76	59.13	25.21	18.51	23.27	46.82	49.25	-23.97
Undetermined organic substance	9.61	13.05	13.86	16.26	10.11	3.55	3.21	2.74	10.31	13.00	+6.65

[1] Numbers in parentheses are based, in part, on interpolated values.
[2] Free amino nitrogen minus (asparagine amide N — 0.8 x glutamine amide N).

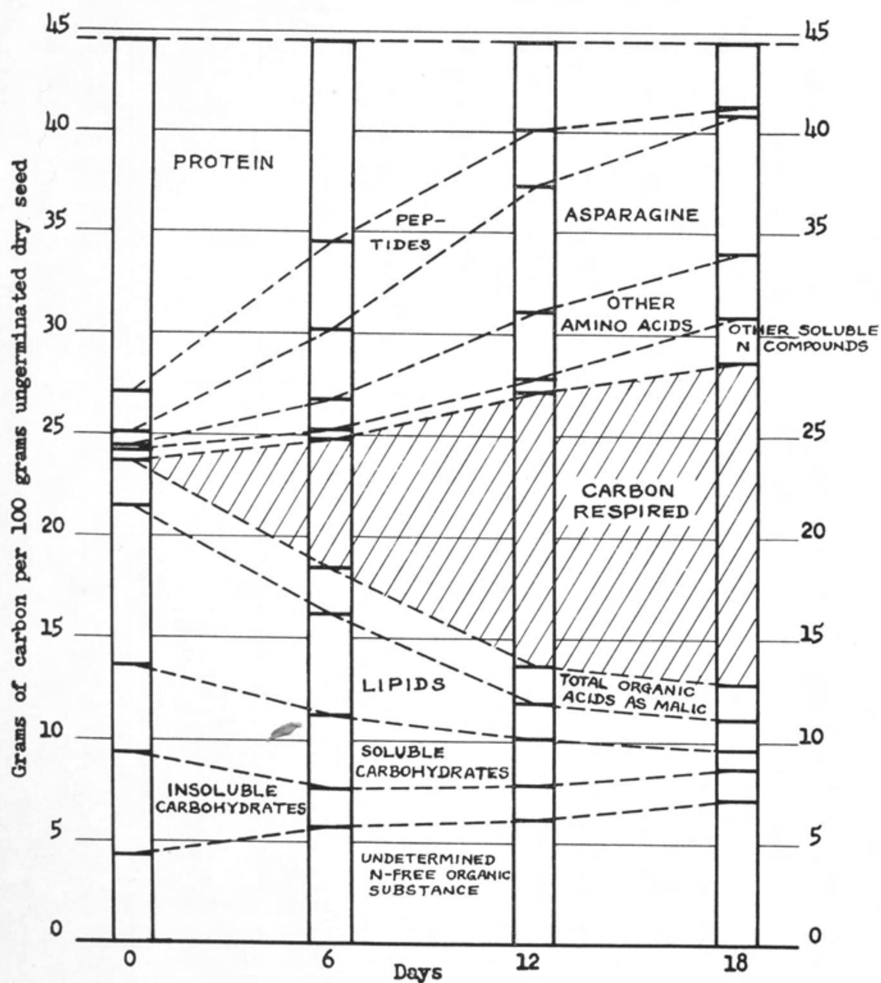


Figure 25. Distribution of carbon among the tissue fractions.

A. Whole seedlings

Table 10. Distribution of Tissue Carbon in Relation to Seedling Growth

Source of Carbon	Seed-1	6-1 total	12-1 total	18-2 total	6-1C	12-1C	18-2C	6-1P	12-1P	18-2P	Net change (totals)
	(Grams of carbon per 100 grams ungerminated dry seed)										
Protein (N x 2.83)	17.32	9.81	4.36	3.07	8.60	2.56	1.34	1.21	1.80	1.73	–14.25
Asparagine ($\frac{48}{14}$ x amide N)	0.74	3.32	6.33	6.91	1.71	1.33	1.03	1.61	5.00	5.88	+6.17
Other amino acids (NH_2-N x 4.4)	0.18	1.58	3.12	3.08	1.10	0.84	0.88	0.57	2.28	2.25	+2.90
Peptides (peptide N x 4.4)	1.94	4.45	2.64	0.40	3.74	1.98	0.22	0.70	0.66	0.18	–1.54
Other soluble nitrogen compounds (N x 2)	0.46	0.40	0.74	2.18	–0.30	–0.52	0.90	0.70	1.26	1.30	+1.72
Total organic acids as malic (40% C)	2.27	2.20	1.74	1.81	1.40	0.80	0.63	0.80	0.94	1.18	–0.46
Lipids (75% C)	7.88	5.05	1.82	1.33	4.65	1.04	0.61	0.40	0.78	0.72	–6.55
Total insoluble carbohydrates (40% C)	5.15	1.94	1.77	1.57	1.33	0.88	0.68	1.94	1.77	1.57	–3.58
Araban (40% C)	1.68	1.46	1.25	1.09	1.14	0.71	0.48	0.32	0.54	0.61	–0.59
Total soluble carbohydrates (40% C)	4.21	3.52	2.22	0.96	1.52	0.41	0.34	2.00	1.81	0.63	–3.25
Total sugars (40% C)	3.40	2.70	1.63	0.68	1.31	0.29	0.22	1.39	1.34	0.46	–2.72
Carbon of undetermined nitrogen-free organic substances (44% C assumed)	4.23	5.74	6.10	7.15	4.45	1.56	1.41	1.23	4.54	5.72	+2.92

of the undetermined nitrogenous substances was assumed to be 40 per cent, or twice the nitrogen content. The factor of 0.75 used for the carbon content of the lipid fraction represents an average of the theoretical values for the most commonly occurring tri-glycerides and free fatty acids, in which the carbon content ranges from 72 to 77 per cent. The factor 0.44, corresponding to the carbon content of cellulose, has been used for the undetermined (nitrogen-free) organic substances. The carbon of the total organic acid fraction was computed by arbitrarily assuming the entire fraction to be malic acid (36 per cent carbon). The carbohydrate carbon was computed with use of the factor of 0.40, the appropriate value for most of the compounds which probably occurred in this fraction; one exception is galacturonic acid which contains 37 per cent carbon, but the error introduced by this discrepancy is small.

DISCUSSION AND INTERPRETATION OF THE EXPERIMENTAL RESULTS

Relationship of Asparagine Accumulation and Growth

The transfer of nitrogen from cotyledons to plant axis and the processes of protein disappearance and asparagine formation seen here with *L. albus* (Figures 10 and 11) are quite in conformity with previous observations on seedlings of species which characteristically accumulate large quantities of asparagine.

Figure 26 shows the relationship of the processes of asparagine formation to several indices of growth. All data used have been plotted on the basis of percentages of the maximum change measured in each growth index during the 24-day period. Comparison of the total asparagine accumulation values (A) with those for linear growth of the hypocotyl and epicotyl portions of the seedling axis suggests that there is a definite physiological relationship in which total asparagine accumulation and growth of the hypocotyl proceed together at roughly the same relative rates, while growth of the epicotyl bears an inverse relationship to the other two processes. After the sixth day, the fresh weight values for the seedling axis very nearly coincided with the values for total asparagine accumulation. The dry weight of the seedling axis, as noted previously, increased more rapidly and attained a maximum sooner than the asparagine which accumulated. If organic solids are plotted instead of dry weight, a nearly identical relationship is observed. This dry weight-asparagine relationship may be interpreted in terms of a piling up of substances which arise by processes not as closely correlated with dimensional growth as asparagine production appears to be. Such substances occur in the soluble carbohydrate fraction of the seedling axis (recall Figure 18A), and might include quantities of asparagine precursors. However, the undetermined (non-nitrogenous) organic substances of the seedling axis increased substantially (2.69 grams) during the period from the 12th to the 18th day, and the decrease in total soluble reducing substances in the axis (2.95 grams), computed as glucose, corresponded roughly to that increase (Table 9). Furthermore, the gain in asparagine in the seedling axis during this period (3.43 grams) corresponds to the sum of asparagine disappearing from the cotyledons (1.88 grams) and the net loss of protein, peptides, and amino acids in the axis tissue (1.33 grams).

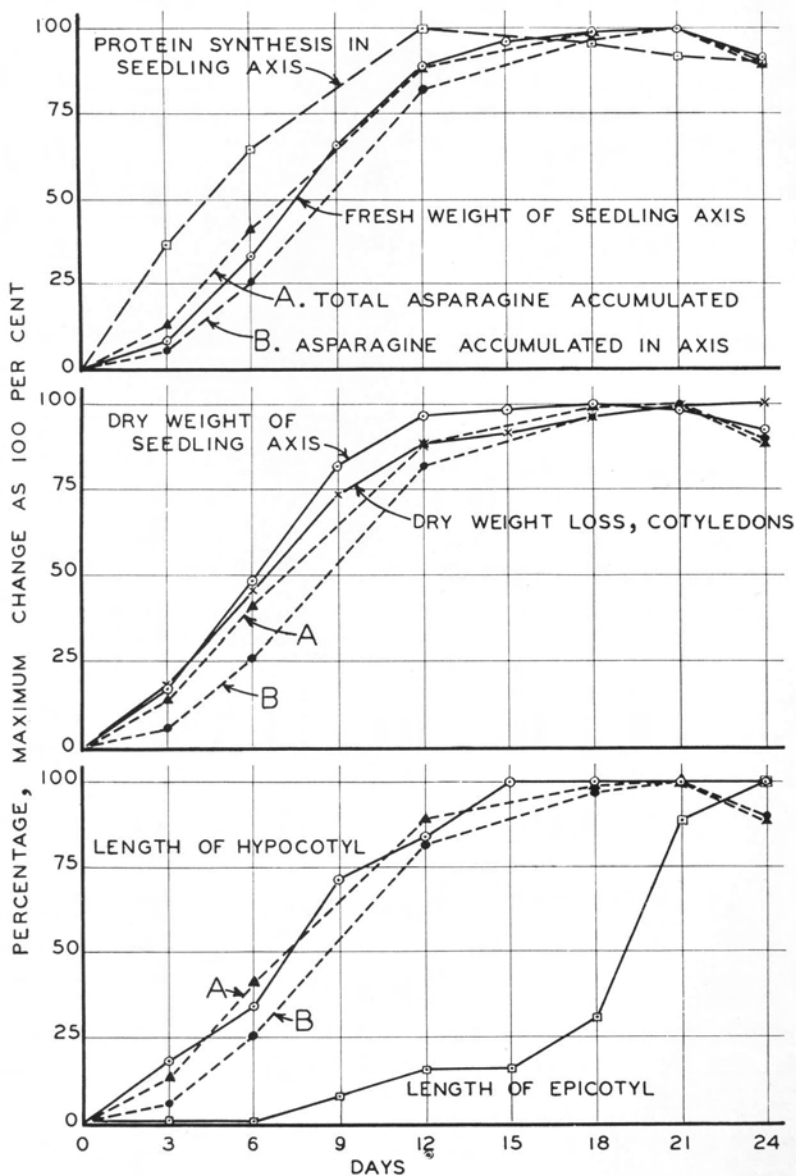


Figure 26. Relationship of asparagine accumulation to several indices of growth.

The discrepancy between dry weight accumulation and asparagine accumulation is much more pronounced if the dry weight curve is compared with the curve for asparagine in the axis alone (B). The general relationship is the same, but the question of synthesis of asparagine in the cotyledons and its subsequent translocation to the axis is raised. An accumulation of 0.6 gram of asparagine nitrogen (above that present in the ungerminated seed) was found in the cotyledons of six-day-old seedlings. Using the figure of 5.4 per cent for the aspartic acid (asparagine) content of the seed protein (Heinrich, 1941), it can be shown from the quantity of protein which disappeared from the cotyledons in the six days, 14.7 grams (Table 9), that only 0.17 gram of the asparagine nitrogen could be in asparagine of primary origin, that which arises directly on hydrolysis of the protein. In general, it has been assumed that the seedling axis, in which most of the growth takes place, is the site of formation of nearly all of the asparagine which is synthesized (i.e., of secondary origin). The present data indicate that a significant proportion of such synthesis took place in the cotyledons, notwithstanding the close correlation of growth and asparagine accumulation.

It is possible to observe a definite sequence of physiological stages characteristic of the accumulation of asparagine in etiolated lupine seedlings. These are: (a) rapid accumulation of asparagine associated with the normal growth and metabolism of the seedlings, with the behavior of illuminated and etiolated seedlings essentially the same; (b) a starvation stage, in which both growth and asparagine accumulation continue, but at a sharply reduced rate, due to a reduction in the quantity and relative availability of respiratory substrates; and (c) physiological breakdown of the tissue with a concurrent disappearance of asparagine, this stage being initiated by the exhaustion of the supply of some essential metabolite. Stage (a) is illustrated in Figure 27 (from Vickery, Pucher, & Deuber, 1942), extending over the first 16 days of growth. Thereafter, the etiolated seedlings underwent severe starvation, as evidenced by the dry weight curve, while the light-grown seedlings were rapidly compensating for dry weight loss by photosynthesis, and had concurrently ceased to accumulate asparagine (see also work of McRary, 1940; Hée & Bayle, 1932).

In the present experiments with *L. albus*, the sequence was rather well defined, and can be related to other processes which took place concurrently. Stage (a) included the period from the beginning of germination until the 12th day, when distinct breaks occurred in the rates of asparagine accumulation, transfer of total nitrogen from cotyledons to axis, decomposition of protein, utilization of the lipid fraction, and disappearance of soluble carbohydrates from the cotyledons. In addition, the high level of soluble reducing substances in the seedling axis began to fall off sharply after the 12th day. Stage (b) extended from the 12th to beyond the 18th day, and was characterized by a continuation of the processes noted under (a), but at much reduced rates associated with the general slowing down of growth and metabolism. Glutamine and free ammonia remained constant during this period, peptide nitrogen decreased, and the soluble nitrogen in the undetermined fraction increased sharply, the increase, however, being largely in the cotyledons. The transition from stage (b) to stage (c) took place in the period between the 18th and 21st days. Stage (c) was characterized by cessation of dimensional

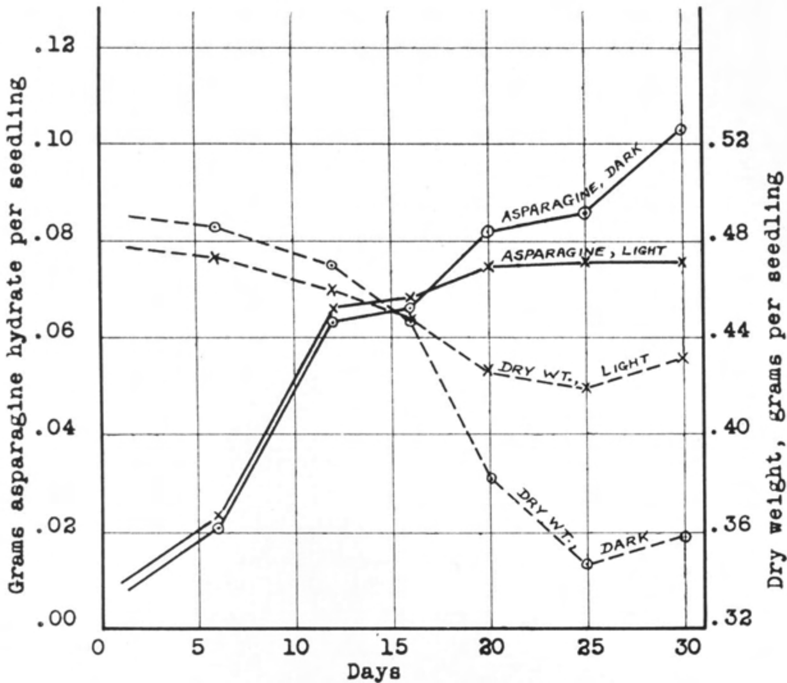


Figure 27. The effect of light on asparagine formation and dry weight changes in Lupinus albus seedlings.

(From: Vickery, Pucher, and Deuber, 1942. Plot of data from Table I, p. 47.)

growth, the disappearance of asparagine from the tissues with a concurrent production of ammonia, loss of water and decrease in dry weight of the tissues, and the development of the flaccid, water-soaked appearance which accompanies a general physiological disruption. By the 24th day, these processes had proceeded to the extent that widespread invasion of the tissues by microorganisms was imminent, and death of the seedlings could be expected to take place within the succeeding day or two.

Some comparisons may be made with the behavior of the seedlings studied by Vickery and Pucher (Figure 2). From the data on *L. angustifolius,* the transition from stage (a) to (b) occurred at about the 8th day, but was less sharply defined than in the case of the present experiments with *L. albus;* the transition to stage (c), however, occurred very rapidly, during the 13th day.

The *angustifolius* seedlings on the 14th day corresponded to the 24-day-old *albus* seedlings. With *Vicia atropurpurea*, the first 16 days of the experiment corresponded to stage (a) and the remaining 10 days of growth lay within the second phase. In the *Cucurbita pepo* study, the entire duration of the experiment corresponded to the first stage in the lupine experiments. However, in view of the fact that it has now been shown that all of the asparagine accumulated in the *Cucurbita* seedlings could have been derived directly from the hydrolysis of the seed protein, these species are more appropriately classified as seedling plants in which no significant secondary formation of asparagine takes place.

It accordingly appears that a definite physiological pattern, with respect to the nitrogen metabolism, occurs in the growth of etiolated seedlings of a given sort, and it seems likely that this pattern is rather closely correlated with the nature of the non-nitrogenous food material stored in the seed. Thus, the form of the asparagine accumulation curve, in relation to the over-all growth, may, on further investigation, prove to be an indicator of the extent to which protein is utilized as a normal respiratory substrate in seed germination and early growth of seedlings.

That secondary formation of asparagine in etiolated seedlings is less directly related to the phenomenon of growth itself than to the respiratory processes is indicated in the recent work of Christiansen and Thimann on the metabolism of sections of etiolated pea stems. Although they showed (1950) that growth inhibition by respiratory enzyme inhibitors was accompanied by a parallel decrease in asparagine production, it was also found (Thimann, Slater, and Christiansen, 1950) that asparagine production was not affected if growth was inhibited by a heavy concentration of mannitol or by calcium or magnesium ions, none of which presumably have a direct effect on the respiratory enzymes.

Since protein synthesis is one of the processes associated with growth, a curve indicating the progress of protein formation in the seedling axis has been included in Figure 26. It is readily apparent that protein synthesis is less closely correlated with asparagine accumulation than are the other growth indices which have been considered. The maximum rate of protein formation occurred within the first three days of growth, and the highest protein content of the seedlings was reached at about 12 days. These maxima occur about six days ahead of the comparable portions of the asparagine curve, and presumably reflect the more intense protein synthesis during the period of rapid cell division than in the later period when growth is primarily by cell elongation.

Some Considerations as to the Source of the Asparagine Nitrogen

It has been assumed that, among other possibilities, the amide and α-amino nitrogen arising by decomposition of protein and peptides may constitute the supply of nitrogen which is readily available for the synthesis of asparagine and amino acids. In consideration of that possibility, Figure 28 was constructed to show the relationship of the disappearance of such "available nitrogen" (utilizing the data of Tables 2 and 4) to the quantity of nitro-

gen utilized in the asparagine synthesis and the production of other free amino nitrogen. Until the 15th day these quantities very nearly coincided, and on the 18th and 21st days the sum of asparagine nitrogen, other free α-amino nitrogen, and free ammonia constituted 93 and 92 per cent, respectively, of the "available nitrogen" which disappeared. At each stage shown in Figure 28, the "available nitrogen" was calculated by adding the peptide nitrogen value for both cotyledons and axis (Table 2) to the appropriate value in Table 4. Thus, it does appear as if the forms of nitrogen which have here been designated as "available" could have contributed nearly all of the nitrogen which accumulated in the asparagine and the free amino nitrogen fraction. There is, of course, theoretical justification for this "available nitrogen" concept in the biochemical scheme that has been proposed for asparagine formation in seedlings. If, as seems to be the case, the nitrogen is made available and is channeled into asparagine formation by the processes of oxidative deamination, transamination, and transamidation, then only α-amino and amide nitrogen should be involved.

It is possible that the equivalence of the amino plus amide nitrogen released from the storage protein and the asparagine amide plus total free amino nitrogen which arise in the developing seedling is a purely fortuitous matter, and that the real situation involves preferential utilization of certain amino acids for respiration and amide synthesis. However, in support of the "available nitrogen" concept, work of Vickery and Pucher on the culture of tobacco leaves should be cited. They found, and confirmed by subsequent experiments, that when leaves were cultured on water, there was an approximate equivalence of the amino nitrogen of the protein which was decomposed and the amide nitrogen of the asparagine which was formed (see Vickery et al., 1937, pp. 797 ff.).

In the data of Dunn and coworkers (1948), if the aspartic and glutamic acids analyzed are considered to be the amides, then 75 per cent of the nitrogen of the asparagine formed in *L. angustifolius* can be accounted for by the nitrogen released on the decomposition of glutamic acid (glutamine), arginine, and the leucines. A similar estimate is obtained if the data of Heinrich (1941) on the amino acid composition of *L. luteus* seed meal are applied to the present experimental data on *L. albus*. Such a computation yields the result that the nitrogen of asparagine, glutamine, arginine, and leucine which would arise as a result of the observed amount of protein decomposition (allowing 11 per cent for resynthesis of seedling axis protein) is equivalent to 85 per cent of the nitrogen of the accumulated asparagine. Unfortunately, the inferences to be made from such fragmentary data as these are entirely speculative, and further consideration of the specific contributions of individual amino acids to the nitrogen of the accumulated asparagine requires a background of careful experimentation, involving at the outset quantitative analyses of the amino acids of the protein, peptide, and free amino acid fractions at successive stages in the growth of etiolated seedlings.

The Source of the Carbon of the Asparagine Synthesized, in Relation to the Sources of Respiratory Carbon Dioxide

In presenting the data on the carbon distribution, the limitations on their accuracy were noted; they should be borne in mind in considering the following discussion.

From the net changes in carbon content calculated for the various tissue components (Table 10), the following summary data were obtained: (The changes listed are based on 18 days of growth from an initial weight of 100 grams of equilibrated dry seed material without seed coats.)

	Grams
Sum of losses of carbon	29.63
Sum of gains of carbon	13.71
Carbon respired	15.92
Sum of protein plus peptide carbon which disappeared	15.79
Gain in asparagine carbon	6.17
Gain in carbon of other soluble nitrogen compounds	4.62
Minimum amount of protein carbon available for respiration, assuming protein as C source for all soluble nitrogen compounds formed	5.00
Loss of insoluble carbohydrate carbon	3.58
Loss of soluble carbohydrate carbon	3.25
Loss of lipid carbon	6.55
Sum of carbon lost from lipids and carbohydrate	13.38
Increase in carbon of undetermined N-free organic substances	2.92
Carbon of lipid and carbohydrate origin available for respiration and synthesis	10.46

From the summary above, it is obvious that protein, to some extent at least, was an obligatory respiratory substrate. If it is assumed that all of the carbon of the asparagine and other nitrogenous compounds which were formed was of protein origin, and the ratio of

$$\frac{\text{protein plus peptide carbon lost}}{\text{asparagine plus sol. N compd. C gained}}$$

is computed for successive periods of growth from the data of Table 10, these values are obtained:

0 – 18 days (over-all)	1.46
0 – 6 days	1.29
6 – 12 days	1.50
12 – 18 days	1.78

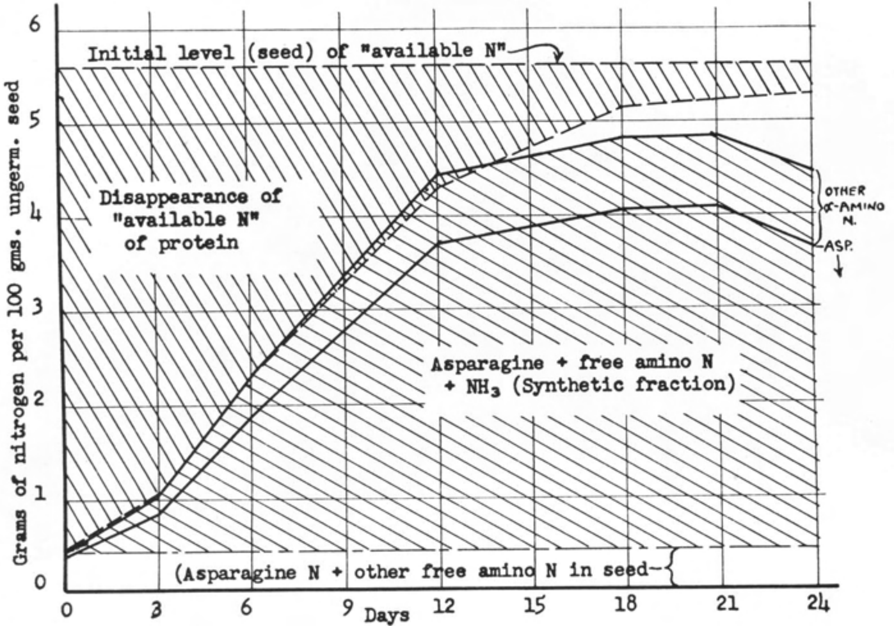


Figure 28. Relationship of the disappearance of "available nitrogen" of protein to the accumulation of asparagine nitrogen and free α-amino nitrogen.

It would appear from this that protein served as a normal respiratory substrate, along with lipids and carbohydrates, during the entire 18-day period of seedling growth.

Table 11 was assembled to show the relationships among protein, lipids, and carbohydrates, of the amounts of carbon which disappeared during the successive periods, using the carbon lost through respiration as one basis of comparison, and the carbon utilized in asparagine formation as another. No inferences can be made as to the channeling of carbon from a given fraction into either respiration or synthesis, but lipids and total carbohydrates supplied carbon in excess of the amount going into the synthesis of asparagine by only 6 and 10 per cent, respectively. The striking increase in the protein-asparagine carbon ratio and decrease in the lipid-carbon ratios reflect the change to protein as the principal source of respiratory carbon on the one hand, and the approaching exhaustion of the carbon available for respiration from the lipid fraction. The carbon from the carbohydrate fractions appears, from the limited data thus far obtained, to have a less direct relationship to respiration and asparagine synthesis than is the case with carbon originating in the protein and lipid fractions. This is indicated in the ratios of Table 11, where it is seen that the disappearances of insoluble carbohydrate during the

zero to 6 day period, and of soluble carbohydrate during the 12 to 18-day period, were the only parts of the over-all carbohydrate metabolism in which the quantities of carbon involved were of a magnitude comparable to the protein and lipid carbon which was metabolized. As noted in the discussion of asparagine in relation to growth, the disappearance of soluble carbohydrate carbon during the 12 to 18-day period was balanced by an increase in the undetermined organic substances in the seedling axis, which consist mainly of materials generally included in the "crude fiber" determination.

TABLE 11. RATIOS OF THE CARBON CONTENTS OF TISSUE FRACTIONS UTILIZED TO CARBON RESPIRED AND TO CARBON OF ASPARAGINE FORMED

R = ratio, $\frac{\text{carbon utilized in metabolism}}{\text{carbon respired}}$

A = ratio, $\frac{\text{carbon utilized in metabolism}}{\text{carbon of asparagine formed}}$

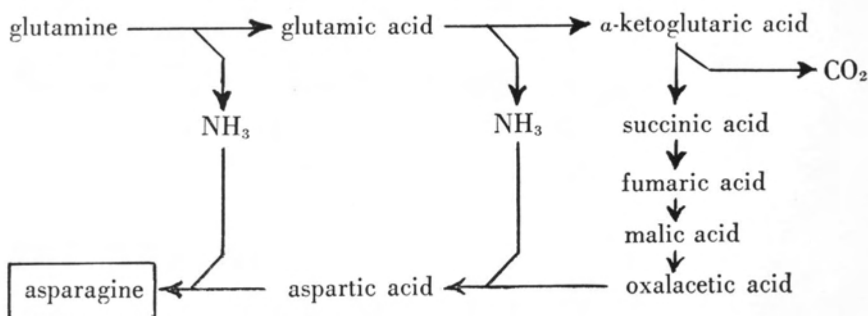
Period of growth, days from beginning of germination:		Over-all 0 - 18	0 - 6	6 - 12	12 - 18
Protein plus peptides	R	0.99	0.79	1.26	1.48
	A	2.56	1.94	2.41	6.09
Lipids	R	0.41	0.44	0.45	0.21
	A	1.06	1.10	1.07	0.84
Total carbohydrates	R	0.43	0.61	0.20	0.61
	A	1.11	1.51	0.49	2.41
Insoluble carbohydrates	R	0.22	0.50	0.02	0.08
	A	0.53	1.24	0.06	0.34
Soluble carbohydrates	R	0.20	0.11	0.18	0.53
	A	0.58	0.27	0.43	2.17

It is implicit in the biochemical relationships of asparagine, as outlined in Figure #1, that any substances which can yield energy by direct participation in any of the reactions of the respiratory cycle must at the same time be potential sources of carbon for the synthesis of asparagine. With that in mind, it appears, on the basis of the evidence at present available, that protein and lipids are the principal respiratory substrates in the metabolism of etiolated white lupine seedlings, that protein is the principal source of asparagine carbon, and that carbohydrates were the least important, as a source of respirable material, of the three major types of stored food.

Presumptive evidence of the origin of the asparagine carbon in the seedling protein has arisen from efforts to synthesize radioactive asparagine by allowing 10-day-old seedlings of *L. angustifolius* to absorb $C^{14}O_2$ in the light for 24 hours (Wilson, 1950). Although slight radioactivity was obtained in the asparagine, other work in the same laboratory showed that un-

der comparable conditions, the sugars produced in tobacco leaves during 24 hours of exposure to $C^{14}O_2$ had a specific radioactivity of about 1,000 times that found in the lupine asparagine.[1] On the basis of these results, Krotkov and Wilson have concluded that sugars were not the source of the asparagine carbon in the lupine seedlings, but that protein may have been the carbon source.[1] Further work (Wilson, Krotkov, & Reed, 1951) seemed to support this contention. From two groups of young tobacco leaves, exposed to NH_4Cl and $C^{14}O_2$ for 11 and 6 days, respectively, asparagine was isolated in which the specific radioactivity was still only about one hundredth of that of the sugars, and similar treatment of eight-day-old seedlings of *L. albus* for three days, and of *L. angustifolius* for six days, yielded asparagine having a maximum specific activity of less than half of that of the tobacco leaf asparagine.

Proof of a claim that protein is the principal source of asparagine carbon requires some knowledge of the specific metabolism of the amino acids in germinating seedlings. Lacking such knowledge, some speculation based on what we do know may be permitted. Particular attention is directed to the primary role claimed for glutamic acid and glutamine in amino acid metabolism. The entire synthesis of asparagine from glutamine according to the reactions indicated in Figure 1, would be assumed to be accomplished thus:

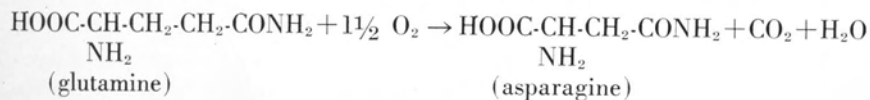


If this particular segment of the general metabolism could be isolated from the rest, that is, if the introduction of intermediates between α-ketoglutaric acid and oxalacetic acid, from sources other than protein, could be prevented, then the asparagine would in fact have been synthesized wholly from glutamine. If, as the best available data indicate, the lupine seed proteins contain 27 or 28 per cent of glutamic acid, as glutamine, then nearly half of the asparagine accumulated might be considered to arise entirely from protein by the above reaction scheme alone. In addition, histidine, proline, ornithine, and possibly lysine, can, in some tissues at least, be metabolized by oxidative reactions leading to the formation of glutamic acid or glutamine (Braunstein, 1947), from which asparagine might be synthesized as above. Again, depending upon the degree of dilution of the protein carbon involved by carbon from non-protein sources, protein might in this case also be the source of the entire asparagine molecule. Valine and the leucines, which together comprise about 10 per cent of the lupine seed protein (Heinrich, 1941), are metabolized by reaction pathways more or less remote from glutamine synthesis (Braunstein, 1947). Leucine and isoleucine, undergoing oxidative catabolism, are thought to yield acetoacetic acid as an intermediate product; the carbon from

[1] Personal communication from Dr. G. Krotkov, April, 1951.

these amino acids might therefore be more likely to be channeled into lipid metabolism than into glutamine and asparagine synthesis. If such were the case, more of the lipids of the seed might be available for complete oxidation, with acetoacetate from the leucines being utilized for synthesis of substances in the lipid fraction of the seedling axis. However, at the very least, and assuming completely random utilization, regardless of source, of all carbon which can be fed into the respiratory-synthetic scheme, protein would furnish much more of the asparagine carbon than any other possible source, simply because it contributes more carbon to the general metabolism than all other sources combined. Furthermore, substantial portions of the total carbohydrates might not enter directly into the reactions of the respiratory cycle at all, but be utilized elsewhere, as in the synthesis of alkaloids (Mothes and Kretschmer, 1946), and cell wall substances, for instance.

In the preceding section, the probability that a considerable amount of asparagine was synthesized in the cotyledons was established. Again utilizing the data of Heinrich on the composition of seed meal of *L. luteus*, it can be shown that the 0.43 gram of nitrogen from asparagine of secondary origin, which accumulated in the cotyledons during the first six days, is considerably more than balanced by the nitrogen of the glutamine made available by protein decomposition. If only glutamic acid, rather than the amide, arose from the protein, 0.38 gram of nitrogen was produced from this source, and if all of the protein glutamic acid was in the form of the amide, 0.77 gram of nitrogen was thereby made available for asparagine synthesis. On the basis of these quantitative relationships, and lacking any knowledge of the rate of translocation of asparagine to the seedling axis, one is tempted to make a further speculation, regarding a special role of glutamine and glutamic acid in the general metabolism of the seedling. The reaction scheme leading from glutamine to asparagine, as written above, includes as one of its steps the decarboxylation of α-ketoglutaric acid, which is one of the three decarboxylations in the respiratory cycle, releasing energy which is ultimately available for use in cellular processes. The question is thus raised as to whether the glutamine →asparagine transformation is not an important source of the energy required in germination and the early stages of seedling growth, particularly as a process linking respiration with protein synthesis. The over-all equation representing such a hypothetical reaction scheme would be



$$\underset{\text{(glutamine)}}{\underset{\displaystyle NH_2}{HOOC\text{-}CH}\text{-}CH_2\text{-}CH_2\text{-}CONH_2} + 1\tfrac{1}{2}\ O_2 \rightarrow \underset{\text{(asparagine)}}{\underset{\displaystyle NH_2}{HOOC\text{-}CH}\text{-}CH_2\text{-}CONH_2} + CO_2 + H_2O$$

with $\triangle F$ equal to approximately –150,000 calories per mole.

The rapid disappearance of lipids and carbohydrates from the cotyledons does not necessarily imply that much of these materials was completely oxidized in that tissue. Since it is well known that the most intense respiratory activity in a plant occurs in the region of most rapid growth, it seems logical to assume that in the cotyledons the lipids and carbohydrates are, for the most part, metabolized only to the extent of breaking them down into fragments of a size which are readily translocated to the rapidly growing tissues of the plant axis. This assumption is supported by the fact that with rapid

disappearance of pectin from the cotyledons, and of "lupeose", as indicated by the rapid drop in the quantity of reducing substances produced by invertase treatment, there was no piling up of free reducing substances in the cotyledons, but there was a pronounced accumulation of them in the plant axis. The high level of free reducing substances in the axis persisted until the total carbohydrates in the cotyledons had practically reached their maximum depletion (Figures 18 and 19).

Some discussion and interpretation of the analytical study of the carbohydrates seem desirable. For the purposes of this discussion, the additional reducing value resulting from treating the water extract of the tissue with invertase is designated the "invertase fraction". From Figure 18, it can be seen that the ungerminated seed contained but a small amount of free reducing sugars, and slightly less pentose, which for the present, is assumed to be mostly in the combined form. The unfermentable fraction in the seed, therefore, would then be considered to consist almost entirely of hexose sugars. The soluble carbohydrate fraction, after subtracting the free reducing sugars and pentose, is presumed to consist wholly of "lupeose" (Schulze and Steiger, 1889), represented in the analytical results as the sum of the invertase fraction and the increased reducing value obtained by dilute acid hydrolysis. There has been no work on lupeose, as such, reported since that of Schulze and colleagues, who purified the sugar, but were unable to obtain it in a crystalline state. They found the purified material to yield, as noted before, 2 molecules of D-galactose, and one each of D-fructose and D-glucose, and to have a specific rotation of $[\alpha]_D = +148.0°$ for the anhydrous substance. The only authenticated crystalline tetrasaccharide known at present is stachyose,

α-galactosyl $\overset{1\text{-}6'}{\text{———}}$ α-galactosyl $\overset{1\text{-}4'}{\text{———}}$ α-glucosyl $\overset{1\text{-}2'}{\text{———}}$ β-fructofuranoside,[1] for which $[\alpha]_D^{20} = +148°$; it is non-reducing, and lupeose is now considered to be identical with it (Pigman and Goepp, 1948, p. 458). The invertase fraction in the ungerminated seed (Figure 18) is equal to roughly three-quarters of the total reducing value attributed to the stachyose after complete hydrolysis, but it has been shown that stachyose, when hydrolyzed by baker's yeast invertase, yields fructose and manninotriose, a reducing trisaccharide having the composition: 4-D-glucose-6-D-galactosyl-galactoside, with the reducing properties due to the glucose group (Pigman and Goepp, 1948, pp. 429, 458). The expected value for the invertase fraction would, therefore, be closer to two-thirds of the stachyose total. However, no investigation has been made of the specificity of the invertase preparation used, and invertase preparations in general are known to be variable in their abilities to hydrolyze polysaccharides (Neuberg and Roberts, 1946). The ratio of "unfermentable sugars" to stachyose, approximately 1 to 4, is readily interpreted: The yeast invertase would split off the fructose group from the tetrasaccharide, and could then, hypothetically, ferment it along with the glucose group from the trisaccharide (manninotriose is known to be fermented by yeast); if the remaining galactose-galactose disaccharide had one free aldehyde group, the reducing value of the yeast-treated extract then would be approximately one-

[1] The structure still requires unequivocal proof.

quarter of that of the tetrasaccharide after dilute acid hydrolysis. This would require that the unfermentable fraction be derived entirely from the stachyose, and that the free reducing sugars consist almost entirely of fermentable substances (galactose is not fermented by yeast under the conditions of the analytical determinations). On the other hand, the high ratio of the invertase fraction to lupeose, might indicate the presence of a larger proportion of fructose, as if the complex sugar of *L. albus* seeds were raffinose, i.e., galactose-glucose-fructose, rather than the tetrasaccharide. Schulze's purified preparations of lupeose, incidentally, came from *L. luteus* and *L. angustifolius,* but not from *albus.* Furthermore, stachyose is frequently found associated with raffinose and sucrose in plants (Pigman & Goepp, 1948, p. 458).

The attainment of a high level of unfermentable reducing substances in the axis of the six-day-old seedlings can hardly be entirely the result of the translocation of the unfermentable fraction of the soluble carbohydrate of the ungerminated seed, since there would necessarily be a parallel translocation of relatively large quantities of galactose, arabinose, and galacturonic acid arising from decomposition of pectin in the cotyledons. At least part of the pectin constituents present in this fraction would represent intermediates in the resynthesis of pectic substances in the seedling axis. Ascorbic acid might also make up a significant portion of the unfermentable fraction.

The complexity of the carbohydrate materials in this species, as compared with a plant material in which transformations involving starch and glucose are predominant, makes it exceedingly difficult to hypothesize a logical scheme of the carbohydrate metabolism on the basis of an analytical study like the present one. It does seem fairly evident, however, that a quantity of starch or starch-like polysaccharides equivalent to the storage carbohydrates of the lupine seed would constitute a much more readily available supply of respiratory substrate. The special contribution of the lupine carbohydrate materials to the process of asparagine accumulation might be thought of as a negative one: by the relatively low availability as respiratory substrates, they promote protein respiration, and consequently, the accumulation of asparagine in the seedlings.

Interpretation of the Data on Organic Acids and Mineral Constituents

In the review section, it was stated that the common plant acids, malic, citric, oxalic, succinic, and the rest of the acids of the respiratory cycle, probably constitute only a small part of the total organic acid fraction of germinating seedlings. Unpublished data of Pucher (1946) on the malic and citric acid contents of blue lupine seedlings provide good evidence in support of this view. Moreover, by analogy with the total quantity of organic acids which can be detected in actively respiring animal tissues, one would expect the total organic acids involved directly in the respiration of seedlings to be of the order of 1 per cent or less of the dry weight of the tissue. For this reason, it is suggested that, in plant tissues in general, any accumulation of material in the organic acid fraction, whether it be known or undertermined acids, in amounts considerably greater than the 1 per cent level, leads to very

doubtful conclusions if one attempts to interpret the over-all organic acid metabolism on the basis of the integrated scheme of reactions of the respiratory cycle.

In the present work, the changes in total organic acids fall into a pattern which corresponds to the dry weight growth curves (Figures 20 and 8). The quantities involved are much in excess of what one would expect if dealing with a question of tissue respiration alone. (See Table 9, in which the total acids are computed as malic acid.) Thus, it must be concluded that the study of total organic acidity contributes little to an understanding of the mechanisms of asparagine formation in the seedlings. If, during the period of most intense respiration and asparagine accumulation, the organic acids of the respiratory cycle were functioning in the axes of the etiolated seedlings only as respiratory catalysts, then a complete analysis of the organic acids should reveal each of the respiratory intermediates in a characteristic and reasonably constant concentration during that period. A fuller understanding of the respiratory system would then require that the concentrations of the various acids be correlated with the relative activities and velocities of the enzyme-catalyzed reactions of the cycle. Notwithstanding that only citric, malic, a single unknown, and phosphoric acids were detected in the present paper chromatographic study, further application of the technique might be expected to reveal the presence, but in much smaller quantities, of other members of the group of respiratory organic acids. Furthermore, the chromatographic study could be extended to include certain other acids which are of interest in addition to the respiratory cycle acids.

The principal organic acidic constituents of the tissue, in addition to the common plant acids, are thought to be ascorbic acid, phytic acid, and pectic acid and free galacturonic acid. These are all considerably less soluble in ether than the common plant acids, and it is not known to what extent they may occur in the ether extract of the acidified tissue, although in view of the presumed high concentration of these acids, they might well comprise a substantial portion of the ether-soluble fraction. They probably make up the bulk of the titratable acidity in a direct acid-base titration of a water suspension of the tissue. Ascorbic acid titrates as a monobasic acid in the range between pH 3.0 and 7.5 in dilute solution (experimentally verified in the present work), as does D-galacturonic acid (Speiser et al., 1945). Pectic acid also titrates in the organic acid range, pH 2.6 to 8.0, but not as a monobasic acid, the degree of dissociation depending on the concentration and the methyl ester content (Speiser et al., 1945). Phytic acid, inositol hexaphosphoric acid, is a potential source of 12 equivalents per mole of titratable acidity. Phytic acid occurs in plants in appreciable quantity as the calcium, magnesium, and potassium salts, and would therefore be expected to account for a considerable fraction of the titration value between the pH limits 2.6 and 8.0. In order to assess the possible relationships of inorganic salts to the acidity titration, the total ash was computed as milliequivalents of potassium carbonate and plotted along with the alkalinity of ash in Figure 22. Comparison of those curves indicates that the titration of inorganic sulfate, chloride, and other strong acid anions would constitute but an insignificant part of the total titration of the water suspension of the tissue, especially since those salts would only begin to be converted to their acids at the lower limit of the titration range, pH 2.6.

Sulfate has been shown by Pucher to be titrated to the extent of less than 4 per cent under the conditions of the total organic acidity titration (Pucher, Wakeman, and Vickery, 1941), but it was found in the present work that about half of any inorganic phosphate present would be titrated under the same conditions.

The basis for the assumption that phytic, ascorbic, and galacturonic acids are important components of the titratable acidity is as follows. The white lupine is, as noted previously, a rich source of pectic acid and therefore of galacturonic acid (Hirst, 1942). The initial stage in the decomposition of pectic acid involves demethylation, which frees carboxyl groups, thereby contributing to the titratable acidity, and subsequently depolymerization takes place, to give free galacturonic acid. Acidity from this source would be expected to increase quite rapidly during the first six days (Figure 19), and thereafter to decrease according to the rate at which the galacturonic acid was metabolized. It has been shown that practically all of the phosphorus in mature seeds is stored in the form of phytin (Earley and deTurk, 1944) (Fontaine et al., 1946), and that a fairly accurate estimate of the phytin content of seeds may be made from the phosphorus content. On this basis, the white lupine seed contains approximately 1.4 per cent of phytic acid.[1] Furthermore, the phytin content has been shown to diminish by one-third to nearly one-half during the first few days of germination of certain legume seeds (Bannerjee and Nandi, 1949). The curve for total phosphorus in the cotyledons of the white lupine seedling (Figure 24) could be considered a rough approximation of the course of phytin disappearance, with the phosphorus accumulation in the axis consisting mainly of inorganic phosphorus along with phosphoric esters. Phytic acid, therefore, should be a major source of titratable acidity in the seed and in the cotyledons during the earliest stages of germination. The maintenance of a fairly high level of titratable acidity in the cotyledons during the first six days could be the result of the balancing of the loss of phytic acid and free phosphoric acid by pectic acid demethylation.

Ascorbic acid exists only in minute quantities in ungerminated legume seeds, but it is synthesized at a very rapid rate during the first few days of germination. For instance, *Vicia faba* seeds contain only about 0.01 per cent of ascorbic acid, but on the eighth day of germination, in the experiments of Shaw and Pascoe (1949), the ascorbic acid content of the seedling axis had reached a maximum value of approximately 1.4 per cent of the dry weight, and by the 25th day it had dropped again to less than 0.1 per cent. The rapid and uniform increase in titratable acidity in the seedling axis in the lupines during the first 12 days (Figure 22) might thus be attributed to the accumulation of ascorbic acid and of galacturonic acid, and the maintenance of a high level of acidity after the 12th day might be due to a lag in the rate of metabolism of the galacturonic acid.

The rough parallelism of the titratable acidity and the total organic acid curve (Figure 22), particularly in the cotyledons, should be noted. In a figurative sense, and on the assumption that the typical, ether-soluble plant acids

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[1] Whether the inorganic phosphate detected in the organic acid fraction of the seed was present as such in the original tissue has not been investigated as yet.

make up only a small part of the total organic acid fraction, the difference in levels of the water and ether extract curves might be thought of as representing a distribution coefficient of the unknown acids (hypothetically consisting mostly of the less ether-soluble phytic, ascorbic, and galacturonic acids) between a water and an ether phase.

The metabolic roles of phytic and galacturonic acids are not at all understood, and it might well be that etiolated seedlings of the white lupine are an excellent material in which to study the metabolism of these widely distributed and quantitatively important constituents of seeds.

Without attempting to discuss the probable role of ascorbic acid as a respiratory catalyst in the seedling metabolism, it is noted that excellent evidence showing that ascorbic acid is synthesized from hexose sugars in cress (*Lepidum sativum*) seedlings has recently been published (Mapson et al., 1949). This fact is cited in further support of the probability that the carbohydrate fractions are not a principal source of asparagine carbon in lupine seedling metabolism.

The increase in total ash content of the seedlings during growth was due, among the elements analyzed, to sodium, magnesium, and calcium, absorbed from the tap water. There were also increases, at a much lower level, of iron, zinc, and copper, the latter two elements probably being absorbed from the culture equipment. The distribution of phosphorus and potassium, and to a lesser extent, calcium and magnesium, corresponded to the distribution of dry weight between the cotyledons and the growing plant axis. The most interesting, but inexplicable, data from the ash analyses are those for manganese, which is found in extraordinarily high concentration in the seeds of *L. albus*, the amount of this "minor" element being as great as that of phosphorus, and as much as magnesium and calcium combined. The amounts of manganese found in the developing axis were minute, corresponding to the quantities normally present in growing plants, while more than half of the manganese content of the seed disappeared from the cotyledons during 18 days of growth, presumably leached out by the water spray in which the seedlings were grown. No explanation can be advanced for this phenomenon.

CONCLUSIONS

The principal conclusion drawn from this work is that the striking accumulation of asparagine in etiolated *Lupinus albus* seedlings appears to be a consequence of a normal, but obligatory, utilization of seed protein as a respiratory substrate. The asparagine which arises by secondary synthesis, in this instance about 90 per cent of the total formed, is thus considered to be a product of protein respiration, and as such, is not metabolized further until acute starvation conditions prevail.

The observations and results which are associated with this principal conclusion are as follows:

1. There is a definite physiological pattern in the accumulation of asparagine in etiolated white lupine seedlings. The pattern is characterized by three successive and more or less definite stages: (a) rapid accumulation of asparagine associated with normal growth and metabolism, (b) a starvation stage, in which both growth and asparagine accumulation continue, but at a sharply reduced rate, and (c) physiological breakdown of the tissue with a concurrent disappearance of asparagine.

2. The first, or "normal metabolism stage", accounts for most of the asparagine formed. There is little difference between the metabolism of illuminated seedlings and that of etiolated seedlings during this stage.

3. Asparagine accumulation is closely correlated with growth in darkness, if considered in terms of expansion of the hypocotyl and transfer of substance from the cotyledons to the developing seedling axis, but bears an inverse relationship to growth of the epicotyl. Asparagine accumulation is less closely correlated with protein formation in the developing seedling than with the other indices of growth.

4. The seed protein is concluded to be the principal source of the asparagine carbon, with protein and lipids possibly of about equal importance as sources of respiratory carbon dioxide. The carbohydrate fractions are apparently less directly related to asparagine formation.

5. The asparagine nitrogen and free amino nitrogen which accumulated were equivalent to the α-amino nitrogen and amide nitrogen of the protein which was decomposed. This is the result to be expected if, in oxidative transformation of the protein amino acids to asparagine, nitrogen transfer is accomplished by the processes of oxidative deamination, transamination, and transamidation. It is implied, furthermore, that the protein nitrogen other than that of the α-amino and amide groups does not provide any significant amount of the asparagine nitrogen. However, the relationship noted may be entirely fortuitous, with the asparagine nitrogen actually coming from selectively utilized amino acids.

6. Glutamine does not accumulate, but behaves as an intermediate in asparagine and protein synthesis. The question is raised as to whether the glutamine→asparagine conversion may be an important source of the energy necessary for seed germination and growth during the "normal metabolism stage". This conversion would account for over 40 per cent of the total asparagine formed during that period.

7. An examination of the results of work with seedlings of other species lends strong support to the view that secondary accumulation of asparagine is a direct consequence of protein respiration, the quantity of asparagine so formed having a direct quantitative relationship to the amount of the protein amino acids oxidized. The presence in the seed of an adequate supply of starch polysaccharides, sucrose, or lipids, or a combination of these reserves, apparently reduces secondary asparagine formation to an insignificant level, without, however, having any effect on the rate or extent of proteolysis.

In the present work, the study of the organic acids has contributed little to a fuller understanding of the process of asparagine formation in lupine seedlings. Citric, malic, and phosphoric acids, and an unknown organic acid were present in detectable quantities in the "organic acid fraction", but unidentified constituents, not members of the respiratory cycle, appeared to be present in major proportions. Observations based wholly or in part on the total organic acidity, in seedlings or in other plant materials, are held to be of little value for interpretation in terms of the integrated series of reactions of the respiratory cycle. However, a biochemical scheme constructed around the tricarboxylic acid cycle provides a satisfactory hypothetical basis for interpreting the experimental data on asparagine accumulation.

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