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THE PENNSYLVANIA STATE COLLEGE
The Graduate School
Department of Agricultural and Biological Chemistry

A CHEMICAL STUDY ON NATURE, CURED AND FERMENTED
PENNSYLVANIA CIGAR-LEAF TOBACCO

A Thesis
by
Renato Marino Royo

Submitted in partial fulfillment
of the requirements
for the degree of
MASTER OF SCIENCE
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Approved:

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INTRODUCTION

Although tobacco is tropical in origin, its culture is world wide. The plant is grown as far north as central Sweden and as far south as Southern Australia and New Zealand. Thus, this plant can be grown successfully under a very wide range of climatic conditions; but on the other hand, the commercial value of the product depends largely on the environment in which it is produced.

Tobacco has been the subject of investigation for many years, but much of the earlier data can no longer be regarded as reliable. The dearth of analytical procedures adequate to cope with the problems presented by the chemical complexity of the tobacco leaf has been a serious obstacle in the pathway of progress along this line.

A marked progress has been made regarding the different chemical changes which the tobacco leaf undergoes in the field, the curing shed and the factory. Many investigators are trying to elucidate the complex nature of the different chemical changes that are constantly going on in the tobacco plant especially during its growth period. Others are endeavoring to advance our chemical knowledge in regard to the process of curing. A knowledge of those chemical changes that take place in the leaves of the tobacco plants when these are detached and allowed to dry with maximal exposure to air is very important, since it represents those changes which affect the physical and chemical properties of the leaf - more particularly those properties which in-

clude the capacity to hold fire and the color, texture, and elasticity.

A number of workers are trying to solve the mystery which involves the fermentation of tobacco. Different theories have been advanced to account for the chemical changes occurring during the fermentation process by different workers on the subject. While certain progress has been made in this particular field, a considerable amount of information remains to be obtained.

More research work along these different lines of investigation; and more attention given to the cultural and fertilizer practices as they are related to quality of the leaf will greatly increase our knowledge of tobacco chemistry and add to the success of the tobacco industry.

REVIEW OF THE LITERATURE

In spite of the almost universal occurrence of organic acids in plants, surprisingly, little trustworthy information is available on the exact identity of the acids found in a given species. Most of the available information about the metabolism of the organic acids has been obtained from studies of the lower forms of plant life, especially molds and yeasts.

It had been pointed out in the reviews of Franzen (10,11,12,13, 14,15) and his co-workers that the older data establishing the identity of the organic acids of the tobacco leaf are very unsatisfactory. The extensive investigations of the Russian workers on cured and fermented tobaccos of the oriental types had recently been summarized on the work of Shmuck (38). These workers on their qualitative investigations identified citric, malic, oxalic, succinic, and fumaric acids on cured and fermented tobaccos. A more recent publication by Vickery, and Pucher (51) contains a very comprehensive review of the literature relating to the organic acids of the tobacco plant.

Pennsylvania cigar-leaf tobacco contains appreciable quantities of malic, citric and oxalic acids; in fact, these actually constitute more than four-fifths of the total ether-soluble acidity of the leaf tissue.

The whole subject of the metabolism of organic acids of green plants has been recently reviewed by Bennett-Clark (7) and reference

should be made to his papers for a comprehensive statement of the present position of our knowledge of the organic acids in plant physiology. The work of Vickery and Pucher (49) on the metabolism of the organic acids of the tobacco plant during culture reveals that the three chief organic acids of the tobacco leaf, malic, citric, and oxalic acids, undergo very little change in absolute amount during culture of the leaves in the light, the total organic acidity remains essentially constant. During culture in the dark, however, quantity of malic acid diminishes and the quantity of citric acid increases considerably, the oxalic acid and the total organic acidity both remain unchanged. A consideration of the possible sources from which the newly formed citric acid may be derived indicates that malic acid is the most probable source. Certain suggestions as to the possible chemical mechanisms to account for the interconversion had been advanced by the authors. The authors also found that oxalic acid decreases; but does not entirely disappear during the development of the plant. Smirnov also found it to be the case with Oriental tobaccos. Malic acid attains its maximum concentration in the young plant and the concentration does not change appreciably in the mature leaf. Concentration of citric acid is also maximal in the young plant, decreasing markedly in the mature fresh leaf and again increasing during the curing process. Malic acid was at all stages of growth the predominating acid of the leaves, oxalic acid being next in order, with citric acid in smallest quantities. The amount of unknown acids was intermediate between the oxalic and citric acids.

In 1914 Garner, Bacon, and Foubert (18) reported data on the malic, citric and oxalic acid content of samples of dried green leaves and cured leaves of several varieties of Connecticut tobacco. The work of Vickery and Pucher (52) on the curing of several varieties of Connecticut tobacco shows that the quantity of malic acid did not change during the first twelve days of curing but subsequently diminished by about 16%. The quantity of citric acid in the leaf increased enormously, at the expiration of twelve days nearly five times as much of this acid was present in the leaf; and at the end of the curing period, the quantity had further increased to 5.8 times the original amount. The synthesis of citric acid was one of the major changes occurring during curing. They present data in this bulletin implying that oxalic acid is a relatively unimportant constituent of the tobacco leaf at any stage of curing. Ruhland regards oxalic acid as of fundamental significance in the general metabolism of many plants being called forth in response to the formation of ammonia as a part of the mechanism to provide for the maintenance of the reaction of the tissue within the proper limits.

Block (8) making a study of the non-volatile organic acids of Pennsylvania cigar-leaf tobacco found that during the curing period the oxalic acid and the citric acids increased in quantity whereas the malic acid decreased. He concluded that the better qualities of cured tobacco was found to have a greater amount of citric acid whereas the quantities of malic and oxalic acids were approximately the same. He found that there was no influence on the citric, malic, and oxalic acid content of the tobacco leaf by varying the fertilizer treatment or the system of rotation.

During the processing of tobacco of the cigar-leaf type certain periods of fermentation are involved. Although the fermentation of tobacco has been the subject of study for more than fifty years, little exact knowledge exists as to the different factors involved. To Jenkins (22) belongs the credit for the first determination of some of the chemical changes occurring during fermentation of cigar-leaf tobacco. Smirnov (40) studied the behavior of the organic acids during the fermentation of tobacco and reached the following conclusions:-

(1) Under anaerobic conditions malic and citric acid practically disappear; in presence of oxygen there is scarcely any change at all.

(2) Fermentation decreases the percentage of organic acids but the change in the citric acid content is greater than that of malic acid.

The work of Mc.Kinstry (27) shows that the normal fermentation is always accompanied by a marked progressive decrease in the total ether-soluble organic acidity and a corresponding rise in the alkalinity of the tissue. A profound diminution of the malic acid and citric acid content of the leaf is associated with a satisfactory fermentation, the oxalic acid present in the tissue is not appreciably altered.

The earliest reference to the addition of yeast to fermenting tobacco was by Koller in 1858 (24). Koller inoculated tobacco with a yeast infusion in an effort to hasten the fermentation. Summaries of the work of several German investigators are to be found in the publication of Wagner (56) and Kissling (23).

Details are given for the use of yeast in numerous ways. Street (43) was the first to report on the use of yeast in fermentation of

tobacco in this country. The addition of living yeast cells to tobacco may be interpreted as either a biological or biochemical method. Yeasts are simple forms of fungi in the classification of plants and parallel reactions are common.

The early work on the chemical factors influencing the quality and burn of the tobacco leaf was concerned mainly with the mineral constituents and the total organic acidity. Schloesing (37) may be called the pioneer in the scientific investigation of the chemical factors affecting the burning qualities of tobacco. He was the originator of the theory of combustibility of tobacco that attributes to potassium salts of organic acids a decidedly beneficial influence to the burning qualities, and to the potassium salts of sulfuric and muriatic acids a very harmful effect inhibiting the burn. He explained this favorable action being due to the selling of the potassium salts of oxalic, citric and malic acids, which, when heated melt before they burn and by further heating yield an inflated and highly porous coal favorable for holding fire. He claimed that there is in general a direct relationship between the alkalinity of the water-soluble constituents of a tobacco ash and its fire holding capacity. The alkalinity of the ash solution is due almost entirely to the potassium carbonate which it contains. The leaf doesn't contain potassium carbonate, but when it is burned, the potassium salts of the organic acids are oxidized to carbonates. So the alkalinity of the soluble ash appears to be a good index to fire holding capacity.

In analyzing the ash of good burning tobacco, van Bemmelen (47) always found a greater quantity of potassium present as the carbonate and lesser quantities as the chloride and sulphate.

Behrens (6) as a result of his investigational work on the burning qualities of tobacco, states that a high content of potassium in combination with citric, malic and oxalic acids largely determines the burning qualities.

Garner (17) made extracts from both good burning and poor burning leaves and concluded that both contained about the same quantities of potassium, but the inferior leaf contained a much higher percentage of mineral acids. Hence, he concluded that the potassium salts of organic acids such as malic and citric are the chief factors controlling the burn.

Haley, Nasset, and Olson on a study of certain constituents of the leaf and their relation to the burning qualities of tobacco (19) arrived at the following conclusions:- (1) The ether-soluble organic acids of the plant appear to occur almost wholly in combination with the alkali earth metals as measured by the alkalinity of the soluble ash and insoluble ash. In practically all cases they found a parallelism between the burning qualities and the alkalinity of the soluble ash. They concluded that the season and the form of potash supplied as fertilizer affected alkalinity of the soluble ash in practically all cases.

Field fertilizers experiments on tobacco has been done by many investigators since early times. The work of Frear (16) Olson (29,30,31, 45), Haley (20,30,45) and Thomas (45) on Pennsylvania cigar-leaf tobacco should be mentioned.

The work done of the effect of fertilization on the organic acids of the tobacco-leaf is very meager, Shmuck (39) presented evidence to show that the citric acid content increased with an increase in ferti-

lization. Nasset (28) studied the effect of fertilization on the organic acid content as well as the effect of the organic acidity on burning quality. Block (8) found no influence on the citric, malic, and oxalic acid content of the tobacco leaf by varying the fertilizer treatment or the system of rotation.

Kurchatov and co-workers (26) and Psarev (31) working with high citric acid Russian tobacco, found that the maximum accumulation of citric acid was obtained by the use of a potash fertilizer. Another Russian worker, Vladimirov (54,55) working with the same type of tobacco, found that an increase in the potassium content of the nutrient medium decreased the citric acid content and the total acidity of the tobacco leaves. He also found that ammonium salts as a source of nitrogen decreased the total acidity and the content of citric acid, while the use of nitrate as a source of nitrogen resulted in increased acidity and citric acid content.

The literature of plant composition, as influenced by the form in which nitrogen is supplied, is very limited. Most investigators have worked from the agricultural point of view and have recorded their results in terms of size, crop yield, luxuriance of foliage, or at best in terms of dry weight of the plants.

Haley and Thomas (45) working on the absorption of plant nutrients in relation to the production of Pennsylvania cigar-leaf tobacco found that the effect of nitrogen on growth and development of tobacco plant depends on the balance of nutrient material present but likewise on the form of nitrogen used. They found that cottonseed meal from standpoint of yield, is more satisfactory as a carrier of nitrogen than NaNO_3 .

They also found that the absorption of nitrogen occurs during the growing season, although the percentage of nitrogen falls at maturity. The clover and alfalfa, when grown in rotation, adds considerably to the quantity of nitrogen available for absorption.

The use of ammonium salts as the source of nitrogen for the growing of tobacco has been extensively studied by Anderson and his collaborators (1,44).

Clark (9) working with tomato plants found an outstanding difference in the relative amounts of organic acids in the tissues when nitrogen was supplied as ammonium nitrogen and when it was supplied as nitrate nitrogen. Furthermore, the distribution of this organic acidity with respect to the relative proportions of oxalic, malic, citric and unknown organic acids was widely different in the two cases. The leaves and stalks of plants grown on ammonium nitrogen contained respectively 71 and 65 milli-equivalents of organic acids per 100 grams of dry tissue, those of the plants grown on nitrate nitrogen contained 153 and 147 milli-equivalents.

Vickery, Pucher and Wakeman (53) very recently worked on the effect upon the composition of the tobacco plant of the form in which nitrogen is supplied. They presented results leading to the conclusion that the tobacco plant is extremely responsive to the nature of the nitrogenous nutriment with which it is supplied. The plants grown with the higher proportions of ammonia in the culture solution contained very little organic acid. Malic acid is the dominant acid in the "nitrate plants", but it appears to be the acid most extensively affected by a change in the nitrogen supply Citric acid appeared to be also extensively affected especially when the amount of ammonium nit-

rogen was increased. The acids of the unknown group in the present case changed remarkably little in absolute quantity over a range of nitrogen nutrition (0 to 60% ammonia), also the oxalic acid was only moderately affected.

The behavior of the alkalinity of the ash is particularly striking. The nitrate plants yielded a highly alkaline ash, the "ammonium nitrogen plants" a moderately alkaline one. The quantity of ash per plant was greatly affected.

The calcium in the ash of the leaves follows a curve that closely resembles that for the organic solids. The variation of calcium as the range of nitrogen nutrition varies from 0 to 80% ammonium nitrogen was not nearly so striking as was the variation in the organic acids. It can be concluded from the data that the calcium absorption at a constant level of calcium supply was little affected by the change in the form of nitrogen in the solution; such differences as were noted can be interpreted only as secondary results of the change in the nitrogen nutrition. Phosphate absorption, like that of calcium, was not greatly affected by an increase in the proportion of ammonium ion available to the plants when the calcium and phosphate were provided at a constant concentration in the several culture solutions.

Records of the analysis of cured tobacco grown in 1927 at the Windsor Tobacco Substation (50) showed that a sample prepared from plants fertilized with NaNO_3 contained 3.5% of nitrate nitrogen, while one from plants fertilized with ammonium sulphate contained 19.5%. Samples from plants that had received various organic nitrogenous fertilizers lay between these extremes. The explanation of the low

nitrate nitrogen content of the sodium nitrate plants is doubtless the leaching of the soluble salt from the unusually heavy rains of that particular growing. Ammonium salts, on the contrary are not readily removed in this way and it is obvious that an extensive transformation to nitrates in the soil must have occurred.

ANALYTICAL PROCEDURES

All samples were analyzed in duplicate and expressed on a moisture free basis.

Determination of Moisture

Moisture was determined by drying a one gram sample to constant weight in a vacuum dessicator containing sulfuric acid, according to the methods of the Association of Official Agricultural Chemists (5).

Determination of Ash

Ash was determined by heating the samples in porcelain dishes for about 16 hours in an electric muffle furnace at 450-500°C.

Determination of the Total Alkalinity of the Crude Ash

Transfer the ash to a beaker and add exactly 50cc. or an excess of N/10 sulfuric acid, heat to boiling and filter. Wash the precipitate thoroughly and titrate with N/10 NaOH solution using methyl orange or xylene cyanide-methyl orange as an indicator. The results are expressed as the total amount of normal acid required to neutralize the total alkalinity of the ash in 100 grams of the original plant material - H₂O free.

Determination of the Total Alkalinity of the Soluble Ash

Add about 150cc. of hot water to the crude ash, filter and wash well with hot water. Titrate the filtrate with N/10 H₂SO₄, using methyl orange or xylene cyanide-methyl orange as an indicator. The results are expressed as the number of cc. of normal acid required to neutralize the alkalinity of the soluble ash contained in 100 grams of the original material - H₂O free.

Determination of the Alkalinity of the Insoluble Ash

The alkalinity of the insoluble ash was calculated by subtracting the alkalinity of the soluble ash from the total alkalinity of the ash. The results are expressed as the quantity of normal acid required to neutralize the alkalinity of the insoluble ash in 100 grams of the original plant material - H₂O free.

Preparation of the Organic Acid Fraction

The organic acids of the tobacco leaf were extracted according to the technique of Pucher, Vickery and Wakeman (32). Two grams of the dried and powdered tissue was acidified to pH 1.0 with sulfuric acid, mixed with 3.5 grams of asbestos, and extracted with the anhydrous ethyl ether in a Soxhlet extraction apparatus for a period of 24 hours. At the end of the extraction period, 25ml. of distilled water was added to the ether-extract, the system was thoroughly agitated to transfer the acids to the aqueous phase, and the ether was carefully distilled. The aqueous solution was cooled in the refrigerator in order to solidify the resinous material which was then removed by filtration through a Gooch crucible. The organic acid extract was made to a definite volume.

Determination of the Total Ether - Soluble Organic Acid Content

The ordinary titration procedure was employed in determining the total ether soluble organic acidity of the sample. A 25ml. aliquot of the organic acid fraction was diluted with 10ml. of distilled water and titrated with .04N NaOH using phenolphthalein as an indicator.

Determination of Oxalic Acid

Oxalic acid was determined by the method of Pucher, Vickery

and Wakeman (32) as follows:

A 25ml. aliquot of the organic acid extract was acidified to Congo Red with 0.5 N HCl. The precipitate that formed was allowed to settle, filtered off on asbestos in a Gooch crucible, and washed with water. A drop of methyl-red was added to the clear filtrate and ammonium hydroxide was added to a faint alkaline reaction. Two to three ml. of glacial acetic acid were added, followed by 5 ml. of 10% calcium chloride solution. After standing at least two hours, the calcium oxalate was filtered on asbestos in a Gooch crucible and was washed with a little very dilute ammonium hydroxide. Crucible and contents were then transferred to a 100 ml. beaker. Five ml. of 50% H_2SO_4 and 20 ml. of water were added; and the solution was heated to boiling and titrated while very hot with 0.02 N potassium permanganate solution.

Determination of Citric Acid

Citric acid, when treated with potassium permanganate and potassium bromide under the proper conditions, is converted into the insoluble substance pentabromoacetone. This reaction was originally employed by Stahre (42) for the qualitative recognition of citric acid, but was placed upon a quantitative basis by the work of Kunz (25) and of Hartman and Killig (21) and it is now widely used for the determination of citric acid. Pucher, Vickery and Wakeman (34) taking advantage of this and other ideas of some Russian workers have developed an accurate volumetric method to determine citric acid in small quantities. In this method, citric acid and malic acids are oxidized simultaneously, and pentabromoacetone which is formed from the citric acid is separated from the malic acid oxidation product by extraction with petroleum

ether, after which the acids are determined separately. The procedure that was used was as follows:

A 5 ml. aliquot of the organic acid solution was diluted to 20 ml. with water and 3 ml. of 50% H_2SO_4 were added. The mixture was boiled gently for eight to ten minutes to expel traces of ether, cooled, and 1 ml. of saturated bromine water was added. After five minutes the solution was filtered with gentle suction through a Gooch crucible into a suction flask marked at 35 ml., and 2 ml., of 1N potassium bromide were added. The temperature of the solution was then adjusted at 20° to 22° for 10 minutes with occasional stirring, and was then chilled to 5° to 10°C and decolorized with 3% H_2O_2 added dropwise with vigorous stirring.

The oxidation mixture was transferred to a 125 ml. pear-shaped separatory flask, and the suction flask was rinsed into the separatory flask with 25 ml. of petroleum ether used in small quantities. The flask was shaken vigorously and the aqueous layer was drawn off. The petroleum ether layer was transferred to a second flask. The aqueous solution was shaken again with 20 ml. of petroleum ether and was then put aside for the determination of malic acid. The two petroleum ether extracts were combined and washed four times with 3 ml. of water and the washings were added to the aqueous extract for the malic acid determination. A 3 ml. quantity of 4% sodium sulfide solution was added to the separatory flask, and the mixture was shaken vigorously. The aqueous layer, which turned reddish, was drawn off into a 50 ml. Erlenmeyer flask, the petroleum ether was then treated a second time with 3 ml. of sodium sulfide, and was washed three times with 3 ml. to 4 ml. of water, the aqueous solution all being combined. To this so-

lution was added 2 ml. of 2 N sulfuric acid and a few quartz pebbles; and the solution was boiled gently for three minutes to expel hydrogen sulfide. It was then cooled to room temperature and sufficient 1.5 N potassium permanganate added to produce a red color permanent for 20 seconds. This usually took about 0.8 to 1.0 ml. of permanganate. The color was discharged by the addition of a small excess of a specially prepared halogen-free hydrogen peroxide (34,35), followed by the addition of 2 ml. of concentrated nitric acid, 3 ml. of standard silver nitrate, 1 ml. of ferric alum indicator solution, and 3 to 4 ml. of ethyl ether. The flask was shaken vigorously to coagulate the silver bromide, and the solution was titrated with ammonium thiocyanate from a micro-burette to a faint salmon-pink color. In as much as the conversion of citric acid to pentabromoacetone is not strictly quantitative, it was necessary to multiply the quantity of citric acid determined by a factor of 1.12.

Determination of Malic Acid

Malic acid was determined by the method of Pucher, Vickery and Wakeman (34). The method described in the present paper depends on the observation that malic acid, when treated with potassium permanganate in the presence of potassium bromide under the proper conditions, is converted into a bromine compound that is volatile with steam. This substance yields an extremely insoluble condensation product when heated with dinitrophenylhydrazine in acid solution. The ratio between the amount of this product and of the malic acid submitted to oxidation is constant, and the quantity can be easily estimated by dissolving the product in pyridine, diluting with water, and making the solution alkaline with sodium hydroxide. The intense blue color formed can be measured with a colorimeter.

The method is as follows:- The aqueous solution, together with the wash fluid from the petroleum ether (obtained as described under the determination of citric acid), was diluted to a 100 ml. A 25 ml. aliquot was transferred to a 300 ml. Kjeldahl flask to which a 25 ml. of water and a few quartz pebbles were added. To insure the destruction of the hydrogen peroxide, about 0.5 ml. of potassium permanganate was added and the solution decolorized by the addition of 2 ml. of sodium sulfite solution. This step has been eliminated in the modifications of the methods recently published (35) by the authors.

The flask was fitted with a bent distillation tube which extended into a 250 ml. wide-mouth Erlenmeyer flask charged with 10 ml. of a freshly filtered 2, 4-dinitrophenylhydrazine solution and 20 ml. of water. The end of the distillation tube was dipped beneath the surface of the reagent in the receiving flask, and the flame of a micro-burner was applied to the Kjeldahl flask. The receiving flask was immersed in a cold water bath and the rate of distillation was adjusted so that the receiving solution did not boil vigorously during the distillation. This last procedure is contrary to the directions as given in the original paper (34), but was advised by the authors in later modifications (35). The distillation was continued until the volume of the solution remaining in the Kjeldahl flask was reduced to somewhat less than 10 ml., usually taking from 12 to 15 minutes. The receiving flask was then removed and the end of the distillation tube rinsed into it.

The orange precipitate was transferred as completely as possible with water to a small No. 2 Gooch crucible furnished with an asbestos mat, and was dried for a short time at 100 to 110°C. Meanwhile the

receiver was thoroughly drained, and the last traces of precipitate were taken up in hot pyridine used in several successive portions, but not more than 3 to 4 ml. in all. The pyridine washings were then transferred to a 25 ml. volumetric flask, the crucible was then fitted into a rubber stopper, carried on a cylindrical funnel -- which is attached to a test tube equipped with a side arm -- and marked at 20 ml. Boiling pyridine was added in small proportions to the crucible; and the contents were gently titrated with a glass rod, after which suction was applied and the pyridine was drawn through each time. Three or four washings usually were sufficient to dissolve and transfer all the precipitate to the 25 ml. flask used for the rinsing of the receiver. After being cooled to room temperature, the solution was made to volume with pyridine and mixed. It was then allowed to settle or was filtered, if necessary, to remove shreds of asbestos.

Either a 2 ml. or a 5 ml. aliquot of the pyridine solution was transferred to a 100 ml. flask and 50 ml. of water were added, followed by a 5 ml. of 5N NaOH solution. The solution was then diluted to the mark, and the blue color was read in a colorimeter by comparison with a standard malic acid solution. This standard malic acid solution containing 10 milligrams per 5 ml. was treated in exactly the same manner as described for the aliquot of the organic acid fraction.

Determination of Potassium

Potassium was determined by the method outlined by Thomas (46): A 2.5 gram sample was weighed into a porcelain crucible and placed in a muffle furnace, regulated so as not to exceed a temperature of 480°C., for 24 hours. The ash was taken up with a 5 ml. of dilute nitric acid and 5 ml. of dilute hydrochloric acid, filtered, and washed with hot

one percent nitric acid. The ashless filter paper and residue were replaced in the crucible and burned in the muffle furnace at a full red heat. This ash was then taken up with an equal mixture of concentrated hydrochloric and nitric acids, boiled, diluted, filtered, and washed with hot 1% nitric acid. The filtrate and washings were made up to a volume of 250 ml. To a 50 ml. aliquot in a porcelain evaporating dish was added 1 ml. of 50% sulfuric acid. The solution was evaporated to dryness on the steam bath, and the dish was then heated over a Meeker burner until the residue was white. The residue was taken up with 1 ml. of concentrated HCl and hot water added with stirring until solution was complete. Then 5 ml. of 2% platonic chloride were added and the solution evaporated on the steam bath just to dryness. The residue was taken up with 25 ml. of acidulated alcohol (2.25 M hydrochloric acid in 80% alcohol) and the mixture was thoroughly stirred. After standing for a half an hour, the contents of the dish were filtered through a weighed Gooch crucible, washed with 80% alcohol, dried at 100°C. for half an hour, and weighed.

Determination of Total Nitrogen

Total nitrogen was determined by the official method of the Association of Official Agricultural Chemists. The Gunning modification of the Kjeldahl method to include nitrate nitrogen (2) was followed up to the point where the material was ready for digestion. After this the Kjeldahl Gunning-Arnold (3) method was followed, with the exception that copper sulfate and a selenized granule were used instead of mercuric oxide or mercury as catalysts.

Determination of Protein Nitrogen

Protein nitrogen was determined by the method employed by a Research Department of one of the large tobacco manufacturers, as follows: One gram of tobacco was placed in a 400 ml. beaker and 100 ml. of 0.5% acetic acid was added. The contents were boiled for five minutes on a hot plate and allowed to cool. The solution was filtered, the filtrate discarded, and the filter washed with hot acetic acid water. The entire mass of tobacco, including the filter paper, was transferred to a 300 ml. Kjeldahl flask and the Kjeldahl Gunning-Arnold procedure, as mentioned above, was followed.

Determination of Non-Protein Nitrogen

The non-protein nitrogen content of the leaf tissue was calculated by subtracting the percentage of protein nitrogen from the total nitrogen of the leaf.

Determination of Calcium

Calcium was determined by the official method of the Association of Agricultural Chemists.

Determination of the volatile organic acidity

The standard method employed by the Research Department of one of the larger eastern tobacco manufacturing companies was used to determine volatile organic acids.

Five ml. of tartaric acid and 100 ml. of carbon-dioxide free water was added to 5 grams of powdered tobacco in a 500 ml. Kjeldahl flask. The still was connected and heat applied to the flask regulating the flame so that the boiling occurred in 5 to 8 minutes. At the expiration of 10 minutes from the time of applying the flame, steam was passed in to the flask and 500 ml. of distillate was collected at the

rate of 10 ml. per minute. The original volume of the solution in the reaction flask was marked and maintained during the distillation. The Distillate was transferred to a 600 ml. beaker and heated to 95°C. 6 drops of phenol red solution was added, and titrated with 0.02N NaOH, using a daylight lamp for illumination. After subtracting the titration value of the blank, the total volatile acids were calculated as acetic acid on a moisture-free basis.

PURPOSE AND PLAN OF THE EXPERIMENTS

EXPERIMENT I

Table I gives definite information as to how the eight outlying plots for the season of 1941 were treated. These plots were localized in different parts of Lancaster County, the center of the tobacco industry in Pennsylvania.

An application of 1000 pounds per acre of a 4-8-12 fertilizer was given to the plots from which samples A and B were taken. The experiment was planned making nitrogen the most important variable. The nitrogen was applied to samples A in a more readily available form (75% NaNO_3 and 25% cottonseed meal) and to samples B in a less available form (75% cottonseed meal and 25% NaNO_3)

A study of the moisture, nitrogen (total, protein, and non-protein) potassium and calcium content was made first on samples of the mature tobacco. This analysis was followed by a study of the soluble and insoluble ash constituents of both ether soluble organic acidity, as well as the three most important non-volatile organic acids found in the tobacco leaf, oxalic, citric and malic, were made of both the mature and cured samples.

The analysis, of samples A were compared to samples B. The differences in the chemical composition of the leaf, when the two different applications of nitrogen made, were studied. The possibility of using this fertilizer as a general treatment of cigar-leaf tobacco is discussed. A study of the organic acid metabolism of the

mature and cured samples is presented.

EXPERIMENT II

Table VII gives definite information as to how certain cured and fermented samples picked from the 1941 crop were treated.

Apparently, samples which had been yeast treated during fermentation seem to be more completely fermented than the untreated samples. The fermentation of the cured samples was carried in two ways; some were yeast treated, while others received no yeast treatment. A comparison of the chemical composition of the yeast treated and untreated samples was made. A study of the alkalinity of the soluble and insoluble ash of the cured and fermented samples (untreated and yeast treated) was likewise made. This was followed by a study of the ether soluble organic acidity, the oxalic, citric and malic acid content of the cured and fermented samples.

Special attention was given to the organic acid metabolism of the cured and fermented samples and a comparison was made between the untreated and yeast treated samples to see if the yeast treatment affected the chemical composition of the leaf.

EXPERIMENT III

The chemical analysis of two cured samples from the crop of 1941 is shown. The "Silvestrius sample" represents a special sample of the 1941 crop and is characterized by a low nicotine content. The usual 4-8-12 fertilizer treatment had been given to both samples. The "Ephrata sample" is characterized by being grown under drought conditions. A comparison of the chemical composition of the leaf of

those two samples grown under entirely different water conditions is made.

EXPERIMENT IV

Table XIII shows the analysis of the soluble and insoluble ash of fermented samples of tobacco of the 1941 crop. An extra sample taken from the market in cigar form, X is analyzed for soluble and insoluble ash and compared to the other fermented samples of the 1941 crop. Analysis of the steam volatile organic acids is also presented.

TABLE I
EXPERIMENT I

Data showing how the samples for Experiment I were treated.

Sample Number	Name of Grower	Location	Treatment	Amount	Planted	Topped	Suckered	Cut
101A	Noah E. Denlinger	Paradise	4-8-12	1000#	5/23-5/24	7/18	8/2	8/8-8/11
101B	Noah E. Denlinger	Paradise	4-8-12	1000#	5/23-5/24	7/18	8/2	8/8-8/11
102A	Charles W. Leferer	Drumore	4-8-12	1000#	6/2-6/3	8/4	8/23	8/25
102B	Charles W. Leferer	Drumore	4-8/12	1000#	6/2-6/3	8/4	8/23	8/25
103A	George Cramer	Pequea	4-8-12	1000#	6/9-6/14	8/11	8/26	8/28-8/30
103B	George Cramer	Pequea	4-8-12	1000#	6/9-6/14	8/11	8/26	8/28-8/30
104A	J. E. Longenecker	Mt. Joy	4-8-12	1000#	6/14	8/12	9/6	9/8
104B	J. E. Longenecker	Mt. Joy	4-8-12	1000#	6/14	8/12	9/6	9/8

A - Ready available nitrogen (75% NaNO_3 and 25% cottonseed meal)

B - Cottonseed Meal (75% cottonseed meal and 25% NaNO_3)

TABLE II

EXPERIMENT I

Composition of Mature Tobacco
from Outlying Plots for the season of 1941

Sample Number	Moisture	Nitrogen			Potassium	Cal- cium	N/K
		Total	Protein	Non- Protein			
	%	%	%	%	%	%	
101A	5.38	3.38	2.22	1.16	3.02	3.30	1.12
101B	6.30	3.66	2.29	1.37	3.41	2.92	1.07
102A	7.20	4.24	2.44	1.80	2.63	2.80	1.61
102B	6.74	4.17	2.36	1.81	2.54	2.93	1.64
103A	5.66	4.69	2.94	1.75	3.09	2.90	1.52
103B	5.98	4.06	2.50	1.56	2.61	3.22	1.56
104A	5.77	4.04	2.26	1.78	2.87	3.53	1.41
104B	5.32	4.23	2.37	1.86	2.73	3.09	1.55

EXPERIMENT I

Discussion:

Due to the limited number of samples studied in all the experiments; it does not seem proper to draw many definite conclusions. It can be said in general, however, that although the number of samples studied was small, they show a general trend of behavior under the specified conditions of the experiment.

Nitrogen, Potassium, and Calcium:

Table I gives definite information as to how the mature samples from the outlying plots of 1941 were treated. Table II presents the analysis of these samples. No appreciable difference was found between samples A and B in the total, protein and non-protein nitrogen. Any difference noticeable is of a minor nature. Although samples A have access to a greater proportion of nitrogen in a more readily available form than samples B; no real difference on the availability of nitrogen, as shown by the chemical analysis of the leaf is demonstrated.

We know that the tobacco plant, as many other plants, take most of its nitrogen from the soil in the form of nitrates. The nitrogen in cottonseed meal and most of the organic nitrogenous fertilizers is supposed to be in an unavailable form and can not be readily utilized by the plant. It is only by complicated decomposition process carried on by soil microorganisms that the nitrogen in this material is finally broken down to the nitrate form and becomes available to the plant.

The nitrogen supplied to samples A is in a readily available form and should give enough supply of nitrogen for the early stages of growth. Nitrogen is absorbed and utilized normally by the growing tobacco plant in the early stages of growth. After the plant is "topped" the absorption and utilization of nitrogen appear to be more harmful than beneficial. The nitrogen applied to samples B proved to be as effective in regard to absorption of nitrogen by the plant, as for sample A.

It is safe to say that the amount of available nitrogen in fertilizer B seems to be sufficient to furnish enough nitrogen for the earliest stages of growth, the plant utilizing the nitrogen from the cottonseed meal as it becomes slowly available due to the action of microorganisms. Also, at the earliest stages of growth the plant could have made use of the nitrates usually present, although in small amounts, in the soil.

It will not make any difference from what source the nitrogen comes as long as the "organic nitrogen" is given enough time to undergo decomposition to the "nitrate form" and become available to the plant. Of course, nitrogen in some readily available form should be applied at planting so that it can be utilized for the earliest stages of growth and avoid nitrogen shortage.

Samples B gave the same nitrogen analysis as samples A due to the fact that there was a general decomposition of the "organic nitrogen" from the cottonseed meal into nitrate form and in this way it became available to the plant. In samples A although a greater proportion of nitrate nitrogen was applied still the amount of protein

nitrogen was greater than the non-protein nitrogen. Nitrate reduction occurs within the plant to "amino nitrogen" which constitute the nitrogen part of the protein molecule in samples B, there are probably three processes occurring simultaneously. A decomposition of the "organic nitrogen" to "nitrate nitrogen" in the soil, followed by the absorption of "nitrate nitrogen" by the plant, and finally the reduction of this "nitrate nitrogen" to the "amino nitrogen" within the plant.

The amount of potassium and calcium absorbed by the two plants, as shown by the analysis of the leaf, does not indicate any difference under these two different conditions of nitrogen nutrition.

Potash is very essential where quality production of cigar-leaf tobacco is desirable. Under normal conditions, nitrogen is absorbed by the plant more readily than potash. An N/K ratio of 1, ordinarily is desirable for the growing of the best quality of cigar-leaf tobacco. With the exception of samples 101A and 101B the N/K ratios are far from unity showing that these applications of nitrogen fertilizer will give an excess of nitrogen in the leaf as compared to potash under the conditions of these experiments. Usually, a concentration of 3.5% total nitrogen in the mature leaf will suffice if the concentration of potassium is in excess of this figure. Some stable forms of nitrogen, such as proteins, should always be present in the leaf since they contribute to certain qualities within the leaf itself. If too high a concentration of proteins is present a satisfactory fermentation may be prevented.

We should emphasize again that no single factor contributes more toward desirable burning qualities of tobacco than the potassium content. The favorable influence of potassium on the burning qualities of tobacco is obtained only when measurable quantities of this element are in combination with ether-soluble organic acids, especially hydroxy acids such as citric and malic acids.

Alkalinity of the Ash

Tables III and IV show the analysis of the alkalinity of the ash of the mature and cured samples. The alkalinity of the soluble ash of the mature samples did not show any considerable difference between the samples which were treated with the available form of nitrogen and those which were treated with the unavailable form. Samples 102A and 102B were subjected to a drought condition and this probably caused the low value. No consistent results could be obtained for the values of the alkalinity of the soluble ash. Apparently, the form of nitrogen applied on a fertilizer does not seem to have an effect on the alkalinity of the soluble ash. The samples, to which nitrogen is applied in an available form, gave a higher value for the total alkalinity than the samples to which nitrogen is applied in an unavailable form. This is naturally due to the greater amount of NaNO_3 applied to samples A. This will provide for a greater amount of sodium ions which will give an increase in the total alkalinity of the ash. The insoluble alkalinity for samples A was also of a higher value than for samples B. This is to be expected since samples A gave a higher

TABLE III

EXPERIMENT I

Alkalinities of the Ash
of the Mature Sample

Sample Number	Ash %	Alkalinity of Ash		Total M.e./100 gms.
		Soluble M.e./100 gms.	Insoluble M.e./100 gms.	
101A	17.57	49.98	199.5	249.51
101B	16.59	56.49	188.43	244.92
102A	13.62	28.58	179.59	208.19
102B	13.64	29.97	171.98	201.5
103A	14.50	48.36	214.67	263.03
103B	14.08	40.88	186.75	227.63
104A	16.60	41.79	258.97	300.76
104B	14.84	40.43	226.27	266.70

✓ 17.29
15.57

TABLE IV

EXPERIMENT I

Alkalinities of the Ash
of the Cured Samples

Sample Number	Ash %	Alkalinity of Ash		Total M.e./100 gms.
		Soluble M.e./100 gms.	Insoluble M.e./100 gms.	
101A	21.95	20.36	292.47	312.83
102A	22.21	12.86	286.89	299.75
102B	21.59	12.86	272.7	285.58
103A	19.86	13.93	282.46	296.39
104A	18.93	19.28	274.87	294.15
104B	20.36	19.28	242.31	261.59

✓ 22.93
20.70

value for the total alkalinity of the ash than samples B. The samples which were supplied with nitrogen in an available form, gave a higher percentage of ash. This is due to the greater amount of mineral matter used in this fertilizer as compared to fertilizer B.

During the curing process, the ash content increased in all cases. This is accompanied by an increase in the total alkalinity of the ash. Samples 104A and 104B did not show any considerable change in the total alkalinity during curing. This is probably due to the fact that in these two samples we have a higher percentage of calcium present, contributing to the greater amount of the total alkalinity. Probably most of this calcium is present in an insoluble form. Calcium in this form is quite inactive and will not undergo any considerable change during the curing process. That the calcium is in an insoluble and inactive form can be shown by the high percentage of oxalic acid given by these two samples. We know that the larger the amount of nitrogen present, the more proteins are formed and of course the more oxalic, acetic, succinic and formic acids are produced in the protein metabolism. Oxalic acid is also a product of carbohydrate metabolism but to a far greater extent a product of protein metabolism. One of the physiological roles of calcium in plant metabolism is to precipitate or neutralize oxalic acid as well as many of the other acids produced within the plant or taken from the soil in surplus quantity. If the calcium is present in an insoluble form it will not be effective in neutralizing other organic acids produced by the protein metabolism, and it

will not take part in the normal reactions of plant metabolism.

In almost every case a direct correlation was found to exist between the amount of potassium present and the alkalinity of the soluble ash. It was found by Haley (19) that not only the amount of potash present but also the form of potash affects the alkalinity of the ash. He concluded that when the plants received muriate of potash, the alkalinity of the soluble ash was less, as a rule, than when the sulphate was used. A profound decrease took place in the alkalinity of the soluble ash during the curing process.

Organic Acid Metabolism:

Tables V and VI show the analysis of the organic acids of the mature and cured samples from the plots of 1941.

Apparently, different applications of forms of nitrogen does not seem to affect the total ether soluble organic acidity of the mature samples. No great difference is noticeable between samples A and B. In the other case, the data presented show that the mature samples B, which had been subjected to an application of an unavailable form of nitrogen (75% cottonseed meal and 25% NaNO_3), gave a higher percentage of oxalic acid in all cases than samples A, which were subject to an application of an available form of nitrogen (75% NaNO_3 and 25% cottonseed meal).

Samples 104A and 104B are very interesting and unusual samples due to its high percentage of oxalic acid. As we know, the larger amount of nitrogen present, the more proteins are formed. These samples gave a high value for the nitrogen content. Oxalic, acetic, succinic and formic acids are products of protein and carbohydrate metabolism.

TABLE V

EXPERIMENT I

Analysis of the non-volatile organic acids
oxalic, citric, and malic-of the mature samples.

Sample Number	Total Ether Soluble Organic Acidity M.e./100 gms.	Oxalic Acid %	Citric Acid %	Malic Acid %
101A	177.77	3.73	2.02	10.10
101B	195.45	4.27	2.82	10.68
102A	211.24	3.22	2.82	8.93
102B	207.94	3.42	2.83	9.13
103A	202.15	2.49	3.59	7.82
103B	202.23	3.97	2.99	7.98
104A	195.95	5.89	3.81	8.07
104B	186.35	7.04	3.83	7.37

15.33 112.24 1134.92
3.43 3.06 8.73

TABLE VI

EXPERIMENT I

Analysis of the non-volatile organic acids-
oxalic, citric and malic - of the cured samples.

Sample Number	Total Ether Soluble Organic Acidity M.e./100 gms.	Oxalic Acid %	Citric Acid %	Malic Acid %
101A	193.99	3.29	4.07	7.43
102A	178.21	3.97	3.74	5.40
102B	167.13	3.81	3.86	6.32
103A	237.42	4.02	4.64	5.45
104A	164.26	5.38	4.93	5.75
104B	181.45	6.98	4.72	5.67

116.66 117.34 1124.03
4.17 4.35 6.01

Apparently, the calcium is in an insoluble form and is not able to neutralize all the acids produced by the plant metabolism. Probably there are many other factors that should be taken into account. We probably have a very intensive carbohydrate and protein metabolism in these two plants, in which case we might have a greater amount of oxalic acid than is normally produced. Perhaps, the amount of calcium present is not sufficiently available to be able to neutralize the excess of oxalic acid. Of course, we should remember that the element calcium has other physiological roles. Calcium is supposed to influence the translocation of carbohydrates and is also active in influencing the physiological availability of other ions. Calcium is also a component part of plant structures and compounds. Oxalic acid is produced in greater quantities than the other acids that are normally produced in protein and carbohydrate metabolism in these two samples. This explains to a certain extent the excess of oxalic acid in the plant but drought conditions should be considered. Due to the intensive metabolism present in these two plants an excess of calcium may be necessary to fully neutralize the effect of the degradation products of protein and carbohydrate metabolism as well as to have enough calcium available to assume its other duties in the plant. Anyway, the case is abnormal.

Malic acid was produced in greater amounts in samples B. Apparently, the form of nitrogen applied seems to affect the quantity of malic acid produced in the leaf. How the form and amount of available nitrogen affect the quantity of malic acid produced is not known, probably in an indirect way by influencing the rate of for-

mation of the malic acid by affecting one or more of the many factors involved in the complex malic acid metabolism of the tobacco plant. The citric acid did not show any consistent changes by the application of the two forms of nitrogen.

The non-volatile organic acids - malic, citric, and oxalic - constitute about 80% of the total ether soluble acidity (27) and nearly about 20% of the total organic solids of the tobacco leaf tissue. The so called "unknown acids" constitute about 20% of the total ether soluble acidity. Of course, this will vary and will depend on the amount of oxalic, citric and malic acid formed. Our idea is to try to reduce the oxalic acid content, and increase the citric and malic acid in the leaf. In trying to do this the amount of unknown acids will be reduced. We want to increase the amount of citric and malic acids, especially a combination of potassium with the two acids, since it is this combination which is so conducive to an even and complete combustion of the tobacco leaf accompanied by a light and coherent composition of the ash.

In general, it should be said that although there is a decided difference in the chemical composition of the organic acids when the two different amounts of available nitrogen are added, the differences are not of such a great nature as to allow for very definite conclusions.

Changes of the organic acids during curing:

During the curing process, apparently the total ether soluble organic acidity underwent no significant change. The oxalic acid

did not seem to take a major part in the "curing process". The paramount changes taking place during curing are the increase in citric acid and the corresponding decrease in malic acid. These changes confirm the findings of Vickery and Pucher (52) and other investigators (8). Three possibilities are given in the literature for the pronounced increase in citric acid during curing: (1) it may have arisen as a by-product of the deamination of amino acids; it may have come from the decomposition of carbohydrates; or it may have been converted from unknown acids precursors. Still, there has been proposed a reasonable hypothesis for the formation of citric acid from malic acid by the mechanism of Knoop and Martius (23a). Vickery and Pucher (49), while studying the chemical changes that occur in tobacco leaves during culture in light and in darkness, showed that there was a considerable decrease in the malic acid content and an increase in the citric acid content when the leaves were cultured in water solution in the dark.

It seems improbable that the decomposition products of the protein metabolism played an important part in the formation of citric acid. That the citric acid might have been formed from the decomposition product of carbohydrate metabolism seems reasonable from a chemical point of view. It has long been known that carbohydrates can be converted into citric acid by molds and the recent experiments of Chrzaszcz and Turkow (8a) are of significance in this connection. Although the view that citric acid formed during curing originates from carbohydrates has much to recommend it, an equally strong argument can be advanced for an entirely different origin of this acid.

The work of Vickery and Pucher (52) showed that the quantity of citric acid which appeared during curing is the same order of magnitude as the unknown acids that disappeared. No evidence of this was found in this investigation. In fact, there was no consistent change with respect to the unknown acids during the curing process. In some cases there was an increase of these acids during curing and in other cases there was a decrease. If it could be shown that these "unknown acids" could be converted into citric acid by natural processes, this view of the origin of the citric acid will receive material support. If however, these acids turn out to be largely substances of a totally different type the idea of carbohydrate origin for the citric acid will become more probable.

No great and no consistent difference was found between the chemical composition of citric, oxalic and malic acid between the cured samples A and B's. Apparently, the amount of available nitrogen applied as a fertilizer does not influence the organic acid metabolism during the curing process.

By the application of this particular fertilizer in which nitrogen was applied in two different proportions, we were able to obtain a higher percentage of citric acid in the cured leaf than in preceding years. The malic acid content was lower, while the oxalic acid content for the 1941 crop was much higher than for the preceding years. (Block (8) found that the better quality cured tobacco was found to have a greater amount of citric acid whereas the quantities of malic and citric acids were approximately the same. So we should try by different fertilizer practices to increase the citric

and malic acids contents, and reduce the amount of oxalic acid in the leaf. With regard to the mature leaves Block (8) found that the better grade of tobacco was characterized by a lower content of citric and oxalic acids than was found in the poorer grades. In the mature samples for the 1941 crop a higher percentage of citric and oxalic acid was found as compared to previous years, and a lower percentage of malic acid.

The problem of determining the effect of a definite application of a fertilizer on the chemical composition of the leaf of a plant is complicated by the existence of several modifying factors. One of the most important factors to be considered in a study of this case is the character of the season, particularly the amount and distribution of rainfall. The character of the weather during the growing and curing season, has an important bearing on the results obtained for any particular year. This is particularly true in case of fertilizer experiments, although it applies somewhat to all field experiments. In case of the tobacco plant the character of the weather modifies not only the physical characteristics of the tobacco leaf, but also affects the availability of the fertilizer which in turn affect the composition of the leaf.

TABLE VII

EXPERIMENT II

Data showing how the samples
for Experiment II were treated

Cured Samples	Fermented Samples
Sample 1 - Box 1-2 Mumma Tobacco Treatment 4-8-12 Kreider Seed	Sample 1- Box 1 (untreated) Mumma Tobacco Treatment 4-8-12 Kreider Seed Sample 2 - Box 2 (Yeast treated) Mumma Tobacco Treatment 4-8-12 Kreider Seed
Sample 14 - Box 3 Mumma Tobacco Treatment 4-8-12 Kreider and Sewarr Seed	Sample 14 - Box 3 (yeast treated) Mumma Tobacco Treatment 4-8-12 Kreider and Sewarr Seed
Sample 8 - Box 4-5 Street Tobacco Treatment 3-6-12	Sample 8 - Box 4 (untreated) Street Tobacco Treatment 3-6-12 Sample 9 - box 5 (yeast treated) Street Tobacco Treatment 3-6-12

TABLE VIII

EXPERIMENT II

Analysis of the alkalinities of the ash
of the cured samples

Sample Number	Moisture %	Ash %	Alkalinity of Ash		
			Soluble M.e./100 gms.	Insoluble M.e./100 gms.	Total M.e./100 gms.
1 & 2	8.36	22.44	79.62	258.2	337.82
14	7.87	19.99	54.70	267.26	321.96
8 & 9	7.86	18.96	27.35	259.95	287.30

TABLE IX

EXPERIMENT II

Analysis of the alkalinities of the Ash
of the fermented samples

Sample Number	Moisture %	Ash %	Alkalinity of Ash		
			Soluble M.e./100 gms.	Insoluble M.e./100 gms.	Total M.e./100 gms.
1	9.16	25.93	61.02	305.22	366.24
2	9.15	25.51	82.5	279.38	361.88
14	9.14	22.52	67.5	274.76	342.26
8	9.12	23.81	25.07	313.92	338.99
9	9.17	20.86	21.43	261.97	283.4

EXPERIMENT II

Alkalinities of the Ash:

Table VII shows how the samples used in the second experiment were treated. The yeast treatment produced tobacco which seemed more completely fermented, as judged by appearance and smoking qualities than the untreated samples.

Tables VIII and IX show the analysis of the soluble and insoluble ash of the cured samples. Specially striking is the high value of the soluble ash of samples I and XIV. This is something we have to look forward since there seems to exist a correlation between the soluble alkalinity of the ash of the samples and their burning qualities. The quantity of alkalinity of the soluble ash is affected by the form and the amount of potash applied as a fertilizer and to a marked extent by the season. The usual range of the alkalinity of the soluble ash of Pennsylvania cigar-leaf tobacco is around 40-50 milli-equivalents per 100 grams of sample. As we see there is a marked difference between the value obtained for samples I and the usual range of the soluble alkalinity of the ash of Pennsylvania cigar-leaf tobacco.

The value obtained for the total alkalinity of the ash for the different samples is about normal; with the exception of sample VIII which gave a lower value. The soluble alkalinity of the ash which gave a lower value. The soluble alkalinity of the ash for this sample was low.

Due to the unusually high value of the soluble alkalinity of the ash of samples I and XIV, a lower value than normal was obtained for the insoluble alkalinity of the ash. Of course, this is desirable to a certain extent.

During the fermentation process an increase in the ash content was found in all cases. This increase in ash should produce a corresponding increase in the alkalinity of the tissue. An increase in the alkalinity of the tissue is usually accompanied by a corresponding decrease in the ether soluble organic acidity in order to maintain the normal constant level of pH necessary for all living tissues. This was found to be the case. McKinstry (27) in his work concluded that the normal fermentation is always accompanied by a marked progressive decrease in the total ether soluble organic acidity and a corresponding rise in the alkalinity of the tissue.

Apparently, the yeast treated samples gave a lower ash content than the corresponding untreated samples. Of course, the yeast treated samples gave a lower value for the total alkalinity of the ash. No consistent results were found in regard to the soluble alkalinity of the ash. In two cases, (samples I and XIV) an increase in the soluble alkalinity of the ash was found when the samples were yeast treated. The untreated samples showed a decrease in the value of the soluble alkalinity from the corresponding cured samples. The untreated sample VIII behaved the same way, but the corresponding yeast treated sample IX failed to give an increase in the soluble alkalinity of the ash. Also,

the total alkalinity of the yeast treated sample IX failed to give an increase in the total alkalinity. This is rather unusual since a "normal" fermentation process is always accompanied by an increase in the total alkalinity. Still, the untreated sample VIII behaved normally as well as the others untreated and yeast treated samples.

The insoluble alkalinity increased during fermentation in every case, the increase shown by the untreated samples being of a higher value than the corresponding yeast treated samples. This is to be expected since the yeast treatment apparently caused a greater increase in the soluble alkalinity which means less insoluble alkalinity of the ash.

Organic Acids Metabolism:

Tables X and XI show the analysis of the total ether soluble organic acidity and of the non-volatile organic acids - oxalic, citric and malic - of the cured and fermented samples. In the first case, a pronounced decrease in the total ether soluble organic acidity was found to accompany the fermentation of the samples studied. Apparently, the yeast treated samples showed a greater decrease in the ether soluble organic acidity. The decrease in the ether soluble organic acidity is in accordance with the total alkalinity increase showed by all the samples.

McKinstry (27) arrived at the same conclusion. His data and the data presented in this investigation in regard to the change in the total ether soluble organic acids during fermentation are at variance with the conclusions drawn by Pucher, Vickery and Wakeman(36).

TABLE X

Experiment II

Analysis of the non-volatile organic acids-
oxalic, citric and malic- of the cured samples

Sample Number	Total Ether Soluble Organic Acidity M.e./100 gms.	Oxalic Acid %	Citric Acid %	Malic Acid %
1 & 2	259.07	2.61	3.43	2.89
14	277.56	2.96	2.61	2.81
8 & 9	269.75	4.32	7.52	4.45

TABLE XI

EXPERIMENT II

Analysis of the non-volatile organic acids-
oxalic, citric and malic- of the fermented samples

Sample Number	Total Ether Soluble Organic Acidity M.e./100 gms.	Oxalic Acid %	Citric Acid %	Malic Acid %
1	225.17	3.37	0.81	1.53
2	230.51	3.61	0.51	1.73
14	214.52	4.08	0.21	1.67
8	228.30	4.02	1.37	0.64
9	213.18	4.12	1.64	0.93

These workers reported that the changes in the concentration of the organic acids of the leaf during fermentation are of a minor nature. As McKinstry explains in his work (27), the conclusions of the Connecticut workers may be applied with propriety to the case-fermented, Connecticut tobacco; this type of leaf is usually subjected to a very mild and very slow fermentation. But our data demonstrates invariably the fact that the normal bulk fermentation of Pennsylvania cigar-leaf tobacco is always accompanied by a pronounced decrease in the organic acidity of the tissue.

The data obtained in this investigation revealed the fact that citric and malic acid, present mainly in the leaf as the potassium salts, are dissimilated to a marked degree during the process of fermentation. This is in accordance with the work of previous investigators (27). This is what to be expected since the citric and malic acids present in the form of potassium salts will satisfactorily serve as an energy source for the microorganisms associated with the normal fermentation process. This has been proved by experiment with pure culture studies; in which it has been demonstrated that the potassium salts or the ammonium salts of citric and malic acids are in a form readily available as an energy source for the rapid proliferating microflora. To this, oxalic acid is an exception since it exists usually in the form of the insoluble calcium salt. The changes showed by the oxalic acid content of the leaf tissue during the fermentation process is not of a consistent nature. In general, there is no appreciable change in the oxalic acid content. The relative inertness of oxalic acid during

fermentation is not surprising. It had been shown previously (48) that this acid occurs mainly in the form of insoluble oxalates in the tobacco leaf. Furthermore, pure culture studies has demonstrated that the oxalate ion - in any chemical combination - will not serve as an energy source for any of the microorganisms associated with the normal fermentation of cigar-leaf tobacco.

The decrease in citric acid during fermentation is of a higher value than malic acid. Smirnov (40) studying the behavior of the non-volatile organic acids of some oriental types of tobacco during fermentation reached the same conclusion.

Apparently, there was no great difference in the chemical composition of the non-volatile organic acids between the untreated and yeast treated samples.

During the fermentation it appears as if the yeast treated samples give a higher percentage of oxalic acid than the untreated. The same thing appears to be true for malic acid. The decrease, or rather the dissimilation of malic acid is more on the untreated samples during the fermentation process. The citric acid did not show any consistent results; the yeast treated samples showing a greater decrease in the citric acid content in one case, and a smaller decrease than the untreated in the other case.

The so-called "unknown acids" present in the tobacco leaf are still a matter of speculation. They can be calculated by subtracting the sum of the percentages of the oxalic, citric, and malic acids from the percentages of the total ether soluble organic acidity. No consistent results could be found in regard to these acids.

Apparently there is no great change on the value of these acids during fermentation. Probably these acids can not be utilized as a source of energy by the microorganisms present in the fermentation process. Any change they undergo during fermentation is probably of a small nature. More work done on these acids might reveal very illuminating and interesting information.

TABLE XII

EXPERIMENT III

Analysis of two cured samples from the 1941 crop,
 Silvestrius and Ephrata, grown under entirely different moisture conditions

Sample	Moisture %	Total Ash %	Alkalinity of Ash			Total %	Nitrogen Protein %	Non Protein %	K %	H/K
			Soluble M.e./100 gms.	Insoluble M.e./100 gms.	Total M.e./100 gms.					
Silves- trius	9.50	20.75	29.98	272.39	302.37	2.90	1.18	1.72	1.47	1.84
Ephrata	5.18	20.80	18.36	285.09	303.45	2.21	1.59	0.62	1.90	1.18

EXPERIMENT III

Table XII shows two samples from the crop of 1941. Their only difference was that the Ephrata sample was grown on a decidedly drought condition. Their fertilizer treatment was the usual 4-8-12. The Silvestrius sample was characterized by its low nicotine content. The analysis is presented to show how a dry season might affect the chemical composition of the tobacco leaf.

The moisture requirements of the tobacco plant are high because of its great leaf area and the desirability of rapid growth. Any factor tending to lower the moisture requirement of the tobacco plant affects the yield and quality of the tobacco leaf.

From the analysis of the two samples it appears that the one which had been subjected to a dry spell gave a lower value for the soluble alkalinity of the ash. The form and amount of potash, and to a certain extent the season, have a great deal to do with the total quantity of the soluble alkalinity of the ash. Of course, the moisture content of both samples were quite different. This is to be expected for the differences in moisture conditions upon which both plants grew.

The total ash content apparently does not seem to be affected by a dry season. The same appears to be true for the total alkalinity of the ash. The total nitrogen is lower in the Ephrata sample, but the protein nitrogen value is higher in this sample. The non-protein nitrogen is lower in the Ephrata sample, that is the sample subjected to a dry spell. This is probably due to the small

amount of water of translocation in this plant. There is probably not enough water in this tobacco plant to accomplish a complete translocation of the nitrates from the soil to the plant and finally to be deposited in the leaf. The leaf when analyzed has to give a higher percentage of protein nitrogen since it is low in non-protein nitrogen.

The tobacco plant is one of the plants which is affected most by any variation in climate and weather. Seasonal variations in weather will naturally affect the quality of the leaf, just as regional differences in climate affect both type and quality. Usually, too low temperatures may prevent the full ripeness of the leaf necessary for the best quality. High temperatures reduce the water content of the plant and favor strong aroma and thickening of the leaf. Optimum moisture conditions, partial shade, and absence of wind favors the production of a large, broad, thin fine-veined, open textured, elastic, light colored, bright leaf with low nicotine content. Less favorable moisture conditions mean a smaller, narrower, denser, heavier, thicker-veined, darker colored, slower burning leaf with more nicotine, stronger aroma - qualities desired in different degrees in cigar filler, cigarettes, pipe smoking, chewing and snuff tobaccos.

Research in recent years has shown how to modify moisture conditions to some extent by fertilizer practices and the use of the right kind of organic matter in the soil. The methods of topping has also been found to have some effect on the moisture relations. It has been found in research work in recent years that a heavy potash fertilization and the use of very limited quantities of chlor-

ides in the fertilizer are both effective in increasing drought resistance. Certain weeds and crop plants preceding tobacco in the rotation are decidedly beneficial, while others are definitely injurious in increasing drought resistance.

So far as our experience shows, from the standpoint of quality of product, tobacco is remarkably sensitive to its environment; and the major problem of the grower is to control the environment of the plant; something we can do only to a very limited extent.

TABLE XIII

EXPERIMENT IV

Analysis of the soluble, insoluble, and total alkalinity
of fermented samples of the 1941 crop
Analysis of the steam volatile organic acids is also shown
The X sample is taken from the commercial market.

Steam volatile organic acids mgm. H.Ac per 5 grams	Sample Number	Total ash %	Total alkalinity of ash M.e./100 gms.	Alkalinity of soluble ash M.e./100 gms.	Alkalinity of insoluble ash M.e./100 gms.
8.80	10A	23.76	328.5	44.83	283.67
10.25	10B	23.95	334.91	46.92	287.99
9.80	10E	23.42	356.31	46.66	309.65
9.10	11C	23.67	308.5	44.83	263.57
8.20	11D	23.35	361.23	44.93	313.61
16.89	X ¹	23.73	374.16	59.01	315.15

¹X represents a sample taken from the Pennsylvania market.

EXPERIMENT IV

Table XIII shows the analysis of the soluble and insoluble alkalinity of the ash of fermented samples of tobacco of the 1941 crop. The X sample was taken from the commercial market and compared to the samples from the 1941 crop.

The value obtained for the alkalinity of the soluble ash represents about the average value for Pennsylvania fermented cigar-leaf tobacco. The cigar samples represented is an exceptionally good grade of tobacco from the Pennsylvania market. Its high value for the alkalinity of the soluble ash indicates its good burning qualities.

The analysis for the steam volatile organic acids for these samples is also shown. The extreme high value of the cigar sample as compared to the other samples should be noticed. McKinstry (27) showed that during the early stages of the fermentation process there is a pronounced increase in the volatile organic acidity. This initial increase is followed by a significant decrease during the remainder of the process. The origin of the volatile organic acids formed during the initial periods of fermentation, as well as the underlying factors involved in their subsequent disappearance are still largely matters of speculation. Many of the volatile organic acids furnish ideal energy sources for the microorganisms associated with the fermentation of tobacco. The possibility that this volatile organic acids, produced as the result of transformations of the more complex leaf constituents, are in turn utilized in the

energy metabolism of the microorganisms is an attractive hypothesis.

Today the subject is a matter of speculation.

SUMMARY

1. Under conditions of Experiment I, in which nitrogen was made the only variable, there was no appreciable difference in the amount of nitrogen (total, protein, non-protein) absorbed as shown by the chemical composition of the leaf between samples A and B.

2. The amount of potassium and calcium absorbed by the two plants under the two different applications of nitrogen fertilization (75% NaNO_3 and 25% cottonseed meal for samples A and 75% cottonseed meal and 25% NaNO_3 for samples B) was apparently the same.

3. Samples A, to which nitrogen was applied in a more readily available form, gave a higher percentage of ash, a higher value for the total alkalinity and the alkalinity of the insoluble ash than the corresponding B samples, to which nitrogen was applied in a less available form. The alkalinity of the soluble ash was found to be about the same in both cases.

During the curing process we have an apparent increase in the ash content, and increase in the total alkalinity of the ash with a corresponding decrease in the ether-soluble organic acidity; and an increase in the alkalinity of the insoluble ash.

4. With the use of these fertilizers, a higher value for oxalic and citric acid and a lower value for malic acid was obtained than for preceding years.

Application of fertilizer B gave a higher percentage of oxalic and malic acid, while no great difference for the values for

citric acid was obtained as compared to the application of fertilizer A.

During the curing process the oxalic acid did not undergo any great change, the citric acid increased considerably while the malic acid underwent a decrease in value.

5. The fermented samples are characterized by a higher percentage of ash content and by an increase in the total, and the alkalinity of the soluble and insoluble ash.

The yeast fermented samples did not show as high increase in the total and insoluble alkalinity of the ash as the untreated samples.

6. The fermentation was accompanied by a decrease in the total ether soluble organic acidity. A profound diminution of malic and citric acid accompanied the fermentation, while the oxalic acid did not undergo any appreciable alteration. The yeast fermented samples gave a higher value for oxalic and malic acid, while the citric acid did not show any great difference between the untreated and yeast treated samples.

7. When a tobacco plant grows on drought conditions, the moisture content of the leaf, the value for the soluble alkalinity of the ash and the total and non-protein nitrogen are lower than when the plant is grown under optimum moisture conditions. The potassium content was found higher; while the ash content and the total alkalinity of the ash did not show any great difference.

BIBLIOGRAPHY

1. Anderson, P.J., Swanback, T.R., and Street, O.E., Conn. Agr. Expt. Sta., Bull. 386 (1936).
2. Association of Official Agricultural Chemists: Methods of Analysis. 4th edition, (1935) page 25.
3. Ibid: page 25
4. Ibid: page 123
5. Ibid: page 336.
6. Behrens, J., Landw. Ver. Sta., 431, 27-30 (1894).
7. Bennet and Clark, T.A., New phytologist, 32, 37,128,197, (1933)
8. Block, S.S., M.S. Thesis, The Pennsylvania State College (1941)
- 8a. Chrzaszcz, T., and Turkow, D., Biochem. Ztschr. 229, 343, (1930)
9. Clark, H.E., Plant Physiol., II, 5 (1936)
10. Franzen, H., and Stern, E.Z., Physiol. Chem. 115, 270, (1921)
11. Franzen, H., and Helwert, F.Z., Physiol. Chem., 122, 46, (1922)
12. Franzen, H., and Keyssner, E., Biochem. Z., 135, 183, (1923)
13. Franzen, H., and Helwert, F., Biochem. Z., 135, 384, (1923)
14. Franzen, H., and Helwert, F., Biochem. Z., 136, 291, (1923)
15. Franzen, H., and Ostertag, R., Biochem. Z., 136, 327, (1923)
16. Frear, William, Pennsylvania State College Agr. Expt. Sta., Bull. 49, (1900)
17. Garner, W.W., U.S. Dept. Agr. Bur. Plant Ind., Bull. 105, (1907)
18. Garner, W.W., Bacon, C.W., and Foubert, C.S., U.S. Dept. of Agr. Bull. 79 (1914)
19. Haley, D.E., Nasset, E.S., Olson, Otto., Plant Physiology, 3, 185-197, (1928)
20. Haley, D.E., Olson, Otto, Plant Physiology, 6, 177-182, (1931)

21. Hartmann, B.G., and Hillig, F., Jour. Assoc. Official Agr. Chem., 10, 264, (1927), 13, 99, (1930)
22. Jenkins, E.H., Conn. Agr. Expt. Sta., Ann. Report for 1892, 28-31, (1893)
23. Kissling, R., Handbuch der Tabakundes, des Tabakbaues, und der Tabakfabrication, Edition V, Berlin, (1925)
- 23a. Knoop, F., and Martius, C., Z. Physiol. Chem. 242, 1, (1936)
24. Koller, J.B.C., Landwirtschaftlicher, U. Teschnischer, Beziehung; 134 pp. Augsburg, (1858)
25. Kunz, R., Arch. Chem., Mikroskop, 7, 285, (1914)
26. Kurchatov, P.W., Lupinovich, I.S., and Busyuk, N.Y., Trudy Veloness Sel'sko Khoz Inst., 5, 61, (1936)
27. McKinstry, D.W., Ph.D. Thesis, The Pennsylvania State College, (1939)
28. Nasset, E.S., M.S. Thesis, The Pennsylvania State College, (1927)
29. Olson, Otto, Penn. State College Agr. Expt. Sta., Bull. 179, (1922)
30. Olson, Otto, Haley, D.E., Penn. State College Agr. Expt. Sta., Bull. 240, (1929)
31. Psarev, G.M., Tabak, 8, 36, (1938)
32. Fucher, G.W., Vickery, H.B., and Wakeman, A.J., J. Ind. Eng. Chem. (anal. ed.), 6, 140, (1934)
33. Ibid: 6, 190, (1934)
34. Ibid: 6, 288, (1934)
35. Ibid: 13, 244, (1941)
36. Ibid: Plant Physiol. 13, 621, (1938)
37. Schloesing, Th., Compt. Rend. Acad. Sci. (Paria) 50, 1027-1030, (1860)
38. Schmuck, A.A., Chemistry of Tobacco and Manufactured Products (in Russian) Krasnodar (1930)
39. Schmuck, A., Bull. Acad. Sci. U.S.S.R. Ser. Biol., 955, (1939)
40. Smirnov, A., U.S.S.R. State Inst. Tobacco Investigations Bull., 34, (1927)

41. Smirnov, A.I., "The Physiological Biochemical Principles of Tobacco Curing and Fermentation" (in Russian) Krasnodar (1933)
42. Stahre, L., Nord. Farmazeutik Tids., 2, 141, (1895)
43. Street, E.O., Report for 1937 of the Tobacco Substation at Windsor, Bull. 410, (1937)
44. Swanback, T.R., and Anderson, P.J., Conn. Agr. Expt. Sta. Bull., 355, 359, (1934)
45. Thomas, J.J., Haley, D.E., Olson, Otto. Penn. State College Agr. and Expt. Sta. Bull. 331, (1936)
46. Thomas, J.J., Ph.D. Thesis The Pennsylvania State College (1935)
47. van Bemmelen, J.M., Landw. Ver. Sta. 37, 409-436, (1899)
48. Vickery, H.B., and Pucher, G.W., Jour. Biol. Chem., 90, 637, (1931)
49. Ibid: 119, 523, (1931)
50. Vickery, H.B., and Pucher, G.W., Conn. Agr. Expt. Sta., Bull. 311, (1930)
51. Ibid: Bull. 323, (1931)
52. Ibid: Bull. 352, (1932)
53. Vickery, H.B., Pucher, G.W., and Wakeman, J.A., Conn. Agr. Expt. Sta. Bull. 442, (1940)
54. Vladimirov, A.V., Chemisation Socialistic Agr. U.S.S.R. 8, 35, (1939)
55. Ibid: Compt. Rend. Acad. Sci. U.S.S.R., 699, (1939)
56. Wagner, L.V., Tabak Kultur, Tabak und Zigarrenfabrikation 248-49, Weimar (1884)

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