

THE NITROGEN DISTRIBUTION IN ALFALFA HAY  
CUT AT DIFFERENT STAGES OF GROWTH

by

Mills Forster Clarke

 Thesis

submitted for

the

Degree of Master of Science in Agriculture

in the

Department of Agronomy.

University of British Columbia

April, 1937.

*Approved, April 27, 1937*

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THE NITROGEN DISTRIBUTION IN ALFALFA HAY CUT AT DIFFERENT  
STAGES OF GROWTH.

INTRODUCTION.

Notwithstanding the fact that quality and feeding value of hay crops in general are dependent upon a large number of factors, each of which is important in itself, protein content is perhaps most commonly used as a measure of general quality. This is particularly true of alfalfa hay since it is notably high in protein content as compared to the hays made from other types of forage plants.

The use of protein content as the main criterion of feeding value is undoubtedly justified, since generally speaking, a high content of digestible protein is inseparably linked with the multitude of factors which collectively are responsible for high quality. Furthermore protein is required in large quantities in the rations of all classes of livestock, hence due consideration must be given to the amounts of this essential and costly nutritive constituent that may be present, when determining the value of any hay.

The expression "crude protein" as usually applied to forage plants and their cured products, includes all the nitrogenous compounds present in the material. Therefore, it offers no information concerning the amount of nitrogen actually present as protein and the amounts present in forms representing different stages of protein synthesis and degradation.

## II

Numerous experiments have shown that the cutting of hay crops at different stages of growth together with the utilization of different methods of curing and storage have produced hays that differed markedly not only in total but also in digestible protein content. The results of such experiments indicate that further information might be gained from a more detailed study of the actual nitrogen distribution in hays produced under varying conditions.

In addition the results of such a study would, in part, be indicative of the nitrogen metabolism of the plants from which the hay was made, since hay is, in reality, plant material with a portion of the water removed. However, owing to the multitude of chemical changes taking place during drying, this material is by no means fully representative of the original plant. For this reason, any deductions that might be made concerning active nitrogen metabolism would have to be interpreted in a very broad and general fashion.

## SCOPE OF THE INVESTIGATION

It had been planned to make a more or less detailed study of the nitrogen metabolism of the alfalfa plant. However, owing to a lack of facilities for studying the plants in the green state, the problem was modified to permit of a detailed chemical study of the plant material in a dried form.

The investigation has been divided into two parts. The first is a review of literature pertaining not only to the analytical methods employed in the study of nitrogen metabolism in plants and plant products, but also to procedures concerned in incomplete protein hydrolysates.

Part II of this paper is devoted to the experimental phases of the problem; it includes an outline of the field experiment together with full details concerning the analytical methods employed. The results obtained are discussed and conclusions drawn with respect to: (1) the nitrogen metabolism of the alfalfa plant at different stages of growth, (2) the relationship between nitrogen distribution and the quality of hay obtained from cuttings made at different stages of maturity, and (3) the suitability of the method of Wasteneys and Borsook for the study of the nitrogen distribution in plant material.

## PART I - REVIEW OF LITERATURE

In the following review no attempt will be made to cover completely the extensive literature dealing with nitrogen metabolism of plants. Discussion will be confined to a consideration of the analytical methods commonly employed for the determination of the various nitrogenous constituents. For reasons to be discussed shortly, this will of necessity include investigations dealing with the preparation of plant material for analysis.

### A. PREPARATION OF PLANT MATERIAL:-

The analytical difficulties encountered in attempts to study the nitrogen metabolism of plants during the course of their entire growing and fruiting periods are many and varied. Consequently the results of different workers disagree to such an extent that it is often difficult to draw significant or definite conclusions.

The findings of numerous investigators have shown very clearly that the methods employed in the preparation of plant material are responsible for tremendous variations in the analytical results obtained. Hence it is of no little importance that the material be handled in such a manner as to insure no change in the proportionate distribution and identity of the nitrogenous compounds.

Drying Material for Analysis - Prior to 1922 much of the work on the investigation of soluble and insoluble nitrogenous

constituents was carried out on dried plant material. Previously, investigators had failed to appreciate fully the effects of the methods of desiccation employed. Chibnall (1922) working with the common bean (*Phaseolis vulgaris*, L.) observed that drying at relatively low temperatures of 30° to 70° C. resulted in considerable proteolysis with consequent increase in the simpler water-soluble nitrogenous products. These were, chiefly ammonia (in the form of ammonium salts or as the amide nitrogen of asparagine) and mono-amino acids; the former being increased from 1 to 5 or 6 percent of the total leaf nitrogen, the amount of increase depending on the conditions of drying. Although the leaf proteins were found to be diminished in amount they were not appreciably changed in character.

Tottingham et al. (1924) and Link, and Schulz (1924) studied the optimum temperature at which autolytic changes in plant tissue were reduced to a minimum. They found that the greatest changes produced either by rapid drying or freezing resulted merely in the coagulation of soluble proteins. Coagulation was found to be greatest at high temperatures; while at lower temperatures (32° to 40° C.) both coagulation and proteolytic changes seemed to occur.

Greenhill (1933) investigated the effects of various drying temperatures upon the analysis of grass samples. Upon drying these in shallow trays for forty-eight hours at a temperature of 40° C., he observed losses in dry matter ranging up to 20 percent. When samples were dried 7 to 14 days, losses in dry matter as high as 12 percent were reported. At the same

time, breakdown of "true protein" ranging up to 16 percent and 28 percent respectively for the two methods was noted, while increases in the amount of pepsin hydrochloric acid soluble nitrogen up to 19 percent and 7 percent respectively occurred.

The effects of drying on the nitrogen metabolism of barley leaves was studied by Richards and Templeman (1936). Samples of fresh leaves were extracted and analyzed immediately after gathering, while duplicate samples were dried, sealed, and analyzed after a period of storage. Two types of hydrolytic changes were observed to have occurred during desiccation: (1) a certain amount of amide gave rise to free ammonia, and (2) protein was hydrolyzed yielding amino acids. As regards the first type of hydrolytic change, the sum of ammonia and amide nitrogen was apparently unaffected by drying. In fresh leaf samples ammonia was found in traces only, hence it was assumed that the ammonia in the dried samples was derived from the amide in the living leaves.

Richards and Templeman consider the hydrolysis of a certain amount of protein, yielding amino nitrogen, as being much more serious than the hydrolysis of the amide nitrogen. The relative change in protein content was found to be negligible, being of the order of 1 percent, but at the same time it resulted in the small amount of amino nitrogen originally present being increased by approximately 15 percent.

Thomas (1927) and Tottingham, Schulz and Leprovsky (1924) have demonstrated that rapid drying methods using temperatures in the neighbourhood of 65° C. combined with

effective aeration result in a very slight alteration of the material being analyzed.

Many investigations have been based on a study of fresh material due to the fact that, unless extreme care is exercised, changes in the proportionate distribution of nitrogen in its various forms will occur during the process of drying the plant tissue prior to extraction. Accordingly in the work of Chibnall (1922), Vickery and Pucher (1931), Nightingale (1927), and Murneek (1926), fresh material is used exclusively.

Extraction of Soluble Nitrogenous Constituents - The aim in extracting plant material is to obtain, in as near to the original form as possible, that portion of the nitrogenous material which is present in a metabolically active form within the plant. In the past there has been very little agreement concerning the most effective method to employ for this purpose. Numerous methods have been proposed, all of which possess certain advantages and disadvantages.

Extraction with hot alcohol in concentrations of 50 to 80 percent has been recommended in certain investigations (Tottingham et al. 1927), for the reason that it stops enzymatic action quickly, precipitates some or all of the proteins, and is a good solvent for the relatively simple nitrogenous compounds. However, the precipitation of soluble proteins by alcohol during extraction is not considered as an advantage by all investigators. Soluble proteins are usually considered as being important constituents concerned in plant metabolism and consequently methods which tend to preserve them intact are used.

Appleman and Miller (1926) found that extracts of potato tubers prepared by the use of hot alcohol yielded practically the same amounts of non-protein nitrogen as did cold water extracts.

Davidson, Clark and Shive (1934) show that hot water will destroy enzymes about as rapidly as will hot alcohol; also, hot water is a slightly better solvent for the simple nitrogenous constituents than is alcohol. By the use of hot water, cell membranes are killed quickly and thereby rendered permeable to the soluble constituents. In the case of certain nitrogen determinations that can be made only on alcohol free samples, the use of hot water further simplifies the subsequent analytical procedure.

Tottingham et al. (1924) and Stuart and Appleman (1935) in making aqueous extracts of soluble nitrogen, ground the plant tissues in a mortar with the aid of sand. The chief disadvantage of such a method lies in the fact that much labor and considerable time is required. Moreover, Robinson (1930) has shown that in the case of plants containing such unstable compounds as some of the cyanogenetic glucosides, the time required for the preparation of an extract by grinding permits of a loss of nitrogen or a change in the distribution of the nitrogenous fractions.

Vickery and Pucher (1931), and Davidson et al. (1934) have endeavoured to eliminate this alteration in nitrogen balance through shortening the period of extraction. This they claim to have effectively succeeded in doing through

boiling of the plant tissue in water for a short period followed by moderate grinding in a mortar without the aid of sand.

Orcutt and Wilson (1936), on the other hand, point out that extraction of soluble material with either hot or cold water or alcohol is objectionable because, in making a quantitative extraction, the final volume is twenty times that of the original material. Since actual amounts of the soluble nitrogen constituents in plant materials are so very low, the further dilution resulting from the previously mentioned methods of extraction increased the care required in analysis. Concentration of the aqueous extract under diminished pressure is sometimes suggested as a means of overcoming this difficulty. Aside from being extremely laborious, this operation leads to further error in that it tends to bring about decomposition such as aminolysis.

In order to eliminate errors arising from the use of solvents in the preparation of extracts of soluble constituents, numerous workers express the sap from fresh tissues by mechanical means. The addition of water is not required hence quantitative extraction is unnecessary. The analytical results are expressed as concentration in the sap, which may be converted to a dry matter basis if desired. Varied applications of the method are found in the work of Greathouse (1932), and Sayr and Morris (1931 and 1932).

The usual procedure is to express the sap through subjecting the material to a pressure of approximately 3000 pounds, following a preliminary grinding in a ball mill. It

is claimed, that when the material has been previously ground in this manner, the concentration of nitrogen does not vary throughout the extraction (Orcutt and Wilson, 1936).

#### B. ANALYTICAL METHODS:-

The methods employed in investigations relative to nitrogen metabolism in plants are based primarily on methods that have been evolved at an earlier time for the study of "pure proteins".

The study of pure proteins has been based on the assumption that the most direct way of obtaining an insight into the probable groupings which occur in the molecule of a complex substance is to break it up into simpler ones whose constitution is already known or may be determined with relative ease.

Various processes have been employed for breaking down the protein molecule, such for instance as acid hydrolysis, fusion with alkalis, the action of enzymes or putrefactive bacteria, and oxidation. The application of this method has for a number of years resulted in the accumulation of a large amount of reliable data relative to the composition of proteins both plant and animal.

Further insight into the composition of proteins is gained from a study of the distribution of nitrogen in the molecule with a view to ascertaining whether it is present in the form of mono- or di-amino acids. This separation depends on the fact that di-amino acids, by virtue of their strongly basic character, are precipitated from solution by the addition

of phosphotungstic acid, whereas mono-amino acids are not. The substance being examined is first hydrolyzed by boiling with hydrochloric acid for several hours. The amount of amide and ammonia-nitrogen is determined by distillation with magnesia in vacuo at 40° C.

The di-amino acid nitrogen is next determined by precipitating the residue in the flask with an excess of phosphotungstic acid and estimating the amount of nitrogen in the precipitate by the Kjeldahl procedure.

The nitrogen combined as mono-amino acids may be determined directly in the filtrate or by the difference between total nitrogen and the sum of the nitrogen fractions determined separately (Haas and Hill, 1912).

With regard to the study of plant proteins, the proteins of seeds alone have been thoroughly investigated. This is easily understood since the seed proteins occur in relatively large amounts and can be isolated with comparative ease.

As to the nature of the proteins and other nitrogenous compounds present in a metabolically active condition within the growing plant there is as yet very little information available. The methods to be discussed shortly are concerned with the study of this material.

Nitrogen Fractions Most Frequently Determined - The determinations made most frequently include: Total nitrogen, including nitrates, free ammonia-nitrogen, amide nitrogen, amino-acid nitrogen, and free nitrate-nitrogen. In some cases both mono- and di-amino acids, and occasionally soluble protein- and proteose-nitrogen are determined. The sum of the

various forms determined is usually subtracted from the total soluble nitrogen and the difference called "Rest", "Residual", or "other" nitrogen. The amount of this fraction varies considerably depending on the methods of analysis and as to its probable significance in the physiological processes of the plant there is little information available.

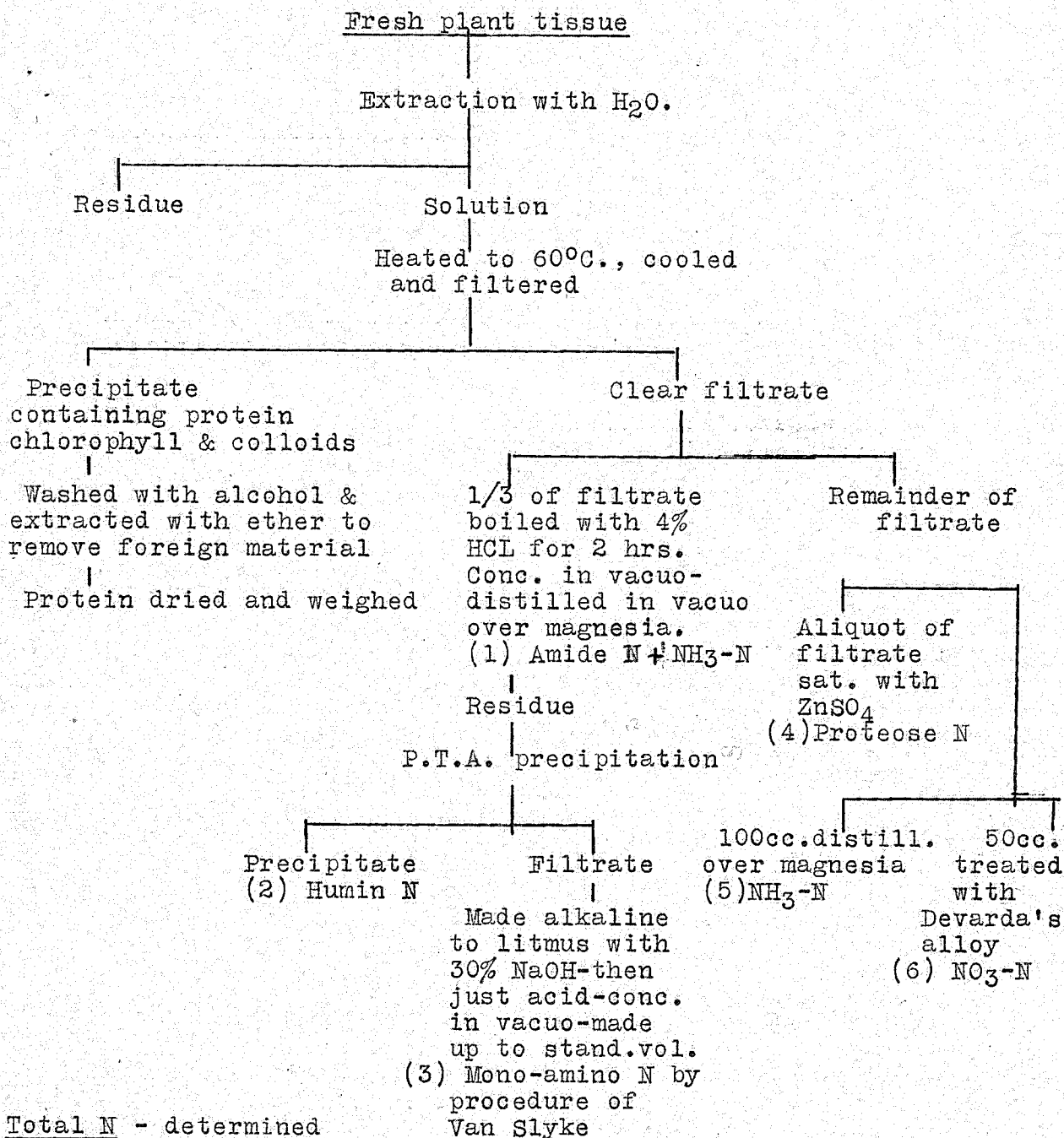
Figs. 1 to 5 inclusive illustrate graphically the procedures used by different workers in an examination of plant material.

With the exception of that of Mulay (Fig. 5) all the schemes agree quite closely with regard to the fractions determined and the order of procedure. Minor differences, however, do occur when one proceeds to the ultimate separation of the different fractions. This can be explained in part through the fact that each procedure has been used on a different type of plant material. Owing to the variable composition of different plant species a procedure that can be used effectively on all types is practically impossible of development.

Aside from the variations in procedure, explanations for which have already been offered, other differences in the methods occur. All schemes (excepting that of Mulay, Fig. 5) exhibit a certain amount of disagreement with respect to the vigor and type of hydrolysis which should be employed. Chibnall (Fig 1) hydrolyzes the water extract following the coagulation of soluble proteins with 4 percent hydrochloric acid for two hours.

Thomas (Figs. 2 and 3) proposes two schemes of analysis

Fig. 1. Scheme of analysis employed by Chibnall (1922) for studying the distribution of nitrogen in the leaves of the Runner Bean.

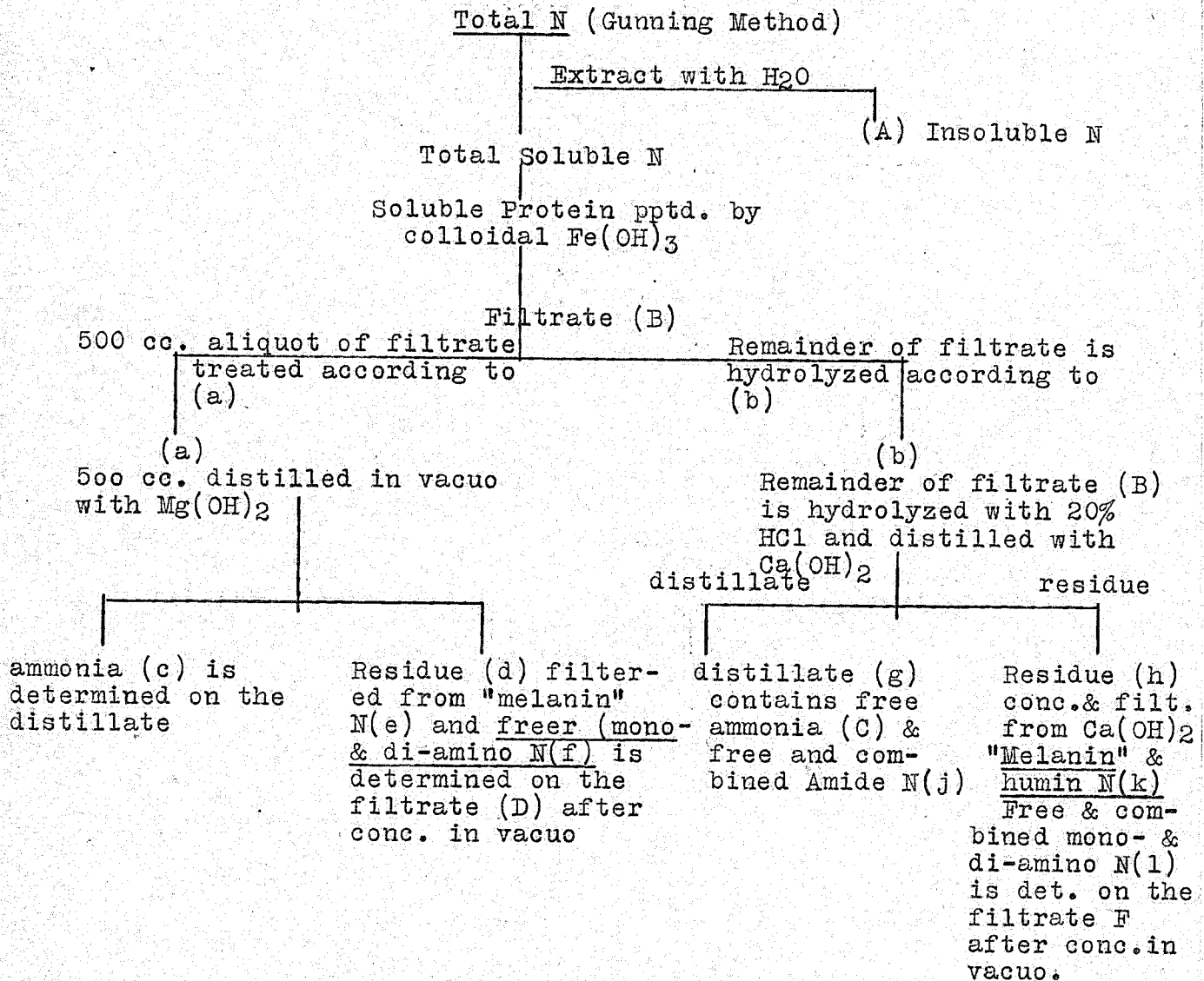


Total N - determined on air dry sample by Kjeldahl method.

Rest N. - Total N minus sum of other N determs.

Fig. 2. Scheme of analysis employed by Thomas (1927) for studying the nitrogenous metabolism of *Pyrus malus*, L.

SCHEME I



N(1) - N(f) = combined mono- and di-amino N.

Fig. 3. Scheme of analysis employed by Thomas (1927) for studying the nitrogenous of *Pyrus malus*, L.

SCHEME II

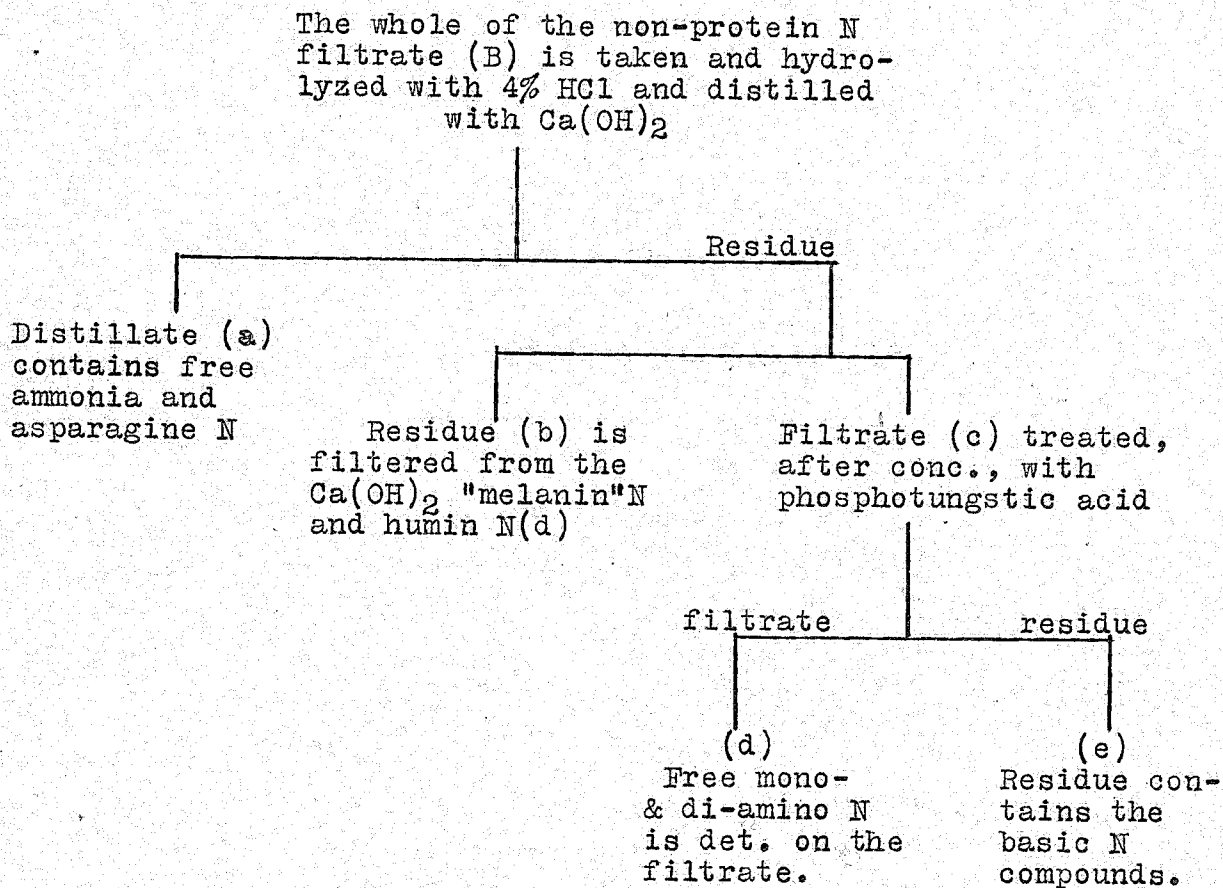


Fig. 4. Scheme of analysis proposed by Orcutt and Wilson (1936) for the study of nitrogen metabolism in the soybean plant.

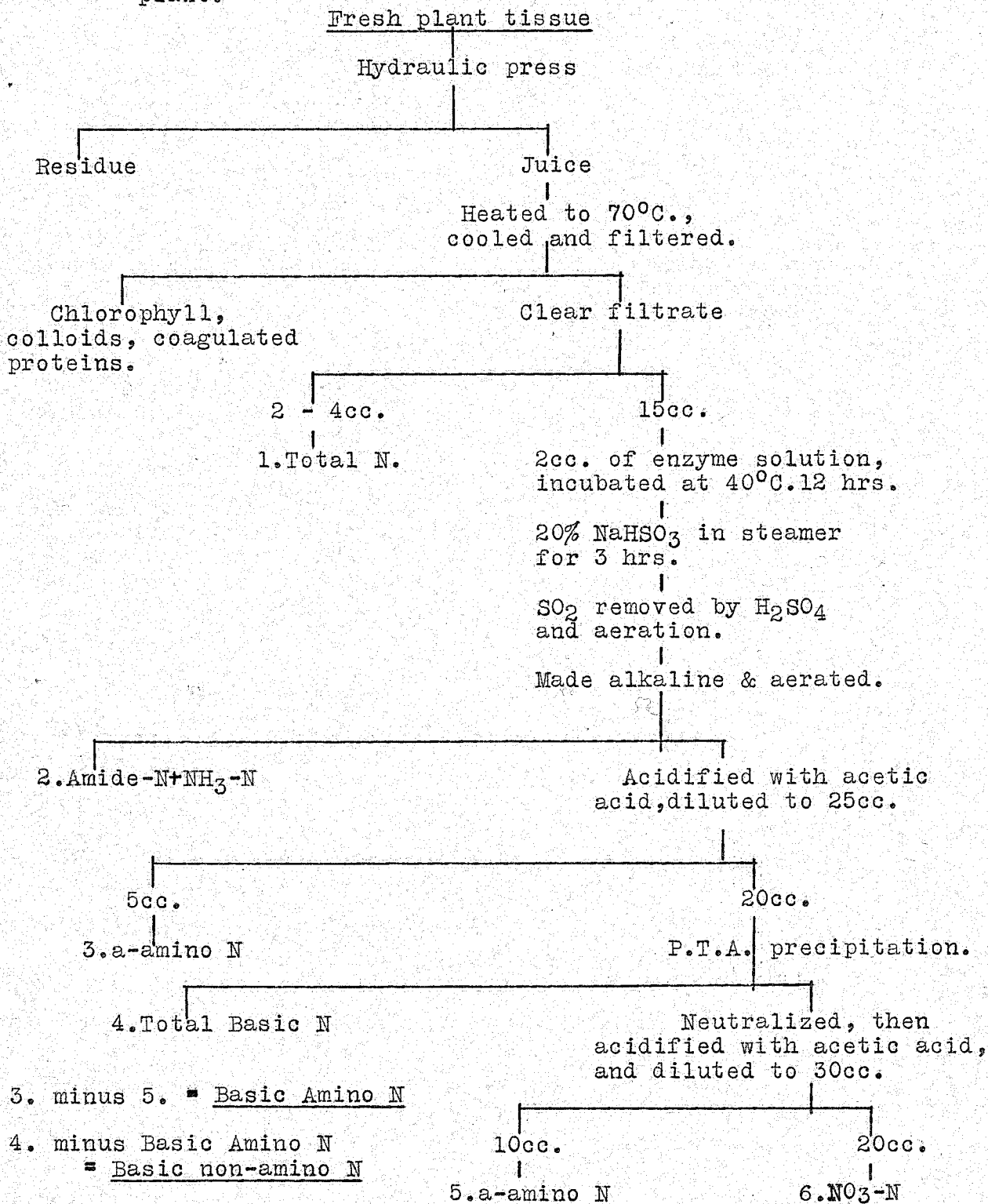
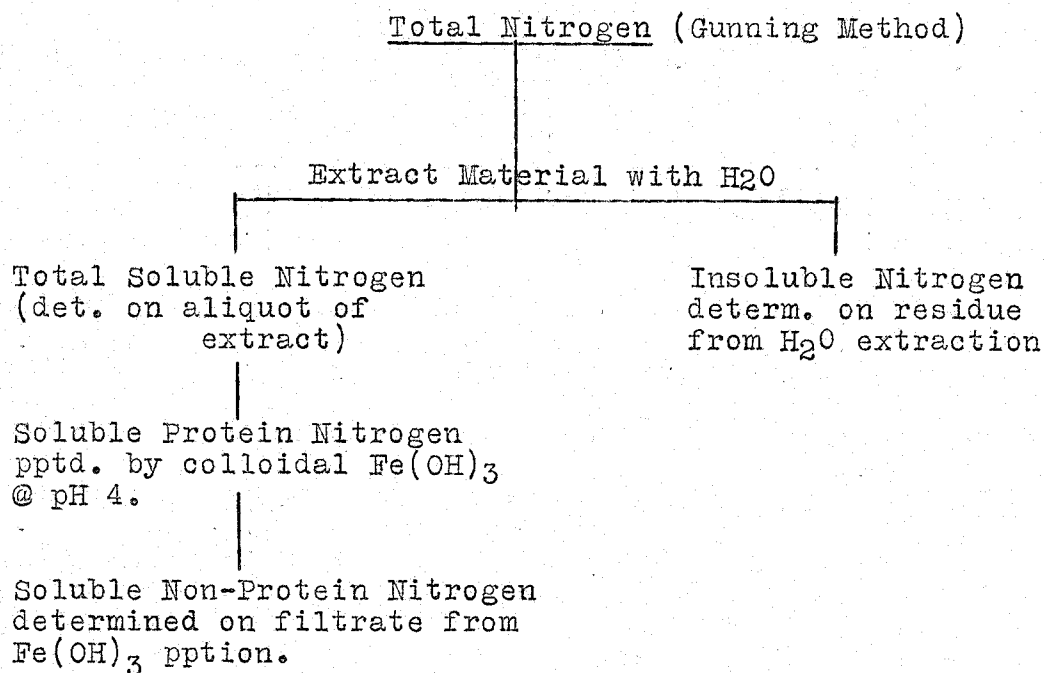


Fig. 5. Scheme of analysis employed by Mulay (1931) for the determination of total, soluble, soluble protein, non-protein, and insoluble nitrogen.



Total Sol. N - Sol. Non-Protein N  
= Sol. Protein N

Insoluble N Sol. Protein N  
= Total Protein N.

6.25 x protein N = Proteins

that differ with respect to the vigor of hydrolysis. In the first procedure (Fig. 2) a portion of the extract previously freed of proteins is hydrolyzed with 20 percent hydrochloric acid for 12 hours. In the second procedure (Fig. 3) the whole of the non-protein nitrogen filtrate is hydrolyzed with 4 percent hydrochloric acid. The difference in the vigor of hydrolysis results in a variation with respect to the distribution of the mono-amino and the di-amino acids, and the substances determined as amide nitrogen. Most of the complex peptide linkings would be decomposed by the stronger hydrolysis, hence the amide nitrogen of this procedure includes the combined as well as the free  $\text{-CO-NH}_2$  groups. The mild hydrolysis employed in scheme II (Fig. 3) gives as amide nitrogen only asparagine and other similarly constituted acid amides, i.e., those having the  $\text{-CO-NH}_2$  groups free. Furthermore in scheme I (Fig. 2) the free amino (both mono- and di-) and also the combined amino nitrogen (both mono- and di-) are determined separately, whereas in scheme II (Fig. 3) compounds containing the combined di-amino nitrogen groups are precipitated with the bases. This latter method will, therefore, give only the free mono-amino nitrogen.

The procedure of Thomas (Figs. 2 and 3) includes also the determination of two not very clearly defined nitrogenous compounds, viz., the so-called "melanin" and "humin" fractions. The term "melanin" is used to denote the nitrogen belonging to the acid pigment-like bodies found in the sap of plants. This fraction is precipitated by calcium hydroxide or magnesium

hydroxide under the conditions defined in the procedure. The nature of the "humins" nitrogen will be discussed later when more detailed consideration is given to the methods of hydrolysis employed by various investigators.

The procedure of Orcutt and Wilson (Fig. 4), although a much more recent development, does not differ fundamentally from the methods of Chibnall and Thomas. The chief difference lies in the fact that hydrolysis is conducted in two stages. The first stage of hydrolysis is accomplished by incubating the material at 40° C. following the addition of a measured amount of enzyme solution, while the second stage is attained through the aid of sodium bisulphite.

The scheme of analysis employed by Mulay (Fig. 5) is not as comprehensive as those discussed above. No attempt, for instance, is made to determine the exact nature of the compounds contained in the water soluble material. This method has been devised, with a view to determining the relationship existing between total soluble, soluble protein, non-protein and insoluble nitrogen within the plant during its growing period.

Total Nitrogen - The total nitrogen content of dried plant material is determined by means of the Kjeldahl method. The chief source of error in this determination arises through the presence of nitrogen in the form of nitrates. This error is usually overcome by fixing the nitrates in combination with an aromatic compound such as salicylic acid. Most widely used of salicylic acid modifications is that proposed by the Association of Official Agricultural Chemists (1925).

Total Nitrogen of Extracts - Very little difficulty is encountered in the determination of total soluble nitrogen when nitrates are absent; the standard Kjeldahl method being quite satisfactory in every respect.

The presence of nitrates in the soluble material, however, gives rise to considerable difficulty. Owing to the presence of large amounts of water in plant extracts, the usual salicylic-thiosulphate method fails to give accurate or consistent results unless certain precautions are taken.

In order to obviate this difficulty Ranker (1925) recommends the evaporation of an exactly neutral portion of the extract to dryness in the Kjeldahl flask prior to making the determination of total nitrogen.

Pucher, Leavenworth and Vickery (1930) in turn have proposed an adaptation of the reduced iron method for the determination of nitrates in order to eliminate the inconvenience involved in evaporating the extract to dryness.

Soluble Protein Nitrogen - The importance to be attached to the determination of soluble protein nitrogen varies considerably in the opinion of different investigators.

Spoehr (1923), for instance does not consider the removal of soluble proteins as being a necessary part of the analytical procedure. Orcutt and Wilson (1936) remove soluble proteins along with chlorophyll and colloidal material by heating the expressed sap of the plant to 70° C. Proteins and other colloids settle out of solution as a coagulum which is readily filtered out upon cooling. The amount of nitrogen removed from the plant tissue by this procedure is not deter-

mined.

Chibnall (1922), on the other hand, determines the soluble proteins of plant extracts gravimetrically. Proteins are removed by warming the extract to 60° C., cooling, and filtering off the coagulum which is then washed with alcohol followed by prolonged extraction with ether. The protein is then dried and weighed.

Reagents for the Removal of Soluble Proteins - The reagents most commonly employed as protein precipitants are, copper hydroxide (Stutzer's reagent), mercuric chloride, colloidal ferric hydroxide, and many acids among which the following are most important, acetic, trichloroacetic, phosphotungstic, tannic, picric, and metaphosphoric acids. The value of any one reagent is determined by its ability to effect a complete separation of the proteins with the removal of as little as possible of the intermediate products of protein degradation. There is, however, considerable difference of opinion as to the most effective reagent for this separation. Thomas (1927) is one of the few workers who maintain that it is extremely doubtful if any one reagent can successfully accomplish the complete exclusion of all products, which are only a little below the original proteins in their complexity. At any rate, the evidence tends to point to the conclusion that a reagent satisfactory for one kind of biological material may be unsuitable for another.

Thomas (1927) strongly advocates the use of colloidal ferric hydroxide as a means of separating proteins from solution. He claims the following advantages for this method:-

(1) it is more convenient and expeditious; (2) it permits all the amino acids to go through, occluding and precipitating none; (3) it effects a sharp separation of true protein from its decomposition products; and (4) the adsorbed proteins can be recovered quantitatively from the residuum.

Hart and Bentley (1915) have shown that Stutzer's reagent may not, in all cases, effect a complete separation of all protein and polypeptide structures from amines and amides. There being a tendency for copper salts under certain conditions to form copper compounds with certain amino acids.

Hiller and Van Slyke (1922) made an extensive study of a number of protein precipitants. Working with Witte's peptone, they found that trichloroacetic acid in solutions more dilute than 5 percent was quite effective as a precipitant for soluble proteins. It was claimed also that trichloroacetic acid permitted all the intermediary products of protein degradation to pass into solution. Tungstic and picric acids were observed to be quite effective as protein precipitants but in addition these reagents had a tendency to remove the intermediate nitrogenous compounds rather completely. Alcohol was found to behave toward Witte's peptone in a fashion similar to tungstic and picric acids. However, it was pointed out that such precipitates were inextricably contaminated with other non-precipitable contents of the solution (e.g., adsorption of free amino acids). Metaphosphoric acid, colloidal iron, and mercuric chloride were intermediate between trichloroacetic and tungstic acids in the completeness with which they precipitated the intermediate products of protein decomposition.

Trichloroacetic acid has been used quite successfully as a precipitant of soluble proteins by Wasteneys and Borsook (1924). The concentration of the reagent used was such that it did not exceed 2 percent of the final concentration of the solution being analyzed.

Proteoses and Simpler Peptides - Plant chemists as a rule have not attached any particular importance to the presence of proteoses and peptones. They have, almost without exception, considered it unnecessary to distinguish these fractions from the soluble proteins.

It is recommended by some workers that the proteoses and simpler peptides be precipitated in common with protein by means of tungstic acid. In blood analysis, Folin and Wu (1919) preferred this reagent to trichloroacetic acid. Rumsey (1923) used sodium tungstate effectively in the removal of proteins and proteoses from flour extracts when the pH was reduced to 2.0 or less. Merrill (1924) found a somewhat higher optimal pH was possible when the above method was employed on bacteriological sera.

Quantitative removal of proteoses was accomplished by Chibnall (1922) following saturation of the protein free plant extract with zinc sulphate. Wasteneys and Borsook (1924), on the other hand, determined proteose nitrogen by difference following saturation with sodium sulphate under rigidly defined conditions.

Amide Nitrogen - This fraction is estimated in terms of the ammonia freed upon the hydrolysis of an aliquot of the plant extract.

The procedure now generally followed (Tottingham et al. 1935), is to hydrolyze with 10 percent sulphuric acid for  $2\frac{1}{2}$  hours rather than with 6 percent hydrochloric acid as used formerly. This concentration of sulphuric acid furnishes approximately the same degree of acidity as 6 percent hydrochloric acid and possesses in addition the advantage of not reducing nitrates. Following hydrolysis the solution is neutralized with an excess of sodium carbonate and the ammonia distilled over. The amount of amide nitrogen is found by subtracting from the total amount of ammonia obtained, the free ammonia nitrogen determined on a corresponding aliquot.

An appreciable source of error encountered in acid hydrolysis is due to humin formation resulting from the condensation of tryptophane, ammonia, and other amino acids, with aldehydic compounds such as glucose. It has been observed that humin production in certain kinds of plant material is not of sufficient magnitude to introduce an appreciable error.

Orcutt and Wilson (1936) found that hydrolysis of soybean juice gave rise to 10 to 15 percent humin. In order to reduce the amount of humin formed they undertook a study of various hydrolytic agents such as sodium bisulphite, zinc amalgam, hydriodic acid, and thio-glycolic acid.

Hydrolysis with 20 percent sodium bisulphite for 3 hours gave a successful hydrolysis of amide nitrogen with only negligible humin formation. However, they observed that the bisulphite had no hydrolyzing effect whatever on peptide linkages.

This difficulty was overcome by conducting a preliminary

hydrolysis with the aid of an enzyme solution which was followed by the bisulphite hydrolysis. This procedure was found to be effective in decomposing peptide linkages completely with the additional advantage of not resulting in humin formation.

Amino Nitrogen - is determined by means of the Van Slyke amino apparatus. Complete descriptions of the apparatus, the technique involved, and the tables necessary for calculating the data are to be found in the standard texts of Physiological Chemistry such as that of Hawk and Bergeim.

If free ammonia is present in the solution in appreciable amounts it must be removed before the determination of amino nitrogen is made.

Stuart (1935) has reported that certain other substances, among which the polyhydric phenols are important, interfere with the Van Slyke determination by yielding excessive amounts of gas which are measured as nitrogen.

Tottingham et al. (1935) recommend distillation at 45° C. with a slight excess of calcium oxide with a view to removing or denaturing excess ammonia and polyhydric phenols.

Ammonia Nitrogen - The amounts of free ammonia occurring in plant extracts are usually very small. The determination is most frequently made to serve as a blank for the amide nitrogen determination.

The method developed by Van Slyke (1911) which involves the distillation of the ammonia in vacuo with alcohol and calcium hydroxide is most frequently used for measuring the amount of nitrogen in this fraction.

Tottingham et al. (1935) point out that the chief source of error arises from the fact that some plants contain appreciable quantities of other volatile bases which are carried over with the ammonia in the usual methods of aeration or distillation.

Nitrate Nitrogen - The methods generally employed for the quantitative estimation of nitrate nitrogen are of three types:-

- (1) Reduction in alkaline solution with Devarda's alloy and determination as ammonia.
- (2) Reduction in acid solution with iron and determination as ammonia.
- (3) Colorimetric methods.

Various modifications of the Devarda's alloy method are to be found in the work of Chibnall (1922), Strowd (1920), and Sessions and Shive (1920).

Vickery and Pucher (1929) have described a procedure for the determination of nitrate nitrogen in tobacco by the reduced iron method.

The phenol disulphonic acid method is the most popular of the colorimetric methods for nitrate determinations. Emmert (1929) has developed a simplified method for use with tomatoe and lettuce while Holtz and Larsen (1929) have proposed a modification for the determination of nitrates in wheat. It might be said at this point that a method which is considered to be applicable to extracts of all types of plants has been developed by Frear (1930).

Basic Nitrogen - This fraction refers to that portion

of the nitrogen which occurs in the form of simpler nitrogenous bases. The exact nature of such bases and the forms in which they are present are not as yet fully understood. No satisfactory method has been devised, as yet, for studying them. It is highly probable that these substances possess considerable physiological significance in the processes of plant metabolism.

The most widely used method for studying basic nitrogen has been by phosphotungstic acid precipitation as originally devised by Osborne and Harris (1903).

Vickery (1927) undertook a comprehensive study of the nitrogen bases of plants. He found, that with plant extracts, the use of phosphotungstic acid gave a very uncertain measure of true basic nitrogen, whereas when used in mixtures of amino acids obtained from the hydrolysis of proteins, it is fairly definite in its action.

### C. DISCUSSION AND CONCLUSIONS:-

It would appear from the foregoing discussion that, while the general method of approach to the study of nitrogen metabolism in plants is the same in practically all investigations; the details of analytical procedure, as adopted by different workers, vary to a remarkable degree. Consequently the results obtained in these investigations are such that it is exceedingly difficult to draw definite or significant conclusions.

The lack of agreement among workers generally with regard to the details of analytical procedure has been due

very largely to the extreme variability in composition and physiological makeup of the plant species each has been working with.

Because of the unstable nature of physiologically active nitrogen compounds and their complex interrelationships within the plant, attempts to use the methods of pure chemistry in their study give rise to considerable difficulty. Therefore, it is probably safe to say that a stereotyped method applicable to all types of plant material is impossible of development.

In nitrogen studies particular emphasis has been placed upon the identification of the type of nitrogenous constituents present. The amounts and particular kinds of amino acids present in the soluble nitrogenous compounds have been determined following the hydrolysis of the material. Comparatively little attention has been devoted to a study of the degree to which these amino acids and amides are condensed. In other words, little importance has been attached to the determination of soluble proteins and compounds such as proteoses and peptones which are only slightly below "true proteins" in complexity. From the standpoint of the active metabolism of the plant the degree to which the simple nitrogenous compounds are condensed is probably of equal, or perhaps even greater importance than the free amino acids.

It is reasonable to expect that the determination of "combined" amino and amide nitrogen will not give a truly representative picture of conditions actually existing within

the plant at any given time. Protein synthesis or breakdown occurs in numerous stages giving rise to compounds of varying complexity. Consequently the breaking down of soluble nitrogen constituents into the simpler amino acids will not give an accurate picture of protein synthesis and degradation.

It may be said, therefore, that the usual methods of studying nitrogen metabolism in plants do not permit of the determination of the nitrogen compounds as they actually exist within the plant at the time of sampling. An extract of the soluble nitrogenous constituents of a plant is, in all probability, representative of an incomplete protein hydrolysate. Consequently methods should be used which permit of studying this material without further modification by hydrolysis.

In doing so more attention should be given to the estimation of the fractions such as proteose, peptone, and sub-peptone which are only slightly less complex than the original proteins. A study of this nature conducted throughout the growing and fruiting periods of the plant would give a clearly defined impression of the relative rates of protein degradation and synthesis at different stages in the plant's life history. This information together with the amounts of the relatively simple forms of nitrogen existing in a free state should give a truer picture of the actual nitrogen "setup" within the plant.

#### D. THE METHOD OF WASTENEYS AND BORSOOK:-

In view of the fact that the methods of analysis usually

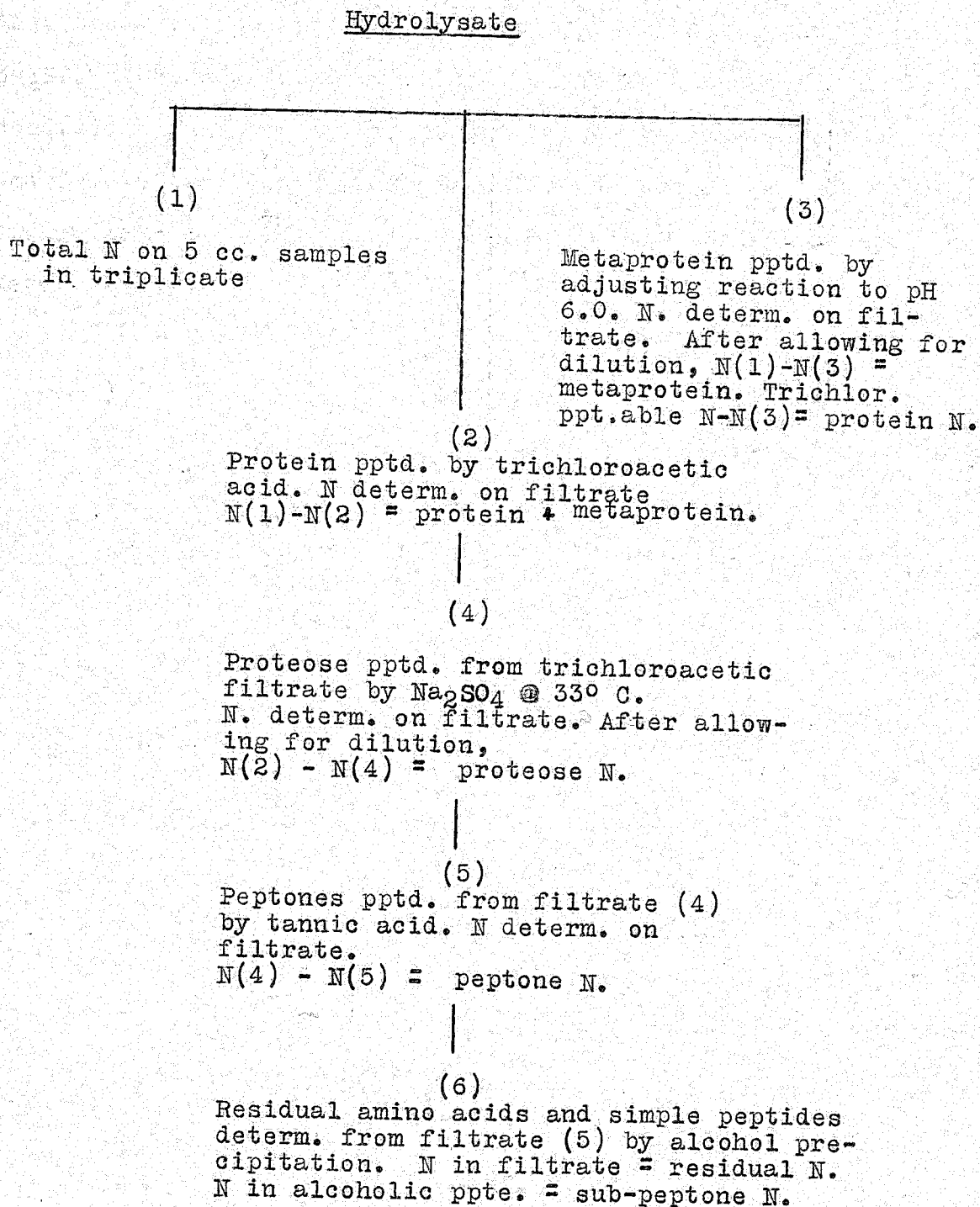
employed do not permit of determining the true relationships existing between the various soluble nitrogenous compounds within the plant, the method of Wasteneys and Borsook (1924) is suggested as a possible means of partially overcoming this difficulty.

Developed primarily for the fractional analysis of incomplete protein hydrolysates, the method of Wasteneys and Borsook has given consistent and accurate results when used by different workers for a number of years. Proof of the value of the method is evidenced by its ever increasing application in the study of the specific changes occurring during cheese ripening (Eagles and Sadler, 1932), (Sherwood and Whitehead, 1934) and (Sherwood, 1935).

Wasteneys and Borsook (1924) state that, "the constituents of an enzymatic hydrolysate of protein can be divided according to their complexity into six fractions; protein, metaprotein, proteose, peptone, sub-peptones, and amino acids". In order to secure more definite information regarding the changes occurring during hydrolysis than is obtained by the usual free amino determinations, they have devised a method for the quantitative estimation of the above fractions.

These workers introduced no new principles but they did so specify the conditions of procedure that quantitative estimation of the above named constituents was possible. The procedure, as originally developed, is represented diagrammatically in Fig. 6. A full description of the method as revised to meet the requirements of the investigation reported herein

Fig. 6. Procedure devised by Wasteneys and Borsook for the fractional analysis of incomplete protein hydrolysates.



will be given later.

Briefly, the procedure involves the precipitation of protein by trichloroacetic acid, of metaprotein by careful adjustment of the reaction, of proteoses by saturation with  $\text{Na}_2\text{SO}_4$  at  $33^\circ \text{C.}$ , of peptones by tannic acid under fixed conditions; and the determination of the residual amino acids and simple peptides by a modification of the alcohol precipitation methods of Folin and Denis (1912) and Van Slyke and Meyer (1912).

## PART II - EXPERIMENTAL

### A. OUTLINE OF FIELD EXPERIMENT:-

The alfalfa used in this investigation was a strain of Ontario Variegated designated as Ottawa, No. 176. The material was grown in duplicate rod rows in the forage crop nursery at the Dominion Experimental Farm Agassiz from seed supplied by the Division of Forage Plants, Ottawa.

The seed was sown in the spring of 1933. Three cuttings of hay were taken from the rows during the two succeeding seasons of 1934 and 1935 and the 1936 material was used for the study reported herein.

Previous to being laid down as a forage crop nursery, the area in which the alfalfa was grown had been in hoed crop for one year. The soil is a well drained, very light sandy loam. Relatively heavy dressings of well rotted barnyard manure were applied to the nursery field in the spring of each year.

Stages of Growth at which Samples were taken - Cuttings of the alfalfa were made at the following stages of growth:-

- (1) Seedling stage
- (2) Pre-bud stage
- (3) Bud Stage
- (4) Tenth-bloom
- (5) Full bloom
- (6) Maturity

In order to obtain material for the seedling stage additional seed was procured. This was sown July 8, 1936 in

quadruplicate rod rows in order to obtain a quantity sufficient for analysis. The sample was cut six weeks after planting. The plants were approximately six inches high and nodulation of the roots had not taken place.

The pre-bud samples were cut at the stage just prior to the formation of flower buds. In order that all samples be cut at a comparable stage of maturity, sampling was carried out at monthly intervals.

Bud stage samples were cut when the flower buds had definitely formed but none had started to open. Owing to the relatively long period during which buds are present before blooming actually starts, this stage is rather difficult to define. Cuttings were made at as nearly the same stage of development as observation would permit.

The tenth-bloom series were cut when approximately one tenth of the flowers were opened out. This stage was comparable to that at which alfalfa hay is usually cut in normal farm practice.

Full bloom samples were taken when no unopened flower buds remained and the earliest flowers were just commencing to wither.

The mature sample was not cut until the seed had ripened. By this time the plants were exceedingly coarse and woody in texture. Also considerable leaf dropping had taken place.

Total Rainfall Between Cuttings - The total amount of rainfall between cuttings at all stages of growth are given

below in Table I. With respect to the first cuttings at each stage of growth it is to be noted that the rainfall figure includes all the precipitation from the first of April, 1936 till the date of sampling. For subsequent cuttings the rainfall is recorded from the time of the preceding cutting. In the case of the seedling stage the rainfall is that which occurred from the date of seeding (July 8) until the time of sampling.

TABLE I. - Rainfall between cuttings.

Stage of Growth	Date of Cutting	Rainfall in inches
Seedling	Aug. 19, 1936	1.42 ‡
Pre-bud	June 6, 1936 July 7, 1936 Aug. 5, 1936 Sept. 9, 1936	10.10 * 4.70 1.28 2.51
Bud	June 11, 1936 July 17, 1936 Aug. 19, 1936	11.18 * 4.90 0.14
Tenth-bloom	June 13, 1936 July 30, 1936 Aug. 27, 1936	11.31 * 4.77 0.50
Full Bloom	June 21, 1936 Aug. 5, 1936	13.22 * 2.86
Maturity	Sept. 9, 1936	18.59 *

‡ From July 8th to August 19, 1936.

\* Total rainfall from April 1st to time of cutting.

Method of Sampling - The two-row plots of alfalfa were divided in such a manner that approximately six feet was allotted to each treatment and each section within the rows was cut only at the stage of growth denoted by its respective label. Consequently samples of any particular stage of maturity taken later in the season were from the same plants as the earlier cuttings of that stage.

Immediately after cutting the samples were transferred to special drying trays and dried indoors. This practice enabled drying to take place at a relatively rapid rate without any appreciable loss in colour or wastage of leaves. The resultant product was a hay of slightly better physical quality than alfalfa hay made under the most ideal field conditions.

Following drying all samples were packed in dustproof cardboard cartons and stored in a dry room.

#### B. ANALYTICAL METHODS:-

Preparation of Material for Analysis - Prior to grinding the samples were subjected to additional drying in an electric oven at a temperature of 45° C. for a period of twenty-four hours. This procedure which reduced the moisture content of the material to approximately 3 percent was found to be necessary in order that all the sample might be ground to a uniform degree of fineness. Grinding was carried out with the aid of a small hand-operated corn mill. The grinding surfaces were set as closely as possible and the resulting material was of approximately the same degree of fineness as

the ground alfalfa meal of commerce. Both leaves and stems were ground together and the product thoroughly mixed to insure a uniform sample. These samples were preserved in tightly stoppered bottles.

Dry Matter - This determination was carried out in triplicate. Aliquots of approximately 0.5 gm. were placed in tared aluminum crucibles, weighed, and dried to constant weight in a water-jacketed oven at a temperature of 98° C.

Total Nitrogen - Quadruplicate one gram samples were analyzed by the Gunning modification of the Kjeldahl method. Distillation was made into 0.07143N hydrochloric acid and the excess acid was titrated with 0.07143N sodium hydroxide using Methyl Red as the indicator. Since careful qualitative tests with acidified diphenylamine reagent failed to reveal any traces of nitrates in the dried material, the determinations of total nitrogen were accordingly not modified to include them.

Extraction of Soluble Constituents - Duplicate consecutive extractions were made on each sample, according to the method of Davidson, Clark and Shive (1934).

50 gms. of the finely ground tissue were placed in a 600 cc. beaker, covered with 400 cc. of boiling distilled water, and heated on a boiling water bath for 20 minutes. The contents of the beaker were then decanted on to an 18-inch square of cotton towelling suspended over a two-litre beaker and expressed by hand. The residue was then transferred to a large mortar and the coarser particles ground to a fine pulp with

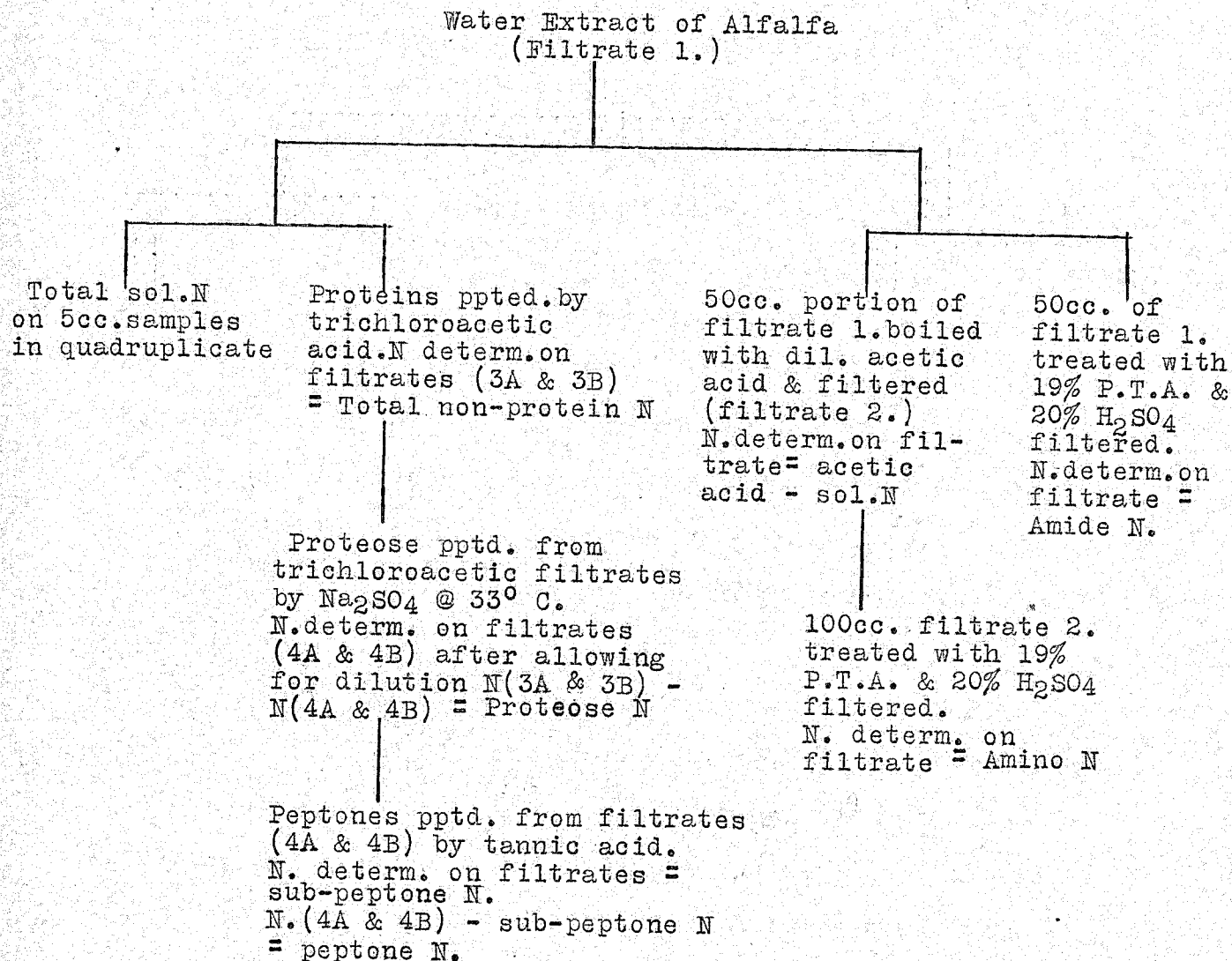
the gradual addition of 200 cc. of boiling water containing 100 cc. of 1 percent phenol. After grinding, the sample was returned to the cloth and again expressed by hand. Following this extraction, the material received a further brief grinding in the mortar with 200 cc. of boiling distilled water.

It was then transferred back to the cloth where wringing and washing were continued until the total amount of extract obtained comprised a volume of 1000 cc. The extract was then filtered through nitrogen-free filter pulp in a Buchner funnel to remove cellular material. A clear reddish brown filtrate free of solid material was thus obtained (filtrate 1.)

Fractionation of Soluble Nitrogen - The procedure used was that of Wasteneys and Borsook as modified by Eagles and Sadler (1932) in the application of the method to a study of nitrogen distribution during cheese ripening. The modification as used by them involved a difference in the size of aliquots and in the actual amounts of the reagents used for each determination. There is too, a slight difference in the conditions under which proteose is determined. Metaprotein was not determined in the method of Eagles and Sadler, nor was any distinction made between sub-peptone and residual amino acids.

The scheme of analysis followed is, for clarity, represented diagrammatically in Fig. 7. It is to be noted that this scheme includes the determination of amino and amide nitrogen. The methods used in their removal are those employed by Eagles and Sadler (1932). No provision, it will be

Fig. 7. Scheme of analysis adopted in this investigation.



Total sol. N - Total Non-protein N  
= Soluble protein N.

recalled, was made for these determinations in the original procedure of Wasteneys and Borsook.

Total Soluble Nitrogen - Quadruplicate 5 cc. portions of the water extract (filtrate 1) were analyzed for total nitrogen by the Kjeldahl method.

Semi-quantitative tests with acidified diphenylamine reagent failed to reveal even traces of nitrate nitrogen in the extracts of any of the samples. It might be added that the nitrate modification of the Kjeldahl method, as proposed by Ranker (1925), was tried but the results obtained were no higher than those from the standard Kjeldahl procedure.

Soluble Protein Nitrogen - This fraction was obtained by difference following the removal of proteins with trichloroacetic acid.

To 150 cc. of the water extract (filtrate 1) were added 30 cc. of 20 percent trichloroacetic acid and the mixture allowed to stand for 1 hour. The solution was then filtered (filtrate 3). The filtrate was divided into two 70 cc. portions (filtrate 3A and filtrate 3B) which were placed in a slowly boiling water bath for 3 hours to decompose the trichloroacetic acid, and to drive off the resulting carbon dioxide and chloroform. Each flask was then cooled to room temperature, filtered and made up to the original 70 cc. volume. Two 5 cc. portions of each filtrate, filtrate 3A and 3B, were taken for the nitrogen determination (total non-protein nitrogen). In this way results in duplicate of duplicate portions were obtained.

Soluble protein nitrogen (trichloroacetic acid pre-

cipitate) was calculated by subtracting the total non-protein nitrogen from the total soluble nitrogen after making corrections for dilution.

Proteose Nitrogen - To the remainder of filtrates 3A and 3B respectively (approximately 60cc.) 22 gm. of anhydrous sodium sulphate were added. The mixtures of salt and solution were held at 35° C. for 1 hour and then filtered into 25 cc. volumetric flasks through a water-jacketed filter maintained at 33° C. (filtrates 4A and 4B). These were at once washed quantitatively into 50 cc. volumetric flasks and made up to the mark. Two 10 cc. portions of each filtrate, filtrate 4A and filtrate 4B, were taken for nitrogen determination.

The difference between these determinations, after allowing for dilution and volume change due to heating, and the nitrogen results of filtrates 3A and 3B respectively is the proteose nitrogen.

In calculating the results of the proteose nitrogen the volume increase due to the heating of the solution to 33° C. must not be overlooked. Correction for this volume change is made by comparing the volume of water at 20° C. with that at 33° C. when saturated with sodium sulphate. Eagles and Sadler (1932) found that 22.5 cc. of water at 20° C. when saturated with sodium sulphate at 33° C., occupied a volume of 25 cc. Therefore, the factor for volume increase due to heating is  $25/22.5$ .

Peptone and Sub-peptone Nitrogen - 25 cc. of each of the filtrates 4A and 4B were pipetted into 250 cc. Erlenmeyer

flasks; to each were added 25 cc. of 2.21 normal sodium hydroxide, and 125 cc. of 20 percent tannic acid dissolved in 0.1 normal sulphuric acid containing 20 percent sodium sulphate. The mixtures were thoroughly shaken and allowed to stand at 20° C. for 3 hours and then filtered. Two 50 cc. portions of each of these tannic filtrates were taken for nitrogen determination.

The nitrogen in the tannic acid filtrate is sub-peptone nitrogen .

The difference between the sub-peptone determinations and the total nitrogen determinations on filtrates 4A and 4B respectively is peptone nitrogen (tannic acid precipitate).

Acetic Acid-Soluble Nitrogen - To 50 cc. of the water extract (filtrate 1) were added 15 cc. of 1 percent acetic acid and 100 cc. of water. The solution was heated in a boiling water bath for 30 minutes, cooled, made up to a volume of 250 cc. and filtered (filtrate 2). Two 50 cc. portions of this filtrate were taken for the determination of acetic acid-soluble nitrogen.

Amino Nitrogen - To 100 cc. of filtrate 2 (acetic acid filtrate) were added 30 cc. of 25 percent sulphuric acid (one volume of concentrated sulphuric acid, four volumes water), 10 cc. water, and 10 cc. of 19 percent phosphotungstic acid. After standing 24 hours the precipitate was filtered, and two 50 cc. portions of the filtrate were taken for the determination of amino nitrogen (phosphotungstic filtrate).

Amide Nitrogen - To 50 cc. of the original water extract (filtrate 1), 30 cc. of 25 percent sulphuric acid,

10 cc. of water, and 10cc. of 19 percent phosphotungstic acid were added. After standing 24 hours the solution was filtered. Four 10 cc. aliquots of the filtrate were taken for the estimation of amide nitrogen (phosphotungstic filtrate) by the Kjeldahl method.

Metaprotein Nitrogen - In the original procedure of Wasteneys and Borsook (1924) this fraction was precipitated by adjusting an aliquot of the original solution to pH 6.0 the point of greatest insolubility of albumen acid metaprotein.

Owing to the fact that the pH of the alfalfa extracts was, on the average, around pH 6.0 metaprotein could not be determined in this way.

Several attempts were made to remove the metaproteins with an iso-electric point of pH 5.4 (Cole, 1933) by adjusting the reaction of the extract to this pH. No results were obtained however.

### C. EXPERIMENTAL RESULTS:-

The nitrogen fractionation results are presented in tables II to VII inclusive. In table II the results obtained from the application of the method of Wasteneys and Borsook to the study of nitrogen distribution in alfalfa are expressed in percent of the absolute dry weight of the material. Table III shows the distribution of acetic acid soluble nitrogen, amino nitrogen, and amide nitrogen in percent. of absolute dry weight. Tables IV and V give the corresponding results of tables II and III in percent of the total nitrogen of each respective sample at the stages of growth defined. Tables VI

TABLE II. Nitrogen Distribution in Alfalfa Hay cut at Different Stages of Growth.

Stage of Growth	Date of Cutting	Total N	Total Water Soluble N	Insoluble Protein N	Soluble Protein N	Total Non-Protein N	Proteose N	Peptone N	Sub-Peptone N
Seedling	Aug. 19, 1936	4.530	1.867	2.663	0.404	1.463	0.148	0.152	1.163
Pre-bud	June 6, 1936	2.885	1.275	1.610	0.219	1.057	0.085	0.108	0.863
	July 7, 1936	4.333	1.923	2.410	0.572	1.351	0.133	0.191	1.027
	Aug. 5, 1936	3.815	1.201	2.615	0.205	0.994	0.099	0.095	0.800
	Sept. 9, 1936	3.152	1.292	1.861	0.267	1.025	0.128	0.108	0.789
Bud	June 11, 1936	2.375	1.060	1.315	0.349	0.711	0.124	0.131	0.456
	July 17, 1936	3.844	1.418	2.426	0.241	1.177	0.128	0.305	0.743
	Aug. 19, 1936	3.665	1.103	2.562	0.377	0.725	0.099	0.069	0.557
1/10 Bloom	June 13, 1936	2.777	1.463	1.314	0.279	1.184	0.141	0.271	0.772
	July 30, 1936	2.861	1.014	1.747	0.089	0.925	0.122	0.122	0.681
	Aug. 27, 1936	3.726	1.416	2.310	0.332	1.084	0.209	0.200	0.675
Full Bloom	June 21, 1936	2.276	0.686	1.591	0.165	0.521	0.084	0.095	0.342
	Aug. 5, 1936	2.660	0.861	1.800	0.137	0.723	0.176	0.101	0.447
Maturity	Sept. 9, 1936	2.223	0.774	1.449	0.086	0.689	0.157	0.122	0.409

Results are expressed in percentage of moisture free material.

TABLE III. Distribution of Acetic Acid Soluble Nitrogen, Amino Nitrogen, and Amide Nitrogen in Alfalfa Hay Cut at Different Stages of Growth.

Stage of Growth	Date of Cutting	Total N	Total Water Soluble N	Acetic Acid Soluble N	Amino (P.T.A. filtrate) N	Amide (P.T.A. filtrate) N
Seedling	Aug. 19, 1936	4.530	1.867	1.631	1.440	1.395
Pre-bud	June 6, 1936	2.885	1.275	1.085	0.942	0.914
	July 7, 1936	4.333	1.923	1.665	1.528	1.468
	Aug. 5, 1936	3.815	1.201	1.166	0.893	0.853
	Sept. 9, 1936	3.152	1.292	1.067	0.955	0.811
Bud	June 11, 1936	2.375	1.060	1.033	0.968	0.691
	July 17, 1936	3.844	1.418	1.279	0.959	0.951
	Aug. 19, 1936	3.665	1.103	0.856	0.708	0.653
1/10 Bloom	June 13, 1936	2.777	1.463	1.401	1.132	0.842
	July 30, 1936	2.861	1.014	1.014	0.912	0.771
	Aug. 27, 1936	3.726	1.416	1.277	0.989	0.961
Full Bloom	June 21, 1936	2.276	0.686	0.653	0.503	0.416
	Aug. 5, 1936	2.660	0.861	0.815	0.793	0.625
Maturity	Sept. 9, 1936	2.223	0.774	0.765	0.723	0.460

Results are expressed in percentage of moisture free material.

TABLE IV. Nitrogen Distribution in alfalfa Hay Cut at Different Stages of Growth.

Stage of Growth	Date of Cutting	Total N (%)	Total Water Soluble N	Insoluble Protein N	Soluble Protein N	Total Non-Protein N	Proteose N	Peptone N	Sub-Peptone N
Seedling	Aug. 19, 1936	4.530	41.21	58.79	8.92	32.29	3.26	3.36	25.66
Pre-bud	June 6, 1936	2.885	44.21	55.79	7.59	36.61	2.96	3.74	29.91
	July 7, 1936	4.333	44.38	55.62	13.20	31.18	3.07	4.41	23.70
	Aug. 5, 1936	3.815	31.47	68.53	5.38	26.06	2.59	2.50	20.97
	Sept. 9, 1936	3.152	40.98	59.03	8.47	32.51	4.06	3.41	25.03
Bud	June 11, 1936	2.375	44.63	55.37	14.68	29.94	5.22	5.52	19.19
	July 17, 1936	3.844	36.89	63.11	6.27	30.61	3.33	7.95	19.33
	Aug. 19, 1936	3.665	30.09	69.91	10.29	19.79	2.70	1.90	15.19
1/10 Bloom	June 13, 1936	2.777	54.75	43.53	10.05	42.63	5.09	9.75	27.79
	July 30, 1936	2.861	42.47	57.53	3.12	32.32	4.25	4.26	23.82
	Aug. 27, 1936	3.726	38.01	61.99	8.91	29.10	5.62	5.36	18.12
Full Bloom	June 21, 1936	2.276	30.12	69.87	7.25	22.87	3.67	4.17	15.03
	Aug. 5, 1936	2.660	32.36	67.64	5.16	27.20	6.61	3.78	16.81
Maturity	Sept. 9, 1936	2.223	34.82	65.18	3.85	30.97	7.06	5.50	18.40

Results are expressed in percentage of the total nitrogen of each respective sample at the stage of growth here defined.

TABLE V. Distribution of Acetic Acid Soluble Nitrogen, Amino Nitrogen, and Amide Nitrogen in Alfalfa Hay Cut at Different Stages of Growth.

Stage of Growth	Date of Cutting	Total N (%)	Total Water Soluble N	Acetic Acid Soluble N	Amino (P.T.A. filtrate) N	Amide (P.T.A. filtrate) N
Seedling	Aug. 19, 1936	4.530	41.21	35.99	31.79	30.79
Pre-bud	June 6, 1936 July 7, 1936 Aug. 5, 1936 Sept. 9, 1936	2.885 4.333 3.815 3.152	44.21 44.38 31.47 40.98	37.61 38.44 30.56 33.85	32.64 35.27 23.41 30.30	31.68 33.88 22.37 25.73
Bud	June 11, 1936 July 17, 1936 Aug. 19, 1936	2.375 3.844 3.665	44.63 36.89 30.09	43.50 33.28 23.37	40.75 24.96 19.31	29.10 24.74 17.80
1/10 Bloom	June 13, 1936 July 30, 1936 Aug. 27, 1936	2.777 2.861 3.726	54.75 35.44 38.01	50.47 35.44 34.26	40.75 31.89 26.53	30.32 26.94 25.79
Full Bloom	June 21, 1936 Aug. 5, 1936	2.276 2.660	30.12 32.36	28.69 30.65	22.12 29.83	18.26 23.48
Maturity	Sept. 9, 1936	2.223	34.82	34.40	32.52	20.68

Results are expressed in percentage of the total nitrogen of each respective sample at the stage of growth here defined.

TABLE VI. Nitrogen Distribution in Alfalfa Hay Cut at Different Stages of Growth.

Stage of Growth	Date of Cutting	Total Water Soluble N(%)	Total non-protein N	Soluble Protein N	Proteose N	Peptone N	Sub-Peptone N
Seedling	Aug. 19, 1936	1.867	78.35	21.65	7.92	8.16	62.27
Pre-bud	June 6, 1936	1.275	82.83	17.17	6.69	8.47	67.67
	July 7, 1936	1.923	70.26	29.74	6.92	9.95	53.39
	Aug. 5, 1936	1.201	82.81	17.11	8.23	7.94	66.64
	Sept. 9, 1936	1.292	79.33	20.67	9.91	8.33	61.10
Bud	June 11, 1936	1.060	67.10	32.90	11.71	12.38	43.01
	July 17, 1936	1.418	82.99	17.01	9.03	21.55	52.41
	Aug. 19, 1936	1.103	65.78	34.22	8.99	6.30	50.49
1/10 Bloom	June 13, 1936	1.463	80.92	19.08	9.67	18.50	52.75
	July 30, 1936	1.014	91.20	8.80	11.99	12.01	67.20
	Aug. 27, 1936	1.416	76.57	23.43	14.79	14.10	47.68
Full Bloom	June 21, 1936	0.686	75.92	24.08	12.19	13.84	49.89
	Aug. 5, 1936	0.861	84.06	15.94	20.41	11.69	51.96
Maturity	Sept. 9, 1936	0.774	87.80	11.07	20.28	15.81	52.85

Results are expressed in percentage of the total soluble nitrogen of each respective sample at the stage of growth here defined.

TABLE VII. Distribution of Acetic Acid Soluble Nitrogen, Amino Nitrogen and Amide Nitrogen in Alfalfa Hay Cut at Different Stages of Growth.

Stage of Growth	Date of Cutting	Total Water Soluble N (%)	Acetic Acid Soluble N	Amino N (P.T.A. filtrate)	Amide N (P.T.A. filtrate)
Seedling	Aug. 19, 1936	1.867	87.34	77.14	74.71
Pre-bud	June 6, 1936	1.275	85.07	73.84	71.67
	July 7, 1936	1.923	86.61	79.46	76.34
	Aug. 5, 1936	1.201	97.11	74.38	71.07
	Sept. 9, 1936	1.292	82.62	73.96	62.81
Bud	June 11, 1936	1.060	97.48	91.32	65.21
	July 17, 1936	1.418	90.21	67.66	67.07
	Aug. 19, 1936	1.103	77.66	64.18	59.18
1/10 Bloom	June 13, 1936	1.463	95.80	77.37	57.55
	July 30, 1936	1.014	100.00	89.99	76.00
	Aug. 27, 1936	1.416	90.13	69.86	67.84
Full Bloom	June 21, 1936	0.686	95.25	73.41	60.62
	Aug. 5, 1936	0.861	94.70	92.18	72.56
Maturity	Sept. 9, 1936	0.774	98.79	93.37	59.39

Results are expressed in percentage of the total soluble nitrogen of each sample at the stage of growth here defined.

and VII contain the results of the water soluble material expressed in percent of the total soluble nitrogen of each respective sample.

In connection with these tables the following definitions must be noted:-

(1) "Insoluble Protein Nitrogen" is calculated by subtracting the total water soluble nitrogen from the total nitrogen value for each sample.

(2) "Soluble Protein Nitrogen". This fraction is obtained by subtracting the nitrogen of the trichloroacetic acid filtrate from total water soluble nitrogen.

(3) "Total Non-Protein Nitrogen" is the total nitrogen of the trichloroacetic acid filtrate.

(4) "Proteose Nitrogen" is obtained by difference following saturation with sodium sulphate under rigidly controlled conditions. The nitrogen of the sodium sulphate filtrate is subtracted from the total non-protein nitrogen (trichloroacetic acid filtrate).

(5) "Peptone Nitrogen". The nitrogen precipitated by tannic acid under the conditions of the experiment. It is determined by subtracting the nitrogen of the tannic acid filtrate from the nitrogen of the sodium sulphate filtrate.

(6) "Sub-peptone Nitrogen". This fraction is represented by the total nitrogen in the tannic acid filtrate. It includes peptides of lesser complexity than the peptones in addition to free amino acids.

(7) "Acetic Acid Soluble Nitrogen" represents the

nitrogen content of the water extract of alfalfa after it has been boiled with very dilute acetic acid.

(8) "Amino Nitrogen". This has been defined as that nitrogen unprecipitated by phosphotungstic acid from the acetic acid filtrate.

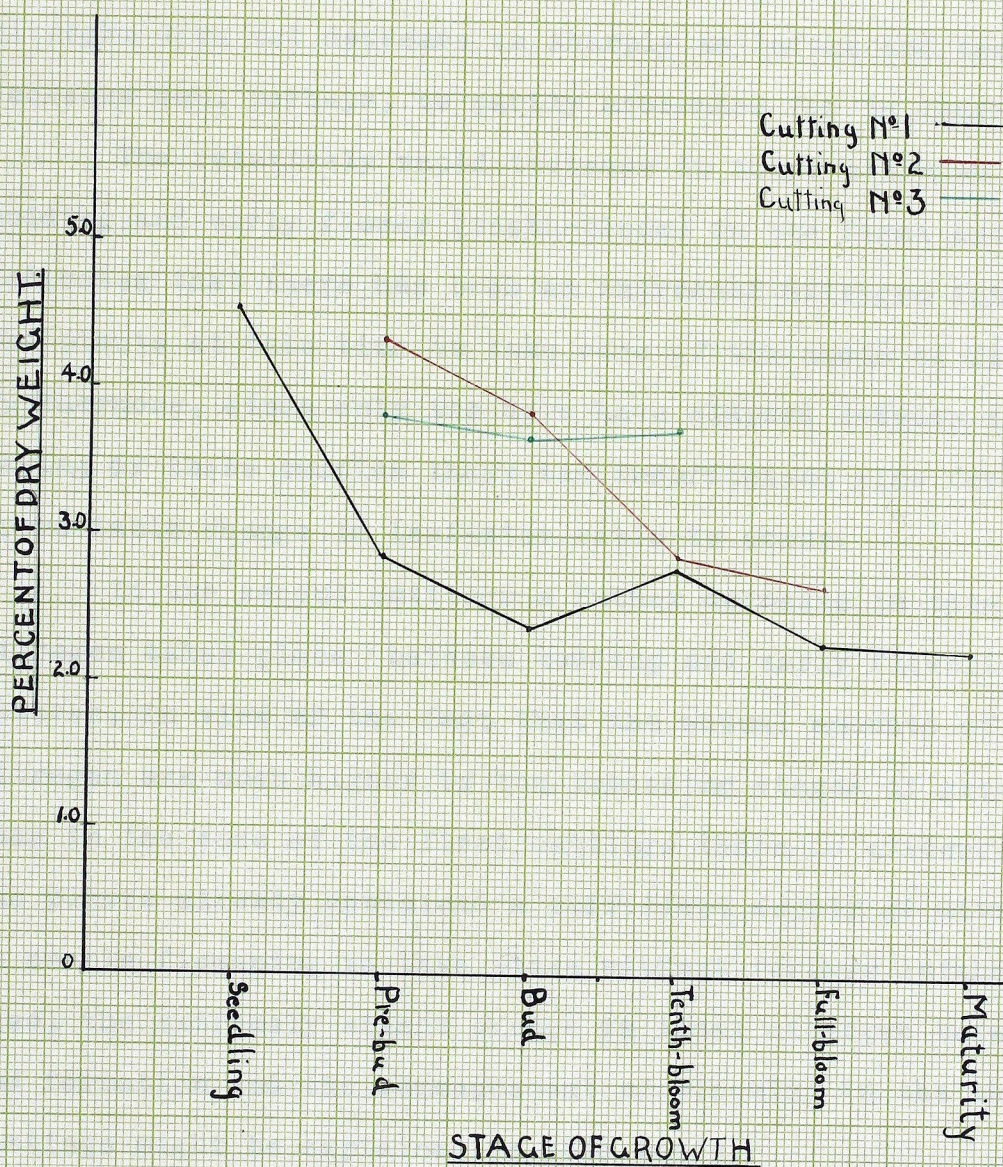
(9) "Amide Nitrogen" is that nitrogen not precipitated by phosphotungstic from the original water extract.

#### D. DISCUSSION AND CONCLUSIONS:-

Total Nitrogen - An examination of the total nitrogen data of Table II when portrayed in graph form (see Fig. 8) shows a relatively steady decrease throughout the growing period. A detailed study of the graphs for the three cuttings indicates that, in the case of the first cutting, a marked decrease in nitrogen occurs during the early period of growth followed by a gradual decrease to maturity, except in the case of the tenth-bloom where a slight increase is noted. The chart for the second cutting, on the other hand, indicates a relatively uniform decrease in nitrogen content throughout the period of study, while the graph covering the third cutting demonstrates very little change in nitrogen content throughout.

The fluctuations in total nitrogen, which are apparent in Fig. 8, may be partially explained in the light of the work of Woodman and Evans (1935), who found that the proportion of leaf in relation to stem falls off as the crop advances in maturity. Since the total nitrogen content of the alfalfa leaf at any stage of growth is, roughly, double that of the

FIG. 8 TOTAL NITROGEN OF ALFALFA HAY CUT AT  
DIFFERENT STAGES OF GROWTH.



stem, a change in the ratio of these two is very significant.

Woodman and Evans observed the rate of decrease of leafiness to be greater before budding than during the period of budding to early flower. This accounts for the sharp decrease in total nitrogen to the tenth-bloom stage in the second cuttings and the decrease from the seedling stage to budding in the first cuttings. From budding onward total nitrogen does not decrease as sharply as before and the decrease can, perhaps, be partly accounted for in leaf-fall.

The relatively rapid increase in total nitrogen between budding and early flower (tenth-bloom) in the first cuttings indicates that there has been an increased nitrogen intake by the plants at this time to take care of the needs of flowering. Furthermore, since only two days have elapsed between the taking of these samples there was insufficient time for an appreciable change in leaf-stem ratio to occur.

In the second cuttings a further decrease in total nitrogen takes place between budding and early flowering. However, in view of the fact that the actual amounts of total nitrogen are much higher in the preceding stages of growth than in the case of the first cuttings there is evidence that the plants already contained a sufficient supply of nitrogen to enable flowering to take place without further intake. Also, the extremely slight change in total nitrogen between bud formation and the commencement of blooming in the third cuttings might be explained on this basis.

The data presented by Woodman and Evans (1935) can also be used in interpreting the increase in total nitrogen content

of the plants at each stage of growth with cuttings made later in the season. They attributed this rise to the fact that later cuts in the season have a distinctly leafier character than the early cuts. The high nitrogen content of the third cuttings together with the negligible fluctuation between different stages of growth indicates not only a high degree of leafiness in the plants but, in addition, that the ratio of leaf to stem does not change appreciably at this time. Observations in the field at the time of sampling showed that the later cuts did not achieve the same degree of vegetative growth as the earlier cuts.

From the total nitrogen data presented in Fig. 8 certain conclusions can be drawn with respect to the quality of alfalfa hay cut at different stages of growth and at different times in the season. The high nitrogen content at the seedling stage is of no practical value since the crop could not be utilized for hay at this stage. This is also partially true of the cutting of hay at the pre-budding stage. Hay cut at this stage would have a very high digestible crude protein content, but the yield of dry matter would be very low and also continued cutting at this stage of growth would tend to deplete the vitality of the stand rather seriously (Woodman, Evans, and Norman, 1933). In the early part of the season it is evident that, from the standpoint of total crude protein, the cutting of hay in the one-tenth bloom stage would give the best results. Later in the season it would probably be permissible to cut at the bud stage in preference to the tenth-bloom, particularly under conditions similar to those at the

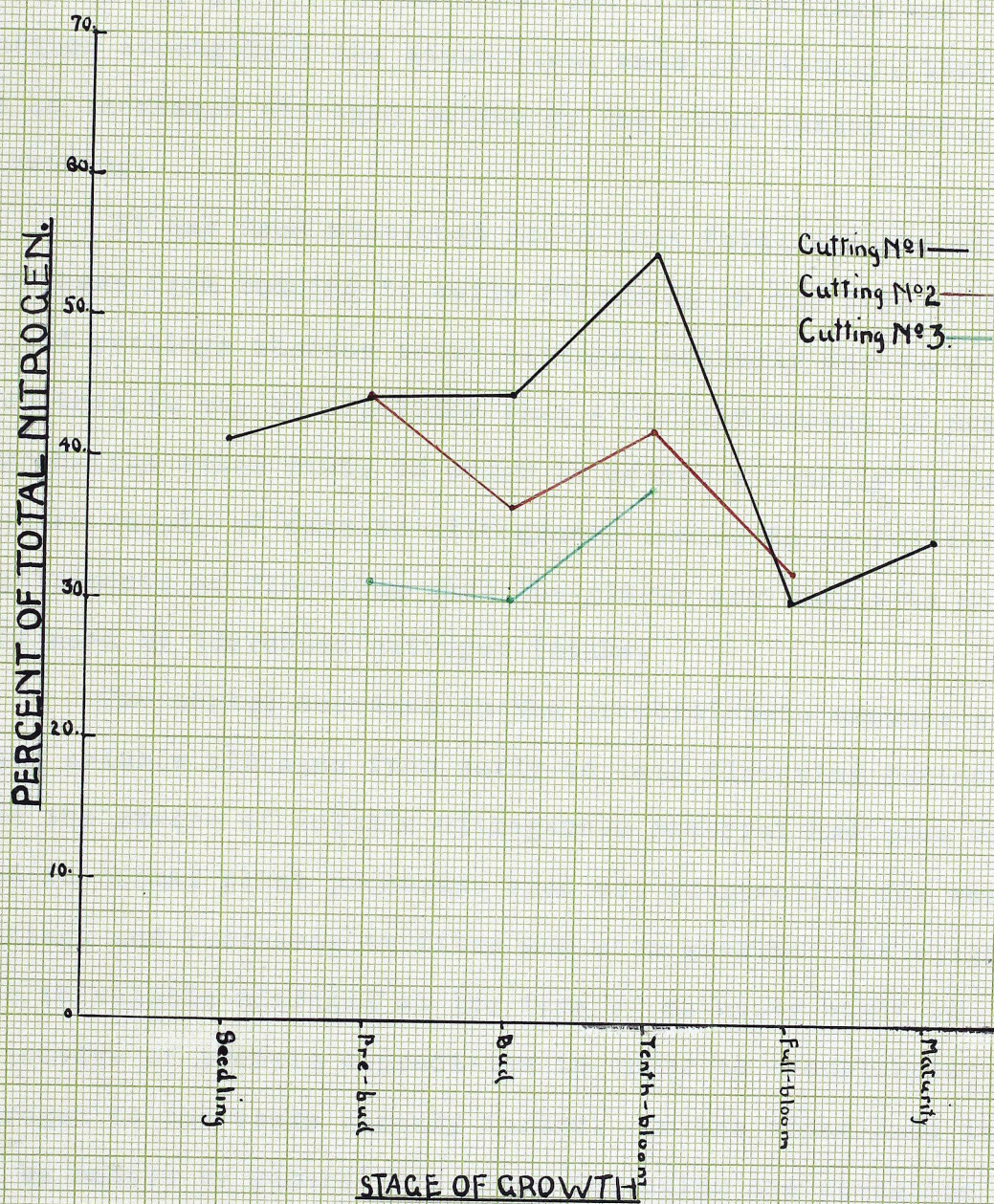
time the second cuttings were taken. The effect of cutting at an earlier stage of growth later in the season would probably not be so harmful to the vigour of the stand as it would be earlier in the season. Later cutting in the early part of the season would permit proper crown and root development to take place. If possible, hay should not be cut in the full bloom stage if a high content of digestible crude protein is to be obtained.

Total Soluble Nitrogen - The results for total soluble nitrogen (see Table IV) are expressed graphically in Figure 9. In order to emphasize the fluctuations between each stage of growth the results are plotted in percent of the total nitrogen of each respective sample.

It is of extreme interest to note that the respective levels of total soluble nitrogen between cuttings, at corresponding stages of growth, are the exact reverse of the order existing for total nitrogen (Fig. 8). The proportionate amounts of total soluble nitrogen, at nearly all stages of growth, are higher in the first cuttings than in the second and third cuttings.

The total soluble nitrogen at the pre-bud and full bloom stages does not follow the general trend for the other stages of growth. Prior to bud development the proportionate amounts of total soluble nitrogen are practically identical in the first and second cuttings. The second cutting at the time of full bloom appears to possess a slightly higher percentage of its nitrogen in a soluble form than the first cutting.

FIG.9- TOTAL SOLUBLE NITROGEN OF ALFALFA HAY  
CUT AT DIFFERENT STAGES OF GROWTH.



Attention must also be drawn to the fact that the proportion of total soluble nitrogen is much higher at tenth-bloom than budding in all three cuttings and in the first and third cuttings soluble nitrogen attains its maximum value at this time. This rise undoubtedly accounts, in part at least, for the increase in total nitrogen (Fig. 8) at the corresponding stage of growth in the first and third cuttings and thus further strengthens the assumption, made previously, that there is an increased nitrogen intake by the plant during early flowering.

It has been mentioned above that total soluble nitrogen increases from budding to early flowering in the case of the second cuttings whereas total nitrogen actually decreases. From this it may be concluded that a certain amount of protein degradation takes place in order to create a readily translocated supply of simpler nitrogenous compounds to fulfill the requirements of flowering when the plants are unable to meet this need by increased nitrogen intake.

The marked decrease in the proportionate amounts of total soluble nitrogen from early to late flowering indicates that the excess of soluble nitrogenous material has been utilized in normal plant processes such as; preparation for seed setting, and increases in fibre and stem growth. The slight rise in soluble nitrogen at the time of maturity might be indicative of protein degradation in order to translocate nitrogen to the seeds already formed. On the other hand it may be caused by general breakdown following the cessation of all growth processes.

With regard to general metabolism the curves for total soluble nitrogen tend to show an increasing rate of synthesis and assimilation up to the period of early flowering. By this time the plants have practically achieved their maximum vegetative growth. From this point onwards a consolidation of soluble materials takes place and active growth processes gradually slow down.

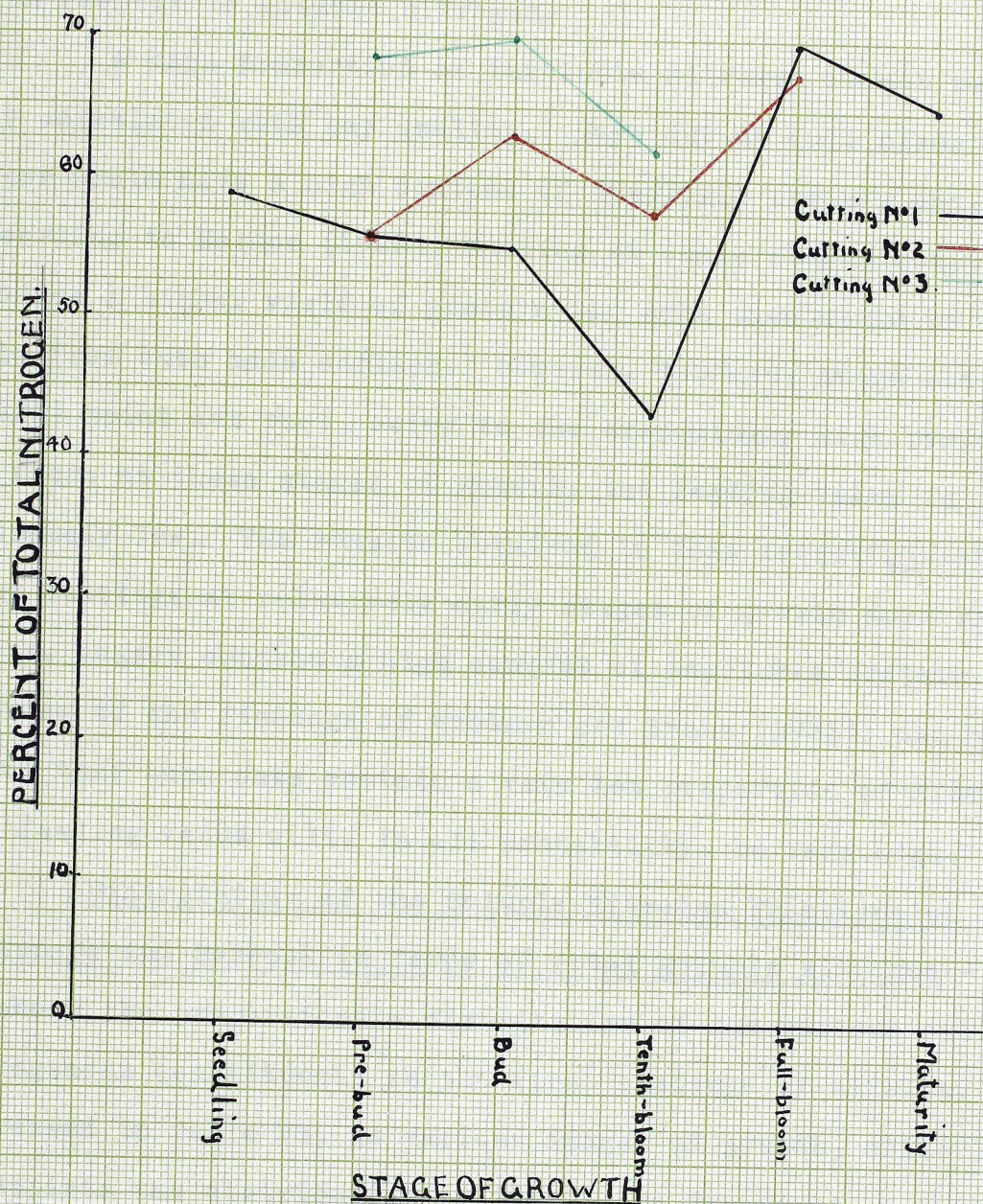
The probable effects of soluble nitrogen on hay quality are not easy to define. The high proportion of soluble nitrogen when the plants have just started to bloom indicates that large leaching losses might be incurred in making alfalfa hay at this stage of growth under field conditions.

As for the influence of soluble nitrogen on the feeding value of alfalfa hay no deductions can be made without further information concerning the exact nature of the compounds contained in this fraction. However, it is generally conceded that the simpler nitrogenous compounds possess a lower nutritional value than the "complete" proteins.

Insoluble Protein Nitrogen - Subtraction of total soluble nitrogen from total nitrogen (see Tables II and IV) gives what might be termed Insoluble Protein Nitrogen. Since it is determined by difference, the curves for this fraction (Fig. 10) will obviously show trends that are intermediate between those for total nitrogen (Fig. 8) and total soluble nitrogen (Fig. 9).

It is apparent that the proportionate amounts of insoluble protein are greater at all stages of growth in cuts made later in the season. This explains the inverse relation-

FIG 10 INSOLUBLE PROTEIN NITROGEN OF ALFALFA  
HAY CUT AT DIFFERENT STAGES OF GROWTH



ship reported previously with respect to total soluble nitrogen.

In general, insoluble protein shows a decrease throughout the periods of active vegetative growth and differentiation. Following the inception of flowering it rises markedly thus validating the assumptions made in the foregoing discussion with respect to soluble nitrogen.

A high proportion of insoluble nitrogen is not necessarily correlated with hay quality. Hays cut at full bloom or later would have a high content of insoluble protein and, according to Nelson (1925), cutting at these stages would produce a higher yield of protein per acre than would be obtainable from cuts made earlier. However, Woodman, Evans and Norman (1933) point out that, owing to the exceedingly high proportion of fibre at these stages of growth, the feeding value is extremely low. The results obtained in this investigation show that cuttings at relatively early stages of growth made later in the season contain a high content of insoluble protein and in view of the findings of Woodman and Evans (1935), who have shown that such cuts contain a very low proportion of fibre, the feeding value will, in all probability, be very high.

Distribution of the Soluble Nitrogen Fractions of Wasteneys and Borsook in Terms of Total Soluble Nitrogen - Fig. 11 shows the proportionate distribution of the various soluble nitrogenous constituents in the first cuttings of alfalfa, as expressed in Table VI, at various stages of growth, while Fig. 12 depicts the distribution of these same fractions at the stages of growth from which second and third cuts were taken.

FIG II - DISTRIBUTION OF TOTAL NON-PROTEIN, SOLUBLE PROTEIN, PROTEOSE, PEPTONE, AND SUB-PEPTONE NITROGEN

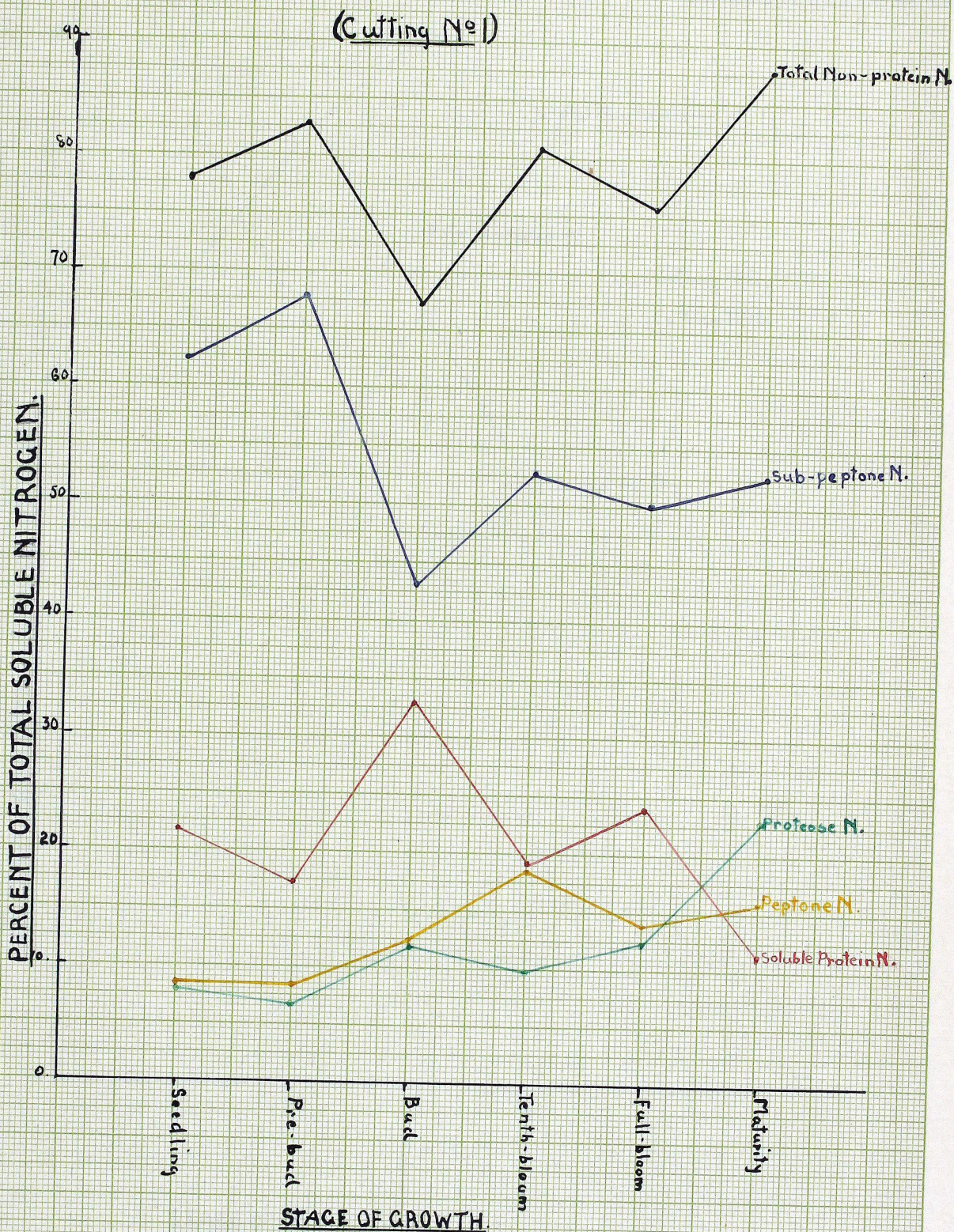
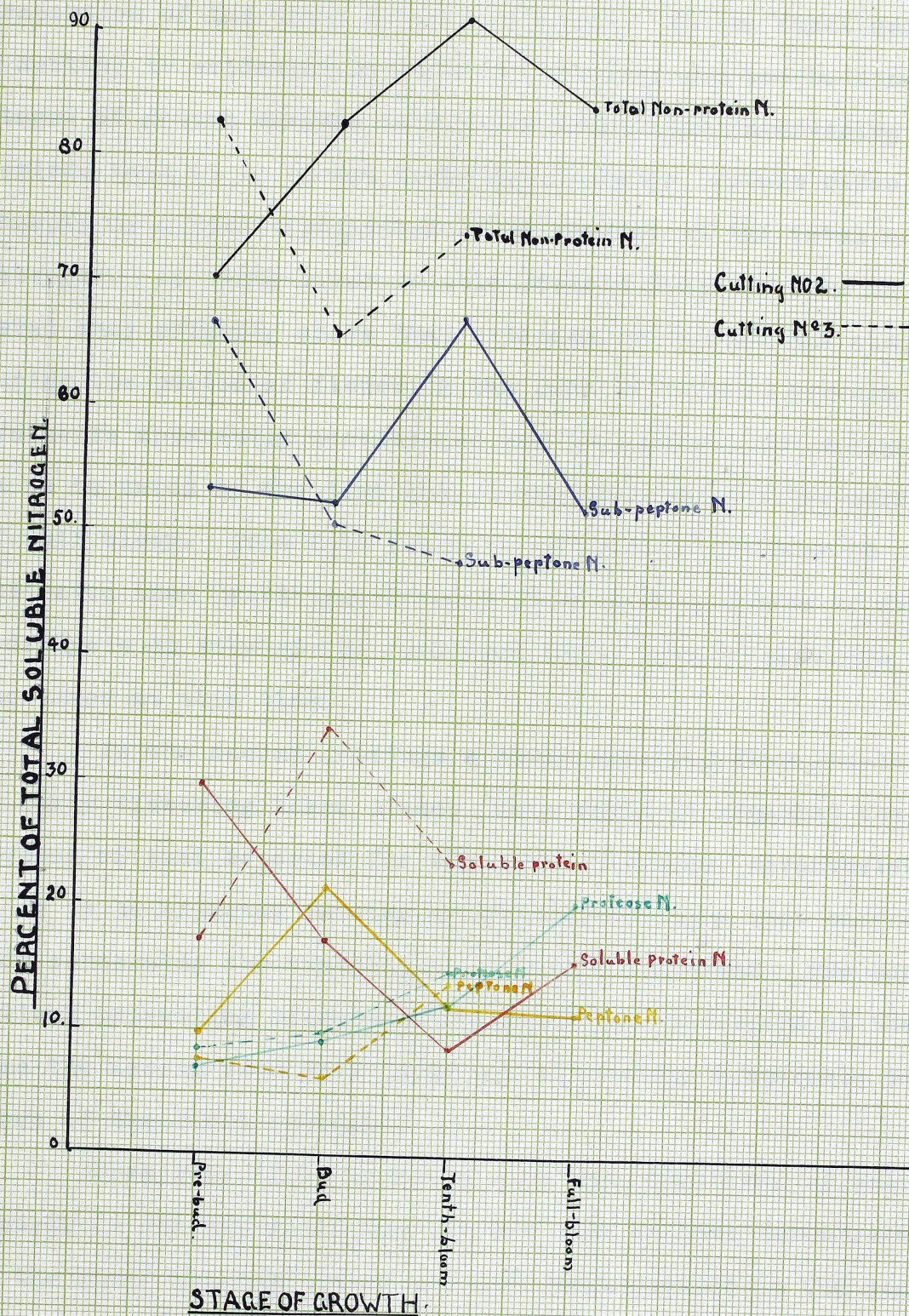


FIG 12-DISTRIBUTION OF TOTAL NON-PROTEIN, SOLUBLE PROTEIN, PROTEOSE, PEPTONE, AND SUB-PEPTONE NITROGEN.



Soluble Protein Nitrogen - It is evident, that at all stages of growth, soluble protein represents a relatively small proportion of the total soluble nitrogen. In the first cuttings a decline is evidenced between the seedling and pre-budding stages of growth whereas during the subsequent intervening period, that is up to bud formation, soluble protein rises very sharply to a peak represented by 33 percent of the total soluble nitrogen. At tenth-bloom soluble protein drops sharply to a level only slightly above that existing in the pre-bud stage. During the period of flowering the rate of soluble protein synthesis apparently accelerated and an appreciable increase is to be noted by the time the plants have reached the period of full bloom. From full bloom to maturity a decrease is indicated thus providing evidence of a certain amount of protein hydrolysis in view of the fact that total soluble nitrogen increases at this time.

In the second cuttings a sharp decrease from pre-budding to early flowering takes place. This trend is somewhat the reverse of that reported for the first cuttings, nevertheless it is to be expected in view of the observations that have been made concerning the fractions previously discussed. The conditions prevailing between tenth-bloom and full bloom at the time the second cuts were taken are apparently comparable to those of the first cuttings.

From Fig. 12 it is to be seen that the trend of soluble protein in the third cuttings is the same as that encountered in the first cuttings which are made much earlier in the season. At this point attention should also be drawn to the fact that

the proportion of soluble protein at this time differs only slightly from that existing in the first cuts.

The high level of soluble protein encountered at the bud stage is rather significant since this period in the plant's life history, as already noted, is generally considered as representing the peak of vegetative growth. Prior to this time the energies of the plant have been devoted chiefly to the production of leaves, stems and roots. Following the appearance of flower buds, however, the plant tends to expend the greater part of its efforts in the formation of fruit. Since this phase of development constitutes a drain on the vegetative resources it is natural that soluble proteins should be diminished in amount.

In so far as quality of hay is concerned it is apparent that the high proportion of soluble protein nitrogen found at the time of budding is coupled with a low content of soluble nitrogen and a reasonably high proportion of insoluble protein. On the basis of previous discussion the value of such a combination is obvious.

Total Non-Protein Nitrogen - This fraction does not warrant detailed consideration since it includes almost all of the soluble nitrogenous material and, for this reason, is obviously complementary to soluble protein nitrogen.

Proteose Nitrogen - In view of the fact that proteoses are only slightly below proteins in complexity, it might be expected that the trends for the two would have a tendency to approximate one another. However, this relationship does not hold, except in two instances. In the first cuttings proteose

follows a trend similar to that exhibited by soluble proteins up to the time of full bloom. Following the period of flowering the situation is reversed and the quantity of protease at maturity is almost double that of soluble protein. An inverse relationship exists from pre-budding to tenth-bloom in the second cuts and from then to full bloom the relationship is fairly constant excepting that the order of magnitude of the two fractions is altered. In the third cuttings the trends of soluble protein and protease follow a course similar to that prevailing in the second cuttings.

It is worthy of note that the type of graph for protease in all cuttings is essentially the same and indicates a distinct increase throughout the growing period; which in turn suggests that this fraction is present as a result of both protein synthesis and degradation. The increase throughout the period of active vegetative growth indicating that proteases may represent one of the steps in protein synthesis; whereas the continued increase following flowering is indicative of protein degradation. However, this conclusion tends to be contradictory to the findings of Chibnall (1922) who observed that protease in the leaves of the common bean (*Phaseolis vulgaris*) tended to decrease with increasing age in common with the leaf proteins. Nevertheless, it is possible that the mature plants analyzed in this investigation had not reached the same stage of degeneration exhibited by those of Chibnall and consequently the increase in protease may be taken as being indicative of the commencement of degeneration.

Peptone Nitrogen - In the first cuttings the curves for proteose and peptone nitrogen are of practically the same order throughout the growing period. However, at tenth-bloom there appears to be an appreciable difference in favour of higher peptone content, while at maturity, the graphs would appear to suggest slightly less peptone nitrogen.

With respect to the third cuttings it will be observed that the graph is of approximately the same order as that of the first cuttings but, in this case, the peptone nitrogen content appears to be slightly lower than that of proteose nitrogen.

The graph covering the second cuttings does not conform to the other curves in that it appears to demonstrate a very definite negative correlation between proteose and peptone at the stages of growth from which comparisons may be made.

In view of the fact that peptone exhibits considerable irregularity throughout the period in which samples were taken it is rather difficult to draw any definite conclusions as to its probable significance in nitrogen metabolism. Furthermore the writer has been unable to find any record in the literature of such a determination having been made on plant material. Consequently no interpretation may be made in the light of previous work.

However, the diversity in the results obtained may be partially explained when it is remembered that the material analyzed had been subjected to a prolonged drying at ordinary temperatures thus permitting enzyme action to proceed unchecked for a considerable length of time. Since, in all probability,

the amounts and combinations of enzymes present will vary considerably depending on the stage of growth and the time of season at which the samples are taken, it is reasonable to expect that the results of their action will be by no means constant.

Nevertheless, peptone, in common with proteose, does show indications of being rather closely correlated with protein synthesis and degradation in all cuttings. Therefore, it is quite possible that determinations on fresh plant material would show this relationship more distinctly.

Sub-peptone Nitrogen - It is quite apparent from an examination of Figs. 11 and 12 that sub-peptone constitutes a very large portion of the total soluble nitrogen in all three cuttings. The trend followed, resembles somewhat that of total non-protein nitrogen. This is particularly true of the first cutting.

Furthermore, the curves for sub-peptone show the trend of protein synthesis and degradation throughout the season. It is very significant that in the very early stages of growth sub-peptone should be at its highest level thus indicating that nitrogen intake is more rapid than protein synthesis. However, by the time flower buds have appeared in the first cuttings, sub-peptone has decreased to the lowest level obtained in any part of the cycle, thereby showing that synthesis has been sufficiently rapid to keep pace with nitrogen intake. Between the appearance of flower buds and the time when the plants are one-tenth in bloom sub-peptone increases in accordance with the

previously noted increases in total soluble nitrogen and peptone nitrogen and the corresponding decreases in soluble proteins and proteoses. This observation is in accordance with the findings of Thomas (1927), who showed that the formation of blossoms in the apple tree resulted in marked increases in the amounts of the simpler forms of nitrogen in the new growth which were derived at the expense of the "reserve" proteins previously accumulated in the older branches.

In the second cuttings, sub-peptone represents a much lower proportion of the soluble nitrogen during the early stages of growth than previously, thereby suggesting the possibility that synthesis had been very rapid during the period following the removal of the first cuttings. An extremely high proportion of the soluble nitrogen is present as sub-peptone at the time of early flowering however, thus substantiating the foregoing deductions relative to bloom formation.

In the third cuttings sub-peptone presents the same picture at the pre-bud stage as was shown in the first cuttings. However, the relationship between budding and the early part of flowering is the reverse of that previously encountered. This observation need not invalidate the assumptions made previously with regard to the need for an abundant supply of relatively simple forms of nitrogen in order to meet the requirements of blooming. On the other hand it suggests the possibility that an abundance of slightly more complex compounds such as proteoses and peptones can meet this requirement. The fact that proteoses and peptones increase furnishes further evidence in support of this supposition.

Distribution of the Soluble Constituents of Wasteney's and Borsook in terms of Maximum Total Soluble Nitrogen - In order that the exact nature of the processes of protein synthesis and degradation might be more clearly described, the variations in the soluble nitrogenous fractions (W. & B.) obtained by calculations based on the data in Table II and expressed in percentage of the maximum total soluble nitrogen, are plotted in Fig. 13. The results expressed in this fashion apply only to the stages of growth from which the first cuttings were taken and do not include the results obtained from subsequent cuttings since they are incomplete with regard to stages of growth. Also the data relative to the seedling stage is omitted in view of the fact that this material was obtained from a different planting. Consequently the conditions under which it was grown would not be quite the same as those prevailing in the case of the other samples.

The curves for total soluble nitrogen, total non-protein nitrogen and sub-peptone show that synthesis and breakdown have alternated throughout the period under discussion. Synthesis has exceeded breakdown from pre-budding to budding and from the early part of flowering to full bloom. Whereas from the time of budding to the early part of flowering and from full bloom until maturity, breakdown overweighs synthesis. Consequently the curves for these fractions might be designated as "percentage synthesis-hydrolysis curves".

In following the curve for soluble protein it is interesting to note that the peak of synthesis is reached at the time

FIG. 13- DISTRIBUTION OF SOLUBLE NITROGEN FRACTIONS OF WASTE NEYS AND BORSOOK IN TERMS OF MAXIMUM TOTAL SOLUBLE NITROGEN.



of budding, which corresponds very closely to the peak of vegetative growth. While from budding to maturity the trend is continually downward thus indicating a steady decrease in anabolic processes.

In so far as proteose and peptone are considered it is rather difficult to draw definite conclusions concerning their relationship to general metabolism throughout the season, in view of the fact that they are present in rather minute quantities. However, it is rather significant that the peak of both proteose and peptone coincides with the peak of greatest breakdown and that this is reached at the next stage of growth after the peak of soluble proteins.

The marked differences occurring between the different stages of growth studied in this investigation suggest that further information might be gained concerning the nitrogen metabolism of the alfalfa plant if a study of this nature were conducted on fresh samples collected at much shorter intervals throughout the life history of the plants. In this way the exact gradations of synthesis and breakdown could be more clearly defined.

The data presented in Fig. 13 show, nevertheless, that the application of the method of Wasteneys and Borsook in this study has made possible the presentation of a relatively clearly defined picture of the trends of synthesis and breakdown throughout the growing period of the plant. This is noteworthy in view of the fact that the amounts of many of the fractions are extremely small and that the relationships existing between them, at any one time, are much more complex than

those to be encountered at any stage in the hydrolysis of pure proteins by defined enzymes.

Acetic Acid Soluble Nitrogen - represents the nitrogen content of the water extract of alfalfa after the nitrogen compounds coagulable by heat and acetic acid have been removed. From Figs. 14, 15 and 16, it is apparent the curves for this fraction are at a much higher level than those for non-protein nitrogen (W. & B.) thus indicating that the removal of proteins by acetic acid, at the concentration used, is far from complete. In the second and third cuttings a relatively constant relationship exists between the two curves, whereas in the first cuttings there is very little agreement.

No information whatever beyond that already provided by total soluble nitrogen can be derived from this data. It should be pointed out, however, that the treatment with acetic acid was carried out primarily as the first step in the amino nitrogen determination.

Amino Nitrogen - This fraction has already been defined as the nitrogen unprecipitated by phosphotungstic acid in the acetic acid filtrate. However, it should not be confused with amino nitrogen as determined by the method of Van Slyke (1912). The estimation of amino nitrogen as made in this study was carried out for the purpose of determining whether or not it might serve as a short-cut analytical method for the estimation of the amount of free amino nitrogen existing in plant extracts. As mentioned previously, the procedure is identical with that used by Eagles and Sadler (1932); which in turn, is based on the decomposition nitrogen of Orla-Jensen (1921). In discuss-

FIG. 14-DISTRIBUTION OF ACETIC ACID-SOLUBLE AMINO, AND  
AMIDE NITROGEN.

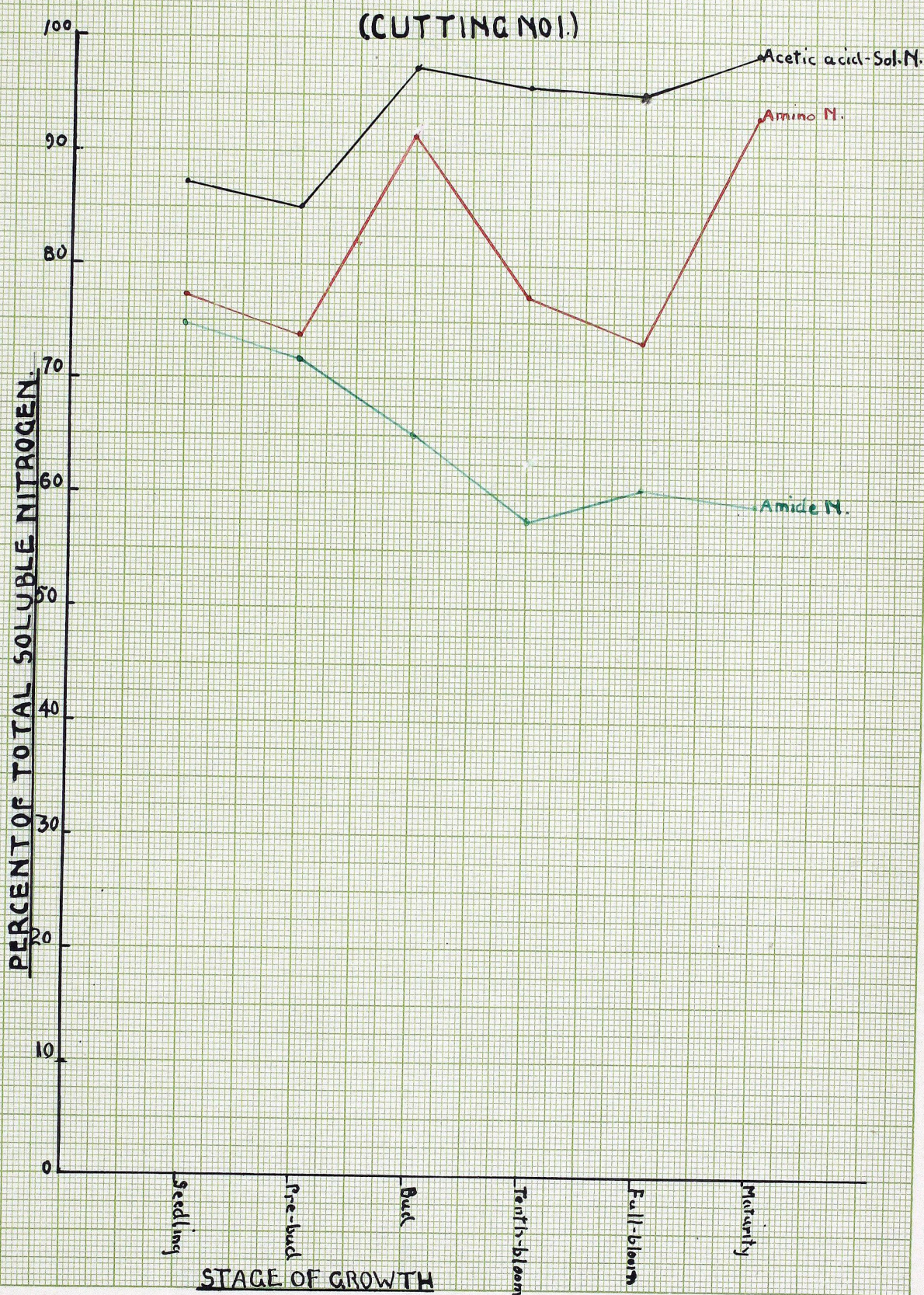


FIG. 15-DISTRIBUTION OF ACETIC ACID SOLUBLE, AMINO, AND AMIDE NITROGEN.

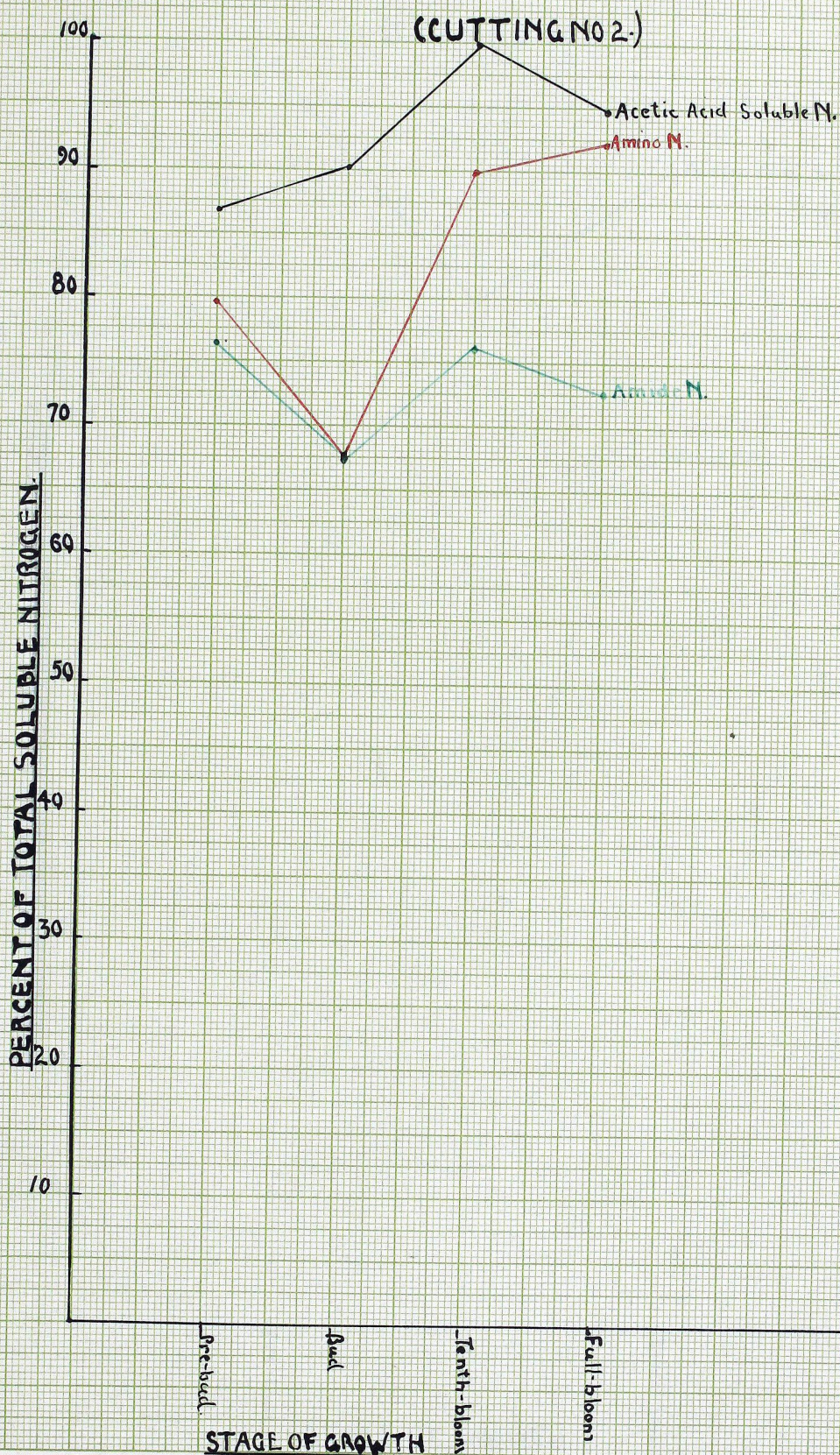
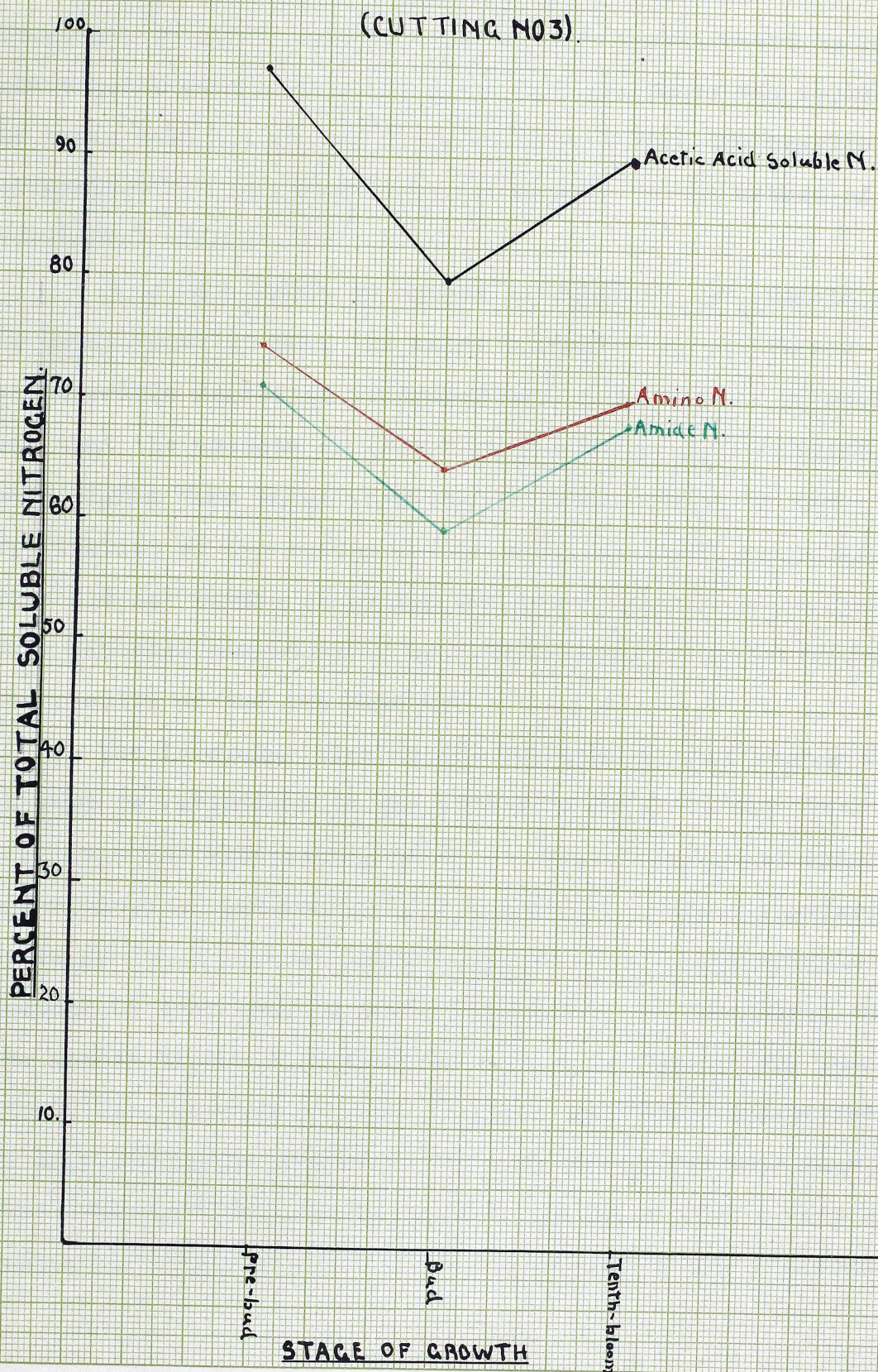


FIG. 16-DISTRIBUTION OF ACETIC ACID-SOLUBLE, AMINO AND AMIDE NITROGEN.



ing the ripening of cheese Orla-Jensen defines the decomposition nitrogen as, "the nitrogen of the protein decomposition products, or amino acids, which are not precipitated by phosphotungstic acid". Consequently this procedure was applied to plant material to determine whether or not the results obtained would approximate those derived by the method of Van Slyke (1912).

From the curves portrayed in Figs. 14, 15, and 16 it is quite apparent that the amino nitrogen results show a considerable degree of irregularity. It should be pointed out that the amounts of this fraction obtained are considerably higher than those obtained by other workers in the application of the method of Van Slyke to plant material. Time did not permit of making Van Slyke determinations on this material consequently no comparisons can be drawn between the results of the two methods.

The irregularities in the results obtained may be due in part to variations in the effects induced by the preliminary treatment with acetic acid. A certain amount of hydrolysis had apparently taken place during boiling because Nesslerization of the filtrates showed the presence of appreciable amounts of free ammonia whereas prior to this treatment only the merest traces were evident. Owing to the extreme complexity of the material at various stages of growth the effects of this extremely mild hydrolysis would tend to vary considerably. Furthermore, Vickery (1927) has shown that a very high degree of inconsistency exists with regard to the compounds

precipitated from plant extracts by phosphotungstic acid. This apparent lack of selective power on the part of phosphotungstic acid is explained by him as being due in part to the existence of certain complex interrelationships between the nitrogenous, and non-nitrogenous compounds present in plant extracts. At any rate the results obtained in this investigation tend to be in accordance with those of Vickery and thereby suggest the need of further study before any definite conclusions may be drawn.

Amide Nitrogen - The amide nitrogen of this investigation is that nitrogen not precipitated in the presence of phosphotungstic acid from the original water extract of alfalfa. The preliminary treatment with dilute acetic acid is omitted consequently the precipitation takes place under different conditions of concentration of the reagents. Possibly, in view of the methods of procedure followed, the amino and amide nitrogen results might be expected to approximate one another. They do not do so in every case, however, nor is there any definite relationship between the amounts of these fractions at all stages of growth in the various cuttings.

Nevertheless, attention should be drawn to the fact that amide nitrogen is present in smaller amounts than amino nitrogen at every stage of growth. Also, at the pre-bud and bud stages of growth in the second cuttings the two fractions very nearly approximate each other in the amounts obtained. At the seedling and pre-bud stages in the first cuts amino nitrogen does not greatly exceed amide nitrogen.

Some importance might, in addition, be attached to the fact that, at the later stages of growth in both the first and second cuttings amino nitrogen is present in much larger amounts than amide. A possible explanation is that the compounds not precipitated by phosphotungstic acid in the amide determination are of the same type as those formed during the processes of protein synthesis. Consequently, the proportion of these compounds would tend to decrease markedly throughout the later stages of growth. In the case of the amino determination, on the other hand, the conditions are such that a number of non-protein nitrogenous compounds, which would otherwise be removed, escape precipitation. Therefore it is possible that, in the later stages of growth, the more or less complex non-protein nitrogen compounds are of a type associated with the early phases of protein decomposition and consequently would be very readily decomposed by the mild hydrolysis with dilute acetic acid. Compounds of a corresponding degree of complexity formed as a result of synthesis would, in all probability, be of a slightly different structure as well as being in very intimate association with other non-nitrogenous products formed at the same time thus tending to render them impervious to the mild hydrolytic treatment.

SUMMARY.

A study has been made of the nitrogen distribution in alfalfa hay cut at varying degrees of maturity throughout the growing period. For the determination of the soluble forms of nitrogen a departure has been made in applying to this study the method developed by Wasteneys and Borsook for the fractional analysis of incomplete protein hydrolysates. In addition, the method evolved by Orla-Jensen and further developed by Eagles and Sadler, for the determination of amino and amide nitrogen has been employed. This was done with a view to determining whether or not the results obtained would approximate those derived by the methods usually employed for the estimation of these fractions in plant material.

The data for total nitrogen shows a relatively steady decrease throughout the growing period. Also, later cuts in the season exhibit a distinctly higher total nitrogen content than earlier cuts at the same stages of growth.

Total soluble nitrogen tends to exhibit considerable fluctuation throughout the growing period. Cuts made later in the season show a progressive decrease in the proportionate amounts of this fraction. Particularly striking is the finding that, in all cuttings, total soluble nitrogen tends to be present in very large amounts during the early part of flowering.

Results obtained from the application of the method of Wasteneys and Borsook make possible the presentation of a relatively clearly defined picture of protein synthesis and degradation throughout the growing period. This data further strengthens the assumption that the period between bud formation and the commencement of flowering represents a very critical stage in the life history of the plant.

No definite conclusions can be drawn from the amino and amide nitrogen results obtained in this investigation. However, there are indications that more consistent results might be obtained following a thorough trial of the method.

The possible relationships between nitrogen distribution and hay quality are also discussed. It is quite evident that, from the standpoint of protein content, later cuts in the season are superior to early cuts at the same stages of growth. Cutting of hay at the full bloom stage and later results in a lowered percentage of total crude protein per ton of hay. The possible effects of the soluble nitrogenous constituents on hay quality are not so clearly defined. However, since the amounts of each soluble constituent are so small it is probably safe to say that, individually, they do not exert any great influence on quality or feeding value.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. G. G. Moe for his support of the work described in this paper. Thanks are also due to Dr. D. G. Laird for assistance in the preparation of the manuscript, and to Dr. B. A. Eagles for much helpful advice with regard to the details of analytical procedure.

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