DISTRIBUTION OF NITROGEN IN THE LEAVES OF CERTAIN SPECIES OF THE ROSACEAE

Thesis for the Degree of M. S.
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John H. Davidson

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DISTRIBUTION OF MERCURY IN THE ISLANDS OF CERTAIN STRIATA OF THE ROSARIAN

By

John W. Davidson

A THESIS

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INTRODUCTION

For the proper functioning of any organism whether plant or animal the chemical and physical condition of the protoplasm must be maintained within certain definite limits. Violation of these naturally prescribed conditions may result in temporary inactivity or death of the organism. The work of Addoms (1), Robbins (46, 47), and Heilbrunn (26) indicate the importance of this fact in their investigations on protoplasm and agents exhibiting a toxic action thereon.

When we realize that proteins are a major portion of all living protoplasm and are believed by some investigators to constitute the greater part of enzymes and by others to be closely associated with enzymes it is quite apparent why protein studies occupy such an important position in present day biological research.

Successful application of anesthetics, disinfectants, fungicides and insecticides may quite largely be attributed to their action on these two vital life systems---protoplasm and enzymes. Bancroft and Richter (3) studying the effects of narcotics upon cellular colloidal systems have shown that anesthesia is a reversible coagulation while disinfection is an irreversible coagulation of the colloidal system within the cell. Also, Bancroft and Rutzler (4) studying the proteins in the sensitive plant, Mimosa pudica, in relation to anesthesia found that some of the proteins

are very close to the state of maximum excitation. Addition of either accumulating or sensitizing agents decreased the sensitivity of the plant to shock. When normal plants not sensitive to external shock were treated with anesthetics they passed through a preliminary stage of excitation when going from the normal state to the state of anesthesia. The action of disinfectants, though similar to anesthetics was more severe. The accumulation of the narcotic upon colloids of the cell eventually reaches such a point that the combined effect of the narcotic and the electrolytes of the cell resulted in coagulating the hydrocolloids. This increase in viscosity slowed down diffusion of essential cell materials and also blocked the surface of active enzymes, which if prolonged resulted in death of the cell.

Heavy metals and some acids are common reagents used as protein precipitants. In studying the toxic action of two common fungicides, Sessions (51) found that sulfur when applied on bean and potato foliage burned at a pH 3 or higher resulted in frequent injury to the host plant. On the other hand if an insoluble copper salt was applied on peach foliage with a pH 4 serious injury occurred. Sulfur sprays on peaches and copper bearing materials on potatoes are the common procedure. Apple foliage (pH 4.7) is tolerant to both materials, but under certain conditions severe injury may result from either.

Upon the brief citations above it may be seen that the

study of proteins and their properties is essential for a better understanding of life and death. With this thought in mind a study of the proteins in Montmorency cherry leaves (Prunus cerasus) sprayed with several of the common fungicides was the motivating desire behind this investigation. The scarcity of investigational work on proteins in the leaves of our common fruit trees and the lack of a workable method for their isolation prevented accomplishment of the desired results. Instead, a study of an exploratory nature was undertaken in the hope that by studying the nitrogen distribution in the cherry leaves by a method similar to that followed by Osborne, Wakeman, and Leavenworth (43) some helpful information might be gained. The results obtained fortified by a review of the literature on the subject of nitrogen distribution in leaves of normal and abnormal plants, particularly those of the Rosaceae family should aid in a better interpretation of future research on this problem.

HISTORICAL

The historical development of nitrogenous fractions found in leaves may best be followed in two sections. (1) A chronological development of the contributions of the more important investigators followed by (2) the development of the methods employed during their investigations. Inasmuch as proteins are more concentrated in seeds of plants most of the early work on this subject has been limited to seed proteins.

I. Investigators And Their Contributions.

Roulle (49) in 1773 announced that the gelatinous matter, protein, which up to that time was known to exist only in the seeds of wheat, was also present in other parts of various plants. Later he isolated a proteinaceous product from the juice of hemlock. In 1789 Fourcroy (23) gave an extensive account of the occurence of coagulable protein in the juice of various parts of many plants, and described the method by which he obtained preparations of what he supposed to be pure plant albumin.

After 1805 no more attention was paid to proteins of green plants due to Einhof's (20) investigations of the seeds of cereals. The relative ease with which proteins could be obtained from seeds emphasized the difficulties of their extraction from green plants with the result that over a century elapsed before further work on leaf proteins

was undertaken.

In 1913 Honcquet (5) presented data showing that the presence of Bacillus mordens within the leaf might be connected with a great variety of irregular foliage types representing curling, wrinkling, and distortions. As this bacteria was a vigorous nitrate reducer it was found that whenever nitrites were detected in leaves bacteria were also present. This occurred every time any irregularity was observed in the foliage. No trace of nitrite could be found in normal leaves. The author concluded that the reducing power of the internal bacteria flora caused nitrogen starvation in the plants affected even when nitrates in the soil were abundant. In a later paper Honcquet (6) suggested that oxidases were produced by the plant to counteract the reducing effect of the invading bacteria. Reported investigations demonstrated that the total nitrogen was always less in diseased plants. One avenue of loss noted was that of ammonia in transpiration water, which occurred only in diseased plants.

Kraus and Kraybill (20) in 1918 introduced a new conception of growth and reproduction in plants based on the ratio of nitrogen to carbohydrates. The plant they chose to work with was the tomato inasmuch as it reacts in a manner similar to fruit trees. They found that (1) the limitation of nitrates resulted in suppression of growth and accumulation of complex carbohydrates. (2) Limitation of carbohydrates even with large quantities of

available nitrates in the soil resulted in decreased growth. (3) A rapid vegetative extension resulted from an adjustment of the carbohydrates and nitrates relative to one another so that both may be utilized in the formation of plant structure. (4) Such a relationship could be secured either by increasing the nitrates without decreasing the carbohydrates, or by decreasing the carbohydrates without increasing the nitrates. To explain this it was suggested by the authors that the higher nitrogen content in tissues tended to have greater water retaining capacity than tissues high in carbohydrate.

The most notable contributions which ushered in a new system of investigating leaf nitrogen were those of Osborne in the United States and Chibnall of Great Britain. Preceding this time the practice of agricultural chemists had been to state the proportion of protein in terms of a total nitrogen determination. In no case had any corresponding quantity of protein been isolated from leaves and identified by suitable chemical methods. Osborne's and Wakeman's work in 1920 (40) with spinach leaves was, therefore, a new approach to this problem. Difficulties involved in extracting and separating the cell proteins from their enveloping walls had previously handicapped such investigations. These investigators found when the cells of spinach leaves were broken by grinding an amorphous mixture in which the components can no longer be

recognized, is obtained. Upon either filtration or centrifugation a fluid and residue were obtained. The former contained cell contents while the residue was composed of cell walls. At least forty per cent of the total spinach nitrogen belonged to the colloidal protein dispersed in the fluid; almost one third to other nitrogenous substances soluble in water and a comparatively small part of the other fourth belonged to chlorophyll and phosphatides.

Chibnall and Schryver in 1921 (15) working with cabbage leaves attacked the problem of separating the cell proteins in a somewhat different manner. With the aid of an ether-water extract acting as a cytolyzing agent, colloidal solutions were obtained from which flocculation of the colloidal protein took place at elevated temperatures. The colloidal precipitates obtained in this manner contained about ten per cent nitrogen while Osborne's precipitate noted above contained fourteen per cent.

Osborne, Wakeman, and Leavenworth first worked on spinach leaves because of the ease in which the nitrogen fractions were extracted and their abundance in the water soluble portion. Later (43) their work on alfalfa leaves gave a somewhat different distribution of nitrogen. The green alfalfa plants were successively extracted with water, alcohol, dilute aqueous alkali, and with dilute alkaline alcohol. Total nitrogen was obtained on an aliquot portion of each extract, as indicated below.

Soluble in water	43.3%
33% alcohol	2.0%
0.3% alcoholic NaOH	39.3%
0.3% aqueous NaOH	7.0%
Extracted residue	5.3%

As most native proteins were quite soluble in aqueous alkali the low figure in this case indicated that most of the metabolically active protein was isolated with the water treatment. When the colloidal contents of the aqueous extract were precipitated by alcohol the chief constituents were found to be protein in combination with a considerable amount of calcium phosphate and other organic salts of calcium.

Schertz in 1931 (50) working along a somewhat different line on the chemical and physiological phases of mottling in leaves made some interesting observations. He concluded that inasmuch as the chloroplast was the birthplace of proteins, color of leaves and protein were intimately related. The figures below on Coleus leaves substantiate this statement.

	Grams N per 100 gm. dry weight
Green leaf	3.35
Mottled Leaf A	1.39
Mottled Leaf B	1.14

Results of nitrogen analyses on leaves in different stages of mottling indicated that such compounds as nucleoproteins, glycoprotcins, phosphoproteins, albumins,

and globulins were rapidly being broken down while the amount of derived proteins, amino acids, prolamines, ammonia compounds and other metabolic products remained practically constant. As the plants became more mottled phospholipins and amines disappeared. This indicated that the plant must be drawing upon its last sources of nitrogen before death ensues. Analyzing a similar series of leaves for, (1) nitrogen in organic combination; (2) nitrogen in ammonia or its combinations; and (3) nitrogen in nitrous or nitric acid showed there was a marked reduction in the total nitrogen content of the leaf with mottling. When the green larvae of Coleus were compared with the mottled it was found that the amount of protein nitrogen, nitrogen as ammonium salts and as nitrates disappeared as the leaves mottled. The greatest decrease was found in the protein nitrogen indicating that the protein compounds were rapidly being broken down. As mottling progressed the inorganic forms of nitrogen were first to disappear followed by the proteins.

In a series of six papers (7-14) published during 1923-24 Chibnall presented considerable information on results and methods of studying the nitrogen metabolism in higher plants. A few of the more pertinent conclusions will be mentioned. (1) Nitrogenous metabolism of the leaf of the runner bean run on the following lines:

Nitric N ⟶ Monoamino N ⟶ Protein ⟶ Other N. (translocated)

2. The nitric and monoamino nitrogen varied with that of the protein nitrogen. 3. In spite of rapid protein degradation at night there was very little change in most of the constituents of the water soluble nitrogen. 4. There was an accumulation of "other nitrogen" late in the growing season. In studying the decomposition of proteins in runner bean plants Chibnall found a large increase in non-protein nitrogen with a corresponding decrease in the protein nitrogen. Free ammonia was one of the main products of protein oxidation, however, it was toxic to plants so that it was probably removed by the formation of asparagine which even in high concentrations was innocuous to plants. In normal plants asparagine did not accumulate which indicated that it might be used as a translocatory substance. Chibnall also noticed an interesting difference between the leaf protein metabolism in normal and abnormal runner bean plants, the abnormality being drought. The leaves from plants affected by drought when detached and placed in water showed considerable increase in non-protein nitrogen, over fifty per cent of the increase being due to amide nitrogen other than asparagine. Normal products of metabolism from leaves treated in a similar manner were not of the amide type at all.

Using nitrogen starved tomato plants Dickerson (19) found that nitrates could be detected in all parts of the plant within twenty-four hours after placing in a

complete nutrient solution. Within thirty-six hours all the plants had considerable nitrite and in certain regions a trace of ammonia. After forty-eight hours there was more ammonia and less nitrites with an occasional crystal of asparagine showing. When three to four days had elapsed there was much less nitrite, a little ammonia and a great increase in amino acids. Simultaneously with this appearance of amino acids starch was disappearing and the chloroplasts of the younger leaves were becoming greener.

Tottingham, Schulz, and Lepkovsky (53) in 1924 compared Chibnall's extraction method with direct water extraction on barberry and sugar beet leaves. The addition of the cytolyzing agent extracted 18 to 20% more non-protein nitrogen. The water extraction was supplemented by Osborne's (43) successive treatment of alcohol, alkali, and acid with the following results:

Water soluble N	1.38%
Alkali soluble N.	0.18%
Acid soluble N	0.25%
Total soluble N	1.75%

These data showed that only a small portion of the exposed protein of the cell failed to dissolve in water. Even this rather drastic acid treatment left about 40% of the cell nitrogen in the tissue. From this, the conclusion was drawn that the residual portion is composed of nitrogenous constituents enclosed by cellulose material and

relatively inert in metabolic processes.

Davies in 1926 (17) obtained the colloidal precipitates from green forage plants following Chibnall's method. He found that in the clovers the protein was difficult to isolate in a pure condition and that it was loosely conjugated with some prosthetic group which contained inorganic compounds.

Mothes (38) found that when there was opportunity for carbohydrate synthesis in sunlight or the leaves had an available supply of glucose in darkness, protein synthesis rather than hydrolysis predominated. The higher the temperature, however, the higher the concentration of glucose which had to be supplied to check proteolysis. On the other hand when carbohydrate reserves became reduced through exposure to darkness there occurred first proteolysis and with further decrease in carbohydrates an increase in amides. Finally ammonia accumulated with eventual injury to the leaf under conditions of extreme carbohydrate deficiency.

Thomas in 1927 (53-57) made a rather complete study of the nitrogen metabolism in the apple (Pyrus malus). He studied the insoluble leaf proteins during the early period of bud formation, at time of maximum active growth, and during declining metabolic activity. From the constancy of the distribution of nitrogen in the proteins there was some justification to conclude that a single protein existed in the leaves. These results indicated

that little if any qualitative change in the nature of the insoluble leaf protein had taken place during its development. From this standpoint it was clear that any relationship between the insoluble proteins and carbohydrates which would affect plant performance was due to quantitative rather than qualitative differences in the nature of the protein.

In another paper of this series Thomas (55) made a quantitative measure of the changes undergone by the various nitrogen fractions throughout a year's cycle. During this study no relationship could be discovered between either; temperature or sunshine, or rainfall or humidity, and any of the nitrogen fractions. Other important deductions from this study were: (1) that the amide nitrogen and the "rest" nitrogen tended to vary inversely as the total water soluble nitrogen, non-protein nitrogen and amino nitrogen, thus supporting Chibnall's (8) contention that amino nitrogen was connected with protein synthesis and the "rest" nitrogen with protein degradation; (2) that proteins were transported to growing parts in the form of amino acids; (3) that there was a definite autumnal migration of nitrogen from the leaves to branches; (4) that nitrates were transformed to amino acids in the fine roots; (5) water soluble nitrogen is only 7 to 10% throughout the cycle; and (6) ammonia nitrogen remains low throughout the cycle.

Thomas (53) also studied the effect of heavy applications of nitrates to the soil finding that in a given period the water soluble nitrogen and non-protein nitrogen in the treated tree increased 43 and 11% respectively; in the untreated tree similar fractions increased only 6 and 3% respectively. Another interesting observation made showed total water-soluble nitrogen and non-protein nitrogen to be decidedly higher in the metabolicly active parts of the treated tree as compared with the untreated tree. This difference was observed throughout the year and corresponds with the relative vigor of the trees.

[illegible]rickson (37) studying nitrogen distribution in pineapple leaves found a rather definite ratio existing between protein and carbohydrate content. When the protein and carbohydrate content were about equal the plant made vigorous growth. Mature leaves were in about normal condition when the carbohydrate content was three to four times as high as the protein content, but the plant showed signs of senescence when carbohydrate content was five to six times that of protein. Chlorotic condition of the leaves was apparent when eight to ten parts of carbohydrate existed to one part protein.

Working with pear leaves in 193[illegible] Lincoln and Mulay (30) found only 10 to 25% of the total nitrogen to be soluble in the expressed sap and this almost wholly in a non-protein form. They, therefore, attempted to peptise

the remaining insoluble nitrogen by extracting the finely divided tissue with various ions or hydrolyze the protein with acid or enzymes. Disturbing factors were the acidity of the tissue, naturally contained tannins, and inability to disintegrate the tissue. In studying the effect of pH on the peptization of cytoplasmic proteins the following figures were obtained.

pH	Reagent	Total N in Extract - %	Total N in Extract Non-protein	Total N in Extract Protein
1	N/10 H_2SO_4	15.8	7.7	8.1
5	HOH	17.45	5.95	11.50
6	N/10 NaOH	19.35	5.65	13.70
7	N/10 NaOH	22.4	4.6	17.8
9	N/10 NaOH	23.35	3.7	23.15
12	0.2 N Na_2CO_3	47.5	5.0	42.5
13	2 N NaOH	89.35	18.5	70.85

It was also shown that the anions of neutral salts differed from one another in their effectiveness to extract cytoplasmic proteins. This was in agreement with the effect on protein solubility by a lyotropic series of ions as presented by Gortner, Hoffman, and Sinclair (24).

Robinson (43) deriving her conclusions from reviewing the literature and by observations made during her own work suggested that proteins appear to provide the fundamental basis for structural individuality. In the cell their colloidal nature provides an ideal reaction medium for vital life processes.

vonEuler and Thunström (31) studying arginine metabolism found no essential difference regarding arginine in chlorophyll defective mutants of barley and the normal plant. On the other hand colorless parts of variegated Pelargonium leaves contained fifty to one hundred times as much free arginine as the green parts.

Hannaker in 1934 (32) studied the seasonal cycles of nitrogenous material during five different periods in apple tree growth. His results from analysing the leaves largely confirms the work of Thoms (53).

Davidson and Shive (11) in 1935 working with peach tissue (Prunus Persica) were forced to modify their methods to include cyanogenetic glucosidic nitrogen. After removal and determination of the hydrogen cyanide they studied the nitrogen partition in the tissue but found considerable variation depending upon growing conditions and activity of plant at time of sampling.

Wann (61) studied protein metabolism in starving barley leaves. He found that during the first twenty four hours the amino and unstable amide (glutamine) fractions rose sharply, followed by a rise in stable amide (asparagine) during the next twenty-four hours, during which time yellowing of the leaf occurred. Free ammonia began to accumulate after forty-eight hours and a concurrent decrease of all other nitrogen fractions occurred. As browning and disintegration of the tissue took place considerable quantities of ammonia

were liberated. It is difficult to say whether ammonia liberation was a cause or an effect. The analyses made in this investigation showed that the breakdown of complex insoluble nitrogenous constituents began at the earliest stages of starvation and indicated that it was a result of protein hydrolysis. No true sparing action of protein by carbohydrates could be detected, since during this initial phase the carbohydrate was at its highest. A continuous hydrolysis of protein seemed rather to be part of the normal mechanism whereby proteins synthesized in the mature leaf were translocated to other parts of the plant. Two reasons have been given by the author to account for the decrease in rate of hydrolysis of proteins as starvation progresses. First, an exhaustion of the proteins associated with the chloroplast most apparent because the other nitrogenous substances in the cell were more resistant to hydrolysis. Second, the slower breakdown in later stages might be caused by the inactivation of proteolytic enzymes by ammonia or other toxic products.

Long and Burneek (31) in 1937 studied the nitrogen content of the strawberry plant (Fragaria virginiana). By analysing the leaves twice a month during the year they found that the nitrogen reserve was built up in the plant as a whole in the autumn and depleted in the spring.

Moreau (38) in 1938 made observations on the proteins of pasturage plants. He found that proteins occurred in the protoplasm of the permanent cells of perennial

rye grass as complexes in which they were chemically associated with water soluble and water insoluble phosphorus substances. "Complex a" consists of a sodium or potassium phosphate protein soluble in water, and inasmuch as sodium and potassium decrease the viscosity of protoplasm the author concluded it was this fraction that determined the yield of soluble proteins by Chibnall's extraction method. "Complex b" was composed of a calcium or magnesium phosphate protein complex. It was much less soluble in water and the effect of calcium and magnesium was to increase the viscosity of the protoplasm rendering this fraction more difficult to extract from leaf tissue. Apparently the ratio of "complex a" to "complex b" in the protoplasm could vary with varying ratio in the vacuole fluids of sodium and potassium to calcium and magnesium. This might account for the differences obtained in nitrogen extraction studies in different plants or even in the same plant but grown under different conditions. Thus the large amounts of oxalic acid in spinach, rhubarb and sorrel would tend to curtail the supply of calcium and magnesium allowing for the relatively high yields of soluble proteins obtained from these plants.

Engard (21) 1930 studied the translocation of nitrogenous substances in Cuthbert raspberry (Rubus strigosus x R. idaeus). He found that the soluble forms of organic nitrogen were most highly concentrated in the subterminal

portions of the plant and that the stem tip and leaves were the seat of protein synthesizing organs of the plant. The concentration of protoplasm was greatest in these organs and the amino acids in these regions were utilized very rapidly. The conclusions drawn from this work indicated that the amount of synthesis of amino acid into protein was dependent upon the amount and activity of protoplasm, all other factors for nitrate reduction being favorable, and was not a function of special organs. There was no well founded reason to assume that the seat of organic nitrogen synthesis was only in the leaves. Nitrogen reduction took place wherever there was sufficient carbohydrate, reducase, a proper pH, proper temperature and essential elements.

Pearsall and Hellikson (44) 1930 studied the effects of light on the nitrogen metabolism in detached leaves of Narcissus. Three noticeable effects of light were observed. (1) An increase in the rate of absorption of inorganic nitrogen, (2) an accelerating of the process of nitrate reduction, and (3) an increasing rate of production of organic nitrogen.

II. Methods.

In dealing with proteins of living plants several special conditions must be considered. The proteins desired are in cells whose walls must be ruptured or otherwise affected before the non-diffusible proteins can be extracted. As soon as the cell walls are ruptured

enzymatic action may occur by stemming the alteration of nitrogen materials in the cell. The extract from living plants contains a great variety of constituents. These must be separated from the protein or other nitrogen fraction in order to obtain preparations of even approximate purity. Methods of accomplishing these conditions used by the various investigators are discussed below.

A. Preservation of Samples.

Although use of fresh material is to be preferred preservation of samples is often necessary. Staining the tissue in chemical baths such as alcohol or other have been reported by Thomas (56) and Lincoln and Wulzy (30) as affecting the amount of extractable protein.

Desiccation has many physical advantages in that the dried product may be easily ground which facilitates more accurate handling. The temperature used and the length of time the tissue is exposed to such treatment affects the distribution of nitrogen in the various fractions. Bottinghem et al. (5) working with sugar beet leaves found that 40°C. permitted extensive solidification in the form and amount of soluble nitrogen. Rapid drying at 55° gave results almost equivalent to fresh leaves. Lincoln and Wulzy (30) dried pear tissue at 50°C. with a resulting increase in soluble nitrogen. When temperatures of 85° were used a marked decrease in extractable protein nitrogen occurred. Hammer (31) found rapid drying of apple leaves at two hour temperatures gave results similar to

fresh tissue.

Results with freezing are varied. Tiemple, Rohlins and Schomerhorn (39) believed freezing followed by immediate analysis gave results on beet leaves equal to fresh leaves. Osborne et al. (43) stored alfalfa plants at -10°C. and recorded results comparable to those obtained from fresh leaves. Lincoln and Duley (30) stated that pear leaves kept for eight weeks at -27°C. were green and in an unaltered condition. Thorne (55) working with apple leaves and Nottingham et al. (53) with barberry and sugar beet leaves found that freezing coagulated the soluble protein thus preventing its complete extraction. Cold, non-freezing, temperatures as a means of preservation resulted in an increase in non-protein nitrogen. When tissues are to be analyzed for hydrocyanic acid use of fresh material is essential according to Levinson and Shive (13).

F. Extraction.

The methods of extraction employed have mostly been modifications of Osborne's (43) grinding procedure. The greatest departure from this has been Thimann's (10) use of a cell plasmolyzing agent such as ethyl ether or butyl alcohol, and then pressing out the major part of the vacuole content. This operation did not rupture the leaf cells and the protoplasm was retained within them. After removal of the vacuole content the protoplasmic material remaining in the residue could be extracted by

grinding with water. This was only applicable on the more succulent types of leaves. Tottingham et al. (53) comparing the two methods with sugar beet leaves found that the non-protein portion extracted by Chibnall's method contained forty to fifty percent of the total nitrogen extracted whereas by the direct water extraction method the non-protein fraction was only twenty-two to thirty-three per cent of the total. Thomas (53) also compared the two methods on apple leaves and found that only two-thirds as much protein nitrogen and non-protein nitrogen were found in the extracts obtained by Chibnall's method.

When more fibrous types of leaves are studied, thorough grinding with sand has been generally employed. Also it has been found that the portion of nitrogenous material extractable with water alone was much less, necessitating the use of some agent to increase the amount of the nitrogen fractions removed. The agents used by Osborne, Wakeman and Leavenworth (43) to accomplish this were 33% alcohol, 0.3% aqueous NaOH and 0.3% alcoholic NaOH. Thomas (53) added two percent phenol but did not state its purpose. Lincoln and Kulay (30) working with pear leaves used water and sodium carbonate for their routine extractions. McColla (33) desired to study protein and non-protein substances from wheat plants. He, therefore, used the protein precipitant trichloracetic acid in the extracting water which separated the soluble nitrogen

into the filtrate leaving all protein material in the residue. Thomas (34) in studying the soluble leaf cytoplasmic proteins remaining in the residue after extraction removed interfering substances first, then hydrolysed the residue containing the impure proteins, determining the nitrogen distribution on the hydrolysate according to the method of Van Slyke, Porter, and Gortner (36).

When only non-protein material was to be studied Bonsen (13) found that with wheat plants autoclaving the tissue at fifteen pounds pressure for five minutes aided extraction and did not affect the non-protein nitrogen.

C. Precipitation of Colloidal Material from Extract.

When both protein and non-protein nitrogen occur in the plant extract some method of separation is necessary if the different fractions are to be studied. The agents used to effect this separation vary as to the purpose of the investigation. Miller and Vanslyke (28) studied various protein precipitants on blood. They found trichloracetic acid was best suited for separating proteins from their intermediate products as it precipitated only proteins. Amino acids were not occluded in the precipitate as they were with some other reagents. The results of Thomas (34) were not in complete agreement with this, but McCalla (33) obtained satisfactory separation by this method in his work. Other precipitating agents mentioned were tungstic acid and picric acid which were

distinguished by the relative completeness with which they precipitated protein intermediate products without affecting the amino acids. Mercuric salts had the advantage that they removed all pigments giving colorless solutions; but the excess mercury was not easily removed, and like copper it also precipitated polypeptides.

For his study of apple leaves Thomas (53) preferred colloidal ferric hydroxide as this reagent did not precipitate any of the amino acids and only a few of the complex proteases approaching protein complexity.

Lincoln and Kidd (30) found that the sum of the fractional nitrogen containing precipitates obtained from heat, colloidal iron and acid to pH 1 were about equivalent to a single treatment of pH 1 on the original pear leaf extract. They stated that the colloidal iron alone was not effective without acidifying the extract and to obtain a good yield the acidity must be increased to such an extent that the acid alone becomes the precipitating agent. Yemm (33) compared heat, colloidal iron, and trichloracetic acid to remove protein from barley leaf extracts, but found little if any difference in the total soluble or the other soluble nitrogen fractions.

D. Nitrogen in the Resulting Residue and Filtrate.

After the proteinaceous substances of the fluid leaf extract had been precipitated, a study of the nitrogen distribution in both the precipitate and filtrate was

generally been made. The nitrogen distribution followed is actually that suggested by Van Slyke (50) for the analysis of pure proteins. Morton and Gortner (38) have pointed out that much valuable comparative data may be secured by the use of this procedure if no attempt was made to assign any part of the nitrogen to a specific amino acid and thereby conclude a certain protein to be present. They pointed out that in plant materials there may be organic nitrogenous compounds which have no relation to the protein molecules such as purine and pyrimidine bases, nitrogenous ~~lipids~~ lipids and pigments as well as other non-protein nitrogenous compounds.

Stuart (52) studied amino nitrogen distribution in leaf extracts of various plants and found several substances which may cause errors in certain phases of Van Slyke's amino nitrogen determination. He found ammonia to be one of the worst offenders. In the free form it was never very abundant in normal plants but frequently studies were made on plants grown under disturbed conditions which favored a breakdown in nitrogen metabolism resulting in the presence of abnormal amounts of ammonia. Another source of ammonia occurred in certain "acid plants" where it may constitute a large proportion of the total soluble non-protein nitrogen. It is, therefore, quite obvious under these conditions that ammonia must be removed prior to any determination employing nitrous acid.

EXPERIMENTAL

Two experiments are reported in this study. Part I was conducted in 1937 and deals with the effect of three different fungicides on the total nitrogen and soluble non-protein nitrogen in cherry leaves. Due to the rather erratic results obtained the 1937 program, Part II, was planned to develop some method of analysis which would give reproducible results and also serve as a method of indicating the nitrogen distribution in the leaves.

Part I

A. Description and Preservation of Leaf Samples.

Leaf samples were obtained from twelve year old Montmorency cherry trees at South Haven, Michigan. The trees were of moderate vigor and had received the regular spring application of ammonium sulfate in May. The leaf samples were gathered in July and early August from the current years growth, the collecting being limited to the south half of the trees. This necessitated some means of preservation until the winter months. Facilities were not available for rapid high temperature drying, while freezing temperatures were easy to secure in a nearby storage and ice plant.

Immediately after collection the leaves, therefore, they were placed in a cold storage room at a temperature of about -4°C. After about a month the leaves were trans-

ferred to a colder room in the Department of Dairy Husbandry, Michigan State College, where the temperature was maintained at or below -13°C. The leaves handled in this manner maintained most of their green color and were apparently well preserved. In some cases where the leaves had been pressed tightly together there was some browning and a little mold growth. Such leaves were avoided in all nitrogen determinations. Except in the case of August 3, 1937 samples, very little spray residue was evident on the leaves.

Samples were collected from duplicate plots receiving similar spray applications of a given fungicide. The three spray materials chosen should accentuate any detectable difference that might exist from the use of different types of fungicides. One set of samples were taken three weeks after spraying, designated as July 1, receiving a total of four applications of the fungicide. The other set August 3, was preceeded one week by a spray application which made a total of five sprays. It was hoped that the July 1 set of samples would show some cumulative effect on the total nitrogen or soluble non-protein nitrogen in the leaves after continued application of the fungicides. During the interval between the collection of the two samples another application of the fungicide was made to see if any effect noted in the first set of samples would be emphasized in the later set. The fungi-

cides employed and the concentrations used are described in the following paragraphs. Table I indicates the treatment received by each sample.

Table I. Showing Treatment Received by Each Sample.

Samples	Plot	Fungicide	Conc. per 100 gal. water	Number of Applications	Date Collected
1, 2	57	Lime sulfur	2½ gal.	4	7/1/37
3, 4	57	Lime sulfur	2½ gal.	5	8/3/37
5, 6,	36	Lime sulfur	2½ gal.	4	7/1/37
7, 8	36	Lime sulfur	2½ gal.	5	8/3/37
9, 10	39	Wettable sulfur	10 lbs.	4	7/1/37
11, 12	39	Wettable sulfur	10 lbs.	5	8/3/37
13, 14	61	Wettable sulfur	10 lbs.	4	7/1/37
15, 16	61	Wettable sulfur	10 lbs.	5	8/3/37
17, 18	26	Bordeaux	4-6-100	3	7/1/37
19, 20	26	Bordeaux	4-6-100	4	8/3/37

Lime sulfur solution of 32° Baume was the common commercial product an approximate analysis being, 14-19% Calcium polysulfide; less than 1% calcium thiosulfate. It is strongly alkaline when applied to foliage but upon oxidation a residue consisting chiefly of elemental sulfur is left. The fate of the sulfur after this is a controversial matter. Young (34) contends that it is oxidized to sulfur dioxide or pentathionic acid while Wilcoxon (32) claims it is reduced to hydrogen sulfide.

The wettable sulfur was a proprietary product

containing 95% sulfur and 5% wetting agent. It may be neutral or slightly acid when applied to the tree, but upon continued exposure to the air would result in products similar to the sulfur residue from lime sulfur solution.

The third material was a copper Bordeaux mixture containing four pounds copper sulfate and six pounds hydrated lime in one hundred gallons of water. The resulting insoluble copper hydroxide is of moderate alkalinity when applied on the foliage, but weathering or plant secretions somewhat alter its composition causing, under certain conditions, severe injury to the foliage.

B. Analytical Methods and Results

Duplicate ten gram samples of the whole leaf were used for moisture determinations on each sample. The leaves were placed in large aluminum weighing pans and left in a vacuum oven at 85°C. until constant weight was obtained. Any difference that might have been due to the spray treatment would probably be masked by the long storage period.

Ten leaves weighing between three and five grams were used for the duplicate total nitrogen determinations. Reported tests with diphenylamine reagent as used by Harvey (25) failed to indicate the presence of nitrates or nitrites in the leaves. The modified Kjeldahl-Gunning (2) method was, therefore, used for determining

the total nitrogen in the leaf tissue.

The soluble nitrogen fractions were obtained by mincing the 25 g. sample of leaves with shears followed by grinding with nitrogen free quartz sand in a mortar and pestel. Water was added until a mass of pasty consistency was obtained. Grinding was then continued until examination revealed no large leaf segments. More water was then added in an attempt to aid the separation of the fluid from the residue by vacuum filtration. Paper pulp on a large Buchner funnel was used as the filtering medium. This filtering process was very slow due to the viscous nature of the extract. Five or six hours were required to obtain 500 ml. of a clear brown filtrate. Filtrates were obtained by this method using water, ether-water and trichloracetic acid as the extracting medium. Tests for protein precipitation such as, heat coagulation, addition of colloidal ferric hydroxide, addition of alcohol, and addition of ammonium sulfate revealed the absence of protein precipitation in the filtrate. It was, therefore, decided to use trichloracetic acid at 3.5% by weight in the extracting liquid and thereby obtain only soluble non-protein nitrogen in the extract. This procedure was then carried out on ten duplicate leaf samples. Total nitrogen was determined on 100 ml. aliquots of the filtrate after evaporating almost to dryness. The combined results of moisture content, total nitrogen and soluble non-protein nitrogen are shown in Table II. A summary of the results for

Table II. Showing Treatment and Analysis of Each of the Twenty Samples.

Sample	Plot	Fungicide	Date Collected	Moisture %	Total Nitrogen % On Dry Weight	Total Nitrogen % On Fresh Weight	soluble Total Nitrogen % On Dry Weight	soluble Total Nitrogen % On Fresh Weight
1	57	Lime Sulfur	7/1/37	53.53	2.44	1.13	0.116	0.054
2	57	" "	7/1/37	47.94	2.08	1.08	0.088	0.046
3	57	" "	8/3/37	51.62	2.29	1.11	0.102	0.044
4	57	" "	8/3/37	56.58	2.55	1.10	0.128	0.055
5	36	" "	7/1/37	47.08	2.01	1.06	0.084	0.044
6	36	" "	7/1/37	44.02	1.77	0.99	0.141	0.079
7	36	" "	8/3/37	50.62	1.76	0.86	0.250	0.113
8	36	" "	8/3/37	49.44	1.52	0.77	0.174	0.088
9	32	Wettable Sulfur	7/1/37	35.97	1.64	1.05	0.224	0.143
10	32	" "	7/1/37	36.54	2.06	1.30	0.093	0.059
11	32	" "	8/3/37	58.76	2.49	1.03	0.260	0.107
12	32	" "	8/3/37	57.50	2.27	0.96	0.090	0.038
13	61	" "	7/1/37	52.64	2.26	1.07	0.173	0.082
14	61	" "	7/1/37	54.71	2.02	1.15	0.192	0.087
15	61	" "	8/3/37	44.11	2.53	1.41	0.068	0.038
16	61	" "	8/3/37	42.19	2.48	1.43	0.254	0.147
17	26	Bordeaux	7/1/37	54.74	2.85	1.29	0.282	0.127
18	26	"	7/1/37	58.43	3.01	1.25	0.268	0.111
19	26	"	8/3/37	37.70	1.99	1.23	0.142	0.088
20	26	"	8/3/37	46.26	2.02	1.09	0.332	0.178

total nitrogen and for soluble non-protein nitrogen is shown in Tables III and IV respectively.

Table III Showing the Percent Total Nitrogen in Cherry Leaves Receiving Different Fungicidal Sprays.

Fungicide	Collection Dates July 1, 1937	Aug. 3, 1937
Lime Sulfur	2.07	2.03
Wettable Sulfur	1.93	2.44
Bordeaux	2.03	2.00

Table IV Showing the Percent Soluble Non-protein Nitrogen in Cherry Leaves Receiving Different Fungicidal Sprays.

Fungicide	Collection Dates July 1, 1937	Aug. 3, 1937
Lime Sulfur	0.197	0.158
Wettable Sulfur	0.170	0.138
Bordeaux	0.275	0.237

Although there was a somewhat definite trend in the 1937 results indicating that more non-protein soluble nitrogen was present in the leaves receiving the Bordeaux fungicide it was quite apparent from the wide variation in the results of duplicate samples that a more dependable method of extraction was necessary before any great significance could be attached to this work. With this view in mind an attempt was made in 1938 to study some of the factors affecting the extraction of nitrogen from the cherry leaves.

Part II. - 1938

A. Description and Preservation of Leaf Samples

Collection and preservation of the leaves was similar to 1937 except that one large sample was obtained from the similar trees which had received a low soluble copper spray treatment throughout the season. These trees were in a more vigorous condition than those of 1937 indicated by the higher total nitrogen figures obtained in 1938.

B. Analytical Methods and Results.

From the uniform leaf samples a large number of leaves were passed through a meat grinder. This procedure broke the leaves up into rather small segments permitting easier handling and giving a more homogenous mass of material. This operation was followed each time a new series of samples was weighed out. It was done as rapidly as possible and whenever weighing was not in progress the large gross sample was kept in the refrigerator to maintain enzymatic action at a minimum.

For the moisture determination triplicate 15 g. samples of the ground leaves were dried in a vacuum oven at 85°C. similar to the 1937 procedure.

The modified Kjeldahl-Gunning method was followed for all nitrogen determinations. Triplicate three gram samples of the ground leaves being used for the total nitrogen determination.

Two phases in particular in the 1937 extraction procedure had been noticeably inefficient. Very little nitrogen was being removed by grinding the leaves with either water or with water and trichloracetic acid. To remedy this defect successive extractions, similar to

those used by Osborne et al. (47) on alfalfa leaves, were tried as a means of ascertaining what treatments were necessary to remove the leaf nitrogen. The other phase needing correction was separation of the fluid extract from the residue. Filtration through paper pulp was very slow frequently requiring five or six hours and in some cases was not complete even after that length of time. Use of linen cloth to effect the separation was slow, incomplete, and not readily adaptable to quantitative technique. Centrifuging as a means of separation, therefore, offered the most promise.

The procedure adopted for 1939 incorporated the two differences noted above. Triplicate twenty-five gram samples of the coarsely ground leaves were macerated to a fine pulp in a large mortar with ten grams of nitrogen free quartz sand and distilled water. The entire sample was then transferred to two large centrifuge tubes and centrifuged until the clear brown supernatant liquid was free of visible solid material. The liquid was then decanted into a 1000 ml. volumetric flask. The entire grinding and extraction process was repeated three times on each sample, the final volume of liquid approaching 1000 ml. Total nitrogen was determined on 500 ml. aliquots after they had been evaporated almost to dryness. The residue was then extracted in a similar manner with 95% alcohol (ethyl) until all the green coloring matter was removed. This required four or five extractions. The entire alcohol extract was evaporated almost to

dryness in a Kjeldahl flask and the total nitrogen determined. The residue from the alcohol extraction was next treated with .2N hydrochloric acid in a similar manner. A fourth extraction with distilled water was made to remove excess acid and combined with the acid extract. The entire light brown solution was evaporated to near dryness in a Kjeldahl flask and the total nitrogen in the extract determined.

The residue left at this time was light brown in color. The next treatment involved three extractions with .2N sodium hydroxide following the same procedure as described above. The extract was dark brown as was also the residue. Total nitrogen was determined on the entire extract, acidified before evaporation to prevent ammonia loss. The results of these treatments on the basis of total nitrogen extracted are shown in Tables V and VI.

Table V. Percent Nitrogen Obtained by the Extractions Indicated. Percentages are Based on Dry Weight of Samples.

Extraction Treatment	Series I. Sample Number			Series 2. Sample Number			Series 3. Sample Number			Series 4. Sample Number			Series 5. Sample Number		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Total Nitrogen	2.59	2.57	2.62	2.44	2.42	2.50	2.45	2.48	2.49	2.46	2.46	2.44			
Water	0.676	0.662		0.757	0.745	0.786	0.843	0.866	0.922	0.775		0.772	0.842	0.856	0.799
Ethyl Alcohol	0;026	0.029		0.039	0.038	0.039	0.033	0.034	0.033	0.109	0.054	0.044	0.042	0.050	0.051
5% NaCl				0.091	0.043	0.039									
.2N HCl				0.030	0.032	0.029	0.049	0.057	0.057	0.034	0.039	0.056	0.033	0,050	0.040
.2N NaOH				0.454	0.428	0.376	0.624	0.591	0.692	0.470	0,498	0.546	0.398	0.397	
Pepsin							0.090	0.102	0.096						
Total N Extracted				1.371	1.286	1.269	1.639	1.650	1.800	1.368		1.418	1.315	1.353	
% Moisture	54.70	54.28	55.12	49.15	48.74		51.99	52.24		57.40	57.45	57.09			

Table VI. Percent Nitrogen Obtained by the Extractions Indicated. Percentages are Based on Fresh Weight of Samples.

Extraction Treatment	Series 1. Sample Number			Series 2. Sample Number			Series 3. Sample Number			Series 4. Sample Number			Series 5. Sample Number		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Total Nitrogen	1.18	1.16	1.19	1.24	1.23	1.27	1.17	1.19	1.19	1.05	1.05	1.04			
Water	0.306	0.299		0.386	0.380	0.401	0.402	0.418	0.440	0.331		0.329	0.403	0.410	0.382
Ethyl Alcohol	0.026	0.029		0.020	0.019	0.019	0.016	0.016	0.016	0.046	0.023	0.019	0.020	0.024	0.024
5% NaCl				0.046	0.022	0.020									
.2N HCl				0.015	0.016	0.015	0.024	0.028	0.027	0.014	0.017	0.024	0.016	0.024	0.019
.2N NaOH				0.232	0.218	0.192	0.298	0.283	0.330	0.201	0.212	0.233	0.190	0.190	
Pepsin							0.043	0.049	0.046						
Total N Extracted				0.699	0.655	0.647	0.783	0.794	0.859	0.592		0.605	0.629	0.648	

Various modifications of the procedure described above were undertaken in an attempt to increase the amount of nitrogen extracted.

It was noted first that when the order of acid and alkaline treatment was reversed a considerable decrease occurred in the amount of nitrogen extracted by both the alkali and acid as shown in Table VII.

Table VII Showing Effect of Order of Treatment on Nitrogen Extraction from Cherry Leaves.

Order of Extraction		% Nitrogen in Extract Sample 1	Sample 2
1	.075 N HCl	.048	.051
2	.075 N NaOH	.120	.110
1	.075 N NaOH	.050	.039
2	.075 N HCl	.024	.033

A second modification involved the digestion of the ground leaf sample with enzymes previous to extraction. This was made in an attempt to determine whether it would have any effect on the availability of the nitrogen in the leaves. In one series a pectin decomposing enzyme sold commercially as "Pectinol" was used on the samples before any extractions had been made. One one-hundredth gram portion of the enzyme preparation containing 0.0 mg. nitrogen was added to the finely ground sample and allowed to digest at 37° for 24 hours. The regular water, alcohol, acid, alkali extraction procedure was then made on the sample. The results are shown in Tables V and VI as

Series 5 and also in Table VIII.

The third modification consisted in treating the residue remaining after complete extraction of one series with 0.10 g. pepsin containing 5.7 mg. nitrogen and digesting the samples for 24 hours at pH 5 in a constant temperature oven at 37°C. The residue was then washed thoroughly with water and total nitrogen determined on the entire extracted liquid.

A still further modification consisted in studying the effect of salt solutions on the further removal of nitrogen. A 5% sodium chloride solution was inserted in the extraction procedure following the alcohol extraction of one series of samples. The same procedure of grinding and centrifuging was followed as with the other samples.

The results from these various extraction procedures are shown in Tables V and VI.

Table VIII Comparison of the Percentages of Nitrogen Extracted by Water, Alcohol, Acid, and Alkali in Terms of Total Nitrogen.

Extraction Treatment	Average of All Normal Treated Samples % Nitrogen	% of Total Nitrogen Normal Treatment	Pectinol Added
Water	.730	31.30	33.41
Alcohol	.043	1.72	1.83
.2 N HCl	.040	1.60	1.64
.2 N NaOH	.513	20.84	19.04
Sum of N Extractions		55.57	59.37
Total N in Leaves	2.40		

DISCUSSION

It was hoped at the outset of this study that the diverse nature of the three fungicidal treatments employed would be manifested in the nitrogen distribution of the leaves treated. In Table III samples receiving similar treatment in the 1937 experiment are averaged together. It is apparent from these figures that there is no appreciable difference between the three fungicides applied to the leaves and their effect on the total nitrogen content.

When a similar compilation is made for the soluble non-protein nitrogen results obtained in 1937, a difference in treatment is evident. This is shown in Table IV. It is quite interesting to note the higher percentage of soluble non-protein nitrogen in the Bordeaux sprayed leaves, while the total nitrogen remained about the same as in the other treatments. As Bordeaux is recognized as the material most likely to cause injury these results are in agreement with the findings of Schertz (50) Chibnall (13) and Wenn (63) who report that the leaves of plants suffering from abnormal growth conditions show a higher soluble, non-protein nitrogen content than normal leaves.

Perhaps the most outstanding observation from the 1938 results is the difficulty with which the nitrogen is extracted from the leaves, the total removed being only slightly over fifty percent of the total nitrogen. This fact is in general agreement with the observations

of Osborne (43), Thomas (5[illegible]), Lincoln and Mulay (3[illegible]), and Tottingham, Schulze, and Lepkovsky (5[illegible]) when they concluded that a rather large portion of the total nitrogen is closely associated with the cellulose or lignin in the leaf cell. More recently Norman (40) in discussing the difficulties in lignin determinations emphasized the fact that the lignin residue from non-woody materials contained nitrogen which is difficult to remove, hydrolytic pretreatment of 5% sulfuric acid for one hour removing only part of it. An investigation on this problem indicated a reaction between the protein and hydrolysable pentose containing constituents.

In Table VIII the difficulty with which removal of the nitrogen has been effected in the leaf cell is shown by averaging together all figures for a given extractant and determining its percentage of the total nitrogen.

From the above extraction results it was apparent that some method of breaking down the cell wall was necessary for further nitrogen removal. Inasmuch as cellulose decomposing enzymes are not available an enzyme known to decompose insoluble pectic constituents was used. The effect of the pectin decomposing enzyme was not very great, in fact the total nitrogen extracted was less than the normal treated samples in spite of the small amount of nitrogen added in the Pectinol preparation. Water soluble nitrogen was somewhat increased but there was a rather large decrease in alkali soluble nitrogen. Table VIII

comprise the portion of treatment with the normal procedure.

Pepsin action on the residue after the series of extractions, had very little effect on the remaining nitrogen. The small amount shown in Tables V and VI can be attributed to the nitrogen content of the pepsin used. The fact that the amount obtained was no greater indicates that the nitrogen remaining in the leaf after the extraction process must have been present in a very inert form. A hydrogen ion concentration lower than pH 5 might have increased the amount of extractable nitrogen. Lincoln and Maley (30), however, found that nearly as much soluble nitrogen was formed from pure leaf tissue with a control at pH 7 as was formed by a proteolytic enzyme at the same hydrogen ion concentration. To eliminate the effect of this pre-treatment on the subsequent extraction the enzyme digestion was carried out on the residue remaining after the regular series of extractions rather than in a manner similar to the "control" digestion.

The one series of extractions with 5% salt solution effected the removal of 1.5% of the total nitrogen. This would indicate that possibly a small portion of the leaf protein is of the globulin type.

Tables V and VI show that the methods employed in 1936 have given much more uniform results than were obtained in 1937. In general the three samples within a series show good agreement and when two series are compared for a given treatment the results appear reproducible.

CONCLUSIONS

1. A study of the extractable nitrogen fractions in the leaves of Prunus cerasus, Montmorency variety, was made in an attempt to find some method whereby the effect of three different fungicides might be determined.

2. Five spray applications of lime sulfur, wettable sulfur or Bordeaux mixture had no apparent differential effect on the total nitrogen content of the leaves.

3. The amount of soluble nitrogen obtained from extraction the leaves with water and trichloracetic acid followed by filtration thru paper pulp gave varied results. A definite trend indicated, however, that more soluble nitrogen was present in the leaves of the Bordeaux sprayed trees.

4. The successive extraction treatment using water, alcohol, acid and alkali removed somewhat more than fifty per cent of the total leaf nitrogen.

5. Use of a pectin decomposing enzyme as a pre-treatment to destroy some of the cell wall material and thus increase the extractable nitrogen resulted in a slight decrease of total nitrogen extracted.

6. Digestion of a series of extracted samples with pepsin failed to increase the amount of nitrogen obtained, indicating that the nitrogen remaining in the leaf is in a very inert form.

7. Five per cent salt solution removed a small percentage of nitrogen indicating the possible presence of the globulin type of protein in the cherry leaf.

8. Although the successive extraction procedure followed does not remove all the leaf nitrogen it would allow a comparative study of an explosive mixture on the nitrogen distribution present in fibrous vegetative samples.

BIBLIOGRAPHY

1. Addoms, R.M. Toxicity as Influenced by the Changes in the Protoplasmic Structure of Root Hairs of Plant. Am. J. Botany 14: 117 (1927)

2. Assoc. Official Agr. Chem. Methods of Analysis. Washington D.C. (1930)

3. Bancroft, W.D., and Richter, G.N. The Chemistry of Disinfection. Jour. Phys. Chem. 35: 511 (1931)

4. Bancroft, W.D., and Rutzler, F.E. Reversible Coagulation in Living Tissue. Proc. Nat. Acad. Sci. 17: 488 (1931)

5. Concquet, P.A. Nitrites and Ammonia in Diseased Plants. Jour. Am. Chem. Soc. 39: 2372 (1918)

6. Concquet, P.A. Oxidases and Diastases in Diseased Plants. Jour. Am. Chem. Soc. 39: 2003 (1917)

7. Chibnall, A.C. Protein Metabolism in the Plant. Chapters VI, VII, IX Yale University Press, New Haven, Conn. (1939)

8. _____ Distribution of Nitrogen in the Leaves of the Runner Bean. Biochem. Jour. 16: 344 (1922)

9. _____ The Effect of Low Temperature Drying on the Distribution of Nitrogen in the Leaves of the Runner Bean. Biochem. Jour. 16: 533 (1922)

10. _____ A New Method for the Extraction of Vacuole and Protoplasmic Material from Leaf Cells. Jour. Biol. Chem. 55: 33 (1923)

11. _____ Diurnal Variations in the Protein Nitrogen of Runner Bean Leaves. Biochem. Jour. 13: 387 (1934)

12. _____ The Role of Asparagine in the Metabolism of the Mature Plant. Biochem. Jour. 13: 395 (1934)

13. _____ Leaf Metabolism in Normal and Abnormal Runner Bean Plants. Biochem. Jour. 13: 405 (1934)

14. _____ Spinacin - A New Protein from Spinach Leaves. Jour. Biol. Chem. 81: 333 (1934)

15. _____ and Schryver, S.B. Isolation of Proteins from Leaves. Biochem. Jour. 15: 60 (1921)

16. Davidson, C.W. and Shive, J.L. Determination of the Nitrogenous Proteins in the Vegetative Tissue of the Beech. Plant Physiol. 10: 73 (1935)

17. Davies, W.L. The Proteins of Green Forage Plants. Jour. Agr. Sci. 15: 280 (1925)

18. Doneen, L.D. Method for Preparation of Green Plant Material for the Extraction of Juices. Plant Physiol. 9: 832 (1934)

19. Eckerson, S.H. Protein Synthesis by Plants. Botan. Gaz. 77: 377 (1924)

20. Einhof, H. Neues Allgem. J. Chem. 1:1 (1903) Taken from Osborne Jour. Biol. Chem. 4: 33 (1921)

21. Eggert, F.J. Translocation of Nitrogenous Substances in the Cuthbert Raspberry. Botan Gaz. 101: 1 (1939)

22. Foreman, F.L. Observations on the Proteins of Pasturage - Phosphorus and Protoplasm. Jour. Agr. Sci. 23: 135 (1933)

23. Fourcroy, A.F. Ann. Chim. [illegible] (17[illegible]) Taken from Osborne Jour. Biol. Chem. 4 : [illegible] (19[illegible])

24. Gortner, R.A., Hoffman, W.F. and Sinclair, W.B. Physico-Chemical Studies on Proteins. Colloid Symposium Monograph 5: 179 (1927) Chemical Catalog Co., New York.

25. Harvey, E.N. Some Observations on the Color Changes of the Diphenylamine Reaction Jour. Am. Chem. Soc. 42: 1245 (1920)

26. Heilbrunn, L.V. The Colloid Chemistry of Protoplasm Chapt. I, II, III, XII. Gebruder Bros., Berlin (1928)

27. Henricksen, H.C. Pineapple Investigations. Porto Rico Agr. Exp. Sta. Rpt. 1927

28. Hiller, A. and Van Slyke, D.D. Protein Precipitants. Jour. Biol. Chem. 53: 253 (1922)

29. Kraus, E.J. and Kraybill, H.R. Vegetation and Reproduction with Special Reference to the Tomato. Oregon Agr. Exp. Sta. Bul. 149 (1918)

30. Lincoln, F.B. and Mulay, A.S. The Extraction of Nitrogenous Materials from Pear Tissue. Plant Physiol. 4: 233 (1929)

31. Long, J.H. and Murneek, A.E. Nitrogen and Carbohydrate Content of the Strawberry Plant. Missouri Agr. Exp. Sta. Res. Bul. 252 (1937)

32. Murneek, A.E. Seasonal Cycles of Nitrogenous Material in Fruit Trees Jour. Pomology Hort. Sci. 12: 177 (1934)

33. McCalla, A.G. Effect of Nitrogen Nutrition on the Protein and Non-protein Nitrogen of Plant. Can. Jour. Res. 9: 542 (1933)

34. McKee, H.S. A Review of Recent Work on Nitrogen Metabolism of Plants. New Phytologist 36: 33 (1937)

35. Michael, G. Relation between Chlorophyll and Protein Decomposition in Yellowing Leaves of Tropaeolum. Z. Botan. 29: 385 (1935) Taken from Chem. Abs. 30: 2225 (1936)

36. Morrow, C.A. and Gortner, R.A. A Study of the Nitrogen Distribution in Different Soil Types. Soil Science 3: 297 (1917)

37. Mulay, A.S. Seasonal Changes in the Composition of the Non-protein Nitrogen in the Current Years Shoot Growth of Bartlett Pear. Plant Physiol. 7: 107 (1932)

38. Mothes, K. Ein Beitrag zur Kenntnis Des N-Stoffwechsels Hoherer Pflanzen. Planta 1: 472 (1926)

39. Nightingale, G.T., Robbins, W.R. and Schermerhorn, L.G. Freezing as a Method of Preserving Plant Tissue for the Determination of Nitrogenous Fractions. New Jersey Agr. Exp. Sta. Bul. 448 (1927)

40. Norman, A.G. Biochemistry of Cellulose, The Polyuronides, Lignin, Etc. Oxford University Press, Oxford (1937)

41. Orcutt, F.M. and Wilson, P.W. Biochemical Methods for the Study of Nitrogen Metabolism in Plants. Plant Physiol. 11: 713 (1936)

42. Osborne, T.B. and Wakeman, A.J. The Proteins of Green Leaves. Jour. Biol. Chem. 4: 1 (1908)

43. Osborne, T.B., Wakeman, A.J., and Leavenworth, C.S. The Proteins of the Alfalfa Plant. Jour. Biol. Chem. 4: 33 (1907)

44. Pearsall, W.H. and Hollingsworth, J.C. The Influence of Light upon Nitrogen Metabolism in Detached Leaves. Ann. Botany 3 n.s.: 601 (1939)

45. Pearsall, W.H. and Price, J. Relation of Nitrogen Metabolism to Plant Succulence. Ann. Botany 48: 23 (1934)

46. Robbins, W.J. The Isoelectric Point of Plant Tissue and Its Importance in Absorption and Toxicity. The University of Missouri Studies 1: 3 (1926)

47. _____ An Isoelectric Point for Plant Tissue and Its Significance. Am. J. Botany 13: 417 (1926)

48. Robinson, W.O. Protein Metabolism of the Green Plant. New Phytologist 21: 117 (1922)

49. Roulle J. Med Chir Pharm. 33: 250 (1773) Taken from Osborne Jour. Biol. Chem. 40: 33 (1951)

50. Schertz, F.M. A Theoretical and Physiological Study of Mottling of Green Leaves. Botan. Gaz. 71: 21 (1921)

51. Sessions, A.C. Fungicide Adjustment. Ind. Engineer. Chem. 21: 937 (1929)

52. Stuart, N.W. Determination of Amino Nitrogen in Plant Extracts. Plant Physiol. 10: 133 (1935)

53. Thomas, W. Nitrogenous Metabolism of *Pyrus malus*. Plant Physiol. 2: 55 (1927)

54. __________ Distribution of Nitrogen in the Insoluble Cytoplasmic Proteins. Plant Physiol. 2: 67 (1927)

55. __________ Partition of the Nitrogen in the Leaves of One and Two Year Branch Growth of *Pyrus malus*. Plant Physiol. 2: 109 (1927)

56. __________ Effect of Applications of Sodium Nitrate on the Total Nitrogen and Its Partition Products in Apple Leaves. Plant Physiol. 2: 245 (1927)

57. __________ The Seat of Formation of Amino Acids in *Pyrus malus*. Science 66: 115 (1927)

58. Nottingham, W.E., Schulze, E.R. and L[illegible]orsky, C. The Extraction of Nitrogenous Constituents from Plant Cells.

59. Van Slyke, D.D. The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino Acids. Jour. Biol. Chem. 10: 15-33 (1911)

60. Vickery, H.B. The Biochemistry of the Nitrogenous Constituents of the Green Plant. Ann. Rev. Biochem. 3: 475 (1934) Stanford University Press, Stanford University, California

61. von Euler, H. and Burstrom, D. Z. Physiol. Chem. 215: 47 (1933) Taken from Vickery Ann. Rev. Biochem. 3: 475 (1934)

30. Bloom, W. and McGilvra, G.M.A. Intracellular Action of Sulfur - Production of H_2S by Sulfured Nerves. Contrib. Mayo Thompson Inst. 3: 13 (1931)

33. Hunt, E.M. Protein Metabolism in Starving Turkey Nerves. Proc. Roy. Soc. (London) B 103: 243 (1937)

34. Young, W.J. The Toxic Property of Sulfur. Ann. Missouri Bot. Garden 1: 403 (1914)

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Davidson

Distribution of nitrogen in the leaves of certain species of the Rosaceae.