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CHEMICAL INVESTIGATIONS OF THE TOBACCO PLANT

V. CHEMICAL CHANGES THAT OCCUR DURING GROWTH

HUBERT BRADFORD VICKERY, GEORGE W. PUCHER,
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With the technical assistance of Laurence S. Nolan

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Hubert Bradford Vickery, George W. Pucher,
Charles S. Leavenworth and Alfred J. Wakeman*

INTRODUCTION

The study from the chemical point of view of the growth of plants is seriously circumscribed by the lack of suitable and accurate methods of analysis. Although the importance of an understanding of the reactions that occur in the cells of a developing plant can hardly be overestimated, the progress that has been made is most disappointing. It is obvious that a knowledge of the qualitative composition of plant tissues must precede the investigation of the quantitative relationships between the constituents; but this knowledge is still so elementary that one must be satisfied, for the most part, with quantitative measurements of groups of constituents rather than with individuals, with "forms" of nitrogen rather than with definite compounds, and with such thoroughly indefinite factors as the water-soluble organic solids, the ether extractives, or the fermentable carbohydrates.

Nevertheless, one has only to turn to the early literature of the subject to appreciate the very real advances that have come since Emmerling made his first attempt in 1880 to solve the problem of the origin of the amino acids found in plant tissues. Emmerling's investigation (11, 12, 13)† represents 20 years spent upon the analysis of a series of bean plants (*Vicia faba, major*) collected in the season of 1880. The time required gives some notion of the labor expended on these analyses. The nitrogen determinations were carried out by the Varrentrap and Will method of combustion with soda-lime, the amino nitrogen determinations by an elaborate but cumbersome modification of the Sacchse-Korman method—the forerunner of the present day Van Slyke method. Few determinations depended on volumetric methods; even ammonia was estimated from the weight of the ammonium chloride produced by allowing a sample of the tissue intimately mixed with lime to stand for three days in an evacuated apparatus that contained an absorption column moistened with

Note: The chemical investigations of tobacco herein described were carried out as part of a general project under the title "Cell Chemistry," by the Department of Biochemistry of the Connecticut Agricultural Experiment Station, New Haven, Conn. The Department has enjoyed the benefit of close coöperation from the Tobacco Substation. The expenses were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

*With the technical assistance of Laurence S. Nolan.

†Numbers refer to bibliography, page 607.

dilute hydrochloric acid. Time was a minor consideration in those days, and Emmerling was a conservative with respect to the adoption of newer and more convenient analytical methods. Nevertheless his results are of great significance. He expressed his data in terms of percentage of the dry substance and also in grams in 1000 plants. The curves that illustrate the accumulation of dry substance—nitrogen, amino nitrogen, and protein nitrogen in leaves, stems, pods, seeds, and roots—furnish a vivid picture of the growth of the plant, and of the migration of organic solids and of nitrogenous substances from the leaves to the seeds as these matured. The interesting part played by the seed pods, as an intermediate storehouse of nitrogenous substances later furnished to the seeds, is particularly well shown.

Emmerling's conclusions in the broader field of general metabolism are still of importance. He pointed out that the reserve protein of the sprouting seed undergoes enzymatic hydrolysis to its components; these migrate, together with nitrogen-free reserve substances (sugar, fat), to the centers of new growth where, under the influence of respiration with its attendant liberation of energy, the nitrogenous products are resynthesized into cell proteins. In the absence or deficiency of nitrogen-free reserve substances, the respiration is maintained at the expense of the nitrogenous components and, as a result, under these circumstances asparagine or glutamine become prominent products of the reactions. These substances in turn may provide a source of the ammonia required for protein synthesis. After the leaves have developed, assimilation of carbon becomes possible, and the incoming nitrogenous substances are rapidly converted to tissue protein inasmuch as energy derived from respiration is readily available. The further development of the plant involves the transport of inorganic nitrogenous substances from the soil, and their transformation into a form (probably ammonia) suitable for assimilation by combination with the carbon compounds produced by the process of oxidation in the leaves, these products then serving for the synthesis of cell protein.

Emmerling's investigations of plant growth are unique for their time. His views on the general subject of nitrogen metabolism are closely related to those of Schulze (32) who had reached similar conclusions from his extensive studies of the growth of seedlings. Most investigators of this period were chiefly concerned with the rate of accumulation of inorganic nutrients in the plant, the interest being mainly confined to the study of the drain upon the soil incident to the growth of the crop, and to the interpretation of the results in terms of the correct practice for the maintenance of fertility. The value of such studies to practical agriculture is obvious and, inasmuch as the chemical methods of ash analysis have long been highly developed, accurate and significant data were readily to be obtained.

Although data referring to the total nitrogen, the organic dry matter, the crude fiber, the ether extract, the carbohydrates, and the crude protein were frequently included in the reports on the study of the inorganic constituents, these factors were arrived at by purely conventional routine methods, and the data seldom admit of interpretation in terms of chemically distinct individual organic substances. Even when data on the nitrate,

the ammonia, and the amide nitrogen are given, critical examination of the methods of analysis that were employed frequently gives rise to doubt that the results have much significance in revealing the details of the physiology of organic growth.

The most important investigations of the inorganic constituents of plants throughout a part or the whole of the growth period have been contributed by experiment station workers in this country and abroad. Schweitzer (33), in 1889 at the Missouri Station, studied the growth of maize by analysis of the plant at approximately weekly intervals from the seed to maturity. He dissected the plant into root, stem, and leaf, and later into husk, tassels, silk, and ear as well, dried the material in an oven to constant weight, and determined the composition of the ash of each part with great care. The data were expressed for the most part in terms of per cent of dry weight, but the quantities in grams per plant were likewise given. Jones and Huston (19) in 1914 published the report of a somewhat similar investigation of the maize plant, the data for which had been collected in 1903 at the Indiana Station. In addition to the fresh and dry weights, and the detailed analyses of the ash of the different parts of the plant, they also determined the nitrogen, the crude fiber, the starch, the "fat", or ether extract, and the so-called "albuminoid" and "amide" nitrogen. The data were expressed in terms of pounds per 10,000 plants, or approximately one acre.

Perhaps the most elaborate of these early studies of plant growth is that of Wilfarth, Römer and Wimmer (51) at Bernburg, Germany, in 1903, who described investigations with wheat, barley, potatoes, peas, and mustard, giving analyses of the parts of the plants for dry matter, starch, potassium, sodium, nitrogen, and phosphorus. The data were expressed as per cent of the dry matter and in pounds per acre.

White (50), working at the Georgia Experiment Station, in 1914 recorded the ash composition of the cotton plant at four stages in its growth in terms of grams per plant and per cent of dry matter.

In recent years Knowles and his collaborators in England have again taken up this type of investigation. They have studied wheat (20) and also the sugar beet (21). Their analyses include data for the ash, and ash constituents, the dry matter, the nitrogen, and, in the case of the sugar beet, for the carbohydrates as well. The data are expressed both as per cent of dry matter, and as grams in a definite number of plants. They likewise provide calculations of the distribution of the more important constituents in the different parts of the plant.

The literature of plant composition at different seasons, as affected by various methods of culture or of fertilization of the soil, is, of course, very extensive. Attention need be drawn only to the work of Shive and his students (34, 38, 39) and to the investigations of the apple tree by Thomas (41) as examples. A full discussion of the early work of this nature on fruit trees is given by Gardner, Bradford and Hooker (15) and by Chandler (6).

The behavior of the nitrogen, dry matter, and of the ash constituents of the leaves of woody plants throughout the growing season has been studied by a number of workers. Tucker and Tollens (42) described a

very thorough and careful investigation of the leaves of *Platanus occidentalis,* in which data were expressed in terms of leaf area, in grams per definite number of leaves, and in per cent of the dry matter. Schulze and Schütz (31) studied the composition of maple leaves collected in the morning and evening at monthly intervals in the growing period, basing their results on the absolute quantity in 200 leaves of equal size. Swart (40) gave data at two stages (early summer and late autumn) for a number of species, and included in his report a very complete review of the earlier investigations on the problem of autumnal migration of nitrogen and inorganic constituents out of the leaves.

More closely allied in point of view with our own work is the investigation of Chibnall (7) who studied the composition of runner bean (*Phaseolus vulgaris v. multifloris*) leaves throughout the growing season, with special attention to the protein and to certain of the simpler nitrogenous constituents. He demonstrated that the leaf protein, although varying quite widely in the relative proportion present at different times, remained practically unchanged in composition. Culpepper and Caldwell (9) have recently studied the composition of the rhubarb plant (leaves, petioles, and root) at various stages of growth. They were particularly interested in the titratable acidity, the organic solids, both soluble and insoluble, and in the nitrogen.

Very little work of the general character of most of the papers mentioned appears to have been done on the tobacco plant. Davidson (10) in 1895 published analyses of Virginia tobacco plants for moisture, ash, and ash constituents at several stages of growth from seedling to maturity, and Carpenter (5) in 1893, in the course of a study of the composition of cured tobacco, gathered a few data on plants grown in North Carolina. No further detailed study of the growth of the tobacco plant has come to our attention until the work of Smirnov and his collaborators at Krasnodar appeared in 1928 (35, 36).

Smirnov's technic differed fundamentally from that of the American investigators. His interest was centered upon the relationship between the composition of the leaf and the surface area, and the changes in this relationship with the growth of the plant. The area of each leaf was determined from the weight of a section cut with a die of known area, the midrib being removed before weighing the fresh leaf. The cut sections, after being weighed, were employed for the determination of the dry weight; the residual material was preserved by treatment with hot alcohol, and was stored for analysis under alcohol. Determinations were made of the water-holding capacity of the dry tissue at 95 per cent humidity, of the peroxidase activity, of the carbohydrates (starch, dextrin, saccharose, maltose, monosaccharides), and organic acids (Fleischer's method (14); see also Vickery and Pucher (44, p. 163)) and of the total nitrogen, protein nitrogen (Barnstein's method (3)), formol titratable nitrogen (Sörensen (37)), ammonia, nicotine, and nitrate nitrogen. The samples examined ranged in age from seedling to mature plants the leaves of which were beginning to turn yellow; both normal and topped plants were employed. The detailed discussion of Smirnov's results will be deferred until after our own data have been presented (see p. 602).

The present study of the growth of the tobacco plant arose from the need of data on the composition, with respect to the carbohydrates, organic acids, and nitrogenous substances, at different stages of development of the plant, in order that material might be more intelligently selected for studies of the metabolism of this plant. We have attempted to collect chemical data from which as complete a picture as possible can be drawn of the synthesis of organic substances in the whole leaf and stem tissue, and later in the pods as well, during growth from the seedling stage to senescence. Many of the methods of analysis employed have been developed in this laboratory for special application to the tobacco plant.

The plants employed were of the variety known as Cuban Shade, or perhaps more generally, as Connecticut shade-grown tobacco. This variety is characterized by a high content of nitrogen in terms of the dry weight of the leaf, and a low content of carbohydrate, and is grown commercially exclusively for the production of cigar wrappers (16). The agricultural conditions under which the plants employed for the experiment were produced are thoroughly standardized and were carefully controlled by members of the scientific staff of the Tobacco Substation at Windsor, Connecticut. We are deeply indebted to them for their coöperation. The plants were grown under shade tents on a soil each acre of which had been dressed with a mixed fertilizer composed of:

2700	lbs.	ground tobacco stems
1000	"	cottonseed meal
100	"	potassium nitrate
200	"	fish meal
200	"	"Calurea"
100	"	precipitated bone
100	"	magnesian lime

The composition of this mixture was such as to supply approximately 200 lbs. of nitrogen, 100 lbs. of phosphoric oxide, 200 lbs. of potassium oxide and 75 lbs. of magnesium oxide per acre.

DESCRIPTION OF COLLECTIONS

A record of the dates at which collections were made, together with the weather conditions, is given in Table 1.

Collection A consisted of seedlings uprooted from the seedbed the day after the field had been set with plants taken from the same bed. The stems were separated from the roots just above the cotyledons. The leaves that were to be extracted were rinsed from adhering sand and dirt in a large vessel of water. The water was subsequently filtered, and the dry weight of the soil collected was subtracted from the weight of the leaves. The leaves that were to be dried were placed in a ventilated oven. After being dried they were carefully agitated to detach any sand, and this was collected and weighed. The corrected weights of the two stem samples were similarly obtained.

Collection B was taken after the seedlings had been set for 19 days. It is to be noted that the leaves of the plants in this sample were newly

developed; the leaves of the seedlings are almost invariably sloughed off after transplantation. The plants varied in size, most being within the range of 5 to 7 grams; a few were considerably heavier, the largest weighing 20 grams. Many plants had been attacked by the potato flea beetle, and a few leaves had been so badly damaged that it was necessary to discard them. Most of the plants had four leaves. The preparation of the samples and the corrections for adhering soil were made as before.

Collection C at 26 days was made by cutting the stem at the ground level. The plants bore four to eight leaves with a number of smaller ones at the top of the stem; this top portion was added entire to the leaf fraction. Corrections for adhering soil were made as before.

TABLE 1

Collection	Days from setting	Date 1933	Weather
A	1	June 1	Overcast.
B	19	June 19	Clear and cool; rain previous day.
C	26	June 26	Overcast; cold nights but clear most of week.
D	35	July 5	Irrigated with equivalent of 1.7 inches of rain previous day. Overcast and cold for two days. Growth normal for season.
E	40	July 10	Cool and mostly fine; plants a little backward.
F	47	July 17	Rainfall during past two months about half normal and plants about one week retarded. Light rain during night before collection.
G	54	July 24	No rain for past week; hot and humid.
H	61	July 31	Very hot; no rain since July 16.
I	75	Aug. 14	Rain during previous night, the first since July 16. Weather overcast.
J	97	Sept. 5	Heavy rain during previous week but fine on date of gathering.
K	110	Sept. 18	Heavy rain Sept. 13 to 17 (2.5 inches)

Collection D at 35 days consisted of plants that bore eight to twelve large, and a number of small leaves. There was very little soil on these plants and this was confined to the lower leaves. The stems had become notably hard and woody.

Collection E at 40 days consisted of rapidly maturing plants. Bottom suckers had been broken off and discarded a few days before the collection. The correction for soil on the leaves was negligible from this point on. Owing to the increased size of the plants, only 16 were taken for Sample I, and 14 for Sample II.

Collection F at 47 days consisted of plants about 4 feet high. The dry season had held the plants back about one week of their normal rate of development. Only 10 plants each for Samples I and II were collected from this point on.

Collection G was made at 54 days, three days after the commencement of the first picking (lowest four leaves) from all the plants in the field save those reserved for this experiment. The weather was hot and humid and the plants were somewhat wilted. A few plants in the field had begun to blossom.

Collection H at 61 days was the first in which an inflorescence sample was taken; this consisted mostly of buds. The weather had been extremely hot for some days and there had been no rain. The second picking of the crop had been completed during the previous week.

Collection I at 75 days consisted of mature plants with well-developed flowers. There had been rain the night before the collection. The gathering of the crop (fourth picking) had been completed.

Collection J, made 97 days from setting, was taken from plants left in the field under a small shade tent for this experiment. A heavy storm a few days earlier had damaged some of the plants severely. Erect and unharmed plants were selected. The seed pods were well developed, but a few blossoms remained on some of the plants.

Collection K at 110 days, the last of the series, was made chiefly to provide data on the influence of the setting of seed. Over 2.5 inches of rain had fallen during the previous five days, and most of the plants were badly damaged in spite of the protection afforded by the shade tent. The best possible selection of plants was made under the circumstances. Many bore fully ripe seed, although a few were still in flower.

In making the collections care was taken to select plants of a uniform state of development as near to that of the average condition of the plants throughout the field as possible. All collections were made at about 9 A. M., the material being then transported to the laboratory at New Haven.

PREPARATION OF MATERIAL

Each collection was subdivided into two roughly equal lots designated Samples I and II. The plants of each sample were dissected into leaf and stem, or later into leaf, stem, and inflorescence or pod portions.[1]

The leaves and stems of Sample I from each collection were separately extracted three times each, successively, with boiling water acidified to pH 3 to 4 with sulfuric acid, according to the technic described in Station Bulletin 324 (45). The complete leaf extracts amounted to from 25 to 40 liters, the stem extracts to from 15 to 30 liters. Previous to extraction, the stems were cut into short lengths and split into several pieces.

Each extract was allowed to stand overnight, and was then decanted from a small quantity of sludge. This sludge was separated by elutriation from any sand that had also settled, and was then washed by centrifugation and dried. When necessary, the sand was collected and weighed, the weight being applied as a correction on the fresh weight of the sample. The extracts were filtered through paper pulp and rapidly concentrated *in vacuo* in stills equipped with vapor coolers (46) until the sediment which separated made further concentration difficult. The sediments were then centrifuged, washed free from nicotine, dried, weighed, and ground for analysis. The solutions were made to a definite volume and preserved with a liberal quantity of toluene. The residues of both leaf and stem tissue were shredded and dried in a ventilated oven, being subsequently weighed and ground to a powder for analysis.

[1] A more elaborate dissection of the plants into lower, middle, and upper leaf and stem portions could not be attempted in the present investigation in spite of the great desirability of data of this sort. The term "stem" in the present publication refers to the main stalk of the plant; to the tobacco technologist, however, the term refers only to the midrib of the leaf.

Sample II from each collection, after dissection into leaf and stem portions, was dried at a temperature of from 80 to 90° and then weighed and ground to a fine powder. The oven employed for the stems was ventilated by a rapid stream of air heated to approximately 100°. Drying was complete within a few hours.

The inflorescence or pod samples consisted of flowers, and more or less well-developed seed pods. Each pod was cut from its pedicel separately, the denuded stalk being added to the stem sample. In all save one case, the inflorescence samples were dried for analysis. This was found to be necessary as the presence of the oil in the maturing seeds rendered hot water extraction impracticable.

The material accumulated for analysis consisted of the following preparations for each of the eleven collections of plants.

Leaf extract	Stem extract
Leaf sludge	Stem sludge
Leaf sediment	Stem sediment
Extracted leaf residue	Extracted stem residue
Dried leaf	Dried stem

In addition, dried inflorescence specimens from both Sample I and Sample II were obtained from the last four collections.

In order to simplify the presentation, it has seemed desirable to combine certain items of the analytical data. The sludges that separated from the leaf and stem extracts obviously consisted to a considerable extent of coagulated protein; this material should therefore be regarded as a part of the insoluble extracted residue, and the data for ash, inorganic solids and nitrogen of the sludges have been combined with the corresponding figures from the analyses of these residues. In the case of the younger plants, the solids and nitrogen of the sludge derived from the leaf tissue formed a very considerable part of the whole. The sludge from the stem extracts was so small, however, that complete analyses could be obtained only for the last three collections.

The sediments that separated from the voluminous leaf and stem extracts, after these had been concentrated to a relatively small volume, obviously represent soluble constituents of the respective tissues. The data for ash, organic solids, and nitrogen have therefore been added to the corresponding data from the analyses of the extracts. The sediments consisted very largely of calcium sulfate, and it was clear that the sulfate radical was derived from the sulfuric acid added to maintain a sufficient acidity during the extraction. The weights of these ashes obviously do not accurately represent the inorganic constituents of the tissue. The assumption was therefore made that the calcium was derived from calcium salts of organic acids and, further, that no significant error would be committed by regarding the whole of the ash from the sediments as calcium sulfate. The quantity of calcium oxide equivalent to this ash weight was therefore calculated, and the result was added to the inorganic solids of the respective extracts. We have previously shown (48) that, when this artifice is employed, the ash determined on dry leaf tissue corresponds closely with the sum of the ashes of the extract and extracted residue.

On standing, the concentrated leaf and stem extracts slowly deposited a further sediment. This was small in amount, and was therefore removed by centrifuging before aliquots were withdrawn from the extracts for analysis; the error involved in neglecting this additional sediment is undoubtedly insignificant.

ANALYTICAL METHODS

Analysis of Dry Leaf, Stem, and Inflorescence Samples

The **moisture content** of the thoroughly mixed specimen was obtained by drying weighed portions in porcelain capsules to constant weight in a vacuum desiccator over sulfuric acid. This usually required four to five days.

The **ash** was obtained by igniting the dried residue in an electric muffle.

Total nitrogen was determined by the Kjeldahl method, with mercury as catalyst, after previous reduction of the nitrate with dilute sulfuric acid and reduced iron powder, according to the method of Pucher, Leavenworth and Vickery (25).

Ammonia nitrogen was determined by distillation in the presence of magnesium oxide *in vacuo*. The apparatus consisted of a 500 cc. Pyrex round-bottomed flask fitted with two necks. A bulb trap was attached to the central and larger neck by a standard taper ground-glass joint, and the vapor tube leading from the trap was bent to descend vertically into the receiver. This consisted of a 25 by 250 mm. heavy-wall test tube graduated at 50 cc., and fitted with a two-hole rubber stopper to accommodate the vapor tube and the wide-bore tube[1] leading to the vacuum pump. The receiver was immersed in a beaker of cold water in lieu of a condenser. The second and smaller neck of the distillation flask was fitted, also by means of a standard taper ground-glass joint, with an air inlet tube bent so as to reach nearly to the bottom of the flask. The air entering through this tube was washed with dilute acid contained in a small gas-washing bottle.

To conduct a determination, a 0.500 gm. sample of the dry powdered tissue was transferred to the distillation flask, and 5 cc. of a 12.5 per cent suspension of light magnesium oxide powder in water were added, together with 30 cc. of water. The receiver was charged with 3 cc. of 0.1 N hydrochloric acid. The apparatus was closed and evacuated, care being taken to allow sufficient air to enter through the air inlet tube to agitate the contents of the flask thoroughly. The air supply was then cut down to a slow current, and a water bath, previously heated to 40°, was raised under the distillation flask so as to cover it almost completely. Distillation was allowed to continue for 15 minutes, when the vacuum was released and the receiver was disconnected. The vapor tube was washed into the receiver with a little water, and 5 cc. of Nessler's solution (22) were

[1] Loss of a little ammonia from the distillate may occur unless the tube leading to the pump is of sufficiently wide bore, or is furnished with a small bulb. This tube should ascend vertically for 6 to 10 cm. above the stopper before being bent. It should invariably be washed back into the receiver at the termination of a distillation.

added to the contents with agitation; the distillate was then diluted to 50 cc. The color produced was read in a Pulfrich photometer with light filter S-43, using an ammonia-free solution that contained 5 cc. of Nessler's reagent diluted to 50 cc. in the blank cell. The ammonia value was read from a calibration chart in which the extinction coefficient was plotted against milligrams of ammonia nitrogen. Blank determinations on the apparatus and reagents were made periodically, and were remarkably constant at 0.006 to 0.008 mg. of ammonia nitrogen. It was found necessary to conduct a blank distillation at the beginning of each day's work and discard the distillate. This served to remove a substance[1] which accumulated in the standing apparatus overnight and gave rise to a slight turbidity with Nessler's reagent.

Quantities of from 0.02 to 2.0 mg. of ammonia can be determined with an error not exceeding 0.005 mg. by this technic, provided photometer cells are available that give readings between 30 and 80 per cent transmission on the instrument. Usually the 5 or 10 mm. cells were used, but a 30 mm. cell was required to read the blank determinations.

Check analyses demonstrated that nicotine is not volatilized under the described conditions in sufficient quantity to interfere with the determination of the ammonia.[2]

Nitrate nitrogen was determined by the method of Pucher, Vickery and Wakeman (27).

Glutamine amide nitrogen[3] was determined by hydrolyzing 0.200 gm. of the tissue in the presence of 10 cc. of a phosphate-borate buffer at pH 7.0 for 2 hours in a boiling water bath. The hydrolysis was carried out in a test tube closed with a stopper that carried a short length of fine-bore tubing to serve as a condenser. The reaction at the end of this operation was in the range pH 6.2 to 6.6. The resulting suspension was transferred with 10 to 15 cc. of water to the ammonia distillation apparatus already described; 2.0 cc. of 1 N sodium hydroxide, and 15 cc. of water were added. The ammonia was then distilled off, and the increase over the quantity of ammonia already present in the tissue was taken as the glutamine amide nitrogen. Owing to the presence of the phosphate buffer, the use of sodium hydroxide was found to be essential to successful quantitative distillation of the ammonia. The presence of magnesium was found to be objectionable because of the possibility that magnesium ammonium phosphate might be formed in sufficient amount to prevent quantitative removal of the ammonia within the time allowed for the distillation.

Asparagine amide nitrogen: An extract of the dry tissue was prepared by suspending 2.50 gm. of the powder in 25 cc. of water in a beaker, and boiling for 3 minutes with constant stirring. The solution was cooled and the aqueous volume made to 50 cc. The suspension was then centrifuged, and aliquots of the clear fluid were withdrawn for determinations of asparagine amide nitrogen, and of the amino and peptide nitrogen. Extracts of the pod tissue were prepared in the same manner from the residues after previous extraction of the substances soluble in ether.

Asparagine amide nitrogen was determined by heating 5 cc. of the extract together with 1 cc. of 6 N sulfuric acid in a 25 by 250 mm. test tube in a boiling water bath for 3 hours. The contents of the tube were transferred to the ammonia distillation apparatus, 15 to 20 cc. of water, 5 cc. of N sodium hydroxide, and 5 cc. of 12.5 per cent suspension of light magnesium oxide were added, and the determination of ammonia was conducted as already described. The asparagine amide nitrogen was calculated by subtracting the sum of the free ammonia nitrogen and of the glutamine amide nitrogen from the ammonia nitrogen found after hydrolysis of the extract with normal acid.

Amino nitrogen was determined by transferring 10 cc. of the extract to the ammonia distillation apparatus where it was freed from ammonia by distillation with magnesium oxide. The residue was treated with 2 cc. of glacial acetic acid to dissolve the magnesia, and was transferred to a 50 cc. flask and made to volume. Amino nitrogen was determined in 5 cc. aliquots of this solution in the Van Slyke manometric apparatus according to the directions of Peters and Van Slyke (24). It should be noted in passing that this method is much superior both in accuracy and convenience to the earlier methods of Van Slyke that involve the special amino nitrogen apparatus.

Peptide nitrogen was determined by transferring 5 cc. of the extract to a 25 by 250 mm. test tube together with 5 cc. of 12 N sulfuric acid; the mixture was heated in a boiling water bath for 6 hours, and was then washed into the ammonia distillation apparatus with 10 to 15 cc. of water; 10 cc. of 5 N sodium hydroxide, and 5 cc. of 12.5 per cent magnesium oxide suspension were added, and the ammonia was distilled off and determined in the usual way. The residue was acidified with 2 cc. of glacial acetic acid and was made to 50 cc. Amino nitrogen was determined as before in this solution. The peptide nitrogen represents the increase in amino nitrogen on hydrolysis with 6 N acid.

The presence of glutamine in the tissues of the tobacco plant renders the results of this method somewhat untrustworthy. Chibnall and Westall (8) have shown that the deamidation of glutamine in hot neutral solution is accompanied by a loss of amino nitrogen. This probably results from ring formation with the production of pyrrolidone carboxylic acid. If, during the preparation of the tissue for analysis (e.g. during drying), a part of the glutamine present has undergone deamidation, this part will be represented in the material subjected to analysis by pyrrolidone carboxylic acid, a soluble substance which, on hydrolysis with strong acid, yields glutamic acid with a resultant increase in the amino nitrogen of the solution. That this source of error may be significant is shown in the discussion of the analysis of the stem samples on a later page.

Total organic acidity and **oxalic acid** were determined as described by Pucher, Vickery and Wakeman (28). According to this method, the

[1] This interfering substance was eventually found to arise from a spontaneous change in the rubber stopper used to close the receiver. The difficulty could be avoided either by boiling this stopper with alkali at the beginning of a day's work, or by simply running a blank distillation. The latter seemed preferable.

[2] An improved modification of this method to determine ammonia is given by Pucher, Vickery and Leavenworth, Ind. and Eng. Chem., Anal. Ed., **7**: 152. 1935.

[3] The data upon which this method to determine glutamine is founded are given by Vickery, Pucher, Clark, Chibnall and Westall (in press). This paper also contains later improvements in technic.

organic acids are extracted from the acidified tissue with ether and transferred to aqueous alkaline solution. The total organic acidity is obtained by titration at the quinhydrone electrode between the limits pH 7.8 and 2.6. Oxalic acid can be titrated only to the extent of 50 per cent under these conditions; the oxalic acid is therefore separately determined in a portion of the organic acid solution, by precipitation as calcium salt, and a correction of the titration value is made. A correction factor is also applied to allow for the fact that malic and citric acids are titrated only to the extent of approximately 90 per cent under the described conditions.

Malic acid was determined by the method of Pucher, Vickery and Wakeman (29). According to this method an aliquot part of the organic acid solution, obtained as described above, is oxidized with potassium permanganate in the presence of bromine; the volatile oxidation product is distilled with steam and converted to its dinitrophenylhydrazone, which is filtered off and dissolved in pyridine. A portion of the pyridine solution is made alkaline with sodium hydroxide, and the blue color produced is read in a Pulfrich photometer. The quantity of malic acid is then obtained from a calibration curve in which the extinction coefficient is plotted against known quantities of malic acid that have been treated in the same manner. The method is capable of estimating from 0.1 to 2.5 mg. of malic acid with an accuracy of ± 5 per cent.

Citric acid was determined by the method of Pucher, Vickery and Leavenworth (26) which is a modification of the pentabromoacetone method of Hartmann and Hillig (18). A portion of the organic acid solution, obtained as described above, is oxidized with potassium permanganate in the presence of potassium bromide, the pentabromoacetone produced is extracted with petroleum ether, dehalogenated with sodium sulfide, and the bromide ion is titrated with silver nitrate. The method is capable of estimating from 1 to 20 mg. of citric acid with an accuracy of ± 5 per cent.

The **ether extract** was determined by drying the sample in an atmosphere of hydrogen for 5 hours at 100° and subsequently extracting with absolute ether for 15 to 20 hours. The residue, after evaporation of the ether, was dried to constant weight in a steam oven.

For the determination of the carbohydrates, an alcohol extract of the dried tissue was prepared. The alcohol was purified before use by distilling absolute alcohol over potassium hydroxide, and the neutral distillate was diluted to 75 per cent by volume with water. Five grams of the dried tissue were placed in a paper thimble and extracted with the diluted alcohol in a continuous extraction apparatus for at least 6 hours. The extract was concentrated *in vacuo* to remove alcohol, and the aqueous residue was made to 100 cc. and preserved with toluene.

Total soluble carbohydrate was determined in 10 cc. aliquots as described by Vickery, Pucher, Wakeman and Leavenworth (48, on pp. 74 and 75). Minor difficulties encountered in the determination of the unfermentable carbohydrate in the solutions prepared with mercuric sulfate led to the development of a modified method of preparing the solutions for this determination.

A 10 cc. aliquot of the solution obtained by alcohol extraction of the tissue was treated with 1 cc. of 5 N sulfuric acid and heated at 75° for 12 minutes to invert the sucrose. The solution was then diluted with 30 to 40 cc. of water, and neutralized to litmus with cold saturated barium hydroxide solution; 10 cc. of 0.2 N oxalic acid were added, and the solution was made to 100 cc., centrifuged, and then poured through a dry filter to remove any turbidity present. A 50 cc. aliquot of the filtrate was gently shaken with 5 gm. of Lloyd reagent in a 125 cc. Erlenmeyer flask for 4 minutes; 3 gm. of permutit were added, and the flask was again shaken gently for 3 minutes. The solution was then centrifuged clear. A blank of 10 cc. of distilled water was carried through the same operations. The total reduction was determined on 2 cc. portions of the Lloyd reagent filtrate by heating with 2 cc. of 0.01 N ferricyanide and 6 cc. of water for 15 minutes in a boiling water bath and proceeding as described by Vickery, Pucher, Wakeman and Leavenworth (48, p. 75).

The **unfermentable carbohydrate** was determined after removal of the fermentable sugar with yeast. A yeast suspension was prepared as described by Vickery, Pucher, Wakeman and Leavenworth (48, p. 76) and 4 cc. were transferred to a centrifuge tube and centrifuged at high speed until the cells were firmly packed together. The fluid was poured off and the residual moisture was removed from the walls of the tube and the surface of the yeast with dry filter paper; 10 cc. of the Lloyd reagent filtrate were added, and the cells were thoroughly mixed with the solution and allowed to react for 10 minutes at room temperature. A blank determination on 10 cc. of the Lloyd reagent blank solution was similarly conducted. After the fermentation was complete, the yeast was centrifuged down, and 2 cc. aliquots of the clear fluid were treated with ferricyanide as before.

Comparisons of the sugar content of solutions as prepared in this way, and of solutions prepared according to the mercuric sulfate clarification technic, showed that equally satisfactory results were obtained by either procedure.

Crude fiber was determined according to the standard conventional procedure (2).

Analysis of Leaf and Stem Extracts

Samples of the extract were centrifuged until perfectly clear bef aliquot parts were withdrawn for analysis; the small quantity of sluc so removed was neglected.

Total solids were determined by evaporating 10 cc. samples in porcelain capsules to dryness on a steam bath. The residue was then dried to constant weight in a vacuum desiccator over sulfuric acid. Constant weight was attained in about 120 hours with the extracts from the younger collections: the older ones required from 15 to 20 days.

The **ash** was obtained by igniting the dry residue in an electric muffle at 560 to 600°.

Total nitrogen was determined in 5 cc. aliquots by the method employed for the dry-leaf samples.

Nicotine was determined in 5 cc. aliquots, after distillation with steam from alkaline solution, by precipitation with silicotungstic acid (1).

Ammonia nitrogen was determined in 5 cc. aliquots of the extract as already described.

Amide nitrogen was determined by hydrolysis with 1 N sulfuric acid for 3 hours in a boiling water bath. To this end, 5 cc. of extract, 5 cc. of water, and 2 cc. of 6 N sulfuric acid were heated as already described in a 25 by 250 mm. test tube for 3 hours. The ammonia was then determined in the usual way. The increase in ammonia brought about by the acid hydrolysis was calculated as amide nitrogen.

The amide nitrogen of these extracts does not include the glutamine amide nitrogen; any glutamine present in the tissue would have been hydrolyzed during the preparation of the extract.

6 N acid hydrolyzable ammonia: The ammonia liberated by hydrolysis for 6 hours with 6 N sulfuric acid was determined in order to obtain evidence for the presence of substances that can be decomposed only by severe hydrolysis with the production of ammonia. Certain of the purines are partially decomposed in this way. The data are not to be considered as determinations of adenine or other purine derivatives; at present we prefer to interpret this factor in a purely empirical manner. To carry out the hydrolysis, 10 cc. of the extract were diluted to 50 cc., and 3 cc. of this were mixed with 3 cc. of 12 N sulfuric acid in a test tube, as already described, and heated in a boiling water bath for 6 hours. The contents of the tube were transferred to the ammonia distillation apparatus, 8 cc. of 5 N sodium hydroxide, 10 cc. of 12.5 per cent magnesium oxide and 10 to 15 cc. of water were added, and the ammonia was distilled *in vacuo* and estimated in the usual way. The increase over the ammonia produced by 1 N acid hydrolysis was calculated and is given as the 6 N acid hydrolyzable ammonia.

The so-called **easily hydrolyzed amide nitrogen** was calculated by subtracting the free ammonia nitrogen of the dry leaf and stem samples (Samples II) from the free ammonia nitrogen of the respective hot water extracts (Samples I).

Analysis of Sediments, Sludges, and Extracted Residues

Total nitrogen was determined by the Kjeldahl method, **moisture** and **ash** by the methods described under analysis of the dry leaf and stem samples.

No attempts were made in the present investigation to obtain a direct measure of the protein nitrogen of the leaves or stems. Although several methods which are supposed to furnish information regarding the protein nitrogen of plant tissue are described in the literature, none of these, in our opinion, provides trustworthy data. For the present, therefore, we have been compelled to assume that the nitrogen which remains insoluble, when a sample of fresh tissue is thoroughly extracted with hot water, is at least a representation of the quantity of protein nitrogen of that tissue. Such results are undoubtedly too high, and it is the purpose of investigations, now being conducted in this laboratory, to arrive at an idea of how much too high they may be. Information already at hand suggests that appreciably more than 10 per cent of the insoluble nitrogen belongs to non-protein substances.

For reasons that have been discussed elsewhere (43), we have also omitted all reference to the basic nitrogen of the extracts from the tissues.

Expression of the Data

The problem of presenting data derived from the analysis of a series of living organisms of different size and age in such a way as to provide a vivid and easily grasped picture of the nature of the changes that have occurred during growth is extremely difficult. The usual bases upon which such analytical data are calculated in terms of per cent all vary during the period of growth, and the only factor that remains constant is the biological unit itself, in this case the individual plant. Calculation of the ratio between, for example, the fresh weight and the total nitrogen shows how this ratio progressively changes during the growth period, but gives no idea whatever of the actual quantities either of nitrogen, or of total plant mass, involved. It refers exclusively to relative concentration of the nitrogen in the plant, and therefore cannot serve as a measure of the growth process. The same restriction applies to calculations on any other of the customary bases of percentage, such as dry weight, residual dry weight, i.e. the dry weight exclusive of the carbohydrates (23), or the total nitrogen. If, however, the biological unit is accepted as a basis of comparison, that is, if the comparisons are made on a basis of quantity of any constituent per individual plant, it is possible to present a picture of the growth process that clearly reveals the quantities involved. Furthermore data so calculated readily lend themselves to recalculation on any percentage basis that may subsequently seem desirable.

The fundamental data of the present investigation have therefore been computed in terms of grams per plant, and the curves plotted from these data show the actual change in the weight of each factor, within the limits of experimental error, as growth progressed. The most serious disadvantage of this method is the inconveniently small magnitude of the quantities involved in the analysis of the earlier collections; this makes graphical presentation on a uniform weight scale difficult. On the other hand, the analyses of these earlier collections, being based upon a much larger number of individual plants, are undoubtedly relatively more accurate than those of the later collections where only a few plants could be dealt with.

Reference should be made at this point to the able presentation given by Gouwentak (17) of the difficulties of the proper expression of the data of plant analysis. Her critique of the methods that have been commonly employed merits the most careful attention. For comparisons between the results of analysis of small numbers of leaves at short time intervals, she prefers to express the data in terms of leaf surface area, and rejects the methods of expression in terms of percentage of the fresh or dry weight. In spite of certain advantages, however, this method is clearly impractical for use in our case.

In addition to expressing the data in terms of weight per plant, in some cases the distribution of certain of the constituents among leaf, stem, and pods has been calculated. These results are presented in a separate section together with calculations of the percentage distribution of the various forms of nitrogen of the leaves and stems. In a few cases, however, it has seemed desirable to give calculations of the percentage distribution of certain constituents, notably the organic acids and carbohydrates, along with the discussion of the weight data.

The data have been plotted in the charts upon a uniform time scale; the scale of ordinates, however, varies from figure to figure, being selected in each case so as to give curves that are as clearly separated from each other as possible. In most cases the points of observation are represented by circles, and the progression of the quantity is represented by a solid line. Occasionally it has been necessary to employ crosses and dotted lines to facilitate the exposition, or to prevent possible confusion of the curves.

The analytical data are collected into tables that appear at the back of the present publication. The second column shows the figure in which each set of data is plotted; the third column indicates to which of the two samples from each collection the data in the following columns refer.

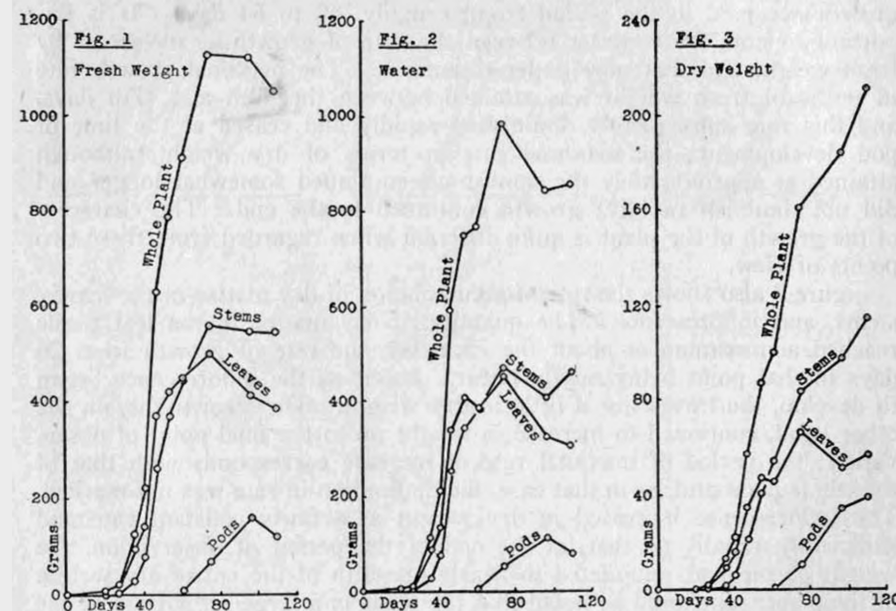
It will be noted that the greater part of the data is derived from the analysis of the dried leaf and stem samples herein designated as Sample II. In certain cases the same constituent was determined both in Sample II and in Sample I which had been subjected to hot water extraction. When this was done the agreement between the results from the early collections was, for the most part, satisfactory, but the agreement between the later collections of only 10 plants per sample was occasionally not good. Although great pains were taken at each of these later collections to obtain 20 plants of uniform size and development for the two samples, it is clearly impossible to rely implicitly on so small a group as 10 plants for such detailed analyses as the present study involves. Furthermore, as the plants aged, individual variations became more pronounced, and the accidents incident to the bad weather of the last few weeks of the season inevitably had their effect upon the results. The assumption involved in such a method of collection is that each lot of 10 plants represents with sufficient accuracy what the next previous lot of 10 plants would have been had they remained in the field. Our experience indicates that this requirement is extremely difficult to satisfy.

In this connection the experience of Knowles, Watkin and Hendry (21) is of interest. They based their data upon collections of 64 plants (sugar beets) but, in order to obtain samples of manageable size, were compelled to divide the individual plants of the older collections into quarters, retaining only one-quarter of each. In spite of the great care with which their material was handled, minor irregularities of the same type as those we have encountered appeared in their data.

THE GROWTH OF THE TOBACCO PLANT

Growth in Terms of Fresh Weight

The fresh weight of one plant, calculated from the average weight of all the plants taken at each collection, is plotted in Figure 1. During the first three weeks after being set, there was very little increase in weight of the tops; the original seedling leaves seldom survive the operation of transplantation, and practically the whole of the weight at 19 days from setting represents the development of the seedling bud. Doubtless considerable growth of the root system occurred in this interval inasmuch as the tops more than trebled their weight in the ensuing week, after the plant had become established. Thereafter the rate of growth rapidly accelerated, the slope of the growth curve reaching a maximum between the 40th and 47th days; subsequently the rate diminished until the plant began to produce seed, when no further increase in fresh weight occurred.



The relative proportions of stem and leaf tissue changed materially during the period of rapid growth. At 35 days only about one-quarter of the total fresh weight consisted of stem tissue, but at 61 days the weight of stem tissue equaled that of the leaves and subsequently exceeded it. The weight of stem tissue reached a maximum at 75 days and thereafter changed but little; the weight of the leaf tissue diminished during the development of the inflorescence. The precise magnitude of these later changes is in some doubt because of the sampling error involved in the selection of the older plants.

The water content of the plants of Sample II of each collection is plotted in Figure 2. The curves are less smooth than those in Figure 1 because

the data are calculated from the analysis of a smaller number of individual plants; they show the rapid rise to a maximum water content at the 75th day, and a subsequent loss of water from the leaves as the inflorescence developed. The water content of the stems somewhat exceeded that of the leaves after the 75th day.

Growth in Terms of Dry Weight

The dry weight per plant calculated from the analyses of Sample II from each collection is shown in Figure 3. After an initial period (40 days) of slowly accelerating rate of growth, the plant began to lay down dry matter at a practically constant rate; the growth curve from 40 to 75 days can be quite satisfactorily smoothed to a straight line that does not depart from the observations by a quantity greater than the experimental error. The two last observations show that the rate of accumulation of dry matter subsequently slowed down somewhat. A careful examination of the growth curve indicates that the maximum rate of growth occurred in the period from, roughly, 40 to 54 days. It is important to note the contrast between the rate of growth as measured by fresh weight and that now under discussion. The maximal growth rate in terms of fresh weight was attained between the 40th and 47th days, and this rate subsequently diminished rapidly and ceased at the time of pod development; the maximal rate in terms of dry weight, although attained at approximately the same time, continued somewhat longer and did not diminish rapidly; growth continued to the end. The character of the growth of the plant is quite different when regarded from these two points of view.

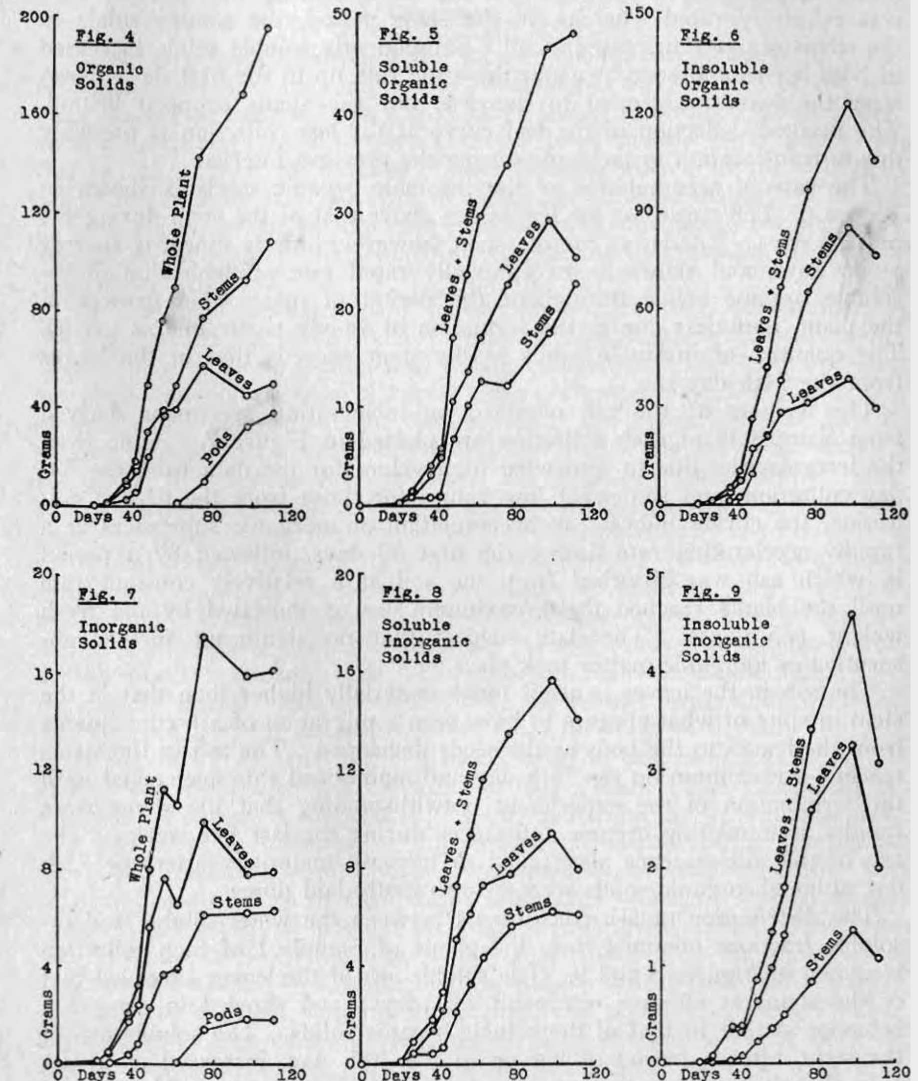
Figure 3 also shows the rate of accumulation of dry matter in the leaves, stems, and inflorescence. The quantity of dry matter in the leaf tissue reached a maximum at about the 75th day, the rate of growth from 26 days to that point being nearly linear. Later, as the inflorescence began to develop, the leaves lost a little in dry weight. The stem tissue, on the other hand, continued to increase in weight up to the final point of observation; the period of maximal rate of increase corresponds with that of the whole plant and, as in that case, the diminution in rate was not marked. The inflorescence increased in dry weight at a fairly constant rate and sufficiently rapidly so that, at the end of the period of observation, the weight of the pods amounted to nearly one-fifth of the entire dry weight of the plant. It should be noted that the seeds in a large proportion of the pods taken at the last collection were far from fully developed, and it is clear, from the tendencies of the curves, that seed development places a heavy drain upon the organic substance of the leaves.

Organic Solids and Ash

The quantities of organic solids, plotted in Figure 4, give curves that closely resemble those of the total dry weight and require little additional comment. The most notable feature is the evidence of continuing accumulation of organic solids at a relatively steady rate long after the total mass of the plant as measured by the fresh weight (see Figure 1) had ceased to increase. The change is due to the development of the in-

florescence and of the stem, these over-balancing the loss of solids by the leaves.

Figure 5 shows the quantities of water-soluble organic solids in the leaves and stems of the plants of Sample I from each collection. A careful



consideration of the data of the last three collections shows that sampling errors render difficult strict comparisons between the plants of Sample I used for water extraction, and those of Sample II which were dried. The earlier collections, however, yielded data that mutually support each other and are consistent. If the curves of Figure 4 are compared with

those of Figure 5, it is clear that the distribution of water-soluble organic solids between leaves and stem followed a course quite different from the distribution of total organic solids. At all save one point (26 days) the leaves contained more soluble organic solids than the stem. Furthermore, the rate of accumulation in the leaves during the first 40 days was relatively rapid whereas, in the same period, the soluble solids in the stems scarcely increased at all. Subsequently soluble solids increased in both leaves and stem at about the same rate up to the 61st day; thereafter the leaves continued to increase, but the stems dropped behind. The marked deflection of the leaf curve at the last collection is probably due to translocation to the seeds during the previous interval.

The rate of accumulation of the insoluble organic solids is shown in Figure 6. The curve for the leaves lies above that of the stem during the first 54 days. The curve for the stem, however, inflects upwards sharply at 40 days, and shows an exceptionally rapid rate of deposition of insoluble organic solids throughout the period of most rapid growth of the plant, doubtless due to the formation of woody tissue in this period. The quantity of insoluble solids in the stem exceeds that in the leaves from the 54th day on.

The weights of the ash obtained on incinerating specimens derived from Sample II of each collection are plotted in Figure 7. Aside from the irregularities due to somewhat high values for the data from the 54-day collection, and somewhat low values for those from the 61-day collection, the curves indicate an accumulation of inorganic substances at a rapidly accelerating rate during the first 40 days, followed by a period in which ash was absorbed from the soil at a relatively constant rate until the plants reached their maximum size as indicated by the fresh weight (75 days). The data suggest that no significant further absorption of inorganic matter took place.

The ash in the leaves is at all times materially higher than that in the stem in spite of what appears to have been a migration of ash constituents from the leaves to the pods as the seeds developed. The ash in the stems reached a maximum on the 75th day and maintained this unchanged until the termination of the experiment notwithstanding that the stems were rapidly accumulating organic substances during the last five weeks. The ash of the inflorescence also failed to increase materially after the 75th day although organic solids were being rapidly laid down.

The distribution of ash constituents between the water-soluble and insoluble fractions obtained from the plants of Sample I of each collection is shown in Figures 8 and 9. The soluble ash of the leaves exceeded that of the stems at all save one point (26 days) and showed in general a behavior similar to that of the soluble organic solids. The soluble ash of the stem, after a period of lag up to the 40th day, increased at a rate only slightly less than that of the soluble ash of the leaves. There is no evidence of a marked drop in soluble ash in the stem at the last collection, and the general behavior of the curves suggests a migration of ash constituents from leaves to inflorescence at this time. The data of Figure 7, derived from the plants of Sample II, support the same view.

The insoluble ash constituents of the leaves also exceeded those of the stem at all times. The curve for the leaves shows the same drop at the time of the final collection as does the curve for soluble ash, again suggesting migration from leaves to inflorescence of inorganic constituents.

Organic Acids

The total organic acidity of the tobacco plant, plotted in Figure 10, represents corrected titration values between pH 7.8 and 2.6 of the organic acids that can be extracted by ether from the acidified tissue. The data are expressed in milliequivalents per plant instead of in grams, as this permits closer comparison of the chemical effects of the different acids; furthermore it is possible to assign an accurate magnitude to the unknown acids when so expressed inasmuch as the factor for the conversion of these unknown acids from equivalents to grams is, of course, not known.

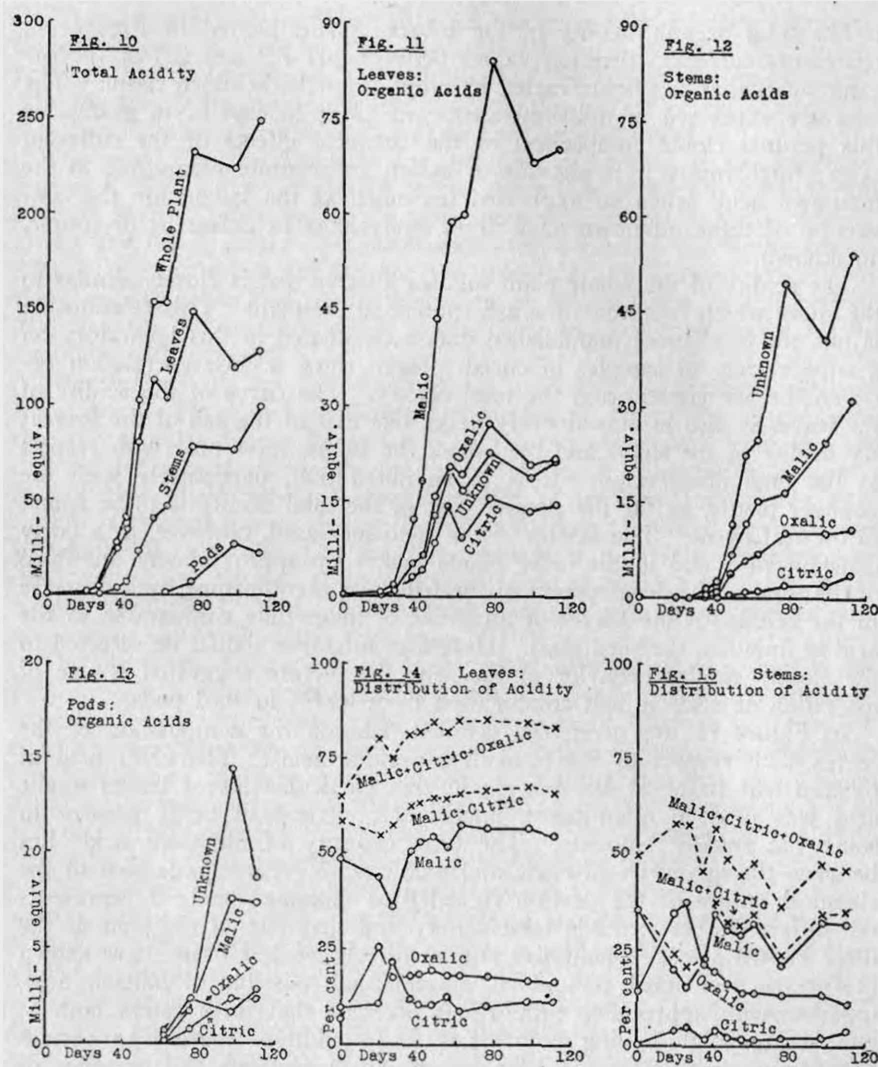
The acidity of the whole plant follows a curve that is closely similar to the curve which represents the ash content of the plant. This relationship is not one of chance; unpublished data accumulated in this laboratory on a wide variety of samples of cured tobacco show a close correlation between the ash content and the total acidity. The curve of the acidity of the leaves is also in almost every detail like that of the ash of the leaves; the acidity of the stems and the ash of the stems differ only with respect to the final observation. It is to be noted that, particularly with the younger plants, by far the greater part of the total acidity is to be found in the leaf tissue. The acidity of the stem increased, however, at a fairly constant rate, and in the older plants makes up approximately one-third of the total. The development of the fruit was accompanied by a decrease in the acidity of the leaves of an order of magnitude comparable to the acidity found in the seed pods. Here also attention should be directed to the closely similar behavior of the ash; the picture suggested is one of migration of acids in salt combination from leaves to seed pods.

In Figure 11 are given the detailed data on the composition of the leaves with respect to the individual organic acids. The chief acid of tobacco leaf tissue is malic acid; in this particular lot of leaves oxalic acid was next in quantitative importance, citric acid being present in somewhat smaller amounts. The total quantity of unknown acids lies between the quantities of citric and oxalic acids. Little is known of the chemical nature of the portion recorded as unknown acid; it represents the difference between the total acidity and that due to the sum of the three known acids. Qualitative studies of tobacco leaf tissue have shown the presence of small amounts of succinic, and possibly of fumaric acid, together with appreciable proportions of acids that form esters both of low and of high boiling points (44). In addition there is a certain quantity of substances the behavior of which suggests the presence of phenolic groups.

A brief study of the acids of the leaves of collection F at 47 days has shown that approximately one-third of the acidity belonging to unknown acids is insoluble in water although soluble in ether. All that can be said, therefore, about the chemical nature of the substances in the

unknown fraction is that the evidence points to the presence in it of a complex mixture of acidic substances.

The rapid increase of malic acid to a maximum value at 75 days was followed by a moderate drop during the period of fruiting. The oxalic acid behaved in a similar manner; the citric acid, however, reached a



maximal value somewhat earlier and, with minor fluctuations, maintained it to the end. The marked drop at 61 days in all of the curves is undoubtedly due to the somewhat low relative weight of the plants of Sample II collected at that time, and may be regarded as sampling error; an

analogous drop was not evident in the data obtained from the leaves of Sample I of the same collection.

The distribution of the acids in the pods (Figure 13) is quite different from that of the leaves. The unknown acids make up the greater part of the acidity; malic acid is present in considerable amount, but oxalic and citric acids are found only in minor quantities.

In order to give an idea of the relative acid distribution of the leaves, the data are plotted in another manner in Figure 14. The quantities shown in solid lines represent the percentage of the total acidity as each of the three known acids. The sum of the malic and citric acid is shown by the lower broken line, and the sum of the malic, citric and oxalic acids by the upper broken line. This line, therefore, is a plot of the relative proportion of unknown acid if the ordinates are reversed and read downwards. The area between the malic acid curve and the lower broken line represents the relative proportion of citric acid; that between the two broken lines, the relative proportion of oxalic acid; and the area above the upper broken line represents the relative proportion of unknown acids.

The variations shown by the curves in the relative proportions of the acids in the young plants are undoubtedly significant. These collections included much larger numbers of plants than did the later collections, so that the sampling error is much smaller. The chemical data are of equal accuracy throughout.

In the youngest plants the proportion of unknown acids (36 per cent) approached the proportion of malic acid (47 per cent); the proportions of oxalic and citric acid were relatively small. During the earliest phases of growth the oxalic acid increased rapidly in relative quantity and the unknown acids decreased; malic acid decreased slightly, and citric acid increased a little. Then, at the point when the seedlings had become established and the period of rapid growth was beginning, citric acid for a short time increased and both malic and oxalic acids decreased. From then on, the relative proportion of oxalic acid stayed constant, citric acid diminished within two weeks to its previous level and thereafter remained constant, and malic acid, at first rapidly and then slowly increased; at 61 days, however, it also reached a level that was maintained to the end of the period studied. If attention is confined to the period after 40 days, which is that in which the most rapid growth of the plant as a whole took place, the relative proportions of the three most important organic acids are practically constant. In other words these three acids are produced in the metabolism of the leaves, during the period of most rapid growth, in amounts that are essentially constant relative to each other. This implies that the unknown acids are likewise produced at a fixed rate. Reference to Figure 10 shows that from 26 to 75 days the total acidity of the leaves increased at a rate that can be satisfactorily expressed by a straight line, but then suddenly began to diminish. Figure 14 shows that, in spite of the sudden cessation of deposition of acid in the leaves, or perhaps the evidence of migration of acid out of the leaves, the relative proportions of the three acids still present remained the same. If organic acids actually were transported out of the leaves in the period after 75 days, it is necessary to suppose that the individual acids

were removed in the same relative proportion as that in which they were present at this time. An alternative to this view is to suppose that the three acids were transformed into non-acidic substances at the same relative rate. Clearly there is a close and intimate interrelationship in the metabolism of these acids in leaf tissue. It is difficult, in the face of these observations, to regard oxalic acid *merely* as an end-product of carbohydrate, or of organic acid oxidation, which is stored in the leaves as the presumably inert calcium oxalate.

The organic acids of the stems are plotted individually in Figure 12. The most striking feature of the diagram is the far more important rôle played by the acids of unknown nature in the stem tissue than in the leaves. The observations after 75 days suggest a degree of relative irregularity that is not nearly so marked in the other data for the stems of these later collections and therefore can hardly be ascribed exclusively to sampling errors. It may be noted, however, that the total acidity of the stems at 75 days (Figure 10) and also the ash content (Figure 7) are both relatively high if the curves for both sets of observations are smoothed.

The malic acid steadily increased in quantity throughout the period of growth and showed no effect of the onset of flowering. The oxalic acid increased to a maximum that was attained at 75 days, and subsequently did not change. Citric acid was present only in small proportion throughout; there is evidence of a slight increase in citric acid in the stems of the last collection.

The relative proportions of the total acidity of the stems present as malic, citric and oxalic acids are plotted in Figure 15. Here again the importance of oxalic acid in the metabolism during the earliest stages of growth is manifest. The proportion of oxalic acid increased and that of malic acid correspondingly diminished during the first 26 days. During the ensuing two weeks the relative proportions of these two acids were reversed, malic again exceeding oxalic; subsequently the malic acid was maintained at approximately a constant level, save for an apparent marked drop at 75 days. The oxalic acid steadily diminished from 35 days to 54 days, although by only a small proportion, but later remained constant. Citric acid apparently plays only a minor part in the metabolism of the stems; there was none detectable in the stems of the seedlings; a small proportion was subsequently slowly formed and remained almost unchanged throughout.

The large proportion of acids of unknown nature in the stem tissue is striking. It is clear that, as growth progressed, these acids increased slowly in relative proportion. The whole question of the acid composition of the stem tissue awaits a more detailed investigation. A brief study of the acids of collection F (47 days) showed that about two-thirds of the unknown acids are insoluble in water although soluble in ether, but no information regarding their chemical nature has yet been obtained.

Carbohydrates

The soluble carbohydrates of the leaves, calculated arbitrarily as glucose, are shown in Figure 16. The total soluble carbohydrate began to increase very rapidly as soon as the plants became established. The curve of fermentable carbohydrate, which probably represents mainly glucose, differs, however, inasmuch as it shows a sequence of changes in rate of accumulation. During the interval from 40 to 61 days the leaves scarcely increased their store of fermentable sugar at all, but then, as the rate of growth diminished, sugar again accumulated.

The chemical nature of the unfermentable carbohydrate has not as yet been established. The quantity formed is at all points less than that of the fermentable sugar save in the leaves in the very earliest stages (see solid line of percentage in Figure 18). This form of carbohydrate does not show any marked fluctuation in rate of accumulation in the leaves during the period of most rapid growth, and consequently the curve showing the percentage of fermentable carbohydrate (Figure 18) drops rapidly in this period—i.e. from 40 to 54 days.

The soluble carbohydrates of the stems are shown in Figure 17. The quantities present in the stems of the very young plants were smaller than those in the leaves but, after 47 days, the quantities materially exceeded those in the leaves. The total carbohydrate increased steadily throughout the period of most rapid growth, a drop in the rate of accumulation being noted only after 61 days when the growth rate of the entire plant had markedly diminished. The fermentable carbohydrate follows the curve of total carbohydrate in every detail, the only apparent check in the rate of accumulation being in the period from 75 to 97 days.

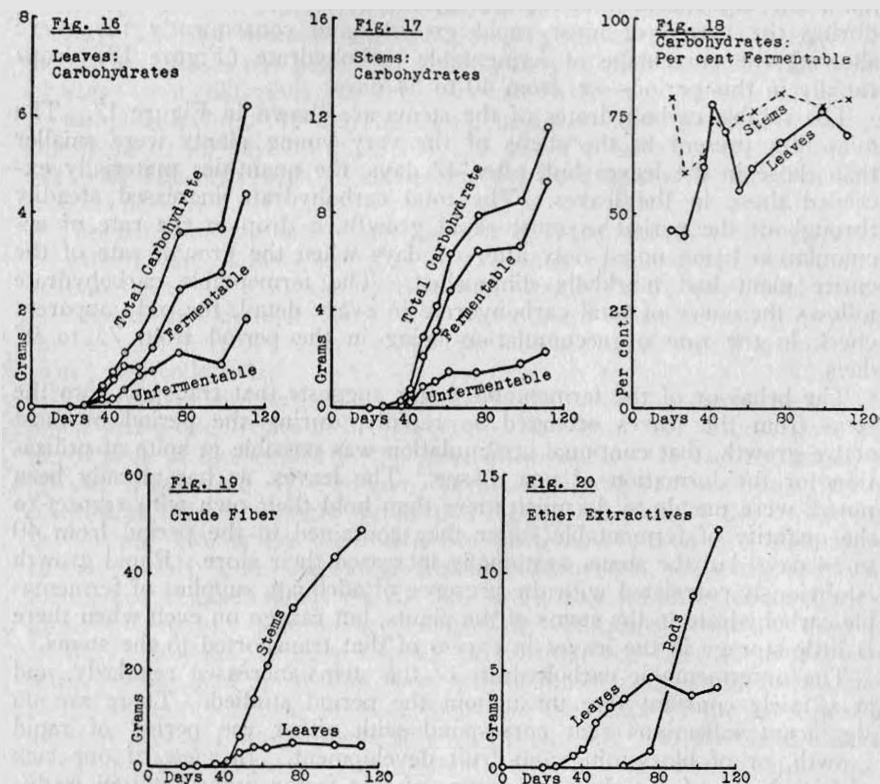
The behavior of the fermentable sugar suggests that transport into the stem from the leaves occurred so rapidly, during the period of most active growth, that continual accumulation was possible in spite of utilization for the formation of new tissue. The leaves, as has already been noted, were unable to do much more than hold their own with respect to the quantity of fermentable sugar they contained in the period from 40 to 54 days, but the stems continually increased their store. Rapid growth is obviously correlated with the presence of adequate supplies of fermentable carbohydrate in the stems of the plants, but can go on even when there is little storage in the leaves in excess of that transported to the stems.

The unfermentable carbohydrate of the stems increased regularly, and at a fairly constant rate throughout the period studied. There are no significant inflections that correspond with either the period of rapid growth, or of blossoming and fruit development. In view of our lack of knowledge of the chemical nature of this factor, interpretation is difficult.

The crude fiber, determined by the conventional procedure, is shown in Figure 19. As might be expected, the crude fiber of the stem mounted rapidly throughout the period of most active growth; it continued to increase, however, even in the plants of the later collections at a rate that was scarcely diminished.

The crude fiber of the leaves behaved in a totally different manner; it accumulated at a slow but steady rate for 54 days and thereafter remained practically constant. Reference to Figure 4 shows that the behavior of the crude fiber is closely like that of the organic solids of the

leaves. The scale on which the crude fiber of the leaves is plotted is too small to show the minor variations clearly, but, if the data are plotted on a sufficiently large scale, a curve is obtained that faithfully follows the irregularities of the curve for the leaves in Figure 4. It may be inferred that the tobacco plant reaches a stage, at the end of approximately two months, at which a fairly definite amount of organic substances has been laid down in the leaf tissue; the subsequent growth of the plant as a whole does not greatly alter the total quantity of organic matter in the leaves, the phenomena then observed are those of interconversion of organic substances rather than accumulation. The contrast with the behavior of



the stem tissue is especially sharp; continued accumulation of organic solids takes place (see Figure 4), especially of the woody tissue upon which the strength of the entire structure depends.

Ether Extractives

The quantities of ether-soluble substances extracted from the dried leaf and pod tissue are plotted in Figure 20. This fraction includes chlorophyll in addition to the hydrocarbons, waxes, sterols, and true fats that make up the bulk of the material. The data show a progressive increase in the ether-soluble substances of the leaves for 47 days, followed by a

period (47 to 75 days) in which the increase is almost linear. Subsequently there is a loss of ether-soluble substances from the leaves, but whether or not this is connected with the development of the inflorescence cannot be determined.

The ether extract of the pod samples reveals the rapid deposition of true fat as the seeds ripen. No data on the composition of fully ripened seed pods were obtained so that the maximum to which this curve may rise is not certain. Our investigations of tobacco seed show (49), however, that the dried seeds contain 42 per cent or more of their weight as ether-soluble substance, most of which is an oil (30). An average plant may be expected to produce approximately 44 grams of seed and, consequently, the curve for the fat content of the ripened pods should ultimately reach a magnitude of about 19 grams.[1]

Nitrogenous Constituents

The total nitrogen content of the plant, plotted in Figure 21, increased with great rapidity from the earliest stage. The fresh weight of the plants at 19 days was only from three to four times as great as that of the seedlings, but the nitrogen content was nearly seven times as great. In the interval from 19 to 26 days the nitrogen content increased fourfold and in the next 9 days it increased nearly fivefold again. During the period of most rapid growth, that is from 40 to 61 days, the plants steadily assimilated nitrogen in the tops at the rate of 1 gram in about 8 days.[2] The apparent loss of nitrogen from the tops in the last period studied is a matter that requires further study.

By far the greater part of the nitrogen taken up by the plant in the early stages of growth is assimilated in the leaves, and at all stages, save in the aging plant, the quantity of nitrogen in the leaves is at least twice as great as that in the stem. The onset of flowering places a relatively sudden check upon the rate of storage of nitrogen in both leaves and stem, and the tendencies of the curves suggest that nitrogen is more or less rapidly translocated from other parts of the plant into the ovules as these ripen.

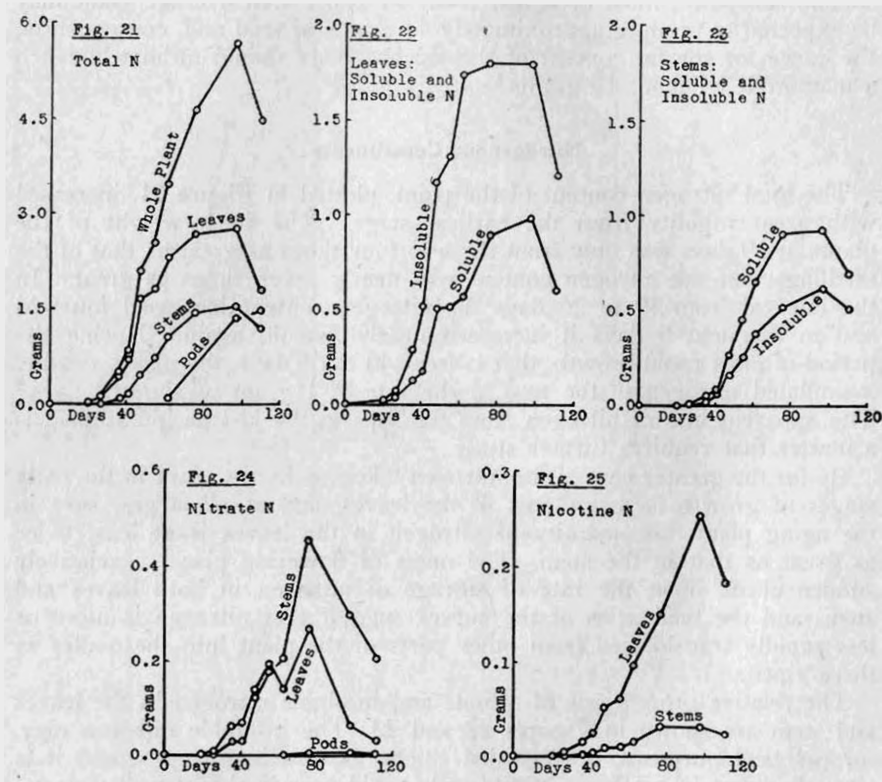
The relative proportions of soluble and insoluble nitrogen in the leaves and stem are shown in Figures 22 and 23. The insoluble nitrogen may, for practical purposes, be regarded chiefly as protein nitrogen, and it is clear that protein synthesis is extremely rapid even in the youngest plants. The 19-day old leaves contained ten times as much insoluble nitrogen as the seedling leaves, and the quantity increased more than fourfold in each of the next two intervals of seven and nine days. During the period of most rapid growth, insoluble nitrogen was laid down in the leaves at the rate of 1 gram in 17 days. At the expiration of 61 days, that is at the beginning of the period of flowering, deposition of insoluble

[1] This admittedly very rough estimate is based on the following figures. An average plant bears approximately 175 pods, each of which contains from 2,000 to 4,000 seeds—3,000 may be taken as an average. One plant therefore produces well over 500,000 seeds. The seeds run from 11,500 to 13,000 to the gram, 12,000 being a rather accurate average. Much of the data for this estimate is contained in Station Bulletin 326 (4); other figures are based on unpublished counts and weights of seeds made by Dr. Th. Berthold at the Tobacco Substation at Windsor, Conn.

[2] This is at the rate of approximately 26.5 lbs. per acre in 8 days on the assumption of 12,000 plants to the acre.

nitrogen in the leaves abruptly ceased and, in the last interval studied, insoluble nitrogen was apparently removed from the leaves.

The soluble nitrogen of the leaves, though less in amount than the insoluble, followed a somewhat similar course during the first 47 days. The rate of accumulation of soluble nitrogen then diminished somewhat, but the curve clearly indicates an accumulation of soluble nitrogen throughout the period in which the insoluble nitrogen of the leaves remained constant. The marked drop in soluble nitrogen at the 110th day is probably associated with the rapid development of the seeds.



The insoluble nitrogen of the stems (Figure 23) did not begin to accumulate in significant amounts until the plants were 26 days old, and the rate of deposition thereafter was much slower than in the leaves. There is no evidence of a marked change in the rate of accumulation of insoluble nitrogen in the stem at the time of flowering, although the rate slowed down slightly. The soluble nitrogen of the stem likewise accumulated slowly at first but, when rapid growth began, it increased more rapidly than the soluble nitrogen of the leaves, overtaking this at 61 days. Subsequently the soluble nitrogen of the stems was practically identical with that of the leaves, the changes being similar in each case.

Nitrate Nitrogen: Figure 24 shows the quantities of nitrate nitrogen absorbed by the plant and stored as such in leaves and stem. More nitrate accumulated in the leaves than in the stem during the first 54 days but, thereafter, the quantity in the stem exceeded that in the leaves; both curves show a steady accumulation of nitrate throughout the period of rapid growth. At the time of flower and fruit development a sudden diminution of the nitrate content in both leaves and stem began, and this continued so rapidly that relatively little nitrate remained in the leaves of the practically mature plants at 110 days. The stem, however, still retained an appreciable quantity at this time. The behavior of the nitrate suggests that, although absorption from the soil continued for at least 97 days, as is shown by Figure 21, towards the end of the growth period either the supply available in the soil was exhausted[1] or transformation of the nitrate into other substances then took place at a rate that exceeded the rate of absorption. The rapid rise in the nitrogen content of the developing pods suggests that the final stage of this transformation consisted in the laying down of protein in the seeds.

Very little nitrate nitrogen accumulated as such in the developing fruit; in fact the quantities found represent little more than traces. This suggests either that the nitrogen is translocated from the leaves into the fruit in some form other than nitrate, or that the chemical transformation of the nitrate in the pods takes place as rapidly as it is supplied from the stem and leaves.

The behavior of the nitrate nitrogen of the tobacco plant is in marked contrast with the behavior of nitrate in the rhubarb plant as observed by Culpepper and Caldwell (9). The nitrate nitrogen increased materially in the petioles of their plants, so much so, in fact, that no less than 1.5 per cent of the dry weight of the senescent petioles might consist of nitrate nitrogen. The leaf blades, on the other hand, did not greatly increase their store, the proportion of nitrate nitrogen in all cases being less than 0.01 per cent of the fresh weight. Our data, when recalculated on the

[1] Mr. O. E. Street of the Tobacco Substation observed the nitrate content of the soil of a field adjacent to that in which the plants of the present investigation were grown during the season of 1933. The fertilizer applied to the two fields was essentially the same, but the crop studied by him was Havana seed tobacco which is grown without a shade tent. Owing to the dry season, the plants were irrigated on July 10 with the equivalent of 1.67 inches of water. The plants in the shade field, used for our experiments, were irrigated in the same manner one week earlier, that is, on July 3 and 4. We wish to express our thanks to Mr. Street for the following data.

Date		Time days	Nitrate nitrogen parts per million	Rainfall during preceding interval inches
June	19	19	48.8	—
"	26	26	35.9	0.08
July	3	33	71.3	0.27
"	10	40	38.2	1.67 (Irrigated)
"	17	47	10.8	1.28
"	24	54	7.0	0.06
"	31	61	18.0	0.76
Aug.	14	75	69.5	1.19
"	21	82	3.0	0.28
Sept.	5	97	6.5	3.16
"	10	110	1.6	2.55

basis of percentage of the fresh weight, show that the nitrate nitrogen of the stems, which amounted to 0.14 per cent of the fresh weight at the seedling stage, dropped in 40 days to the vicinity of 0.05 per cent and there remained, with the exception of a sudden increase to 0.08 per cent at 75 days, possibly due to the rain which fell the day before this collection was made. The nitrate nitrogen of the tobacco leaf tissue started at 0.08 per cent of the fresh weight and, with the exception of the 75-day collection, dropped in almost linear fashion to 0.008 per cent at 110 days.

Nicotine Nitrogen: The quantities of nicotine nitrogen are shown in Figure 25. The synthesis of nicotine began very early in the development of the plant, and accumulation in the leaves proceeded rapidly throughout the period of growth. The data indicate a drop in nicotine content in the plants of the last collection in conformity with the drop of total nitrogen that apparently occurred at this time. Nicotine accumulated slowly in the stems throughout the period of rapid growth, but storage in the stems ceased after 75 days.

Ammonia and Amide Nitrogen: The determination of amides in plant tissue rests upon a difference in the stability of the amide nitrogen of asparagine and of glutamine to hydrolyzing agents. The amide nitrogen of pure glutamine is quantitatively split off as ammonia when glutamine is heated for two hours at 100° in a solution buffered at pH 6 to 7, whereas the amide nitrogen of asparagine is scarcely affected under these conditions. In order to hydrolyze the amide nitrogen of asparagine completely, it is necessary to heat the substance at 100° for three hours with 1 N sulfuric acid. Certain other substances behave in a similar manner, and two of these, urea and allantoin, if present in substantial amounts in a plant extract, would make the accurate determination of asparagine and glutamine by hydrolytic methods difficult, if not impossible. The determination, according to the methods we have adopted for the analysis of tobacco leaf and stem tissue, involves the assumption that asparagine and glutamine are the only amides present, and that no interfering substances occur along with them. These assumptions are probably justified in the case of tobacco leaf tissue. A careful search for allantoin in an extract of tobacco leaves obtained from plants of the same type as those employed in the present investigation failed to reveal its presence. The method employed was one that had succeeded in showing considerable quantities of allantoin in an extract of tobacco seed, and it is felt, therefore, that the quantity of allantoin in tobacco leaf tissue is probably so small that interference from this source with the amide determinations is not to be feared. The absence of substantial quantities of urea is inferred from the observation that the quantity of ammonia produced by hydrolysis of the tissue at pH 7 for two hours is not increased by longer heating. Under the conditions adopted for hydrolysis of glutamine amide nitrogen, only about 20 per cent of the nitrogen of urea is converted to ammonia; but further conversion is brought about by longer heating.

The question of the presence of amides other than glutamine and asparagine in the tobacco plant is, however, more difficult to answer. As will be shown, the behavior of the tobacco stem tissue suggests the possible

presence of an unstable amide other than glutamine. In view of this it is necessary for the present to employ the terms "glutamine amide nitrogen" and "asparagine amide nitrogen" in the following discussion in a somewhat broad sense; although in certain cases there is reason to believe that the two forms of nitrogen so designated are really derived from these two substances, in others this is by no means certain; consequently the terms are to be understood to mean a form of nitrogen which behaves in the same way as asparagine, or glutamine amide nitrogen respectively, behaves.

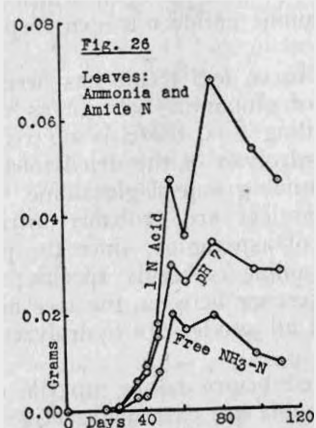
That asparagine actually occurs in tobacco leaf tissue has been shown by direct isolation (45); the presence of glutamine has not as yet been confirmed in this manner. Notwithstanding this, there is no reasonable doubt that the ammonia produced by hydrolysis of the dried tobacco leaf tissue at pH 7 arises mainly from the amide group of glutamine. In fact the determinations of glutamine in the leaf are probably intrinsically more accurate than the determinations of asparagine, since the property employed for the determination of glutamine is highly specific; the asparagine determinations rest upon a difference between the free ammonia and the acid hydrolyzable ammonia, and all amides are hydrolyzed under the conditions adopted.

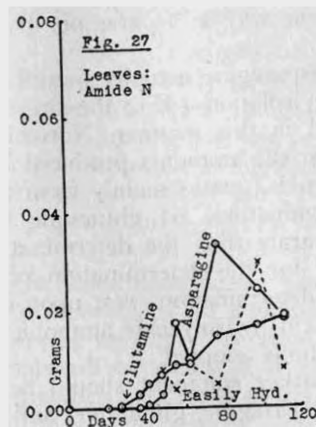
One further comment should be added before taking up the data in detail. Asparagine, glutamine, and ammonia are extremely active metabolites in plant tissue. The proportions of the amides present are rapidly influenced by changes in the proportion of ammonia in the plant, and chance differences in the supply of ammonia from the soil in which the individual plants grew would have an effect upon the amides. Irregularities in the curves for the amides are therefore to be expected. These irregularities are not to be regarded as errors; they represent rather the marked variability of the amides in response to conditions that could not be controlled in a field experiment.

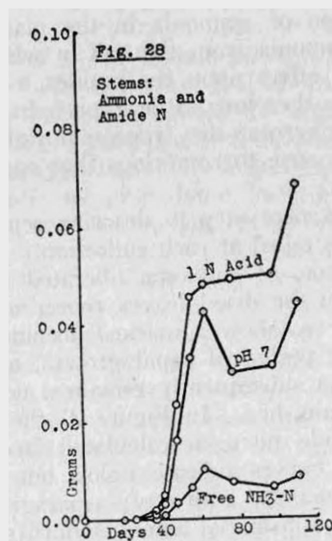
In discussing the data obtained, it is necessary to describe separately the results from the two sets of samples taken at each collection. Figure 26 shows the quantities of free ammonia, of ammonia liberated by hydrolysis at pH 7[1], and by 1 N acid, in the dried leaves represented by Sample II of each collection. The curves show a marked accumulation of ammonia, and of amides, during the period of rapid growth, and indicate that the quantity of free ammonia subsequently remained constant for several weeks and finally slowly diminished. In Figure 27 the quantities of glutamine and asparagine amide nitrogen calculated from the curves of Figure 26 are plotted. These curves indicate a slow but steady accumulation of glutamine beginning from the 26th day; asparagine apparently did not begin to accumulate in substantial amounts until several weeks later, but was then rapidly formed in considerable amounts. Towards the end of the experiment the quantity of asparagine diminished.

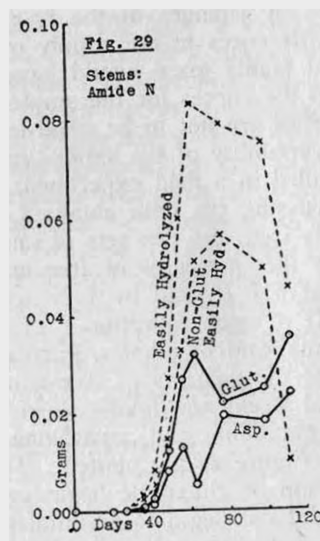
[1]To avoid possible misunderstanding, it may be necessary to recall that the buffer solution actually used was at pH 7.0. After being mixed with the tissue and heated for two hours the reaction was in the range pH 6.3 to 6.6 in most cases. The exact reaction at which this hydrolysis is conducted makes little difference so long as it is above pH 6.2 and below pH 7. In this connection see Vickery, Pucher, Clark, Chibnall and Westall (in press).

Plotted on the same figure in broken line is a curve which represents the "easily hydrolyzed amide nitrogen" of the leaves. This quantity is obtained by subtracting the free ammonia of the dried leaf samples from the free ammonia of the hot water extract made from the leaves of Sample I of each collection. Granting that the leaves of Sample II and those of Sample I are comparable, it represents the ammonia set free from some









unstable substance during the operation of preparing the hot water extract. Up to 47 days this curve is practically identical with that of the glutamine amide nitrogen; subsequently the agreement between the two curves is not good, although they intersect each other in a manner that strongly suggests that they represent what is fundamentally the same thing. The reasons for placing more reliance upon the accuracy of results from the early collections have been discussed elsewhere, and there

seems little reason to doubt that the so-called "easily hydrolyzed amide nitrogen" of tobacco leaves arises from glutamine.

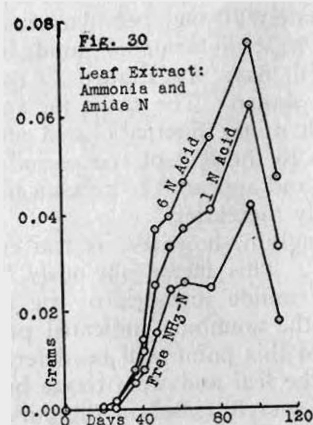
The ammonia and amide nitrogen of the dried stem samples are plotted in Figure 28. Free ammonia accumulated rather slowly in the stems, reaching a maximum of only approximately 10 mg. per plant in 61 days, and thereafter diminishing slightly. The two forms of amide nitrogen, however, mounted rapidly after the 35th day. In Figure 29 the asparagine and glutamine amide nitrogen are shown. The curve for asparagine amide nitrogen indicates, although with minor fluctuations, a steady accumulation of asparagine from 35 days to the end of the period studied. Glutamine formed more rapidly at first, and appeared to reach a maximum in 54 days that did not change materially thereafter.

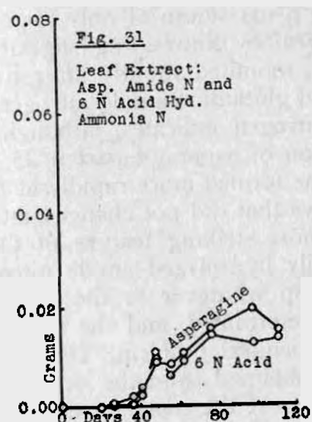
The most striking feature of the diagram, however, is the curve for the "easily hydrolyzed amide nitrogen". This factor obviously bears no relationship whatever to the glutamine amide nitrogen of the stems as directly determined, and the origin of the ammonia indicated presents a difficult chemical problem. Discussion of this point will be deferred until the data obtained from the extracts of the leaf and stem tissue have been described. At the moment, attention is merely called to the curve which represents the difference between the "easily hydrolyzed amide nitrogen" and the glutamine amide nitrogen. This curve is designated "non-glutamine, easily hydrolyzed amide nitrogen". It rises rapidly to a well-defined maximum at 61 days which is maintained until 97 days, and then falls to a very low figure.

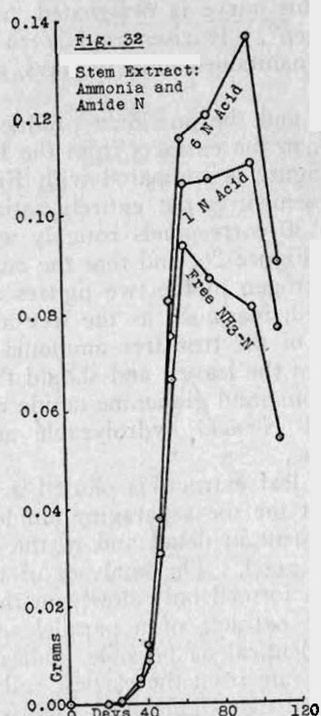
Figure 30 shows the free ammonia, and the ammonia produced by 1 N and by 6 N sulfuric acid hydrolysis, in the extracts from the leaves of Sample I of each collection. If this figure is compared with Figure 26 it will be noted that, although the agreement is not entirely satisfactory, the curve for free ammonia in Figure 30 corresponds roughly with that for the pH 7 hydrolyzable ammonia in Figure 26, and that the curves for the 1 N acid hydrolyzable ammonia nitrogen in the two figures are also reasonably close. This is to be expected, inasmuch as the free ammonia curve in Figure 30 represents the sum of the true free ammonia and of the "easily hydrolyzed amide nitrogen" of the leaves, and should therefore be the same as the sum of the free ammonia and glutamine amide nitrogen shown in Figure 26. Similarly the 1 N acid hydrolyzable ammonia nitrogen should be the same in each case.

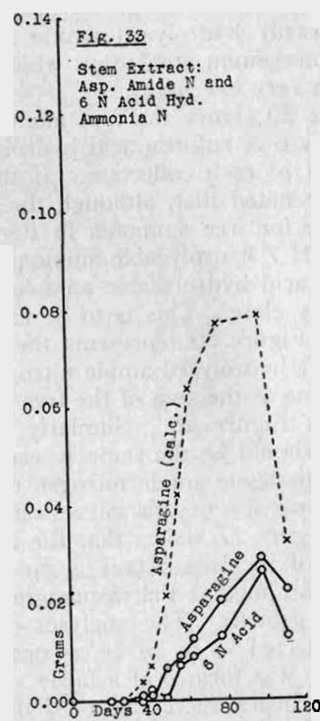
The asparagine amide nitrogen of the leaf extracts is plotted in Figure 31. Comparison of this curve with that for the asparagine amide nitrogen of Figure 27 shows that the agreement in detail and in the general behavior of the two curves is far from good. The analyses of the dry leaf samples suggest that asparagine was formed only slowly in the early stages of growth. The analyses of the extracts of a parallel series of leaves, selected so as to be as nearly identical as possible, indicate that asparagine was formed at a fairly steady rate from the earliest collections, that it reached a maximum at 97 days and then diminished slightly. The discrepancies between the analyses of these sets of samples clearly indicate that the sampling error plays a very important part when dealing with such highly reactive metabolites as the amides.

The difficulty that arises from the lack of agreement between the results of the analyses of the two sets of samples is intensified by the extraordinary results obtained from the stems. Figure 32 shows the ammonia









and 1 N acid hydrolyzable ammonia in the water extract from the stems, and the solid line in Figure 33 shows the asparagine amide nitrogen calculated from these data. It happens that the asparagine amide nitro-

gen so found agrees reasonably well with the data from the dry stem samples plotted in Figure 29, particularly in the case of the younger plants.

Another method to arrive at an estimate of the asparagine amide nitrogen of the stem tissue is to subtract the sum of the free ammonia and the glutamine amide nitrogen, determined in the dry stem samples, from the ammonia produced by 1 N acid hydrolysis of the stem extract—that is, to subtract the data plotted in the curve designated pH 7 in Figure 28 from the data designated 1 N acid in Figure 32. The results of this calculation are plotted in the broken line in Figure 33. Clearly, there is no relationship whatever between the asparagine so calculated and that derived from analyses of the stem extract itself or of the dried tissue. Mutually consistent results are obtained from each set of samples, but it is not possible to employ data for the amides obtained from one set in calculations of results from the other set.

Both the free ammonia and the 1 N acid hydrolyzable ammonia nitrogen of the stem extract are enormously greater than the analogous data from the dried stem samples. In both cases the data are consistent, and are fitted by fairly smooth curves; the two sets of curves simply bear no relationship to each other, in spite of the fact that they ostensibly represent measures of the same thing. It should perhaps be emphasized that the data in Figure 28, with the exception of the curve for free ammonia, were obtained from analyses of hot water extracts of the previously *dried* tissue, that is to say, the gross discrepancy between the data of Figures 28 and 32 is entirely a matter of whether the stem tissue was dried or not before being extracted with boiling water.

It is apparently necessary to assume that the stem tissue contains a substance which is decomposed with the production of ammonia, when the tissue is extracted with boiling water at pH 4, and the extracts are thereafter concentrated to small volume, and that this substance is converted, during the process of drying the tissue, into a substance that no longer gives off ammonia on being boiled with water.

It is of interest to inquire into the possibility that this hypothetical substance may be glutamine itself, that is, to assess the degree of probability that the glutamine determinations in the stem tissue may be very seriously too low.

In the first place, the storage of glutamine in stem tissue in considerable quantities under favorable conditions is a phenomenon that occurs in the tomato plant (47); storage of glutamine in considerable amounts may also occur in the fleshy root of the beet (unpublished observations). Consequently one would be inclined to expect larger amounts of glutamine in the stem tissue of the tobacco plant than in the leaf. The glutamine data of Figure 29, if they are worth anything at all, indicate that this is indeed the case (cf. Figure 27).

Glutamine is decomposed with the production of ammonia when its aqueous solution is heated to 100°. The speed of the decomposition varies with the reaction of the solution, being somewhat slower in the vicinity of the isoelectric point (ca. pH 5), but decomposition is complete at pH 7 in two hours. The other product of the decomposition is

probably pyrrolidone carboxylic acid (8) as is shown by the diminution of the free amino nitrogen of the solution. Pyrrolidone carboxylic acid is converted into glutamic acid by boiling its solution for several hours with acid of 1 N strength or greater. If, then, glutamine is present in fresh tobacco stem tissue one would expect: First, that a loss of part, or perhaps even all, of the glutamine should take place during the drying of the tissue at 80 to 100°, particularly if this required a long time; second, that a production of free ammonia equivalent to the glutamine decomposed in the drying tissue should occur; third, that a soluble substance which yields amino nitrogen on acid hydrolysis should be demonstrable in quantities commensurate with the loss of glutamine.

Unfortunately we have no data on the glutamine content of tobacco stem tissue before and after being dried. Nevertheless, data to be presented in a forthcoming publication show that it is possible to dry slices of the fleshy root of the beet plant in the same drying-oven equipment as was used for the tobacco stems, with a loss of not more than from 10 to 15 per cent of the glutamine of the tissue as determined directly on the fresh material. It therefore seems improbable that much of the glutamine of the stems was destroyed during drying. This view is supported by the data for the free ammonia of the dried stem tissue (Figure 28); at only one point (61 days) was there as much as 10 mg. of free ammonia nitrogen per plant, whereas at this same time the glutamine determination indicated the presence of 32 mg. of glutamine amide nitrogen, but over 80 mg. of easily hydrolyzed amide nitrogen. The order of magnitude of the free ammonia nitrogen of the dried tissue, then, is not in accordance with the view that any large proportion of the glutamine was destroyed during drying, unless the somewhat improbable assumption is made that the ammonia produced was driven out of the tissue at the temperature of the oven.

It will be necessary to anticipate a little in order to correlate the possibility of loss of glutamine during drying with the production of a substance that yields amino nitrogen on acid hydrolysis. In Figure 36 the data for the peptide nitrogen of the stems are plotted. The observations were made on hot water extracts of the dried tissue, and it should be noted that the scale of ordinates is half that of Figure 29. Unfortunately it is impossible to discriminate between actual peptide nitrogen and the amino nitrogen resulting from the hydrolysis of pyrrolidone carboxylic acid. It is clear, however, that, if the true peptide nitrogen of the stem tissue is quite low, there is room for quantities of amino nitrogen arising from pyrrolidone carboxylic acid of an order of magnitude comparable with those shown in the curve for "non-glutamine easily hydrolyzed amide nitrogen" in Figure 29. This argument, therefore, leads to the result that it is possible that the determinations of glutamine in the stem tissue are serious underestimates of the quantity actually present. The argument is weak, however, inasmuch as there is no good reason to assume that the peptide nitrogen of the stems is in fact very low; greater significance is probably to be attached to the results of the free ammonia determinations in the dried stem tissue.

We are therefore driven to the provisional assumption of a third source of amide nitrogen in the stem tissue, and to the view that the curve designated "non-glutamine easily hydrolyzed amide nitrogen" in Figure 29 furnishes an approximate idea of the quantity present at each stage of growth.

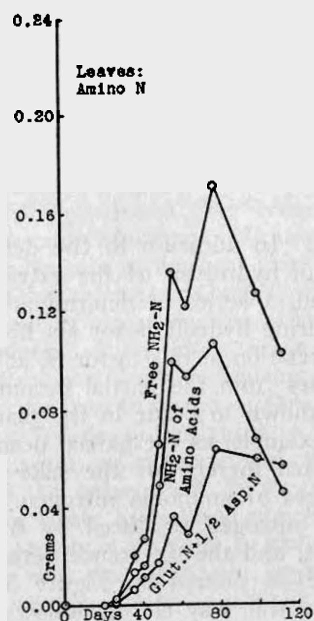
Manifestly the investigation of the amides of the tobacco plant is still in a very elementary state. The methods of analysis hitherto developed have validity only if certain assumptions regarding the chemical composition of the tissue are justified; that this is not the case with respect to the stem tissue is evident, and it is possible that some doubt may be properly attached to the results of the analysis of the leaf tissue as well. If the present investigation does no more than to make this clear, the labor expended will be well repaid.

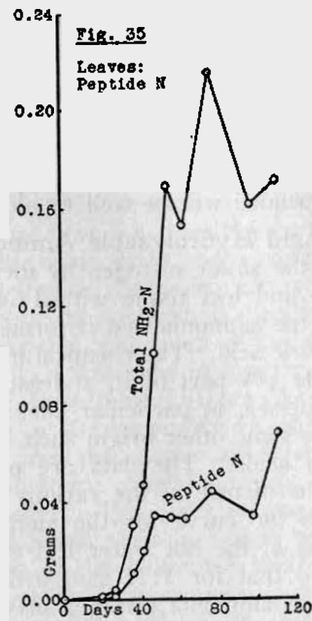
6 N Acid Hydrolyzable Ammonia: In addition to the determinations of the amide nitrogen by means of hydrolysis of the extracts from the stem and leaf tissue with 1 N acid, a series of determinations was made of the ammonia that is formed during hydrolysis for six hours with 6 N sulfuric acid. The chemical interpretation of this factor is, at present, impossible. A part of it, at least, arises from the partial decomposition of the purines, in particular adenine, known to occur in the plant; part may have some other origin such, for example, as the partial deamination of amino acids. The data are presented merely for the sake of completing the picture of the various sources of ammonia nitrogen. Figure 30 shows the curve for the ammonia nitrogen produced by 6 N acid hydrolysis of the hot water leaf extract, and the difference between this curve and that for 1 N acid hydrolysis is plotted on Figure 31. The quantity of ammonia nitrogen arising in this way closely follows, and is nearly identical with, the quantity apparently present as asparagine amide nitrogen. Figures 32 and 33 show similar data for the hot water stem extract, and here again the behavior of the quantities involved closely resembles the asparagine amide nitrogen.

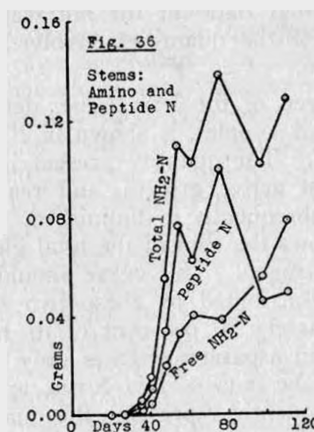
Amino Nitrogen: The amino nitrogen of the leaf tissue, determined upon hot water extracts of the dried leaf samples, is shown in Figure 34 by the curve designated "free NH_2-N". The quantity present increased very rapidly during the period of most active growth, and reached its maximum at the time of blossoming; subsequently it diminished. Plotted on the same diagram is a curve that shows the sum of the total glutamine nitrogen and one-half the asparagine nitrogen. This curve should represent the quantity of amino nitrogen contributed by these two amides[1], inasmuch as glutamine yields approximately 90 per cent of its nitrogen when treated with nitrous acid (8), and asparagine reacts only with its amino group. The difference between the data of this curve, and those of the free amino nitrogen curve, consequently represents the quantity of amino nitrogen that arises from the amino acids themselves, irrespective of the amides. The curve plotted from these differences shows an accumulation of amino acids, during the period of rapid growth, that proceeded to a maximum at 54 days and was maintained at this level for

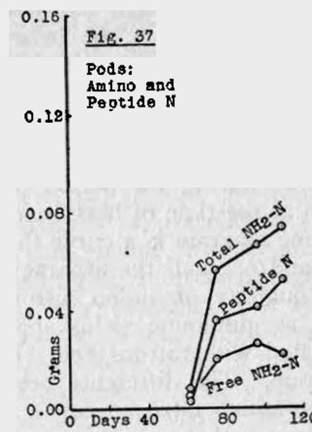
[1] The assumption is made that no significant proportion of the glutamine was hydrolyzed during the drying of the leaf samples.

three or four weeks. The period of development of the fruit, however, corresponds with a rapid diminution of the amino acids of the leaves. This behavior is strongly suggestive of translocation of amino acids as such from the leaves to the fruit. In this connection it is interesting to note that the sum of the glutamine and asparagine in the leaves did not









markedly diminish during this same period—a result that is contrary to the view that these amides are of especial significance as translocatory substances for nitrogen. Too much emphasis should not be placed upon this result at the moment, however; more comprehensive data than those at present in hand will be needed to clarify the situation completely.

In Figure 35 are plotted the results of the determination of the amino nitrogen, after hydrolysis with 6 N acid, of an extract from the dried leaf samples; the curve is designated "total NH_2-N". It represents the sum of the amino nitrogen of the amino acids and amides already present together with the amino nitrogen liberated by the hydrolysis of what are presumably soluble peptides. Beneath it are plotted the results obtained by subtracting the data for the free amino nitrogen. The curve shows that soluble peptides accumulated but slowly in the leaves as growth progressed; from the 47th to the 97th day the quantity present did not change significantly. There is evidence of a slight rise in the leaves of the final collection; in any case, however, peptide nitrogen does not appear to be an especially active component in the metabolic processes of the leaves.

Figure 36 shows the free and total amino nitrogen determined in a hot water extract of the dried stem tissue, together with the peptide nitrogen. The free amino nitrogen, although always present in smaller amount in the stems than in the leaves, rose fairly rapidly through the period of rapid growth, was then maintained at a constant level for several weeks and finally continued to rise through the period of blossoming. The total amino nitrogen behaved in a manner closely like that of the leaves; it rose rapidly to a maximum at 75 days and then fell slightly. The peptide nitrogen rose to a maximum at 75 days and then fell off materially.

A calculation of the free amino nitrogen in the dried stems due to the amides glutamine and asparagine leads to figures which are irreconcilable with the data for free amino nitrogen—in every case quantities are obtained that exceed the free amino nitrogen of the stems as directly determined. This discrepancy furnishes a check upon the determinations of the two amides in the dried stem extract which strongly suggests that the recorded data for the amides are considerably too high and serves further to emphasize the inadequacy of the analytical methods for amides when applied to tobacco stem tissue.

The amino and peptide nitrogen of a hot water extract of the dried, fat-free pods are plotted in Figure 37. The rapid accumulation of peptide nitrogen so that, in the last collection, the quantity was approximately equal to that in the stems is of interest. It should perhaps be pointed out that this peptide nitrogen does not represent the protein of the developing seeds, as the protein of the seeds was not extracted by water under the conditions adopted; it probably represents soluble compounds of amino acids synthesized in preparation for the laying down of the seed protein.

RELATIVE DISTRIBUTION OF THE CONSTITUENTS OF THE TOBACCO PLANT

The analytical data hitherto discussed have for the most part referred to the absolute quantity of each constituent, usually expressed in grams, in a single plant. This method of presentation has furnished a picture of many of the chemical details of the growth of the tobacco plant, but has

given little direct information regarding the distribution of the various factors in the different parts of the plant, or of the quantitative relationships between these factors. It is customary to express analytical data obtained from plants in terms of percentage of some quantity such as the fresh or dry weight; that is to say, the relative concentration of the various factors is calculated in terms of a quantity which is assumed to be constant. But, clearly, every quantity that might be employed as a basis for the calculation varies as the plant grows, and one cannot assume that the manner of variation of a given factor, and of the factor taken as the basis of computation of the percentage, is in every case similar. Irregularities in a curve of percentage may therefore be due to a change in either or both of the quantities involved, and consequently become difficult to interpret.

Accordingly, the calculations given below of the distribution of the various components have been restricted for the most part to cases in which the fundamental biological unit, the single plant, is still the dominant factor in the situation, as our aim is to provide as vivid a picture as possible of the chemical changes that occur during the growth of the individual plant.

Figure 38 shows the distribution of the fresh weight of one plant as between leaves, stem, and pods. The relative mass of leaf tissue increased from 68 per cent to 85 per cent of the whole in 26 days after setting, but then diminished fairly rapidly so that, at 61 days, the relative proportions of leaf and stem tissue were approximately equal. The stem reached its maximum relative proportion at the same time and thereafter remained substantially constant, the diminution in relative weight of the leaves being compensated by the increase in the relative weight of the blossoms and pods.

The general distribution of the dry weight of the plant (Figure 39) is very closely like that of the fresh weight, but there are differences in detail. The dry weight of the seedling leaves was 77 per cent of the whole, being 10 per cent greater than the fresh weight, but, at 26 days, when it reached its maximum proportion, it was only 2 per cent greater than the fresh weight. The curves of dry weight intersect at 57 days, and thereafter the proportion of dry stem tissue remained substantially constant, while the pods increased and the leaves decreased. The lowest proportion reached by the leaves was 27 per cent of the whole, being about 7 per cent less than the lowest proportion reached by the fresh leaves.

Figure 40 shows the distribution of the organic solids and ash of the plant. The curves for the organic solids, as might be expected, are closely like the curves showing the distribution of dry weight. The curves for the distribution of the total ash of the plant differ markedly, however, and are plotted in broken lines on the same figure to permit close comparison. The ash in the leaves at all times exceeded the ash in the stems, and only in the leaves of the last two collections was the leaf ash as little as one-half the total ash of the plant. Throughout the period of most rapid growth more than two-thirds of the total ash was found in the leaves.

The distribution of the titrable organic acidity of the whole plant is shown in Figure 41. The proportion of the total organic acids in the

leaves rose from 82 per cent to nearly 94 per cent in the 26-day-old plants, and then diminished almost linearly to 51 per cent in the leaves of the last collection. The proportion of the acidity in the stems rose to about 30 per cent at the time of blossoming, and then remained constant during the greater part of the period of pod development. Comparison of Figure 41 with the curves for the distribution of the ash in Figure 40 shows a marked similarity between the two sets of curves. This furnishes another example of the intimate relationship between the acidity and the ash content of the tobacco plant to which reference has already been made.

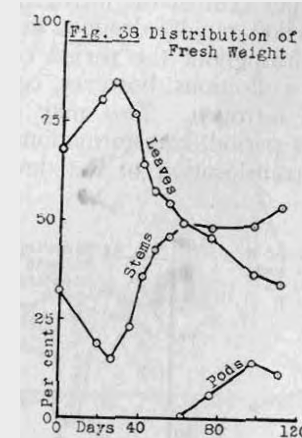


Fig. 38 Distribution of Fresh Weight

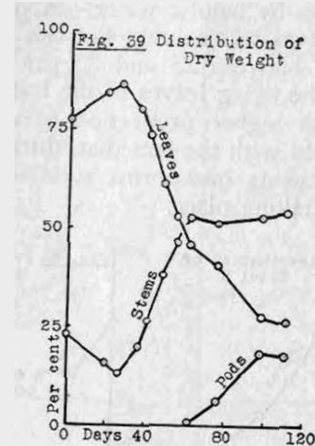


Fig. 39 Distribution of Dry Weight

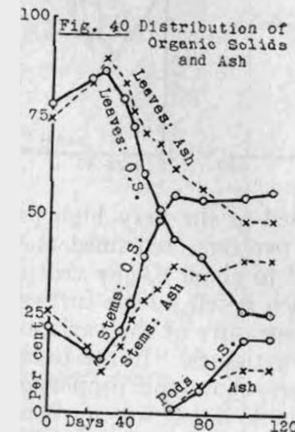


Fig. 40 Distribution of Organic Solids and Ash

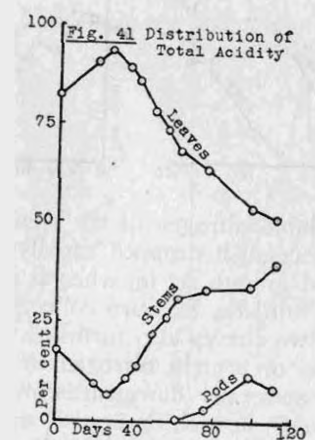
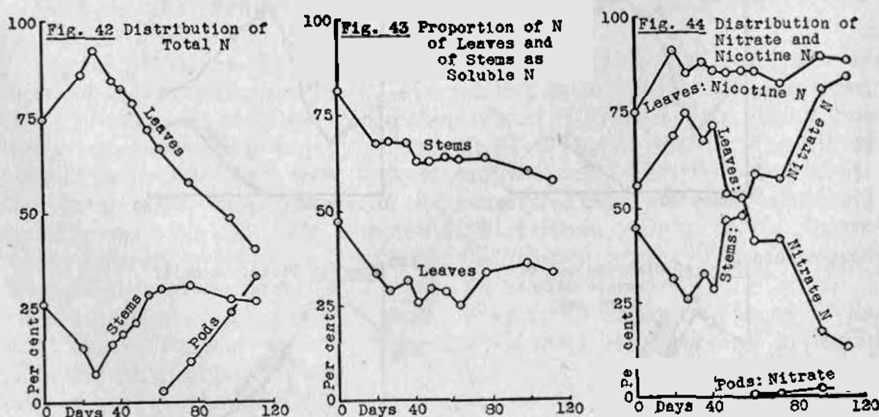


Fig. 41 Distribution of Total Acidity

It will perhaps have been noted that in every case considered the change in distribution resulting from the development of the fruit has resulted in a maintenance of the proportion present in the stem at approximately the level attained at 61 days, while the proportion in the leaves diminished. The distribution of the nitrogen of the plant, shown in Figure 42, strikingly illustrates this because of the high proportion of the nitrogen found in the pods as these develop. The nitrogen of the leaves increased at the

start, reaching nearly 93 per cent of the whole at 27 days; it then diminished fairly rapidly to 40 per cent at the last collection. Meanwhile the stem nitrogen reached a maximum of only about 30 per cent at 61 days and thereafter diminished slightly. The nitrogen of the pods, however, increased with great rapidity to approximately 33 per cent at the final observation, but the slope of the curve suggests that the pods of still older plants would contain an even larger share of the total nitrogen.

In Figure 43 are plotted two curves that show the relative proportions of the total nitrogen of the leaves and stem respectively that are brought into solution by boiling water. About 47 per cent of the nitrogen of the seedling leaves was soluble, but this proportion rapidly dropped to a level that varies between 25 and 30 per cent throughout the period of rapid growth. The aging leaves of the last three collections, however, contained a somewhat higher proportion of soluble nitrogen. This may perhaps be associated with the fact that, during this period, transformations of the leaf constituents into forms suitable for translocation to the developing seed were taking place.

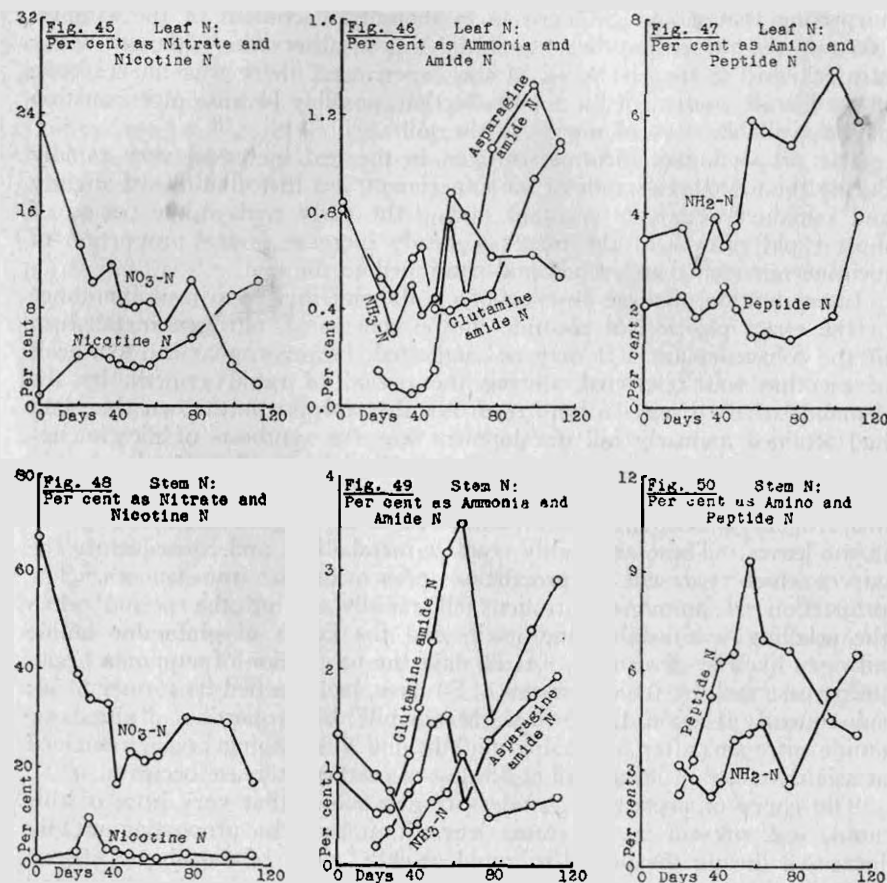


The soluble nitrogen of the stems started at the very high proportion of 82 per cent; it dropped rapidly to 67 per cent, remained at this level until rapid growth set in, when it dropped to about 63 per cent and there remained until the last two collections when it fell a little further.

These two curves also furnish a rough measure of the variations in the proportion of protein nitrogen in the two tissues. If the ordinates are reversed and read downwards, the curves show the proportion of the total nitrogen in each tissue that was insoluble in hot water. Much of the insoluble nitrogen of the leaf tissue is certainly protein nitrogen, and it seems probable that this is also true of the insoluble nitrogen of the stem. It is clear that the proportion of protein nitrogen in the leaves rapidly increased during the time the plant was establishing itself, but remained relatively constant during the period of rapid growth. The proportion of protein nitrogen in the stem likewise increased at the start, but remained quite constant throughout the growth period; towards the end there was a minor increase in protein nitrogen.

Figure 44 shows the relative distribution of the nitrate nitrogen in the plant. The seedlings contained nearly as much of their nitrate in the stem as in the leaf, but the relative proportion in the leaf rapidly increased during the first 26 days. Subsequently it fell away so that in the final collections nearly the whole of the nitrate of the plant was present in the stem. The pods at no time contained more than a very small proportion of the whole.

Plotted on the same figure is a curve which shows the proportion of the total nicotine of the plant found in the leaves; throughout most of the



period studied this varied between 85 and 88 per cent. The constancy of the proportion in the leaves is remarkable in view of the rapidly changing relative proportions of leaf and stem tissue as the plant grows, and raises an interesting question with regard to the kind of cells that are capable of synthesizing nicotine.

The distribution of the total nitrogen of the leaf among the various forms that were determined analytically is shown in the next three figures. Figure 45 shows the relative proportions of nitrate and nicotine nitrogen.

The nitrate of the seedling leaves was very high, amounting to 23 per cent of the whole. This rapidly diminished so that the nitrate in the oldest leaves was approximately 2 per cent of the total nitrogen. The curve shows several irregularities, notably at 35 and 75 days. Although the possibility that these may be in part due to sampling error is not excluded, it happens that each of these collections was made the day following a change in the conditions of the culture of the plants. The field was heavily irrigated on the 34th day, and on the 74th day the first rain in several weeks fell. Under these circumstances it is perhaps not surprising that a sudden increase in the nitrate content of the samples taken on the succeeding days should have been observed. Although heavy rain fell during the last week of the experiment, there was no response in the nitrate content of the final collection, possibly because of exhaustion of the available store of nitrate in the soil.

The proportion of nicotine nitrogen in the leaf increased very rapidly during the first three weeks of the experiment, but then diminished slightly and remained relatively constant during the early part of the period of most rapid growth of the plant; a steady increase in the proportion of nicotine nitrogen then ensued and continued to the end.

Interpretation of these observations is difficult in view of our ignorance of the exact position of nicotine in the scheme of nitrogen metabolism of the tobacco plant. It may be suggested, however, that the synthesis of nicotine was restricted, during the period of rapid growth, by the demands of the tissues for nitrogen in other forms; only when the plant had attained a nearly full development was the synthesis of nicotine resumed at a relative rate that resembled the initial rate of synthesis.

Figure 46 shows the relative proportions of three closely related forms of nitrogen—ammonia, glutamine amide, and asparagine amide nitrogen—in the leaves. These are highly reactive metabolites, and consequently the curves which represent the proportions present are far from smooth. The proportion of ammonia nitrogen fell rapidly during the period when the seedling was establishing itself, and the curve of glutamine amide nitrogen likewise descended. At 26 days the proportion of ammonia began to increase and, by the expiration of 54 days, had reached its former level; subsequently it again diminished. Meanwhile the proportion of glutamine amide nitrogen, after increasing slightly and diminishing again, remained at a fairly constant level until at 75 days a marked increase occurred.

The curve of asparagine amide nitrogen shows that very little of this amide was present in the young leaves, but that the proportion rapidly increased during the period of rapid growth. The relationships between the quantities of asparagine and of ammonia are in almost every detail what is to be expected if the synthesis of asparagine is regarded as being brought about by an increase in the free ammonia. Moreover, the decrease in free ammonia at the end may very probably be attributed to transformation into one or the other, or perhaps both, of the amides. These observations, then, are consistent with the view that amides are synthesized by the plant as a protective measure.

The proportions of amino and of peptide nitrogen are shown in Figure 47. The data for the amino nitrogen are somewhat irregular, but indicate an increase of free amino nitrogen, during the period of most rapid growth, to a level nearly twice as great as that at the start; this higher level is maintained to the end. The peptide nitrogen, on the other hand, remained, with minor variations, at a constant level of approximately 2 per cent until the last collection, when it suddenly increased sharply. This change is probably associated with the rapid translocation of nitrogen from leaf to seed pod that took place at this time.

The distribution of the nitrogen of the stem is shown in Figures 48, 49 and 50. The nitrate nitrogen in the tiny stems of the seedlings amounted to no less than 65 per cent of the total nitrogen—an obvious case of storage under what were designedly the most favorable possible conditions for growth. During the period of establishment of the plant, the nitrate diminished very rapidly indeed to the vicinity of 20 per cent. Comparison of Figure 48 with Figure 45 shows that the stems also exhibit the relatively higher proportion of nitrate at 35 and at 75 days that characterized the leaves at these points—a further support for the explanation already suggested.

During the period of rapid growth, nitrate increased in relative proportion in the stem, but dropped in the stems of the last collection, possibly due to leaching of the soil by the heavy rains of the last interval. At all times, however, the nitrate made up a considerably larger part of the nitrogen of the stem than it did of the nitrogen of the leaves.

The nicotine nitrogen of the stem is, save for the observation at 26 days, relatively constant throughout at the low level of approximately 2 per cent. In this respect the behavior of the stem nicotine contrasts sharply with that of the leaf nicotine (Figure 45).

The curves in Figure 49 are presented purely with the object of completing the picture. No interpretation can at present be assigned to them as the data represent the results of analytical determinations carried out in routine fashion on the stem tissue in spite of the fact that these indirect methods are probably not applicable in this particular case. Attention should be directed to the fact that the rise and fall of the ammonia nitrogen is accompanied by analogous changes in the curve labeled "glutamine amide N", but no direct inference of a relationship between these phenomena is warranted until the chemical nature of the amide nitrogen of the stem tissue is more fully known.

The proportions of amino and of peptide nitrogen in the stem tissue are shown in Figure 50. The amino nitrogen dropped in the young plants until rapid growth began, when the proportion increased. The low value for the amino nitrogen at 75 days is difficult to explain and, with the exception of this one point, the increase continued until the termination of the experiment. The relative proportion of amino nitrogen in the stem tissue is of about the same order of magnitude as the proportion in the leaf tissue.

The peptide nitrogen rapidly increased from a low value at the beginning to a maximum approximating 8 per cent at 54 days; subsequently it fell to about 4 per cent at the end of the experiment. The behavior of the proportion of this form of nitrogen in the stem is entirely different from that in the leaf, and the possibility that some of the amino nitrogen

produced by strong acid hydrolysis may have arisen from pyrrolidone carboxylic acid has already been pointed out. Whatever its origin, however, the relative proportion is much greater in the stem, and the alterations in this proportion, as growth progressed, are in striking contrast to the constancy of the proportion in the leaf. If the peptide nitrogen of the stem may be regarded as a measure of the quantity of partially constructed protein in transit from the leaves to the growing points, it is clear that the maximal amount was present in the stem tissue during the period of most rapid growth of the plant as a whole.

THE WORK OF SMIRNOV

Smirnov and his collaborators (35, 36), as has already been mentioned, have also investigated the composition of the tobacco plant at various stages in its growth. Their experimental technic and chemical methods differ fundamentally in many respects from our own and, moreover, the variety of tobacco plant they employed was likewise widely different, being a high-carbohydrate, low-nitrogen type. Their data are expressed for the most part in terms of grams per unit leaf area, and consequently there is no way in which direct comparisons with ours can be made. Fortunately, however, they likewise calculated the proportions of some constituents in terms of percentage of the dry weight of the leaf, and these calculations enable us to point out certain of the contrasts between the two varieties of tobacco grown under widely different conditions.

An important consideration in a comparison such as this is the relative length of the growing season at Krasnodar and in Connecticut, and the time required for the plants to reach maturity. It is difficult to correlate these factors. Smirnov refers to the leaves of his plants at 90 days as technically ripe, while the 110-day-old leaves were beginning to turn yellow. The lower leaves of Connecticut shade tobacco are regarded as mature in about 50 days—in fact the first picking of the crop from the plants in the field used in our experiments was made on the 51st day, and the fourth and last picking had been completed by 75 days. But even at 110 days of age the leaves of our plants had not begun to turn yellow. This is an instance of the fundamental difference between the two types of tobacco plant under discussion.

Smirnov's plants at 50 days had begun to form flower buds and at 63 days were in flower. A few flower buds had formed in the field from which we drew our samples at 54 days, but flowering had not become general until 61 days had elapsed. In view of this, the life cycle—at least with respect to reproduction—is apparently not unduly different in the two localities; the great difference is in the use to which the respective crops are put.

In Figure 51 is shown the percentage of total soluble carbohydrate in the leaves of our samples of tobacco calculated on the basis of dry weight. The curve drops sharply during the period of establishment of the plant, rises as rapid growth began, but changes to only a minor degree through the period of most rapid growth. As the plants reached their full size, however, the percentage of carbohydrate rapidly increased to a high level. Smirnov's data, calculated on the same basis, are plotted in broken lines in the same figure. The soluble carbohydrate of his young plants rapidly increased to a very high level, and then diminished again during the period of rapid growth preceding blossoming. The curve for the topped plants oscillates about a level of approximately 10 per cent. The behavior of the two types of plant is thus quite different. Smirnov's plants at all times, save in the 110-day collection, contained a much higher concentration of soluble carbohydrates than ours.

The proportion of nitrogen in the leaves of our plants, calculated as percentage of the dry weight, is shown in Figure 52. The proportion dropped slightly in the first period of study, doubtless due to the rapid metabolism of the large quantities of nitrate in the seedling leaves and the increase in non-nitrogenous constituents, and then remained at a relatively constant level of approximately 5 per cent until the plants were nearly full grown. The level dropped in the leaves of the last two collections very materially, probably due to withdrawal of nitrogenous substances and translocation to the fruit.

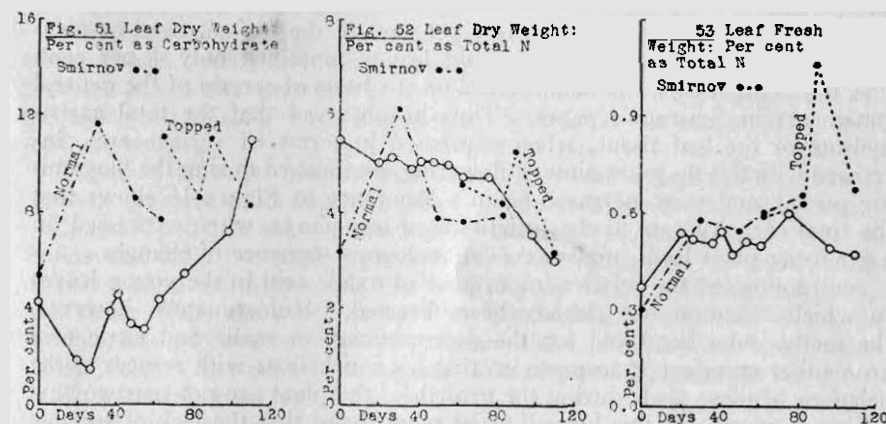


Fig. 51 Leaf Dry Weight: Per cent as Carbohydrate

Fig. 52 Leaf Dry Weight: Per cent as Total N

53 Leaf Fresh Weight: Per cent as Total N

Smirnov's seedlings were much lower in total nitrogen than ours, but rapidly increased to the extraordinary figure of 6.2 per cent at 30 days; they then dropped again to a fairly constant proportion of about 4 per cent during the period of rapid growth and beginning of flowering. Comparison of Figures 52 and 51 illustrates clearly the fundamental difference in type of the two varieties of tobacco under discussion to which attention has already been drawn. Interestingly enough, the topped plants studied by Smirnov approached very closely to our own normal plants both in nitrogen content, and in the behavior of the nitrogen in the aging leaves.

In Figure 53 the total nitrogen of the leaves calculated as per cent of the fresh weight is plotted. This curve, unlike that of percentage on dry weight, starts at a low level in the seedlings and rapidly rises as the plants established themselves. During the early part of the growth period, the proportion oscillated about a mean value slightly above 0.5 per cent, and then gradually diminished to slightly less than 0.5 per cent. One of

the values, that at 75 days, is extraordinarily high, the rest, however, are remarkably constant. Smirnov's data, plotted on the same figure, indicate that, when calculated in this manner, his leaves were richer in nitrogen than ours in all save the first case. He likewise encountered one sample in the aging plants with an extraordinarily high nitrogen content. Further investigation will be required to decide whether the aging leaves do in fact pass through a stage of unusually high nitrogen content. We are inclined, however, to attribute the high value in our own case to a sudden influx of nitrate, due to the rain which fell after a prolonged dry period during the 74th day of our experiment.

Further detailed comparisons of Smirnov's data with our own is perhaps superfluous. Clearly the two types of tobacco are widely different in their metabolism, and this is illustrated most forcibly by the difference in nicotine content. The highest proportion of nicotine nitrogen attained by our plants was slightly more than 10 per cent of the total nitrogen; Smirnov's oldest plants reached the astonishing figure of 34.2 per cent of the total nitrogen as nicotine nitrogen. These plants had been topped which, of course, profoundly altered the normal course of leaf metabolism, and perhaps a comparison is not strictly justifiable. Nevertheless his normal plants at 63 days contained 16.3 per cent of the leaf nitrogen as nicotine nitrogen, whereas our 61-day-old leaves contained only 4 per cent.

A few comparisons can be instituted on the basis of certain of the general statements in Smirnov's paper. Thus he observed that the total carbohydrate of the leaf tissue, when expressed in terms of surface area, increased with age up to the time of flowering, diminished during the blossoming period, and then increased again. Reference to Figure 16 shows that the total carbohydrate of the leaf tissue of our plants, when expressed on a gram per plant basis, underwent an analogous sequence of changes.

Smirnov noted the relative importance of oxalic acid in the young leaves to which attention has already been directed. Unfortunately, however, the methods he employed for the determination of malic and citric acid are neither specific nor accurate, so that his conclusions with respect to the behavior of these acids during the growth of the plant are not trustworthy. Smirnov recognized this himself as he pointed out that the lack of suitable methods of analysis made it impossible to obtain a clear picture of the changes in the organic acids during the vegetative period of the leaves.

SUMMARY

The rate of growth of the tobacco plant has been investigated by means of detailed chemical analyses of the leaves, stems, and fruit of a series of collections taken at frequent intervals from the seedling stage to the point at which the seed pods were well advanced in the process of ripening. The variety studied was Connecticut shade-grown tobacco; the plants were grown under normal agricultural conditions under a shade tent as part of a general crop.

The collections of plants were divided into two roughly equal lots, and were then dissected into leaf, stem, and later, inflorescence, or pod portions.

The leaves and stems of one lot were separately extracted with boiling water, those of the other lot were at once dried in an oven. The pods were dried.

Analyses of the extracts, residues from extraction, and of the dried samples were carried out by methods many of which have been specially developed in this laboratory for application to the tobacco plant. The results of the analyses were calculated on a basis of grams per individual plant, and are plotted in the figures on a uniform time scale. Ratios between certain of the constituents and distributions of some of them in the three main parts of the plant have also been calculated.

The rate of growth, as measured by the increase of fresh weight of the tops, was very slow during the first three weeks while the seedling was establishing itself. The rate then rapidly accelerated to a maximum between the 40th and 47th days; thereafter it diminished in a regular fashion until seed production began. The relative proportions of leaf and stem tissue changed profoundly during the growth period. At 35 days, the stems weighed one-third as much as the leaves, but after 61 days exceeded the leaves in weight. The leaves of the older plants diminished in weight as the inflorescence developed.

Measured in terms of dry weight, the rate of growth was different in many details. After an initial period of slow acceleration, the rate of growth became practically constant in the period from 35 to 75 days, and subsequently diminished more slowly than did the rate as measured in terms of fresh weight.

Together with the results that show the broad aspects of the rate of growth are presented data that show the rates of accumulation in leaves, stem, and pods of the organic solids, the ash, the water-soluble organic solids and ash, the total organic acidity, the quantities of malic, citric, oxalic, and of unknown acids, the total and the fermentable carbohydrates, the crude fiber, the ether extractives, the nitrogen both soluble and insoluble, and of the nitrate, nicotine, ammonia, asparagine amide, glutamine amide, amino and peptide nitrogen. Where possible, attempts have been made to correlate certain of the data with each other, and especial attention has been given to the effect of the onset of the reproductive period.

It is not feasible to draw many detailed conclusions from a single experiment that represents the effects of but one growing season. Attention may be directed, however, to a few of the results that appear to have general significance.

The data on the organic acids are the first that have hitherto been obtained by accurate and trustworthy methods from which conclusions can be drawn regarding the behavior of these substances in a growing plant. A rapid accumulation of a high relative proportion of oxalic acid in the very young tobacco plants was observed. This is a phenomenon already noted by Smirnov and, if oxalic acid may be regarded as an end-product, suggests an extremely rapid rate of metabolism in these plants. The most significant result, however, is the observation that the three chief acids—malic, citric, and oxalic—maintained a nearly constant ratio to one another in the leaves from the 40th day to the end of the period of observation (110 days), although the total quantity of organic acids present increased

about 400 per cent in the interval between 40 and 75 days and then sharply decreased. This implies that the quantitative relationships of the three acids to one another were not affected either by rapid deposition in the leaves or by withdrawal from them. Manifestly the metabolism of these three acids is closely related, and it is clear that oxalic acid shares proportionately with the others in the chemical changes. No definite evidence was secured, however, that connects the organic acid metabolism with either the carbohydrate or the protein metabolism of the plant.

Malic acid was at all stages of growth the predominant acid of the leaves, oxalic being the next in order, with citric in smallest quantity. The amount of unknown acids was intermediate between the oxalic and citric acids. In the stems, the unknown acids predominated, malic and oxalic acids being present in considerably smaller amounts; citric acid was invariably present, but in only small quantities. In the pods also, the unknown acids predominated, malic acid coming next in quantity; traces only of oxalic and citric acids were present.

The investigation of the amide nitrogen showed that our knowledge of the substances in the tobacco plant which produce ammonia on mild hydrolysis with acids is far from complete. The results with the leaf tissue could, for the most part, be satisfactorily interpreted on the assumption that asparagine and glutamine are the only amides present. The stem tissue, on the other hand, may contain a considerable proportion of an unstable amide-like substance in addition to these; if this is so, the accurate determination of glutamine and asparagine by the methods employed is impossible.

The growth of the plant as a whole can be roughly divided into three periods. The first is the period of from three to four weeks during which the seedling established itself in the soil but increased little in weight. The dry matter, organic acids, ash, carbohydrates and nitrogen in all forms increased in absolute quantity per plant, but the relative distribution of the individual organic acids, and of the forms of carbohydrate and of nitrogen underwent considerable changes. Thus, the malic acid diminished, and the oxalic and citric increased when calculated as percentage of the total acidity. The proportion of the total soluble carbohydrate as fermentable carbohydrate diminished. Calculated as percentage of the total nitrogen, the nitrate nitrogen dropped profoundly, and the ammonia, amide, and amino nitrogen diminished; the nicotine nitrogen increased.

During the period of rapid growth, which extends roughly from the 35th to the 75th day, organic and inorganic substances accumulated in the plant with surprising speed, but the alterations in relative proportions were less striking. The individual organic acids were remarkably constant in both leaf and stem. The proportion of fermentable carbohydrate in the leaves dropped temporarily during the time of most rapid growth, but soon recovered its earlier level, while the proportion in the stem steadily, though slowly, increased. The relative proportions of the more active nitrogenous metabolites, i.e. the nitrate, ammonia and amide nitrogen, underwent material fluctuations, but in general the nitrate in the leaf diminished, that in the stem increased. The nicotine of the leaves increased, that of the stem remained constant, but the relative proportions of the total nicotine in leaf and stem tissue were unchanged; the amides and ammonia of both leaf and stem increased.

In the final period, that of reproduction, which began at approximately the 61st day, the leaves decreased both in fresh and in dry weight; the stems remained constant in fresh weight, but increased in dry weight; the organic acids and the ash of both leaves and stems increased, but the relative proportions of the individual acids remained approximately steady; the soluble carbohydrates remained constant during the early part of the reproductive period, but then increased. The total nitrogen of the plant appeared to decrease somewhat at the end of the period of reproduction, but additional evidence will be required to make this certain. The quantity of nitrate nitrogen diminished sharply in both leaf and stem tissue; the quantity of nicotine nitrogen in the leaves increased, that in the stems remained constant; the amides and ammonia in general decreased in amount. The relative proportion of nitrate decreased, that of the nicotine of the leaves increased, while that of the stems remained constant; the proportions of amide nitrogen and of ammonia nitrogen increased.

The most striking feature of the final period was, of course, the evidence of translocation of organic and of inorganic substances from other parts of the plant, particularly the leaves, into the developing seed pods. Approximately one-fifth of the organic solids of the plant were ultimately located in the fruit, and nearly one-third of this consisted of ether-soluble material, mostly true fat. There was also a marked storage of nitrogen in the seed pods, which at the end amounted to nearly one-third of the nitrogen of the entire plant. Much of it was doubtless in the form of seed protein. The translocation of organic acids, probably combined as salts of inorganic cations, into the fruit was by no means a minor matter as about one-tenth of the organic acidity was ultimately found therein. The picture presented is very clearly one of transformation of the plastic materials of the leaves and to lesser extent of the stems into the stable reserves of nutriment for the succeeding generation.

BIBLIOGRAPHY

1. Assn. Official Agr. Chem., Methods of Analysis, 66. 1925.
2. Assn. Official Agr. Chem., Methods of Analysis, 117. 1925.
3. Barnstein, F., Landw. Vers. Sta., **54**: 327. 1900.
4. Berthold, T., Conn. Agr. Expt. Sta., Bul. **326**: 406. 1931.
5. Carpenter, F. B., N. C. Agr. Expt. Sta., Bul. **90a**. 1893.
6. Chandler, W. H., Fruit Growing. (Boston) 1925.
7. Chibnall, A. C., Biochem. Jour., **16**: 344. 1922.
8. Chibnall, A. C., and Westall, R. G., Biochem. Jour., **26**: 122. 1932.
9. Culpepper, C. W., and Caldwell, J. S., Plant Physiol., **7**: 447. 1932.
10. Davidson, R. J., Va. Agr. Expt. Sta., Bul. **50**. 1895.
11. Emmerling, A., Landw. Vers. Sta., **24**: 113. 1880.
12. Emmerling, A., Landw. Vers. Sta., **34**: 1. 1887.
13. Emmerling, A., Landw. Vers. Sta., **54**: 215. 1900.
14. Fleischer, E., Arch. Pharm., **205**: 97. 1874.
15. Gardner, N. R., Bradford, F. C., and Hooker, H. D., The Fundamentals of Fruit Production. (New York) 1922.

16. Garner, W. W., Bacon, C. W., and Bowling, J. D., Indus. and Engin. Chem., **26**: 970. 1934.
17. Gouwentak, C. A., Rec. Trav. Bot. Néerland., **26**: 19. 1929.
18. Hartmann, B. G., and Hillig, F., Jour. Assoc. Off. Agr. Chem., **10**: 264. 1927.
19. Jones, W. J. Jr., and Huston, H. A., Ind. Agr. Expt. Sta., Bul. **175**. 1914.
20. Knowles, F., and Watkin, J. E., Jour. Agr. Sci., **21**: 612. 1931.
21. Knowles, F., Watkin, J. E., and Hendry, F. W. F., Jour. Agr. Sci., **24**: 368. 1934.
22. Koch, F. C., and McMeekin, T. L., Jour. Amer. Chem. Soc., **46**: 2066. 1924.
23. Mason, T. G., and Maskell, E. J., Ann. Bot., **42**: 189. 1928.
24. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, **2**: 385. (Baltimore) 1932.
25. Pucher, G. W., Leavenworth, C. S., and Vickery, H. B., Indus. and Engin. Chem., Anal. Ed., **2**: 191. 1930.
26. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S., Indus. and Engin. Chem., Anal. Ed., **6**: 190. 1934.
27. Pucher, G. W., Vickery, H. B., and Wakeman, A. J., Jour. Biol. Chem., **97**: 605. 1932.
28. Pucher, G. W., Vickery, H. B., and Wakeman, A. J., Indus. and Engin. Chem., Anal. Ed., **6**: 140. 1934.
29. Pucher, G. W., Vickery, H. B., and Wakeman, A. J., Indus. and Engin. Chem., Anal. Ed., **6**: 288. 1934.
30. Roberts, W. L., and Schuette, H. A., Jour. Amer. Chem. Soc., **56**: 207. 1934.
31. Schulze, B., and Schütz, J., Landw. Vers. Sta., **71**: 299. 1909.
32. Schulze, E., Ztschr. physiol. Chem., **24**: 18. 1898.
33. Schweitzer, P., Missouri Agr. Expt. Sta. Bul. **9**. 1889.
34. Sessions, A. C., and Shive, J. W., Soil Sci., **35**: 355. 1933.
35. Smirnov, A. I., with collaborators, U. S. S. R. State Inst. Tobacco Investigations, Bul. **46**. (In Russian) 1928.
36. Smirnow, A. I., Erygin, P. S., Drboglaw, M. A., and Maschkowzew, M. T., Ztschr. wiss. Biol., Abt. E, Planta, **6**: 687. 1928.
37. Sörensen, S. P. L., Biochem. Ztschr., **7**: 45. 1908.
38. Stahl, A. L., and Shive, J. W., Soil Sci., **35**: 375. 1933.
39. Stahl, A. L., and Shive, J. W., Soil Sci., **35**: 469. 1933.
40. Swart, N., Die Stoffwanderung in ablebenden Blättern. (Diss. Jena) 1914.
41. Thomas, W., Plant Physiol., **7**: 391. 1932.
42. Tucker, G. M., and Tollens, B., Jour. Landw., **48**: 39. 1900.
43. Vickery, H. B., Plant Physiol., **2**: 303. 1927.
44. Vickery, H. B., and Pucher, G. W., Conn. Agr. Expt. Sta., Bul. **323**. 1931.
45. Vickery, H. B., and Pucher, G. W., Conn. Agr. Expt. Sta., Bul. **324**. 1931.
46. Vickery, H. B., and Pucher, G. W., Indus. and Engin. Chem., Anal. Ed., **6**: 372. 1934.
47. Vickery, H. B., Pucher, G. W., and Clark, H. E., Sci., **80**: 459. 1934.
48. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., Chemical Investigations of the Tobacco Plant. Carnegie Inst. Wash., Pub. **445**. 1933.
49. Vickery, H. B., Wakeman, A. J., and Leavenworth, C. S., Conn. Agr. Expt. Sta., Bul. **339**: 625. 1932.
50. White, H. C., Ga. Expt. Sta., Bul. **108**. 1914.
51. Wilfarth, H., Römer, H., and Wimmer, G., Landw. Vers. Sta., **63**: 1. 1906; translated by Emslie, B. L., and published by Vinton and Co. (London) 1906.

APPENDIX

After the manuscript of the present bulletin had been prepared for the press, we received the valuable papers of Vladescu (Vladescu, I., Buletinul cultivarei si fermentarei Tutunului, **23**: 231, 359. 1934) on the assimilation of mineral and organic substances during the development of the tobacco plant. This extensive study, carried out at the Institute for the Culture and Fermentation of Tobacco in Băneasa, deals with the composition of the tobacco plant throughout the life cycle, particular attention being paid to the inorganic constituents.

The plants studied by Vladescu were of the variety Molovata, a small-leaved plant with slender stems especially suited for chemical investigations. The behavior of these plants under the agricultural conditions that obtain in Roumania differs fundamentally from that characteristic either of Connecticut tobacco or the Russian variety studied by Smirnov at Krasnodar. After passing through the blossoming stage (46th to 56th days from setting) the plants begin to lose in fresh weight owing to the ripening of the basal leaves (66 days). The ripening process gradually extends up the plant, and is accompanied by marked losses of water and even of organic matter, possibly due to processes analogous to those noted by the present writers during the curing of Connecticut tobacco leaves (Carnegie Inst. Wash., Pub. **445**: 1933). A renewed reproductive activity begins at about the 96th day from setting and results in a distinct increase in the fresh and dry weight of the plants as well as in the quantities of inorganic constituents; but at the expiration of 119 days the fresh and dry weight has again diminished.

Vladescu's papers deal explicitly with the matter of calculation of the results of chemical analyses of plants during the growth cycle. He points out that calculation in terms of percentage of the fresh or dry weight may readily lead to contradiction and error. The calculation in terms of absolute weight in a fixed number of plants (he uses 100 plants), however, leads to definite and easily appreciated results, and is much to be preferred for studies of growing plants.

His work is divided into two main parts. In the first part he deals with plants taken from the seedbed at frequent intervals during the first four weeks of vegetation. The plants had attained a size suitable for transplantation in 17 days and at that time bore 4 to 5 leaves and were 12 cm. high. The entire plants, including the roots, were removed from the beds, and washed free from soil; superficial moisture was removed and, after being weighed, the material was dried at 105°. The dry tissue was then analyzed for total nitrogen, protein nitrogen, nicotine, and ash, and the ash was analyzed for calcium, magnesium, iron, manganese, potassium, phosphorus, and silicon by well known and accurate methods. The results are expressed in tables and graphs which show both percentage of dry weight and the absolute quantity in 100 plants.

Aside from the evidence of rapid assimilation of nitrogen and inorganic substances, the most important observation is that nicotine is elaborated at a constantly increasing rate even from the earliest stage, and forms a very appreciable part of the non-protein nitrogen. It is unfortunate that determinations of nitrate nitrogen were not included.

The second part of Vladescu's work deals with analyses of plants after transplantation to the field. Four agricultural technics were employed in the culture of these plants. Two equal areas of the experimental plot were fertilized with manganese applied at the rate of 16 kilos of manganese

sulfate per 1000 sq. m. in addition to the usual fertilizer. The suckers were removed from half of the plants on each area at the proper time. The final data therefore include observations on plants stimulated by the administration of manganese, on normal plants, and on plants under both conditions of nutriment from which the suckers had been removed.

The curves which show the rate of accumulation, in the plants with and without manganese, of fresh and dry weight, and of water, all indicate a rapid rise to a maximum at approximately the 65th day from setting. At this time the seed capsules were formed and drying of the basal leaves had begun. During the following 30 days a diminution in weight occurred. An increase to a second but lower maximum followed during the next 10 to 14 days, this second maximum being much more pronounced in the case of the plants from which suckers had been removed.

The weight of the ash and, indeed, of several of the ash constituents showed a similar, though in some cases less striking sequence of changes: iron, manganese, and silicon gave no distinct maxima, but the curves reveal marked inflections at the critical points. Vladescu interprets these changes in terms of positive and negative migration of the various constituents, and points out that negative (downward) migration of iron, manganese, and silicon apparently does not occur.

The behavior of the nitrogen in Vladescu's plants is of particular interest because of the contrast to the behavior in Connecticut tobacco. The curves as drawn in his paper indicate a rise to a maximum at about 65 days (end of flowering) followed by a drop, and then a second rise to a sharp maximum at about 108 days. The first maximum nitrogen content per individual plant is 1.73 gm. which is very much less than the maximal nitrogen content (5.12 gm.) of our plants attained on the 75th day. The nitrogen of the plants then drops to 1.38 gm. at about 89 days, rises to 1.76 gm. at 108 days and finally drops sharply.

A careful study of the distribution of the points from which Vladescu plotted his curve, and a consideration of our own difficulties in selecting average plants so as to minimize the sampling error leads us to wonder if Vladescu's data provide as clear a demonstration of a negative migration after the first period of flowering as he would imply. There is, unfortunately, no information in his papers regarding the number of plants taken at each collection and the magnitude of the probable error cannot, therefore, be assessed.

Vladescu's data on the nicotine content of his plants, however, give evidence of changes far greater than any possible experimental or sampling error. The nicotine content reached a maximum at 65 days and then steadily diminished to a value at 100 days of less than half the maximum. The normal plants, that is, those from which suckers had not been removed, then increased materially in nicotine, doubtless due to the elaboration of alkaloids in the newly developing parts; the others, however, maintained the nicotine level unchanged through this period.

The quantities involved indicate that at the point of maximal nicotine content (65 days) Vladescu's plants contained 0.0511 gm. of nicotine nitrogen per plant of fresh weight 610 gm. Our plants at 61 days contained 0.113 gm. in a plant of 954 gm. fresh weight. But at 61 days our

plants had only begun to pass into the blossoming phase; at the period when blossoming was over and seed pods were developing (75 days), our plants contained 0.182 gm. of nicotine nitrogen in a plant of 1,110 gm. fresh weight, and subsequently the nicotine nitrogen increased to 0.287 gm. (97 days) with only a minor increase in fresh weight.

No phenomenon at all analogous to the profound drop in nicotine content (from 0.051 gm. at 65 days to 0.028 gm. at 100 days) noted by Vladescu occurred in our plants, and this is of great importance in the interpretation of the physiological function of nicotine in the plant. We have elsewhere (Carnegie Inst. Wash., Pub. **445**. 1933) pointed out that the nicotine content diminishes slightly during the curing of detached tobacco leaves. But the rapid decrease that occurred in the leaves of the Roumanian plants indicates that the destruction of the alkaloid in the drying leaves still attached to the plant is a much more striking phenomenon and suggests the occurrence of a series of chemical changes, the interpretation of which might throw much light on the function of nicotine in the tobacco plant.

TABLE 2. FRESH WEIGHT, WATER CONTENT, AND DRY WEIGHT
(Figures are grams in one plant)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Number of plants		I	440	380	80	22	16	10	10	10	11	10	10
Fresh weight													
Leaves		I	1.04	4.81	20.91	91.14	132.7	362.1	391.2	476.6	480.3	455.6	401.9
Stem		I	0.477	1.17	2.76	31.69	73.94	246.1	353.4	465.6	565.1	591.1	511.0
Pods		I								11.7	64.8	173.1	120.9
Plant		I	1.517	5.98	23.67	122.8	206.6	608.2	744.6	953.9	1110	1220	1033
Number of plants		II	274	222	87	20	14	10	10	10	10	10	10
Fresh weight													
Leaves		II	1.174	4.93	16.66	99.51	150.2	376.1	453.7	431.4	523.2	374.4	367.2
Stem		II	0.558	1.185	2.97	29.81	84.5	276	384	430.7	551.5	501.8	575.7
Pods		II								8.77	71.5	147.9	122.1
Plant		II	1.732	6.12	19.63	129.3	234.7	652.1	837.7	870.9	1146	1024	1065
Average fresh weight													
Leaves	1	I+II	1.1	4.9	18.7	95.3	142	369	422	454	501	415	384
Stem	1	I+II	0.5	1.2	2.86	30.7	79.2	261	368	448	558	546	543
Pods	1	I+II								10.2	68.1	160	121
Plant	1	I+II	1.6	6.1	21.6	126	221	630	790	912	1128	1122	1049
Water content													
Leaves	2	II	1.09	4.36	15.0	89.2	134	341	407	386	457	323	310
Stem	2	II	0.54	1.09	2.71	27.2	78.6	254	343	378	468	404	461
Pods	2	II								7.5	60.3	114	83
Plant	2	II	1.63	5.45	17.7	116.4	213	595	750	771	985	841	854
Dry weight													
Leaves	3	II	0.078	0.528	1.70	10.3	16.0	35.5	46.7	45.9	66.6	51.6	57.2
Stem	3	II	0.023	0.099	0.257	2.58	5.85	21.9	40.7	52.9	83.4	97.8	115
Pods	3	II								1.2	11.1	34.2	38.9
Plant	3	II	0.101	0.627	1.96	12.9	21.9	57.4	87.4	100	161	184	211

TABLE 3. ORGANIC SOLIDS AND INORGANIC SOLIDS (ASH)
(Figures are grams in one plant)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Organic solids													
Leaves	4	II	0.0630	0.427	1.316	8.495	13.55	29.91	39.03	39.36	56.69	43.88	49.35
Stem	4	II	0.0179	0.0787	0.2126	2.219	5.062	19.58	37.17	49.02	77.24	91.58	108.5
Pods	4	II								1.146	9.78	32.30	37.04
Plant	4	II	0.0809	0.5057	1.5286	10.714	18.612	49.49	76.20	89.526	143.71	167.76	194.89
Inorganic solids (ash)													
Leaves	7	II	0.0153	0.101	0.381	1.774	2.478	5.601	7.615	6.546	9.922	7.739	7.866
Stem	7	II	0.0053	0.0204	0.0449	0.361	0.788	2.324	3.555	3.916	6.166	6.223	6.316
Pods	7	II								0.106	1.384	1.865	1.849
Plant	7	II	0.0206	0.1214	0.4259	2.135	3.266	7.925	11.17	10.568	17.472	15.827	16.031
Soluble organic solids													
Leaves	5	I	0.029	0.205	0.705	3.17	4.84	10.25	13.6	17.0	22.9	29.0	25.2
Stem	5	I	0.0085	0.0296	0.832	0.867	0.920	6.72	9.99	12.5	11.9	17.4	22.8
Plant	5	I	0.0376	0.234	1.54	4.04	5.76	17.0	23.6	29.5	34.8	46.4	48.0
Insoluble organic solids													
Leaves	6	I	0.0218	0.212	0.926	4.31	7.29	16.8	20.7	28.3	31.0	37.9	29.5
Stem	6	I		0.051	0.094	1.02	2.56	9.46	21.6	38.0	65.4	85.0	76.5
Plant	6	I	0.023	0.263	1.02	5.33	9.85	26.2	42.3	66.3	96.4	123.0	106.0
Soluble ash													
Leaves	8	I	0.0398	0.087	0.401	1.42	2.20	5.09	6.09	7.25	7.72	9.35	7.95
Stem	8	I	0.0069	0.021	0.476	0.406	0.695	2.16	3.22	4.36	5.66	6.21	6.14
Plant	8	I	0.0467	0.108	0.878	1.82	2.89	7.25	9.31	11.6	13.4	15.6	14.1
Insoluble ash													
Leaves	9	I	0.0023	0.028	0.072	0.358	0.321	0.628	0.980	1.30	2.58	3.23	2.00
Stem	9	I		0.001	0.003	0.026	0.075	0.217	0.359	0.497	0.822	1.35	1.05
Plant	9	I	0.0023	0.029	0.075	0.384	0.396	0.845	1.34	1.80	3.40	4.58	3.05

TABLE 4. ORGANIC ACIDS

(Figures in the upper section of this table are milliequivalents of acid in one plant; figures in the lower section are percentages of the total organic acidity)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Total acidity: Leaves	10	II	0.111	1.02	4.21	24.5	34.6	79.6	113	104	148	120	128
Stem	10	II	0.024	0.111	0.291	2.92	5.74	22.4	40.5	47.1	77.5	76.8	99.4
Pods	10	II								1.36	6.43	25.9	21.3
Plant	10	II	0.135	1.13	4.50	27.4	40.3	102.0	153	152	232	223	249
Leaves: Malic acid	11	II	0.054	0.446	1.50	12.3	18.3	43.3	58.3	59.8	84.1	68.0	70.3
Citric acid	11	II	0.011	0.108	0.905	2.98	3.69	8.27	14.2	9.0	15.3	13.1	14.6
Oxalic acid	11	II	0.008	0.259	0.745	4.40	6.32	15.6	20.7	19.2	27.0	20.6	21.8
Unknown acid	11	II	0.039	0.208	1.06	4.80	6.30	12.5	20.1	16.5	22.5	18.4	21.4
Stem: Malic acid	12	II	0.0084	0.022	0.045	0.650	1.96	6.68	11.7	15.0	16.3	24.5	31.1
Citric acid	12	II	0.000	0.0041	0.014	0.048	0.203	0.477	0.719	0.829	1.65	1.67	3.36
Oxalic acid	12	II	0.0035	0.038	0.110	0.622	1.12	3.66	5.49	6.48	10.2	10.2	10.8
Unknown acid	12	II	0.012	0.047	0.123	1.60	2.46	11.6	22.6	24.8	49.4	40.5	54.2
Pods: Malic acid	13	II								0.28	1.70	7.58	7.41
Citric acid	13	II								0.13	0.67	2.31	2.40
Oxalic acid	13	II								0.37	2.30	1.65	2.82
Unknown acid	13	II								0.58	1.75	14.3	8.64
Distribution of acidity in leaves													
% Malic acid	14	II	48.5	43.7	35.6	50.3	52.8	54.4	51.5	57.3	56.5	56.6	54.9
% Citric acid	14	II	9.59	10.6	21.5	12.2	10.7	10.4	12.5	8.61	10.3	10.9	11.4
% Malic + citric acid	14	II	58.1	54.3	57.1	62.5	63.5	64.8	64.0	65.9	66.8	67.5	66.3
% Oxalic acid	14	II	6.99	25.3	17.7	17.9	18.3	19.5	18.2	18.3	18.1	17.1	17.0
% Malic + citric + oxalic acid	14	II	65.1	79.6	74.8	80.4	81.8	84.3	82.2	84.2	84.9	84.6	83.3
Distribution of acidity in stem													
% Malic acid	15	II	35.4	19.9	15.3	22.3	34.1	29.8	28.8	31.9	21.0	31.9	31.2
% Citric acid	15	II		3.73	4.91	1.66	3.54	2.13	1.77	1.76	2.13	2.18	3.38
% Malic + citric acid	15	II	35.4	23.6	20.2	23.9	37.6	31.9	30.5	33.6	23.1	34.0	34.6
% Oxalic acid	15	II	14.5	34.4	37.7	21.3	19.4	16.4	13.6	13.8	13.2	13.3	10.8
% Malic + citric + oxalic acid	15	II	49.9	58.0	57.9	45.2	57.0	48.3	44.1	47.4	36.3	47.3	45.4

TABLE 5. CARBOHYDRATES, ETHER EXTRACTIVES AND TOTAL NITROGEN

(Figures not otherwise designated are grams in one plant)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Leaves													
Total carbohydrate	16	II	0.0033	0.0098	0.0251	0.397	0.725	1.16	1.47	1.98	3.62	3.68	6.24
Fermentable carbohydrate	16	II		0.0045	0.0112	0.247	0.570	0.825	0.819	1.22	2.46	2.81	4.39
Unfermentable carbohydrate	16	II		0.0053	0.0139	0.149	0.155	0.337	0.651	0.760	1.15	0.87	1.85
% Fermentable	18	II		45.9	44.6	62.2	78.6	71.1	55.7	61.6	68.0	76.4	70.4
Stem													
Total carbohydrate	17	II		0.0048	0.0070	0.296	0.820	2.92	4.19	6.31	7.93	8.47	11.6
Fermentable carbohydrate	17	II		0.0038	0.0042	0.191	0.614	2.08	3.13	4.79	6.43	6.60	9.21
Unfermentable carbohydrate	17	II		0.0010	0.0028	0.105	0.206	0.832	1.06	1.52	1.50	1.87	2.37
% Fermentable	18	II		79.2	60.0	64.5	74.9	71.2	74.7	75.9	81.1	77.9	79.4
Crude fiber													
Leaves	19	II		0.0297	0.112	0.733	1.21	3.20	4.20	4.01	4.96	4.49	4.31
Stem	19	II				0.536	1.30	6.07	14.2	20.9	33.0	43.0	48.3
Ether extractives													
Leaves	20	II	0.0037	0.025	0.099	0.614	0.938	2.34	3.08	3.54	4.61	3.75	4.17
Pods	20	II								0.86	0.941	8.92	12.2
Total nitrogen													
Leaves		II	0.0043	0.0265	0.0868	0.506	0.806	1.77	2.30	2.15	3.16	1.83	1.74
Stem		II	0.0012	0.0041	0.0089	0.084	0.166	0.529	0.825	0.911	1.51	1.04	1.22
Pods		II								0.072	0.450	1.21	1.43
Plant		II	0.0055	0.0306	0.0957	0.590	0.972	2.30	3.13	3.13	5.12	4.08	4.39
Total nitrogen													
Leaves	21	I	0.0032	0.0243	0.100	0.420	0.694	1.68	1.84	2.32	2.69	2.78	1.80
Stem	21	I	0.0011	0.0040	0.0081	0.076	0.148	0.451	0.724	1.04	1.44	1.55	1.21
Pods	21	I								0.10	0.50	1.37	1.49
Plant	21	I	0.0043	0.0283	0.1081	0.496	0.842	2.13	2.56	3.46	4.63	5.70	4.50

TABLE 6. NITROGENOUS CONSTITUENTS

(Figures are grams in one plant)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Soluble N: Leaves	22	I	0.0015	0.0081	0.0291	0.131	0.175	0.503	0.524	0.579	0.899	0.991	0.603
Insoluble N: Leaves	22	I	0.0017	0.0162	0.0709	0.289	0.520	1.17	1.32	1.74	1.79	1.79	1.20
Soluble N: Stem	23	I	0.0009	0.0027	0.0055	0.0512	0.0928	0.282	0.461	0.659	0.918	0.928	0.696
Insoluble N: Stem	23	I	0.0002	0.0012	0.0026	0.0244	0.0549	0.169	0.263	0.383	0.518	0.620	0.510
Nitrate N: Leaves	24	II	0.0010	0.0035	0.0088	0.058	0.067	0.141	0.195	0.142	0.329	0.063	0.032
Stem	24	II	0.0008	0.0016	0.0030	0.028	0.027	0.123	0.177	0.206	0.454	0.299	0.205
Pods	24	II								0.0017	0.0078	0.0088	0.0064
Nicotine N: Leaves	25	I	0.00003	0.001	0.0046	0.0165	0.0241	0.0542	0.0621	0.097	0.150	0.255	0.184
Stem	25	I	0.00001	0.0001	0.0008	0.0023	0.0041	0.0094	0.0101	0.0163	0.0322	0.0316	0.0249
Leaves													
Free NH_3–N	26	II	0.000036	0.00012	0.00027	0.00252	0.00306	0.0080	0.0204	0.0172	0.0196	0.0115	0.0094
pH 7 hydrolyzed NH_3–N	26	II	0.000071	0.00026	0.00067	0.0056	0.0083	0.0152	0.0297	0.0266	0.0345	0.0289	0.0285
1 N acid hydrolyzed NH_3–N	26	II		0.00030	0.000755	0.00593	0.00898	0.0181	0.0474	0.0363	0.0683	0.0535	0.0470
Asparagine amide N	27	II		0.00004	0.00008	0.00031	0.00067	0.0029	0.0177	0.0097	0.0338	0.0246	0.0185
Glutamine amide N	27	II	0.000035	0.00014	0.00040	0.0031	0.00525	0.0072	0.0093	0.0094	0.0149	0.0174	0.0191
Easily hydrolyzed amide N	27	I+II	0.000087	0.00023	0.00060	0.0032	0.0051	0.0081	0.0041	0.0086	0.0053	0.0302	0.0084
Stem													
Free NH_3–N	28	II	0.000016	0.000038	0.0000692	0.000291	0.000719	0.00357	0.00633	0.0106	0.00757	0.00652	0.00861
pH 7 hydrolyzed NH_3–N	28	II	0.000027	0.000060	0.000122	0.00124	0.0034	0.0158	0.0329	0.0425	0.0299	0.0316	0.0443
1 N acid hydrolyzed NH_3–N	28	II		0.000068	0.000155	0.00188	0.00489	0.0235	0.0456	0.0481	0.0493	0.0499	0.0682
Asparagine amide N	29	II		0.000008	0.000033	0.00064	0.00149	0.0077	0.0127	0.0056	0.0194	0.0183	0.0239
Glutamine amide N	29	II	0.000011	0.000022	0.000053	0.00095	0.00268	0.0122	0.0266	0.0319	0.0223	0.0251	0.0357
Easily hydrolyzed amide N	29	I+II	0.000027	0.000067	0.00026	0.0037	0.0080	0.0269	0.0596	0.0830	0.0789	0.0747	0.0456
Non-glutamine amide N	29	I+II	0.000016	0.000045	0.00021	0.0028	0.0053	0.0147	0.0330	0.0511	0.0566	0.0496	0.0099

TABLE 7. NITROGENOUS CONSTITUENTS

(Figures are grams in one plant)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Leaf extract													
Free NH_3–N	30	I	0.00012	0.00035	0.000875	0.00573	0.00812	0.0161	0.0245	0.0258	0.0249	0.0417	0.0178
1 N acid hydrolyzed NH_3–N	30	I	0.000165	0.00050	0.00156	0.00782	0.0121	0.0256	0.0334	0.0372	0.0413	0.0620	0.0336
6 N acid hydrolyzed NH_3–N	30	I	0.00017	0.00061	0.0018	0.00832	0.0146	0.0373	0.0399	0.0467	0.0561	0.0753	0.0477
Asparagine amide N	31	I	0.000045	0.00015	0.000685	0.00209	0.00398	0.0095	0.0089	0.0114	0.0164	0.0203	0.0158
6 N acid hydrolyzed NH_3–N	31	I	0.000005	0.00011	0.00024	0.0005	0.0025	0.0117	0.0065	0.0095	0.0148	0.0133	0.0141
Stem extract													
Free NH_3–N	32	I	0.000043	0.00011	0.000329	0.0040	0.00875	0.0305	0.0659	0.0936	0.0865	0.0812	0.0542
1 N acid hydrolyzed NH_3–N	32	I	0.000058	0.000158	0.00052	0.0052	0.0101	0.0371	0.0748	0.106	0.107	0.110	0.0768
6 N acid hydrolyzed NH_3–N	32	I	0.000064	0.000187	0.000525	0.00536	0.0121	0.0377	0.0820	0.115	0.120	0.136	0.0901
Asparagine amide N	33	I	0.000015	0.000048	0.00019	0.0012	0.0013	0.0066	0.0089	0.0124	0.0205	0.0288	0.0226
Asparagine amide N (calc.)	33	I+II	0.000031	0.000098	0.000397	0.00395	0.00670	0.0213	0.0419	0.0635	0.0771	0.0784	0.0325
6 N acid hydrolyzed NH_3–N	33	I	0.000006	0.000029	0.000005	0.00016	0.0020	0.0006	0.0072	0.009	0.013	0.026	0.0133
Leaves													
Free amino N	34	II	0.00015	0.00098	0.00244	0.0199	0.0269	0.0664	0.1360	0.122	0.171	0.127	0.103
Glutamine N+½ asparagine N	34	II		0.00032	0.00088	0.0065	0.0112	0.0173	0.0363	0.0285	0.0636	0.0594	0.0567
Amino N of amino acids	34	II		0.00066	0.00156	0.0134	0.0157	0.0491	0.0997	0.0935	0.1074	0.0676	0.0463
Total amino N	35	II	0.00024	0.00159	0.0040	0.0307	0.0469	0.1018	0.1705	0.154	0.216	0.162	0.172
Peptide N	35	II	0.00009	0.00061	0.0016	0.0108	0.0200	0.0354	0.0345	0.033	0.045	0.035	0.069
Stem													
Free amino N	36	II		0.00013	0.00024	0.0018	0.00433	0.0201	0.0334	0.0400	0.0387	0.0555	0.0789
Total amino N	36	II		0.00022	0.00053	0.00616	0.0147	0.0547	0.110	0.103	0.138	0.102	0.128
Peptide N	36	II		0.0009	0.00029	0.00436	0.0103	0.0345	0.0769	0.063	0.100	0.0464	0.049
Pods													
Free amino N	37	II								0.00489	0.0201	0.0259	0.0220
Total amino N	37	II								0.0081	0.0562	0.0669	0.0741
Peptide N	37	II								0.0032	0.0360	0.0410	0.0521

TABLE 8. DISTRIBUTION DATA

(Figures are per cent on bases indicated)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Fresh weight, plant													
Leaves, fresh weight	38	II	67.8	80.5	84.8	76.9	64.0	57.7	54.2	49.6	45.7	36.6	34.5
Stem, fresh weight	38	II	32.2	19.5	15.2	23.1	36.0	42.3	45.8	49.5	48.1	49.0	54.0
Pods, fresh weight	38	II								0.9	6.2	14.4	11.5
Dry weight, plant													
Leaves, dry weight	39	II	77.2	84.2	86.7	79.8	73.1	61.8	53.4	45.9	41.4	28.0	27.1
Stem, dry weight	39	II	22.8	15.8	13.3	20.2	26.9	38.2	46.6	52.9	51.8	53.2	54.5
Pods, dry weight	39	II								1.2	6.8	18.8	18.4
Organic solids, plant													
Leaves, organic solids	40	II	77.9	84.4	86.1	79.3	72.8	60.4	51.2	44.0	39.5	26.2	25.3
Stem, organic solids	40	II	22.1	15.6	13.9	20.7	27.2	39.6	48.8	54.8	53.7	54.6	55.7
Pods, organic solids	40	II								1.2	6.8	19.2	19.0
Ash, plant													
Leaves, ash	40	II	74.3	83.2	89.5	83.1	75.9	70.7	68.2	61.9	56.8	48.9	49.1
Stem, ash	40	II	25.7	16.8	10.5	16.9	24.1	29.3	31.8	37.1	35.3	39.3	39.4
Pods, ash	40	II								1.0	7.9	11.8	11.5
Organic acidity, plant													
Leaves, acidity	41	II	82.2	90.3	93.5	89.4	85.8	78.0	73.8	68.4	63.8	53.8	51.4
Stem, acidity	41	II	17.8	9.7	6.5	10.6	14.2	22.0	26.2	31.0	33.4	34.4	39.9
Pods, acidity	41	II								0.6	2.8	11.8	8.7
Total nitrogen, plant													
Leaves, total nitrogen	42	I	74.4	85.9	92.5	84.7	82.4	78.9	71.9	67.0	58.1	48.8	40.0
Stem, total nitrogen	42	I	25.6	14.1	7.5	15.3	17.6	21.1	28.1	30.0	31.1	27.2	26.9
Pods, total nitrogen	42	I								3.0	10.8	24.0	33.1

TABLE 9. DISTRIBUTION DATA

(Figures are per cent on bases indicated)

Collection	Fig.	Sample	A	B	C	D	E	F	G	H	I	J	K
Time (days)			1	19	26	35	40	47	54	61	75	97	110
Leaf N as soluble N	43	I	46.9	33.3	29.1	31.2	25.2	29.9	28.5	25.0	33.4	35.7	33.5
Stem N as soluble N	43	I	81.8	67.5	67.9	67.4	62.7	62.5	63.7	63.4	63.7	59.9	57.5
Nitrate N, plant													
Leaves, nitrate N	44	II	55.5	68.6	74.6	67.4	71.3	53.4	52.4	40.6	41.6	17.0	13.2
Stem, nitrate N	44	II	44.5	31.4	25.4	32.6	28.7	46.6	47.6	58.9	57.4	80.6	84.4
Pods, nitrate N	44	II								0.5	1.0	2.4	2.4
Plant nicotine N in leaves	44	I	75.0	90.9	85.2	87.8	85.5	85.2	86.0	85.6	82.3	88.9	88.0
Leaf total N													
Nitrate N	45	II	23.2	13.2	10.1	11.5	8.31	7.97	8.48	6.60	10.4	3.44	1.84
Nicotine N	45	I	0.94	4.11	4.60	3.93	3.47	3.23	3.37	4.18	5.58	9.17	10.2
Ammonia N	46	II	0.837	0.453	0.311	0.498	0.379	0.452	0.887	0.800	0.620	0.628	0.540
Glutamine amide N	46	II	0.813	0.528	0.461	0.613	0.651	0.407	0.404	0.437	0.472	0.951	1.10
Asparagine amide N	46	II		0.151	0.0922	0.0613	0.0831	0.164	0.770	0.451	1.07	1.34	1.06
Amino N	47	II	3.49	3.70	2.81	3.93	3.34	3.75	5.91	5.68	5.41	6.94	5.92
Peptide N	47	II	2.09	2.30	1.84	2.13	2.48	2.00	1.50	1.54	1.42	1.91	3.96
Stem Total N													
Nitrate N	48	II	66.7	39.0	33.7	33.3	16.3	23.2	21.5	22.6	30.1	28.8	16.8
Nicotine N	48	II	0.908	2.50	9.87	3.03	2.77	2.08	1.39	1.57	2.23	2.04	2.06
Ammonia N	49	II	1.33	0.927	0.777	0.346	0.433	0.674	0.767	1.16	0.501	0.627	0.705
Glutamine amide N	49	II	0.916	0.536	0.596	1.13	1.61	2.31	3.22	3.50	1.48	2.41	2.92
Asparagine amide N	49	II		0.195	0.371	0.762	0.897	1.46	1.54	0.615	1.28	1.76	1.96
Amino N	50	II		3.17	2.69	2.14	2.61	3.80	4.05	4.39	2.56	5.33	6.47
Peptide N	50	II		2.19	3.26	5.19	6.20	6.52	9.31	6.91	6.62	4.46	4.01
Dry weight leaves as carbohydrate	51	II	4.23	1.86	1.48	3.86	4.53	3.27	3.15	4.31	5.43	7.13	10.9
Dry weight leaves as nitrogen	52	II	5.51	5.02	5.11	4.91	5.04	4.98	4.92	4.68	4.74	3.55	3.04
Fresh weight leaves as nitrogen	53	II	0.366	0.538	0.521	0.509	0.536	0.470	0.507	0.498	0.604	0.489	0.474