CHEMICAL INVESTIGATIONS OF THE TOBACCO PLANT

XI. Composition of the Green Leaf in Relation to Position on the Stalk

by Hubert Bradford Vickery

THE CONNECTICUT AGRICULTURAL
EXPERIMENT STATION
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INTRODUCTION

The Problem of Sampling Tobacco Leaves

In studies of the effect of administering chemical substances to leaves, it is necessary to know the composition of the tissue both before and after the treatment. As a rule, the leaves are detached from the plant so as to isolate the system and the treatment is applied; the treated leaves are then subjected to chemical analysis. The problem is to determine what the composition of these leaves was before treatment. Where the effect upon the composition is a large one, or where a substance not initially present is introduced, only approximate information regarding the initial composition may be essential; but where the chemical changes produced are small, and especially when metabolic transformations of the substance introduced occur, it is necessary to have accurate knowledge.

It has been customary in this laboratory to approach this problem by making use of sets of samples of leaves collected according to statistical principles in such a way as to be as nearly identical in composition as possible (1). It is assumed that the variation in composition among individual leaves is made up of three components, one attributable to variation between plants, one to variation associated with leaf position on the plant, and a third random component which includes variation arising from such minor uncontrolled factors as chance differences in the supply of nutrients to the individual plants, differences in their exposure to light and air, or the occurrence of occasional small injuries by disease organisms or insects. The leaves are cut from the plants and distributed to the samples in such a way that each plant used and each leaf position on the plant is equally represented in each sample. With sets of 10 samples of 20 leaves, 10 leaves being taken from each of 20 plants, it is easily possible to attain coefficients of variation (standard error times 100 divided by the mean) as small as 2 per cent for the fresh weight of the samples and 1.5 per cent for their total nitrogen content. Such coefficients set the limits within which the assumption of identity among the samples is probably true.

Of such a set of samples, one or more is used for the determination of the initial composition, others for suitable controls, and the rest for the experimental treatment. If the data are then expressed in terms of a fixed quantity of initial fresh weight of tissue, subtraction of the observation on the control sample from that on the experimental sample gives the magnitude of the change due to the treatment. This general technique has served well in many studies of the metabolism of the organic acids in the tobacco leaf. It suffers, however, from one serious defect. The quantities of substrate necessary for the preparation of the culture solutions are substantial and, when rare or valuable substances are to be studied, the problem of obtaining sufficient material becomes serious. Accordingly, it has become desirable to devise a sampling technique which will make less extravagant demands for reagents. This has become the more necessary with the availability today of numerous substances labeled with radioactive isotopes, especially that of carbon, for only minute quantities of these substances are ordinarily obtained. Examination of the distribution of the isotope after a labeled substrate has been administered to a leaf furnishes valuable information upon the nature of the metabolic transformations that have occurred under the influence of the enzyme systems of the cells.

For many experimental investigations, it suffices to treat a single leaf of the tobacco plant with the substrate under investigation since one leaf provides sufficient material for many subsequent analytical determinations. How, then, can one determine the composition of this leaf before treatment?

There are three general methods by which moderately satisfactory information can be obtained. If only one or at most a few analytical determinations are required, for example, the rate of uptake of oxygen or the content of one of the more easily determined substances, a few small discs of constant area can be cut with a punch from the lamina, avoiding the larger veins, and these can be analyzed. Meanwhile the rest of the leaf, or discs cut from it, are subjected to treatment and subsequently prepared for analysis. This method, although convenient, is subject to certain inherent errors. It is frequently assumed that the discs cut from the lamina in fact represent a fair sample of the composition of the leaf as a whole. This may not be true. Dr. R. N. Jeffrey presented unpublished data at the Cigar Manufacturers Symposium and Research Seminar in Washington in 1958 which showed that the amount of nicotine per unit area in a single tobacco leaf may vary between wide limits (2- to 3-fold) depending on whether the discs are taken from the base, the middle or the tip of the leaf. Furthermore, the fresh weight per unit area likewise varied with the location of the discs, and leaves from different positions on the plant differed among themselves with respect to the distribution of alkaloid. Analogous observations with respect to the distribution of chlorogenic acid in the tobacco leaf have been recorded by Zucker and Ahrens (2). Thus, unless the discs cut at the start of the experiment are precisely matched by discs cut from symmetrical positions at its close and the substance under investigation is in fact symmetrically distributed, the measurements may be seriously in error if the observed changes are attributed to an alteration in composition of the leaf as a whole. The

composition of the entire leaf is not at all necessarily represented by the composition of selected small areas.

An even more subtle source of error in the disc-sampling method may develop when the treatment involves shrinking of the leaf area as, for example, in studies of chemical changes that occur during curing. In this event, it is essential to determine the shrinkage and correct the data obtained from the analysis of discs cut after this has occurred.

A second method which has also been widely used is the so-called half-leaf method. The entire lamina is cut from one side of the midrib and analyzed to give the composition at the start. The remaining half-leaf with its midrib intact is then treated, and at the end of the treatment the lamina from the other side is removed and analyzed. It is assumed that the mechanical damage done to the leaf has no significant effect upon the biochemical behavior of the remaining tissue and that the composition of the two sides of the leaf was initially identical. Among the difficulties of interpreting such experiments is the calculation of the results. If calculated in terms of percentage of the dried or fresh weight, it is assumed that the treatment altered neither of these quantities. Yet respiration continued throughout the treatment (and photosynthesis if the experiment involved exposure to light) thus altering the quantity of organic solids present, changes in the content of water inevitably occur, and the treatment itself may have introduced a significant amount of extraneous material. Unless only major alterations in composition are of importance, this method also leaves something to be desired from the standpoint of accuracy.

A third method that has also been used involves the collection of three successive leaves from the stalk of the plant. The upper and lower of these are then separately analyzed and the intermediate leaf is subjected to treatment. Alternatively, the dried tissue of the upper and lower leaves is pooled, and analysis of the pool is assumed to represent the composition of the intermediate leaf before the treatment. If separate analyses of the upper and lower leaves are made, the arithmetic mean of the data yields an estimate that presumably represents the composition of the intermediate leaf.

This method possesses the advantages that the leaf subjected to treatment is not damaged in any way and its fresh weight before treatment is accurately known. Its major deficiency is the uncertainty of our knowledge with respect to the way in which the composition of the leaf may be expected to vary in relation to its position on the stalk. It is this aspect of the problem with which the present paper is concerned. It is desired to establish the variation in composition of the leaves of the tobacco plant as a function of position. Plots of the data should then yield curves the form of which should make it possible to select leaves for experimentation which in fact have a composition that approaches closely to the arithmetic mean of the composition of the leaves above and below them.

DESIGN OF EXPERIMENT

The plants were grown at the farm of the Tobacco Laboratory of this Station in Windsor from seed that represented a selection from the strain known as Connecticut 49 and designated Strain 7 D of the Consolidated Cigar Company. This strain is characterized by resistance to black root-rot and weather fleck. There had been 7 or 8 generations of selection at the time the seeds were planted in the spring of 1959. The plants were set under a shade tent on June 4th in Field 13 (Merrimac sandy loam) which had been fertilized at the rate of 3360 pounds per acre with a mixed fertilizer that provided 185 pounds of nitrogen, 134 pounds of phosphoric oxide, and 182 pounds of potassium oxide per acre. Collection of the samples was made beginning at 9:20 A.M. on July 28. At this time, 5 of the 10 vigorously growing plants used were in bud, and a few of the leaves on some plants were slightly flecked.

A row of 10 uniformly developed plants was selected and the small basal leaf (leaf position 1), which was damaged on most of the plants, was discarded. Collection of the samples was then made as shown in Table I so that Sample 1 consisted of all of the leaves in positions 2 and 3 from all 10 plants, Sample 2 of all of the leaves from positions 4 and 5 and so forth. Sample 12 consisted of the small remaining leaves from all of the plants; these ranged from 2 to 6 in number and averaged 4 per plant. The flower buds were not taken.

TABLE I. Position and weights of 20-leaf samples from 10 plants.

Sample	Leaf Position	Fresh Weight	Equilibrated Dry Weight gm.
1	2 and 3	346.3	26.70
2	4 and 5	415.1	35.40
3	6 and 7	443.4	40.45
4	8 and 9	482.8	46.92
5	10 and 11	469.0	48.02
6	12 and 13	424.7	46.57
7	14 and 15	409.2	49.92
8	16 and 17	353.2	46.40
9	18 and 19	286.9	39.59
10	20 and 21	217.9	32.50
11	22 and 23	171.2	26.39
12	small remaining leaves	180.4	29.45

The samples were at once placed in tight polyethylene bags and taken to the laboratory in New Haven where they were weighed and immediately dried at 80° C. until the midribs were crisp. The dry leaves were then broken into fragments, placed in tared bottles, and exposed in an air-conditioned room to a humidity of 50 per cent and a temperature of 23° C. until they attained constant weight (about 14 days). The final weighings were made to the nearest centigram. The material was then ground to a powder and was kept in closed bottles in the air-conditioned room, being removed only for short periods when taken to the balance to weigh out portions for analysis.

The use of two successive leaves rather than single leaves per sample is justified since the main consideration was to establish the form of the curves which represent the composition. The precise composition is, of course, valid only for this particular set of samples, but the form of the curves is probably characteristic of the species. Furthermore, the use of two-leaf instead of single-leaf samples diminished the analytical labor by one-half without significant loss in the applicability of the data.

The analytical methods used have been described in previous Bulletins of this series (3, 4). The determinations of nicotine and nitrate nitrogen, and also of the inorganic components by spectroscopic methods, were made by the Department of Analytical Chemistry of this Station. All analytical data were first expressed in terms of percentage of the equilibrated dry weight, or for organic acids as milliequivalents per 100 gm. of dry weight. The results were then recalculated as described below.

The fresh weights of the 20-leaf samples are plotted in Fig. 1. The mean fresh weight of two leaves per plant is one-tenth of the figure on the ordinate. The abscissa shows the sample number. The position on the stalk of the two leaves collected from each plant in each sample is obtained by multiplying the number of the sample by 2; the product gives the position of the lower of the two leaves in the sample. Sample 4, for example, represents the 8th and 9th leaf positions. It will be noted that the weight of the samples increases up to the 8th and 9th leaf position and then diminishes along a fairly smooth curve. Sample 12, however, which represents all of the small leaves remaining on the plants above the 23rd position (an average of 4 per plant) does not fall on the smooth curve, and many instances will be seen in the data to be presented where the results for this sample are out of line. This is to be expected since these small leaves are not precisely related as a continuous function to the other samples either in terms of number of leaves per plant or position of insertion on the stalk.

Calculation of the Data

There are a number of useful ways to express analytical data obtained on leaf tissue, the commonest perhaps being as a percentage of the dry weight. This method might be suitable in the present instance since the samples were all collected at the same time and were not subjected to any treatment which would alter their composition before being dried in preparation for analysis. Where such treatments are applied, loss of organic solids by respiration during the period of the treatment, and increase in solids as a result of the treatment alter the basis of calculation of percentage and introduce a systematic error which must be corrected before valid comparisons of composition before and after treatment can be made. To avoid this difficulty, it has been cutomary in this laboratory to express the data in terms of the fresh weight at the time of collection, since this quantity is measured with accuracy. Any convenient fixed weight of tissue may be chosen as the basis of the calculation, and the present data have been arbitrarily calculated in terms of the amount of analytical component, expressed in grams or in milliequivalents where more suitable, per kilogram of initial fresh

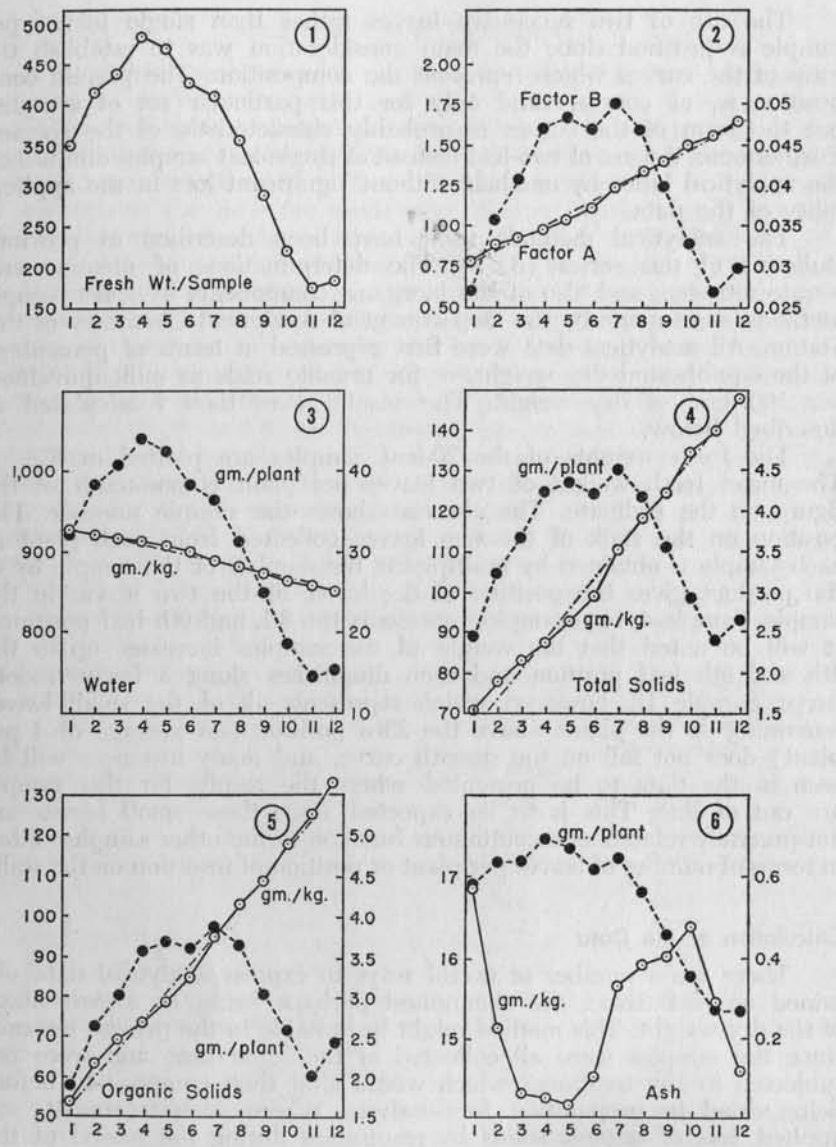

Plate I

Fig. 1: Fresh weight per 20-leaf sample. The ordinate is in grams and the abscissa is the sample number explained in Table I. Fig. 2: Factors A (ordinate at left) and B (ordinate at right); for explanation see the text. The regression line for Factor A is indicated in part by a light dot and dash line. Fig. 3: Water content in grams per kilogram of fresh weight of the samples (ordinate at left, solid line, open circles), and in grams per plant (ordinate at right, dashed line, filled circles). This quantity is the mean weight of water in the 20 leaves of the sample from 10 plants. The regression of water in grams per kilogram on sample number is indicated in part by a light dot and dash line. Fig. 4: Total solids in grams per kilogram of fresh weight (ordinate at left, and regression on sample number as light dot and dash line), and in grams per plant (ordinate at right). Fig. 5: Organic solids in grams per kilogram of fresh weight (ordinate at left and regression on sample number as light dot and dash line), and in grams per plant (ordinate at right). Fig. 6: Ash in grams per kilogram of fresh weight (ordinate at left) and in grams per plant (ordinate at right).

weight of the samples. This quantity is obtained by calculating from the data in Table I for each sample what the equilibrated dry weight would have been if the sample had weighed exactly one kilogram when the leaves were picked. These weights, when divided by 100, give the factors (more fully discussed on p. 36) used to convert the analytical data expressed as a percentage of the equilibrated dry weight to grams per kilogram of initial fresh weight. A plot of these factors (open circles, solid line), referred to for convenience as Factor A, is shown in Fig. 2. The points fall along a curve which is nearly a straight line and closely fit the regression line the equation of which is $y = 0.0794x + 0.667$ where y is the value of Factor A and x is the sample number, that is, the position of the lower leaf in the sample divided by 2. Portions of the regression line are indicated lightly in Fig. 2. Inasmuch as Factor A yields data in units of concentration in the fresh weight, the factor for Sample 12 lies accurately on the straight line although the fresh weight of this sample, as shown in Fig. 1, is out of line with the weights of the other samples.

In addition to information on the concentration of the various components in the leaf tissue, it is also desirable to have information on the absolute amounts present in the leaves in the designated positions on an individual plant, in other words, in terms of a biological unit. The factor to convert the percentage of a component in the equilibrated dry weight to the actual amount present in the two leaves collected from the successive positions is equal to the equilibrated dry weight per sample divided by 1000. The factors for the successive samples are plotted as a dashed line (filled circles) in Fig. 2 as Factor B (ordinate on the right). They fall upon a curve that increases to a moderately flat maximum extending from the 8th to the 16th leaf position (Samples 4 to 8) and then decreases. The factor for Sample 12 is out of line with the others as would be anticipated, for although labeled as a plot of factors, this curve is in fact a plot of the equilibrated dry weight, the data for which are given in Table I. However, it should be noted that the quantity obtained by multiplying Factor B by 1000 is not an exact measure of the true dry weight of the tissue from the two successive leaves in the designated positions. The samples had been equilibrated with air at a relative humidity of 50 per cent at 23° C. and thus still contained from 8.3 to 9.7 per cent of water. The true dry weight of the samples is shown in Fig. 4 as the total solids.

COMPOSITION AS A FUNCTION OF LEAF POSITION

Gross Composition

The water content of the successive samples is plotted in Fig. 3. The curve for gm. per kilogram (ordinate at left) is nearly a straight line which slopes downwards to the right; the lowest leaves are highest in water content, the upper leaves highest in concentration of total solids. The data conform closely to the regression equation $y = -7.10x + 939$[1] which is indicated where possible as a light dot and dash line in the figure. Division of the numbers on the ordinate by 10 gives the percentage of water in the fresh leaves; if the regression line is taken as the most accurate expression of the data as a whole, the bottom leaves contained about 93 per cent of water and the small top leaves about 85 per cent.

Fig. 3 also shows a plot of the amount of water per plant in the two successive leaves which make up the series of samples. The curve (ordinate at the right) resembles that for the fresh weight of the samples (Fig. 1) in close detail as would be anticipated since water makes up so large a part of the fresh weight.

The curve of the total solids in terms of gm. per kilogram (determined by drying analytical samples at 110°) plotted in Fig. 4 approximates closely to a straight line the equation for which is $y = 7.13x + 60.9$ which is also plotted in the figure. However, it will be noticed that there is evidence of a minor sinuosity in the curve for the samples from the lower half of the plant. The explanation is clear if Fig. 6 is consulted. The concentration of ash components in the lowest leaves is high, but drops in the leaves from the 6th to the 12th position and then increases. The large scale upon which the ash data are plotted in Fig. 6 exaggerates this effect, but the correlation between the rise and fall of the weight per kilo of the ash with the slight sinuosity in the curve for total solids is obvious. The amount of solids per plant is also plotted in Fig. 4 (ordinate at the right). It rises to a flat maximum in the leaves from the 8th to the 16th position.

Fig. 5 shows the difference between the concentration of total solids (Fig. 4) and of the ash of the samples (Fig. 6), a quantity that is referred to as the organic solids. Strictly speaking, the data so obtained are underestimates of the true organic solids, because the weight of the ash in each case includes carbonate approximately equivalent to the alkalinity of the ash. This carbonate represents the chemical product of the combustion of the salts of the organic acids, and is thus in turn equivalent to the carboxyl groups which are ionized

[1] In this and in all subsequent regression equations, X is the sample number. To obtain the position of the lower of the two leaves in the sample, multiply the sample number by 2.

The analytical data in terms of concentration for all of the curves shown in the Figures are collected in Table IV at the end of this Bulletin. To obtain the amount of any component in the two leaves from a single plant, it is necessary to divide the quantity given in the Table by Factor A and multiply the result by Factor B. To aid in such calculations, the ratio of Factor A to Factor B is shown in Table IV as Factor C. Any item in the Table when multiplied by Factor C gives a product in the units grams per plant.

at the pH of the cell contents. Carbonate of this origin is part of the true "organic solids" of the leaf tissue. The correction has not been made in the present instance since its mean value is 5.6 gm. with a standard error of 0.5 gm. Accordingly, the shape of the curve for corrected organic solids would not be altered to any notable extent although the curve would be placed approximately 5.6 gm. higher on the scale of ordinates. The important point in the present connection is that the concentration of organic solids can be closely represented by a straight line the equation of which is $y = 7.09x + 45.8$.

The organic solids in terms of grams per plant are also shown in Fig. 5 and follow a curve similar in shape to that for the equilibrated dry weight (Factor B in Fig. 2) as would be expected.

The concentration of inorganic components, that is, the ash, is plotted in Fig. 6 on a scale 20 times greater than that used for Figs. 4 and 5. The curve follows a complex course, for the concentration in the lowest leaves is high, but drops in the larger leaves in the middle of the plant (6th to 12th positions) and rises again to a sharp maximum at the 20th position. The concentration in the small top leaves falls away again.

The plot of the weight of ash components per plant shows that the quantity of inorganic material in the fully expanded leaves from the 4th to the 14th position is high and fairly constant. Above this position, the weight of ash in the leaves diminishes as the size of the leaves decreases.

Nitrogen and Protein Content

The total nitrogen content is shown in Fig. 7. The plot of the concentration does not give a straight line, but rather appears to follow a slightly sinusoidal course. However, for the present purposes the curve may be considered as consisting of two straight lines indicated in the figure, one of which closely approaches the data for the leaves from the first to the 16th position and a second which rises somewhat more steeply and approximates to the concentration in the leaves from the upper part of the plant. The regression equations for these lines are, respectively, $y = 0.38x + 1.09$ and $y = 0.72x - 1.53$. Samples 1 and 3 depart somewhat widely from the first of the regression lines, but the other data fall closely upon them. Thus it would appear that the concentration of nitrogen in tobacco leaves increases somewhat more rapidly in relation to position in the upper leaves than it does in the lower leaves.

The curve for total nitrogen in terms of grams per plant shows a smooth curvilinear increase to a fairly flat maximum extending from the 10th to the 18th leaf positions and then falls slightly. The amount of nitrogen in the sample representing the group of small top leaves is greater than that in the two leaves immediately below them.

The plot of the concentration of protein nitrogen in Fig. 8 follows a curved line that appears to be slightly concave upwards. Although it would be possible to represent the data fairly satisfactorily by three regression lines that intersect at points represented by Samples 6 and 9, and each with a slightly increased slope, it is clear that the relation of protein concentration to leaf position is a somewhat complex one. It would

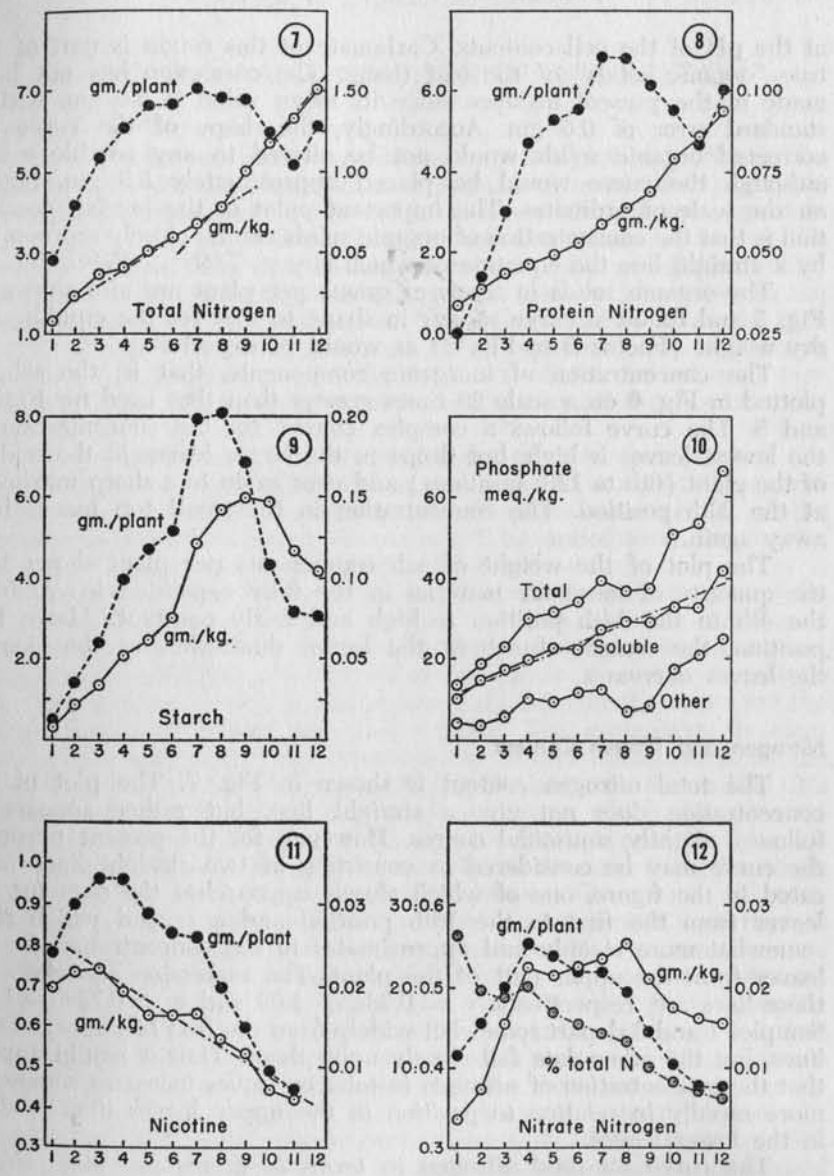

Plate II

Fig. 7: Total nitrogen in grams per kilogram of fresh weight and grams per plant. (The conventions for location of the ordinates and the symbols used are given in the legend of Plate I). Fig. 8: Protein nitrogen. Fig. 9: Starch. Fig. 10: Phosphate. The upper curve shows phosphate calculated in milliequivalents per kilogram from spectrographic determinations of total phosphorus. The middle curve shows determinations of water-soluble phosphate by a specific method for the phosphate ion. The lower curve shows the differences between the data plotted in the other two curves. Fig. 11: Nicotine. Fig. 12: Nitrate nitrogen. A third curve to show the percentage of the total nitrogen present as nitrate nitrogen is superposed. The ordinates are at the extreme left and the points are shaded circles.

be surprising if this were not the case. The protein content of tobacco leaves has been shown by Zucker (5) to be greatly influenced by the conditions, especially the supply of light, under which the plants were grown. Furthermore, the protein content of young expanding upper leaves is continually increasing while that of mature leaves low on the plant is continually diminishing as they approach senescence. Under such circumstances a rectilinear relationship between protein concentration and leaf position is not to be anticipated.

The curve for the amount of protein per plant rises to a narrow maximum at leaf positions 14 to 16 and then falls. The rising part of the curve is not smooth above the 8th position. The protein content of the topmost small leaves is high.

Starch

Tobacco leaves of the varieties used in Connecticut for the production of cigar wrappers are usually low in starch content. For example, the field-grown plants of the season 1949 used for a study of the curing process (3) contained as little as 0.6 gm. of starch per kilogram in the bottom 4 leaves although the collection was made late in the morning of a bright day. Samples of leaves representing groups of 10 successive fully expanded leaves from plants of strain Connecticut 49 grown in the greenhouse have usually been found to contain about 0.7 gm. per kilogram. The leaves from the lowest position on the plants used for the present study were also low in starch content, but as is shown in Fig. 9 the concentration increased rapidly to a maximum of 6 gm. per kilogram in the leaves in the 16th position and decreased only moderately in the higher positions. The curve is a complex one; it follows a nearly straight line course up to the 12th position and then rises more steeply to a rounded maximum. The concentration of starch in green tobacco leaves doubtless varies between fairly wide limits in response to weather conditions and exposure of the leaves to light in the period immediately before collection, and the present data cannot be taken as representative of the behavior of the leaves of this variety of tobacco in any generalized sense. They merely show the response of this particular set of plants on the day these samples were collected. However, the data do suggest that samples of three successive leaves taken from rather low on the plant might be acceptable in experiments in which the initial concentration of starch is important.

The curve for the amount of starch per plant rises to a sharp maximum in the leaves at the 14th to 16th positions and then falls away steeply in the smaller leaves at the top.

Phosphorus

In Fig. 10 are plotted three curves which give the distribution of phosphoric acid between water-soluble and insoluble forms. The units are milliequivalents of phosphoric acid per kilogram of leaf tissue. The data for total phosphate (top curve) represent phosphoric acid equivalent to all of the forms of phosphorus present since they are calculated from the determinations of total phosphorus obtained

by spectroscopic methods (see Fig. 28). The data for soluble phosphate (middle curve) represent colorimetric determinations of phosphate ion in water extracts of the dried leaf tissue. The lowest curve shows the difference between the two other determinations, and its irregularities are to a considerable extent attributable to the errors inevitable in data so calculated. Of these curves, that for the soluble phosphate is the most important in connection with the study of the metabolism of the acidic components of the tobacco leaf, and it is clear that, despite minor irregularities, the data can be fairly well expressed by a straight line the equation for which is $y = 2.48x + 8.94$.

The curve for "other phosphate" represents estimates of soluble organic phosphorus compounds that do not respond to the colorimetric reagent for the phosphate ion, and also includes any insoluble inorganic phosphates present in the dried tissue. It would appear that the concentration of these forms of phosphorus is fairly constant at from 5 to 10 meq. per kilogram in the leaves up to the 18th position, but increases moderately in the youngest and most actively growing leaves. The soluble organic phosphorus compounds include a number of phosphorylated intermediates in glycolysis such as phosphorylated carbohydrates and derivatives of these, and several coenzymes of the greatest importance in metabolism. The curve shows the maximal possible concentration of such substances present in the leaves.

A curve for the soluble phosphate in terms of grams per plant is not shown, but this quantity increased from 0.3 meq. per plant in the lowest leaves to a broad rounded maximum at about 1 meq. per plant in the leaves in the 8th to the 16th positions and then fell away to 0.5 meq. in the leaves at the top of the plant.

Nicotine

Fig. 11 shows that the concentration of nicotine rises slightly in the leaves from the base to the 6th position and then diminishes along a somewhat irregular curve which is moderately closely approximated by a regression line plotted from the equation $y = -0.037x + 0.48$. The curve for amount per plant indicates that the largest quantity is contained in the large leaves in the 6th to 8th position, but that the amount per leaf also diminishes in a moderately regular manner in the leaves from this point to the top of the plant. Nicotine is synthesized for the most part in the roots of the tobacco plant (6), and the curves apparently illustrate its accumulation in the leaves in an amount which is roughly in proportion to leaf size. It should perhaps be emphasized at this point that the present data refer to the nicotine content of *intact* plants. As is discussed more fully below, the distribution of nicotine in the tobacco plant is fundamentally altered if the top of the plant is removed a few weeks before the samples are collected.

Nitrate Nitrogen

Tobacco for use as cigar wrappers is grown in Connecticut under conditions of liberal fertilization with nitrogen. Accordingly, the leaves are usually characterized by the presence of a high proportion of nitrate nitrogen. Fig. 12 shows that the concentration of nitrate nitrogen

in the leaf rises steeply from the base of the plant to a fairly flat maximum from the 8th to 16th positions and then diminishes somewhat in the upper leaves. The amount per plant reaches a maximum at the 8th position and then diminishes towards the top of the plant. The significance of the data can perhaps be more fully appreciated from a third curve plotted as a dash and dot line (shaded circles) which shows the nitrate nitrogen as a percentage of the total nitrogen of the samples. The ordinate for this curve is at the extreme left of Fig. 12, and the curve shows that no less than 26 per cent of the total nitrogen of the basal leaf consisted of nitrate nitrogen, and that in the large leaves in the middle of the stalk the percentage ranged from 20 down to 15 per cent of the total nitrogen. An even more striking result is obtained if the nitrate nitrogen is calculated as a percentage of the non-protein nitrogen, that is to say of the soluble nitrogen in the leaves. The curve is not plotted, but can be approximately represented by a straight line that slopes from about 50 per cent of the soluble nitrogen in the basal leaves to about 35 per cent in the top leaves. The mean value is 45.3 ± 10.6 per cent of the soluble nitrogen.

Organic Acid Components

Inasmuch as the organic acids in a plant tissue are to a considerable extent although not completely neutralized at the pH of the cell contents by the basic ions which are also present, titration of the alkalinity of the ash furnishes a quantity that is related to the total organic acid content. This quantity is plotted in Fig. 13 in terms of milliequivalents of alkalinity per kilogram of fresh weight of leaves. The curve follows a complex course similar to that of the ash in Fig. 6, the alkalinity being high in the leaves at the bottom of the plant, low in the leaves at the 10th to 12th position and rising to a maximum at the 16th position and then diminishing again. The curve for the magnitude of the alkalinity per plant is simpler in form and shows that the alkalinity is high in the lower leaves and then diminishes toward the top of the plant. This is also true of the weight of the ash (Fig. 6).

In Fig. 14 an attempt is made to relate the complex data for the alkalinity of the ash to the inorganic and organic solids of the leaves. The upper curve (open circles) shows the ratio of the alkalinity to the weight of the ash, the units being meq. of alkalinity per gram of ash. From the 4th leaf position to the 14th, the proportion of the ash weight that is present as titratable basic components follows a fairly smooth curve which approximates to a straight line and slopes downwards. The data for the leaves in higher positions yield a curve which is irregular: nevertheless the relationship as a whole can be approximated by a curve which drops rather steeply along a course which is concave upwards. Also shown in Fig. 14 is a plot of the ratio of the alkalinity of the ash to the organic solids, likewise expressed in meq. per gram (closed circles). The data are indicated on the ordinate at the right. The curve is smooth, slopes fairly steeply downwards to the right, and is also concave upwards. It suggests that the part of the true organic acid acidity which is measured by the alkalinity of the ash is a smoothly diminishing fraction of the organic solids.

The organic acids present in plant leaves may be roughly classified

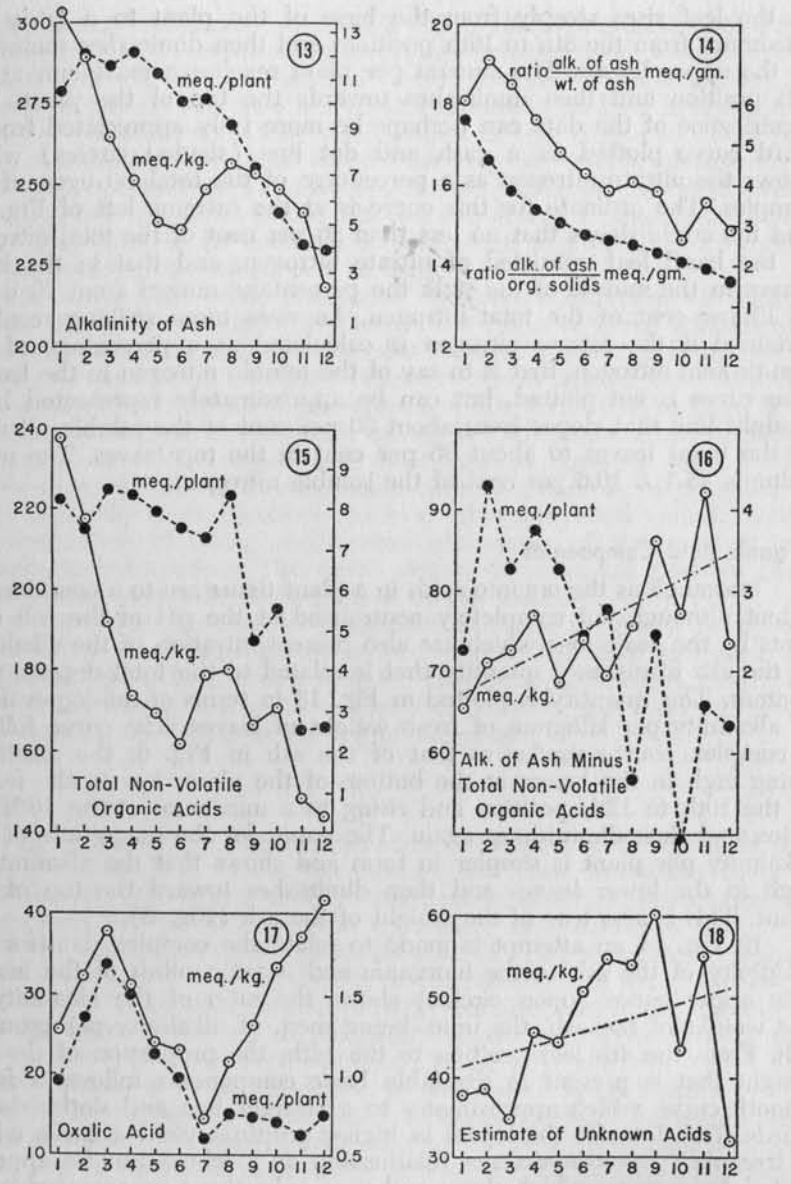

Plate III

Fig. 13: Alkalinity of ash in milliequivalents per kilogram of fresh weight. Fig. 14: Ratio of the alkalinity of the ash to the weight of ash (ordinate at left) and to the weight of organic solids (ordinate at right). Fig. 15: Total non-volatile organic acids. Fig. 16: Excess of alkalinity of the ash over the total non-volatile organic acids. Fig. 17: Oxalic acid. Fig. 18: Excess of alkalinity of the ash over the sum of the non-volatile organic acids and oxalic acid.

into three groups. There is generally a minute proportion of volatile acids, usually formic and acetic acids with occasional traces of higher homologues. In addition, there is a fairly large group of acidic substances characterized by the presence of aromatic rings which are often substituted with phenolic hydroxyl radicals; such substances as chlorogenic acid are typical of this group. Finally there is a major group of non-volatile organic acids, many of which contain α-hydroxyl groups. Malic acid is frequently the component of this group present in largest amount, and citric acid is also usually present in substantial quantities. In addition to these, there are various but as a rule small to trace amounts of oxalic, lactic, succinic, fumaric, malonic, glyceric, glycolic, and glyoxylic acids; quinic, and shikimic acids; aspartic, and glutamic acids; and even smaller amounts of α-keto acids such as pyruvic, oxaloacetic, and α-ketoglutaric acids. Small proportions of acidic phosphorylated substances are also present. Tartaric acid and isocitric acid, although uncommon, are found in some species in astonishingly large amounts.

In the tobacco leaf, malic acid is the predominant acid of the non-volatile group, and citric acid is next to it in amount present. Oxalic acid is also found, although usually not in soluble form in the leaf as prepared for analysis since, as a rule, there is sufficient calcium present to fix it as calcium oxalate. In addition, there are a number of substances present in little more than traces, and these are usually determined together and designated the minor acids. This group has been found to consist of aspartic and glutamic acids together with at least two acidic polypeptides containing one or other of these amino acids,[2] glycolic acid, D-glyceric acid (7), quinic acid (8), and succinic acid. Several of these are extremely reactive substances in metabolism and the amounts present are often minute. In addition to these components, malonic acid has been demonstrated to be present (9) in several varieties, and pyruvic and α-ketoglutaric acid quite possibly occur in traces. The presence of a few other acids—e.g., fumaric acid and chlorogenic acid (2)—has been reported, and presumably still others will be found. However, the acids mentioned are the ones which make up by far the greatest part of the non-volatile organic acids in this plant.

The analytical method which has been developed in this laboratory for the determination of these substances (4) depends upon the adsorption of the acids from a water extract of the dried leaf upon the anion exchange resin Dowex 1. The column of resin is then treated with a gradually increasing concentration of formic acid which elutes the acids in succession, the minor acids first, followed by the trace amount of succinic acid, then the major component malic acid and finally the citric acid. Phosphoric acid is eluted together with citric acid, and if fumaric acid is present, it can sometimes be detected beyond citric acid on the curve which shows the titrations of the successive fractions after the formic acid has been evaporated. The sum of the titrations of all of the fractions gives the value for the so-called "total non-volatile organic acids" and this quantity, as determined in the present set of samples, is plotted in Fig. 15. In terms of concentration in the fresh tissue, the total organic acids are highest in the

[2] Unpublished observations in this laboratory by Dr. G. A. Barber.

leaves in the lowest position on the stalk, but the concentration falls away rapidly in the leaves up to the 12th position, and then rises somewhat in the next few positions before dropping in the topmost leaves. The form of the curve is in general similar to that for the concentration of ash components and for the alkalinity of the ash (Figs. 6 and 13).

The curve for the amount of organic acids in the leaves shows that the amount per plant is at a high and nearly constant level in the leaves from the base to the 16th position, but then diminishes rapidly in the upper leaves.

As has been pointed out, the alkalinity of the ash is a function of the total amount of base-binding substances in the tissue, that is to say of the true total organic acidity. It is a measure of the sum of the carbonates and oxides which remain when the salts of the acidic substances are burned. Precisely what fraction of the sum of the acidic groups present is neutralized by the inorganic basic components is unknown. At the pH of extracts of the dried leaf tissue, strong acid groups are completely neutralized, weak ones only partially. Thus the alkalinity of the ash must be less than the true total acidity. Furthermore, no account is taken of organic basic substances, for example nicotine, which also have a role in the acid-base equilibria of the cells. Nevertheless, if the fairly reasonable assumption is made that the fraction of the true total acidity neutralized by inorganic bases varies between narrow limits from sample to sample, the rise and fall of the alkalinity of the ash with leaf position can be interpreted as moderately valid criteria of the increase and decrease in the concentration of total acid components.

In an effort to obtain some information on the relative quantities of acidic substances not accounted for by the easily determined group of non-volatile soluble organic acids, a plot is shown in Fig. 16 of the difference between the alkalinity of the ash and the total non-volatile organic acids. This quantity should be an approximate measure, although doubtless an underestimate, of the organic acids that were not extracted by water, or were so extracted and adsorbed on Dowex 1 resin, but were not eluted by formic acid. Among such substances would be oxalic acid, aromatic organic acids which are held strongly by the resin, the so-called pectic acids, and presumably also the base-binding groups present in water-insoluble substances, especially the proteins. The curve for the concentration of this group of acidic substances is highly irregular, but a regression line plotted from the equation $y = 1.56x + 65.6$ suggests that the concentration in the leaves increases by some 20 meq. per kilogram between the basal and the top leaves. In terms of amount per plant, however, the quantity diminishes from about 4 meq. to about 1 meq. from the bottom to the top leaves.

The substances in this group make up a substantial part of the total acidic material in the plant, the mean value being approximately 30 per cent of the alkalinity of the ash. Oxalic acid is doubtless the major single component of this group, and the concentration of this acid is plotted in Fig. 17. The determinations were made by a method that depends upon precipitation as calcium oxalate from an acid extract of the dried leaves and titration of the liberated oxalic acid with permanganate. The concentration of oxalic acid follows a curve somewhat similar in form to that of the alkalinity of the ash; it rises to a maximum

in the leaves in the 6th position, falls to a minimum at the 14th position and rises again in the smaller leaves towards the top of the plant. The curve for quantity per plant resembles it in form although there is no clearly marked increase in the amount of oxalic acid in the upper leaves.

Oxalic acid is widely regarded as an end-product of the organic acid metabolism in leaves. It is known that it can arise in the tobacco leaf, among other possible sources, from the oxidation of glycolic acid (10), and, in view of the presence of considerable amounts of calcium, much of it is probably accumulated as the insoluble calcium salt once it is produced. Its presence in high concentration in the young rapidly expanding leaves at the top of the plant where organic acid metabolism is proceeding at high intensity is perhaps not surprising, but that it should rise to a narrow maximum in the leaves in the 6th position and then diminish sharply is a matter that is less easy to account for.

In Fig. 18 the differences between the data of Fig. 16 and the concentration of oxalic acid (Fig. 17) are plotted. Analytical errors are magnified in such difference data, and the curve as a result is highly irregular; it is quite possible that some of the points plotted are far from the true value. Nevertheless, a regression line has been calculated from the equation $y = 0.87x + 40.5$, and it seems clear that whatever the chemical nature of the acidic substances represented, the concentration in the upper leaves is somewhat greater than in the lower. The line represents a rough estimate of the concentration of organic acids of completely unknown nature, and it would seem that a quantity of the order of 15 per cent of the acidity represented by the alkalinity of the ash is to be attributed to the substances in this group. Since the alkalinity of the ash is itself an underestimate of the total amount of acidic substances in the tissue, it is clear that other and for the most part unknown acids form a substantial part of the organic material present; among these chlorogenic acid[3] is doubtless an important component.

Phosphorylated substances such as glucose 6-phosphate, and adenosine triphosphate presumably contribute to some extent to the total base-binding capacity of tobacco leaf tissue. However, the curve for "other phosphate" in Fig. 10 sets an upper limit to the possible magnitude of this contribution. Save in the youngest leaves in the 20th position and above, it cannot be greater than 10 meq. per kilogram, or not more than 5 per cent of the alkalinity of the ash.

Determination of Citric Acid

When a sample of the water extract of the dried leaves is washed into a column of Dowex 1 with water, and the acids adsorbed on the resin are eluted with increasing strengths of formic acid, citric acid is found in a group of fractions that are removed towards the end of the process. These fractions are evaporated to dryness to expel the formic acid and titrated, phenolphthalein being used as the indicator.

[3] Chlorogenic acid was not determined in these samples because the samples had been dried. Chlorogenic acid is not stable during the drying operation. (Personal communication from Dr. M. Zucker.)

The fractions are then pooled, and citric acid is determined in the solution by oxidative bromination to pentabromoacetone which is extracted by an organic solvent and debrominated, the bromide ion being subsequently determined. The phosphoric acid present is determined by a specific colorimetric method in a small portion of the pooled fraction. At the end-point of the titration with the indicator used, all three carboxyl groups of citric acid are neutralized by the base, but only two of the three base-binding groups of phosphoric acid have been neutralized. This fortunate circumstance permits an accounting to be made of the acid composition of these fractions.

The sum of the titrations of all of the fractions gives a quantity referred to as the "citric acid peak," since a plot of the titrations rises from the baseline to a moderately symmetrical peak. The colorimetric determination of the phosphoric acid gives the acidity attributable to this component, and two-thirds of this quantity is the proportion that was titrated. Accordingly, the sum of the separately determined citric acid and two-thirds of the phosphoric acid should equal the acidity in the "citric acid peak." As a rule, there is a small deficit which presumably represents the presence of a trace of some other organic acid together with the analytical error associated with the numerous titrations.

The data for the citric acid peak plotted in Fig. 19 show that the sum of the concentrations of citric and phosphoric acids is low in the leaves up to the 6th position, but then rises rapidly to a maximum at the 20th position. The curve for the amount per plant rises to a maximum at the 16th position and then falls away in the upper leaves. The analytical determinations of citric acid itself are plotted in Fig. 20 and give a smooth curve which has a rounded minimum near the 6th leaf position and a sharp maximum at the 20th position. The form of this curve is similar to that for the alkalinity of the ash (Fig. 13), although the relative height and positions of the minimum and maximum are quite different. The curve which shows the amount of citric acid per plant closely resembles that for the concentration although it is somewhat less smooth, and the maximum is located at a leaf position somewhat lower on the plant.

The complexity of this curve clearly presents a problem to one who wishes to select three successive leaves so that the mean between the upper and lower of the three shall represent the composition of the intermediate leaf with respect to citric acid. It would seem that selection of leaves at the 3rd, 4th, and 5th positions or, alternatively, leaves in the 12th to the 18th positions would come nearest to meeting the requirements of this method of sampling. Selection of leaves from the base or from positions high on the plant would clearly be contraindicated.

Fig. 21 is a plot of two-thirds of the phosphoric acid found in the pooled fractions that represent the citric acid peak. The data suggest that in the lower leaves there is a higher concentration of phosphoric acid than of citric acid. This is emphasized by comparison of the curve for meq. per plant in Fig. 21 with that in Fig. 20.

A curve which gives the deficit between the combined concentrations of citric and phosphoric acid and the total titratable acidity of the fractions in the citric acid peak is not shown. In the leaves up to the 14th position, this quantity was essentially constant with a mean

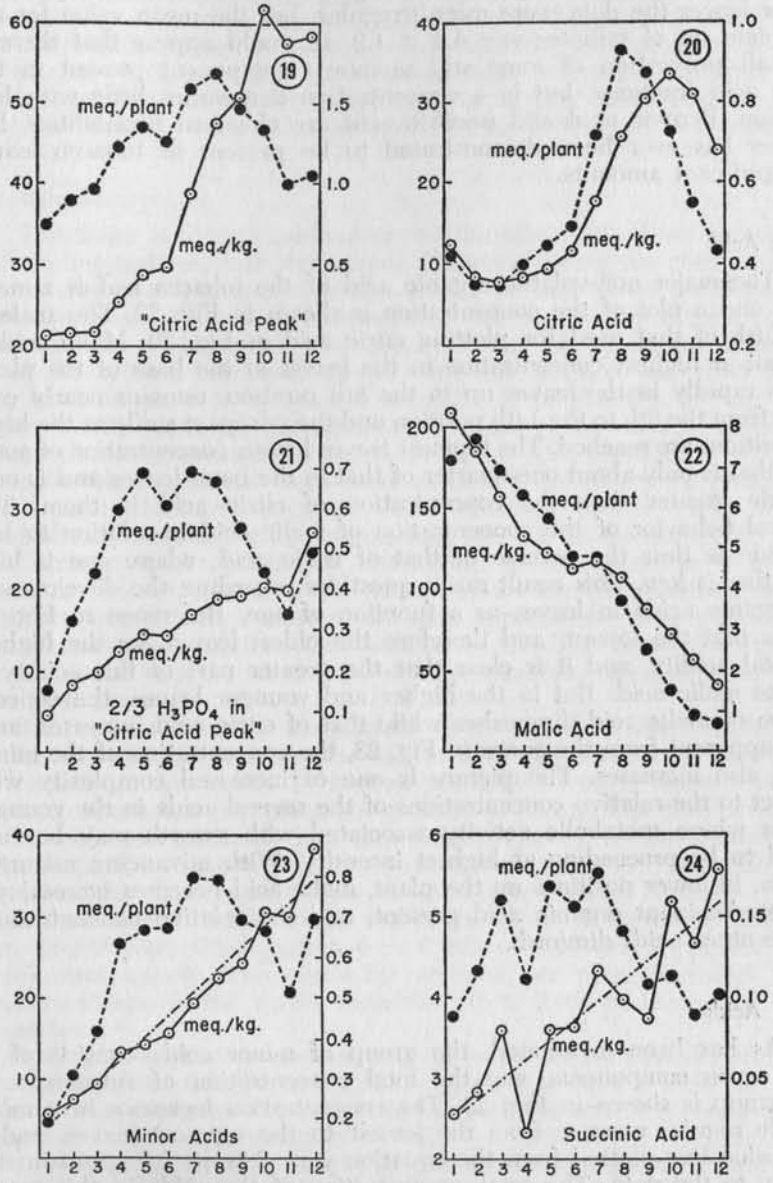

Plate IV

Fig. 19: "Citric acid peak," a quantity which includes both citric acid and water-soluble phosphoric acid. Fig. 20: Citric acid. Fig. 21: Two-thirds of the amount of phosphoric acid present in the fractions which contain citric acid. For explanation, see the text. Fig. 22: Malic acid. Fig. 23: Minor acids. For list of these acids, see the text. Fig. 24: Succinic acid.

value of 4.6 meq. per kilogram and ranged between 4 and 5 meq. In the upper leaves the data were more irregular, but the mean value for the complete set of samples was 4.8 ± 1.9. It would appear that there is a small proportion of some still unknown component present in the critic acid fractions, but in a concentration that varies little with leaf position. Pyruvic acid and isocitric acid are chemical possibilities, but neither has ever been demonstrated to be present in tobacco leaves in significant amounts.

Malic Acid

The major non-volatile organic acid of the tobacco leaf is L-malic acid, and a plot of the concentration is shown in Fig. 22. The scale is one-fifth of that used for plotting citric acid in Fig. 20. Malic acid is present at highest concentration in the leaves at the base of the plant, drops rapidly in the leaves up to the 8th position, remains nearly constant from the 8th to the 14th position and then drops steadily as the higher positions are reached. The topmost leaves have a concentration of malic acid that is only about one-quarter of that in the basal leaves and is only a little greater than the concentration of citric acid in them. The general behavior of the concentration of malic acid in relation to leaf position is thus the reverse of that of citric acid; where one is high the other is low. This result raises questions regarding the development of organic acids in leaves as a function of age. Reference to Fig. 15 shows that the lowest, and therefore the oldest leaves are the highest in total acidity, and it is clear that the greater part of this acidity is due to malic acid. But in the higher and younger leaves, the concentration of malic acid diminishes while that of citric acid increases, and, as is apparent from the curve in Fig. 23, the concentration of the minor acids also increases. The picture is one of increased complexity with respect to the relative concentrations of the several acids in the younger leaves where metabolic activity associated with growth may be supposed to be proceeding at highest intensity. With advancing maturity, that is, in lower positions on the plant, malic acid becomes increasingly the predominant organic acid present, and the relative concentrations of the other acids diminish.

Minor Acids

As has been mentioned, the group of minor acids consists of at least seven components, and the total concentration of substances in this group is shown in Fig. 23. The concentration increases in a moderately regular manner from the lowest to the topmost leaves, and a regression line plotted from the equation $y = 2.8x + 1.2$ approximates closely to the data. The total concentration of the acids in this group in the upper leaves is about the same as that of citric acid.

The curve for the amount per plant rises steeply to a maximum at the 14th to 16th positions, but falls away rather slowly in the upper leaves.

Succinic acid can be determined by the standard chromatographic procedure with only moderate accuracy since the amounts present in the tobacco leaf are small. As a result, the curve for the concentration

of succinic acid in Fig. 24 is highly irregular. Nevertheless, a regression line has been calculated from the equation $y = 0.25x + 2.3$ and shows that the concentration of succinic acid increases moderately with leaf position on the plant. The curve for amount per plant indicates that the leaves in the middle positions contain slightly more succinic acid than the basal or the top leaves, but that the differences are small.

Inorganic Components

The major inorganic components of the tobacco leaf are potassium, calcium, magnesium, and phosphorus. Curves showing the concentration and the amount per plant in the leaves in successive positions are plotted in Figs. 25 to 28. The quantities are the concentrations of the element, not of its oxide. The concentration of potassium increases rather slowly to a maximum at the 16th leaf position and then drops slightly. The curve is somewhat irregular from the 6th to the 14th positions. The curve for the amount per plant is, however, a smoothly rising and falling one with a maximum at the 10th position. If analyses of single tobacco leaves were used to detect deficiency of potassium in the plant, the curve suggests that misleading results might be secured if care were not taken in selecting the leaf to be examined.

Calcium, unlike potassium, diminishes in concentration from the lowest to the top leaves of the plant. There are irregularities, but a regression line to which most of the data approximate closely has been plotted from the equation $y = -0.12x + 2.7$. The curve for amount per plant also shows that the lower leaves contain more calcium than the upper ones.

The concentration of magnesium (Fig. 27) diminishes slightly in the leaves from the base to the 8th to 10th positions, and then rises moderately in the leaves in higher positions along a smooth curve. In terms of amount per plant, magnesium is constant in the leaves up to the 14th position, but then diminishes. The curve for phosphorus (Fig. 28), unlike that in Fig. 21, is plotted in grams per kilogram and indicates that the concentration of total phosphorus increases fairly regularly from the bottom to the top of the plant. A regression line has been plotted from the equation $y = 0.041x + 0.11$ and expresses most of the data closely. The curve for amount per plant increases to a broad maximum in the leaves from the 8th to 14th positions and then diminishes.

Although sodium is present only in small concentrations, and the data are irregular, the plot in Fig. 29 suggests that there is little variation in the sodium concentration in relation to leaf position. A regression line derived from the equation $y = 0.00035x + 0.097$ is for practical purposes a horizontal straight line. The plot of the amount of sodium per plant is also highly irregular, but indicates that leaves low on the plant are slightly richer in sodium than the upper leaves; the differences are, however, small.

Fig. 30 shows the data for the concentration of iron. Despite the irregularities, some of which may well arise from contamination of the leaf surfaces with dust blown from the field, the curve indicates that the lowest leaves are the richest in iron, and that the concentration diminishes to a minimum at about the 12th leaf position and then in-

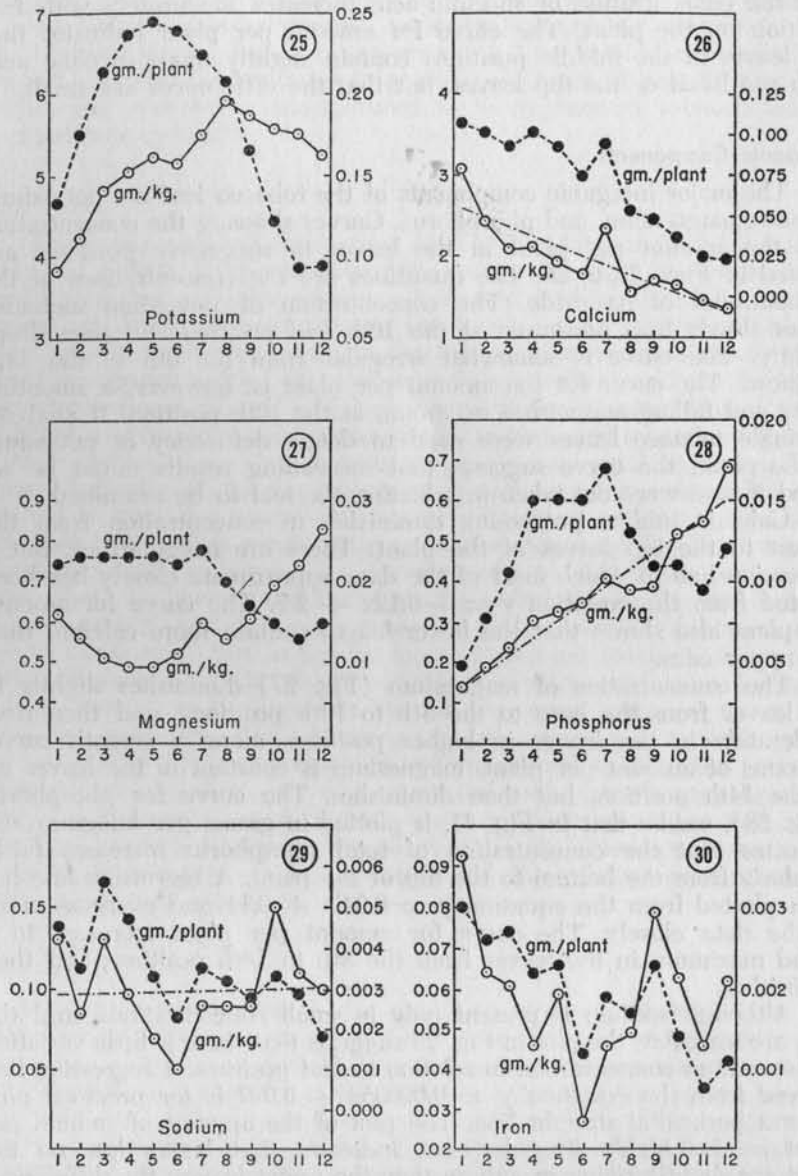

Plate V

Fig. 25: Potassium. Fig. 26: Calcium. Fig. 27: Magnesium. Fig. 28: Phosphorus. Fig. 29: Sodium. Fig. 30: Iron.

TABLE II. Concentration of trace elements in tobacco leaves as a function of position on the plant.

The data are in grams or milligrams per kilogram of fresh weight.

Sample	Aluminum (gm.)	Zinc (gm.)	Copper (mgm.)	Boron (mgm.)
1	0.013	0.056	1.5	4.8
2	0.035	0.038	1.0	4.8
3	0.023	0.064	1.6	3.6
4	0.022	0.088	1.6	3.4
5	0.013	0.043	1.5	3.3
6	0.013	0.033	1.3	3.0
7	0.019	0.045	1.7	3.4
8	0.014	0.049	2.2	3.9
9	0.011	0.051	6.2	4.6
10	0.013	0.069	3.3	4.8
11	0.012	0.035	3.5	3.2
12	0.010	0.049	3.7	4.9

creases somewhat in the higher positions. On the other hand, the curve for amount per plant suggests that there is no significant increase in the higher leaves; the amount per plant diminishes fairly steadily with leaf position.

The data for aluminum, zinc, copper, and boron have not been plotted, but are shown in Table II. If the apparently high value for aluminum in Sample 2 is disregarded, the remainder of the data have a mean value of 0.015 gms. per kilogram. Nevertheless, the trend of the data suggests that aluminum is a little higher in the leaves low on the plant than in the upper leaves. The data for zinc are somewhat irregular, but the mean value of 0.048 gms. per kilogram is a fair estimate of the concentration of zinc in these samples; there is little indication of a regular trend in the data. The data for copper and boron are expressed in milligrams per kilogram of fresh weight (i.e., parts per million). The concentration of copper appears to increase moderately in the leaves above the 16th position although it is fairly constant in the lower leaves. The data for the concentration of boron also suggest only a small variation with position; the mean value is 4.0 ± 0.7 mgs. per kilogram.

DISCUSSION

Previous Studies

Many investigators have been concerned with the composition of the tobacco leaf in relation to its position on the stalk. Several workers have made analyses of samples collected from the base, the middle, and top of the plant with the object of establishing the variation in the concentration of certain important components in the fresh leaf as influenced by agricultural practices or varietal differences. The data are, almost invariably, expressed as percentage of the dry weight. Illustrative of this approach is the paper of Bovay (11), who studied the effect of topping on the concentration of nitrogen, proteins, nicotine, potassium, calcium, and several other components. The major result appears to have been that nicotine accumulates to a high concentration in the

upper leaves of the plants from which the tops had been removed. Gugnoni (12) made similar analytical determinations on samples from Kentucky and bright tobacco grown in Italy with the object of comparing these varieties. Drake and Scarseth (13) examined the potassium, calcium, and magnesium content of bottom and top leaves of a Turkish variety of plants grown in pots as influenced by the supply of potassium.

Another approach to the problem is illustrated by the paper of Darkis, Dixon, Wolf and Gross (14) in 1936. They carried out an extensive series of analyses of flue-cured tobacco grown in North Carolina under standard conditions during four crop years. The leaves were harvested by the priming method as they attained maturity, and were flue-cured and graded. The samples examined consisted of whole leaves, including midribs, and represented the several grades recognized by the industry, these grades in turn being representative of groups of leaves taken from eight successive regions of the stalk. Thus, although the data cannot be precisely correlated with position in terms of leaf number, the relative position of the groups of leaves is established. Determinations were made of total, water-soluble and amino nitrogen, of nicotine, soluble ash, calcium, potassium, petroleum-ether extract, total sugar, and total non-volatile acidity, the data being reported in percentage of the dry weight. The authors' primary concern was to establish relationships between quality, as represented by the grade, and chemical composition. Inasmuch as the leaves of highest quality are derived from the middle portion of the stalk, they pointed out that the sugar content, which was high in the middle leaves in relation to the lower and upper leaves, and the several forms of nitrogen, which were relatively low in the middle leaves, were indicative to some extent of quality. Total acidity, soluble ash, and potassium were also relatively low in the leaves from the middle region of the plant. The nicotine content was low in the leaves in the grades from near the base and increased towards the top of the plant. Calcium varied in the reverse direction.

A somewhat similar study made in 1944 on a cigar type of tobacco is that of Hanmer, Street, and Anderson (15). These workers were concerned with the composition of cured Havana seed tobacco as a function of leaf position. The plants were topped according to usual agricultural practice about three weeks before harvest, and the leaves were cured on the stalk. A representative group of 36 plants was selected from each of two strains, and the leaves from each of 16 successive positions were pooled. The midribs were removed, and the samples of laminar tissue were dried and prepared for analysis. Determinations were made of ash, pH, potassium, calcium, magnesium, total nitrogen, protein nitrogen, nicotine, and ammonia, and were expressed as percentage of the dry weight. Plots of the tabulated data show that the ash content diminished along a smooth curve that was slighly concave upwards, that potassium increased to a rounded maximum at the 9th to the 12th leaf positions, and that calcium diminished and total nitrogen increased with position along fairly smooth curves that approximate to straight lines. Nicotine increased from about 1 per cent in the basal leaf to about 3.5 per cent in the leaves at the 10th to 14th position, but decreased a little in the topmost leaves.

A somewhat similar analytical study is that of Bortner, Wallace, and Hamilton (16) who determined potassium, nitrogen, and alkaloids in samples of 10 varieties of topped and stalk-cured Burley tobacco from each of two farms. The samples represented the blade tissue from the "trash" or bottom leaves, the "lugs," the "leaf," and the "tip," these terms representing groups of leaves from successive positions on the stalk. The extensive data scarcely admit of brief summarization save to point out that the nitrogen content increased from the base upwards, and that nicotine was at maximal concentration in the "leaf" group.

The data of none of these investigations are strictly comparable with the results of the present study. In several instances where fresh leaves were examined, only bottom, middle, and top leaves were analyzed. In the more elaborate investigation on topped tobacco plants of Darkis et al., the leaves had been flue-cured with resultant changes in composition, and in that of Hanmer, Street, and Anderson where the complete sequence of individual leaves was examined, the tobacco had been cured on the stalk. This is an operation which gives an opportunity for migration of components into and out of the blade tissue and also brings about many other fundamental chemical changes.

Vladescu's Analytical Studies

Only one comprehensive report has been noted in which a detailed examination of the composition of the green leaves from all positions on the stalk of a normally growing tobacco plant has been attempted. In a series of three papers published in 1937 in the *Zeitschrift für Untersuchung der Lebensmittel,* Vladescu (17) gave the results of an extensive series of analyses of the leaves in each successive position on the plants of three varieties of tobacco grown in Romania. In his experiments with leaves from the variety Molovata, a high quality cigarette type of oriental origin, samples were taken from 200 plants of the 1932 crop at the time of full blooming. These samples consisted of the pooled leaves from each of the 20 leaf positions. The data were reported in terms of percentage of the dry weight, and in some instances also in terms of grams per 100 grams of fresh weight, and in grams in 100 leaves so that certain comparisons with the present data are possible. Figs. 31 to 34 show the comparisons of the fresh weight of one leaf, the total solids, the total nitrogen, and the protein nitrogen. The abscissa in each figure is leaf position, and the ordinate of the data for the Connecticut tobacco is the mean value of the 20 leaves represented by each sample collected for the present work. The curves for the Molovata variety are plotted from the data for each alternate leaf in Vladescu's tables. The weights per single leaf (Fig. 31) show that the Romanian variety is a much smaller plant than the present strain of Connecticut 49, and that the leaf of maximal weight occurs lower on the plant. However, the forms of the two curves are closely similar. Presumably because Vladescu's data refer to thoroughly mixed samples taken from 200 plants, rather than from 10, the curve is almost free from minor irregularities.

Fig. 33 (below Fig. 31 in Plate VI), which gives the total solids, shows that the Romanian leaves, although smaller than those from Connecticut, are considerably richer in solid matter and therefore cor-

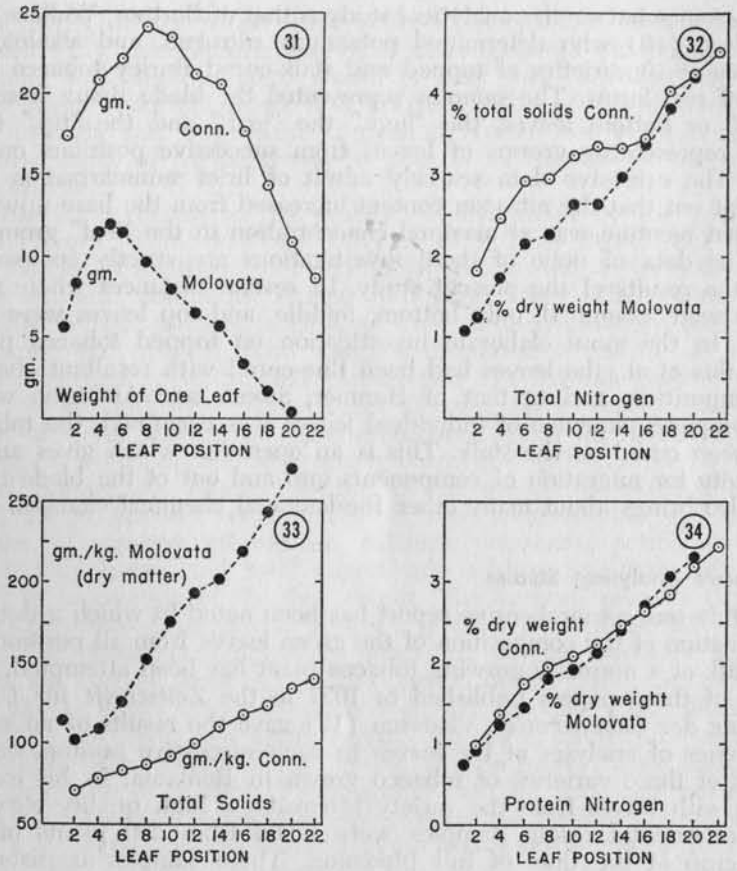

Plate VI

Fig. 31: Comparison of the weight of one leaf of Connecticut 49 tobacco with the weight of one leaf of Romanian Molovata tobacco. The abscissa is leaf position on the plant. Fig. 32: Comparison of total nitrogen as per cent of dry weight in the two kinds of tobacco. Abscissa, leaf position. Fig. 33: Comparison of total solids of the two kinds of tobacco in the units grams per kilogram of fresh weight. Abscissa, leaf position. Fig. 34: Comparison of protein nitrogen of the two kinds of tobacco in the units per cent of the dry weight.

respondingly lower in water content in the fresh condition. The Romanian data have been plotted in grams of dry matter per kilogram of fresh weight. A minor technical question arises with respect to the precise meaning of the term *"Trockengewicht"* which has been translated "dry matter." Vladescu's samples were weighed immediately after collection to obtain the fresh weight, cut into small pieces, and then dried by exposure in the greenhouse, again weighed, and ground to powder for analysis. The samples were thus in an air-dried condition and, whether or not fully equilibrated, contained a moderate percentage of water. Information in his tables shows that this ranged from about 2

to 4 per cent. The data are thus not strictly comparable with the present results which are expressed in the units grams of total solids (free from water that is removed in the oven at 110°) per kilogram of fresh weight. If the units were precisely the same, the data for the Romanian tobacco would be placed a little lower on the diagram although the smoothness of the curve and its slope would probably not be greatly altered. The slope of the curves is the most significant difference between the two kinds of tobacco. As a cigarette type, it is likely that the Romanian tobacco was considerably higher in carbohydrates, especially starch, than the Connecticut cigar type, and it would also appear that the concentration of such components increased more rapidly in the upper leaves than was observed in the present study. Unfortunately, Vladescu did not publish data for carbohydrates in his material.

Curves which show the total nitrogen content of the leaves studied in the two investigations are shown in Fig. 32 (upper right in Plate VI). In this instance, the Romanian data give total nitrogen as a percentage of the *"Trockensubstanz,"* and other evidence in the paper indicates that the samples used for this determination were dried at 95°. The figures are thus reported as per cent of dry weight and are comparable with the data for the present samples when these have been recalculated in terms of percentage of the analytically determined total solids. Comparison of the curve for the nitrogen content of Connecticut tobacco in Fig. 32 in these units with the curve for the same data calculated in terms of grams of nitrogen per kilogram of fresh weight as plotted in Fig. 7 furnishes an excellent example of the advantage of the method of reporting data used in the present paper. The curve in Fig. 32 shows many irregularities[4] whereas that in Fig. 7 is smooth, and the data as a whole can be closely approximated by two regression lines of slightly different slope.

[4] The irregularities may be to a considerable extent ascribed to the errors involved in calculating results in terms of percentage of the dry weight. This quantity is determined experimentally and is subject to many sources of error. When plant tissues are dried either in an oven or by exposure to dry air at moderate temperatures, especially if a large mass of material is being dealt with, there is no easily applied criterion of when the drying operation is complete. Destruction of unstable components may occur if the period at high temperature is too prolonged, and drying of midribs or stalks may be incomplete if sufficient time is not allowed. Success in the operation thus depends upon judgment and experience. Drying in the air is especially objectionable since enzyme activity is not promptly stopped and extensive chemical changes are certain to occur.

It is customary for investigators to grind the dried tissue to a powder that is carefully mixed in preparation for analysis. Serious difficulties attend this operation. Thoroughly dried plant tissue is usually extremely hygroscopic, and increase in weight from the absorption of water is inevitable. In humid weather with some tissues this may be so extreme that the material becomes rubber-like and difficult or even impossible to grind. The dry, ground material from different samples may absorb different amounts of water from the air, thus introducing an error when what are supposed to be equal amounts of two samples are weighed out for analysis. On a humid day, thoroughly dried samples may take up water during the operation of weighing out portions, so that accurate weights cannot be obtained.

Most of these sources of error can be eliminated, or at least their effects can be minimized, if the dried tissue is equilibrated with the air of a room at controlled temperature and humidity, subsequently ground in the same room and kept

Both sets of data shown in Fig. 32 indicate that the leaves of the tobacco plant become richer in nitrogen the higher the position on the plant. A similar conclusion can be drawn from the data of Hanmer, Street, and Anderson (15), and further evidence is to be seen in the data of Bovay (11), and of Bortner, Wallace, and Hamilton (16) already referred to.

Fig. 34 shows a comparison of the protein nitrogen content of the Romanian and the Connecticut varieties of tobacco, both expressed in similar units. The protein nitrogen content of the Romanian tobacco in terms of dry solids is closely similar to that of the tobacco grown in Connecticut in spite of the smaller size of the leaves of the Romanian variety. However, in view of the method used for drying the Romanian samples, it is quite possible that some of the protein was hydrolysed during the operation and that all of the data are a little low.

The Problem of Selecting Samples from the Growing Plant

Even a cursory inspection of the figures in the present paper shows that two fundamental questions must be answered before one may intelligently pick three successive leaves from a tobacco plant with the object of using the middle leaf for experimental treatment, and the others as controls on its initial composition. Aside from decision on the analytical methods to be used, the first question involves a decision on the units in which the analytical data are to be expressed. Several choices are possible. Since the initial fresh weight will certainly be obtained before treatment, and this weight can be secured with accuracy if only moderate precautions are taken, it is a simple matter to use the fresh weight as the basis of the comparison of the initial and final composition of the tissue. If the samples are to be dried for analysis, equilibration at a controlled temperature and humidity is essential if high accuracy is sought, but determination of the equilibrated dry weight permits calculation of the final results either in terms of concentration in a fixed amount of fresh weight or in terms of the dry solids. If the drying operation is inadmissible, analysis of the fresh tissue, or of suitably prepared extracts from it leads also to expression in terms of initial fresh weight. For some investigations, on the other hand, it may be preferable to express the results in terms of a single leaf; this method has advantages when, for example, a radioactive substance has been administered, and the uptake is accurately known from measurements on the solution from which the radioactive substance was absorbed.

Having made a decision on the analytical methods to be used, and on the method of calculating the data, it is next necessary to determine the location on the plant from which the three successive leaves are to

in it in closed containers. These containers are taken from the room only for short periods when portions are weighed for analysis. The moisture content of the equilibrated sample is determined, and analytical data can then be calculated in terms of the true dry weight if this form of report is desired. However, expression of the final results in terms of the initial fresh weight, or of a biological unit (per plant or per leaf), with use of the technique described in this paper, is to be preferred since most of the sources of error mentioned are avoided entirely.

be taken. If the results are to be expressed in terms of fresh weight, the curves which show the variation in concentration as a function of leaf position aid in the selection. Clearly, one should take the leaves from a position where the component in which one is interested has been found to vary as a straight line function, or as nearly as possible to a straight line function, of leaf position. If this desired position can be found, the error in the assumption that the initial composition of the middle leaf is the arithmetic mean of the composition of the control leaves is minimized.

On the other hand, if the data are to be expressed in terms of a biological unit, that is per leaf, the curves for composition in amount per plant should be consulted. If selection is made from a position

TABLE III. Mode of variation in composition of tobacco leaves as a function of position on the plant.

Fig. No.	
	Concentration of component varies as a straight line function of position.
2	Ratio of equilibrated dry weight to fresh weight (Factor A)
3	Water content
4	Total solids
5	Organic solids
7	Total nitrogen[5]
10	Soluble phosphate
23	Minor non-volatile organic acids
24	Succinic acid
26	Calcuim
28	Total phosphorus
29	Sodium
	Concentration of component varies in a simple curvilinear manner with position.
8	Protein nitrogen
14	Ratio of alkalinity of ash to organic solids
19	"Citric acid peak"
22	Malic acid
25	Potassium
27	Magnesium
	Concentration of component varies in a complex curvilinear manner with position: maxima and minima occur.
6	Ash
9	Starch
13	Alkalinity of ash
15	Total non-volatile organic acids
17	Oxalic acid
20	Citric acid
30	Iron

[5] The total nitrogen varies approximately in linear fashion from the basal to the 16th leaf position. Above this position it also varies approximately in linear fashion, but with a slightly increased slope.

where the amount per plant of the component of interest varies as a straight line function of position, and if the analyses of the control leaves are calculated on the same basis, a close approximation to the initial composition of the treated leaf can be found.

In Table III are listed the components of the tobacco leaf that have been found to vary approximately in linear fashion when the data are calculated in terms of concentration in the fresh leaf. Also shown are the components that vary along fairly smooth, but moderately curved lines, and components that vary along complexly curved lines that pass through a minimum and a maximum. Even with these components, however, it is usually still possible to select a position on the plant where the concentration of the component may be expected to vary nearly linearly for a sufficient number of leaf positions to permit a reasonably valid selection of leaves for experimental treatment.

Where data are to be calculated in terms of amount per single leaf, selection of the position for taking the samples is a little more difficult. Most of the components that have been examined vary in a manner which gives a curve rising to a more or less rounded or sometimes flattened maximum and then diminishing. Selection from a position fairly low on the plant as a rule may be expected to give three leaves that vary linearly in composition, and in some instances the maximum is so broad that selection may be made from this region. Nevertheless, it is obvious that for the highest accuracy it is necessary for an investigator to establish the mode of variation with leaf position of the components in which he is interested for the variety and crop of tobacco available to him. The present data can merely suggest the approximate region on the plant where rectilinear variation in composition is probably to be found.

Physiological Considerations

In the selection of samples of tobacco leaves for physiological or biochemical experiments, it is important not only to proceed in such a way that sufficient information can be obtained on the initial composition of the treated leaf; it is even more important to select for experimental treatment a leaf that is best adapted with respect to its composition and biochemical behavior to illustrate or demonstrate the phenomenon under investigation. It is strikingly apparent, from inspection of the Figures, that a tobacco leaf taken from one level on the plant may differ widely in composition from a leaf taken from another level. The investigator must therefore decide at the outset whether he is concerned with the behavior of the young rapidly expanding tissue of leaves from near the top of the plant, with that of fully expanded and "mature" leaves from an intermediate position, or with that of old and possibly senescent leaves from its base.

The necessity for the exercise of judgment on such matters may be illustrated by some data from the literature. Zelitch (18) found that a young tobacco leaf that weighed 2.4 gm. respired at the rate of 26.3 μmoles of oxygen per hour per gram of fresh weight, a fully expanded one weighing 14.8 gm. at the rate of 7.6 μmoles. The ratio of glycolic acid oxidase activity to oxygen uptake in these two leaves

was 2.3 for the small leaf and 11.2 for the more mature one. Obviously for studies of glycolic acid oxidase activity, a fully expanded leaf is to be preferred to a small one from the top of the plant; whereas if the concern is with observations on a vigorously respiring system, the young leaf would be chosen. In experiments upon the capacity for oxidative phosphorylation by particles obtained from spinach leaves, Zelitch and Barber (19) found that glycolate is oxidized at about twice the rate by particles from mature leaves as by particles from young leaves, but that the reverse is the case for the oxidation of succinate and malate. The protein content of the particles from the two sources was not significantly different. Clayton (20), when studying the pentose cycle activity in extracts from tobacco leaves, noted that this activity is highest in seedling leaves or in the upper three to five leaves of the growing plant, but that leaves at or below the midstalk position are practically devoid of it.

Such deep-seated differences in the behavior of enzyme systems in the leaves from different locations on the plant doubtless reflect far more subtle differences in composition than have been detailed by the analytical studies of this paper. They serve to show, however, that there is no substitute for good judgment supported by careful experimental testing in the choice of suitable leaf material for investigation. Because a phenomenon has been detected in, for example, seedling leaves is no compelling reason for the assumption that it will be found in fully developed or senescent leaves of the same species. An assertion about the behavior or composition of a tobacco leaf is greatly impaired in significance unless information is also provided regarding its approximate position on the stalk and thus regarding its relative age.

The wide differences in composition of tobacco leaves at different levels of insertion on the stalk of a normally growing plant raise many questions not only for the investigator concerned with the selection of leaves for his experiments, but also for the tobacco technologist. There are two main procedures for harvesting tobacco leaves. In the priming method, the leaves are picked beginning at the base of the plant, two or three leaves being taken from each plant as they are judged to have become mature. Successive pickings are taken at intervals of a few days, and the leaves are prepared for the curing process. The other method involves cutting the entire plant, allowing it to wilt for a few hours and then hanging it by its stalk in the barn to cure. Nevertheless in spite of the intervals of time allowed for the leaves to become mature between pickings in the first process, and quite obviously in the case of stalk-curing, there will be wide differences in the composition of leaves from different positions, and these differences will be reflected in the value of the individual leaves for commercial purposes. The elaborate grading processes that are employed in the industry doubtless have their origin in the recognition of these differences. Darkis et al. (13) have emphasized for North Carolina tobacco the close correlation between the recognized grades and the level on the plants from which the leaves were taken, the leaves of highest value coming from about the middle of the plant. This is in general also true of cigar type tobacco as grown in Connecticut.

Comment on the Data

Some further clarification may be welcome of the meaning of the quantity designated Factor A plotted in Fig. 2. Factor A is obtained by solving the proportion

$$\text{Fresh weight} : \text{Equilibrated dry weight} = 1000 : X$$

The value of X thus gives the equilibrated dry weight if the sample of leaves had weighed exactly 1000 gm. when collected. When a portion of the equilibrated leaf tissue is analyzed and the result is calculated as a percentage P, it is possible to set up the proportion

$$P : 100 = Y : X \qquad \text{whence } Y = (P \times X)\ /100$$

and, since X = 1000 (Equilibrated dry weight / Fresh weight) the relationship,

$$Y = P \cdot \frac{\text{Equilibrated dry weight}}{\text{Fresh weight}} \cdot \frac{1000}{100}$$

is obtained. Accordingly, to simplify the calculation of the data, the quantity 10 times the ratio of the equilibrated dry weight to the fresh weight is calculated once for all for each sample. This is Factor A and, when multiplied by the percentage of any component, it gives Y, the amount of that component in 1000 gm. of fresh leaf tissue. Factor A is thus a ratio, and is ten times as great as the fraction of the fresh weight that consists of equilibrated dry solids. Figure 2 shows that this fraction varies as a straight line function of leaf position on the tobacco plant; it is 0.075 (or 7.5 per cent) of the fresh weight at the base of the plant and 0.162 (or 16.2 per cent) of the fresh weight at the top.

The data for the ash content of the present samples plotted in Fig. 6 resemble the results for water-soluble ash of Darkis et al. (13). These workers record a mean soluble ash content of 12.6 per cent in the so-called "scrap trash" or lowest leaves, but the soluble ash dropped to 9 per cent in the so-called "best leaf" grade at about the middle of the plant (approximately the 9th position), and then increased to 10.5 per cent in the "tips." Thus in leaves picked from the plant at successive intervals of time as they reached the stage of technical maturity, the soluble ash content in the leaves from an intermediate position on the plant passed through a minimum.

The present results as well as those of Darkis et al. are quite different, however, from the data for ash of Hanmer, Street, and Anderson (15). Plots of their results give slightly curving lines which are concave upwards and drop from 34 per cent and 40 per cent of the dry weight in the basal leaves of the two sets of plants examined to about 17 per cent in the top leaves of both sets. There is no indication whatever of the complexity that characterizes the curve in Fig. 6.

The extraordinarily high ash content they observed in the lower leaves is a clear indication that inorganic substances migrated from the stalk into the leaf during the process of curing. Green tobacco leaves may be expected to contain from 17 to 20 per cent of their dry weight as ash; and only a small apparent increase could occur

when detached leaves are cured, this being entirely due to the loss of organic substances. For the ash content to attain the high values observed in the experiments of Hanmer et al., transfer of inorganic components from the stalk must have occurred.

Determinations of the alkalinity of the ash have been extremely useful in the interpretation of the data of experiments in which a salt of an organic acid has been administered to tobacco leaves by the excised leaf culture technique. The *increase* in alkalinity of the ash has been found to be a valid measure of the amount of the salt taken up, as has been shown in experiments in which the leaves were cultured in solutions of sodium tartrate over a wide range of pH reactions (21). Nevertheless, although the interpretation of a *change* in alkalinity of the ash under such experimental conditions is a simple matter, interpretation of the significance of the alkalinity of the ash itself is not. This may be illustrated by data from a paper by Pucher, Vickery, and Wakeman (22) in which the relationship between the ash constituents and the organic acids and bases of a series of samples of cured and fermented tobacco was considered. The data were calculated in terms of milliequivalents per 100 gms. of dry tissue. A series of samples from the 1927 crop had the following mean composition:

Calcium	161
Potassium	128
Magnesium	90
Nicotine[6]	13.7
Ammonia[6]	18.6
Total positive ions	411
Total inorganic negative ions	42
Excess positive ions	369
Organic acidity	245

The total inorganic negative ions were calculated from the determinations of phosphorus, nitrate, sulfur, and chloride in the samples, it being assumed that all of the phosphorus and chloride, and 70 per cent of the sulfur functioned as base-binding groups. No account was taken of organic bases other than nicotine and ammonia since, aside from the proteins, these are doubtless the major components of this class present in the tissue. The value they gave for organic acidity represents a quantity which, although determined by another method, is analogous to the data for the total non-volatile organic acids in the present paper save that oxalic acid is included. Clearly, at most only 66 per cent of the positive ions potentially present are neutralized by the organic acids in this group, and since at the pH of the green leaf only about 70 per cent of the malic and citric acid is present in fully ionized form, the excess of potential positive ions is even greater

[6] The values for nicotine and ammonia suggest that the share in the acid-base equilibrium taken by organic bases is small in comparison with the inorganic bases. The high value for ammonia in the samples is characteristic of cured and fermented tobacco (3). Much of it is derived from the hydrolysis during fermentation of the asparagine which is formed during curing.

than this estimate would indicate. Although this view of the situation is doubtless greatly over-simplified, the data serve to emphasize the fact that far more information than is at present available is required before an exact accounting can be given of the relationship between the excess of positive ions, which is approximately determined as the alkalinity of the ash, and the base-binding components of the tissue. The curve plotted in Fig. 18 draws attention to the inadequacy of our present knowledge of the chemical nature of the base-binding groups in the tobacco leaf, for it shows estimates of the organic acidity of unknown nature in this tissue that are not only substantial but are minimal at best.

The distribution of nicotine in the leaves of the tobacco plant is a matter of considerable importance both from the practical and the physiological points of view. Not only are there wide species and varietal differences in the chemical nature and amounts of the alkaloids which accumulate in tobacco leaves, but agricultural practices also exert a

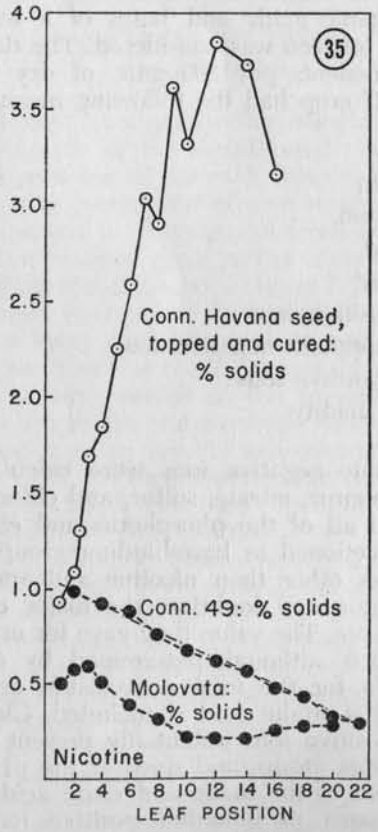

Fig. 35

Nicotine content of green leaves of normal plants of the variety Connecticut 49 and of the normal plants of the Romanian variety Molovata compared with the nicotine content of the leaves of topped Havana seed plants cured on the stalk.

profound influence. This is well illustrated by two earlier papers (14, 15) that have been referred to above. Vladescu's investigation (17) dealt with the distribution of a number of components in the leaves of normal plants grown according to standard Romanian methods. His data for nicotine are plotted in Fig. 35 in which the abscissa is *leaf position* and the ordinate percentage of nicotine in the dry solids. Molovata tobacco is apparently a variety characterized by low nicotine content since the basal leaves contained only about 0.5 per cent and the proportion dropped to 0.25 per cent in the leaves in the 10th position. The nicotine content rose only slightly in leaves in higher positions on the plant although the plants were approaching maturity being in full flower.

The nicotine content of the present samples is also plotted in Fig. 35 in the same units. The basal leaves in sample 2 contained 1 per cent of the dry solids as nicotine, and the proportion diminished along a nearly straight line to 0.33 per cent in the leaves at the top of the plant. The observations conform closely to the regression line plotted from the equation $y = -0.072x + 1.07$. The nicotine content of the cured leaves from the topped Havana seed plants studied by Hanmer, Street, and Anderson (15), also plotted in Fig. 35, presents a totally different picture. They found that nicotine accumulated in the upper leaves to an extraordinary extent. The data for only one of the two strains they examined are shown since both behaved in essentially the same way. The observations of Bovay (11) on a variety of tobacco grown in France reveal the same type of behavior. Whereas the basal leaves of his normal plants contained 1.45 per cent of nicotine and the top leaves 1.66 per cent, the basal leaves of the topped plants contained 2.1 per cent and the top leaves 6.26 per cent. Tso and Jeffrey (23) have also noted that removal of the tops of tobacco plants of the Robinson broadleaf type brings about a three- to fivefold increase of the total alkaloid content of both root and shoot.

That nicotine is synthesized almost exclusively in the roots of the plant and subsequently translocated to the leaves was pointed out in 1942 by Dawson (6) who had studied the nicotine content of tobacco scions grafted on tomato rootstocks. Nevertheless, a small proportion of the alkaloid is synthesized in the leaves, and subsequent investigations by Tso and Jeffrey (24) and by Dawson have shown that if the terminal bud of such grafted scions is removed, the accumulation of nicotine in the scion is stimulated. In a recent study, Dawson and Solt (25) have found that the small proportion of the total alkaloid content of the shoot (of the order of 1 to 3 per cent of the alkaloid present) that is synthesized in the shoot is substantially increased if the plants are topped. Synthesis in the root of intact plants is also stimulated by the topping operation. Accordingly, the behavior of the nicotine in plants that have been topped, as illustrated by the upper curve in Fig. 35, is perhaps not surprising. This operation obviously elicits a profound response which extends throughout the organism. Data which suggest that the amount of alkaloid in the plant may be increased as much as tenfold by topping have recently been published by Tso, McMurtry, and Sorokin (26).

SUMMARY

For many kinds of experiments, it is desirable to select a single leaf from a tobacco plant for treatment and use the leaves immediately above and below it as controls from which the initial composition of the treated leaf may be ascertained. If the selection is made from a region on the plant where the composition with respect to the components of interest in the proposed experiment varies as a straight-line function of leaf position, the initial composition of the leaf to be treated can be calculated with considerable accuracy. As an aid in locating the position on the plant where this requirement is most likely to be satisfied, the composition of the green leaves from successive positions on tobacco plants of the variety Connecticut 49 has been determined. The data are expressed in the units grams, or milliequivalents for acidic components, per kilogram of initial fresh weight, and also in terms of grams or milliequivalents per plant. Each sample consisted of two adjacent leaves in the same position taken from each of ten vigorously growing plants at the time that flower buds were beginning to develop, and the set of samples represented each pair of successive positions on the entire length of the stalks. Data for the organic solids, ash, total nitrogen, protein nitrogen, and nitrate nitrogen, for the major individual organic acids and the alkalinity of the ash, for nicotine, and for the more important inorganic components are presented as curves in which the concentration and the quantity of the individual components are both plotted as functions of leaf position. From the forms of these curves, it is possible to select a region of the plant at which certain of the individual components vary with position in approximately straight-line fashion for a sufficient distance to permit a satisfactorily accurate calculation of the initial composition of an intermediate leaf from the composition of the leaves above and below it. Nevertheless, this position must be selected with due attention to the units in which the data are to be expressed. Although there are a number of components which, when expressed in terms of concentration in the fresh weight, vary in an approximately straight-line fashion from the base to the top of the plant, others vary in this way only for short distances. When expressed in terms of amount per leaf, most components vary in a manner which gives a curve that rises to a more or less broad maximum in the middle region of the plant and then diminishes in the upper leaves. However, selected portions of these curves are also in most instances nearly straight lines.

Citric acid has been found to be low in concentration in the basal leaves, but rises to a maximum near the top of the plant. Malic acid, on the other hand, is highest in concentration in the basal leaves and lowest in the top leaves. The concentration of oxalic acid follows a complex curve being low in the basal leaves, passing through a maximum and a minimum in the intermediate leaves and rising to its highest concentration in the topmost leaves. The group of minor acids and succinic acid are low in concentration in the basal leaves and increase in approximately straight-line fashion in relationship to leaf position. Nicotine is highest in concentration in the basal leaves and diminishes in concentration in the upper leaves, the curve which expresses the data being close to a straight line. Attention is drawn to the effect of topping the plants on the concentration of nicotine. This operation greatly increases the concentration of nicotine in the upper leaves.

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TABLE IV. Composition of tobacco leaves as a function of their position on the stalk.

The data are expressed in the units grams per kilogram or milliequivalents per kilogram of fresh weight of the leaves. These data can be converted to grams or milliequivalents per plant by multiplication by Factor C which is the ratio of Factor A to Factor B.

Fig.	Component	1	2	3	4	5	6	7	8	9	10	11	12
2	Factor A	0.771	0.853	0.912	0.972	1.024	1.097	1.220	1.314	1.380	1.492	1.541	1.633
2	Factor B	0.0267	0.0354	0.0405	0.0469	0.0480	0.0466	0.0499	0.0464	0.0396	0.0325	0.0264	0.0295
	Factor C	0.0346	0.0415	0.0416	0.0482	0.0468	0.0424	0.0409	0.0353	0.0287	0.0217	0.0171	0.0180
3	Water, gm.	929	922	917	912	908	901	890	882	876	866	860	852
4	Total solids, gm.	70.7	78.1	83.0	87.9	92.5	99.0	110.2	118.5	124.4	134.4	139.9	148.0
5	Organic solids, gm.	53.9	62.9	68.7	73.7	78.3	84.5	94.6	102.6	108.4	117.4	124.5	133.4
6	Ash, gm.	16.9	15.1	14.3	14.2	14.2	14.5	15.7	15.9	16.0	16.4	15.4	14.6
7	Total nitrogen, gm.	1.29	1.91	2.42	2.60	3.01	3.35	3.70	4.13	5.04	5.71	6.35	7.07
8	Protein nitrogen, gm.	0.69	1.09	1.46	1.73	1.94	2.20	2.71	3.14	3.55	4.31	4.82	5.49
9	Starch, gm.	0.33	0.85	1.35	2.05	2.47	3.01	4.83	5.70	5.99	4.92	4.67	4.18
10	Total phosphorus as phosphate, meq.	13.6	18.4	23.3	30.1	32.0	34.0	39.8	36.9	37.9	50.5	53.9	66.7
10	Water soluble phosphate, meq.	9.2	14.5	17.3	19.9	22.7	22.3	27.5	29.8	29.7	33.0	33.0	41.8
11	Nicotine, gm.	0.70	0.73	0.74	0.68	0.63	0.63	0.63	0.57	0.54	0.45	0.42	
12	Nitrate nitrogen, gm.	0.34	0.38	0.46	0.53	0.51	0.53	0.54	0.55	0.51	0.48	0.46	0.46
13	Alkalinity of ash, meq.	303	289	265	250	238	236	248	256	253	248	241	218
15	Total non-volatile organic acids, meq.	238	218	192	173	169	162	178	180	166	171	149	144
17	Oxalic acid, meq.	27.5	32.2	37.8	31.2	24.0	23.1	14.7	21.8	26.1	33.4	37.1	41.6
19	"Citric acid peak," meq.	21.4	21.7	21.9	25.6	28.9	29.7	38.9	47.7	50.9	61.8	57.6	58.3
20	Citric acid, meq.	12.1	8.4	7.9	8.3	9.4	11.5	17.6	26.3	30.4	33.3	32.1	24.0
21	2/3 of phosphoric acid in citric peak, meq.	4.6	8.3	9.9	12.5	14.6	14.3	16.9	18.9	19.2	20.2	19.7	26.9
22	Malic acid, meq.	208	186	157	132	123	113	116	106	86	71	56	42
23	Minor acids, meq.	5.5	7.5	9.6	13.2	14.3	15.8	19.5	22.4	24.3	30.6	30.0	38.4
24	Succinic acid, meq.	2.6	2.8	3.6	2.3	3.6	3.7	4.3	4.0	3.8	5.2	4.7	5.6
25	Potassium, gm.	3.9	4.2	4.8	5.1	5.2	5.2	5.5	5.9	5.7	5.6	5.5	5.3
26	Calcium, gm.	3.1	2.4	2.1	2.1	1.9	1.8	2.3	1.5	1.7	1.6	1.5	1.3
27	Magnesium, gm.	0.62	0.56	0.51	0.49	0.49	0.52	0.60	0.56	0.61	0.69	0.74	0.83
28	Phosphorus, gm.	0.14	0.19	0.24	0.31	0.33	0.35	0.41	0.38	0.39	0.52	0.55	0.68
29	Sodium, gm.	0.13	0.09	0.13	0.10	0.07	0.05	0.09	0.09	0.09	0.15	0.11	0.10
30	Iron, gm.	0.092	0.064	0.061	0.046	0.049	0.027	0.046	0.049	0.079	0.063	0.046	0.062